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Interactions of the Fluorescent Anion 1-Anilino-8-Naphthalene Sulfonate with Membrane Charges in Human Red Cell Ghosts

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Summary. The interaction of the fluorescent anion 1-anilino-8-naphthalene sulfonate (ANS) with erythrocyte membranes was studied as a function of the charges in the membrane. The membrane charges were altered by including in the incubation medium the organic ions tetraphenylboron (TPB^-), tetraphenylarsonium (TPA^+) and tetraphenylphosphonium (TPP^+) which differ in charge but have similar structure and high affinity for the membrane. TPB^- decreases whereas TPA^+ and TPP^+ increase ANS fluorescence arising from the membrane. The fluorescence changes are accompanied by variations in the affinity and capacity of the membrane for ANS. The fluorescence arising under conditions where all the ANS present is within the membrane (by extrapolation to infinite membrane concentration) does not vary with these ions. Salicylate and SCN^- which inhibit anion permeability also decrease ANS fluorescence with no apparent change in quantum yield. These results indicate that ANS exists as an anion in the membrane, associated with the regions involved in ion permeability, and that the concentration of ANS in the membrane is a function of the concentration of all the ions present in the medium and their relative affinities for the membrane. Thus, variations in ANS fluorescence reflect redistributions of the ANS ions following changes in electrostatic interactions with the membrane fixed charges.

The permselectivity of the erythrocyte membrane for anions over cations has been related to the presence of fixed positive charges [20, 21]. These charges are thought to be NH_3^+ groups because the pH dependence of permeability shows a pK of about 9, and amino reagents decrease anion and increase cation permeability [22]. According to the fixed-charge hypothesis [20–22], these membrane charges control the concentration of diffusible ions in the membrane phase. The concentrations and activities of ions in the membrane have been estimated on the basis of kinetic measurements assuming Donnan equilibria between the membrane and the external solutions and the presence of positive fixed charges [20]. However, direct

measurements of the concentration of diffusible ions in the membrane phase are hindered by the difficulties in distinguishing between the ions within the membrane and those in solution. A more direct approach would be feasible if an ion could be used whose properties change when it interacts with the membrane in a way such that a measurable parameter would indicate its concentration in the membrane phase. In this respect, certain fluorescent ions seem to offer special advantage because their fluorescent properties are extremely sensitive to the environment [6, 24]. Thus, 1-anilino-8-naphthalene sulfonate (ANS)[1] is an anion which becomes highly fluorescent when it interacts with certain proteins and membranes [2, 3, 5, 6, 9, 23, 25] but is practically non-fluorescent in water, so that under certain experimental conditions it is possible to measure the fluorescence arising solely from those molecules that are in the membrane.

The present investigation was carried out to study the possibility that ANS interacts with erythrocyte membranes in an electrostatic form; that is, could ANS fluorescence be used to follow variations in the concentration of mobile ions within the membrane? ANS fluorescence was studied using organic ions, which presumably have a high affinity for the membrane, in an attempt either to change the total amount of anions within the membrane by adding a cation, or to change the relative distribution of the different anions within the membrane by adding an anion. Evidence is presented which suggests that ANS exists as a mobile anion in the membrane and that variations in the distribution of membrane charges are reflected by variations in ANS fluorescence. A preliminary report of this paper has been previously presented [8].

Materials and Methods

Blood was drawn into heparin from healthy male adults and used immediately to prepare hemoglobin-free ghosts as described previously [19] and frozen-thawed to eliminate any permeability barriers. Prior to use, the ghosts were washed three times and suspended at a concentration of 5–10 mg protein/ml in the same buffer used in the incubations, usually 20 mM Tris-Cl at pH 7.5 unless otherwise specified. Experiments repeated with ghosts used on the day of preparation but not frozen-thawed show the same qualitative behavior. Membrane protein was measured according to the method of Lowry, Rosebrough, Farr and Randall [17] using crystalline bovine serum albumin (Sigma Chemical) as standard. Fluorescence measurements were done on an Aminco Bowman Spectrophotofluorometer with a 150 W Hanovia Xenon Arc lamp and an RCA IP 21 photomultiplier tube. The composition of the incubation mixtures is given in the figure legends. Titrations were done by adding concentrated aliquots to the same cuvette, and where necessary the concentrations were corrected for changes in total

1 Abbreviations used: ANS, 1-anilino-8-naphthalene sulfonate; TPB^-, tetraphenylboron; TPA^+, tetraphenylarsonium; TPP^+, tetraphenylphosphonium.

