Interaction of Fluorescent Probes with Membranes. I. Effect of Ions on Erythrocyte Membranes*

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ABSTRACT: 1-Anilinonaphthalene-8-sulfonate, a fluorescent probe, does not fluoresce in water but fluoresces strongly in organic solvents and when bound to certain native proteins. The presented studies indicate that 1-anilinonaphthalene-8-sulfonate binds with enhanced fluorescence to hemoglobin-free rabbit erythrocyte membranes. This binding is sensitive to the cation concentration of the suspending medium. Thus 1-anilinonaphthalene-8-sulfonate binds to hemoglobin-free erythrocyte membranes in 20 mosm Tris-HCl buffer (pH 7.4) to the extent of 1.05×10^7 molecules/cell with a statistical binding constant, $\bar{K}_{app}$ of 4.3×10^{-5} M. Addition of 300 mosm NaCl or 3.0 mosm $CaCl_2$ increases the number of 1-anilinonaphthalene-8-sulfonate molecules bound to 3.14×10^7 and 2.83×10^7 molecules per cell, respectively, while the $\bar{K}_{app}$ values decrease to 3.45 and 2.70×10^{-5} M, respectively. Addition of NaCl to hemoglobin-free erythrocyte membranes suspended in hypotonic 20 mosm Tris-HCl buffer (pH 7.4) leads to volume changes of the membranes which indicate an osmotic response and thus the restoration of the membrane's impermeability to cations. An attempt is made to corrolate these volume changes with the effect of the ions on the membrane *per se* which bring about the enhanced binding of 1-anilinonaphthalene-8-sulfonate. Results indicate that the addition of low concentrations of cations leads to shielding by the ion atmosphere, of the electrostatic repulsion of ionic groups of membrane phospholipids which could explain the enhanced binding of 1-anilinonaphthalene-8-sulfonate and the restoration of cation impermeability. Studies on the binding of 1-anilinonaphthalene-8-sulfonate to detergent micelles indicate that 1-anilinonaphthalene-8-sulfonate is a probe for hydrophobic–hydrophylic interphases containing neutral or cationic hydrophilic groups.

Many of the structural and functional properties of biological membranes depend upon the presence within the membrane of apolar regions. Thus the cellular permeability to different molecules has been correlated with the solubility of these molecules in apolar solvents (Hober, 1945, and references therein). The formation of such hydrophobic regions is likely to be the result of the presence in membranes of high concentrations of amphipathic (Hartley, 1936) phospholipid molecules which in aqueous solutions are oriented so that the fatty acid chains are directed away from water in close van der Waals contact, whereas the polar groups are directed so that they have maximum contact with water (Abramson *et al.*, 1964; Lenard and Singer, 1966). In addition, the participation of hydrophobic bonding in the stabilization of the native conformation of proteins implies that these molecules might also be involved in the formation of the apolar regions of membranes (Kauzmann, 1957).

It is important therefore to ascertain the properties of such apolar regions. In general this has been approached indirectly by following the changes in the permeability properties of the cell under varying conditions. A more recent approach has been to determine the exchange of a lipid-soluble membrane component such as cholesterol between a membrane and a lipoprotein or other apolar entity (Bruckdorfer *et al.*, 1968).

Edelman and McClure (1968) have defined fluorescent probes as small molecules which undergo changes in one or more of their fluorescent properties as a result of noncovalent interactions with proteins. Thus, molecules like 1-anilinonaphthalene-8-sulfonate, 2-*p*-toluidinylnaphthalene-6-sulfonate, and several dansylamino acids are nearly nonfluorescent in aqueous solution but become highly fluorescent upon binding to certain proteins (Weber and Laurence, 1954; McClure and Edelman, 1966; Chen, 1967; Aronson *et al.*, 1968). It appears that the increase in quantum yield is the result of changes in microscopic polarity in the vicinity of the probe. Strong fluorescence has been correlated with binding of the probe to hydrophobic sites of the proteins (Laurence, 1952; McClure and Edelman, 1966).

The present report is an extension of the use of these molecules to study the properties of apolar regions of biological membranes. Binding of 1-anilinonaphthalene-8-sulfonate to hemoglobin-free rabbit erythrocyte membranes[1] has been studied as a function of ionic composition of the suspending media. The behavior of 1-anilinonaphthalene-8-sulfonate in the presence of detergent micelles was also studied as a possible model for the interaction of the dye with the membranes.

Experimental Procedure

Fluorescence measurements were performed in a Farrand

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[1] The term hemoglobin-free rabbit erythrocyte membranes refers to the isolated intact plasma membrane of the erythrocyte usually referred to as the erythrocyte "ghost" or stroma. The volume response observed is therefore a change in the water content of this intact vesicle.

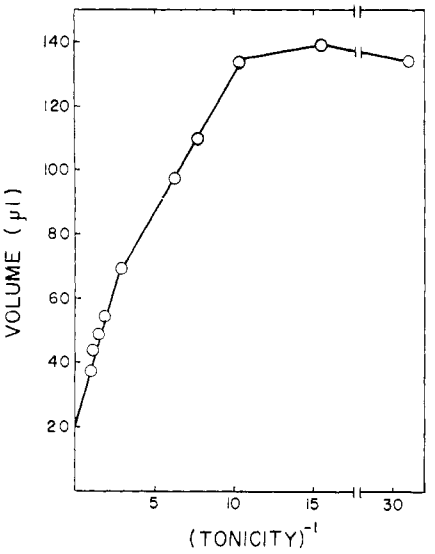

FIGURE 1: Osmotic responsiveness of hemoglobin-free rabbit erythrocyte membranes. Packed cell volume corrected for extracellular volume is plotted against tonicity; 310 mosm NaCl is assumed to have a tonicity of 1. (See section on Experimental Procedure for details.)

