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TEMPERATURE EFFECTS ON 1,8-ANILINONAPHTHALENE SULFONIC ACID FLUORESCENCE WITH SARCOLEMMA VESICLES

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Summary

Increased temperature produces a red shift and decreased fluorescence intensity of the emission peak of 1,8-anilinonaphthalene sulfonic acid (ANS) in suspensions of biomembrane vesicles. These changes have been attributed to a conjectured increase in polarity of the microenvironment of ANS. If the conjecture is correct, fluorescence lifetimes must be decreased with warming.

We showed that ANS binds to both protein and lipid portions of sarcolemma, that there are two kinds of sarcolemma-lipid ANS-binding sites, and that there are three fluorescence lifetimes of excited sarcolemma-bound ANS. The three fluorescence lifetimes were unchanged on warming, or decreased too little to account for the observations. Fluorescence lifetime data were consistent with the notion that the effect of increasing temperature is to decrease the amount of ANS bound to sarcolemma.

From studies of liposomes prepared from lipid extracts of sarcolemma, and of proteins from sarcolemma it was deduced that warming reduced the amount of ANS bound to both of these sarcolemma components, probably mainly by reducing binding capacity. There might also be a shift of affinities such that the ratio, K_A sarcolemma lipid/K_A sarcolemma protein, is larger at higher temperature.

Except at very small concentration ratios of ANS/sarcolemma, more than twice as much ANS was bound to sarcolemma lipids as to proteins.

Abbreviations: ANS, 1,8-anilinonaphthalene sulfonic acid; L-ANS, sarcolemma lipid-bound ANS; P-ANS, sarcolemma protein-bound ANS; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid.

Introduction

The fluorophore 1,8-anilinonaphthalene sulfonic acid (ANS) has been used for studies of biomembranes. Its more obvious properties, first described by Weber and Laurence [1], are that its quantum yield increases markedly and its emission peak shifts toward blue as it is dissolved in solvents of decreasing polarity or when it is in aqueous solution containing certain proteins with which it associates in an unknown way.

There have been four previous reports of temperature effects on ANS in systems of biomembranes [2—5]. The reports agree that fluorescence intensity decreases as temperature increases, usually with a shift toward the red end of the spectrum. The results have usually been interpreted as indicating that the environment in the immediate vicinity of membrane-bound ANS becomes more polar as temperature increases.

We have shown previously [6] that ANS binds to sarcolemma proteins and to sarcolemma lipids. Sarcolemma protein-bound ANS, hereafter referred to as P-ANS, has a higher fluorescence quantum yield than does sarcolemma lipid-bound ANS, hereafter referred to as L-ANS. The steady-state fluorescence emission peak of P-ANS is about 20 nm further toward blue than that of L-ANS. The capacity of sarcolemma lipids to bind ANS is at least twice that of sarcolemma proteins. The steady-state emission spectrum of ANS in suspensions of sarcolemma vesicles shifts toward red as total ANS concentration is increased. At relatively low total ANS concentrations the shift is due to increased binding to lipids. At very high total ANS concentrations the shift is due to the contribution of free ANS.

These observations at constant temperature (10°C) suggest a possible alternative explanation for the effect of temperature on the steady-state emission spectra of ANS with suspensions of biomembranes. If at higher temperature there were simply less membrane-bound ANS, there would be decreased fluorescence emission intensity and there would be a red-ward shift, as has been reported. The distinction between this alternative explanation and that proposed previously (that the immediate environment of membrane-bound ANS becomes more polar as temperature rises) could be made by studies of fluorescence lifetime spectra. If the latter explanation is correct there will be a decrease in fluorescence lifetime. If our possible alternative is correct the lifetimes of the components will be unchanged.

To distinguish between the two possibilities we undertook a study of temperature effects, mainly over the range from about 10 to 40°C, on ANS fluorescence lifetime curves, ANS steady-state fluorescence emission spectra, and ANS binding in the presence of sarcolemma vesicles, and of proteins or liposomes prepared from these vesicles.

Materials and Methods

Details of methods were as described previously [6].

Plasma membranes, sarcolemma, were prepared from hindleg muscles of male rats as described previously [7]. In most studies we used suspensions of

sarcolemma vesicles. In others we used: (a) liposomes prepared from sarcolemma lipids, or (b) protein recovered from the lipid-poor residue after chloroform/isopropanol extraction of sarcolemma.

ANS was obtained from Eastman Organic Chemicals and used without further purification.

Fluorescence emission spectra were obtained on the American Instrument Co. (Aminco-Bowman) spectrofluorimeter, Model J4-8912. Excitation was usually at 385 nm. Cuvettes contained constant volumes of 250 mM sucrose in 1 mM HEPES, pH 7.4, at room temperature, and constant concentrations of KCl in a given series of cuvettes, usually 100 mM final, never less than 80 mM. Temperature was controlled by circulating methanol.

Fluorescence lifetimes were measured by nanosecond fluorimetry in an Ortec Model 9200 nanosecond fluorimeter, and the curves were analyzed as described previously [6].

Results and Discussion

Temperature effect on emission peak of ANS-sarcolemma vesicle fluorescence

As temperature increased from about 10°C to about 40°C there was a shift of the wavelength of the emission peak toward red, the magnitude of which depended on the ratio of ANS to membrane protein. For convenience ratios are considered in two groups: those below 400 nmol ANS/mg membrane protein and those above 1000 nmol ANS/mg protein (Fig. 1A). At the lower ratio, the 30°C rise in temperature caused a shift of 10 nm in emission peak. At the higher ratio the shift was 15 nm.

At 10°C emission peak increased toward red, from its lowest wavelength at about 475 nm, as the ratio of ANS/sarcolemma vesicles increased (Fig. 1B). The shift was slight for ratios less than 1000 nmol ANS/mg protein, but increased sharply at higher ratios.

The range of useful concentrations of ANS was limited at the high end by evidence of excimer formation; that is, at 10^{-4} M ANS the emission peak shifted toward red and the fluorescence intensity was sometimes less than that at $3 \cdot 10^{-5}$ M ANS with the same concentration of sarcolemma. In some experiments there appeared to be excimer formation even at $6 \cdot 10^{-5}$ M ANS, a concentration often used in published studies. The highest ratios of ANS/sarcolemma vesicles were achieved, therefore, by reducing the concentration of sarcolemma to 1 μg protein/ml, or less. The lower limit of ANS concentration was set by the sensitivity and noise of the detecting instrument. It was difficult to obtain reliable spectra when 10^{-7} M ANS was used with sarcolemma vesicle protein concentrations in the range of 1—10 μg/ml, particularly at higher temperatures, although this concentration of ANS gave satisfactory spectra when sarcolemma vesicle concentration equaled or exceeded 100 μg protein/ml.

