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CORRELATIONS BETWEEN STRUCTURE AND SPECTROSCOPIC PROPERTIES IN MEMBRANE MODEL SYSTEMS TRYPTOPHAN AND 1-ANILINO-8-NAPHTHALENE SULFONATE FLUORESCENCE IN PROTEIN–LIPID–WATER PHASES

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SUMMARY

The general aim of our current work is to calibrate spectroscopic techniques, useful for the structural analysis of biological membranes, against model systems of well-defined structure. In this study 1-anilino-8-naphthalene sulfonate (ANS) and tryptophan fluorescence and X-ray diffraction analyses were carried out on a variety of protein–lipid–water phases. The fluorescence parameters—emission spectrum, quantum yield and efficiency of energy transfer—were found to display a remarkable correlation with the structure of the phases, and with the nature of the two main types of protein–lipid interactions, namely electrostatic and hydrophobic. The sites of fixation of ANS in the different phases are discussed.

INTRODUCTION

A great emphasis is placed in many quarters on the use of spectroscopic techniques as tools for the structural analysis of biological membranes. Nevertheless the conclusions drawn from optical rotatory dispersion (ORD), circular dichroism (CD) and fluorescence experiments are so confusing and controversial that the attempt to put forward structural interpretations of spectroscopic data seems premature in systems as complex as biological membranes. We believe that spectroscopic studies of model systems of well-defined structure, focusing on the interactions of proteins, lipids and water, may provide insight into the correlation between spectral and structural parameters relevant to the problem of membranes. We have recently described[1] the structure of a variety of protein–lipid–water phases and the structure dependence of the CD spectra associated with the aromatic and the heme chromophores of the protein molecules. We report here on the fluorescence properties of the same phases (and of some lipid–water phases) associated with intrinsic and extrinsic chromophores—tryptophan and 1-anilino-8-naphthalene sulfonate (ANS). More precisely we have determined the emission spectrum and quantum yield of ANS fluorescence,

Abbreviations: ORD, optical rotatory dispersion; CD, circular dichroism; ANS, 1-anilino-8-naphthalene sulfonate.

which are dependent upon the polarity of the environment of ANS[2]. We have also examined the quenching of tryptophan fluorescence by ANS and the efficiency of energy transfer from tryptophan to ANS, both of which are functions of the orientation and distance between the two chromophores[3]. We show that these parameters display remarkable correlations with the structure of the phases, and more specifically with the nature of the protein–lipid interactions—namely electrostatic and hydrophobic.

EXPERIMENTAL

The sources of lipids and protein, as well as the preparation of the phases, are described in our previous article[1]. The concentration of ANS was kept low, in order to avoid perturbations of the structure of the phases. The partition coefficient K (the ratio of ANS concentration between the lipid-containing phase and water) was determined by dispersing the phase in excess water, in the presence of a fixed amount of ANS, separating the phase by centrifugation, and determining ANS concentrations in the phase and in water. In all cases, K was found to be independent of ANS concentration in water in the range $2 \cdot 10^{-5} < [\text{ANS}] < 8 \cdot 10^{-4}$. The values of K are given in Table II.

Absorption measurements were carried out with a Cary 14 spectrophotometer. The fluorimeter used in this work was described elsewhere[4]. The spectroscopic study was made on pure nonturbid phases contained in a sample holder formed by two fused silica plates separated by spacers of variable thickness. The sample was oriented at 45° of the incident beam and the fluorescence measured at 90°. Due to the high concentration of the absorbing material all the experiments were performed on very thin preparations (a few micra thick).

The contribution of each component to the total absorption was determined as follows (see Fig. 1): (a) the protein contribution was determined from the absorption

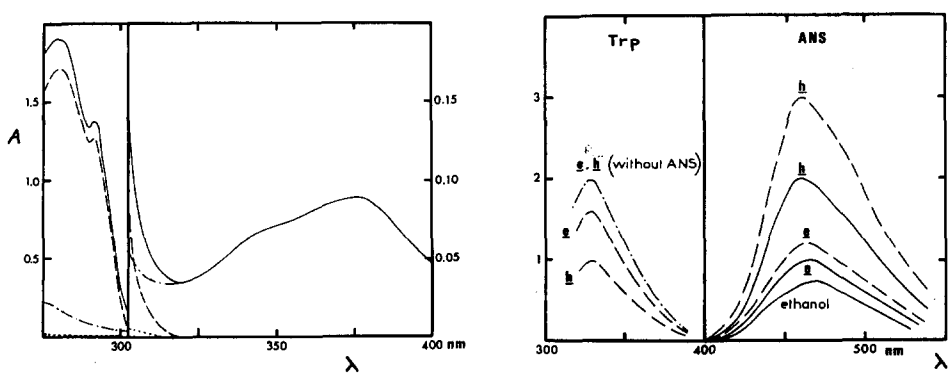

Fig. 1. Absorption spectra of the lamellar phase type *e* of the system lysozyme–cardiolipin–water (see text, Table II and Fig. 5). ———, absorbance of the phase. Partial absorbance of the components: — — —, lysozyme; -------, cardiolipins; –·—·–, ANS.

