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The Interaction of 1-Anilino-8-naphthalenesulfonate with the Membranes of the Sarcoplasmic Reticulum and Various Lipid Compounds

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Summary. (1) The enzymatic removal of lipids from the vesicular membranes of the sarcoplasmic reticulum does not interfere with the fluorescence of the 1-anilino-8-naphthalenesulfonate (ANS) vesicular complex. (2) The fluorescence intensity of the ANS vesicular complex is considerably (50%) reduced by oleic acid (0.5 mM) because it displaces ANS from its binding sites. (3) Stearic acid, which also combines with the membranes, interferes neither with ANS binding nor with ANS fluorescence. (4) Of all lipid compounds tested, oleylamine produces the most pronounced fluorescence enhancement of ANS. (5) The complexes formed between oleic acid and cetyltrimethyl ammonium salts or between oleic acid and polylysine produce a much higher fluorescence enhancement than the isolated components. (6) Low concentrations of ether added to ANS-containing vesicular suspensions reduce their fluorescence intensity. It returns to the initial intensity when the ether is removed. (7) A small cyclic change of the fluorescence of the vesicular ANS complex takes place during active calcium uptake.

The dye 1-anilino-8-naphthalenesulfonate (ANS) has been found to combine with various proteins, lipids and lipoproteins, especially with those of biological membranes (Tasaki, Watanabe, Sandlin & Carnay, 1968; Chance, Azzi, Mela, Redda & Vainio, 1969; Vanderkooi & Martonosi, 1969). The adsorption of the dye to these compounds results in a strong enhancement of its fluorescence. Concomitantly the maxima of the emission spectra are shifted towards shorter wavelengths. Similar changes in the fluorescence of ANS-containing solutions occur when their dielectric constant is reduced. Therefore, it has been assumed that the dye interacts with apolar regions of the surface of the protein molecules or the lipid micelle.

Because of these properties, ANS has been used in recent studies as a probe to detect structural change in various membranes. Chance *et al.* (1969) found that the fluorescence of a dye-containing suspension of mitochondria

increases on addition of calcium or of the local anesthetic butacaine. Tasaki *et al.* (1968) reported that during excitation of the squid axon a minute increase in the fluorescence of the adsorbed dye can be observed.

This report deals with the interaction of ANS with the sarcoplasmic membranes whose ANS-fluorescence-enhancing properties have been studied recently by Vanderkooi and Martonosi (1969). The authors attribute the fluorescence enhancement largely to the interaction of the dye with the membrane phospholipids. In this study, evidence is presented that the fluorescence arises from specific interactions of the dye with the membrane protein. The reduction of the fluorescence which occurs when oleic acid or dodecyl sulfate is bound by the sarcoplasmic membranes can be attributed to a displacement of the dye from amino groups of the membrane protein.

Materials and Methods

The sarcoplasmic vesicles were prepared from rabbit skeletal muscle as described by Hasselbach and Makinose (1963). The lipid compounds were dissolved in alcohol and a small volume of the solution (~0.04 ml) was added to the assay medium (4 ml). Homogeneous suspensions of glycerol tristearate and cholesterol were prepared by ultrasonication. All experiments were performed in media containing 0.2 M KCl, 20 mM phosphate, 10 mM Mg^{++}, pH 6.5, and 0.1 to 0.5 mg vesicular protein/ml. Deviations are given in the legends.

The fluorescence measurements were made with a Hitachi-Perkin Elmer Spectrophotofluorimeter. Emission and excitation spectra were recorded for all ANS-containing mixtures. All data presented were measured at a wavelength of 360 nm for excitation and 470 nm for emission. The data are given as relative fluorescence intensities. The intensities are corrected for the absorption of the exciting light by the free dye in the solution. The correcting factors were obtained from the decrease of the fluorescence intensity of a vesicular suspension with increasing concentration of the dye.

