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Fluorescent Probes of Biological Membranes

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Abstract. Fluorescent and phosphorescent probes that have readily interpretable emission properties can be specifically inserted into biological macromolecules to reveal facets of their structure and dynamics: (1) *Proximity.* Singlet–singlet and triplet–singlet energy transfer can serve as spectroscopic rulers in the 10–65 Å range, whereas triplet–triplet transfer can be used to show that two groups are less than about 12 Å apart. (2) *Rotational mobility.* Nanosecond fluorescence polarization measurements can reveal whether a macromolecular system has any modes of flexibility in times of nanoseconds. (3) *Polarity.* The presence of mobile dipoles in the environment of certain chromophores is reflected in their fluorescence quantum yield and emission spectrum.

We have synthesized a number of new fluorescent probes for biological membranes. Anthroyl stearic acid (I), dansyl phosphatidyl ethanolamine (II), and octadecyl naphthylamine sulfonic acid (III) are readily incorporated into bilayer vesicles composed of phosphatidyl choline. The emission spectra of these probes in the vesicles indicate that the chromophore of I is located in the hydrocarbon region, that of II is located in the glycerol layer, and that of III is located at the aqueous interface of the bilayer. Thus, fluorescent chromophores can be selectively placed in different transverse regions of a model membrane system.

Biological membranes contain highly organized molecular assemblies that mediate important processes such as energy transduction, active transport, nerve impulse conduction, sensory reception, and hormonal integration. The molecular structure and mechanism of action of membrane systems pose challenging problems that require a variety of incisive experimental approaches for their solution. There has been much recent interest in the use of fluorescence spectroscopy to study conformational changes in biological membranes.[1–7] Fluorescence techniques appear promising in this regard because the emission properties of many chromophores are responsive to the nature of their near and distant environments. In this article, we briefly review the use of fluorescence spectroscopy in revealing aspects of the structure and dynamics of biological macromolecules, namely proximity, rotational mobility, and polarity. We then describe the design, synthesis, and properties of a number of new fluorescent probes which are analogs of membrane lipids. These probes exhibit selective affinity for different transverse regions of phosphatidyl choline bilayers.

Spectroscopic Rulers. Under suitable conditions, electronic excitation energy can be transferred between chromophores separated by distances of the order of 35 Å.[8–10] The transfer is expressed in terms of a quenching of the fluorescence of the energy donor and a shortening of the lifetime of its excited state. The

energy acceptor exhibits a concomitant increase in its fluorescence intensity. Förster proposed that this kind of energy transfer occurs by a dipole–dipole resonance interaction of the donor and acceptor chromophores, and derived an explicit expression relating the transfer rate to geometric and spectroscopic factors.[11] Some essential features of Förster's theory have been verified by studies of singlet–singlet energy transfer in model systems in which an energy donor is separated from an energy acceptor by a molecular spacer of defined length: (1) the transfer rate depends on the inverse sixth power of the distance between the donor and acceptor;[9,10] (2) the transfer rate is proportional to the overlap of the emission spectrum of the energy donor and the absorption spectrum of the energy acceptor.[12] *These studies suggest that singlet–singlet and triplet–singlet energy transfer*[13] *can be used to deduce proximity relationships in the 10–65 Å range in biological macromolecules and organized molecular assemblies.*[9,14] Optimal use of this method requires that a single donor and a single acceptor chromophore be located at unique sites, as exemplified by a recent energy-transfer study of the tertiary structure of yeast phenylalanine transfer RNA.[15] For distances shorter than about 12 Å, the appropriate spectroscopic ruler is triplet–triplet energy transfer,[16] which is mediated by an exchange interaction that requires electron overlap and is expressed in terms of a sensitized phosphorescence.[17]

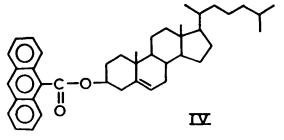
Rotational Mobility. Recent advances in the generation and detection of nanosecond light pulses have made it feasible to directly determine whether a macromolecule has any modes of flexibility in times of nanoseconds.[14,18] Excitation of a fluorescent chromophore on a macromolecule with a very short pulse of y-polarized light produces an ensemble of excited chromophores in which the transition moments are preferentially aligned along the y-direction. The macromolecules then undergo Brownian motion and their orientations become randomized. *The rate of randomization, and consequently the time-dependence of the emission anisotropy, depends on the size and shape of the macromolecule and also on its degree of flexibility during the excited state lifetime of the fluorescent chromophore.* Measurements of this kind have shown, for example, that chymotrypsin behaves as a rigid, compact particle in the nanosecond time interval.[14] In contrast, anti-dansyl antibody exhibits a significant degree of flexibility.[19]