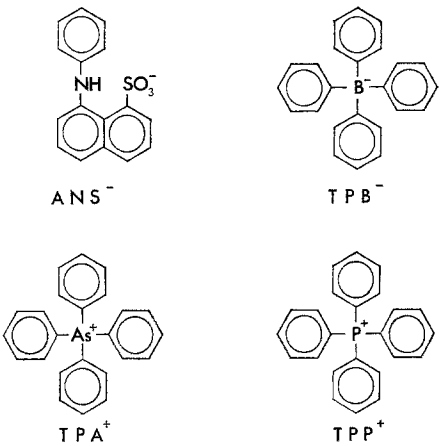

Fig. 1. Structural formulas of ANS and tetraphenyl derivatives. Note the similarity in structure between the tetraphenyl ions but their different charge

volume during the titration. Initial volume in the cuvette was 2 ml. Under the conditions used in these experiments, fluorescence arising from free ANS in H_2O was negligible. The excitation and emission spectra are not corrected for variation in the sensitivity of the instrument with wavelength. Since in some experiments phosphate buffer was used rather than Tris (*see* Fig. 3), it should be understood that the excitation and emission spectra of ANS were the same in both conditions. Except where indicated, excitation was at 380 nm and emission at 470 nm. The slit arrangements used gave band widths at half-height of 15–20 nm. All experiments were done at room temperature (23 °C). The suspensions were stirred magnetically with a circular fly inside the cuvette. The mixing time was about 2 sec. The Mg salt of ANS was a gift from Dr. L. Stryer. The Na salt of tetraphenylboron (TPB^-) and the Cl salts of tetraphenylarsonium (TPA^+) and tetraphenylphosphonium (TPP^+) were obtained from K & K Laboratories. The structures of ANS and the tetraphenyl derivatives used are shown in Fig. 1.

Determinations of $\bar{K}_{app}$ (average apparent dissociation constant) were done by the double reciprocal plot method [3, 10, 23] assuming no interaction between sites, using the equation $\frac{1}{F}=\frac{\bar{K}_{app}}{F_\infty}\cdot\frac{1}{[ANS]}+\frac{1}{F_\infty}$ where F is the fluorescence at a given ANS concentration and F_∞ is the maximum fluorescence when all the sites are occupied. In a double reciprocal plot of fluorescence against [ANS], $\bar{K}_{app}$ will be given by the slope of the curve divided by its intercept at the ordinate. The plots were linear over the range of concentrations used.

Results

The addition of Hb-free ghosts to a solution containing ANS results, as shown in Fig. 2, in a rapid increase in fluorescence intensity representing the interaction of the dye with hydrophobic regions of the membrane [9, 23]. The magnitude of the fluorescence increase appears proportional to the amount of ghosts added, and further additions will show similar increases

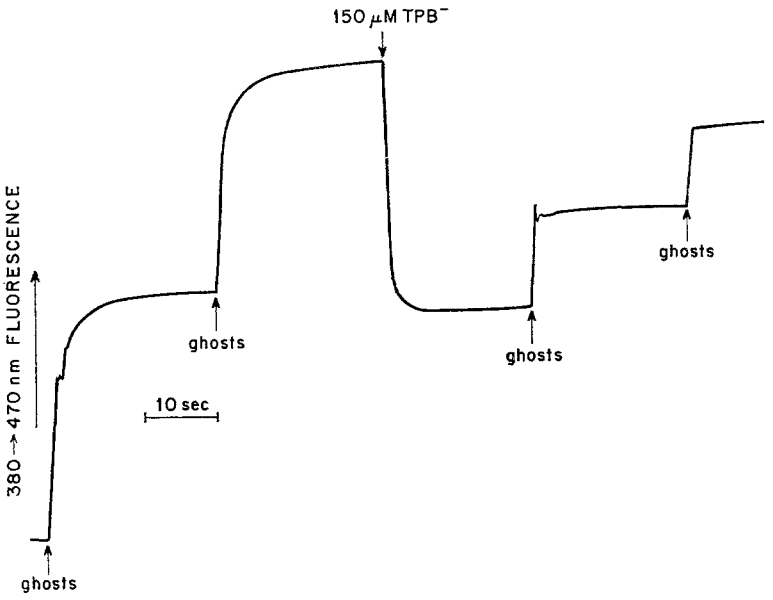

Fig. 2. Kinetics of the interaction of ANS with erythrocyte membranes. Effect of TPB^-. Experimental conditions: 20 mM Tris-Cl, pH 7.5, 50 μM ANS. Each addition of frozen-thawed ghosts consisted of 50 μliters containing 356 μg ghost protein. An upward deflection means an increase in fluorescence. TPB^- was added at the final concentration indicated. See text for discussion