Fig. 2. Emission spectra of tryptophan and ANS in the lamellar phases type *e* and *h* of the system lysozyme–cardiolipin–water (see text, Table II and Fig. 5) and of ANS solution in ethanol (1 mM). Ordinates: corrected fluorescence intensities divided by the absorbance of the chromophore at the excitation wavelength. ———, spectra excited at 320 nm, where only ANS absorbs (see Fig. 1); –·—·– and — — —, spectra excited at 305 nm, where both ANS and lysozyme absorb (see Fig. 1). Note that ANS fluorescence is more intense if the excitation wavelength is 305 rather than 320 nm, and that tryptophan fluorescence is quenched by ANS.

at 280 nm (at this wavelength the contributions of lipids and ANS are small, and can be easily calculated); (b) the lipid contribution was calculated from the lipid content of the phase; (c) the contribution of ANS was determined either by subtraction of the protein and lipid contributions from the total absorption, or directly from absorption in the range 320–400 nm where only ANS was found to absorb (Fig. 1) and extrapolating to lower wavelengths using the known absorption of ANS in solvents of different polarities.

The quantum yield of ANS fluorescence was determined by excitation at different wavelengths between 320 and 400 nm (see Fig. 2) and absolute values were obtained by comparing the integrated fluorescence energy to that observed in ethanol ($\lambda^E_{max} = 468$ nm, quantum yield = 0.37) or in butanol ($\lambda^E_{max} = 464$ nm, quantum yield = 0.56) (see ref. 2). The fluorescent parameters involving tryptophan were determined by excitation in the range 300–320 nm—under our experimental conditions the absorption of the protein is too high at its maximum (280 nm). At 300–320 nm both the protein and ANS absorb significantly; the absorption of the lipid is smaller, but it was taken into account as well (see Fig. 1). The degree of quenching was calculated by comparing the intensity of tryptophan fluorescence in the presence and in the absence of ANS. The efficiency of energy transfer was determined from the enhancement of ANS fluorescence as compared to that expected in the absence of transfer (Fig. 2).

RESULTS

Lysozyme–water phase

Several fluorescence parameters were measured with solutions of lysozyme in water, to which ANS was added at the molar ratio of 1:2.5, similar to the ratio adopted in the protein–lipid–water phases. The results are given in Table I. In dilute solutions both the spectrum and the quantum yield of ANS fluorescence are similar to those observed in water. As the protein concentration increases, λ^E_{max} is shifted to the blue and the quantum yield increases; at the same time the efficiency of the energy transfer (and concurrently the quenching of tryptophan fluorescence) increase.

TABLE I

FLUORESCENT PARAMETERS OF LYSOZYME IN WATER SOLUTIONS

Abbreviations: c_p, lysozyme weight concentration; λ^E_{max}, wavelength of the maximum of the emission spectrum; ET, efficiency of energy transfer from tryptophan to ANS.

c_p	*Quantum yield (ANS)*	λ^E_{max} *(ANS) (nm)*	*Fluorescence quenching (Trp) (%)*	*ET (%)*
0.005	0.005	510	0	0
0.01	0.007	500	0	0
0.05	0.05	475	< 10	< 10
0.25	0.13	472	30	30
0.50	0.15	471	50	50
0.75	0.20	470	70	70

Furthermore no circular dichroism was found to be associated with the ANS absorption bands at any protein concentration. These results confirm and extend previous observations[2] that lysozyme displays a small affinity for ANS. Indeed it should be noted that with other proteins, which strongly bind ANS, the quantum yield is high even at low protein concentration[2,5], and a strong circular dichroism is induced in the absorption band of ANS[6].

It may thus be concluded that in dilute lysozyme solutions ANS preferentially remains embedded in water, and that as the amount of water decreases the ANS molecules are somehow forced into a microenvironment of low dynamic polarity, perhaps some hydrophobic niche on or near to the surface of the protein. The high values of the efficiency of the energy transfer are consistent with the vicinity of the ANS molecules to the tryptophan residues[2,3].