The chemicals used were obtained from the following sources: lecithin, lysolecithin and ANS magnesium salt from Serva (Heidelberg, Germany); oleic acid from Merck (Darmstadt, Germany); oleylamine from Ega-Chemie Germany; ANS sodium salt from Eastman Kodak; and phospholipase A from Boehringer (Mannheim, Germany).

ANS binding was determined in the standard medium containing 0.8 to 1.2 mg protein/ml and 0.05 or 0.1 mM ANS. The protein was precipitated by centrifugation at 40,000 rpm for 1 hr. The dye concentration in the supernatant was determined spectrophotometrically at 270 nm. The measurements were corrected for the absorption of supernatants of dye-free suspensions with and without fatty acids.

Results and Discussion

A vesicular suspension containing 1 mg protein/0.1 mM ANS fluoresces at pH 6.5 as intensely as a solution of the same dye concentration in ~90% alcohol. Since in the aqueous vesicular suspension approximately 70% of the dye remains free in the solution (Table 1), and therefore does

Table 1. *ANS binding to sarcoplasmic vesicles and its reduction by oleic and stearic acids. Standard medium; 15 °C*

Preparation	Concentration of free ANS (μM)	Bound ANS (μmoles/mg protein)		
		No addition	With oleic acid (500 μM)	With stearic acid (500 μM)
1	52	0.05	—	—
	29[a]	0.03	—	—
2	69	0.03	0.015	—
	85	—	—	—
3	72[b]	0.07	—	—
	85[b]	—	0.04	—
	55	0.05	—	—
	70	—	0.03	—
4	75	0.028	—	0.021
	72	0.035	—	0.038

[a] Total concentration of ANS in medium: 0.05 mM.
[b] Protein concentration: 0.40 mg/ml. Otherwise, 0.8 to 1.0 mg/ml.

not contribute to the fluorescence, the dielectric constant at the binding site of the dye must be considerably lower than 30.

The same fluorescence enhancement observed for native vesicles is produced by vesicular preparations from which the lecithin and cephalin fraction have been removed by phospholipase A digestion in the presence of albumin [(Fiehn, 1969) (Fig. 1)]. These two lipids comprise more than 80% of membrane lipids. The neutral lipids, cholesterol and glycerides which remain in the membrane after enzymatic delipidation should give rise to only a very weak fluorescence enhancement (Table 2). In contrast, the phospholipids lecithin and cephalin that are hydrolyzed by phospholipase A produce a considerable fluorescence enhancement, in agreement with Vanderkooi and Martonosi (1969) who first reported that these compounds can enhance ANS fluorescence. According to the data of Table 2, the ANS lipid fluorescence can contribute maximally to about 40 to 50% of the total vesicular fluorescence. However, a contribution of the phospholipids to the vesicular ANS fluorescence can hardly be reconciled with the finding that the vesicular fluorescence does not decline when the phospholipids are removed by phospholipase A treatment in the presence of albumin. Two explanations seem possible:

1. The apolar environment in the membrane to which the dye is bound belongs solely to the membrane protein and not to the membrane lipids.