Temperature effect on intensity of ANS-sarcolemma vesicle fluorescence

As temperature increased from about 10 to 40°C there was a continuous decrease in fluorescence intensity (Fig. 2). The decrease in intensity overall was by about 50% for the 30°C rise in temperature, and was independent of ANS/sarcolemma vesicles ratio.

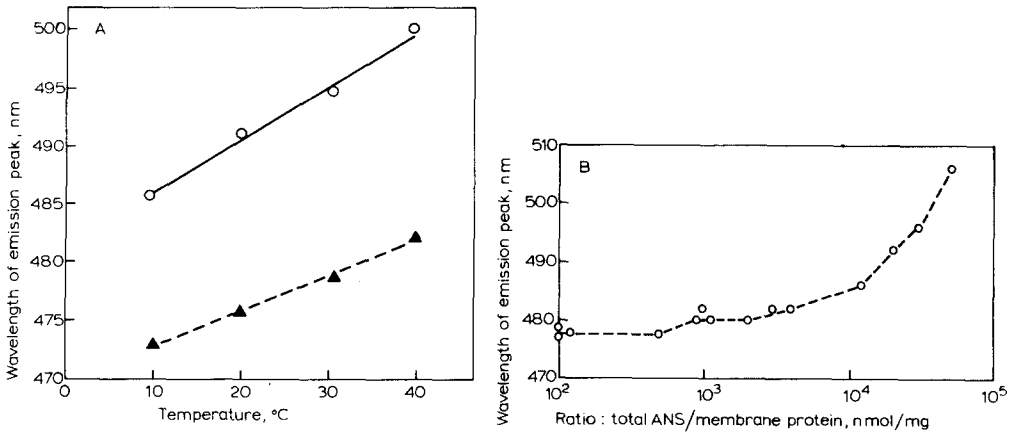

Fig. 1. (A) Effect of temperature on wavelength of fluorescence emission peak of ANS in suspensions of sarcolemma vesicles. Excitation at 360 nm. Upper line (○): ANS/membrane protein ratio greater than 1000 nmol/mg. Lower line (▲): ANS/membrane protein less than 400 nmol/mg. Five sets of experiments for each line. Each point is the mean of five. (B) Effect of ANS/membrane protein ratio on wavelength of fluorescence emission peak of ANS in suspensions of sarcolemma vesicles at 10°C.

The decrease in fluorescence emission intensity was not due to decreased absorbance; absorbance of ANS-sarcolemma vesicles suspensions was not affected by temperature over the range 10—40°C.

The decrease in fluorescence intensity was not due to a change in pH of buffer with temperature. A series of experiments was carried out at constant temperature, 25°C, with sarcolemma vesicles in a series of buffer (Tris) solutions from pH 6.8 to pH 8.2, to cover the range of pH occurring when solutions of Tris, made to pH 7.4 at 25°C, are subjected to temperatures from 6 to 40°C. Fluorescence intensity was not altered by pH over this range.

Nor was there a decrease in fluorescence emission intensity of ANS when

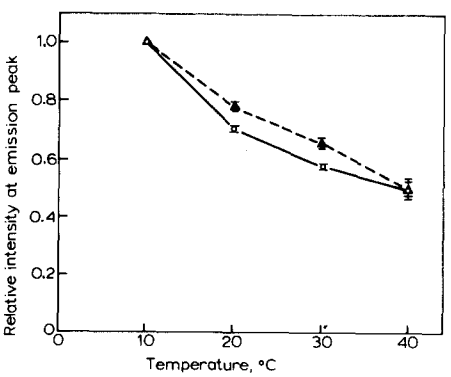

Fig. 2. Effect of temperature on fluorescence intensity at peak emission of ANS in suspensions of sarcolemma vesicles. Same experiments as in Fig. 1A. ○———○, ANS/membrane protein greater than 1000 nmol/mg; ▲———▲, ratio less than 400 nmol/mg. Range of ±1 S.E. indicated.

temperature was raised from 10 to 40°C if there were no sarcolemma vesicles present.

Effect of temperature on fluorescence lifetimes of ANS-sarcolemma vesicles and ANS-liposomes and ANS-proteins from sarcolemma

Observed fluorescence lifetime curves were normalized to unit area; that is, they were transformed to probability density functions, designated *r(t)*. *r(t)* for ANS with sarcolemma vesicles or with lipids or proteins from sarcolemma at 40°C always had higher peaks, peaked at shorted elapsed time, and fell initially more rapidly from the peak than did the spectra at 10°C (Fig. 3).

The raw fluorescence lifetime curve has two components, one is light scattered from the excitation source, the other is true fluorescence:

$$r(t) = ps(t) + (1 - p)f(t) ,$$

where *s(t)* is the light scatter function normalized to unit area, *f(t)* is the fluorescence component normalized to unit area, and *p* is the fractional contribution made by light scatter.

A set of these functions, *s(t)*, *r(t)*, and the derived *f(t)*, appears in Fig. 4.

s(t) had a small half-width and a high and early peak compared to those properties of *r(t)* or *f(t)*. For that reason, the larger *p*, that is, the larger the contribution by *s(t)*, the higher and earlier was the peak of *r(t)*. One reason for the observed effects of increased temperature on *r(t)* was that *p* increased as temperature increased. At a concentration of sarcolemma vesicles correspond-

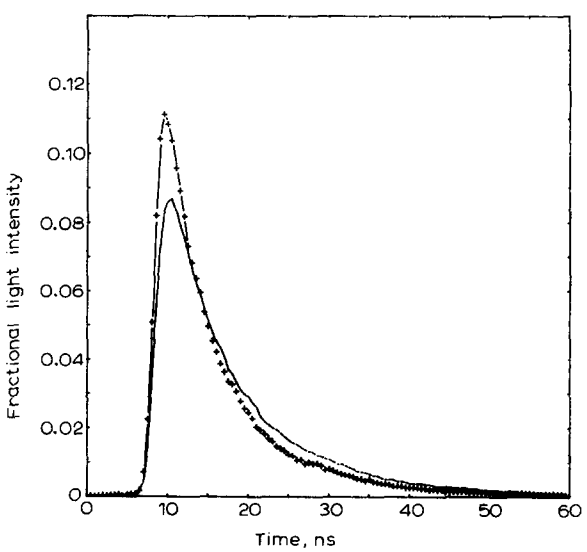

Fig. 3. Effect of temperature on normalized raw fluorescence lifetime spectrum of ANS in suspension of sarcolemma vesicles. Spectra normalized to unit area but not corrected for contributions from light scattered from the excitation source. Sarcolemma protein, 0.1 mg per cuvette; ANS, 30 nmol per cuvette; volume, 1.1 ml. ———, 10°C, +———+, 40°C.

