# Interaction of 8-Anilino-1-naphthalenesulfonate with Rod Outer Segment Membrane†

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ABSTRACT: The interaction between bovine rod outer segment (ROS) membrane and a fluorescent probe, 8-anilino-1-naphthalenesulfonate (Ans), has been studied to investigate the visual excitation process and structural aspects of the membrane. Binding of the probe to the membrane is indicated by its fluorescence enhancement and a blue shift of the emission band. When ROS membrane is bleached, a further increase in fluorescence and blue shift can be observed. These phenomena are attributed to local conformational changes in the opsin part of the molecule upon bleaching. The possibility of energy transfer from bound probe to 11-*cis*-retinal has been excluded by lifetime measurements. Ans binds to both lipids and rhodopsin. The relative contribution to fluorescence of Ans bound to protein and lipid was assessed on the basis of fluorescence lifetime and steady-state emission spectra. The lifetime spectrum of lipid-poor rhodopsin has a time constant of ~14 ns and that of lipid, ~4 ns. These time constants do not change with pH or upon illumination. About 30 molecules of Ans are bound per molecule of rhodopsin, and the calculated dissociation constant for binding is $3.2 \times 10^{-6}$ M. Measurements of energy transfer from tryptophan residues to bound Ans molecules show no differences in either transfer efficiency or distances between them when rhodopsin is bleached. However, these values vary with pH, indicating that the nature of the light-induced conformational change is different from that of the pH-induced change in the secondary structure of the rhodopsin protein (revealed in the circular dichroism and fluorescence measurements). Temperature dependence of Ans fluorescence intensity shows two reversible thermal transitions in ROS membranes, one at ~7 °C and the other at ~36 °C. These phenomena have been attributed to the phase transitions of the lipid at the two temperatures. The fluorescence of bound Ans is enhanced by lowering the pH and by the presence of $Na^+$ or $Ca^{2+}$ in the order $Ca^{2+} \gg Na^+$. The pronounced effect of $Ca^{2+}$ cannot be correlated with changes in ionic strength but suggests a specific effect of the divalent ion on membrane conformation.

In photoreceptor cells of vertebrates, the conversion of light energy into neural excitation occurs within the specialized membranes of outer segments. The initial action of light absorption by rhodopsin in the rods is to isomerize the chromophore 11-*cis*-retinal to an all-trans configuration (Wald, 1968). The precise mechanism by which the initial excitation of rhodopsin is converted into an electrical signal is unknown. Rhodopsin is the major protein component of the rod outer segment (ROS). This integral membrane protein is organized within an array of disk membranes stacked within an outer enveloping plasma membrane. A part of the rhodopsin molecule is in close contact with the hydrophobic interior of the lipid bilayer of the disk membranes (Blaurock & Wilkins, 1972; Chabre, 1975). It has been proposed that rhodopsin mediates the permeability of the outer segment disk membrane to a transmitter substance, possibly calcium ions (Yoshikami & Hagins, 1971; Hagins, 1972; Hagins & Yoshikami, 1974).

It is generally thought that conformational changes in rhodopsin of the ROS upon illumination may be correlated with changes in permeability of the disk membrane and thus play a central role in visual excitation (Abrahamson & Wiesenfield, 1972; Hagins, 1972; Williams, 1975; Daemen & Bonting, 1977). Many investigators have observed a light-induced change in the circular dichroism spectrum of rhodopsin in detergents (Schichi, 1969; Rafferty et al., 1977; Stubbs et al., 1976). However, there is no evidence of a change in the peptide region on bleaching ROS fragments unexposed to detergents. The conformational stability of rhodopsin is enhanced in the ROS membrane (Zorn & Futterman, 1971; Schichi, 1973; Rafferty et al., 1977). The presence of phospholipids associated with rhodopsin was found to be essential in maintaining a regenerable conformation of rhodopsin in recombinant membranes (Hong & Hubbell, 1973; O'Brien et al., 1977).

In an attempt to understand the properties of ROS membrane and the changes that occur in its components upon illumination, we have utilized a fluorescence probe, 8-anilino-1-naphthalenesulfonate (Ans), to monitor the microenvironment of the bound probe. Ans has a low fluorescence yield in aqueous solution, but its fluorescence is enhanced when it is bound to hydrophobic sites on membranes and proteins (Radda, 1971). The probe has been used extensively to study the properties of proteins (Brand & Gohlke, 1972; Johnson et al., 1979; Weber et al., 1979) and membranes (Phillips & Cramer, 1973; Cramer et al., 1973; Prasad et al., 1977; Andley & Singhal, 1977; Andley et al., 1981). Fluorescence spectra, quantum yield, polarization, and decay times are sensitive to the microenvironment of the Ans molecule (Brand & Gohlke, 1972; Radda, 1971; Stryer, 1965). To our knowledge, Ans has never been used to study the structural aspects of ROS membranes or the dynamics of photoexcitation of the rhodopsin molecule. However, Wu & Stryer (1972) have successfully employed specifically labeled fluorescent chromophores to deduce proximity relationships within bovine rhodopsin. Despite certain advantages of the use of covalently bound probes, if they are linked to a functionally important site they are likely to interfere significantly with the biochemical properties of the system. On the other hand, for noncovalently bound probes such as Ans the perturbation will be smaller, and by utilizing the change in the spectral properties of the molecule, one can follow the nature of the probe–membrane interaction. We have studied in detail the

