# Location and interaction of N-(9-anthroyloxy)-stearic acid probes incorporated in phosphatidylcholine vesicles

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The set of n-(9-anthroyloxy)-stearic acid (n-AS) probes (n = 2, 3, 6, 9, 12) was studied in small unilamellar vesicles of dipalmitoylphosphatidylcholine by nuclear magnetic resonance spectroscopy (anisotropic diamagnetic effects and relaxation experiments) and in multilamellar vesicles by differential scanning calorimetry. From our results it can be concluded that (i) the probes are located at a graded series of depths, (ii) the induced perturbations on the membrane are of long range, (iii) 2-AS is adsorbed on the membrane interface and (iv) incorporation of 9-AS induces the greatest membrane perturbation.

## Introduction

The fluorescent n-(9-anthroyloxy)-stearic acids (n-AS) are the set of probes most widely used for obtaining information on molecular aggregates such as micelles and membranes [1—3]. For these probes, there is now evidence that the anthroyloxy fluorophores are located at a graded series of depths inside a membrane, depending on its substitution position (n) in the aliphatic chain [1—3]. According to its primary use, most of the structural information available for these probes was obtained from fluorescence studies [4,5], but a few other techniques such as X-ray diffraction [6], infrared spectroscopy [7] and nuclear magnetic resonance [8,9] were also reported.

The validity of using molecular probes to obtain structural information is dependent on the knowledge of their location and induced perturbations on the system. It is the purpose of the present work to elucidate these two questions in a model system of dipalmitoylphosphatidylcholine (DPPC) vesicles. A systematic study of the set of n-AS probes (n = 2, 3, 6, 9, 12) was carried out by nuclear magnetic resonance spectroscopy (NMR) in small unilamellar vesicles (SUV) and by differential scanning calorimetry (DSC) in multilamellar vesicles (MLV). Information on location was obtained by NMR in two different ways: from the variation of spin-lattice relaxation times ($T_1$) of the probes in the presence of a paramagnetic agent and from the diamagnetic anisotropy effect induced by the probe on the lipid resonances. The structural perturbations induced by the n-AS on the lipid, upon probe incorporation, were evaluated from the alterations in the lipid spin-spin relaxation times ($T_2$) as obtained by NMR, and also from DSC data.

While some of these points (e.g., locations) were addressed previously via fluorescence techniques, the wide interest on these probes prompted us to a study via NMR spectroscopy to confront the

results, and obtain information on structural details of their incorporation in the membrane only addressed by this spectroscopic approach.

## Materials and Methods

### Materials

DPPC was obtained from Sigma (St. Louis, MO), and its purity was checked by thin-layer chromatography, the $n$-AS probes ($n$ = 2, 3, 6, 9, 12) from Molecular Probes (Eugene, OR) and $GdCl_3$ from Koch-Light Lab. (Bucks, U.K.). They were used as received. The solvents used were of spectroscopic grade.

### Preparation of phospholipid vesicles

The lipid samples for NMR and microcalorimetry measurements were prepared by combination of chloroform solutions of the phospholipid and the appropiate amount of probes. The organic solvent was evaporated under a stream of $N_2$ and the last traces of solvent were removed by a further 3—5 h evaporation under high vacuum. After the addition of twice-distilled water (in some experiments $GdCl_3$ was also included), multilamellar vesicles (MLV) were formed by vortexing and keeping the samples some degrees above $T_c$. Mixing was continued until a homogeneous and uniform suspension was obtained. SUV were formed from MLV by sonication in a Branson sonicator. Usually ten cycles of 20 s were performed until no significant decrease in scattering intensity of the suspension was obtained. Suspensions were cooled over ice during sonication. The vesicles were then submitted to a high speed centrifugation to separate titanium particles. Final concentrations used were $10^{-2}$ M in lipid and $2 \times 10^{-3}$ M in probe.

