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THE STUDY OF LIPID–PROTEIN INTERACTIONS IN MEMBRANES BY FLUORESCENT PROBES

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SUMMARY

1. We report the fluorescence of 1-anilinonaphthalene-8-sulfonate from 220 mμ up in various solvents, in association with lecithin and lysolecithin and bound to membranes of human erythrocytes.

2. The membrane spectra indicate that there is transfer of electronic excitation energy from some of the tryptophans of membrane proteins to membrane-bound 1-anilinonaphthalene-8-sulfonate.

3. Our findings raise the possibility that some or all of the membrane-bound 1-anilinonaphthalene-8-sulfonate is associated with membrane proteins; the dye can therefore not be assumed to act solely as a probe for possible lipid regions in membranes.

INTRODUCTION

The binding of membrane lipids to membrane proteins appears to involve extensive apolar interactions[1], but little is known about the relationships between these membrane components. Indeed, there are good reasons to suppose that not all membranes are alike in this respect and that the lipid–protein associations in a given membrane are responsive to its biological activity. These problems have recently become approachable: it is possible to insert into membranes certain apolar molecules whose physical properties are influenced by, and therefore reflect, their immediate surroundings. At present, such probes fall into two categories: (a) molecules bearing unpaired electrons in nitroxide radicals, which can be monitored by their electron spin resonance, *e.g.*, TEMPO (ref. 2); spin-labeled phosphatides[3]; (b) fluorescent probes such as 1-anilinonaphthalene-8-sulfonate (ANS)[4–11], which transmit information about their micro-environment through characteristic features of their fluorescence spectra. The probe approach has the great advantage of allowing one to follow rapid structural changes accompanying biological activity[5,9–11], but it must be recognized that various probes may inform about only restricted membrane regions and also that they might act as structure perturbants.

Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

In this report we will describe the effects of the insertion into erythrocyte membranes of one probe, ANS, upon the fluorescence characteristics of both the probe and the membrane proteins. Aqueous solutions of ANS have a low fluorescence quantum yield, while solutions of the dye in less polar, organic solvents fluoresce strongly. Hence, the fluorescence enhancement observed when ANS binds to certain proteins and to membranes is thought to indicate sequestration of the dye in regions of low polarity[4–15]. Such regions may represent specific peptide conformations. For example, the conversion of the soluble polypeptide, poly-L-lysine from α-helical or "unordered" to the β-form is accompanied by a large enhancement of the fluorescence of 2-*p*-toluidinyl-naphthalene-6-sulfonate, a dye closely related to ANS[16].

Another useful feature of ANS and related dyes is their absorbance peak near 350 mμ, which overlaps the region of tryptophan fluorescence. One might expect measurable transfer of electronic excitation energy from membrane tryptophan to dye molecules inserted close enough to these amino acids. Energy transfer from the fluorophores of membrane proteins to ANS and other appropriate, lipophilic dyes might thus inform about the lipid–protein associations in membranes. To investigate these possibilities, we have obtained the fluorescence spectra of ANS from 220 mμ up in various solvents in association with certain phosphatides bound to erythrocyte membranes.

The data, as reported for apomyoglobin by STRYER[13], indicate transfer of excitation energy from membrane proteins to membrane-bound ANS. This raises the possibility that some or all of the membrane-bound ANS is associated with membrane proteins and that the dye is not a reliable probe for possible lipid regions in membranes. We suggest that a number of known, membrane-active compounds might be more useful probes for the study of lipid–protein interactions in membranes by fluorescence techniques.

MATERIALS AND METHODS

Membranes

Membranes of human erythrocytes were prepared by the method of DODGE *et al.*[17]. The membranes contained no spectroscopically detectable hemoglobin. Small samples of the membranes were stored in 0.25 M sucrose at −28°. For fluorescence measurements the membranes were washed twice with 0.1 M phosphate buffer (pH 7.5) (Spinco rotor 40, 20000 rev./min = 36190 × g_{max}, 4°) and then suspended in the same buffer.

ANS

The sodium salt of 1-anilinonaphthalene-8-sulfonic acid (Eastman Kodak No. T484) was purified by recrystallizing 4 times from water[14]. The purified material was protected against exposure to light and was stored at 4°. At the concentrations employed (maximum 30 μM), ANS did not lyse intact erythrocytes nor protect against osmotic hemolysis.