Lipid–water phases

A study of the fluorescence of ANS incorporated into a variety of lipid–water phases is in progress in this laboratory. A few preliminary results obtained with the lamellar phase (Lα) are relevant to the protein–lipid–water systems. The phase Lα consists of stacked and equidistant lipid lamellae, separated by water layers[7]. The lipid lamellae are filled by liquid hydrocarbon chains, and the water–hydrocarbon interface is covered by the polar groups of the lipid molecules; the thickness (d_1) of the lipid layer and the area (S) available to each polar group on the interface can be determined[7]. We have shown previously[8] that when the lipid molecules bear a net

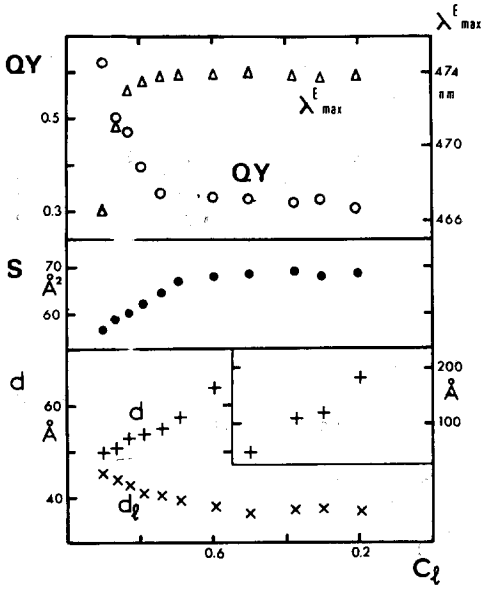

Fig. 3. Phase Lα of the lipid–water system containing 90% lecithin and 10% phosphatidic acid with added ANS ([lipid]/[ANS] = 50). Repeat distance (d), thickness of the lipid lamellae (d_1), area per polar group at the lipid–water interface (S), quantum yield (QY) and maximum of emission spectrum (λ^E_{max}) of ANS fluorescence, as a function of the lipid concentration (c_1). Note that as c_1 decreases d_1 and QY decrease, S and λ^E_{max} increase, in the range $c>0.7$; at $c<0.7$ all the parameters become constant.

TABLE II

X-RAY DIFFRACTION AND FLUORESCENCE PARAMETERS OF VARIOUS PHASES

Nomenclature of the phases: lipid–water systems, Lα lamellar; protein–lipid–water systems: L*e*, lamellar type *e*; L*h*, lamellar type *h*; $_2$H, two-dimensional hexagonal; $_2$T, two-dimensional square. c_p and c_l are the weight concentrations of protein and lipid; d is the repeat distance of the lamellar phases; d_p and d_l are the partial thickness of the protein and lipid lamellae (see ref. 1). K is the partition coefficient of ANS between the phase and water. Other notations as in Table I. In all the phases containing lysozyme the molar ratio of lysozyme to ANS is 2.5:1.

System	*Phase*	c_p	c_l	d (Å)	d_p (Å)	d_l (Å)	$\frac{[Lipid]}{[ANS]}$	K	*Quantum yield (ANS) ± 0.05*	λ^E_{max} *(ANS) (nm)*	*Fluorescence quenching (Trp) (%) ± 10*	*ET (%) ± 10*
Cardiolipin–water	Lα		0.70	50.0		35.0	40	9	0.20	470		
Lysozyme–cardiolipin–water	L*e*	0.38	0.34	92.0	29.0	34.8	45	65	0.50	461	20	10
	L*h*	0.52	0.31	77.0	35.0	27.0	30	190	0.95	458	50	50
Phosphatidyl inositol–water	Lα		0.70	56.5		39.5	40	9	0.30	468		
Lysozyme–phosphatidyl inositol–water	L*e*	0.33	0.35	106.0	29.5	39.5	45	45	0.60	460	25	20
	L*h*	0.42	0.39	74.0	27.0	32.5	40	180	0.95	458	50	45
	$_2$H	0.51	0.26				30	220	0.95	458	40	35
	$_2$T	0.38	0.35				45	280	0.95	458	30	30

electrical charge, the thickness of the water layer can be increased almost indefinitely; as the amount of water is increased, d_1 decreases and S increases slightly at first, and then becomes constant when the water content reaches approx. 30 % (see Fig. 3).