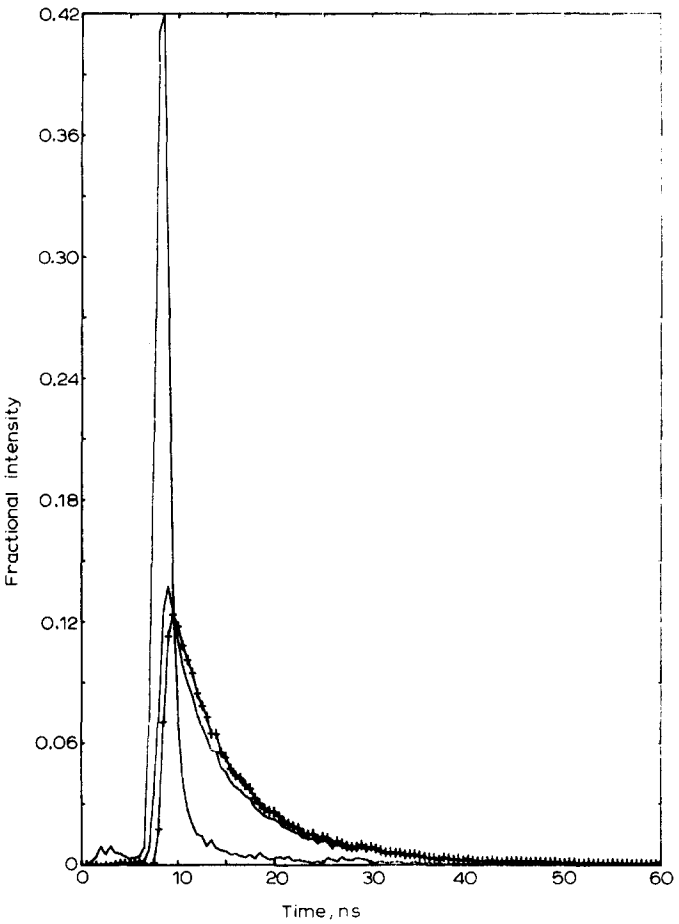

Fig. 4. Effect of light scatter from excitation source on the fluorescence lifetime spectrum. Tallest curve: Light scatter function, $s(t)$, suspension of sarcolemma vesicles without ANS. Smaller continuous curve: Raw spectrum, $r(t)$, 30 μM ANS in sarcolemma vesicle suspension, 0.1 mg protein/ml. +———+, lifetime spectrum corrected for light scatter, $f(t)$. Temperature 40°C. Each curve normalized to unit area.

ing to about 0.1 mg protein per cuvette and an ANS concentration of 30 nmol per cuvette, p was 0.08 at 10°C and 0.15 at 40°C. At a concentration of sarcolemma vesicles corresponding to about 1 mg protein per cuvette and an ANS concentration of 10 nmol per cuvette, p was 0.22 at 10°C and 0.41 at 40°C.

The increase in p was not due to an increase in light scattering with temperature. Total scattered light accumulated per second of real time, during which data were collected, averaged 2.2 at 10°C and 1.6 at 40°C for the smaller sarcolemma vesicle concentration, and 15 at 10°C and 13 at 40°C for the larger sarcolemma vesicle concentration. The increase in p with temperature occurred because the contribution by true fluorescence decreased. At an emission wavelength of 475 nm, the integral of the raw fluorescence lifetime spectrum per second of real time averaged 28 at 10°C and 10.6 at 40°C for the smaller mem-

brane vesicle concentration with 30 nmol ANS per cuvette, and 67 at 10°C and 32 at 40°C for the ten times larger membrane vesicle concentration with 10 nmol ANS per cuvette. The steady-state fluorescence emission intensity at a given wavelength is in fact the integral over all time of the fluorescence lifetime spectrum at that wavelength. These changes in integral s^{-1} did correspond to the temperature-induced decrease in relative fluorescence emission intensity observed in the steady state.

When lifetime curves were corrected for light scatter the peaks decreased and shifted toward longer time. Nevertheless the differences between *f(t)* at 10 and 40°C were not eliminated. At 40°C the peak remained higher and there remained a more rapid initial fall from peak.

f(t) is itself the convolution integral of a relatively brief function, the absorption function, *a(t)*, and the true fluorescence decay curve of the fluorophor, *h(t)*, the unit impulse response. *h(t)* is considered to be generated by independent fluorescence emissions in parallel; hence it is a sum of exponential terms, each weighted by its fractional contribution. Since *s(t)* and *a(t)* decay rapidly, if *h(t)* contains a component with a relatively long lifetime that lifetime will dominate the tail of the raw curve, *r(t)*, and can be determined by analysis of the terminal slope of a semilogarithmic plot of *r(t)*. It is therefore important to note at once that such terminal slopes were indistinguishable among all preparations, yielding an average lifetime, τ, of about 16 ns (range: 14—17 ns, 12 sarcolemma vesicle preparations and three each of sarcolemma vesicle liposomes and sarcolemma vesicle proteins). This terminal slope was not affected by temperature. Therefore, in subsequent solutions for *h(t)* we required that one term have a τ of 16 ns.

f(t) for P-ANS was fitted by convolution of *a(t)* with an *h(t)* with two lifetimes. At 10°C the minor component, about 15%, had $\tau = 4$ ns; the other had $\tau = 16$ ns. At 40°C fits were obtained either with the same two τ values, but with the minor component increased to about 25%, or with a smaller τ, 3 ns, contributing about 18%, and the same larger τ. The 16-ns component was similar to that we found for ANS bound to bovine serum albumin, 17 ns. For that reason, and because this τ was only a minor component of L-ANS, we attributed the 16-ns component to ANS bound to membrane proteins.