Polarity. The emission properties of a variety of chromophores, such as 1-anilino-8-naphthalene sulfonate (ANS), are markedly sensitive to the polarity of their environment.[20–24] In going from water to a hexane-like environment, the fluorescence quantum yield of ANS increases from 0.004 to 0.98, and the emission maximum shifts from 515 to 454 nm.[21] The striking effect of solvent on the quantum yield of ANS is not yet adequately understood. On the other hand, the dependence of the emission maximum on solvent polarity can readily be interpreted in terms of a reorientation of the solvent shell around the chromophore when it is in a polar medium.[25] The more dipolar excited state of ANS interacts with a polar solvent so as to further align the solvent dipoles, resulting in the emission of a photon of lower energy. The dipoles in the vicinity of the chromophore must be mobile in times of the order of a nanosecond for this effect to occur.[26] Thus the emission spectrum of the chromophore reflects the dynamic polarity of its environment.

Design of Membrane Lipid Probes. Fluorescence techniques are most informative when responsive chromophores are specifically located at defined sites of interest. There are a number of methods for the specific insertion of fluorescent chromophores into proteins, some of which should be applicable in labeling membrane-bound proteins. The high reactivity of catalytic residues at the active sites of enzymes can be exploited, as in the formation of a fluorescent anthraniloyl derivative of chymotrypsin.[27] Advantage can be taken of specific noncovalent interactions, as in the binding of chromophoric sulfonamides to the zinc atom at the active site of carbonic anhydrase,[28] or of fluorescent haptens to the combining sites of specific antibody.[29] Furthermore, fluorescent maleimides and aziridines react preferentially with sulfhydryl residues in proteins.[30] In contrast, there are no known methods for the specific covalent attachment of fluorescent chromophores to lipid regions of biological membranes. Thus, techniques need to be devised for the specific labeling of lipid regions if fluorescence methods are to be used to fullest advantage in studies of biological membranes.

We have approached this problem by synthesizing a number of fluorescent analogs of membrane lipids. The aim has been to prepare fluorescent probes which will sample different transverse regions of the lipid bilayer structure shown in Fig. 1. Several criteria entered into the design of these probes, which consist of a fluorescent chromophore attached to a lipid moiety (Fig. 2). (1) The lipid portions were chosen to have high affinity for bilayer regions. Each probe has a long alkyl chain to promote its interaction with the phospholipid molecules in the bilayer. (2) The probes have distinctly nonpolar and polar regions to orient them with respect to the inside and outside of the bilayer. The chromophoric part of each probe is then expected, on the basis of steric factors, to be located in a specific transverse region of the bilayer. (3) The emission spectra of the chromophores are responsive to the polarity of their environment.

We report here the synthesis and properties of 12-(9-anthroyl)-stearic acid (I), *N*-dansyl phosphatidyl ethanolamine (II), and *N*-octadecyl naphthyl-2-amine 6-sulfonic acid (II). These probes were incorporated into phosphatidyl choline vesicles[31] which are known to have the bilayer structure depicted in Fig. 1. The locations of the chromophoric portions of these probes in the phosphatidyl choline bilayers were inferred from their emission properties.



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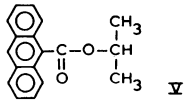

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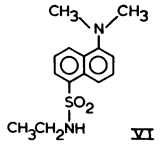
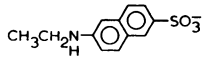