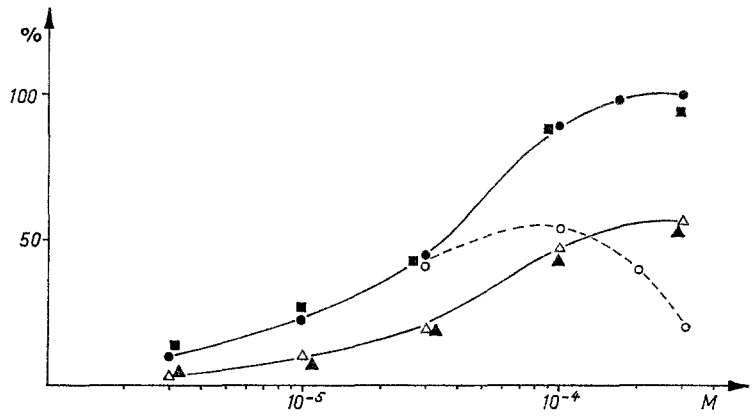

Fig. 1. The increase of the fluorescence intensity of the ANS vesicular complex with rising ANS concentration. Ordinate: the maximal fluorescence intensity produced by normal vesicles has been taken as 100. Abscissa: total ANS concentration (M). ●, normal vesicles (0.5 mg/ml); ■, delipidated vesicles (0.5 mg/ml); △, normal, and ▲, delipidated vesicles in the presence of 1 mM oleate; – – – uncorrected fluorescence intensity of normal vesicles

This assumption contradicts the statement of Vanderkooi and Martonosi (1969) that the ANS fluorescence by the sarcoplasmic membranes may be attributable largely to membrane phospholipids. However, their statement is at variance with their own observation that phospholipase C digestion reduces the fluorescence intensity maximally by 40 to 50%. Therefore, a considerable contribution of the membrane protein to the enhancement of the fluorescence must be assumed. Furthermore, the role of the membrane protein is not disproved by the finding that trypsin digestion does not reduce ANS fluorescence (Vanderkooi & Martonosi, 1969). Since only 30% of the vesicular protein becomes soluble during trypsin digestion, it is very likely that the hydrophobic sites which are essential for ANS fluorescence are located in the insoluble residue.

2. The adsorbed dye fluoresces in combination with both membrane components. In this case, however, the decrease of the fluorescence which should be caused by the removal of the lipids has to be compensated for by an increase of the fluorescence of the membrane protein:ANS complex. The latter condition makes the second version rather unlikely.

As to the requirements a structure must fulfill to enhance ANS fluorescence, the weak ANS fluorescence observed in suspensions of neutral lipid shows that factors in addition to hydrophobicity must be involved.

Table 2. *The ANS fluorescence of sarcoplasmic vesicles, sarcoplasmic lipids, a. d va. ious lipid analogues*[a]

Preparation	Concentration (mg/ml)	Relative fluorescence[b] (%)
Normal vesicles	0.3	100
	0.1	38
Delipidated vesicles	0.3	95
Vesicular lipid extract	0.4	120
	0.15	40
Lecithin	0.3	85
Glycerol tristearate	0.9	10
Cholesterol	1.0	12
Oleic acid	1.4	12
	0.28	2.5
Oleylamine	0.17	230
Cethyltrimethyl ammonium bromide	0.24	150
Butylamine	7.3	1.5
Polylysine	0.5	1.5
Polylysine + oleic acid	0.5 + 0.12	13
Polylysine + oleic acid	0.5 + 0.28	30

[a] The concentrations of the lipids that can contribute markedly to the fluorescence of the sarcoplasmic membranes have been chosen so that a direct comparison with the normal vesicles is possible. For instance, 0.15 mg of vesicular lipid extract corresponds to the lipid content of 0.3 mg of vesicular protein. Furthermore, since under the condition applied the fluorescence intensity is proportional to the concentrations of the enhancing agents (Vanderkooi & Martonosi, 1969), the data can be compared. For glycerol tristearate: cholesterol oleate mixtures with ANS, the maxima of the emission spectrum has been found between 500 and 470 nm depending on the concentration of the lipid compounds. The intensity at 470 nm is maximally 30% lower than at the emission peak. The intensity of the ANS fluorescence of oleylamine and cethyltrimethylammonium bromide in aqueous solutions is not at all or only very little enhanced by the addition of salts.

[b] Relative fluorescence of solvent: 1.5%. All data are related to the fluorescence of a suspension containing 0.3 mg/ml. The solvent contained 0.2 M KCl, 20 mM phosphate (pH 6.5), 20 mM $MgCl_2$, and 0.1 mM total final ANS concentration. The lipids dissolved in alcohol were added to the solvent and sonicated if necessary to obtain homogenous suspensions.

Total concentration of ANS in medium: 0.05 mM.