Lecithin and lysolecithin

These phospholipids were obtained from the Sigma Chemical Comp., St. Louis, Mo., U.S.A. The lysolecithin was shown to be pure using thin-layer chromatography

on silica gel. The lecithin (from egg yolk) was used after further purification using chromatography on 1-mm layers of silica gel. Stock emulsions of the phosphatides (500 μM) were prepared in 0.1 M phosphate buffer (pH 7.5) by 2-min sonication with a Branson sonifier (Model S125, Branson Instrument Co., Danbury, Conn.) using a micro tip with 2 A output at 20 kcycles.

Human serum albumin

The crystalline material from Sigma Chemical Comp. was used without further purification.

Fluorescence spectroscopy

Most of our fluorescence spectra were obtained with a Perkin–Elmer MPF-2A recording spectrophotofluorimeter at 25–29°. The recorded spectra were not corrected for the spectral dispersion of excitation energy, nor for the spectral characteristics of the detection system. The spectral bandwidth for both excitation and emission spectra obtained with this instrument were maintained below 8 mμ and are specified in the text. In order to obtain an accurate measure of the spectral position of the various excitation and emission bands under study and also of their relative intensity, a number of spectra were obtained manually on a Zeiss spectrophotofluorimeter (ZFM 4C with double-monochromator M 4 Q III) which had been calibrated for the spectral dispersion of excitation energy and for the spectral characteristics of its detection system.

Since ANS absorbs strongly in the spectral regions of tyrosine and tryptophan excitation (ε 265 mμ = 19200 cm^2/mmole), and also in the region of tryptophan emission (ε 350 mμ = 6200 cm^2/mmole), the addition of ANS to a protein solution or membrane suspension might diminish the observed tryptophan fluorescence of the proteins in two ways: (a) by transfer of electron excitation energy to protein-bound ANS molecules; (b) artifactually, by decreasing the flux of exciting quanta reaching the protein fluorophores and by absorbing some of the light emitted by these fluorophores, that is, by "inner filter effects". Where necessary we have corrected for these artifacts using calibration curves which predict the decrement of amino acid fluorescence due to absorption of light by ANS. These curves were obtained using ANS, tyrosine and tryptophan under conditions where there is no energy transfer.

Fluorescence microscopy

It is conceivable that ANS might bind only to limited patches on cell membranes. Therefore, we have examined erythrocyte membranes suspended in buffer solutions with and without ANS by phase-fluorescence microscopy. A Zeiss Standard Universal fluorescence microscope was employed. In the presence of the dye the cell peripheries fluoresced a bright, bluish-green, when excited in the ultraviolet. No inhomogeneities of staining were observed at the maximum resolution attainable (magnification, 1250 ×). No fluorescence was observed in the absence of ANS.

RESULTS

Fig. 1 presents uncorrected excitation spectra of ANS in a series of ethanol–water mixtures and illustrates the increase in fluorescence with increasing apolarity,

as previously described[13,15]. When corrected for the spectral variation of exciting intensity, the band near 280 mμ (272 mμ, corrected), corresponding to the major absorption band, is found to have 1.9 times the intensity of the maximum at 367 mμ

