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FLUORESCENCE OF 1,8-ANILINONAPHTHALENE SULFONIC ACID BOUND TO PROTEINS AND TO LIPIDS OF SARCOLEMMA

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Summary

Relative contributions to fluorescence of 1,8-anilinonaphthalene sulfonic acid-sarcolemma by 1,8-anilinonaphthalene sulfonic acid bound to proteins and to lipid were assessed on the basis of fluorescence lifetime and steady-state emission spectra. The lifetime spectrum of lipid-poor proteins prepared from sarcolemma had a major time constant of 16 ns; that of lipids extracted from sarcolemma had a major time constant of 7 ns and a minor one of 4 ns. In sarcolemma, components having all three time constants were observed with weighting factors 0.33, 0.6 and 0.07, respectively, indicating the fraction of 1,8-anilinonaphthalene sulfonic acid bound to protein and to lipid. Steady-state emission spectra of 1,8-anilinonaphthalene sulfonic acid-sarcolemma were resolved into and resynthesized from contributions by 1,8-anilinonaphthalene sulfonic acid bound to proteins and to lipids. The latter was at least two thirds of total 1,8-anilinonaphthalene sulfonic acid bound. Results of analyses of spectra obtained with various concentrations of sarcolemma and 1,8-anilinonaphthalene sulfonic acid suggest that the proteins have a higher affinity, but only about half the binding capacity for 1,8-anilinonaphthalene sulfonic acid as do lipids.

Introduction

Fluorescence emission intensity of 1,8-anilinonaphthalene sulfonic acid (ANS) is enhanced when it is added to biomembranes; observations were reported in 1969 [1—3] and have since been confirmed by many. There is a somewhat similar enhancement of its fluorescene when ANS is added to lipo-

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Abbreviations: ANS, 1,8-anilinonaphthalene sulfonic acid; ATPase, adenosinetriphosphatase (EC 3.6.1.3); HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

somes (for example, see refs. 3 and 4), and, of course, it has been known since Weber and Laurence [5] first observed this interesting property that its fluorescence is enhanced when ANS is added to solutions of certain proteins.

The question is, in the presence of biomembranes does ANS bind to lipids, to proteins, or to both, and, if to both, in what proportions? Evidence for binding to membrane lipids and to membrane proteins has been reported. Among the pioneering papers in the field, Vanderkooi and Martonosi [3] reported that addition of phospholipase C, but not of trypsin, decreased fluorescence intensity of ANS in a suspension of vesicles of sarcoplasmic reticulum; hence they proposed that ANS bound to lipids, not to proteins of that membrane. On the other hand, Hasselbach and Heimberg [6], on the grounds that ANS binds to aliphatic amines, proposed that ANS binds to membrane proteins, not to lipids. This proposal was buttressed by observations of enhanced ANS fluorescence with vesicles of sarcoplasmic reticulum even after treatment with phospholipase A [7].

We have taken a different approach to the problem. As reported in this paper, our analysis of fluorescence lifetime spectra of ANS bound to vesicles of sarcolemma from rat hindlimb muscles yielded three lifetimes, of which two, about 7 and 16 ns, together accounted for about 95% of the total. We prepared liposomes from lipids extracted from sarcolemma vesicles. The major lifetime of ANS fluorescence with suspensions of the liposomes was about 7 ns. From the protein residue after lipid extraction we prepared solutions of membrane protein. The major lifetime of ANS fluorescence in these solutions was about 16 ns. This suggested that ANS bound to both proteins and lipids of sarcolemma. But how much of each?

We propose that an answer can be obtained by analysis of steady-state fluorescence emission spectra of ANS in suspensions of sarcolemma vesicles. For this purpose an observed steady-state fluorescence emission spectrum is viewed as the sum of three independent spectra: that of ANS bound to membrane proteins, of ANS bound to membrane lipids, and of free ANS, or

$$I(\lambda) = I_P(\lambda) + I_L(\lambda) + I_F(\lambda) \tag{1}$$

where $I(\lambda)$ is relative fluorescence intensity as a function of wavelength, and subscripts P, L, and F refer, respectively, to the three species of ANS: protein-bound, lipid-bound, and free.

Each of these component spectra can be expressed as a function of four factors. For the case of protein-bound ANS, for example, these factors are: (a) the quantum yield, ϕ_P, of that species of ANS, (b) the probability density function of wavelength, $h_P(\lambda)$, of that species of ANS, (c) the concentration, c_P, of that species of ANS, and (d) an instrument factor α, common to all species of ANS, which depends on lamp intensity, instrument configuration, etc., and which relates fluorophor concentration to observed relative intensity. The probability density function of wavelength is independent of this instrument factor, and of quantum yield and fluorophor concentration. It is obtained by dividing each observed intensity by the total area under the curve; for example,

$$h_P(\lambda) = I_P(\lambda) / \int_0^\infty I_P(\lambda)\, d\lambda \tag{2}$$

From these considerations, substitutions into Eqn. 1 give the observed fluorescence emission spectrum as an explicit function of the concentration of each species of ANS,

$$I(\lambda) = \alpha[\phi_P h_P(\lambda) c_P + \phi_L h_L(\lambda) c_L + \phi_F h_F(\lambda) c_F] \quad (3)$$

Quantum yields were obtained from fluorescence lifetime spectra of ANS-membrane proteins, and of ANS-membrane lipids, for ϕ_P and ϕ_L, respectively, and a value from the literature was used for ϕ_F. The three probability density functions were obtained experimentally from appropriate preparations and calculated as defined by Eqn. 2. The concentration of free ANS is

$$c_F = c_T - (c_P + c_L) \quad (4)$$

where c_T, total ANS concentration, is known.

Hence, there are three unknowns in Eqn. 3: α, c_P, and c_L. Their values can be obtained by setting up three simultaneous equations of the type of Eqn. 3, each at a different λ. This was done; that is, the concentration of ANS bound to sarcolemma lipids and the concentration bound to sarcolemma proteins were obtained.