The data presented in Table 2 and Fig. 2 show that the simplest model compounds which produce a similar or even more intense fluorescence enhancement than the vesicles are primary and quarternary amines with

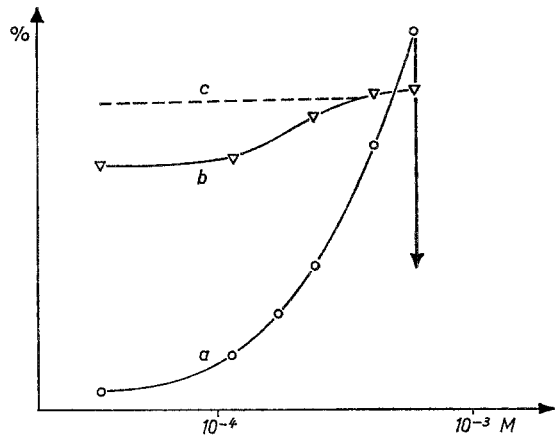

Fig. 2. Fluorescence enhancement of ANS produced by oleylamine. Ordinate: fluorescence intensity. Abscissa: oleylamine concentration (M). (a) relative fluorescence intensity; (b) specific fluorescence intensity: relative fluorescence intensity divided by the oleylamine concentration; (c) specific fluorescence intensity corrected for the absorption of the free dye: total dye concentration, 0.1 mM; →, addition of sodium dodecyl sulfate (1 mM final concentration)

long aliphatic chains like oleylamine or cethyltrimethylammonium salts. A somewhat more complicated model substance is the polylysine:oleic acid complex. Although its constituents only weakly support the ANS fluorescence, the complex produces a considerable fluorescence enhancement. Like the fluorescence of the ANS vesicular complex, the fluorescence of the oleylamine:ANS complex and of the ANS polylysine:oleic acid complex is drastically reduced when the pH of the suspension increases from 6 to 11 (Fig. 3). In contrast, the fluorescence of the ANS: cethyltrimethylammonium complex is nearly pH independent. On the basis of these observations, the most likely site for ANS binding is an amino group in an apolar surrounding. Since the removal of the lipids does not alter the vesicular ANS fluorescence, the ANS binding is most likely localized in the membrane protein itself. This conclusion is further supported by the decrease of the ANS protein or ANS amine fluorescence caused by oleic acid or dodecyl sulfate (Figs. 1 & 2). Both compounds are bound in large quantities by intact and delipidated vesicles (Heimberg, 1969), and, as illustrated in Table 1, oleic acid considerably reduces the binding of ANS to the vesicles. This explains the reduction of the fluorescence as a competition between the negatively charged dye and aliphatic acids. This effect seems to be rather specific since stearic acid, whose binding to the vesicles is identical to that of oleic acid (Heimberg, 1969), neither reduces dye binding nor markedly reduces the fluorescence