### Instrumentation

FT-NMR spectra were carried out in a Bruker CXP300 at 45°C, and the instrumental parameters used allowed 1.5 Hz resolution. Spin-lattice relaxation times ($T_1$) of the aromatic probe protons were acquired from the 180/deg-$\tau$-90° pulse sequence and the values of $M_0$ and $T_1$ were recovered from the best fit of a minimum of 10 experimental intensities to equation $M_z = M_0 (1 - 2e^{-\tau/T_1})$. The uncertainty in $T_1$ arising from this procedure is shown for each experiment in the

Results section. Line widths ($T_2$) were evaluated from graphical analysis of the spectra.

DSC was performed in MLV vesicles with the same lipid probe ratio used in the NMR experiments. The vesicles were left for about 30 min at room temperature for equilibration and 15 $\mu$l aliquots of the suspensions were sealed in small aluminium pans and scanned in a Perkin-Elmer DSC-4 calorimeter using a reference pan containing water. The heating rate was 4°C/min in all the experiments. Peak areas were measured by weighing paper cut outs of the peaks. The instrument was calibrated using indium as a standard. Data was obtained from the thermograms as previously described [10].

## Results

### Nuclear magnetic resonance

The NMR spectrum of SUV vesicles of DPPC is shown in Fig. 1, where the resonances assignment was made accordingly to McLaughlin et al.
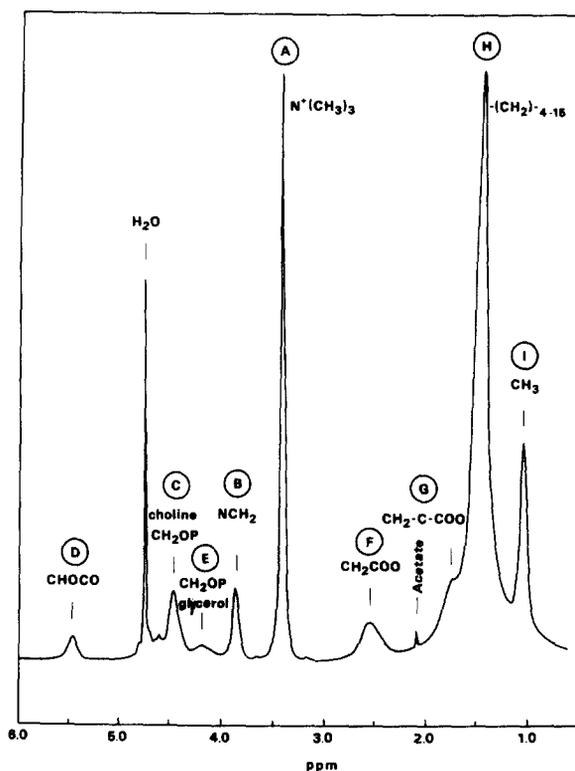