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interaction of Ans with suspensions of ROS membrane under various conditions. We have utilized energy-transfer (tryptophan to bound Ans) measurements as a spectroscopic ruler to calculate transfer efficiency and distance between tryptophan residues of rhodopsin and the bound probe. The relative contribution to the fluorescence of Ans bound to proteins and to lipid of the membrane has been assessed on the basis of lifetime and steady-state emission spectra. The fluorescence of Ans in ROS suspensions appears to be a useful and sensitive indicator of structural changes of the membrane components that may be induced by illumination and by other environmental conditions, such as salts, pH, and temperature.

## Materials and Methods

*Preparation of Materials.* Rod outer segments were isolated from fresh bovine retinas by using the procedure of Papermaster & Dreyer (1974). After isolation on sucrose density gradients, the ROS membranes were washed extensively with deionized water to remove peripheral proteins, suspended in 5% sucrose, and sonicated for 1 min (Heat Systems Ultrasonics Model 185 fitted with the microtip) at 4 °C. All procedures were carried out in dim red light (Kodak Series 1 filter) and at 4 °C. The ROS's were stored at −70 °C until used. The rhodopsin content was determined by dissolving 0.1 mL of ROS sample in 50 mM cetyltrimethylammonium bromide containing 0.1 M hydroxylamine and by measuring the absorption of this solution at 500 nm in the dark and after bleaching, assuming the molar extinction coefficient of rhodopsin to be 40600 (Wald, 1968).

Lipids were extracted from ROS membranes according to the procedure of Borggreven et al. (1970) with a 2:1 chloroform–methanol mixture. The extract of the lipids was separated from the residue by centrifugation, the procedure being repeated 3 times. The lipids were dried under a stream of $N_2$, resuspended in sucrose solutions, and sonicated for 1 min. ROS protein was obtained from the residue after removal of the lipids by washing with a buffer containing 1 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7, and 250 mM sucrose. Since this sample was not completely free of lipid, we will henceforth refer to it as "lipid-poor" ROS protein.

*Fluorescence Measurements.* Steady-state fluorescence was routinely measured at 20 °C with a Hitachi Perkin-Elmer MPF 11 spectrofluorometer with a differential corrected spectra unit attachment. A small volume of a stock solution of 8,1-Ans (Eastman Kodak) was added to a suspension of washed ROS membranes in the dark at room temperature. Fluorescence of Ans was excited at 370 nm and measured between 400 and 600 nm. Low concentrations of Ans (2–50 μM) were used throughout. Fluorescence intensities were corrected for scattering by the ROS suspension and for inner-filter effects according to the method of Chignell (1972). In experiments involving temperature variation, the temperature of the Ans–ROS mixture was regulated by a circulating water bath.

Tryptophan fluorescence and tryptophyl-sensitized Ans fluorescence were measured in the corrected mode of the spectrofluorometer by using a 300-nm cutoff filter in the emission beam. For estimation of the maximal transfer efficiency from tryptophyls to Ans, the tryptophyl values were corrected for the inner-filter attenuation of the excitation and fluorescence by Ans by taking into account the 90° angle between the excitation and fluorescence in the spectrofluorometer. The dissociation constant and number of Ans binding sites (expressed in moles per rhodopsin) were determined by the method of Zierler (1977).

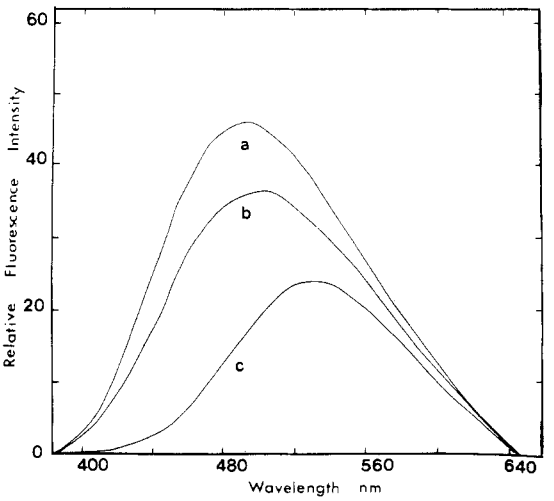


FIGURE 1: Emission spectra of Ans in buffer solution (bottom curve) and in suspensions of ROS membrane in the dark (middle curve) and after bleaching (top curve). The medium contained 25 mM Hepes buffer, pH 7. Ans concentration was 40 μM. ROS membrane protein concentration was 60 μg/mL.