To fit *f(t)* for L-ANS by convolution of *a(t)* and *h(t)* required three lifetimes, 4, 7, and 16 ns at 10°C, and either the same set of τ values or 3, 6, and 16 ns at 40°C. At 10°C, the 16-ns component contributed 10—15% of the total; at 40°C its contribution was further reduced. The 4-ns component contributed about 10% at 10°C, about 65% at 40°C. Haynes and Staerk [8] reported a trend, but not observed in all their experiments, toward decreased fluorescence lifetimes of ANS bound to lipid vesicles as temperature increased from 15 to 50°C, but this range included the phase transition temperature of their preparations. Our results could be consistent with theirs, but it is also possible that lifetimes of L-ANS were not affected by temperature, but that smaller lifetime became more prominent.

At both 10 and 40°C *r(t)* for sarcolemma vesicles-ANS could be matched excellently by the spectrum of ANS in a mixture of liposomes and proteins from sarcolemma (Fig. 5). These fits suggest that *h(t)* for sarcolemma vesicles-ANS contains three terms, two with time constants characteristic of L-ANS and

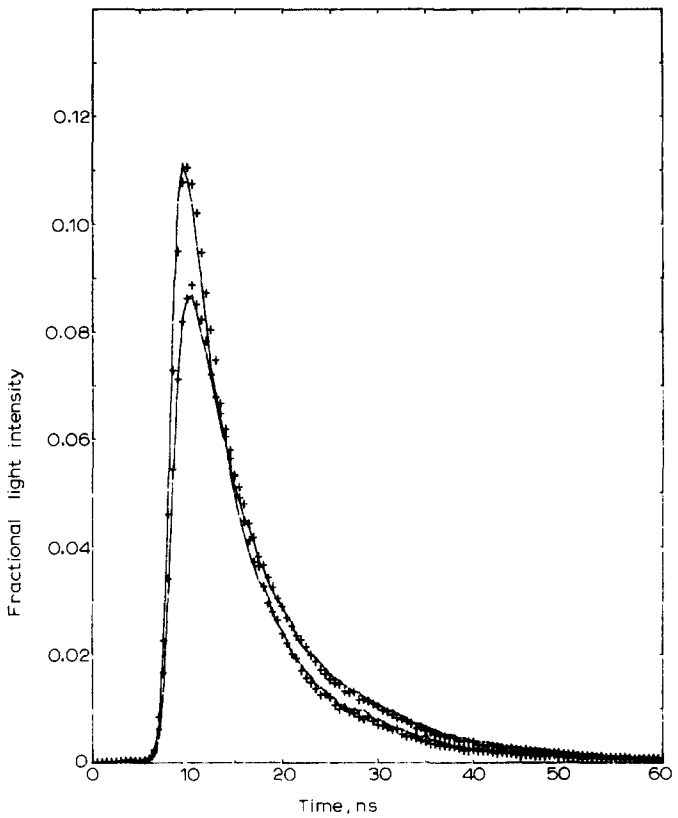

Fig. 5. Comparison of fluorescence lifetime spectrum of ANS in suspension of sarcolemma vesicles with spectrum of ANS in mixture of liposomes from sarcolemma and proteins recovered from sarcolemma. Curves normalized to unit area but not norrected for contributions by light scattered from the excitation source. Continuous curves, ANS in sarcolemma suspensions; + + +, ANS in mixture. Upper set, 40°C; lower set, 10°C.

one with a time constant characteristic of P-ANS. At 10°C, $h(t)$ for ANS-sarcolemma was fitted by the following function (Fig. 6):

$$[h(t)]_{10°C} = (a_1/4)\, e^{-t/4} + (a_2/7)\, e^{-t/7} + (a_3/16.25)\, e^{-t/16.25}$$

At 40°C the choice of fit was ambiguous; the function was fitted by either set of liposome functions at 40°C plus a third term with a lifetime of about 16 ns:

$$[h(t)]_{40°C} = (b_1/4)\, e^{-t/4} + (b_2/7)\, e^{-t/7} + (b_3/16.25)\, e^{-t/16.25}$$

or,

$$[h(t)]_{40°C} = (c_1/3)\, e^{-t/3} + (c_2/6)\, e^{-t/6} + (b_3/16.25)\, e^{-t/16.25}$$

where $(c_1 + c_2) = (b_1 + b_2)$, and $(4b_1 + 7b_2) = (3c_1 + 6c_2)$; that is, the mean τ is the same for the two equations at 40°C.

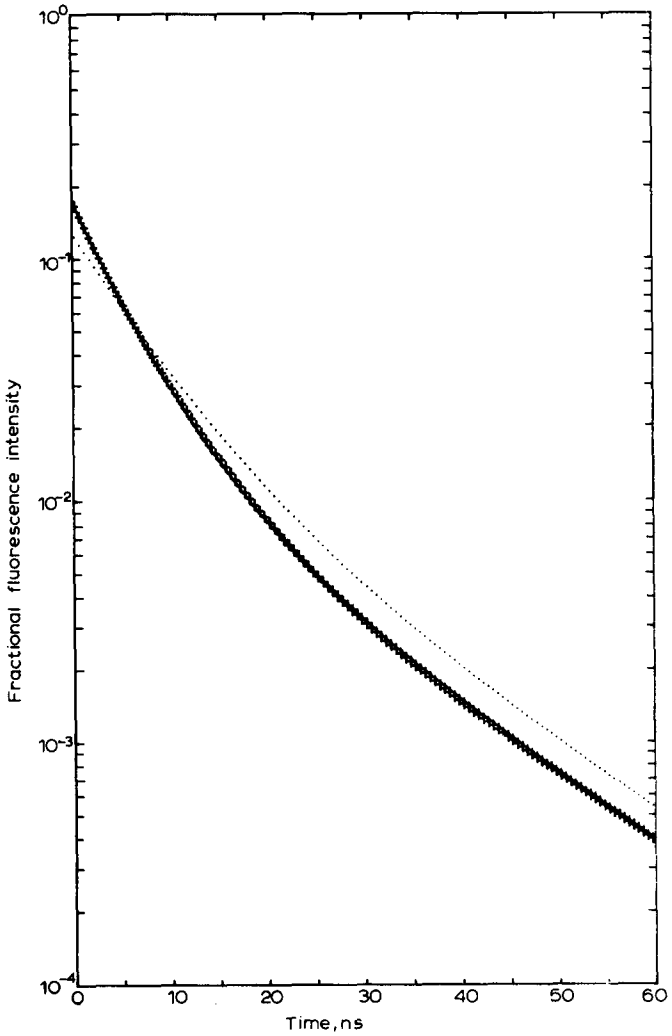

Fig. 6. Deconvolved fluorescence lifetime spectrum, $h(t)$; ANS in suspension of sarcolemma vesicles. Corrected for light scatter and deconvoluted. 30 μM ANS; sarcolemma protein 0.1 mg/1.1 ml. · · · · · ·, 10°C, ——— and + + +, the two equations at 40°C given in the text. Note that the two curves at 40°C are virtually indistinguishable, and that the final slope is the same in all curves.