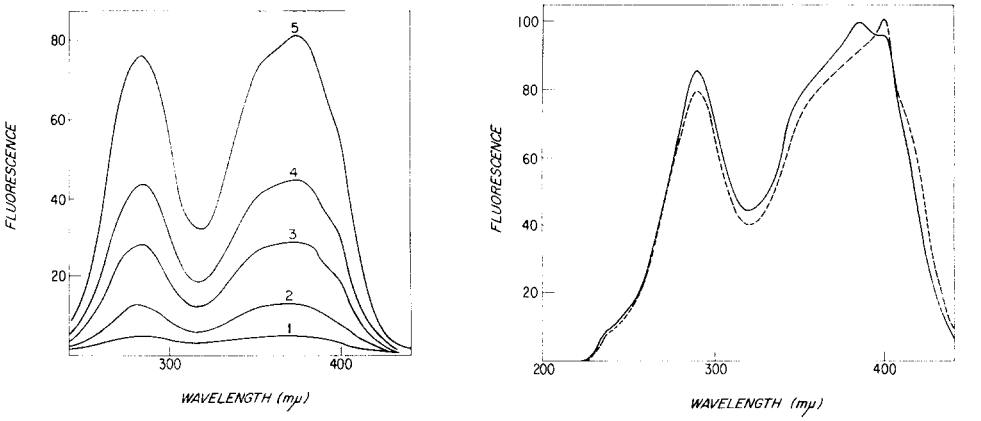

Fig. 1. Excitation spectrum of ANS in various ethanol–water mixtures. 1, 38% ethanol; 2, 57% ethanol; 3, 77% ethanol; 4, 87% ethanol; 5, 96% ethanol. ANS concentration 20 μM. Emission at 480 mμ. Excitation bandwidth 2 mμ. Spectra not corrected.

Fig. 2. Excitation spectra of ANS associated with (a) lecithin (– – –) and (b) lysolecithin (— — —). Spectra normalized setting amplitude of maximum near 380 mμ to 100 fluorescence units. Phosphatides suspended in 0.1 M phosphate buffer (pH 7.5) by sonication at 0°, for 2 min using a Branson sonifier (model No. S125), micro tip at an intensity of 2 A (20 kcycles). Phosphatide concentration 11 μM. ANS concentration 30 μM. Emission at 480 mμ. Excitation bandwidth 4 mμ, emission bandwidth 4 mμ. Spectra not corrected.

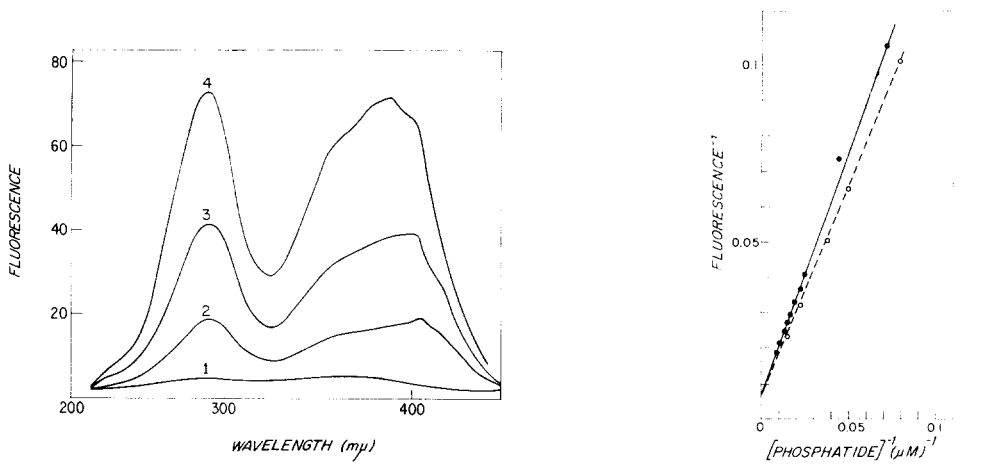

Fig. 3. Excitation spectra of ANS associated with lysolecithin at various concentrations. 1, 30 μM ANS; 2, 30 μM ANS + 4 μM lysolecithin; 3, 30 μM ANS + 16 μM lysolecithin; 4, 30 μM ANS + 38 μM lysolecithin. Emission 480 mμ. Excitation bandwidth 4 mμ, emission bandwidth 4 mμ. Spectrum not corrected.

Fig. 4. Plots of $1/F$ vs. $1/[\text{phosphatide}]$ for lysolecithin (●—●) and lecithin (○---○). Phosphatides dispersed in 0.1 M phosphate buffer (pH 7.5) as in Fig. 2. 1 μM ANS. Data derived from duplicate excitation and emission spectra. For excitation spectra, emission was measured at 480 mμ and the excitation and emission bandwidths were 4 and 8 mμ, respectively. For emission spectra, excitation was at 286 mμ with identical bandwidths.

in 96% ethanol. Its position changes little with solvent composition, whereas the longer wavelength maximum shifts from 355 to 367 mμ with decreasing polarity, at the same time resolving into several bands. There is also a corresponding blue-shift of the fluorescence emission peak, as reported elsewhere[13,15].