Fluorescence lifetime measurements were carried out on an Ortec 9200 photon-counting nanosecond fluorometer according to the method of Yguerabide (1972). Excitation was selected by a Corning 7.54 filter and emission by a CS 3-68 filter. The samples were suspended in a medium containing 250 mM sucrose and 1 mM Hepes titrated to pH 5. The data were analyzed by the method of moments (Isenberg & Dyson, 1969).

*Analytical Procedures.* Gel electrophoresis of ROS proteins was performed by the method of Fairbanks et al. (1971). The gels were stained with Coomassie blue and scanned on a Gilford spectrophotometer equipped with a gel scanning accessory. Protein was determined according to the method of Lowry et al. (1951). Circular dichroism (CD) spectra of ROS suspensions were measured by using a 0.5-mm pathlength cell on a Cary 60 spectropolarimeter with CD attachment.

## Results

$NaDodSO_4$–polyacrylamide gel electrophoresis of ROS membrane showed only one protein in significant amounts, migrating with an apparent molecular weight of 37000. On the basis of the similarity of this molecular weight with that reported by Papermaster & Dreyer (1974), this protein band can be assigned to rhodopsin. Only a few minor components other than rhodopsin were revealed on the gel scans, which indicates the purity of the ROS preparation. Since at least some of the rhodopsin was bleached (because the animals were not dark adapted before enucleation), the spectral ratios $A_{280}/A_{500}$ and $A_{400}/A_{500}$ of the solubilized ROS preparation were corrected for bleaching according to the procedure of De Grip et al. (1972). The corrected ratios were 2.4 and 0.22, respectively. This preparation was used for studying the interaction of the fluorochrome Ans with ROS membranes.

*Effect of Illumination on the Fluorescence Spectrum of Membrane-Bound Ans.* Figure 1 shows the emission spectrum of *Ans* before and after addition to a suspension of ROS in 25 mM Hepes–NaOH buffer at neutral pH. The emission spectrum of Ans is blue shifted, and there is a ~2-fold increase in its fluorescence intensity in the presence of ROS membranes in the dark (curve b). Upon bleaching of the ROS membranes under room light, a further increase in the fluorescence intensity of bound Ans and a further shift to the blue (from 500 to 490 nm) was observed (curve a, Figure 1). (The spectra shown in Figure 1 have been corrected for the inner-filter

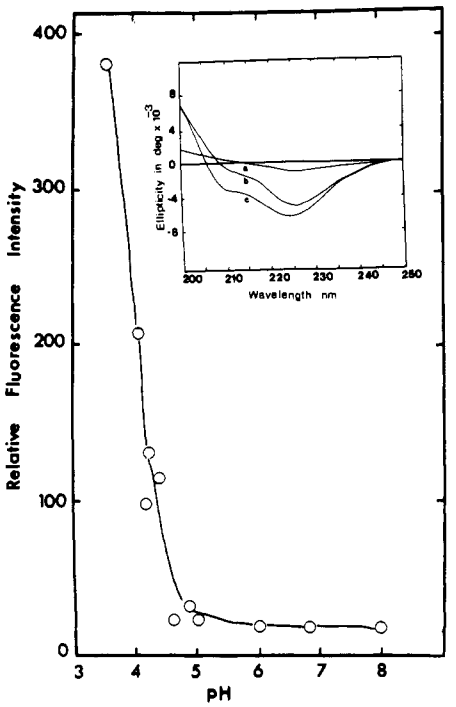


FIGURE 2: Effect of pH on fluorescence intensity of Ans at 500 nm in suspension of ROS membrane. pH was varied by using buffers of different pH. Ans concentration was 10 $\mu$M. ROS membrane protein concentration was 60 $\mu$g/mL. The inset shows CD spectra of ROS membrane suspensions at different pH: (a) pH 4.1; (b) pH 5.2; (c) pH 7.0.

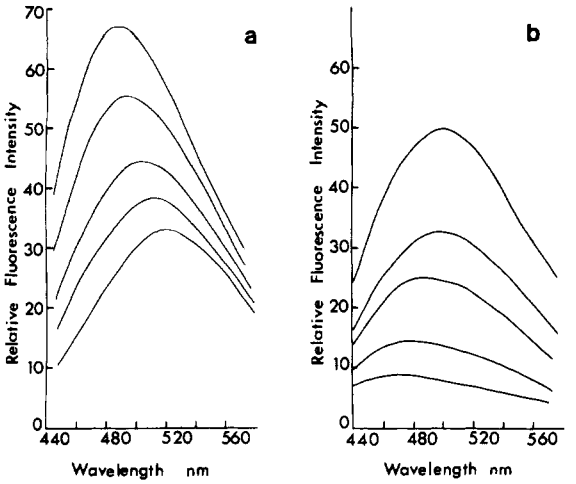


FIGURE 3: (a) Fluorescence emission spectra of Ans–ROS membrane at constant Ans concentration (50 $\mu$M). ROS membrane concentration was varied [from highest to lowest curve ($\mu$g of protein/mL): 90, 63, 45, 36, and 18]. (b) Fluorescence emission spectra of Ans–ROS membrane at constant membrane concentration (60 $\mu$g of protein/mL). The Ans concentration was varied (from highest to lowest curve: 50, 30, 15, 10, and 5 $\mu$M).

attenuation of the exciting light and the fluorescence signal by the added chromophore retinal. Therefore, the enhancement of fluorescence intensity of Ans cannot be due to decreased absorption of retinal at 500 nm on bleaching.)