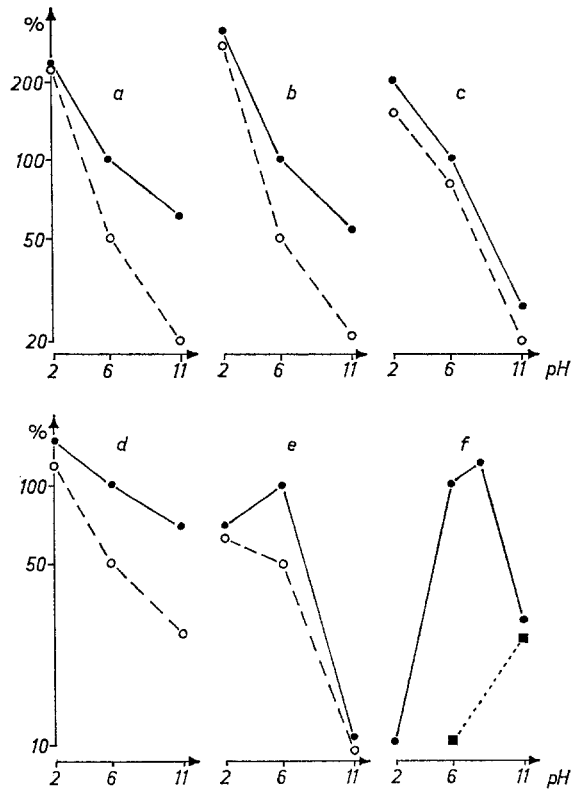

Fig. 3. The pH dependence of the fluorescence intensity of ANS lipid and membrane complexes. Ordinate: relative fluorescence intensity (the fluorescence at pH 6 has been taken as 100%). Abscissa: pH. •——•, without oleic acid; ○----○, 0.5 mM oleic acid present. (a) Normal sarcoplasmic vesicles (0.5 mg/ml); (b) delipidated sarcoplasmic vesicles (0.5 mg/ml); (c) total lipid extract (0.5 mg/ml); (d) egg lecithin (0.5 mg/ml); (e) oleylamine (0.2 mg/ml); (f) polylysine:oleic acid complex (0.5 mg/ml:0.28 mg/ml), ▪------▪, polylysine alone

of the ANS vesicular complex. Oleic acid, as well as dodecyl sulfate, reduces the fluorescence of the ANS vesicles complex maximally by 50%. The similar reduction is observed for the ANS:oleylamine and the ANS: lecithin complexes. Obviously the aliphatic acids can only partially displace the dye from lipid micelles. The residual fluorescence is obviously the fluorescence of the triple complex ANS:fatty acid:NH_2R. (The fluorescence of such a triple complex is not necessarily smaller than that of a two-component complex; i.e., the fluorescence of the cethyltrimethylammonium: ANS complex increases by 50% on addition of oleic acid.)

ANS is not only displaced from its binding site at the protein by the addition of oleic acid to the vesicular suspension but also if fatty acids are

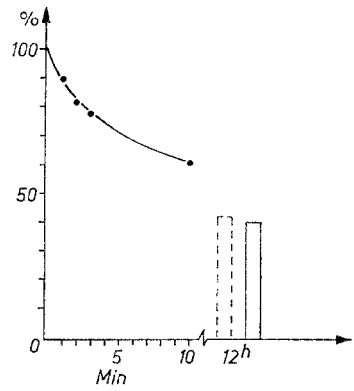

Fig. 4. Decrease of the fluorescence intensity of the ANS vesicular complex produced by phospholipase A digestion. Abscissa: time. ⊏⊐, fluorescence intensity after 12-hr digestion; ⊏⊐, no further decline when 1 mM oleic acid is added to a 12-hr digestion. Protein concentration 0.5 mg/ml

produced in the membrane itself by lipolysis. As shown by Fiehn and Hasselbach (1969), both splitting products of phospholipase A action, fatty acids and lysolecithin, remain in the membrane. As would be expected for a displacement of ANS during phospholipase A digestion, the fluorescence considerably declines and no further decrease occurs when oleic acid is added (Fig. 4).

It can be assumed that the blockage of amino groups in the membrane protein by fatty acids as revealed by the reduction of ANS fluorescence is causally linked to the formation of leaks for calcium ions in the vesicular membranes since the leaks are produced by phospholipase A hydrolysis or by attachment of oleic acid (Fiehn & Hasselbach, 1969).

Although the changes of the fluorescence of the ANS membrane complex as produced by oleic acid can be understood, two other interesting observations remain unexplained.