ANS was incorporated into the Lα phase of a variety of lipid–water systems, at 25°. The partition coefficient is strongly dependent on the electrical charge of the lipid since ANS is negatively charged. So with cardiolipin and phosphatidyl inositol, which are negatively charged, $K = 9$ (see Table II); for egg lecithin, devoid of net electrical charge, $K = 250$. Furthermore if the electrical charges of cardiolipin are neutralized with Ca^{2+} the value of K is raised from 9 to 90.

X-ray diffraction and fluorescent studies were carried out at the molar ratio of lipid to ANS of 50:1, as a function of the water content. Under these conditions cardiolipin and phosphatidyl inositol yield the Lα phase only at $c_1 < 0.7$ with d_1 and S independent of c_1. At higher lipid concentrations other phases are present. With a mixture of egg lecithin and phosphatidic acid (90:10, by wt.) the phase Lα is found at $c_1 < 0.95$. ANS fluorescence, studied on aliquots of the X-ray samples, displays a striking concentration dependence in the dry end of the egg lecithin–phosphatidic acid–water phase diagram and a strong correlation with the variations of d_1 (and S) (see Fig. 3). This correlation is further confirmed by the observation that as d_1 becomes independent of c_1, the quantum yield becomes constant.

The fact that the fluorescent parameters of ANS are independent of the water content over a wide concentration range ($0.7 > c_1 > 0.05$) indicates that the preferential affinity of the lipids for ANS is high. Besides the high quantum yield suggests that ANS is embedded in a region of low polarity. Since ANS is not soluble in paraffins its most likely location is at the interface: the correlation between S and the quantum yield confirms that this is the case, and suggests furthermore that the ANS molecules are forced deeper into the hydrocarbon region as the packing of the polar groups becomes denser.

Lysozyme–lipid–water phases

Several phases were observed with these systems, whose structure was described previously[1].

The lamellar phases consist of lipid bilayers, with intercalated polar sheets containing protein and water (see Fig. 5). Two types of lamellar structures may be distinguished[1]: those stabilized mainly by electrostatic interactions (type *e*) and those in which hydrophobic interactions exist as well (type *h*). The difference between the two resides in the thickness of the lipid layer, which for type *e* is identical to, and for type *h* is considerably smaller than that observed in the absence of protein. Moreover in phases of type *e* the protein can be separated from the lipid by dispersion in saline water (NaCl, 1 M) while in phases of type *h* the separation requires salting out the lipid by divalent cations[1]. The two-dimensional phases are characterized by two-dimensional lattices, either hexagonal or square[1]. The precise distribution of the components in these phases has not yet been determined, although the sensitivity to electrolyte is akin to that of the lamellar phase type *h*.

ANS was incorporated into several protein–lipid–water phases; the partition coefficient is quite high in all the cases (see Table II). The X-ray and fluorescence studies were carried out at the molar ratio of lysozyme to ANS of 2.5:1. The structure of the phases was determined by X-ray diffraction; the fluorescence measurements

were performed on aliquots of the X-ray samples. The results are given in Table II.

The fluorescence of tryptophan was studied in all the phases in the absence of ANS; it was found to be only slightly dependent on the nature of the phase (see Fig. 2).

In all the phases the fluorescence spectra of ANS are shifted to the blue, as compared to ANS in water and in the lysozyme–water system (see Tables I and II), and the quantum yield is high. Besides, the phases of type *h*, both lamellar and two-dimensional, are clearly distinguishable from the phases of type *e*, as in the former the blue shift, the quantum yield and the energy transfer are higher than in the latter.

DISCUSSION

The location of ANS in the various phases may now be discussed. The notion put forward by other workers[9] will be adopted here, that the emission spectrum and quantum yield of ANS fluorescence are directly related to the polarity of the micro-environment of the ANS molecules: the lower the polarity the more pronounced the blue shift and the higher the quantum yield of the emission spectrum.

Our present data, namely the high partition coefficient, the blue shift and the high quantum yield of the ANS emission spectrum, the constancy of the fluorescence parameters as the thickness of the water layer becomes greater than approx. 20 Å (see Fig. 3), clearly show that in the lamellar phase Lα of the lipid–water systems the ANS molecules are embedded in the lipid leaflet. Moreover the known poor solubility of ANS in water and in paraffins, the amphipatic character imparted on ANS by the small polar group and the fairly bulky nonpolar moiety (see Fig. 4) suggest that the fixation sites are localized at or near the lipid–water interface. It should be noted that from the operational standpoint "interface" means here the ill-defined region perturbed by the hydrocarbon–water interaction. The remarkable correlation of the fluorescence parameters with the area *S* (see Fig. 3) indicates that with an increase of *S* the polarity of the microenvironment of ANS decreases; this can be interpreted as if the ANS molecules were displaced deeper into the hydrocarbon leaflet when *S* decreases.