Fig. 1. NMR ($^1$H) spectrum of DPPC (SUV) at 45°C. Sodium acetate was used as internal standard.
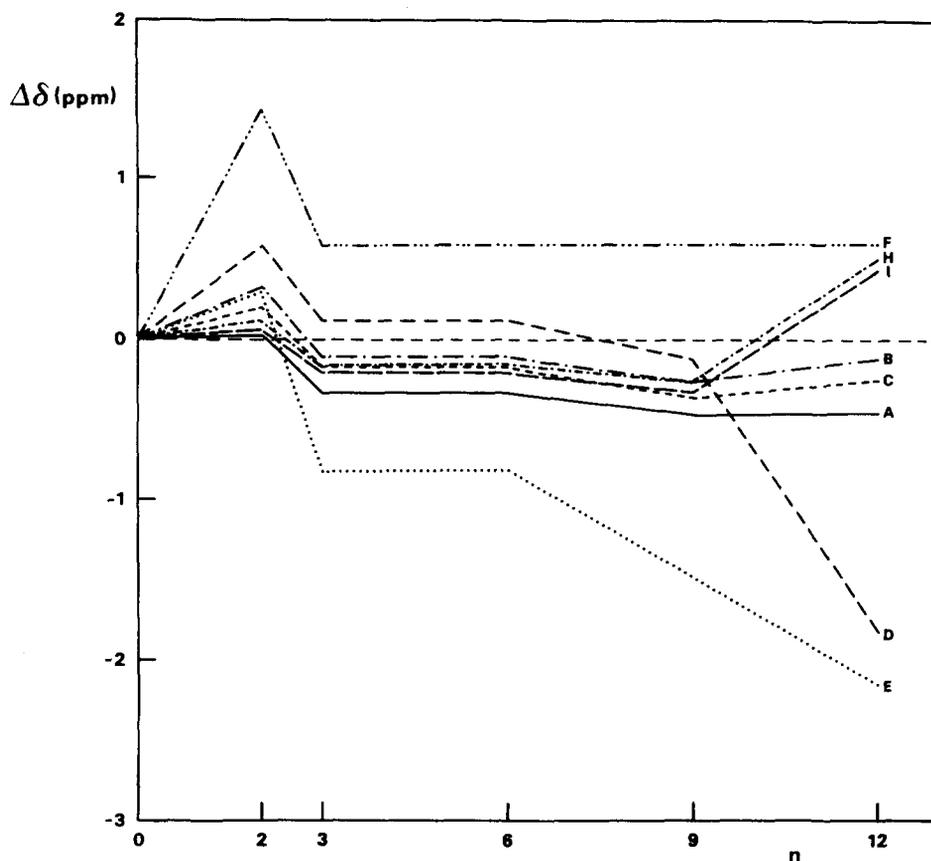
Fig. 2. Chemical shift variations ($\Delta\delta$) of the lipid protons of DPPC upon incorporation of $n$-AS probes ($n$ = 2, 3, 6, 9, 12). (A) —N$^+$(CH$_3$)$_3$, (B) —CH$_2$N—, (C) —CH$_2$OP— (choline), (D) —CHOCO—, (E) —CH$_2$OP— (glycerol), (F) —CH$_2$COO—, (G) —CH$_2$—C—COO—, (H) —(CH$_2$)$_n$— and (I) —CH$_3$.

TABLE I

Nuclear magnetic resonance line widths (Hz) of dipalmitoylphosphatidylcholine SUV vesicles resonances in the presence of $n$-AS probes ($n$ = 2, 3, 6, 9, 12).

|  | CH$_3$ | (CH$_2$)$_{4-15}$ | CH$_2$COO | N$^+$(CH$_3$)$_3$[a] | NCH$_2$ | CH$_2$OP (choline) | CHOCO |
|---|---|---|---|---|---|---|---|
| DPPC | 22 | 37 | 65 | 5 | 20 | 34 | 34 |
| 2-AS | 25 | 67 | 77 | 7 | 17 | 31 | 35 |
| 3-AS | 33 | 102 | 75 | 8 | 24 | 36 | 48 |
| 6-AS | 39 | 91 | 72 | 8 | 20 | 31 | 34 |
| 9-AS | 46 | 187 | 67 | 12 | 24 | 31 | 53 |
| 12-AS | 34 | 94 | 84 | 16 | 24 | 46 | 46 |

[a]Average value of two peaks due to choline in the outer and inner monolayers.

[11]. In the presence of the n-AS probes, the lipid resonances underwent a strong diamagnetic aniso-tropic effect due to the aromatic anthroyloxy moiety of the probe (Fig. 2). The different lipid resonances were distinctly affected upon incor-poration of the different n-AS probes, e.g., the presence of 12-AS induces positive (upfield) shifts on the resonance of the aliphatic protons of the lipids, whereas a strong negative (downfield) effect was observed for the glycerol moiety. In addition, the effects were shown to be probe-dependent, only 3-AS and 6-AS showed a similar behavior (Fig. 2).