*Number of Ans Binding Sites.* The number of Ans binding sites ($n$) on the ROS membrane and the dissociation constant ($K_D$) were determined according to the method of Zierler (1977). This method is based on determining initial slopes of direct plots of fluorescence intensity vs. the concentration of the fluorochrome at constant membrane protein concentration [Pr]. The slope of this line is $S_{[Pr]}$. In another set of experiments, the Ans concentration was held constant ($[A]_1$ and $[A]_2$ in two sets of titrations) and the protein concentration

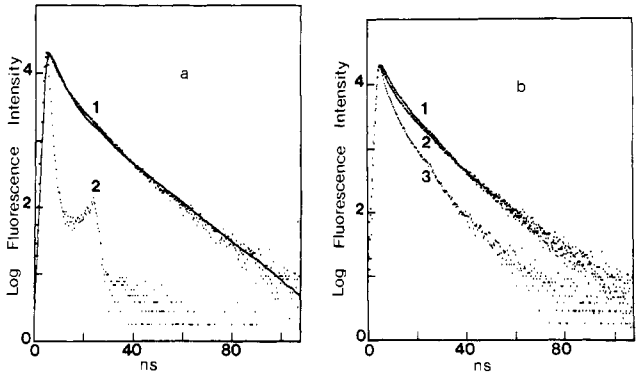


FIGURE 4: (a) Fluorescence decay curve of (1) Ans–ROS membrane in the dark and after bleaching (the solid line superimposed on curve 1 is the curve calculated by using parameters obtained from the method of moment analysis) and (2) excitation pulse. (b) Fluorescence decay curves of (1) Ans-lipid poor protein, (2) Ans–ROS membrane, and (3) Ans-lipid. See Table I for lifetime results.

Table I: Fluorescence Decay Parameters of 8,1-Ans-Labeled ROS Membrane Components[a]

| material | $A_1$ | $\tau_1$ (ns) | $A_2$ | $\tau_2$ (ns) |
|---|---|---|---|---|
| Ans–membrane (dark) | 0.842 | 4.04 | 0.158 | 14.65 |
| Ans–membrane (bleached) | 0.864 | 4.15 | 0.136 | 15.29 |
| Ans–protein (lipid poor) | 0.805 | 4.64 | 0.195 | 13.18 |
| Ans–lipid | 0.95 | 2.53 | 0.05 | 11.79 |

[a] $A_1$ and $A_2$ are the amplitudes of the major and minor decay components, respectively. $\tau_1$ and $\tau_2$ are the corresponding lifetimes. All values were obtained from two exponential method of moment analyses. Measurements were made at different pH values. We did not observe any significant change in either the lifetime or amplitude ($A_1$ or $A_2$) of the individual components with changes in pH.

varied. The initial slope of the line (at low protein concentrations) was determined and designated $S_{[A]_1}$, where $[A]_1$ is a given concentration of Ans. The dissociation constant $K_D$ was determined from

$$K_D = \frac{S_{[A]_2} - S_{[A]_1}}{S_{[A]_1}/[A]_1 - S_{[A]_2}/[A]_2}$$

and the number of binding sites is

$$n = [S_{[A]_1}/S_{[Pr]}] + [K_D(1/S_{[Pr]})(S_{[A]_1}/[A]_1 - S_{[Pr]}/[Pr])]$$

For ROS membrane, $n$ was determined to be 30 mol of Ans bound/mol of rhodopsin as calculated from the concentration of protein. $K_D$ was $3.18 \times 10^{-6}$ M at pH 7.

*Effect of pH.* The fluorescence intensity of Ans bound to ROS is also sensitive to the pH of the suspending medium. Figure 2 shows that at pH values between 8 and 6, the fluorescence of bound Ans is low, but as the pH falls below 4.5, the fluorescence intensity is enhanced dramatically. The emission maximum of bound Ans is at 500 nm between pH 8 and 6 and is shifted to the blue (490 nm at pH 5, 480 nm at pH 4.5, 470 nm at pH 4.0, and 468 nm at pH 3) as the pH is lowered from 6 to 3. The inset shows the CD spectra of ROS between 200 and 250 nm in buffers of different pH. The magnitude of the CD band at 223 nm decreases as the pH falls below 4.5, and at pH 4.1 no distinct CD spectrum is observed.

*Steady-State Emission Spectra.* At a given concentration of Ans (50 $\mu$M) the fluorescence emission spectra shifted to the blue and the peak intensity increased as the concentration of ROS membrane increased. In the experiment illustrated in Figure 3a the emission peak shifted from 520 to 490 nm as ROS membrane protein concentration was increased from 18 to 90 $\mu$g/mL. At higher concentration of ROS membrane, the spectra shifted to as far as 470 nm.