VI



VII

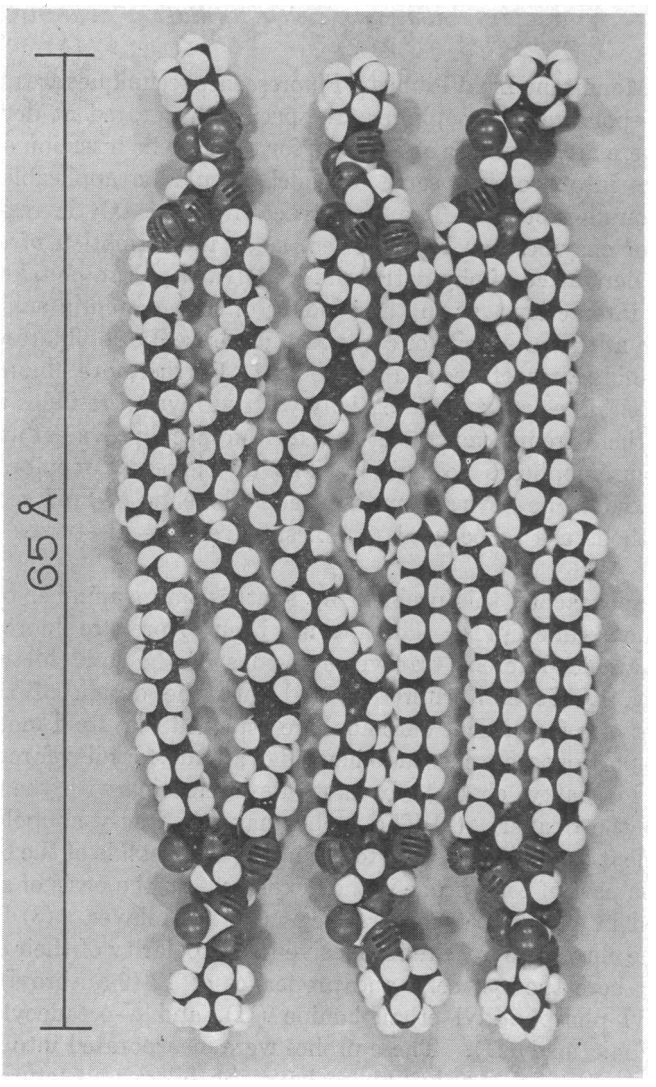
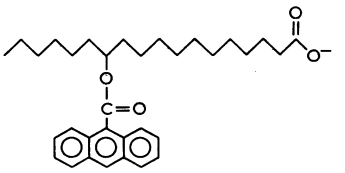


FIG. 1. Molecular model of a phosphatidyl choline bilayer.

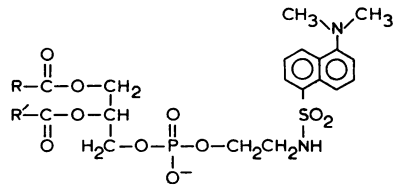
The role of the lipid moiety in determining the position of the chromophore was evaluated by studying the interactions of short-chain derivatives (V, VI, VII) of the fluorescent groups with the phosphatidyl choline vesicles.

Materials and Methods. 12-(9-Anthroyl)-stearic acid (AS, I): A dry benzene solution containing 4 g (13 mmol) of 12-hydroxystearic acid and 4 g (17 mmol) of anthracene-9-carboxylic acid chloride was refluxed 4 hr. After cooling, a yellow precipitate was discarded and the solvent was removed under reduced pressure. The product was eluted from a silica gel column with mixtures of chloroform and hexane. Recrystallization yielded white crystals which melted at 78–79°C (uncorrected). The absorption maxima in ethanol were at 381 (7540), 361 (8370), 344 (5680), and 328 nm (2850 M^{-1} cm^{-1}). *Analysis.* Calculated for $C_{33}H_{44}O_4$: C, 78.5; H, 8.8%. Found: C, 78.4; H, 9.0%.

Cholesteryl ester of 9-Anthroic acid, (IV) was synthesized from anthracene-9-



I



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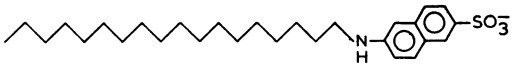

III

FIG. 2. Fluorescent probes designed to sample different regions of phospholipid bilayers. I, anthroyl stearic acid (AS); II, dansyl phosphatidyl ethanolamine (DPE); III, octadecyl naphthylamine sulfonate (ONS). R and R′ in II are long-chain fatty acid residues.

carboxylic acid chloride and cholesterol by the method of Latt, Cheung, and Blout.[8] The white needles melted at 201–203°C (uncorrected; literature[8] mp 201–202°C). The absorption maxima in ethanol were at 381 (7580), 361 (8500), 344 (5680), and 328 nm (2840 M^{-1} cm^{-1}).

Isopropyl ester of 9-Anthroic acid (V): A mixture of 1.0 g of anthracene-9-carboxylic acid chloride in 5 ml of isopropyl alcohol was refluxed for 5 hr, cooled, and washed with 0.1 N $NaHCO_3$. The solvent was removed and the residue taken up in petroleum ether for recrystallization. The product, a white crystalline material with a melting point at 99°C (uncorrected), had absorption peaks in ethanol at 381 (7230), 361 (8140), 344 (5560), and 328 (2790 M^{-1} cm^{-1}). *Analysis.* Calculated for $C_{18}H_{16}O_2$: C, 81.8; H, 6.1%. Found: C, 82.2; H, 6.2%.