until all the ANS is bound. The increments are the same whether ghosts are added to an ANS solution or ANS is added to a membrane suspension. Kinetically, the fluorescence response is biphasic in the sense that there is an initial fast component, followed by a slower increase in intensity. The first component is completed faster than the mixing time of the cuvette (1–2 sec); the slow component has a half-time of about 6–8 sec. Similar kinetics have been reported in ghosts and mitochondria [3, 9] and seem to depend on the structure of the fluorescence probe used and the integrity of the membrane. When TPB^-, an anion which appears to have a high affinity for membranes [14, 16, 18] is added, fluorescence is seen to decrease sharply and further additions of the same amounts of ghosts now show smaller increases in fluorescence. Furthermore, the response changes from a biphasic to a monophasic fast increase, indicating that the slow phase is the main component affected by TPB^-. The overall fluorescence change is the same whether TPB^- is added before or after the ghosts, although in the presence of TPB^- each response is monophasic.

The decrease in ANS fluorescence intensity induced by TPB^- only occurs in the presence of ghost membranes; TPB^- has no effect on ANS

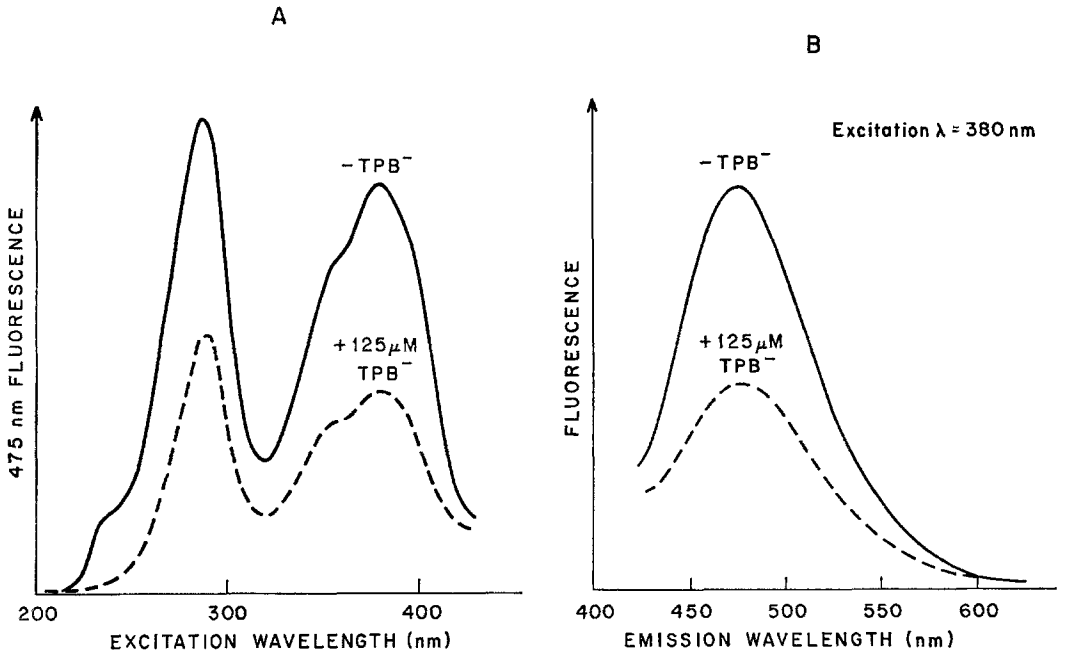

Fig. 3A and B. Excitation and emission spectra of erythrocyte membranes in the presence of ANS. Experimental conditions: 136 mM Na phosphate pH 7.4, 20 μM ANS and 150 μg/ml ghost protein. Fluorescence is in arbitrary units. The dotted lines are the spectra after addition of 25 μliters of NaTPB to a final concentration of 125 μM. The spectra are not corrected for variation of sensitivity of the instrument with wavelength

fluorescence in water or ethanol, suggesting that the effect is caused by an interaction of TPB^- with the membrane.