Fig. 2 shows the excitation spectra of ANS associated with lecithin and lysolecithin. The band at 275 mμ (corrected) has about 1.5–1.8 times the intensity of the peak at 375 mμ (corrected). For the concentrations illustrated, 11 μM, lecithin is micellar, while lysolecithin is not[18]. Fluorescence enhancement is detected even at 1 μM concentrations of the two phosphatides. The differences near 400 mμ, between the excitation spectra of ANS bound to lysolecithin and lecithin (Fig. 2), thus relate to the micellar state. Above the critical micelle concentration, the excitation spectra of ANS bound to lysolecithin and lecithin are similar in shape. The spectral effect of the micellar transition of lysolecithin is shown in Fig. 3.

Plots of $1/F$, the reciprocal of fluorescence intensity, *vs.* the reciprocal of the phosphatide concentration, are linear for lecithin above 1 μM, and for lysolecithin above 15 μM, and have identical $1/F$ intercepts at $1/[\text{phosphatide}] = 0$ (Fig. 4). These uncomplicated isotherms indicate that at large phosphatide excess the ANS fluorescence is identical in lecithin and lysolecithin micelles.

The data indicate that fluorescence enhancement can come about by association with molecular lysolecithin as well as by insertion of the dye into phosphatide micelles. However, the two types of binding can be distinguished spectroscopically. The spectra of ANS associated with phosphatides in micellar suspensions are similar to those found with organic solutions of the dye.

When excited by ultraviolet light, erythrocyte ghosts (without ANS) exhibit a fluorescent emission maximum at 338 mμ (corrected). This is characteristic of tryptophan in an apolar milieu. There is no evidence of tyrosine fluorescence at 310 mμ, and the shape of the emission spectrum is identical with excitation at 270

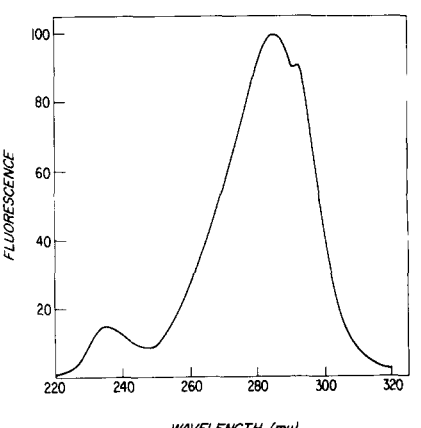

Fig. 5. Excitation spectrum of erythrocyte membranes without ANS. Protein concentration 45 μg/ml. Emission 338 mμ. Excitation bandwidth 4 mμ. Spectrum not corrected.

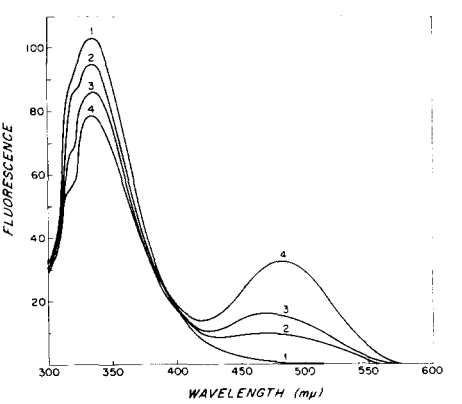

Fig. 6. Emission spectra of erythrocyte membranes alone (1), and in the presence of 20 μM ANS (2), 40 μM ANS (3) and 80 μM ANS (4). Protein concentration 9 μg/ml. Excitation bandwidth 3 mμ, emission bandwidth 4 mμ. Excitation at 286 mμ. Spectra corrected. The shoulders near 320 mμ are due to Raman scattering by water.

and 290 mμ. This phenomenon is found in many globular proteins and in the plasma membranes of Ehrlich ascites carcinoma[19]; it indicates a transfer of excitation energy from tyrosine to tryptophan.

The excitation spectrum of erythrocyte membranes (without ANS) is shown in Fig. 5. The band at 235 mμ (not corrected) is common to all aromatic amino acids. The contributions of tyrosine and tryptophan to the main excitation band (maximum at 275 mμ, corrected) are clearly discernible.