Means and ranges of the values for the coefficients in three preparations (30 μM ANS; sarcolemma, 0.8—1.0 mg protein/ml) were:

a_1 = 0.04 (0.02—0.07), a_2 = 0.55 (0.45—0.60), a_3 = 0.41 (0.33—0.50),

b_1 = 0.31 (0.2—0.4), b_2 = 0.4 (0.35—0.50), b_3 = 0.29 (0.25—0.31),

c_1 = 0.12 (0.10—0.15), c_2 = 0.60 (0.59—0.60) .

The coefficients (the a, b and c values) have physical meaning. Each is the ratio of the concentration of that component to the total concentration of

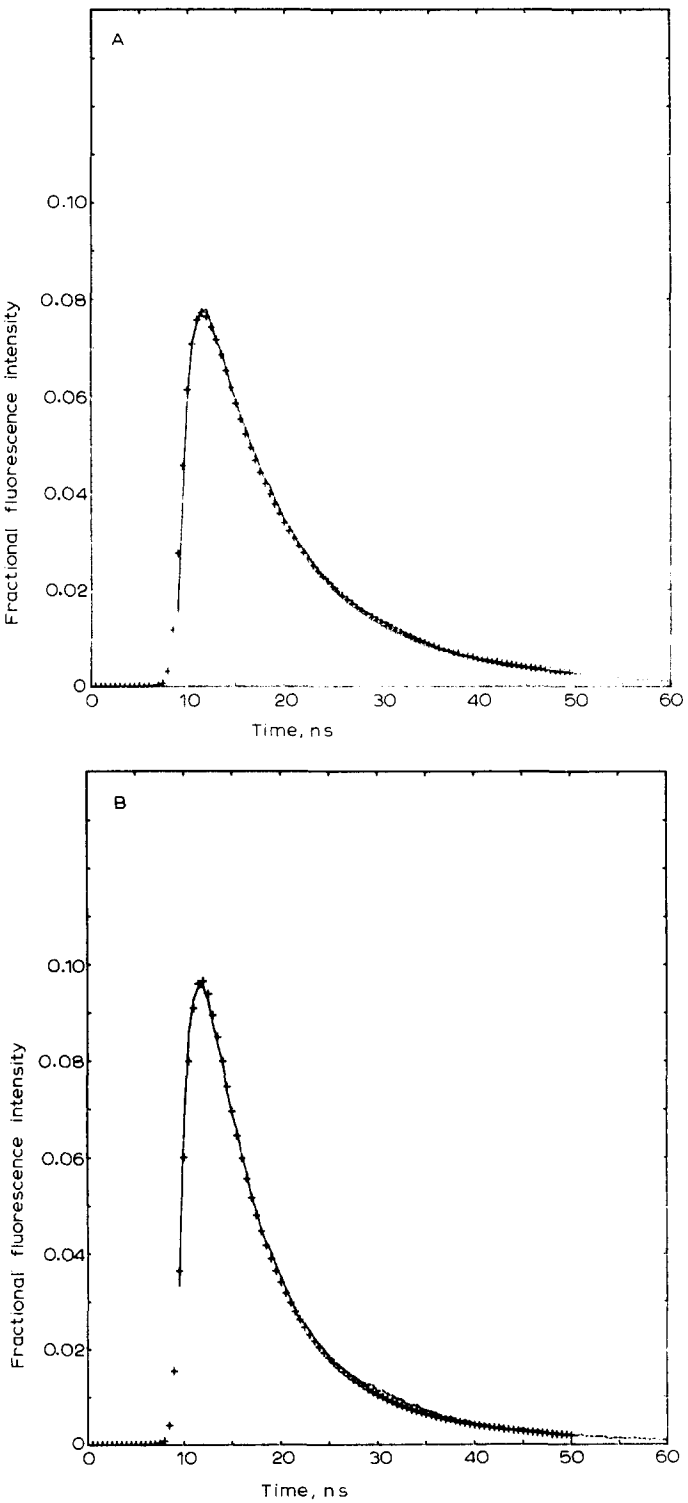

Fig. 7. Effect of temperature on fluorescence lifetime spectra of ANS in suspensions of sarcolemma vesicles, and comparison with reconvoluted functions. Continuous line, observed spectrum corrected for contributions by scattered light from the excitation source and normalized to unit area; + + +, reconvoluted curve. (A) 10°C. (B) 40°C.

bound ANS, c_B; e.g., $a_3 = c_P/c_B$ at 10°C. Hence, c_P, the concentration of protein-bound ANS in sarcolemma, at the concentrations used in these experiments, decreased from about 40% of the total membrane-bound ANS at 10°C to about 30% at 40°C.

Fig. 7 compares the observed spectra corrected for light scatter, the $f(t)$ values, and the convolution intergrals of these $h(t)$ values on the ANS absorption function.

From the coefficients and time constants given above, mean quantum yield, $\overline{Q}$, of membrane-bound ANS can be calculated:

$$\overline{Q} = (1/\tau_0) \sum_{i=1}^{n} a_i \tau_i ,$$

where τ_0 is the natural fluorescence lifetime of ANS. Reported values for τ_0 cover a wide range. We have used Stryer's [9] calculations of quantum yield of ANS in 1-butanol and our data on the fluorescence lifetime of ANS in 1-butanol to calculate τ_0, 22 ns. In three sets of preparations of sarcolemma vesicles, the mean quantum yield of bound ANS ranged from 0.44 to 0.51 (mean 0.47) at 10°C, and from 0.37 to 0.42 (mean 0.40) at 40°C. The mean quantum yield decreased by 16% (individual values: 12, 17, and 19%) when temperature was raised from 10 to 40°C. This is not enough to account for the observed temperature-induced decrease in steady-state fluorescence emission intensity, which was by about 50%. We conclude from this that some factor other than decreased quantum yield accounted for most of the observed fluorescence emission intensity observed when the ANS-sarcolemma vesicle suspensions were warmed.