Fig. 3 shows excitation and emission spectra of ghosts suspended in a solution containing ANS. As previously reported [9, 26], the interaction of ANS with ghosts results in the appearance of a fluorescence peak in the 470 nm region corresponding to the ANS in hydrophobic regions of the membrane (Fig. 3B). The dotted lines in Figs. 3A and 3B show the excitation and emission spectra after addition of TPB^-. The disappearance of the shoulder in the 250 nm region in Fig. 3A is due to a filter effect as TPB^- absorbs strongly in this region. The fluorescence decrease induced by TPB^- is not accompanied by shifts in the excitation or emission wavelengths of the ANS associated with the ghosts.

The decreased fluorescence intensity in the presence of TPB^- may be caused by a decrease in the number of ANS molecules within the membrane and/or to a decrease in the quantum yield resulting from some alteration in the environment of the ANS within the membrane. One approach toward

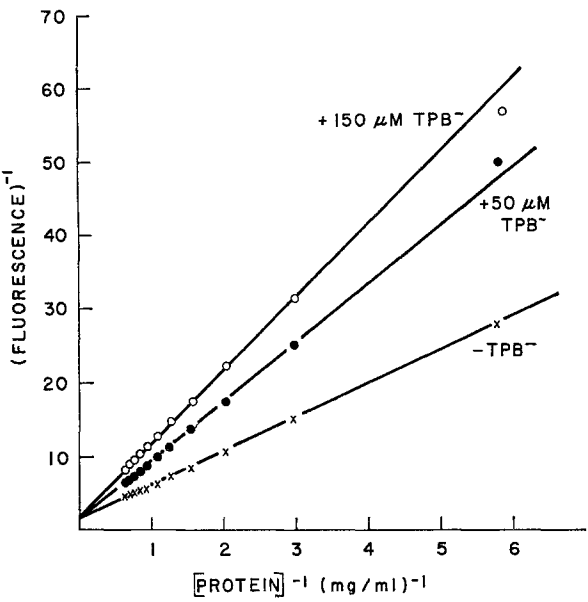

Fig. 4. Double reciprocal plot of ANS fluorescence vs. ghost protein concentration. Effect of TPB^-. Experimental conditions: 20 mM Tris-Cl, pH 7.5, 25 μM ANS. Where indicated, 50 or 150 μM NaTPB was present during the titration. Ghosts were added in 50-μliter increments. Ghost protein was varied from 0.173 to 1.532 mg/ml. In this and the following figures, fluorescence is in arbitrary units

distinguishing between these possibilities can be made graphically by extrapolating to conditions when all the dye is associated with the membrane [3, 5, 23]. Double reciprocal plots of ANS fluorescence vs. the concentration of membrane have been found to be linear [3, 23, 26]; when extrapolation to infinite membrane concentration is made, the intercept should correspond to the fluorescence when all the dye is within the matrix [5] and a relative measure of average quantum yield may be obtained. This method has been used to distinguish between a change in fluorescence caused by variations in quantum yield and changes in the number of ANS molecules within the membrane, since the former should have a different intercept whereas the latter will have the same intercept but a different slope. The results of such an experiment carried out at two different concentrations of TPB^- are shown in Fig. 4. It is apparent that TPB^- decreases ANS fluorescence, but the fluorescence at infinite membrane concentration remains the same. These results indicate that alterations in fluorescence seen under these conditions reflect, primarily, changes in the number of ANS molecules within the membrane. It should be pointed out that this type of extrapolation assumes that the curve will not bend at high membrane concen-