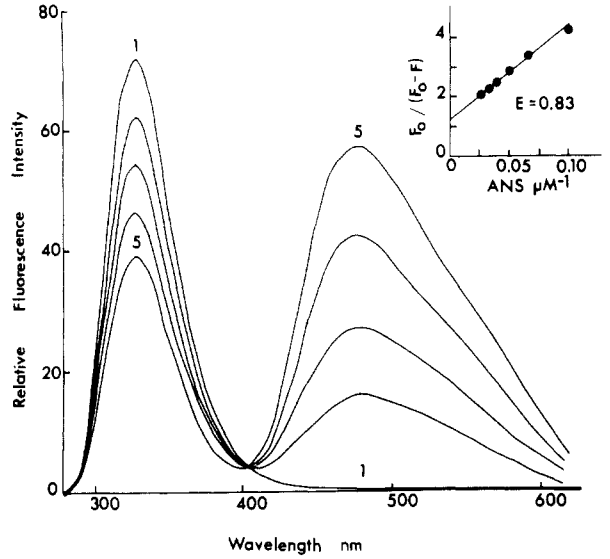


FIGURE 5: Corrected fluorescence spectra of ROS membranes. (Curves 1–5) 0, 5, 10, 15, and 20 μM Ans, respectively. Half-bandwidths of excitation and emission were 6 nm. The medium contained 250 mM sucrose and 1 mM Hepes titrated to pH 5. The inset shows the double-reciprocal plot of the decrease of tryptophyl fluorescence against the concentration of Ans. $F_0$ and $F$ denote tryptophyl fluorescence in the absence and presence of the added Ans. Excitation and emission wavelengths were 295 and 330 nm, respectively. The spectra have been corrected for the inner-filter attenuation of the exciting light and of the fluorescence signal by the added Ans. Membrane protein concentration was 60 μg/mL.

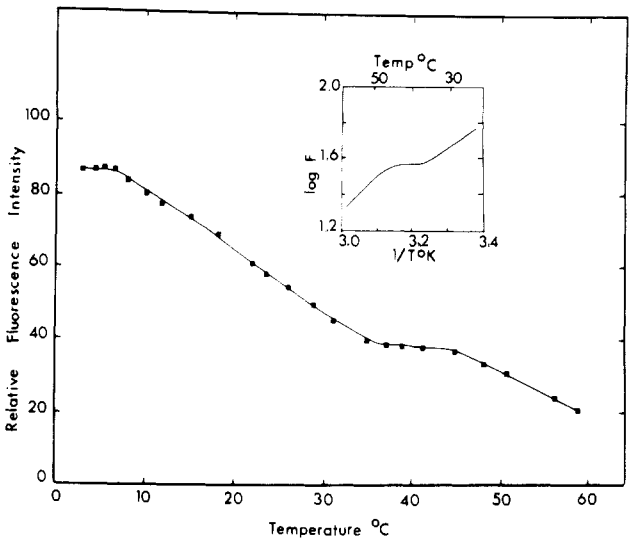


FIGURE 6: Variation of fluorescence intensity of Ans in suspensions of ROS membrane with temperature. The medium contained 1 M NaCl and 25 mM Hepes buffer, pH 7. Ans concentration was 40 μM; ROS membrane protein concentration was 60 μg/mL. The inset illustrates the transition at 34–36 °C in a plot of log fluorescence intensity vs. inverse of absolute temperature between 20 and 60 °C.

In Figure 3b, the emission spectra of Ans are shown at a given concentration of ROS membrane (60 μg of protein/mL) as the concentration of Ans was increased. The emission maximum shifted to a longer wavelength, from 470 to 515 nm, with increase in fluorescence intensity as the Ans concentration was increased from 5 to 50 μM.

*Fluorescence Lifetime Measurements.* Fluorescence decay curves for Ans bound to the membrane and for extracted ROS lipids and proteins are shown in Figure 4. Analysis of the curves was done by the method of moments assuming the decay curve to consist of two components. Results of the analyses (Table I) show that there is no significant change in the fluorescence lifetime of the membrane-bound Ans when the ROS are bleached. These results further show that when lipid is depleted from the membrane, the long component is enhanced, whereas when protein is depleted, the short component is enhanced. Thus, it can be inferred that the long component of the decay curve of Ans–ROS membrane corresponds to the lifetime of Ans bound to the membrane proteins and the short component represents the lifetime of Ans bound to membrane lipids. The observed difference in the lifetime of Ans bound to lipids extracted from the membrane and in the intact membrane is likely to be due to a change in the nature of the Ans binding site in lipid after extraction.

*Energy Transfer.* Figure 5 shows the emission spectra of ROS membranes excited at 295 nm, where tryptophan absorbs, both in the absence of Ans (curve 1) and in the presence of increasing amounts of Ans (curves 2–5). In the absence of Ans, fluorescence is emitted by tryptophan residues only with a band maximum at 330 nm. On addition of increasing amounts of Ans, the tryptophyl fluorescence band at 330 nm is quenched, and a second fluorescence band appears with a maximum at 470 nm. Since free Ans does not fluoresce under these instrumental conditions, the 470-nm band represents tryptophan-excited Ans fluorescence. The inset shows the double-reciprocal plot of the decrease in fluorescence intensity of tryptophan vs. Ans concentration. The intercept on the ordinate gives the reciprocal maximal transfer efficiency corresponding to a state of complete occupation of all Ans binding sites (Wallach et al., 1970). From such a plot the maximal transfer efficiency was calculated to be 0.83 at pH 5 (data shown in Figure 5) and 0.55 at pH 7.