To test validity of Eqn. 3 and its components, the values of α, c_P and c_L were inserted into the equation with the known ϕ and h values and synthetic fluorescence emission spectra were generated for comparison with observed. Agreement was excellent.

Materials and Methods

Plasma membranes, sarcolemma, were prepared from hindleg muscles of male rats, 150—200 g body weight (CD strain, Charles River) by the method of Schapira et al. [8] with modifications described previously [9]. In brief, rats were killed by cervical fracture, hindleg muscles placed in cold 250 mM sucrose solution, minced, and homogenized in same solution at 4°C. After low speed centrifugation, the pellet was extracted in the cold overnight in 0.5 M LiBr, followed by a wash in 0.6 M KCl. The washed high speed pellet of this extract was placed on a discontinuous sucrose density gradient, centrifuged at 100 000 × g at 4°C for 2 h. The fraction at the interface between 8.5 and 30% (w/v) sucrose was taken to be sarcolemma. Electron microscopy showed vesicles heterogeneous in size, about 0.1—3 μm in diameter. No mitochondria were observed. The fraction bound insulin and ouabain specifically by the usual criteria, was enriched in $(Na^+ + K^+)$-ATPase, 5′-nucleotidase and adenylate cyclase and contained a specific D-glucose transport system. It was poor in $(Mg^{2+} + Ca^{2+})$-ATPase.

The sarcolemma fraction was harvested, suspended in 250 mM sucrose in 1 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4, and stored at −85°C. (In earlier experiments the buffer was 1 mM Tris, but when it was discovered that Tris, even at 0.1 mM, quenches ANS fluorescence we abandoned its use.) Protein was determined by the method of Lowry et al. [10].

For the preparation of lipid-poor protein and liposomes, the sarcolemma fraction was lyophilized and the dry residue homogenized in chloroform/

isopropanol (7 : 11, v/v). The low-speed sediment of the extract was suspended in and dialyzed against three changes of 250 mM sucrose in 1 mM HEPES, pH 7.4. About half the material originally designated 'protein' by the Lowry method entered this aqueous phase. The latter scattered very little light in the cuvette in the nanosecond fluorometer compared to light scattering by suspensions of sarcolemma vesicles or of liposomes. Because this protein was in an aqueous phase and scattered little light we shall refer to it as a solution of sarcolemma protein, although it was not a true solution because protein sedimented during 30 min of centrifugation at 100 000 $\times g$. Liposomes were prepared from the low-speed supernatant of the chloroform/isopropanol extracts as described by Czech [11]. Solvents were evaporated under a stream of N_2. Sucrose solution was added and the mixture sonicated intermittently for 1 min at 25°C with the microtip of a Branson sonifier. Protein content of these liposomes was less than 5% of that of the original sarcolemma preparation, and the quantity of liposomes added to cuvettes was expressed in terms of mg protein of sarcolemma from which it was derived.

ANS was obtained from Eastman Organic Chemicals and used without further purification. (Twice-recrystallized ANS prepared as the Mg^{2+} salt had more complex absorption and emission spectra.)

For studies of fluorescence, final volume in cuvettes was always 1.1 ml. In a given series the volume of ANS or its diluent (water), the volume of membrane component or its diluent (250 mM sucrose in 1 mM buffer) and the volume of 150 mM KCl were constant. The final concentration of KCl was never less than 80 mM, and usually was about 100 mM. Preliminary experiments confirmed the observations of Vanderkooi and Martonosi [3] and of Rubalcava et al. [12] that salt enhanced ANS fluorescence and demonstrated little or no additional enhancement when KCl concentration was increased from 80 to 120 mM.

Fluorescence emission spectra were examined in the American Instrument Co. (Aminco-Bowman) spectrophotofluorometer, Model J4-8912, with an ellipsoidal condensing mirror, xenon lamp with magnetic arc stabilizer, and with ratio compensation for altered intensity of excitation with spectral changes. The photomultiplier tube was RCA 4837 in early experiments. This tube peaks at 450 nm, and its response is attenuated sharply above 535 nm. In later experiments the broad spectrum tube R446 was used. Glan prisms were kept in place, one at the excitation window and one at the emission window, in all but the earliest experiments. Usually the analyzer and polarizer were parallel. In some later studies in which it was crucial to eliminate Raman scatter, the polarizer was perpendicular to the excitation source (which was partially polarized by the diffraction grating and mirror system) and the analyzer was perpendicular to the polarizer (i.e. parallel to the excitation source). Excitation wavelength was 385 nm. Temperature was 10°C.

Fluorescence lifetimes were measured by nanosecond fluorometry. The basic instrument was an Ortec Model 9200 nanosecond fluorometer. Excitation was by high voltage arc in air at about 30 kHz. There is a nitrogen peak at 382 nm, conveniently close to the absorption maximum from which ANS fluoresces. Visible light in the excitation spectrum was cut off by a Corning 7-54 filter. Emission passed through a diffraction grating to an RCA-8850 photomultiplier tube. Recording channels were set at 0.5-ns intervals. The Ortec Model 9351

cuvette housing was modified by substitution of an Aminco Model 4-8262 cuvett chamber milled to form conduits for circulation of temperature-controlled fluid and mounted on a perforated air nozzle to permit air flow in order to prevent fogging of the cuvette. Temperature was 10°C.

Analysis of fluorescence lifetime spectra. Lifetime spectra were analyzed by our method (unpublished). Briefly, an observed spectrum is considered to be made of two contributions, scattered light and true fluorescence. The true fluorescence function is the convolution integral of the probability density function of fluorescence lifetimes of the fluorophor, which we wish to determine, and a function we designate the absorption function. The light scatter function was obtained from suspensions of sarcolemma vesicles or of liposomes or from solutions of sarcolemma proteins, whichever was appropriate, all without ANS. The absorption function was obtained by deconvolving from the observed lifetime spectrum of ANS in 1-butanol the single lifetime. The tail of the observed spectrum of ANS-sarcolemma, or of ANS-liposome, etc., plotted semilogarithmically, was best-fitted to an exponential, from which the largest time constant was obtained. Deconvolution was by our numerical method [13], with the modification that the function was required to be a sum of exponentials operating in parallel, of which the largest time constant was that determined from the tail of the original function, as described above. The function obtained by deconvolution was tested by reconvolution until agreement with the observed spectrum was acceptable. We required only that the agreement look satisfactory to us. We did not subject it to autocorrelation tests used by others in analysis of fluorescence decay curves because we do not have the on-line capacity for that analysis. An example of our results is illustrated, and suggests that we are not likely to be sufficiently inexact in our estimate of the deconvolution to damage our conclusions.