***N*-dansyl phosphatidyl ethanolamine (DPE, II):** A solution of 140 mg of phosphatidyl ethanolamine (prepared from hen egg yolk[32]) in chloroform was added to 55 mg of dansyl chloride and 0.3 ml of triethylamine in 1.0 ml of chloroform at room temperature. After several hours, the product was eluted from a column, consisting of Silicar CC7 and Celite, with a mixture of chloroform and methanol. The purified dansyl phosphatidyl ethanolamine was stored in chloroform at −20°C. *Analysis:* Calculated for an estimated molecular weight of 1000: N, 2.93; S, 3.36%. Found: N, 2.98; S, 3.40%.

***N*-dansyl ethylamine (VI):** Dry monoethylamine was bubbled for 10 min into 20 ml of chloroform containing 1.0 g of dansyl chloride at room temperature. After several hours the chloroform solution was washed several times with dilute aqueous HCl to remove unreacted amine. The chloroform solution was dried with Na_2SO_4 and the solvent was removed. The green residue was recrystallized from diethyl ether to give yellow-green crystals which melted at 136–138°C (uncorrected). The material had an absorption maximum in ethanol at 334 nm (4600 M^{-1} cm^{-1}). *Analysis.* Calculated for $C_{14}H_{18}N_2SO_2$: C, 60.4; H, 6.5; N, 10.1. Found: C, 60.2; H, 6.6; N, 9.9.

***N*-octadecyl naphthyl-2-amine 6-sulfonic acid (ONS, III):** An aqueous solution (200 ml) of 12 g (50 mmol) of 2,6-naphthylamine sulfonic acid (sodium salt), 40 g (400 mmol) of sodium bisulfite, and 30 g (110 mmol) of octadecylamine was stirred and refluxed for 20 hr. After cooling, the precipitate was filtered and a portion was dissolved in 50 ml of boiling methanol. Upon addition of 5 ml of concd HCl the octadecylamine

salt of the product precipitated and was immediately filtered from the hot solution. The triethylamine salt of the product was formed by boiling the octadecylamine salt in a hexane solution containing 10% triethylamine. The triethylamine salt, which is insoluble in hexane, was filtered. The sulfonic acid was then formed by washing a chloroform solution of the triethylamine salt three times with 2.5 N aqueous HCl. The *N*-octadecyl naphthylamine sulfonic acid precipitated and was crystallized from chloroform–methanol 2:1 containing a trace of water. The sulfonic acid decomposed near 290°C and had an absorption maximum in methanol at 349 nm (950 M^{-1} cm^{-1}). *Analysis.* Calculated for $C_{28}H_{45}NSO_3$: C, 70.7; H, 9.5; N, 2.9; S, 6.7%. Found: C, 70.4; H, 9.4; N, 2.7; S, 6.7%.

***N*-ethyl naphthyl-2-amine 6-sulfonic acid (VII):** Monoethylamine was slowly bubbled for 20 min into a refluxing aqueous solution (150 ml) containing 10 g (40 mmol) of 2,6-naphthylamine sulfonic acid (sodium salt) and 30 g (300 mmol) of sodium bisulfite. After 20 hr of refluxing, excess amine was removed by steam distillation. The solution was cooled and filtered. Crystallization of the precipitate from methanol–water–concd HCl 1:1:0.1 yielded clear plates which turned brown near 290°C. The absorption maximum in methanol was at 349 nm (738 M^{-1} cm^{-1}). *Analysis.* Calculated for $C_{12}H_{13}NSO_3$: C, 57.3; H, 5.2; N, 5.6; S, 12.7%. Found: C, 53.2; H, 5.7; N, 4.7; S, 12.1%.

Formation of phosphatidyl choline vesicles:[31] Phosphatidyl choline was prepared from hen egg yolk.[32] An aliquot of a chloroform solution containing the fluorescent probe was added to 10 mg of phosphatidyl choline in chloroform. The molar ratio of fluorescent probe to phosphatidyl choline was 1:50. A 0.1% aqueous dispersion consisting of the phosphatidyl choline and probe was formed by adding 10 ml of buffer and swirling the mixture. The buffer and solution used throughout this work was 0.1 M NaCl, 0.01 M Tris·HCl at pH 8.5. The dispersion was sonicated for 1 hr under nitrogen in a stainless steel centrifuge tube immersed in a well-stirred ice bath. The power of the Branson Sonifier (Model W185D) was set at 4. The sonicated mixture was centrifuged at 100,000 $\times$ *g* for 30 min to give a sufficiently clear solution for fluorescence spectroscopy.