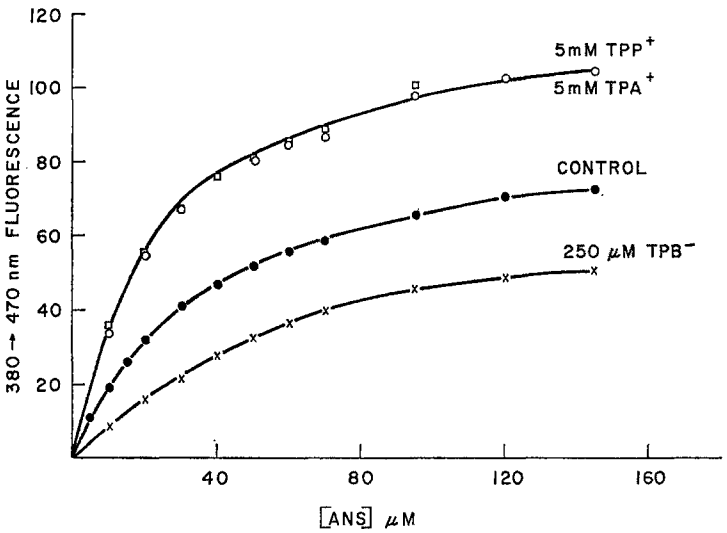

Fig. 5. Effect of tetraphenyl ions on ANS fluorescence in ghosts. Experimental conditions: 20 mM Tris-Cl, pH 7.5. Ghosts: 51 μg protein/ml. The indicated final concentrations of TPB^-, TPA^+ and TPP^+ were present at the beginning of the titration

trations. Also, the ANS concentration at which the titration is done may alter the results. It was found, for instance, that if this titration was carried out at ANS concentrations below 5 μM, TPB^- shows a different intercept, presumably because under these conditions only the regions with highest affinity for ANS are involved and a different average quantum yield is observed.

As discussed earlier, the total number of negative ions in the membrane is determined by the total number of positive charges. When more than one mobile anion is present, the distribution of the different anions within the matrix will depend on their relative affinities for the membrane cations. An increase in the number of positive charges increases the number of all anions in the membrane, whereas inclusion of other anions at constant ionic strength alters only their relative distributions in the membrane without affecting the total number of negative charges. If the effect of TPB^- on ANS fluorescence in ghosts is considered to occur because of the exclusion of negatively charged ANS molecules from the membrane by competition between TPB^- and ANS for the positive fixed charges in the membrane, changing the charge on the TPB^- would result in an increase rather than a decrease in fluorescence since the affinity and/or capacity for ANS would be greater because of an increased number of positive charges in the membrane. The effects of TPA^+ and TPP^+ on ANS fluorescence are shown in Fig. 5. The negatively charged TPB^- decreases fluorescence, whereas the

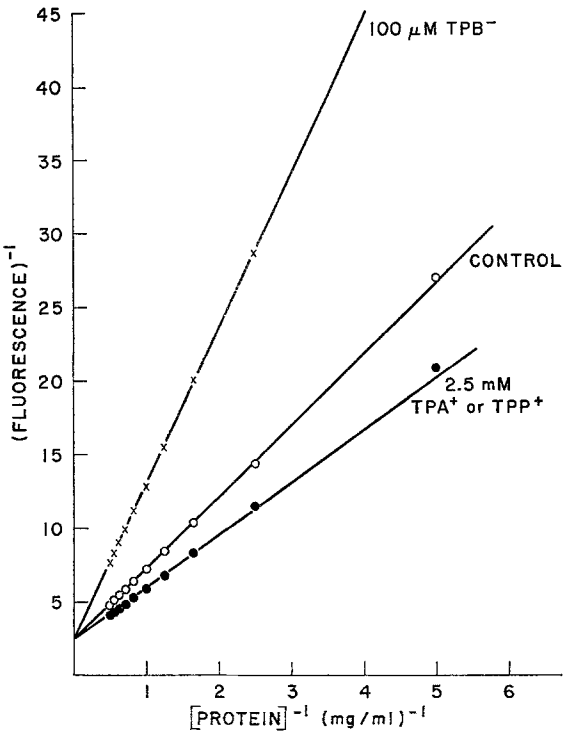

Fig. 6. Double reciprocal plot of ANS fluorescence vs. ghost protein concentration. Effect of tetraphenyl ions. Experimental conditions: 20 mM Tris-Cl, pH 7.5, 20 μM ANS. Ghost protein was varied from 0.2 to 2 mg/ml. The indicated final concentrations of the tetraphenyl ions were present at the beginning of the titration

positively charged TPA^+ and TPP^+ increase fluorescence regardless of the ANS concentration. Thus, the direction of the fluorescence change depends solely on the sign of the charge of the ion that interacts with the membrane. It should be noted that the concentration of the added cations required to give a fluorescence change equivalent in magnitude to that caused by TPB^- is about an order of magnitude larger. As in the case of TPB^-, TPA^+ and TPP^+ change only the fluorescence intensity without shifts in the emission wavelengths. A double reciprocal plot of fluorescence against membrane concentration (Fig. 6) indicates that the cations do not affect the relative quantum yield but promote the solubility of more ANS molecules within the membrane phase.