Results and Discussion

Fluorescence lifetime spectra

Fluorescence lifetime spectra of ANS in suspensions of sarcolemma vesicles, in suspensions of liposomes from sarcolemma, and in solutions of protein from the residue after lipid extraction of sarcolemma were corrected for the contribution by scattered light and normalized to unit area (Fig. 1) to provide probability density functions of fluorescence lifetimes, $f_S(t)$, $f_L(t)$, and $f_P(t)$, respectively. The liposome function had the highest peak and the largest contribution by fast components. The protein function had the lowest peak and the largest contribution by slow components. The sarcolemma function was intermediate.

The tails of the three functions ultimately decayed with a similar time constant, about 16 ns. Since this time constant was the major component of the protein function, it is attributed to ANS bound to sarcolemma protein. Its persistence in the liposome preparation is probably due to the presence in the lipid extract of about 5% of the original membrane 'Lowry positive' material, which may be protein.

A solution of sarcolemma proteins was added to a suspension of liposomes from sarcolemma and shaken gently for 45 min at 37°C. The concentration of protein was selected to be the same as the concentration of protein in a suspen-

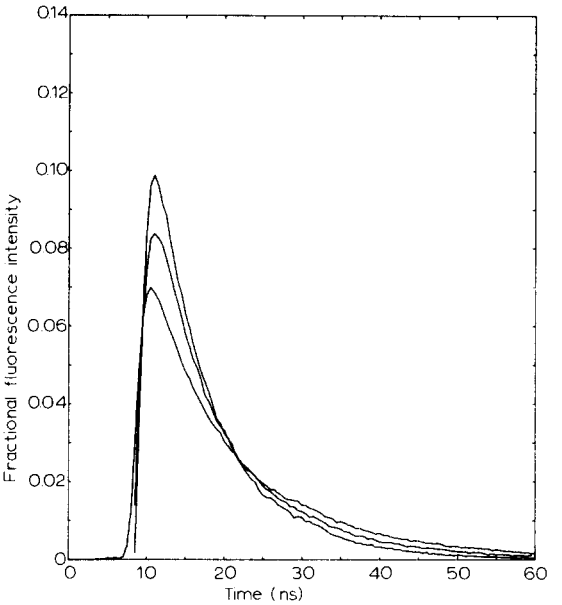


Fig. 1. Fluorescence lifetime spectra; probability density functions of fluorescence lifetimes; fractional fluorescence emission intensity as a function of time. From highest to lowest peak: ANS in suspension of liposomes from sarcolemma, ANS in suspension of sarcolemma vesicles, ANS in solution of proteins soluble after lipid extraction of sarcolemma. Observed spectra were corrected for light scatter; the spectra shown represent apparent true fluorescence.

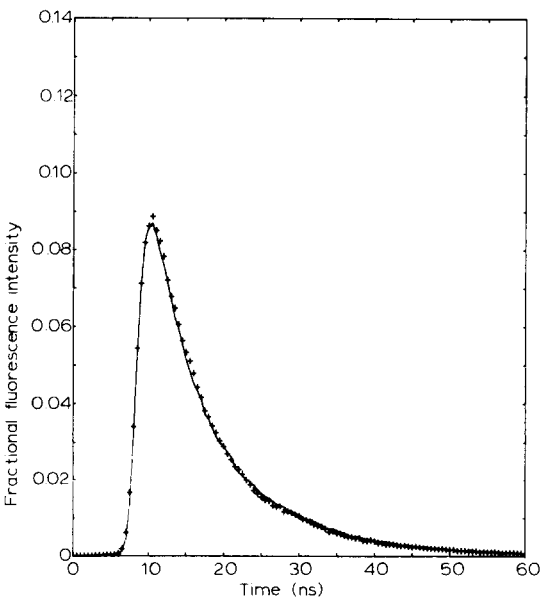


Fig. 2. Fluorescence lifetime spectra; probability density functions of fluorescence lifetimes. Continuous line: ANS in suspensions of sarcolemma vesicles (cuvette 1). +++, ANS plus a mixture of proteins and liposomes from sarcolemma (cuvette 2). The mixture contained a solution of sarcolemma proteins designed to make the protein content of cuvette 2 the same as that of cuvette 1. The liposome content of the mixture represented the lipid extracted from the amount of sarcolemma in cuvette 1. Close similarity of the two spectra suggests that ANS bound to both components contributes to the original sarcolemma lifetime spectrum.

sion of sarcolemma vesicles from which the liposomes and protein had been prepared. The liposome concentration was selected to be that quantity of lipid which had been extracted from sarcolemma vesicles identified by the same protein concentration. The fluorescence lifetime spectrum of ANS in this mixture of protein was barely distinguishable from the spectrum of ANS in sarcolemma vesicles (Fig. 2).

Since we knew from the separate studies of liposomes and of proteins that ANS bound to both with different distributions of fluorescence lifetimes (cf. Fig. 1), the study illustrated in Fig. 2 suggested that in sarcolemma vesicles ANS binding to lipid and to protein components was approximately additive, and that the fluorescence lifetime spectrum of ANS-sarcolemma was a weighted sum of the spectra with liposomes and protein. Accordingly, we calculated the time constants from fluorescence decay of ANS with sarcolemma liposomes and with sarcolemma proteins and attempted to fit the fluorescence lifetime spectra of ANS-sarcolemma by using those time constants.