Temperature effect on binding of ANS to sarcolemma and to liposomes and proteins from sarcolemma

We have discussed elsewhere [6] theoretical and practical limitations of methods used to estimate binding parameters of ANS to membrane components. Because of these limitations we are unable to calculate numerical values for the binding parameters of ANS-sarcolemma vesicles. However, we can draw semiquantitative or qualitative conclusions concerning temperature effects on ANS bindings.

Fig. 8A shows a plot of relative fluorescence emission intensity, I, at peaks of steady-state emission spectra, against total ANS concentration over the range 10—60 μM, above which there was evidence of excimer formation. Notice in Fig. 8A that I at 40°C is about half of I at 10°C at each ANS concentration. This occurred in each of 25 experiments. In none of them did I plateau with ANS concentration at 10°C, although it appeared to in some at 40°C, and usually appeared to be closer to a plateau at 40°C than at 10°C. Since at plateau, all ANS-binding sites are occupied, this observation indicates that the capacity of sarcolemma vesicles to bind ANS was less at 40°C than at 10°C. If the mean quantum yield of sarcolemma vesicles-bound ANS at the two temperatures were the same, we could conclude that ANS-binding capacity at 40°C was substantially less than half of that at 10°C. However, since the mean

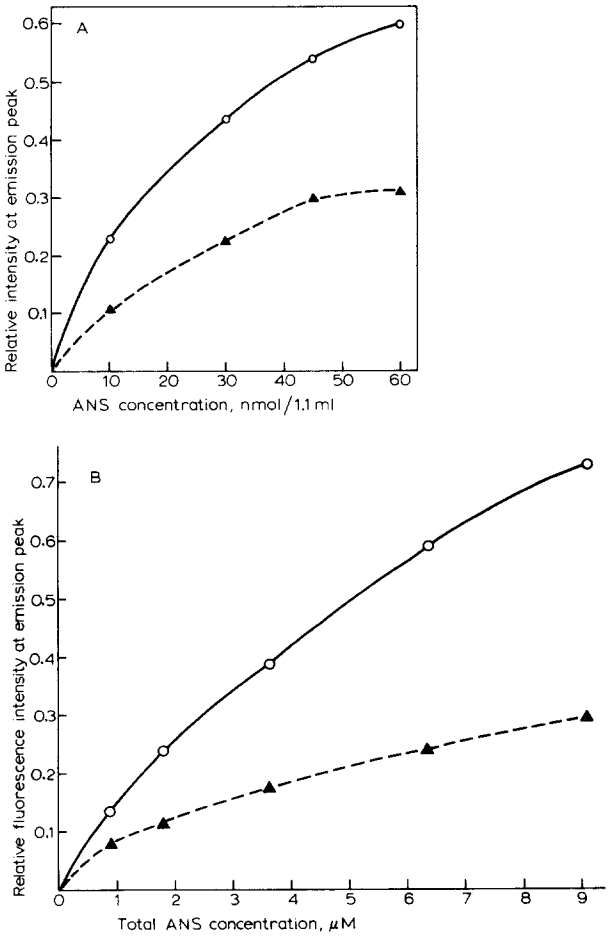

Fig. 8. (A) Effect of temperature on steady-state fluorescence emission intensity at peak emission of ANS in suspensions of sarcolemma vesicles. Sarcolemma protein concentration constant, 0.54 mg/1.1 ml; ANS concentration varied. Upper curve (○), 10°C; lower curve (▲), 40°C. (B) Effect of temperature on steady-state fluorescence emission intensity at peak emission of ANS in suspensions of sarcolemma vesicles with low concentrations of ANS. Sarcolemma protein concentration, 0.069 mg/1.1 ml. Upper curve (○), 10°C; lower curve (▲), 40°C.

quantum yield was reduced by 16% at 40°C, the reduction in capacity may not have been greater than 50%. In any case, it is safe to conclude that the major cause of thermally induced decreased ANS binding to sarcolemma vesicles was decreased capacity.

Fig. 8B shows a plot of I against total ANS concentration over the range 1—9 μM to emphasize that the curves rose more rapidly at low than at higher ANS concentrations, and revealed a knee at low concentrations, more prominent at 40°C than at 10°C. Such curves cannot be simulated by non-cooperative systems in which there is only one kind of binding site, nor by systems in which there are two kinds of binding sites in which capacities and affinities differ only by a small factor. Their simulation requires a system in which one

of the capacities is relatively small, and in which the affinity for that site is relatively large, so that the small capacity is nearly fully occupied at low concentrations of ANS.

This set of restrictions does not apply to the major component of ANS binding to sarcolemma proteins or to sarcolemma lipids, since the total ANS binding capacities of sarcolemma protein and of sarcolemma lipids did not appear to differ sufficiently. It is suggested that the component with small capacity and large affinity is the minor component of ANS bound to sarcolemma lipids, with a fluorescence lifetime of about 4 ns. A relative increase in this component, indicated by results of analysis of fluorescence lifetime curves, can account for the increase in prominence of the knee seen on warming (Fig. 8B).

Plots of ANS-liposome fluorescence emission intensity against total ANS concentration were similar to those for ANS-sarcolemma vesicles shown in Fig. 8A, except that the curves at 40°C tended to plateau earlier, at 30 μM ANS. Here, too, the ratio $I(40°C)/I(10°C)$ was less than 0.5 at all but the smallest ANS concentrations, suggesting that the rise in temperature reduced the capacity of liposomes from sarcolemma to bind ANS.

Proteins extracted from sarcolemma appeared to be relatively homogeneous with respect to ANS binding. That is, fluorescence lifetime data showed little contamination of the 16 ns lifetime component attributed to protein by the 4 ns lifetime component attributed to lipid. Therefore we were able to quantitate binding parameters, as follows.

Sarcolemma proteins, with bound ANS, sedimented during 30 min of centrifugation at 100 000 $\times g$. Free ANS concentration in the supernatant was measured by absorption at 360 nm. In two of three experiments differences between free and total ANS concentrations were sufficient to yield acceptable calculations of the concentration of bound ANS. In the third experiment nearly all ANS was free and the concentration of bound ANS could not be estimated reliably.