Fluorescence spectroscopy: Fluorescence emission spectra were obtained on a recording spectrofluorimeter described previously.[21] The emission spectra and maxima have not been corrected for the variation with wavelength in the sensitivity of the detection system. The relative sensitivity of the instrument is 2.33 at 420 nm, 1.92 at 440 nm, 1.60 at 460 nm, 1.30 at 480 nm, 1.03 at 500 nm, 0.80 at 520 nm, and 0.62 at 540 nm. The excitation wavelength was 330 nm.

Nanosecond emission kinetics were measured using a single-photon counting apparatus described in detail by Yguerabide, Epstein, and Stryer.[19] The exciting light pulse had a measured full width at half-height at 2.5 nsec. The finite duration of the exciting light pulse was taken into account by means of a convolution integral in deriving excited-state lifetimes from the observed emission kinetics.[33] Nanosecond data were accumulated in the pulse height analyzer until the peak channel contained about 25,000 counts. Solutions of chromophores in organic solvents were deoxygenated by bubbling nitrogen into the cuvet for at least 10 min; the lifetime was not lengthened on further flushing with nitrogen. All spectral measurements were performed at 22 $\pm$ 1°C.

Results. Anthroyl stearic acid (AS, I): The fluorescence emission spectrum of AS is markedly dependent on the polarity of the solvent (Fig. 3*a*). The emission maximum (λ_{max}) shifts from 444 nm in hexane to 464 nm in methanol. The fluorescence emission spectrum of AS in phosphatidyl choline vesicles is shown in Fig. 3*b*. The position of the emission maximum indicates that *the environment of the anthroyl chromophore in the phosphatidyl choline bilayer is distinctly nonpolar.* The λ_{max} of AS in the phosphatidyl choline vesicle is intermediate between that of AS in hexane and benzene (Fig. 4). If the emission spectra of IV and V in various organic solvents are taken as the standard[34] (Fig. 4), the degree of polarity is like that of benzene.

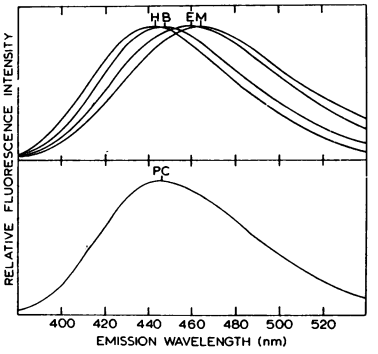

FIG. 3. (*a*) Fluorescence emission spectra of 2.7×10^{-5} M AS in hexane (H), benzene (B), ethanol (E), and methanol (M). (*b*) Emission spectrum of 2.7×10^{-5} M AS in phosphatidyl choline vesicles (PC) in aqueous buffer. The emission spectra are normalized to the same peak intensity.

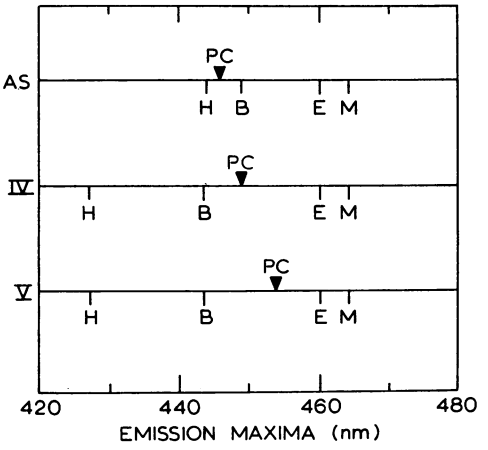

FIG. 4. Position of the fluorescence emission maxima of 2.7×10^{-5} M AS, IV, and V in hexane (H), benzene (B), ethanol (E), methanol (M), and phosphatidyl choline vesicles (PC) in aqueous buffer.

The nanosecond emission kinetics of the anthroyl chromophore (Fig. 5) are also highly informative because of their sensitivity to solvent polarity. As the solvent polarity decreases in going from methanol to hexane, the excited-state lifetime of AS increases from 1.6 to 8.5 nsec. In the phosphatidyl choline vesicle, AS has a lifetime of 12.4 nsec. Thus, the fluorescence emission kinetics also reveal that the anthroyl group of AS is located in a nonpolar region of the