When ANS is added to erythrocyte ghosts and these are irradiated at 286 mμ, the uncorrected excitation maximum of membrane protein, there is a decrease of tryptophan fluorescence beyond what can be accounted for by the absorption of light by added dye. There is also appearance of fluorescence with maximal emission at 480 mμ, due to ANS. These effects are shown in Fig. 6. The shape of the emission spectrum is the same whether excitation is at 266 (the absorption maximum of ANS in water), 270, 286, or 295 mμ. The data thus indicate transfer of electronic excitation energy from membrane tryptophan to bound ANS.

With increasing ANS concentration there is progressive diminution of tryptophan fluorescence which is due in part to absorption of exciting and emitted light by unbound dye. After correcting for this, one arrives at the actual quenching of tryptophan fluorescence by bound ANS. This is shown in the plot of the reciprocal fluorescence decrement *vs.* reciprocal ANS concentration in Fig. 7. The quenching limit appears to be approx. 26 % of tryptophan fluorescence. Since there are about 200 nmoles of tryptophan per mg of membrane protein[20], this quenching value is compatible with about 50 nmoles of ANS bound per mg protein; this figure is in accord with the values of 45 found derived from studies of the enhancement of ANS fluorescence under corresponding conditions[8].

The presence of energy transfer is seen from the excitation spectrum of membrane-associated ANS (emission at 480 mμ) (Fig. 8), when this is compared on the one hand to the excitation spectra of ANS in apolar environments (Figs. 1, 2 and 3), and, on the other, to that of the complex of ANS with serum albumin shown in Fig. 9. The intensities of the short-wavelength excitation maxima of the ANS–membrane and ANS–albumin complexes relative to those of the 370–380-mμ peak are high

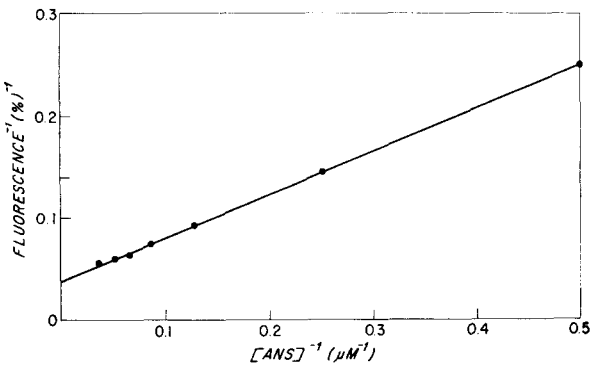

Fig. 7. Plot of reciprocal fluorescence decrement at 338 mμ *vs.* reciprocal of ANS concentration. Excitation at 286 mμ. Each point corrected for the absorption of light by free ANS at 286 mμ and 338 mμ at the concentrations employed. Protein concentration 9 μg/ml. Excitation bandwidth 3 mμ, emission bandwidth 4 mμ.

(2.6 and 2.9, respectively) compared with the ratios found in 96% ethanol (1.9) and in the ANS–phosphatide complexes (1.5–1.8). These findings are consistent with energy transfer from aromatic protein fluorophores to ANS[13]. In the 350–380-mμ region the spectra of the complexes of ANS with erythrocyte membranes resemble those obtained with organic solvents and micellar phosphatides and suggest contribution of at least three absorption bands.

The enhancement of ANS fluorescence by erythrocyte membranes in 0.1 M phosphate buffer (pH 7.5) is rather small. At equivalent protein concentrations, the