The apparent interchromophore separation, $R$, between the membrane tryptophan and bound Ans molecules was determined from transfer efficiencies ($E$) in dark and bleached ROS membranes by using the relationship given by Förster (1966)

$$R = R_0(E^{-1} - 1)^{1/6}$$

where $R_0$ is the distance at which the transfer efficiency is 50% (critical transfer distance)

$$R_0 = (JK^2Q_0\eta^{-4})^{1/6}9.79 \times 10^3 \text{ Å}$$

where $Q_0$, $K$, $J$, and $\eta$ indicate quantum yield of the donor emission in the absence of the acceptor, the orientation factor, the overlap integral between donor emission and acceptor absorption on the wavenumber scale, and the refractive index of the medium, respectively. $J$ was determined according to the method of Yagi et al. (1977), $\eta$ was 1.4 (Wu & Stryer, 1972), $Q_0$ was 0.06 (Ebrey, 1971), and $K^2$ was assumed to be $^2/_3$ in randomly oriented pairs of donor and acceptor (Stryer, 1978). For ROS membranes, $R_0$ was found to be 14.8 Å. This value was used to calculate $R$, the mean apparent distance separating the membrane tryptophan and bound Ans molecules. At neutral pH, $R$ was calculated to be 14.0 Å in the dark and 14.1 Å in the bleached membranes. At pH 5, $R$ was calculated to be 11.0 Å.

*Effect of Temperature.* Figure 6 shows the variation in fluorescence intensity of Ans in suspensions of ROS membrane when the incubation temperature was raised from 3 to 60 °C. The ROS–Ans mixtures were incubated at each temperature for 5 min, and the fluorescence emission spectrum of Ans was measured. The fluorescence intensity of Ans at 480 nm remains almost constant as the temperature is increased from 3 to 7 °C; beyond that temperature, it decreases rapidly until a temperature of ~34 °C is reached. Beyond this level the fluorescence remains almost constant up to ~42 °C and then declines rapidly as the ROS is incubated from 42 to 60 °C. The emission spectrum of Ans is red shifted by ~2 nm for every 10 °C rise in the incubation temperature. The two

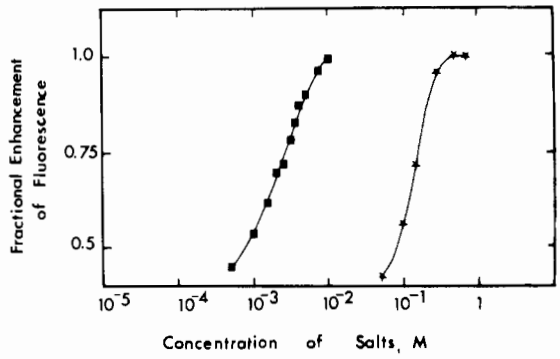


FIGURE 7: Effect of NaCl and $CaCl_2$ on the enhancement of Ans fluorescence by ROS membrane. The solution contained 25 mM Hepes buffer, pH 6.8, 25 μM Ans, 60 μg/mL ROS membrane protein, and added salts in the concentrations indicated in the figure. Fractional enhancement was calculated by taking the fluorescence intensity with saturating concentrations of cations as unity. NaCl (★); $CaCl_2$ (■). Ans fluorescence was measured at 490 nm.

breaks (at 7 °C and 34–36 °C) in the fluorescence vs. temperature curve can be observed when Ans concentration is high (40–50 μM) and in the presence of a high concentration of NaCl (1 M). At low concentrations of Ans and NaCl, these transitions are not sharp. When the ROS's were first incubated at 40 °C with Ans and then cooled to 3 °C, the temperature-induced changes in fluorescence intensity showed the same pattern, indicating that the transitions are reversible. Since there is no change in the pH of the medium within the temperature range studied, changes in fluorescence intensity of bound Ans with temperature cannot be ascribed to a change in pH of the medium. The inset in Figure 6 shows a plot of reciprocal absolute temperature vs. log of fluorescence intensity between 20 and 60 °C. The transition in the curve at ~36 °C appears more clearly in this plot.

*Effect of Salt.* The fluorescence intensity of Ans bound to ROS membranes is enhanced in the presence of low concentrations of salts. A 3-fold increase in fluorescence intensity is observed in the presence of NaCl or $CaCl_2$ (Figure 7). The saturating concentration of the cations is 400–500 mM for the monovalent cation ($Na^+$) and 4–5 mM for the divalent cation ($Ca^{2+}$). A 15-nm blue shift of the emission spectrum of bound Ans is also observed in the presence of both monovalent and divalent cations.