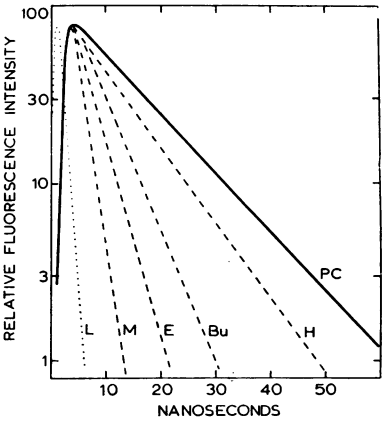

FIG. 5. Nanosecond emission kinetics of 2.7×10^{-5} M AS in deoxygenated methanol (M), ethanol (E), butanol (Bu), hexane (H), and phosphatidyl choline vesicles (PC) in air-equilibrated aqueous buffer, following excitation by a nanosecond light pulse (L). The decay curves are normalized to the same peak intensity. The excited-state lifetimes are 1.6 (M), 3.4 (E), 5.6 (Bu), 8.5 (H), and 12.4 (PC) nsec. The lifetimes of IV and V are similar to those of AS.

phosphatidyl choline bilayer. Indeed, the long lifetime of AS in the vesicles, which is greater than that of AS in hexane, indicates that the excited state of the anthroyl group in the bilayer is more protected against quenching processes than even in hydrocarbon solvents.

The hydroxystearic acid moiety was replaced by cholesterol (IV) or isopropanol (V) to determine whether substituents affect the location of the anthroyl chromophore in the bilayer. The emission spectra of IV, V, and AS in phosphatidyl choline vesicles are appreciably different (Fig. 4). In the vesicles, the polarity of the environment of the anthroyl group decreases in going from V (where the substituent is an isopropyl group) to IV (where the substituent is cholesterol) to anthroyl stearic acid. Thus the stearic acid moiety has a significant effect in specifying the location of the anthroyl chromophore of AS in the phosphatidyl choline bilayer.

Dansyl phosphatidyl ethanolamine (DPE, II): The λ_{max} of DPE varies from 443 nm in hexane to 514 nm in methanol (Fig. 6). The emission maximum of

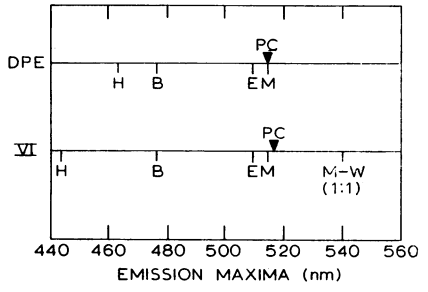

FIG. 6. Position of the fluorescence emission maxima of 2.7×10^{-5} M DPE and VI in hexane (H), benzene (B), ethanol (E), methanol (M, a 1:1 mixture of methanol and aqueous buffer (M-W, 4×10^{-4} M) chromophore, and phosphatidyl choline vesicles (PC) in aqueous buffer. The excited-state lifetime of DPE in the vesicles is 12.2 nsec.

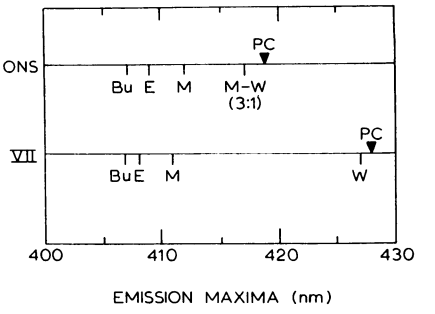

FIG. 7. Position of the fluorescence emission maxima of 2.7×10^{-5} M ONS and VII in butanol (Bu), ethanol (E), methanol (M), a 3:1 mixture of methanol and aqueous buffer (M-W), aqueous buffer (W), and phosphatidyl choline vesicles (PC) in aqueous buffer. The excited-state lifetime of ONS in the vesicle is 12.7 nsec.

DPE in phosphatidyl choline vesicles is at 515 nm, which corresponds closely to that of DPE or VI in methanol. Thus, the environment of the dansyl chromophore in the phosphatidyl choline bilayer is quite polar, similar to that of methanol.

The emission spectrum of VI in phosphatidyl choline vesicles is nearly the same as that of DPE. This finding suggests that the location of the dansyl chromophore in the vesicles is largely determined by the properties of the dansyl group itself, rather than by the phospholipid moiety.

Octadecyl naphthylamine sulfonic acid (ONS, III): The λ_{max} of the chromophore shifts from 407 nm to 427 nm in going from butanol to water (Fig. 7). In the phosphatidyl choline vesicles, the emission maximum of ONS is at 419 nm, close to that observed for the chromophore in a 3:1 mixture of methanol and

water. Thus, the environment of the naphthylamine sulfonate chromophore in the phosphatidyl choline bilayer is polar, intermediate between that of methanol and water.

The length of the hydrocarbon substituent has a significant effect on the position of the naphthylamine sulfonate chromophore. In the phosphatidyl choline vesicles, the chromophore in VII (the ethylamine derivative) exhibits an emission maximum characteristic of an aqueous environment (Fig. 7). This finding suggests either that VII is not firmly bound to the vesicle, or that the chromophore in VII occupies a more external position in the bilayer than does ONS. Thus, the C_{18} hydrocarbon chain anchors the chromophore to the bilayer.