In order to obtain quantitative estimates of affinity constants and the number of bound ANS molecules under the conditions mentioned above, binding isotherms were done following the methods used by others [3, 9, 10, 23]. The Table shows the values of the average apparent dissociation

Table. *Effect of tetraphenyl ions on the average apparent dissociation constant for ANS in human erythrocyte ghosts. Incubation medium: 20 mM Tris-Cl, pH 7.5, containing 0.1–0.2 mg ghost protein/ml. Volume of mixture was 2 ml*

Ghost preparation [a]	$\bar{K}_{app}$ [b] (μM)	
Non F–T ghosts	54.1 ± 0.6	(2)
F–T ghosts	31.3 ± 3.5	(5)
F–T ghosts + TPB^- (250 μM)	83.8 ± 1.5	(2)
F–T ghosts + TPA^+ or TPP^+ (5 mM)	25.4 ± 0.8	(2)

[a] Non F–T ghosts are ghosts used immediately after preparation, washed three times in 20 mM Tris-Cl, pH 7.5, without freeze-thawing. F–T ghosts were stored frozen after preparation and washed three times in 20 mM Tris-Cl, pH 7.5, after thawing before the titration. The indicated concentrations of TPA^+, TPP^+ or TPB^- were present at the beginning of the titration.

[b] Fluorescence was measured as a function of ANS concentration which varied from 0–120 μM, and $\bar{K}_{app}$ was determined as described in the text from double reciprocal plots of fluorescence against [ANS]. Numbers in parentheses represent numbers of separate measurements. The average values of $\bar{K}_{app}$ are given together with the range.

constant ($\bar{K}_{app}$) for ANS calculated by means of double reciprocal plots of fluorescence against ANS concentration. The value of $\bar{K}_{app}$ obtained for non-frozen-thawed ghosts is in good agreement with published values [3, 9, 23]. Freeze-thawing increases the affinity of the ghosts for ANS presumably by increasing the availability of hydrophobic regions for which ANS has higher affinity than the hydrophilic anions present. The increase of $\bar{K}_{app}$ in the presence of TPB^- reflects the competition between TPB^- and ANS to neutralize the positive charges in the membrane. $\bar{K}_{app}$ decreases only slightly in the presence of TPA^+ and TPP^+, which is consistent with an overall increase of anions in the membrane owing to an increase in positive charges.

In addition, ANS binding was also studied using Scatchard plots [3, 9, 10]. These plots were found to be linear when the concentration of ghosts at which the ANS titration was carried out was kept below 0.2 mg protein/ml. At higher ghost concentrations, the Scatchard plots became curved or showed two or more linear regions with different slopes. This may perhaps reflect the heterogeneity of different membrane regions with respect to their affinity for ANS. The range of values determined for n (maximum number of nmoles of ANS bound/mg protein) is between 25 and 60 for frozen-thawed ghosts in 20 mM Tris-Cl, pH 7.5, when the plots are linear, in agreement with the published values [3, 9, 23]. However, the variability and curvature of these plots suggests that this should be taken only as an approxi-