The true fluorescence functions of ANS in sarcolemma, sarcolemma liposomes, and sarcolemma proteins, $h_S(t)$, $h_L(t)$, and $h_P(t)$, respectively are concealed in the fluorescence spectra as components of convolution integrals:

$$f_S(t) = A(t) * h_S(t)$$

$$f_L(t) = A(t) * h_L(t)$$

$$f_P(t) = A(t) * h_P(t)$$

where $A(t)$ is the ANS absorption function, obtained by deconvolution of the lifetime spectrum of a standard solution of ANS in 1-butanol.

Instead of obtaining the h values by deconvolution, techniques for which have been criticized [14], we convoluted on $A(t)$ function of the type

$$\sum_{i=1}^{n} (a_i/\tau_i)\, e^{-t/\tau_i}$$

where $0 < a_i$ and $\Sigma_{i=1}^{n} a_i = 1$. This is the probability density function for a set of independent, parallel fluorescence decays. First the τ and a values were obtained approximately by numerical deconvolution of $f_L(t)$ and $f_P(t)$. Both $h_L(t)$ and $h_P(t)$ had a fast time constant of 4 ns and a slow time constant of about 16 ns. In addition, $h_L(t)$ had as its major component an intermediate time constant of 7 ns. The set of three $h(t)$ values corresponding to the three $f(t)$ values of Fig. 2 is displayed in Fig. 3.

We required that the sarcolemma function be of the form

$$(a_1/4)\, e^{-t/4} + (a_2/7)\, e^{-t/7} + (a_3/\tau_P)\, e^{-t/\tau_P}$$

where τ_P, usually about 16 ns, was the time constant of the best-fitted exponential to the tail of each observed $f_S(t)$. Since the sum of the a values is unity, there are only two degrees of freedom in selecting parameters to fit the curves. Fig. 4 displays an example of the agreement between the convoluted function, $A(t) * h_S(t)$ and the normalized observed spectrum corrected for light scatter, $f_S(t)$. We make no claim that the chosen value of $h_S(t)$ is the best possible fit, only that it is an acceptable fit and that the fluorescence lifetimes

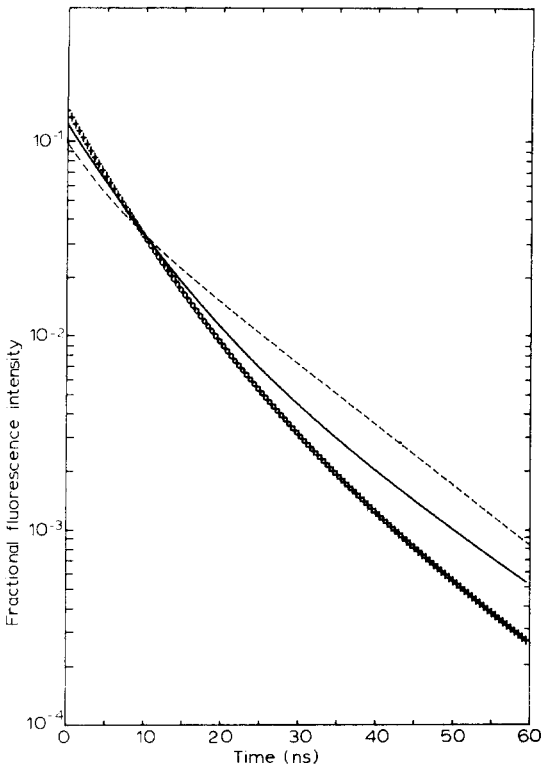


Fig. 3. Deconvolved fluorescence lifetime spectra; probability density function of fluorescence lifetimes. Semilog plot. From highest to lowest zero-time intercept: ANS in suspension of liposomes from sarcolemma (+++), ANS in suspension of sarcolemma vesicles (———), ANS in solution of proteins from sarcolemma (- - - - -). The equations are of the form $\Sigma_{i=1}^{n} (a_i/\tau_i)e^{-t/\tau_i}$. Parameters were:

	a_1	a_2	a_3	τ_1	τ_2	τ_3
ANS-liposomes	0.15	0.65	0.20	4	7	15
ANS-protein	0.15	0	0.85	4	—	14
ANS-sarcolemma	0.07	0.6	0.33	4	7	16.25

can be consistent with the interpretation that ANS binds to both lipids and proteins of sarcolemma.

The a values have physical meaning. If the total concentration of sarcolemma-bound ANS is c_B, then,

$$a_i = c_i/c_B$$

where each numerator c is concentration of one kind of membrane-bound ANS. In the previous paragraph we associated a_3 with the longest time constant, τ_P, about 16 ns, which was the major component of the lifetime spectrum of protein-bound ANS. From Fig. 3, a_3 was about 0.33. This means that this major component of protein-bound ANS constituted about 33% of total sarcolemma-bound ANS under the condition in which fluorescence lifetimes were studied. We associated a_2 with the intermediate τ, 7 ns, the most common component of liposome-bound ANS. From Fig. 3, this lipid-bound ANS constituted about 60% of total sarcolemma-bound ANS under these conditions. The remaining 7% of sarcolemma-bound ANS had a τ of about 4 ns.

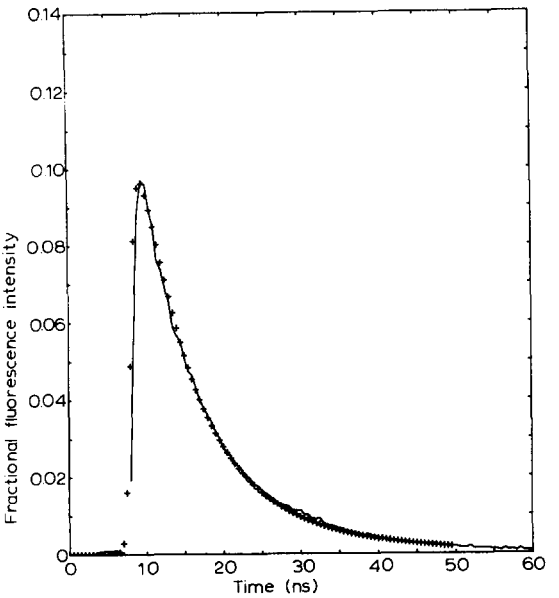


Fig. 4. Comparison of calculated function, $A(t) * h_S(t)$, with $f_S(t)$, the normalized observed fluorescence lifetime spectrum of ANS in suspension of sarcolemma corrected for light scatter. Parameters of $h_S(t)$ are those given for ANS-sarcolemma in legend to Fig. 3. Continuous line, $f_S(t)$. +++, convoluted function.