Discussion. The emission properties of the probes in phosphatidyl choline vesicles show that the fluorescent group of AS is in a benzene-like environment, that of DPE is in a methanol-like environment, and that of ONS is in an environment which has a polarity intermediate between that of methanol and water. It is evident that the chromophoric portions of these probes sample different transverse regions of the phosphatidyl choline bilayer. *We infer that the fluorescent moieties of AS, DPE, and ONS probe the hydrocarbon, glycerol, and aqueous interface regions, respectively, of the bilayer membrane, as shown in Fig. 8.*

The interaction of these probes with biological membranes will almost certainly be more complex than with the phosphatidyl choline model system. The first problem will be to introduce these probes, which are quite insoluble in water, into the biological membrane. One way in which this might be accomplished is to disperse the probe in phospholipid vesicles and allow it to diffuse into the intact membrane system. This process might be facilitated by the presence of protein factors that have been shown to catalyze the exchange of phospholipids.[35] Another possible means of introducing the fluorescent probe into some biological membranes is by biosynthetic incorporation, as has been accomplished for a spin-labeled fatty acid similar in structure to AS.[36] A second potential problem in using these fluorescent probes in more complex systems is that they may bind to numerous sites. It will be of interest to see whether probes like AS, DPE, and ONS exhibit a preferential affinity for the lipid component of membranes which are also rich in protein.

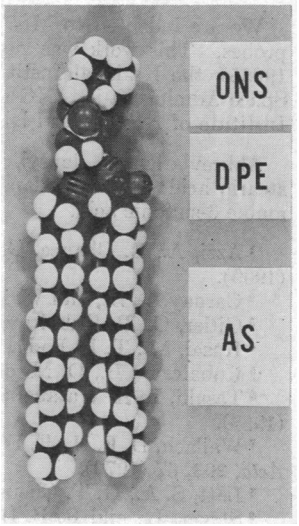

FIG. 8. Proposed locations of the chromophoric portions of ONS, DPE, and AS in the phosphatidyl choline bilayer.

The present studies provide a rationale for the design of new fluorescent membrane probes. The experimental strategy is to attach a fluorescent chromophore to a long-chain lipid which serves to anchor the probe in the bilayer regions, orients it with respect to the inside and outside, and places it at a defined transverse location. The differences in position of the chromophoric portion of AS compared to that of IV and V, and of ONS relative to that of VII, illustrate the contribution of the

lipid part in determining the location of the fluorescent moiety. However, the polarity of the chromophoric portion of the fluorescent lipid must also be considered in relation to the polarity of the region to be probed. For example, the dansyl group of ω-dansyl-heptadecanoic acid is *not* located in the hydrocarbon region of phosphatidyl choline vesicles, as might be expected solely on the basis of the likely orientation and position of the fatty acid moiety.

The probes presented here will probably be useful in nanosecond fluorescence polarization studies of rotational motions in biological membranes, since their lifetimes of about 12 nsec are appropriate for this purpose. Second, the probes absorb in the spectral region of tryptophan emission, and consequently they can serve as acceptors in energy-transfer experiments which seek to establish proximity relationships. Third, molecular models suggest that the transition moments of AS and ONS may be parallel to the direction of the hydrocarbon chain in the bilayer. If so, emission dichroism studies would be informative in revealing changes in the orientation of the lipid region. Finally, we note that membrane probes that are sensitive to pH, redox potential, and the concentration of specific ions such as Ca^{++} might be synthesized using the principles described in this paper.

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Abbreviations: dansyl, 5-dimethylaminonaphthalene 1-sulfonyl; AS, 12-(9-anthroyl)-stearic acid; DPE, *N*-dansyl phosphatidyl ethanolamine; ONS, *N*-octadecyl naphthyl-2-amine 6-sulfonic acid.