## Discussion

In suspensions of ROS membranes, the fluorescence of bound Ans is considerably enhanced and blue shifted, indicating that the environment within the membrane is less polar than its aqueous surroundings. It is now fairly well established that Ans molecules transmit information about their microenvironment through characteristic features of their fluorescence spectra. A detailed study (Kosower et al., 1975) of the fluorescence behavior of Ans derivatives has revealed that two emitting states are involved in this process: a nonplanar singlet state and a planar intermolecular charge-transfer state, with $\lambda_{max}$ at 520 and 460 nm, respectively. The relative population of the two states depends mostly on the solvent polarity and viscosity of the medium. In a nonpolar environment the emitting species is mostly nonplanar, whereas in the polar medium the molecule is in the charge-transfer state and the fluorescence is quenched via an efficient internal conversion of the excited state to the ground state. Thus, the Ans fluorescence spectrum in a given medium can be considered as a composite spectrum of the two emitting states. The enhancement of fluorescence intensity of Ans upon binding to proteins or membranes arises predominantly from a nonplanar state, in contrast to the situation in polar medium (e.g., aqueous solution) in which a weak red-shifted emission occurs (Weber et al., 1979).

The most important finding in this study is that there is a further enhancement and blue shift in the emission spectra of Ans upon bleaching ROS membranes. The question is, "what change in the membrane could lead to this observation?" Probably bleaching produces an even more hydrophobic environment in the vicinity of bound Ans. The increase in intrinsic tryptophan fluorescence of ROS membranes upon bleaching observed by Chiba et al. (1978) was attributed to local conformational changes in rhodopsin. These authors also suggested that the environment of tryptophan residues of the unbleached rhodopsin molecule is cooperatively modified, upon bleaching, to a more hydrophobic environment. Further indication of a change in the protein conformation was obtained from the observation of birefringence transients in the intact retina (Liebman et al., 1974). Thus, the fluorescence change of the bound Ans very likely monitors the changes in protein conformation upon bleaching.

Since the fluorescence intensity is sensitive to the pH of the medium, it is pertinent to discuss the effect of pH on the emission properties of Ans. Although change in pH could considerably alter the surface charge (discussed later) and the overall structure of the membrane (either could influence the Ans fluorescence), the simpler approach we made was to monitor the protein structure by CD measurements at various pH values. pH-dependent CD spectra (Figure 2) indicate that a decrease in pH is followed by the partial loss of helical structure of the protein; eventually, near pH 4, the membrane tends to aggregate. Thus, the fluorescence enhancement caused by the decrease in pH (from 7.0 to 4.5) is partly due to a change in conformation of the protein. This would affect the environment of the bound probe by decreasing the polarity of the environment, by decreasing the orientation constraint (Radda, 1971), or by a change in the geometry of the bound Ans molecules at low pH (Penzer, 1972). The fluorescence enhancement may also be due to a change in the degree of mobility of water molecules near the Ans binding site due to protein conformational change (Johnson et al., 1979; Weber et al., 1979).

In view of its amphiphilic nature, Ans is known to be a specific probe for apolar–polar interfaces. Such interfaces could be due to phospholipids or to hydrophobic regions in rhodopsin protein. Steady-state emission spectra (Figure 3) clearly indicate that Ans is bound to both protein and lipid. The arguments in favor of this are similar to those described by Zierler & Rogus (1978). Their reported results for sacrolemma vesicle–Ans complex are essentially the same as ours—a blue or red shift in the emission spectra depending upon the dye/membrane ratio. Had there been only one component, lipid or protein, for Ans binding, there would be little or no spectral shift with altered fluorescence intensity.

Further evidence of at least two major binding regions for Ans (protein and lipid) was obtained from lifetime measurements. The higher lifetime can be attributed to lipid-poor protein and the lower, to lipid. The observed fit of the decay curves, with the assumption of the presence of two components, suggests the presence of a third component, a minor one that we did not analyze. We observed a difference in the values, significant for lipid, between separated components and membrane suspension (Table I). This indicates that the microenvironment of Ans, that is, the structures of the lipid and protein components, suffers a small change when the components are isolated from the membrane.

Wu & Stryer (1972) used specific fluorescent probes, which

have emission maxima in the wavelength range of the absorption maximum of 11-*cis*-retinal, to study energy transfer from the probe to 11-*cis*-retinal. Since Ans in the presence of ROS membrane fluoresces in this same wavelength region, such an energy transfer may be expected to occur. However, we have ruled out this possibility on the basis of lifetime measurements, which show that the lifetime of bound Ans fluorescence does not change upon bleaching, in contrast to what was observed by Wu & Stryer (1972) in their studies.

Although Ans binds to both protein and lipid components of the ROS membrane, the linearity of the double-reciprocal plot of fluorescence intensity vs. protein concentration in our study (data not shown) indicates that fluorescence enhancement of each bound Ans molecule is the same, irrespective of site (Radda, 1971). This is supported by the fact that our results involving energy transfer from aromatic residues to Ans show a distinct isoemissive point (Figure 5).