The source of this component, whether lipid or protein, is not known. However, because it dominated the spectrum of ANS-liposomes under other experimental conditions (increased temperature), it is attributed to lipid-bound ANS. Thus, from analysis of fluorescence lifetime spectra we deduced that the ratio of total lipid-bound ANS to protein-bound ANS in suspensions of sarcolemma is about 2 under the conditions in which lifetime spectra were obtained.

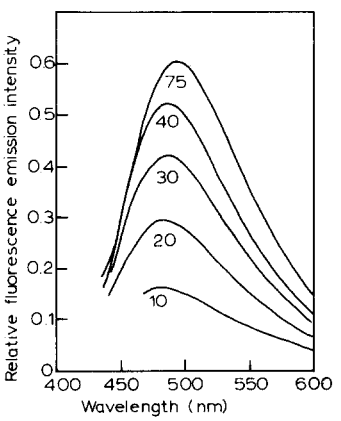
It is difficult to compare our estimate of fluorescence lifetimes with those reported by others because the biological preparations are different and the methods of analyses of the spectra are different. The first report of fluorescence lifetime spectra of ANS and biomembranes was by Brocklehurst et al. [15] in 1970. In non-energized, heavy mitochondria the decay curves were said to have two components with lifetimes of 5 and 9 ns. However, their illustration (their Fig. 8) shows that the tail of the lifetime spectrum fell with a half-time at least 10 ns, giving a τ of at least 14 ns. In 1971 Wahl et al. [16] obtained fluorescence lifetime spectra of ANS with suspensions of membrane fragments from the electric organ of *Electrophorus electricus*. These workers corrected for background light and deconvoluted to obtain the true lifetime function, which they resolved into a sum of three exponentials with time constants of 2, 8, and 17 ns at 20°C, somewhat similar to the set we found. However, the distribution was different. The term with the largest τ contributed about 60% of the total, nearly twice as much as we found for sarcolemma. That lifetime spectra from different membranes should have different weighting factors for the time constants is not surprising, since membranes differ in the relative lipid and protein content. Haynes and Staerk [4] reported a comprehensive study of fluorescence lifetime spectra of ANS with phospholipid vesicle membranes. They report but a single lifetime for each condition

studied, but do not describe the method by which it was calculated. This lifetime ranged from 5.4 to 9 ns depending on temperature, kind of fatty acid, phospatidic acid or cholesterol content, and the kinds of ions and their concentrations in the suspending solution. The results bolster our confidence that the long lifetime of ANS with sarcolemma is sassociated with protein-bound, not lipid-bound, ANS.

Steady-state fluorescence emission spectra

In a series of cuvettes at a given concentration of sarcolemma vesicles, steady-state fluorescence emission spectra shifted to the right and peak intensity increased as total ANS concentration increased (Fig. 5), until ANS reached 100 μM, at which there was evident excimer formation (intensity decreased and peak shifted further to the right). In the experiment illustrated in Fig. 5 the emission peak shifted from 480 to 493 nm as ANS concentration increased from 10 to 75 μM. In other experiments, at lower ratios of ANS/membrane protein, the emission peak was at 475 nm.

The companion experiment is one in which at constant ANS concentration in a series of cuvettes, sarcolemma vesicle concentration was increased. In this case, the spectrum shifted to the left and peak intensity increased as sarcolemma vesicle concentration increased (Fig. 6). At the lowest membrane concentration, emission peak was 535 nm. It shifted to 487 nm at the highest membrane concentration illustrated in Fig. 6, but in similar series, in which



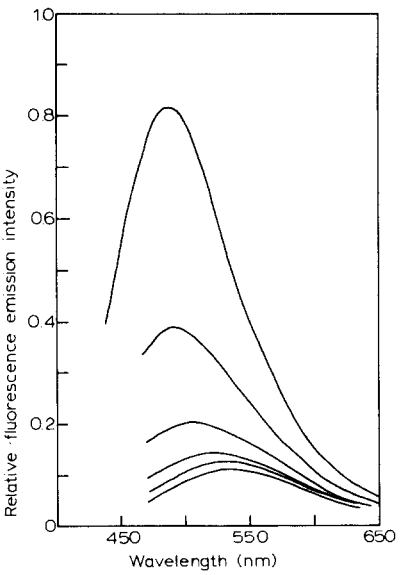


Fig. 5. ANS-sarcolemma vesicles. Steady-state fluorescence emission spectra. Relative fluorescence intensity as function of wavelength. Constant sarcolemma concentration, varying ANS concentration as indicated on curves (in nmol/ml). Membrane protein, 4 μg/ml. Note not only increase in peak intensity but shift to right as ANS increased.

Fig. 6. ANS-sarcolemma vesicles. Steady-state fluorescence emission spectra. Relative fluorescence intensity as function wavelength. Constant ANS concentration (30 nmol/ml). Varying sarcolemma protein concentration from highest to lowest (μg/ml): 16.8, 2, 1, 0. Note not only increase in peak intensity but shift to left as protein concentration increased.