The effect of the probe on the lipid resonance line widths was obtained from the previous ex-periments, and the results are shown in Table I. Large increases in line widths are observed, and this effect is also probe-dependent, i.e., there is no systematic contribution to this effect from the probe intrinsic resonances in the methyl and methylene regions. The pattern of variation is similar to the one obtained by Podo and Blasie [8], with the exception of the $N^+(CH_3)_3$ resonance where no alteration induced by the 12-AS probe was observed.
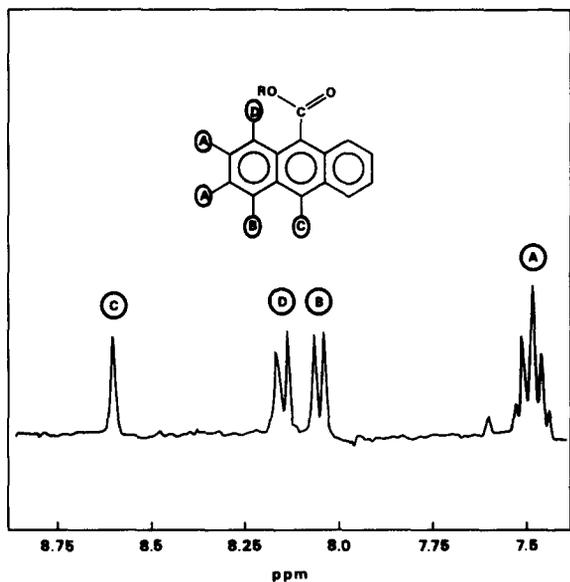


Fig. 3. Aromatic region of the $^1$H-NMR spectrum of 12-AS in CDCl$_3$.
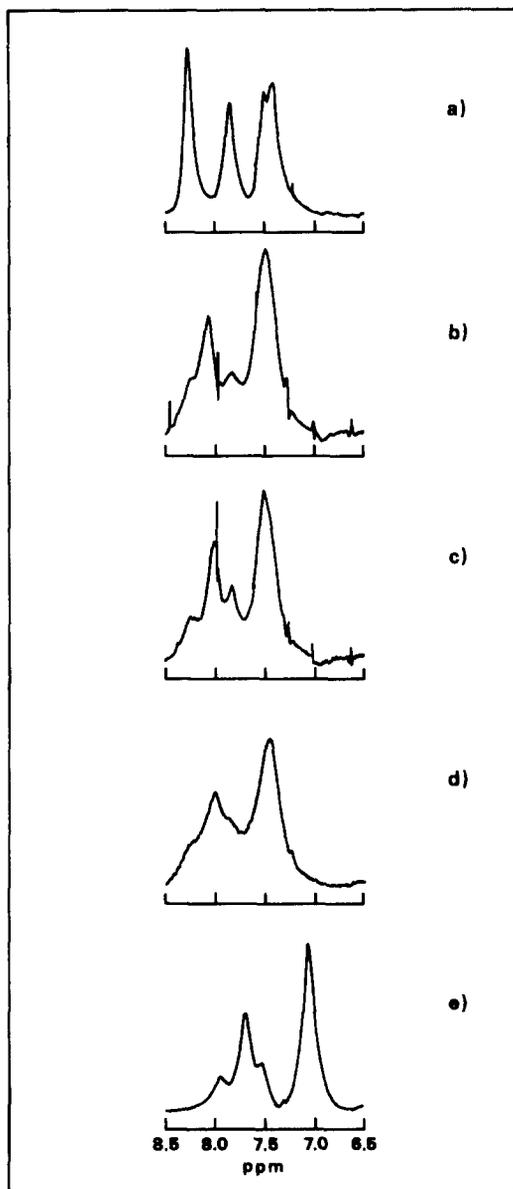


Fig. 4. Aromatic region of the $^1$H-NMR spectra of (a) 2-AS, (b) 3-AS, (c) 6-AS, (d) 9-AS and (e) 12-AS incorporated in DPPC (SUV) at 45°C. Sodium acetate was used as internal standard.

The aromatic proton NMR spectrum of the probe in CDCl$_3$ and its assignment is shown in Fig. 3. As expected, large alterations were observed when the probe is incorporated in the lipidic li-quid crystal [12], as shown in Fig. 4, and no

TABLE II

$T_1$ (ms) at 45°C of the aromatic protons of n-AS probes in the absence and in the presence of $Gd^{3+}$.

| $Gd^{3+}$ (molar) | 2-AS | 3-AS | 6-AS | 12-AS |
|---|---|---|---|---|
| 0 | 521 ± 10 | 519 ± 3 | 466 ± 7 | 536 ± 16 |
| 2 × $10^{-3}$ | 55 ± 10 | 298 ± 11 | 349 ± 5 | 427 ± 5 |
| 10 × $10^{-3}$ | — | 74 ± 2 | 141 ± 7 | — |
| 30 × $10^{-3}$ | — | — | 51 ± 2 | 97 ± 7 |

spectral assignment was attempted in this medium. All the probes show a similar spectrum with a single peak at higher field and three other peaks at lower field, with the exception of 2-AS, where three main peaks are apparent.