The maximal transfer efficiency, calculated from the results of energy transfer from tryptophan to bound Ans, is high; consequently, the calculated distance between them is low. The transfer efficiency is even higher at lower pH, as expected from the increase in the number of bound Ans molecules with increasing hydrogen ion concentration. In analyzing energy transfer data from other membrane systems, Radda (1971) suggested that at low pH, Ans molecules may bind to sites that are further away from the protein, possibly to lipids. Since we did not observe any significant increase in the proportion of bound Ans molecules in the lipid component upon lowering pH (Table I), it is likely that the protein provides the sites for increased binding of the dye. It should be recognized that energy-transfer measurements, used commonly in various membrane systems, can provide only some qualitative average values for the interchromophoric distance. Due to a lack of precise knowledge of the molecular orientation of these chromophores, it is impossible to estimate accurately these values from the calculated efficiency of excitation energy transfer. However, the energy-transfer data in our study can be utilized to distinguish between pH-dependent and light-induced conformational change of rhodopsin. In the former, the interchromophore distance changes because of a conformational change of the protein backbone, whereas in the latter it remains the same, indicating that any light-induced conformational change is likely to be in the microenvironment of the probe.

We have used a characteristic property of lipids, the lipid phase transition, to study the effect of temperature on Ans binding to ROS membrane. Similar studies with the Ans probe and *Escherichia coli* membranes have revealed the phase transition temperature of the lipids (Traüble & Overath, 1973). At the NaCl concentration of 1 M, most of the protein binding sites of Ans on the ROS membrane are saturated, and some Ans binding to the lipids may occur as in *E. coli* membrane. Figure 6 distinctly reflects two phase transition temperatures for the ROS membrane, 7 °C and 34–36 °C. Miljanich et al. (1978), using differential scanning calorimetry, reported that both bovine ROS membrane and its extracted phospholipids show one relatively sharp phase transition, at ~7 °C. Our results with Ans fluorescence support this finding and further indicate a transition of membrane lipids at 34–36 °C.

At low concentrations of Ans and NaCl, the sharp changes in fluorescence intensity at the transition temperatures were not observed. Schichi (1973) has shown that the stability of opsin conformation to thermal denaturation at 64 °C is enhanced when opsin is associated with the phospholipids. Thus the phase transition of the ROS membrane between 5 and 45 °C, as reflected by bound Ans fluorescence, is not likely to be caused by a change in opsin conformation. It is probable that the two breaks in the fluorescence vs. temperature curve represent a lipid phase transition of the ROS membrane phospholipids.

Metal cations are known to increase Ans fluorescence in biomembranes by increasing the binding of Ans to the membrane (Vanderkooi & Martonosi, 1969; Rubalcava et al., 1969). The effect, in general, is due to shielding of the surface negative charges by the added salts, thereby reducing the electrostatic repulsion between the negatively charged Ans and the membrane residues. The enhancement due to $Na^+$ was found to saturate at a concentration higher than that of $Ca^{2+}$. In other words, at the same ionic strength $Ca^{2+}$ produced a greater enhancement of Ans bound to ROS membrane than did $Na^+$. The plot of fluorescence intensity vs. $Ca^{2+}$ concentration (not shown) indicates the presence of two classes of binding sites for $Ca^{2+}$ as suggested by Daemen et al. (1977). These phenomena have been reported for low-density lipoprotein (Ghosh et al., 1974) and microsomal membrane (Gomperts et al., 1970; Vanderkooi & Martonosi, 1969). It is generally agreed that the enhancement of Ans fluorescence by the addition of cations to membranes is entirely due to the creation of additional probe binding sites. The relative effectiveness of $Ca^{2+}$ compared to $Na^+$ can be due to various factors: a screening effect of the cations reducing the negative surface potential of the bound Ans (Vanderkooi & Martonosi, 1969), preferential affinity of the membrane for $Ca^{2+}$ (Daemen et al., 1977), chelation of $Ca^{2+}$ to phospholipids (Ghosh et al., 1974), or effective ordering of the water molecules by $Ca^{2+}$ (Johnson et al., 1979). In the absence of detailed studies of the effect of cations, particularly $Ca^{2+}$, on ROS membrane, a definitive interpretation cannot be made.

In conclusion, our use of Ans as a fluorescence probe has provided some information regarding this important photosensitive membrane, ROS. Light-induced conformational change of rhodopsin, the proximity relationship between the bound probe and tryptophan of membrane protein, temperature-induced lipid phase transition, and the strong affinity of this membrane for calcium ion may have long-range significance in understanding the structure–function relationship of the membrane. Our study further indicates that, with proper choice of probes and with adequate instrumentation, one could derive additional and more specific information; for example, energy transfer with lipophilic fluorescent probes (which can accept electronic excitation energy from tyrosine or tryptophan of membrane protein) and lipid–protein interaction in relation to membrane structure and/or function could be conveniently assessed. Even with Ans, measurements of emission polarization of the bound probe would provide information regarding the fluidity of the region where Ans is bound and the change that occurs upon illumination.

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