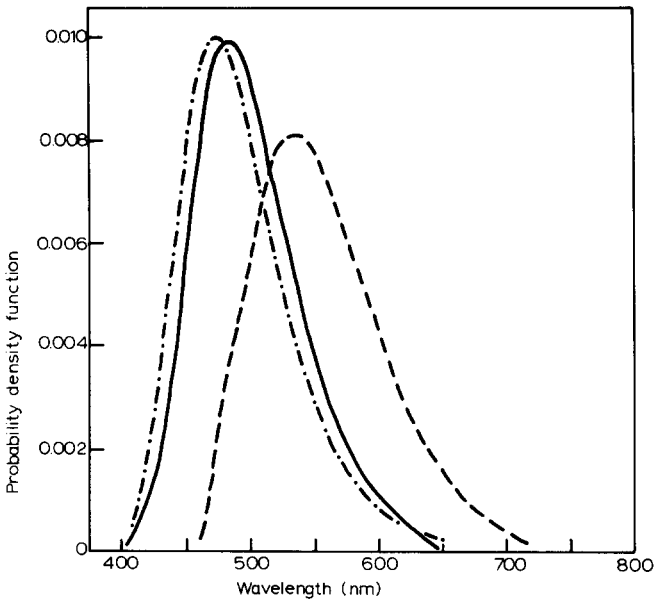


Fig. 7. Probability density functions of wavelength; normalized steady-state fluorescence emission spectra. From left to right: - - - - -, h_P; ———, h_L; - - - - -, h_F.

sarcolemma concentrations were increased by a further factor of about 10, peak emission shifted to as low as 475 nm.

Steady-state fluorescence emission spectra were obtained of ANS in solutions of sarcolemma proteins, in suspensions of liposomes from sarcolemma vesicles, and in aqueous solutions. Normalized spectra, the probability density functions of wavelength, designated $h_P(\lambda)$, $h_L(\lambda)$, and $h_F(\lambda)$, respectively, appear in Fig. 7. Emission peaks were 472, 489, and 535 nm, respectively. Since the liposomes retained some of the original protein, it is possible that the peak of $h_L(\lambda)$ would be even further to the right if all proteins were removed. There is a suggestion from studies of fluorescence lifetimes that there may have been a lipid component among the proteins, so that true $h_P(\lambda)$ might be even further to the left. It is clear that the emission spectrum of ANS-sarcolemma can run the gamut from a peak nearly as blue as that of $h_P(\lambda)$ to one nearly as red as that of $h_F(\lambda)$, depending on the ratio of ANS to sarcolemma concentrations. This suggests that the distribution of ANS among the various species of bound ANS and between free and bound ANS changes as the ratio of ANS to sarcolemma changes. It is evident that some ANS must be bound to protein, otherwise peak emission could not be to the left of that of $h_L(\lambda)$, 489 nm. It is also evident that at very high ratios of ANS to sarcolemma, despite the small quantum yield of free ANS, so much ANS is free that it can dominate the spectrum.

If there were only two kinds of ANS, protein-bound and free, the graded shifts in emission peak shown in Figs. 5 and 6 could not occur. If this were the case, the equation for emission intensity as a function of wavelength, analogous to Eqn. 3, would be

$$I(\lambda) = \alpha[\phi_P h_P(\lambda) c_P + \phi_F h_F(\lambda) c_F]$$

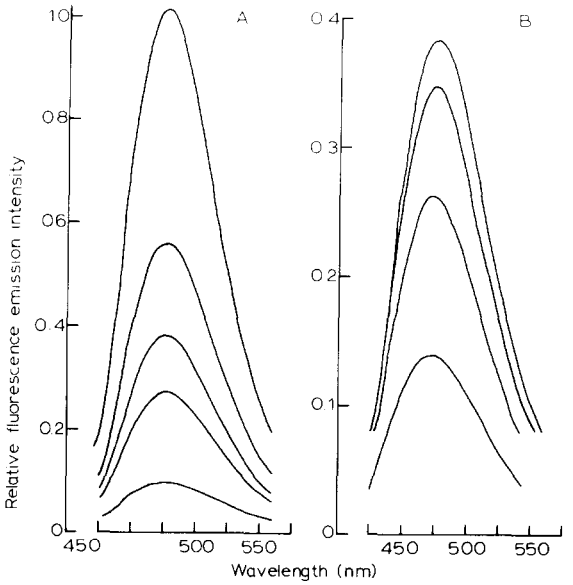


Fig. 8. ANS-bovine serum albumin solution. (A) Constant ANS concentration (10 nmol/ml), varying albumin concentration (10, 30, 40, 50, 100 μg/ml). (B) Constant albumin concentration (40 μg/ml), varying ANS concentration (1, 2.5, 5, 10 nmol/ml). Note that, in contrast to results shown in Figs. 5 and 6, there was little or no spectral shift with altered fluorescence intensity. This suggests homogeneity of binding sites for ANS-albumin but heterogeneity for ANS-sarcolemma.

Because ϕ_P/ϕ_F is about 200, protein-bound ANS dominates the spectrum until the ratio of free/bound ANS gets to be greater than 200. For that reason intensity falls but the spectrum does not shift as the ratio ANS/sarcolemma is increased until very high ratios are reached. This was indeed the behavior of ANS in solutions of protein, either protein from sarcolemma or bovine serum albumin (Fig. 8), in a series of cuvettes in which the ANS/protein ratio was varied. However, this was not the behavior of ANS in suspensions of sarcolemma vesicles. Hence, it seemed likely that at least two kinds of bound ANS are required to account for the observed emission spectra.

Accordingly, as described in Introduction, sets of three simultaneous equations were solved for the three unknowns, α, c_P, and c_L. (Specifically, an observed $I(\lambda)$ from a cuvette containing ANS in a suspension of sarcolemma vesicles was digitized at 5-nm intervals. Eqn. 3 was used. The left-hand side was the numerical value of I at a selected λ. The right-hand side contained the known values of h_P, h_L, and h_F, each at the same selected λ, the known values of the quantum yields, ϕ_P, ϕ_L, and ϕ_F, the known value of total ANS, c_T, and the three unknowns.) The values of α, c_P, and c_L were substituted in Eqn. 3, and an $I(\lambda)$ was then generated synthetically over a range of wavelengths. Examples appear in Fig. 9. Agreement was pleasing, and argues that, since we can generate emission spectra like those observed in response to altered ratios of ANS to sarcolemma protein, our conjecture about the composition of those spectra is not contradicted; that is, we can fit observed spectra on the assumption that they are made up of variously weighted contributions from two kinds of bound ANS, protein-bound and lipid-bound, and from free ANS.