[1] Azzi, A., B. Chance, G. K. Radda, and C. P. Lee, *Proc. Nat. Acad. Sci. USA*, **62**, 612 (1969).
[2] Carnay, L. D., and W. H. Barry, *Science*, **165**, 608 (1969).
[3] Gitler, C., B. Rubalcava, and A. Caswell, *Biochim. Biophys. Acta*, **193**, 479 (1969).
[4] Kasai, M., T. R. Podleski, and J. P. Changeux, *FEBS Lett.*, **7**, 13 (1970).
[5] Rubalcava, B., D. M. deMuñoz, and C. Gitler, *Biochemistry*, **8**, 2742 (1969).
[6] Tasaki, I., A. Watanabe, R. Sandlin, and L. Carney, *Proc. Nat. Acad. Sci. USA*, **61**, 883 (1968).
[7] Wallach, D. F. H., E. Ferber, D. Selin, E. Weidekamm, and H. Fischer, *Biochim. Biophys. Acta*, **203**, 67 (1970).
[8] Latt, S. A., H. T. Cheung, and E. R. Blout, *J. Amer. Chem. Soc.*, **87**, 995 (1965).
[9] Stryer, L., and R. P. Haugland, *Proc. Nat. Acad. Sci. USA*, **61**, 883 (1968).
[10] Bücher, H., K. H. Drexhage, M. Fleck, H. Kuhn, D. Möbius, F. P. Schäfer, J. Sondermann, W. Sperling, P. Tillman, and J. Wiegand, *Mol. Cryst.*, **2**, 199 (1967).
[11] Förster, T., in *Modern Quantum Chemistry—Istanbul Lectures*, Section IIIB, pp. 93–137, edited by Sinanoglu O. (Academic Press, 1965).
[12] Haugland, R. P., J. Yguerabide, and L. Stryer, *Proc. Nat. Acad. Sci. USA*, **63**, 23 (1969).
[13] Galley, W. C., and L. Stryer, *Biochemistry*, **8**, 1831 (1969).
[14] Stryer, L., *Science*, **162**, 526 (1968).
[15] Beardsley, K., and C. R. Cantor, *Proc. Nat. Acad. Sci. USA*, **65**, 39 (1970).
[16] Galley, W. C., and L. Stryer, *Proc. Nat. Acad. Sci. USA*, **60**, 108 (1968).
[17] Ermolaev, V. L., *Soviet. Phys. Usp.*, **6**, 333 (1963).
[18] Wahl, P., *Compt. Rend.*, **263D**, 1525 (1966).
[19] Yguerabide, J., H. F. Epstein, and L. Stryer, *J. Mol. Biol.*, **51**, in press (1970).
[20] Weber, G., and D. J. R. Laurence, *Biochem. J.*, **56**, 31P (1954).
[21] Stryer, L., *J. Mol. Biol.*, **13**, 482 (1965).
[22] McClure, W. O., and G. M. Edelman, *Biochemistry*, **5**, 1908 (1966).

[23] Turner, D. C., and L. Brand, *Biochemistry*, **7**, 3381 (1968).
[24] LePecq, J. B., and C. Paoletti, *J. Mol. Biol.*, **27**, 87 (1967).
[25] Lippert, E., *Z. Elektrochem. Z.*, **61**, 962 (1957).
[26] Ware, W. R., P. Chow, and S. K. Lee, *Chem. Phys. Lett.*, **2**, 356 (1968).
[27] Haugland, R. P., and L. Stryer, in *Conformation of Biopolymers*, Vol. 1, pp. 321–335, edited by Ramachandran, G. N. (New York: Academic Press, 1967).
[28] Chen, R. F., and J. C. Kernohan, *J. Biol. Chem.*, **242**, 5813 (1967).
[29] Parker, C. W., T. J. Yoo, M. C. Johnson, and S. M. Godt, *Biochemistry*, **6**, 3408 (1967).
[30] Haugland, R. P., Ph.D. thesis, Stanford University (1970).
[31] Huang, C., *Biochemistry*, **8**, 344 (1969).
[32] Singleton, W. S., M. S. Gray, M. L. Brown, and J. L. White, *J. Amer. Oil Chem. Soc.*, **42**, 53 (1965).
[33] Hundley, L., T. Coburn, E. Garwin, and L. Stryer, *Rev. Sci. Instrum.*, **38**, 488 (1967).
[34] The emission spectra of IV, V, and AS are virtually superposable in either methanol or ethanol. However, in hexane and benzene, two differences are evident: (*a*) λ_{max} of IV and V are at shorter wavelengths than those of AS (Fig. 4); (*b*) the emission spectra of VI and V exhibit fine structure, whereas that of AS is structureless. The methyl ester of AS, in contrast to AS, has emission properties like those of IV and V. The anomalous emission properties of AS in hexane and benzene probably arise from an interaction of the anthroyl chromophore with the carboxyl group.
[35] Wirtz, K. W. A., and D. B. Zilversmit, *Biochim. Biophys. Acta*, **193**, 105 (1969).
[36] Keith, A. D., A. S. Waggoner, and O. H. Griffith, *Proc. Nat. Acad. Sci. USA*, **61**, 819 (1968).