Relaxation times $(T_1)$ were measured for these distinct peaks in the presence and in the absence of a paramagnetic agent $(Gd^{3+})$ and the results are shown in Table II. No significant differences in the $T_1$ values were found for the distinct peaks in each probe; in this way, the values presented in Table II were determined considering the intensities of the global aromatic region of the probe in the spectra. $T_1$ values in the absence of the paramagnetic agent are similar for all probes, the one for 6-AS being slightly lower. The lanthanide induces a greater decrease in $T_1$, when the substitution position of the chromophore in the aliphatic chain $n$ is smaller, this effect being also lanthanide concentration-dependent.

TABLE III

Differential scanning calorimetry data for DPPC (MLV) in the absence and in the presence of the n-AS probes[a].

| | ΔH (kcal/mol) | $T_c$ | $T_w$ |
|---|---|---|---|
| DPPC | 8.4 | 41.2 | 0.8 |
| 2-AS | 5.5 | 40.0 | 6.3 |
| 3-AS | 4.9 | 38.9 | 9.8 |
| 6-AS | 4.5 | 38.9 | 8.0 |
| 9-AS | 3.5 | 39.3 | 8.1 |
| 12-AS | 5.9 | 37.6 | 10.0 |

[a]ΔH, enthalpy change of the phase transition; $T_c$, gel to liquid-crystal phase transition temperature (°C); $T_w$, transition width (°C).

### Differential scanning calorimetry

DSC data for the DPPC (MLV) incorporated with n-AS probes are presented in Table III, and the expected alterations were observed showing the perturbations induced in the membrane structure. The ΔH values for the 2-AS and 12-AS are similar and a minimum is apparent for the 9-AS probe. The gel to liquid-crystal phase transition temperature $T_c$ values, are displaced from the one obtained for the pure lipid, 2-AS being now the less affected. The transition width, $T_w$, is identical for 6-AS and 9-AS ($\cong 8.1$°C) and for 3-AS and 12-AS ($\cong 9.9$°C), whereas $T_w$ for 2-AS is significantly lower.

### Discussion

#### Ring current effects

When using extrinsic probes, the induced perturbation on the system is always a matter of concern [4]. The intrinsic low sensitivity of NMR spectroscopy implies a high probe to lipid ratio, and this value is orders of magnitude greater than the ones used in fluorescence [13]. In this situation, the existence of domains of probe in the lipid matrix is expected; for n-AS, probe aggregation was reported from fluorescence data when the probe to lipid ratio is above 0.02 [14]. Considering the small range of the ring current effect this would lead to the existence of two peaks for the lipids, one of them corresponding to the lipid molecules away from the probe. In the present study, this effect was not apparent, and an identical observation was explained on the basis of fast exchange between sites (i.e, near and away from the probe), on the NMR time scale [8].

Ring current effects are a well-known approach to obtain information on probe locations [15], a positive (upfield) shift being an indication that, e.g., the lipidic proton in the membrane is located over the aromatic probe ring. From Fig. 2, where the shifts induced upon incorporation of the n-AS probes on the lipid resonances are shown, it can be inferred that the 12-AS is solubilised in the bilayer center. Positive shifts are observed for the alkyl protons of the lipid, namely for the terminal methyl groups, and as expected, negative shifts are obtained for the remaining protons, the effect being stronger for the ones corresponding to the glycerol backbone. The 3-AS and 6-AS probes have an identical pattern and can be located on the glycerol region, the 9-AS probe showing a transition behavior between the former ("surface probes") and the 12-AS ("inner probe"). For 2-AS, positive variations were observed for all resonances, pointing out that this probe is sitting on the membrane interface, and is apparent that this probe has peculiar properties within the set, as will be emphasized later.