For the two experiments in which protein-bound ANS concentration was calculated, Scatchard plots were linear (Fig. 9). ANS binding capacity averaged

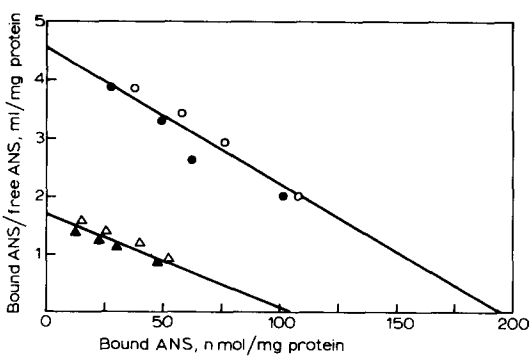

Fig. 9. Effect of temperature on Scatchard plots of ANS in solutions of proteins recovered from sarcolemma. Sarcolemma protein concentrations were 0.05 (open symbols) and 0.1 mg/1.1 ml (filled symbols). Circles, 10°C; triangles, 40°C. Lines are best fits. ANS binding capacity; 10°C, 189 nmol/mg sarcolemma protein; 40°C, 106 nmol/mg sarcolemma protein. Association constants: 10°C, $2.4 \cdot 10^4$ M^{-1}; 40°C, $1.6 \cdot 10^4$ M^{-1}. Parameters calculated from individual experiments are given in the text.

183 nmol/mg protein at 10°C and 104 at 40°C. Association constants averaged $2.5 \cdot 10^4$ M^{-1} at 10°C and $1.7 \cdot 10^4$ at 40°C.

In the third experiment, in which the concentration of bound ANS could not be determined because nearly all ANS was free, it was still possible, on the following basis, to calculate association constants but not capacities. From the equilibrium mass action equation for the homogeneous case, in which observed fluorescence intensity I is a linear function of bound ANS concentration,

$$\frac{1}{I} = \frac{1}{\beta n[P]} + \left(\frac{1}{\beta n[P]K_A}\right)\frac{1}{c_F}$$

where c_F is free ANS concentration, n is capacity, $[P]$ is protein concentration, K_A is the association constant, and β relates I to bound ANS concentration. In the case at hand total ANS concentration, c_T, is substituted for c_F. In this experiment K_A was $3 \cdot 10^4$ M^{-1} and was not affected by temperature. Since in this experiment fluorescence emission intensity at 40°C was only 40—50% of that of 10°C it is likely that capacity was decreased by about 50% by the increase in temperature.

Thus, the major effect of increased temperature over this range on ANS binding to sarcolemma proteins was to reduce capacity to about half, with possibly a minor reduction in affinity.

Steady-state emission spectra contain information about relative capacities for ANS of proteins and liposomes prepared from sarcolemma. We showed previously [6] that fluorescence emission intensity of ANS in the presence of sarcolemma vesicles could be expressed as a sum of contributions of protein-bound, liposome-bound, and free ANS: $I_P(\lambda)$, $I_L(\lambda)$, and $I_F(\lambda)$, respectively. The contribution at a given wavelength by protein-bound ANS, for example, is

$$I_P(\lambda) = \alpha\phi_P c_P h_P(\lambda) \qquad (1)$$

where α is an instrument factor, ϕ_P is quantum yield, c_P is the concentration of protein-bound ANS, and $h_P(\lambda)$ is the probability density function of wavelength of protein-bound ANS. Similar equations apply to $I_L(\lambda)$ and $I_F(\lambda)$. At 10°C, $h_P(\lambda)$ peaked at 472 nm, $h_L(\lambda)$ at 489 nm, and $h_F(\lambda)$ at 535 nm. (See Fig. 7 in ref. 6 for illustration of the three probability density functions.)

Equations of the type of Eqn. 1 have several useful applications. For one thing, we can compare c_L and c_P by dividing I_L by I_P to obtain

$$\frac{c_L}{c_P} = \left(\frac{\phi_P}{\phi_L}\right)\left(\frac{h_P(\lambda_1)}{h_L(\lambda_2)}\right)\frac{I_L(\lambda_2)}{I_P(\lambda_1)},$$

where λ_1 and λ_2 need not be the same. It is convenient to select λ_1 and λ_2 so that $h_P(\lambda_1) = h_L(\lambda_2)$. This condition is nearly met at the peaks of h_P and h_L at $\lambda_1 = 472$ and $\lambda_2 = 489$ nm. The ratio ϕ_P/ϕ_L is the ratio τ_P/τ_L, where τ_P is the fluorescence lifetime of sarcolemma protein-bound ANS and τ_L is the mean fluorescence lifetime of sarcolemma liposome-bound ANS. τ_P was about 16 ns, independent of temperature; τ_L was 6.65 ns at 10°C and 5.17 ns at 40°C.

Substitution of these values into the equation for c_L/c_P gives:

$c_L/c_P = 2.4\ I_L(489)/I_P(472)$, at 10°C

and

$c_L/c_P = 3.1\ I_L(489)/I_P(472)$, at 40°C

It was observed experimentally (30 μM ANS; sarcolemma material equivalent to 0.1 mg original sarcolemma protein) that the ratio I_L/I_P was approximately unity at both temperatures. Therefore, there was about 2.4 times as much ANS bound to sarcolemma liposomes as bound to proteins at 10°C, and an even greater amount at 40°C. Since these ratios were obtained in experiments with high ANS concentrations, they largely reflect relative capacities, and since the relative capacity of proteins to bind ANS at 10°C averaged about 190 nmol ANS/mg protein, sarcolemma liposomes bound about 460 nmol ANS/mg protein equivalent. At 40°C the capacity of proteins was about 100 nmol ANS/mg protein, so that the capacity of liposomes to bind ANS at 40°C was about 300 nmol ANS/mg protein equivalent.

From steady-state fluorescence emission spectra we can also deduce something about relative affinities of ANS for sarcolemma vesicle lipids compared to sarcolemma vesicle proteins, and something about a change in this relationship upon warming.

From Eqn. 1 and from the notion that the observed steady-state fluorescence emission spectrum of ANS-sarcolemma vesicles, $I_T(\lambda)$, is the sum of contributions by I_P, I_L, and I_F,

$$I_T(\lambda) = \alpha\{\phi_P c_P h_P(\lambda) + \phi_L c_L h_L(\lambda) + \phi_F c_F h_F(\lambda)\} \tag{2}$$

To exploit Eqn. 2 to deduce relative affinities recall the following: the ratio of the quantum yields ϕ_P/ϕ_L at 10°C is 2.4, which is about the same as the ratio of ANS-binding capacities of sarcolemma vesicle lipids/sarcolemma vesicle proteins. If affinities were identical we expect peak emission of ANS-sarcolemma vesicles to lie at a wavelength midway between those of peak h_P and peak h_L, shown previously [6] to be 472 and 489 nm, respectively. At small ANS concentrations, peak I_T was near 472 nm, suggesting that ANS-sarcolemma vesicle protein affinity exceeded that of ANS-sarcolemma vesicle lipids. At 40°C the peak I_T was always to the right of that at 10°C at any ANS concentration (see Fig. 1), as if the rise in temperature produced a relative increase in affinity of sarcolemma vesicle lipids for ANS.