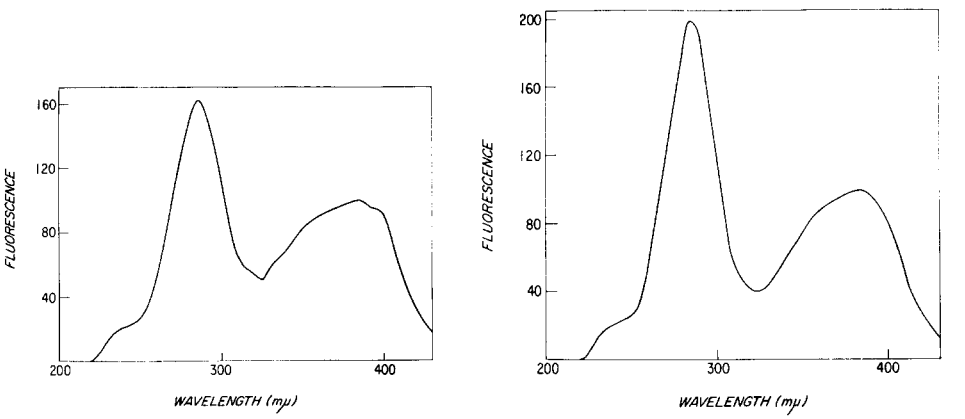

Fig. 8. Excitation spectrum of erythrocyte membranes in the presence of 12 μM ANS. Protein concentration 42 μg/ml. Emission 480 mμ. Excitation bandwidth 2 mμ, emission bandwidth 4 mμ. Spectrum not corrected.

Fig. 9. Excitation spectrum of bovine serum albumin in the presence of 1 μM ANS. Protein concentration 5 μg/ml. Emission at 480 mμ; excitation bandwidth 4 mμ, emission bandwidth 8 mμ. Spectrum not corrected.

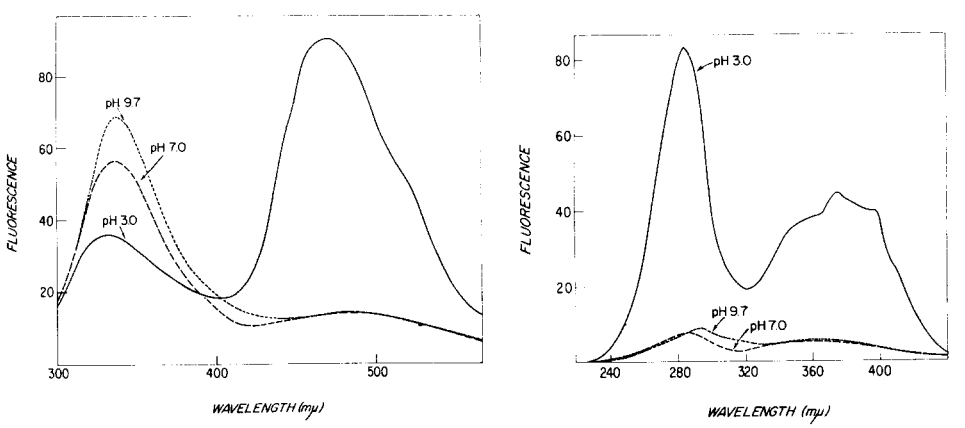

Fig. 10. Emission spectra of erythrocyte membranes as a function of pH. (a) – – – –, 0.2 M Na_2CO_3–$NaHCO_3$ buffer, pH 9.7; (b) — — —, 0.1 M phosphate buffer, pH 7.5; (c) ———, 0.1 M citrate (Sörensen) buffer, pH 3.0. Protein concentration 9 μg/ml, ANS concentration 8 μM. Excitation at 286 mμ. Excitation bandwidth 2 mμ, emission bandwidth 4 mμ. Spectra not corrected.

Fig. 11. Excitation spectra of erythrocyte membranes as a function of pH. (a) – – – –, pH 9.7; (b) — — —, pH 7.5; (c) ———, pH 3.0. Conditions as in Fig. 10.

fluorescence at 480 mμ (excitation at 286 or 380 mμ) of serum albumin is 8.5 times greater than that of membranes. Further, expressed in terms of their lipid content, 43%, the fluorescence enhancement of the membranes is about one-sixth that of lysolecithin. These facts speak for a more limited number of binding sites than what would be expected in a micellar model.

When the pH of a membrane suspension is dropped from 7.5 to 3.0, the ANS fluorescence at 480 mμ (excitation at 286 mμ) increases about 7-fold (Fig. 10). There is a concomitant diminution of tryptophan fluorescence indicating increased energy transfer from this fluorophore to ANS. The data points to the appearance of new binding sites, as proposed by others[7]. These sites might be associated with the refolding of some membrane peptide chains into β-structure, which occurs at low pH (ref. 19). The fluorescent enhancement at low pH is also evident in the excitation spectrum (Fig. 11). The persistence of energy transfer is apparent from the large amplitude of the 285-mμ band relative to the peak at 380 mμ.