## $T_2$ of the lipid protons

Resonance line widths in lecithin vesicles are dependent on the vesicle size [11,16], its value being inherent to the method of preparation of the vesicles. Nevertheless, this fact does not invalidate the comparison of selective broadening due to the incorporation of different probes in identical lipidic systems.

The spin-spin relaxation rate, in this work evaluated from the resonance line widths, is in these systems essentially related to segmental reorientation of the C—C bond axes [8]. From Table I, it can be concluded that strong restrictions to this type of bilayer dynamics is introduced by the probes. As previously reported, the dynamic perturbation induced by a probe extends to all the bilayer far away from its location [8]. In agreement, we found that the methylene resonances are affected even by an external probe such as 2-AS, this effect being also apparent when lowering the probe to lipid ratio. Maximum line widths variations were observed for the methyl and methylene groups of the lipidic chain, the increase noticed on the methylene resonances by 9-AS being the greater in the set of probes. This

fact is rationalized considering that the 9-AS probe accommodation in the membrane is a very unfavorable thermodynamic process when compared, e.g., with the 12-AS probe. In agreement, theoretical calculations have shown that two probe locations are energetically favorable in membranes [17], in the bilayer interior (i.e., 12-AS), or near the interface (i.e., 2-AS, 3-AS or even 6-AS). In this way, the 9-AS probe, being incorporated in the palisade structure of the chains, would not fit in any of these situations.

## $T_1$ of the aromatic probe protons

Due to its nature (dipole-dipole), the interaction of the lanthanide ion on the spin-lattice relaxation rate of the probe protons has an explicit distance dependence $(r^{-6})$ [18], allowing it to be used to evaluate distances on a molecular scale. The lanthanide ion in solution or bound to the interface [19], induces a monotonically decreasing effect of $T_1$ on the set of probes, according to its substitution position (Table II). These NMR data provide evidence, in agreement with previous information from fluorescence spectroscopy [1—3], that these probes are located at a graded series of depths in the membrane. The assumption of a well-defined location is certainly a limiting situation, the present results being evidence that the distribution is centered at increasing distances with increasing n. It should be noticed that the variation of a single carbon position in the chain, from 2-AS to 3-AS, induces a very large variation in the $T_1$ value. This observation is eventually due to the contribution of close interactions in the case of 2-AS, and the specificity of this probe is again emphasized by this spectroscopic approach, when compared to fluorescence techniques.

The spin-lattice relaxation operates in an analogous way to the electronic (dipolar) energy transfer interactions in photophysics [20]. Its application to the geometry of the present experiment, i.e., ions in solution and bound to the interface, and the probe embedded in the membrane, would imply that for the same lanthanide concentration, the relationship $\ln(1/T_1 - 1/T_1^0) = -\alpha \ln d$ would hold, where $T_1$ and $T_1^0$ are the relaxation times in the presence and in the absence of $Gd^{3+}$, and $d$ is the probe distance to the membrane interface. Assuming n (the substitution posi-

tion of the chromophore) as a measure of this distance, linear relationships were obtained but with a slope ($\alpha = 1$) far from the value of $\alpha = 3$ expected for the rapid-diffusion limit [21,22], imposed by the NMR time scale of the experiment. This discrepancy would point out that the absolute sequence of distances is not strictly correlated to $n$, but the eventual existence of probe domains [14] precludes a clear interpretation. However, as previously stated, the qualitative discussion, i.e., evidence for a graded series of depths according to $n$, is not hampered by this fact.

*DSC experiments*

The calorimetric data provide evidence for the strong perturbation induced on the membrane (Table III). The $\Delta H$ value for the 12-AS probe is identical within experimental error to the one previously reported [2]. In the present work a deviation is observed for 2-AS, with a $\Delta H$ value similar to the internal 12-AS probe. This probe is the one for which the minimum perturbation is apparent. The phase transition enthalpy decreases in the sequence 3-AS, 6-AS and 9-AS, and a sharp decrease is observed for this last probe. This fact is inherent to the location of 9-AS in the middle of the palisade structure of the membrane, as previously described in this work from the $T_2$ results obtained for the lipid.