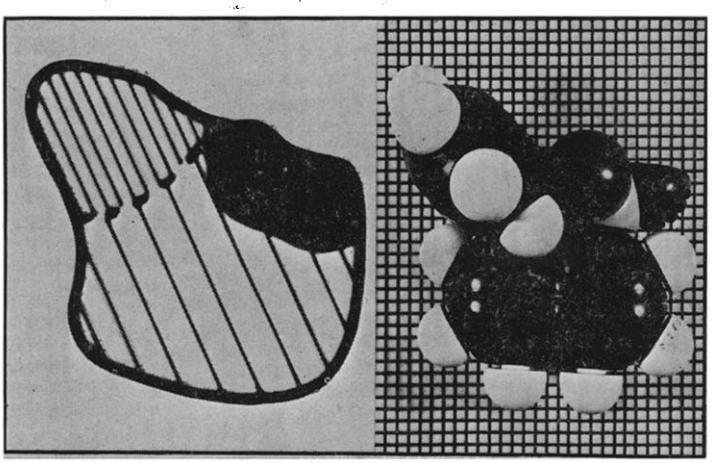

Fig. 4. Molecular model of ANS, and schematic representation as used in Fig. 5. The black area is the polar region of the molecule.

For all the protein–lipid–water phases the high values of the partition coefficient as compared with the low affinity of ANS for lysozyme, indicate that ANS is localized in the lipid moiety or in the areas of protein–lipid contacts. The fluorescent properties of the two types of phases, displaying only electrostatic (type *e*) or also hydrophobic (type *h*) protein–lipid interactions, are sharply different.

The emission spectrum and the quantum yield of ANS fluorescence for the lamellar phase of type *e* are similar to those of the Lα lipid–water phases (for example see Fig. 3 at c_1 approx. 0.90). On the contrary these parameters take quite different values in lysozyme solutions, even at the highest concentration (see Table I). It may thus be inferred that in the phases of type *e* ANS is indeed located at the lipid–water interface. Furthermore, since the fluorescence parameters indicate that the polarity of the microenvironment is lower than in the lipid–water phase (see Table II) it appears that the sites of fixation are regions where *S* is slightly smaller than the average. Thus we tentatively identify these sites with the areas of protein–lipid close contacts where the negative charges of the lipids are neutralized by the positive charges of lysozyme (see Fig. 5).