We showed previously [6] that Eqn. 2 could be used, first, to calculate values of α, c_P, and c_L, and second, to synthesize steady-state fluorescence emission spectra for comparison with observed spectra. There are three unknowns in Eqn. 2: α, c_P, and c_L. c_F is the known c_T minus the sum of the unknowns c_P and c_L. Hence, from a set of three simultaneous equations, established by using values of an observed $I_T(\lambda)$ at three values of λ, the unknowns are determined. The values for α, c_P, c_L, and c_F are then inserted into the right-hand side of Eqn. 2 and a synthetic $I_T(\lambda)$ is generated for comparison with observed. (Once α has been determined for one spectrum of a family of spectra at different membrane or ANS concentrations and at different temperatures, it is applied to all spectra of that family. Thus, for the subsequent spectra there are but two unknowns, c_P and c_L.) At 10°C the fit was excellent. It was also excellent at 40°C (Fig. 10). This fit suggests that Eqn. 2 is valid. Although values of c_P and c_L can be calculated by this method, the uncertainties are such that precision of their ratios is questionable. Nevertheless, it is worth mentioning that c_L was never less than twice as great as c_P for all spectra peaking at or above 480 nm. For spectra peaking close to 475 nm, c_P exceeded

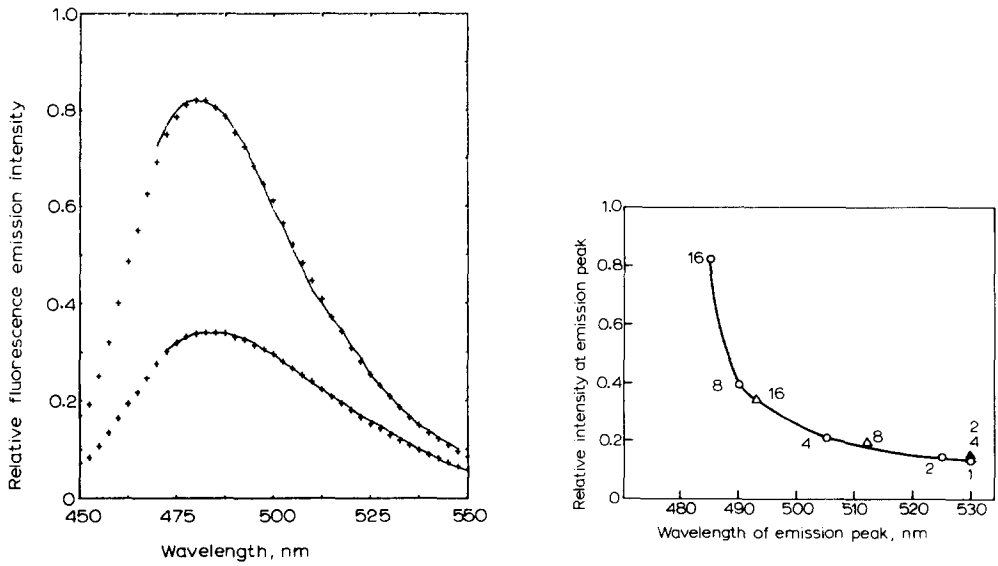

Fig. 10. Syntheses of steady-state fluorescence emission spectra. Continuous lines, observed spectra at 10°C (upper curve), and at 40°C. + + +, spectra synthesized by use of Eqn. 2. 30 μM ANS; sarcolemma, 16 μg protein/ml.

Fig. 11. Effect of temperature on relative fluorescence emission intensity of ANS in suspensions of sarcolemma vesicles as a function of wavelength of the emission peak. ○, 10°C; △, 40°C. Numbers are sarcolemma concentrations as mg protein/ml in temperature-paired experiments.

c_L, in agreement with the analysis presented earlier. For any given concentration of ANS and sarcolemma, the sum of c_P *and* c_L was less at 40°C than at 10°C, and the ratio c_L/c_P was always greater at 40°C, in agreement with results of analysis of lifetime curves.

Data presented suggest that elevation of temperature from 10 to 40°C reduced the capacity of sarcolemma to bind ANS. If this conjecture is correct, it should be possible to mimic data from experiments conducted at 10°C by data from experiments conducted at 40°C simply by adding more sarcolemma in the latter. Fig. 11 shows the results of a series of experiments conducted at constant ANS concentration and at varying sarcolemma vesicle concentration at about 10°C and at about 40°C. Relative fluorescence intensity at the emission peak was plotted against the wavelength of emission peak. At the lower temperature as sarcolemma concentration decreased, intensity decreased and the peak shifted toward red along a curve concave upward. At the higher temperature, every corresponding point fell below and to the right of the point at the lower temperature, but the curve at the higher temperature coincided with that at the lower temperature. That is, the effect of increased temperature was similar in this respect to a decrease in sarcolemma concentration at constant ANS. This coincidence is consistent with the hypothesis that the major effect of increased temperature was decreased capacity of sarcolemma to bind ANS.

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References

1 Weber, G. and Laurence, D.J.R. (1954) Biochem. J. 56, 31p
2 Kasai, M., Changeux, J.-P. and Monnerie, L. (1969) Biochem. Biophys. Res. Commun. 36, 420–427
3 Vanderkooi, J. and Martonosi, A. (1969) Arch. Biochem. Biophys. 133, 153–163
4 Weidekamm, E., Wallach, D.F.H. and Fischer, H. (1971) Biochim. Biophys. Acta 241, 770–778
5 Augustin, J. and Hasselbach, W. (1973) Eur. J. Biochem. 35, 114–121
6 Zierler, K. and Rogus, E. (1979) Biochim. Biophys. Acta 514, 37–53
7 Cheng, L.C., Rogus, E.M. and Zierler, K. (1977) Biochim. Biophys. Acta 464, 338–346
8 Haynes, D.H. and Staerk, H. (1974) J. Membrane Biol. 17, 313–340
9 Stryer, L. (1965) J. Mol. Biol. 13, 482–495