Minimum transition width $T_w$ was obtained for 2-AS, intermediate values for 6-AS and 9-AS, and maximum identical values for 3-AS and 12-AS. Lower values for $T_w$ indicate that the probe moiety is less effective at reducing the cooperative unit involved in the transition [23]. From our results, this would point out that this effect is less relevant when the probe is in the outer region of the membrane (2-AS), or in the intermediate acyl region (6-AS and 9-AS).

**Concluding remarks**

The $n$-(9-anthroyloxy) stearic acid probes ($n$-AS) were studied in DPPC model membranes, by proton magnetic resonance and by DSC, in order to assess its location, and obtain information on the induced perturbations on this model system.

The results obtained regarding locations are in agreement with previous reports using fluores-

cence techniques, i.e., they are located at a graded series of depths in the membrane [1—3]. In addition, from this study, it was concluded that (i) 2-AS is a peculiar member of the family of probes, as the chromophore is adsorbed on the membrane interface. In contrast, 3-AS, which has the chromophore displaced only a carbon-carbon length in the aliphatic chain, shows properties typical to the other members of the series; (ii) the 9-AS probe is located in the middle of the palisade structure of the membrane and can not reach the bilayer center — apparently for this case the greatest perturbation is induced on the membrane; and (iii) the induced perturbations on the membrane are not localized near the solubilization site of the probe, but extend far away in the transverse direction of the membrane, i.e., an inner probe such as 12-AS affects the membrane dynamics of the lipid up to the interface.

**References**

1 E. Blatt and W.H. Sawyer (1985) Biochim. Biophys. Acta 822, 43—62.
2 D.A. Cadenhead, B.M.J. Kellner, K. Jacobson and D. Papahadjopoulos (1977) Biochemistry 16, 5386—5392.
3 E. Blatt, R.C. Chatelier and W.H. Sawyer (1984) Photochem. Photobiol. 39, 471—483.
4 F. Grieser and C.J. Drummond (1988) J. Phys. Chem. 92, 5580—5591.
5 M.N. Berberan-Santos and M.J.E. Prieto (1987) J. Chem. Soc. Far. Trans. II 83, 1391—1409.
6 W. Lerslaner, J.E. Cain and J.K. Blasie (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 1499—1503.
7 J. Villalaín, J.C. Gómez-Fernández and M.J.E. Prieto (1988) J. Colloid Interface Sci. 124, 233—237.
8 F. Podo and J.K. Blasie (1977) Proc. Nat. Acad. Sci. U.S.A. 76, 1032—1036.
9 D. Fornasiero, F. Grieser and W.H. Sawyer (1988) J. Phys. Chem. 92, 2301—2305.
10 A.W. Eliasz, D. Chapman and D.F. Ewing (1976) Biochim. Biophys. Acta 448, 220—230.
11 M. McLaughlin, F. Podo and J.K. Blasie (1973) Biochim. Biophys. Acta 330, 109—121.

12  E.D. Becker (1980) High resolution NMR, Academic Press, New York, pp. 179—180.

13  E.L. Wehry (Ed.) (1976) Modern Fluorescence Spectroscopy, Vol. 2, Heyden, London, Chap. 3.

14  A.G. Lee (1976) Biochim. Biophys. Acta 448, 34—44.

15  J.C. Eriksson (1963) Acta Chem. Scand. 17, 1478—1481.

16  F. Podo and J.K. Blasie (1976) Biochim. Biophys. Acta 419, 1—18.

17  J.A. Marqusee and K.A. Dill (1986) J. Chem. Phys. 85, 434—444.

18  I.D. Campbell and R.A. Dwek (1984) Biological Spectroscopy, Benjamin/Cummings Publ. Co., London, Chap. 6.

19  J. Westman and L.E.G. Eriksson (1979) Biochim. Biophys Acta 557, 62—68.

20  U. Gösele (1977) Physics 85B, 317—322.

21  D.D. Thomas, W.F. Carlsen and L. Stryer (1978) Proc. Nat. Acad. Sci. U.S.A. 75, 5746—5750.

22  K. Kinosita, T. Kouyama and A. Ikeganni (1987) J. Chem. Phys. 86, 3273—3282.

23  H.L. Hinz and J.M. Sturtevant (1972) Chem. Phys. Lipids 12, 117—122.