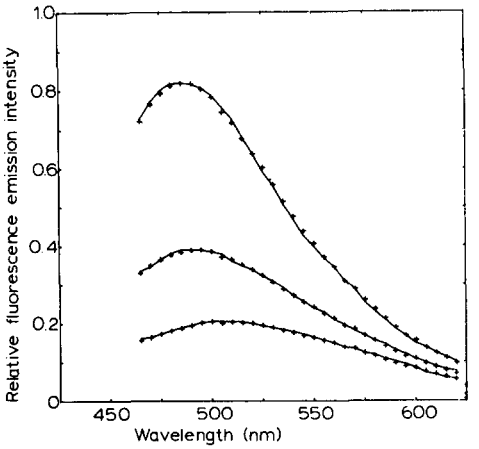


Fig. 9. ANS-sarcolemma vesicles. Synthesis of steady-state fluorescence emission spectra. Relative fluorescence intensity as function of wavelength. Continuous lines, observed spectra; 30 nmol NAS/ml; protein concentration, from highest to lowest, 16, 8, and 4 μg/ml; +++, synthesized spectra.

Over the range examined, the calculated value of ANS bound to sarcolemma lipid, c_L, was always at least twice as great as the calculated value of ANS bound to sarcolemma protein, c_P.

Note that in Eqn. 3 and its empirical solution only two quantum yields, ϕ_P and ϕ_L, were used even though fluorescence decay spectra required three lifetimes. From the decay spectra, ANS bound to sarcolemma lipids had two lifetimes, about 4 and about 7 ns. In order to reduce the number of parameters requiring solution we made the simplifying approximation that, for purposes of analysis of steady-state emission spectra, lipid-bound ANS could be treated as a single species. If there are two lipid-bound ANS species, as the decay spectra suggest, the one with a lifetime of 4 ns is only about 10% of the total lipid-bound ANS. If there is only one $h_L(\lambda)$, and we can distinguish only one, then the factor $\phi_L c_L$ in Eqn. 3 can be equated to

$$\phi_L c_L = \phi'_L c'_L + \phi''_L c''_L$$

where the primes and double primes refer to two distinct kinds of membrane lipid-bound ANS, and $c_L = c'_L + c''_L$. If $c''_L = 0.1\ c'_L$ and $\phi''_L = 4/7\ \phi'_L$, the error in our simplification, that $\phi_L c_L = \phi'_L c'_L$, is about 5%. If we have overestimated ϕ_L by 5%, we have underestimated c_L by 5% because we could treat $\phi_L c_L$ as a unit in the solution of the set of three simultaneous equations.

Critique of methods intended to measure the concentration of membrane-bound ANS

Our determination of c_P and c_L from Eqn. 3 revealed that most of the ANS bound to sarcolemma is lipid bound. However, since solutions by sets of three simultaneous equations are inherently associated with large variance, the results were not suitable for analysis such as by Scatchard plots or by Hill plots, designed to give solutions for the binding parameters: capacities and affinities of membrane proteins and lipids for ANS and indices of cooperativity.

Nor were we successful in measuring the total concentrations of bound

ANS, $c_B = c_P + c_L$. In this case, the problem was to convert observed intensity of fluorescence emission to concentration of bound ANS, c_B. Even though the literature contains reports of such conversion by three kinds of methods, none of these methods was satisfactory in our hands.

The first method is based on physical separation of membrane fragments from the aqeuous phase, either by centrifugation [2,3,6,17] or by filtration [3,18], with measurement of ANS in the aqueous phase (free ANS) by absorption at 360 nm. We used both forms, ultracentrifugation [3] and filtration through Amicon centriflo cones [18]. The main problem is that in order to approach closely saturation of membrane binding sites one must use high ratios of ANS/membrane. Under such conditions nearly all ANS is free. The calculated concentration of bound ANS is a small difference between two large numbers, total and free ANS, and is subject to large error. Although it is possible to measure the concentration of bound ANS by this method under conditions in which a substantial fraction of total ANS is bound, one cannot use the relationship between bound ANS concentration and fluorescence emission intensity under these conditions to extrapolate to conditions at high ANS concentration and estimate bound ANS from observed fluorescence emission intensity. The reason is that the relationship between fluorescence emission intensity and concentration of bound ANS is not linear. There are three classes of quantum yields for fluorescence of sarcolemma-bound ANS. Since the relative concentrations of lipid-bound and protein-bound ANS vary as total ANS concentration changes, the non-linear relation predicted by Eqn. 3 follows. (An important inference from Eqn. 3 is that if more than one form of the fluorophor contributes non-negligibly to fluorescence emission intensity, the relationship between intensity, $I(\lambda)$, and total concentration of bound fluorophor is not linear. Only if there is a single form of bound ANS and if the contribution of free ANS is negligible does Eqn. 3 reduce to the linear expression

$$I(\lambda) = \alpha \phi_B h_B(\lambda) c_B$$

where the subscript B refers to bound fluorophor).

The second method for measuring bound ANS concentration is based on addition to the cuvettes, after fluorescence emission of membrane-bound ANS has been measured, of enough albumin to bind all the ANS [1]. It is assumed that (a) fluorescence emission intensity of this mixture represents that intensity which would have occurred had the membranes bound all the ANS and (b) fluorescence emission intensity is a linear function of membrane-bound ANS. We have already seen that assumption b is false. Assumption a is false because the quantum yield of ANS-albumin is not the same as that of ANS-sarcolemma.

The third method is based on plots of reciprocal fluorescence emission intensity against reciprocal total ANS concentration from experiments with constant total ANS and varied membrane concentration. Such plots are reported to be linear [18]. The intercept on the reciprocal intensity axis occurs at infinite protein concentration and is taken as the reciprocal of intensity when all ANS is bound. In our experience such plots were uniformly linear with remarkable correlation coefficients, never less than 0.999. Yet in about one-third of the experiments at 10°C the extrapolated intercept was negative,

which yields a physically inadmissible result. Accordingly, the theoretical basis of this method was examined and it was concluded that the double reciprocal plot cannot be analytically a straight line if the classical mass action equilibrium is obeyed [19]. The linear extrapolation is false and can lead to unbounded error, as it did in our case.