1. If small amounts of ether (2 to 7%) are added to vesicular suspensions the ANS fluorescence is considerably reduced. A concentration of 2% (v/v) ether already produces a drop of the relative fluorescence intensity by ~20% without any observable change in the activity of the calcium-dependent ATPase or in calcium transport. When the ether is removed by washing or evaporation, the ANS fluorescence rises again and it reaches values somewhat higher than those of the untreated preparation. Even the fluorescence decrease (~50%) produced by higher concentrations of ether

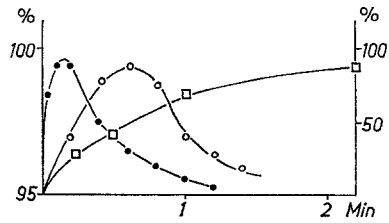

Fig. 5. Transient increase of the fluorescence intensity of the ANS vesicular complex during calcium uptake. Ordinate: increment of the fluorescence intensity. The maximal fluorescence intensity reached after the addition of 0.1 mM Ca^{++} to the assay medium is taken as 100%. The ATP concentration in the usual assay medium is 10 mM. •——•, first addition of Ca^{++} (0.1 mM); ○——○, second addition of Ca^{++} (0.2 mM). Left ordinate: □——□ calcium uptake. Reaction mixture used for fluorescence measurements and calcium uptake: 0.2 M KCl, 20 mM phosphate, 10 mM Mg^{++}, 10 mM ATP; pH 6.5; $T=20$ °C. Vesicular protein 1 mg/ml. The suspension contained 0.2 mM Ca^{++} in the uptake experiment

(7 to 10% v/v), which makes the vesicular membranes leaky (Fiehn, 1969), is reversible.

2. If calcium or ATP is added to a vesicular suspension containing 0.2 M KCl, 20 mM phosphate buffer and 10 mM Mg^{++}, pH 6.5, no change in the ANS fluorescence can be detected. If, however, calcium (0.1 mM) is added to the ATP-containing suspension (10 mM), a small cyclic change is observed (Fig. 5). The initial rise of the fluorescence intensity is followed by a slow decline. As long as ATP is present, the cycle can be repeated. If one of the essential components of the system (magnesium as activator, phosphate as calcium-precipitating agent, or ATP as energy donator) is omitted, no fluorescence change occurs on addition of calcium. For instance, if phosphate is replaced by Tris chloride, no transient fluorescence is observed when magnesium (5 mM) or even high concentrations of calcium (2 mM) are added. Under the conditions where the cyclic fluorescence change occurs, calcium is transported into the vesicles and the calcium-dependent ATPase is activated (Hasselbach & Makinose, 1963). The initial rate of calcium uptake is low (0.12 μmole calcium mg protein^{-1} min^{-1}), and the cycle time is required for the storage of 80% of the calcium present in the system. No reasonable explanation has been found for the relationship between the activity of the calcium-transport system that slowly declines from its initial value and the slowly rising and falling fluorescence change.

Although the ATP-dependent translocation of calcium can hardly be understood without assuming a structural change in the membrane, many trials have been undertaken in vain to obtain results supporting the assumed

structural change (*cf.* Mommaerts, 1967, and Vanderkooi & Martonosi, 1969). The observed cyclic change during calcium uptake may support the occurrence of some structural change that interferes with the interaction of ANS and the membrane protein.

Conclusions

The fluorescence of ANS is enhanced if it is bound to basic sites located in a hydrophobic surrounding. Such hydrophobic regions are present in micelles of phospholipids, in aliphatic amines, and in complexes of aliphatic acids with basic polypeptides as well as in the lipid-free membrane protein of the sarcoplasmic reticulum. In support of this type of interaction, negatively charged detergents displace ANS from the complexes and thereby reduce their fluorescence intensity.

In the sarcoplasmic membrane, the fluorescence reduction produced by oleic acid or dodecyl sulfate is accompanied by a large increase in permeability of the membranes for calcium. In contrast, a fluorescence reduction of the same magnitude produced by small amounts of ether does not interfere with any vesicular function. The amplitude of the cyclic change of ANS fluorescence that has been observed during ATP-driven calcium uptake amounts to only a few percent of the functionally irrelevant ether-induced alteration. Obviously a rational correlation between ANS fluorescence and membrane functions remains to be found.

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