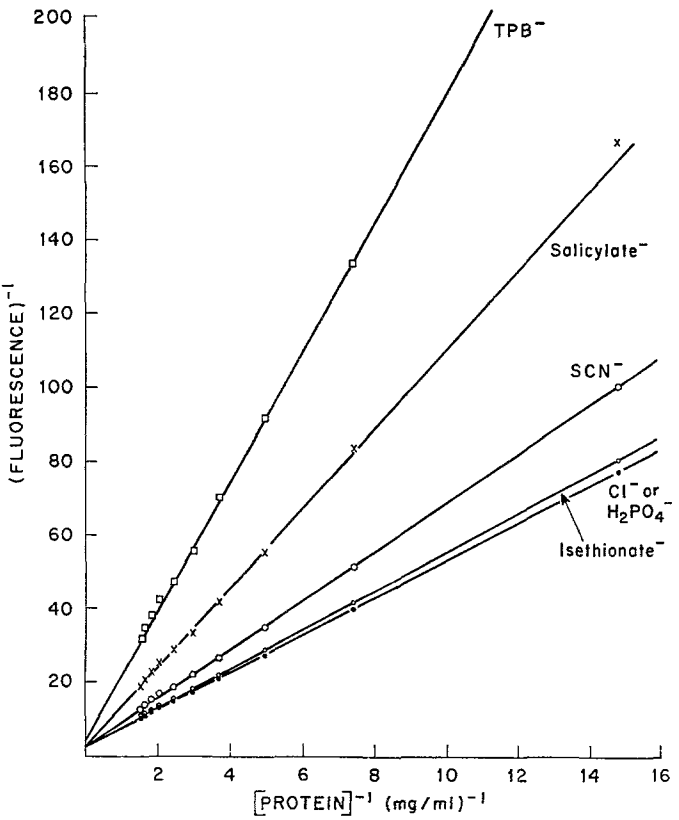

Fig. 7. Effect of anions on ANS fluorescence in ghosts. Experimental conditions: 20 mM Tris-Cl, pH 7.5, 25 μM ANS. Where indicated, 20 mM NaX was included, where X represents the anion, except for TPB^-, which was 0.2 mM. Ghost protein was varied from 67.5 to 675 μg/ml

mate value subject to the experimental conditions under which it is obtained. Nevertheless, TPB^- consistently decreased n while TPA^+ and TPP^+ had the opposite effect. The values of $\bar{K}_{app}$ calculated from the Scatchard plots for each condition are within the range of the values given in the Table.

Passow [22] and Wieth [27] have shown that certain anions act to decrease the permeability of erythrocyte membranes to Cl^- and $SO_4^=$. In order to see if these effects could be correlated with changes in the membrane ions as reflected by ANS fluorescence, a comparison of the effect of a variety of anions on ANS fluorescence in ghosts was done. Fig. 7 shows that SCN^- and salicylate decrease ANS fluorescence compared to Cl^-, phosphate and isethionate. It is interesting to note that the order of the effectiveness in decreasing ANS fluorescence is the same as that found in inhibition of anion transport [27], salicylate being more effective than SCN^-. The results of the double reciprocal plots (Fig. 7) suggest that the effect of

the various anions is not on quantum yield but on the membrane concentration of ANS resulting from a redistribution of the anions within the matrix based on their relative affinities for the membrane phase.

Discussion

The high sensitivity of the fluorescence parameters of ANS and related compounds to the polarity of the environment in which they are dissolved has been used to study the microenvironment of proteins [5, 6, 24], enzymes [6, 24], and membranes [2, 3, 9, 23, 25, 26]. ANS fluorescence in membranes also appears to be very sensitive to electrostatic interactions. Cations and low pH increase ANS binding and fluorescence in ghosts [9, 23] and in sarcoplasmic reticulum membranes [25]; divalent and trivalent cations are, respectively, more effective than monovalent cations [11]; negatively charged fatty acids have the opposite effect [13]. ANS fluorescence changes with voltage steps in squid axon, and the direction of the change depends on the polarity of the voltage step [4]. Cationic and neutral detergent micelles bind ANS with enhanced fluorescence, but anionic detergents do not [23]. The partition coefficients of ANS for phospholipid-water systems decrease as the negative charges in the phospholipid increase [12]. The direction of the change in ANS fluorescence on energization of the mitochondrial membrane [1, 2] is reversed in sub-mitochondrial particles [1] which have an "inside-out" membrane and possibly a membrane potential in the opposite direction [16, 18].

The variety of factors that may affect the fluorescence properties of ANS and the complexity of biological membranes makes the interpretation of these phenomena difficult. Fluorescence may change for two general reasons: (a) a change in the quantum yield owing to a change in the environment of the ANS molecules in the membrane; (b) a change in the affinity and/or capacity of the membrane for ANS would change the number of ANS molecules in the membrane. It is the latter possibility that suggests the usefulness of ANS to study the membrane charges, since a change in the electrostatic interaction between the membrane and ANS will be reflected by a different affinity and, thus, different binding. Previous studies in erythrocyte and sarcoplasmic reticulum membranes have shown that changes in the number of molecules of ANS in the membrane accompany the fluorescence changes with cations and fatty acids [3, 9, 11, 13, 23, 25]. In the present work, evidence is presented indicating that ANS contributes a significant fraction of the negative charges associated with the fixed positive charges in the ghost membrane. The magnitude of the fraction of negative