Inferences concerning relative capacities and affinities between ANS and membrane proteins and ANS and membrane lipids

Despite our failure to obtain sufficiently sensitive measurements of bound ANS concentrations we can make helpful inferences about the binding parameters.

Fig. 10 is a plot of relative fluorescence emission intensity at the emission maximum as a function of total ANS concentration. The two points at which intensity of ANS-sarcolemma proteins was measured nearly coincide with corresponding points on the curve for ANS-sarcolemma lipids. The concordance of the points argues that the ratio of bound ANS concentrations is the reciprocal of the ratio of quantum yields, as the following argument shows.

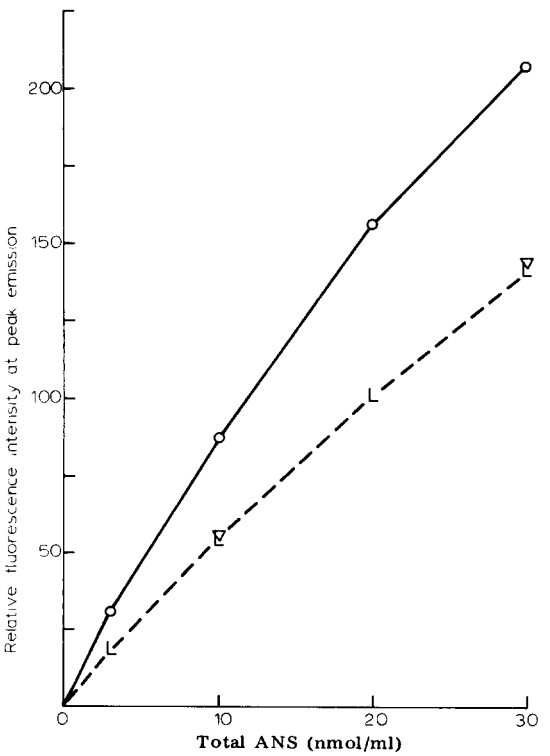


Fig. 10. Relative fluorescence emission intensity as a function of total ANS concentration at constant protein concentration. ○———○, sarcolemma; L- - - - -L, liposomes from sarcolemma; ▽, protein from lipid-extracted sarcolemma. Wavelengths of peak emission (nm): sarcolemma, 475—478; protein, 470—472; liposomes, 485—487. Sarcolemma protein concentration, 0.1 mg/ml; protein concentration, 0.1 mg/ml; liposomes equivalent to lipid extracted from sarcolemma vesicles at protein concentration of 0.1 mg/ml.

From the considerations that led to Eqn. 3,

$I_L(\lambda) = \alpha\phi_L h_L(\lambda)c_L$

and

$I_P(\lambda) = \alpha\phi_P h_P(\lambda)c_P$

where c_L is the concentration of liposome-bound ANS and c_P is the concentration of protein-bound ANS.

Notice in Fig. 7 that at its peak, 489 nm, h_L is approximately the same amplitude as h_P at its peak, 472 nm. Notice in Fig. 10 that I_L is approximately equal to I_P, where each intensity was read at its peak, near 489 and 472 nm, respectively. It follows that for the case of ANS-liposomes and ANS-proteins,

$c_L/c_P \simeq \phi_P/\phi_L \simeq 2.2.$

This ratio is not the ratio of c_L/c_P in sarcolemma. That ratio varies with total ANS concentration, as it must in order to account for the spectral shift. In Fig. 9, the fits to the observed spectra did require different ratios of c_L/c_P; the ratio decreased as sarcolemma concentration increased, as peak emission shifted toward blue.

In Fig. 10, the concentration of bound ANS is increasingly dependent upon binding capacity, rather than upon affinity, as total ANS concentration increases. Hence, the fact that the intensities of protein-bound and lipid-bound ANS are about the same at 30 μM ANS, argues not only that c_L/c_P is approx. 2, but that the binding capacity of lipids is about twice as great as that of proteins prepared from sarcolemma.

There is additional information in Fig. 10. The sum of intensities of protein-bound ANS and lipid-bound ANS exceeds the observed intensity of ANS-sarcolemma at the corresponding total ANS concentration. This is true even when intensities are compared at the same wavelength. This is not due to quenching, which does not occur until higher ANS concentrations are reached. There are several possible explanations. One is that the binding behavior of lipids and/or of proteins toward ANS is different in native sarcolemma than in the separated components. This difference is quite likely for the case of proteins, because the protein fraction used here was only about half the original membrane protein and represented proteins with particular physical properties; they were not precipitated after chloroform/isopropanol extraction of sarcolemma. There could also be differences between liposomes from sarcolemma and lipids in complete sarcolemma. A second possibility is that binding sites available on the separate components are occluded in complete sarcolemma. This must be true particularly for membrane proteins; most of the potential binding sites must be buried in or posted antipodally in the membrane. Thus, it can be argued that the relative concentration of protein-bound ANS to lipid-bound ANS is even less in complete sarcolemma, suggesting that the capacity to bind ANS may be substantially greater than twice as much by lipid than by protein in complete sarcolemma.

We can draw inferences about relative affinities by examining the wavelength of peak emission as a function of total ANS concentration, at constant sarcolemma concentration. Since the emission peak of the ANS-protein solu-

tion occurred at 472 nm and that of ANS-liposomes at 489 nm, and since the quantum yield ratio and concentration ratio, c_L/c_P, were approximately reciprocal at higher ANS concentrations, we would expect the emission peak of ANS-sarcolemma to fall midway between the two wavelengths, at about 480 nm, if the affinities were nearly the same. However, at low ratios of ANS to sarcolemma the emission peak was 475 nm, close to that of protein-bound ANS. This argues that the affinity between ANS and sarcolemma protein is substantially greater than that between ANS and sarcolemma lipids.

Thus, although we do not know the values of capacities and affinities, and we do not know if there is cooperativity with respect to ANS binding, we can infer that the capacity of sarcolemma lipids to bind ANS is at least twice as great as the capacity of sarcolemma proteins, and that the association constant between sarcolemma proteins and ANS is substantially greater than that between sarcolemma lipids and ANS.

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