The fluorescence properties of ANS incorporated into the protein–lipid–water

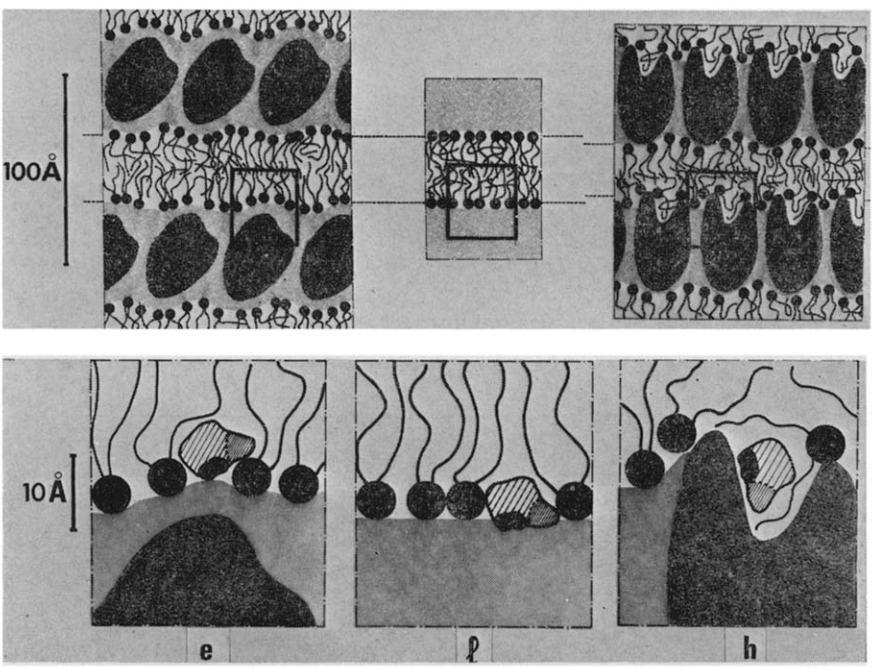

Fig. 5. Lysozyme–lipid–water system; structure of the lamellar phases. The sections are perpendicular to the plane of the lamellae. The densely and lightly hatched areas represent the protein molecules and the water regions (both of arbitrary shape). Note that the thickness of the lipid leaflet is the same in phase *e* and in the lipid–water phase *l*, and that it decreases in phase *h*. This shrinkage is interpreted to involve hydrophobic contacts between the protein and the lipid, shown in the figure. The lower frames are enlarged representations of the areas of the upper frames enclosed in the rectangles, and show the position of the ANS molecules. In phases *e* and *l* the ANS molecules are located at the lipid–water interface, more deeply embedded in the hydrocarbon regions in phase *e* than in phase *l*. In phase *h* the ANS molecules are in closer contact with a hydrophobic region of the protein molecules (see text).

phases of type *h* indicate a microenvironment even less polar than that in phase *e* (see Table II). Although at very high concentration, and thus at high d_1, the fluorescence parameters of the Lα lipid–water phase could well take values similar to those of phases *h* (extrapolate the data of Fig. 3 towards $c_1 = 1$), the location of ANS at the interface is unlikely in phases *h* where d_1 is in fact considerably smaller than in the lipid–water phase (see Table II). Consequently the most likely location of ANS is the region of hydrophobic contacts between the protein and the lipids (see Fig. 5).

These locations of ANS are consistent with the efficiency of the energy transfer from tryptophan to ANS observed with the different phases (Table II). According to FÖRSTER's[3] theory the efficiency of energy transfer (ET) between two fluorescent chromophores has a simple expression.

$$\mathrm{ET} = 100 \times \frac{R^{-6}}{R^{-6} + R_0^{-6}}$$

where R is the distance between the chromophores and the parameter R_0 is a function of the overlapping of the emission spectrum of the donor and the excitation spectrum of the acceptor, of the refractive index of the medium and of the relative orientation of the chromophores. Since the quantum yield of tryptophan fluorescence (in the absence of ANS) is the same in all the phases (see Fig. 2), the ratio R/R_0 can be evaluated for the various phases if the assumption is made that the refractive index is the same, the mutual orientation of the chromophores is random, and the tryptophan residues of each lysozyme molecule are assimilated to a single chromophore. The results (R/R_0 varies from 1.44 to 1.00 as the energy transfer varies from 10 to 50 %) show that R is smaller in the phases of type *h* than in those of type *e*, in agreement with the notion that in the latter type of phase ANS is inserted into a hydrophobic niche of lysozyme (see Fig. 5).

ANS was chosen here because of its widespread use as a conformational probe in biological systems[9]. The most interesting conclusion of our work, which lends itself to straightforward applications to biological membranes, is that ANS fluorescence is sensitive to the nature of the protein–lipid interactions. Nevertheless it must be noted that this conclusion can be reached only in cases in which the affinity of ANS for the interfaces between the polar and the hydrophobic regions is much higher than that for the protein molecules. The situation is likely to be more complex in membranes and more generally in systems containing more than one protein. The next step will be to test other fluorescent probes whose site of attachment is more specifically defined—fully embedded in the hydrocarbon matrix, covalently bound to the polar groups of the lipid or to specific residues of the proteins. However, it will be essential in all cases to calibrate these probes with model systems of well-defined structure.

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REFERENCES

1 T. GULIK-KRZYWICKI, E. SHECHTER, V. LUZZATI AND M. FAURE, *Nature*, 223 (1969) 1116.
2 L. STRYER, *J. Mol. Biol.*, 13 (1965) 482.
3 TH. FÖRSTER, *Radiation Res. Suppl.*, 2 (1960) 326.
4 M. IWATSUBO AND A. DI FRANCO, *Bull. Soc. Chim. Biol.*, 47 (1965) 891.
5 G. WEBER AND L. B. YOUNG, *J. Biol. Chem.*, 239 (1964) 1415.
6 S. R. ANDERSON, *Biochemistry*, 8 (1969) 4838.
7 V. LUZZATI, in D. CHAPMAN, *Biological Membranes*, Academic Press, New York, 1968.
8 T. GULIK-KRZYWICKI, A. TARDIEU AND V. LUZZATI, *Mol. Cryst. Liquid Cryst.*, 8 (1969) 285.
9 L. STRYER, *Ciba Foundation Symp. on Molecular Properties of Drug Receptors*, 1970, in the press.