Shift of pH from 7.5 to 9.7 also produces significant differences in the excitation spectra of ANS and in the emission spectra of tryptophan. The displacement to the red and greater intensity of the aromatic excitation band reflects titration of tyrosine hydroxyl groups. At pH 9.7 the tryptophan fluorescence at 338 mμ (excitation at 286 mμ) is greater than at pH 7.5, but the intensity of ANS fluorescence is the same. Energy transfer is apparently more efficient between tyrosines and tryptophans at the higher pH, but not between the latter and ANS.

DISCUSSION

When ANS and related dyes are bound by certain proteins[12–16], detergent micelles[8], phospholipid micelles[6], and membranes[4–11], their fluorescence is enhanced over that observed in water, and their emission maxima are shifted to the blue. Both phenomena are similar to what is observed when these dyes are transferred from water to less polar solvents. These spectral effects correlate with those resulting from variation of solvent polarity[13,15]. ANS and similar dyes can therefore serve as sensitive indicators of the polarity of their micro-environments. Our study does not deal explicitly with this application of fluorescent probes to the analysis of membrane structure, but indicates that, with adequate instrumentation, one can derive additional and more specific information from their use.

First, our work on lysolecithin indicates that the fluorescence of ANS is altered not only when it is partitioned into a phase of low polarity, but also by molecular associations with appropriate hydrophobic substances. The differences in the fine structure of the 350–400-mμ region of the excitation spectra of molecular and micellar lysolecithin reflect those modes of interaction.

Second, our studies show that in erythrocyte membranes, just as in apomyoglobin[13], there is significant transfer of energy absorbed by tyrosine molecules to tryptophan and from tryptophan molecules to ANS.

The matter of energy transfer has recently been reviewed with special emphasis on its study with fluorescent probes[15]. In the present case, we are presumably dealing with singlet–singlet transfer, where return of the energy donor, tryptophan, to the ground state is coupled to excitation of the energy acceptor, ANS. Energy transfer can be large when there is significant overlap between the emission spectrum of the acceptor; this is the case with typtophan and ANS.

An important aspect of energy transfer between the protein chromophores of membranes and fluorescent probes is the fact that the efficiency of transfer depends upon the distance between the energy donor and acceptor. Thus, if we assume that quenching of tryptophan fluorescence by ANS is due only to energy transfer, the maximum quenching would correspond to a transfer efficiency of 26% and would be compatible with a donor–acceptor distance of about 40 Å, assuming a single binding site[21,22]. Unfortunately, there is good reason to suspect multiple ANS binding sites in various membrane proteins, at protein–lipid interfaces and possibly in regions containing only lipid.

However, the results suggest that measurement of energy transfer between the chromophores of membrane proteins and suitable energy acceptors might permit one to map out some of the proximity relations between membrane proteins and lipids. The conditions for the precise application of such "spectroscopic rulers" to macromolecules, as specified by STRYER[15] are: (a) presence of only single, specific donor–acceptor pairs, (b) calibration of the relationship between transfer efficiency and inter-chromophore distance and (c) information about relative orientations of donor and acceptor. These requirements, particularly the first, are too stringent at present for membrane biology, but then the spatial resolution required to gain valuable information about lipid–protein interactions in membranes and their alterations with biologic activity, is currently less than that needed for the structural analysis of proteins.

The study of lipid–protein interactions by energy-transfer techniques suggests the use of lipophilic fluorescent probes, which can accept electronic excitation energy from tyrosines and tryptophans of membrane proteins and whose mode of association with the membranes and effects on membrane function and/or structure can be assessed independently. With this information one could use the probes either as perturbants or to report perturbations caused by other agents. Low absorbance at about 280 mμ and high membrane affinity are desirable to minimize filter effects by unbound probe molecules.

Many substances which potently influence membrane structure and/or function might serve as useful fluorescent probes, and of these, the polyene vitamins and antibiotics appear particularly promising. Thus, vitamin A and its relatives are excited maximally near 327 mμ and fluoresce near 510 mμ (ref. 23). The polyene antibiotics (amphotericin, filipin, nystatin, *etc.*) are related to vitamin A through their conjugated double bond systems. Filipin, the best characterized, is a conjugated pentaene, which absorbs maximally near 340 mμ and fluoresces in the green[24]. Conjugated tetraenes, pentaenes and hexaenes have relatively low absorbances near 280 mμ (ref. 25).