charge contributed by ANS depends on the other ions present in the incubation medium. Anions with high affinity for the membrane charges will compete with ANS, displacing it from the membrane and thus decreasing the fluorescence, while addition of cations to the membrane increases the total negative charges associated with them and consequently increases the ANS concentration in the membrane as measured by fluorescence. It is unlikely that quantum yield changes occur in the experiments presented here since the fluorescence changes are only in intensity and are unaccompanied by shifts in the emission peaks (Fig. 3) which are usually associated with changes in quantum yield [24]. Furthermore, the maximum fluorescence values obtained by extrapolation to infinite membrane concentration (Figs. 4, 6, 7) show no change under the different conditions used. These results support the conclusion that the observed changes are caused by a change in the number of bound ANS molecules following variations in the membrane charges rather than changes in the environment of the ANS molecules. The fact that the anion (TPB^-) is more effective than the cations (TPA^+ and TPP^+) in changing the fluorescence may be related to the existence of fixed positive charges in the membrane which may define the relative ease with which the ion could enter the matrix.

It should be mentioned that TPB^- is a reagent used to precipitate K^+ in solution and is also known to have a high affinity for protonated amino and ammonium groups including those associated with long organic chains [7]. K^+ determinations in the membranes used in the present work showed less than 0.5 nmoles of K^+ per mg protein, making unlikely the possibility that the major effect of TPB^- on ANS fluorescence is associated with K^+ in the membrane.

One of the difficulties in the interpretation of ANS studies with membranes is the lack of detailed information on the structure of the membrane and the location of the ANS within it. ANS is an amphipathic molecule and thus most likely to interact at polar-apolar interfaces. X-ray diffraction studies on bimolecular leaflets of fatty acids or phospholipids show that the dye is located at the lipid-water interface, with the sulfonate group in the region of the polar heads and the naphthalene ring buried in the hydrocarbon core [15]. In erythrocyte membranes, the location of ANS cannot as yet be specified, but energy transfer measurements between ANS and tryptophan in erythrocyte ghosts have shown an average distance of approximately 40 Å between tryptophan residues and ANS [26]. The biphasic increase in fluorescence on addition of ghosts (Fig. 2) has been reported before [3, 9] and interpreted to represent interaction at "surface" and "inner sites" (fast and slow components, respectively) because the slow

component disappears when the ghosts are sonicated. This would seem to agree with the effect of TPB^- in decreasing mainly the slow component (Fig. 2) if ANS interacts primarily at the surface when the TPB^- is within the membrane. However, more information, unavailable at the present, is necessary to evaluate this interpretation.

ANS fluorescence arises from those molecules that are in a region of low polarity. If these ANS molecules are interacting with the membrane fixed charges, it would seem that the latter could be closely associated with hydrophobic regions. On the other hand, these positive fixed charges have been associated with the ion-permeable channels. As Passow has pointed out [20], the nature of these ion-permeable channels in red cell membranes is not known but has been assumed to be aqueous in the calculations of anion concentrations in the membrane. If this is the case, it would seem that the ion-permeable channels could be of a polar-apolar nature of the type where the amphipathic ANS is likely to interact. The association of ANS with the ion-permeable channels is suggested by preliminary experiments that show that ANS decreases $SO_4^=$ permeability in erythrocytes and that ANS fluorescence decreases when the membranes are treated with dinitrofluorobenzene or pronase, which are known from Passow's work to decrease anion permeability. Also, it is interesting to note that anions which have been found to inhibit anion permeability [22, 27] decrease ANS fluorescence with the same order of effectiveness (Fig. 7), supporting the idea that the inhibition is caused by a decrease in the membrane concentration of the anion owing to competition with the inhibitor anions.

The preceding discussion suggests the possibility of using ANS and other fluorescent ions to investigate the relationship between ion permeability and specific properties of the membrane. However, it must be noted that any attempts to correlate a functional state of the membrane with ANS fluorescence experiments must take into account the possibility that only a fraction of the fluorescing ANS molecules may be associated with the functional regions. Also, although less likely, effects of ANS molecules in polar environments – and thus, non-fluorescent – are possible.

Finally, there are difficulties in comparing phenomena occurring in intact red cells (with which the permeability experiments are done) with those in Hb-free ghosts, but the fluorescence studies with ANS are necessarily confined to ghosts because of light absorption by hemoglobin.

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