In addition, there are several potent membrane-active drugs, which at low levels, absorb to membranes as if partitioning into a lipid phase[26], and also have fluorescence properties compatible with energy-transfer probes. Of particular interest are the phenothiazines (*e.g.*, chlorpromazine) and the reserpines. The former have excitation maxima near 320 mμ, fluoresce at 490 mμ (ref. 27) and have an absorption minimum at 280 mμ. Reserpine which has an excitation maximum at 300 mμ and fluoresces at 375 mμ (ref. 27) is suitable as an acceptor of excitation energy from tyrosine.

The above reagents, selected from the many, readily available membrane-

active substances, appear more suitable as probes of membrane structure than ANS and related dyes. More specific probes, such as conjugated polyene phosphatides, should be accessible to synthesis.

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REFERENCES

1 D. F. H. WALLACH, *J. Gen. Physiol.*, 54 (1969) 38.
2 W. L. HUBBELL AND H. MCCONNELL, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 12.
3 A. D. KEITH, A. S. WAGGONER AND O. H. GRIFFITH, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 819.
4 I. TASAKI, A. WATANABE, R. SANDLIN AND L. CARNAY, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 883.
5 B. CHANCE, A. AZZI, L. MELA, G. RADDA AND H. VAINIO, *Fed. European Biochem. Socs. Letters*, 3 (1969) 10.
6 J. VANDERKOSI AND A. MARTONOSI, *Arch. Biochem. Biophys.*, 133 (1969) 153.
7 R. FREEDMAN AND G. RADDA, *Fed. European Biochem. Socs. Letters*, 3 (1969) 150.
8 B. RUBALCAVA, D. M. DE MUNOZ AND C. GITLER, *Biochemistry*, 8 (1969) 2742.
9 A. AZZI, B. CHANCE, G. K. RADDA AND C. P. LEE, *Proc. Natl. Acad. Sci. U.S.*, 62 (1969) 612.
10 L. PACKER, M. P. DONOVAN AND J. M. WRIGGLESWORTH, *Biochem. Biophys. Res. Commun.*, 35 (1969) 832.
11 M. KASAI, J. P. CHANGEUX AND L. MONNINE, *Biochem. Biophys. Res. Commun.*, 36 (1969) 420.
12 G. WEBER AND D. J. R. LAURENCE, *Biochem. J.*, 56 (1959) 31.
13 L. STRYER, *J. Mol. Biol.*, 13 (1965) 482.
14 W. O. MCLURE AND G. M. EDELMAN, *Biochemistry*, 5 (1966) 1908.
15 L. STRYER, *Science*, 162 (1968) 526.
16 J. LYNN AND G. D. FASMAN, *Biochem. Biophys. Res. Commun.*, 33 (1968) 327.
17 J. T. DODGE, C. MITCHELL AND D. HANAHAN, *Arch. Biochem. Biophys.*, 100 (1963) 119.
18 L. SAUNDERS, *Ann. Pharm. Franç.*, 24 (1966) 89.
19 D. F. H. WALLACH AND P. H. ZAHLER, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 1552.
20 S. A. ROSENBERG AND G. GUIDOTTI, *J. Biol. Chem.*, 243 (1968) 1985.
21 T. FORSTER, *Ann. Phys.*, 2 (1948) 55.
22 L. STRYER AND R. P. HAUGLAND, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 719.
23 W. A. HAGINS AND W. H. JENNINGS, *Discussions Faraday Soc.*, 27 (1959) 180.
24 M. E. BERGY AND T. E. EBLE, *Biochemistry*, 7 (1969) 653.
25 R. T. HOLMAN, *Methods Biochem. Anal.*, 4 (1957) 99.
26 P. SEEMAN, *Ann. Rev. Neurobiol.*, 9 (1966) 146.
27 S. UNDENFRIEND, D. E. DUGGAN, B. M. VASTA AND B. B. BRODIE, *J. Pharmacol. Exptl. Therap.*, 120 (1957) 26.