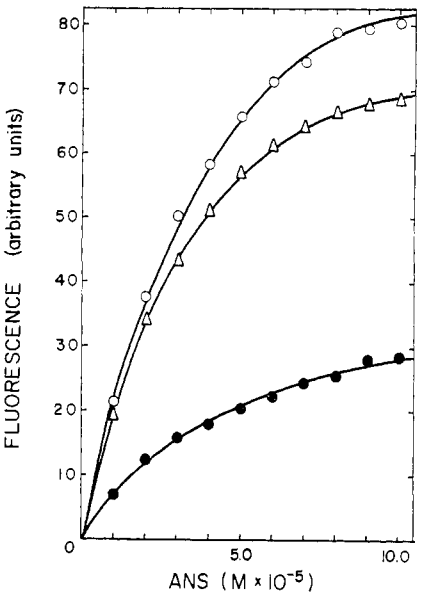

FIGURE 2: The variation in fluorescence intensities with 1-anilinonaphthalene-8-sulfonate concentration of hemoglobin-free erythrocyte membranes suspended in 20 mosm Tris-HCl buffer (pH 7.4). No additions (●); with added 300 mosm NaCl (○); with added 3.0 mosm $CaCl_2$ (△). The cell suspension (3.0 ml; 0.77 mg of protein) was placed in quarz fluorometer cell and ANS was added with an Agla micrometer syringe. The suspension was stirred with a micromagnetic bar for 1 min between additions. Final 1-anilinonaphthalene-8-sulfonate concentrations are shown.

spectrofluorophotometer. The excitation wavelength, 350 mμ, was selected by a monochromator and Wratten glass filter 18A, from the emission of a xenon light source. The fluorescence filter was a Wratten gelatin filter 2A. Emission was determined at 470 mμ. The sodium salt of 1-anilinonaphthalene-8-sulfonate was twice recrystallized from water (McClure and Edelman, 1966).

Hemoglobin-free rabbit erythrocyte membranes were prepared essentially by the procedure of Dodge *et al.* (1963) except that 20 mosm Tris-HCl buffer (pH 7.4) was used in the hemolysis steps; mosm[2] is used throughout as defined by Dodge *et al.* (1963). To preclude the peroxidation of the hemoglobin-free erythrocyte membrane lipids, the rabbits received *per os* 25 mg of α-tocopheryl acetate dissolved in corn oil at weekly intervals. In addition, the hemoglobin-free erythrocyte membranes were stored at 4° and used within 3-days preparation.

Volume Changes of the Hemoglobin-Free Erythrocyte Membranes. The determination of volume changes as a function of NaCl concentration presented difficulties since at low cation concentrations [^{14}C]inulin and [^{14}C]serum albumin gave erratic results in the determination of extracellular trapped volumes. These difficulties were absent when Blue Dextran 2000 (Pharmacia Fine Chemicals) was used. The procedure followed was to mix 0.5 ml of a suspension of equal volumes of hemoglobin-free erythrocyte membrane pellet and 20 mosm Tris-HCl buffer (pH 7.4) with 5.0 ml of a solution containing the appropriate NaCl concentration and 2.0 mg of Blue Dextran 2000. The cells were allowed to equilibrate for 20 min at 25 ± 2° and were then centrifuged in graduated hematocrit tubes at 12,000*g* for 30 min in an International microcapillary centrifuge-type MB using rotor 905. The solution above the pellet was carefully removed and the volume of the pellet was noted. The pellet was then dissolved in 2% sodium dodecyl sulfate and the concentration of Blue Dextran 2000 was determined from its absorbance at 630 mμ. This allowed readily for corrections to be made of the extracellular volume.

Detergents. Sodium dodecyl sulfate and cetyltrimethylammonium bromide were high-purity lots obtained from the British Drug Houses Ltd. Triton X-100 was obtained from Rohm & Haas de México. These detergents gave a critical micelle concentration in aqueous solutions, determined by surface tension (Chávez, 1967) of 5.25, 1.02, and 0.1 mM, respectively. These are in agreement with previously published values (Mukerjee and Mysels, 1955; Scott and Tartar, 1943; Gonick and McBain, 1947).

Results

Volume Changes of the Hemoglobin-Free Erythrocyte Membranes. The volume changes of the hemoglobin-free erythrocyte membranes upon addition of NaCl are shown in Figure 1, plotted according to Ponder and Barreto (1957). No change in volume was observed between 10 and 30 mosm NaCl (tonicities of 10–30). Two linear regions were found between 30 and 110 mosm and between 110 and 310 mosm (reciprocal toncities of 2.8–10 and of 1.0–2.8, respectively) indicating that the membranes are behaving as osmometers. These results are presented mainly as reference for the following studies on 1-anilinonaphthalene-8-sulfonate binding.

Binding of 1-Anilinonaphthalene-8-sulfonate to the Hemoglobin-Free Erythrocyte Membranes. Since 1-anilinonaphthalene-

[2] The milliosmolar concentration was calculated by totalling the concentration of all ionizable species in the solution, neglecting deviation of the salts from ideal behavior. The concentration of 310 mosm was considered isotonic with the intact erythrocyte.

TABLE I: Relative Fluorescent Intensities of 1-Anilinonaphthalene-8-sulfonate Bound to Hemoglobin-Free Erythrocyte Membranes in Different Salt Solutions and to Detergent Micelles.

System	Rel Fluorescent Intensity[a]
Hemoglobin-Free Erythrocyte membranes[b]	100
+300 mosm NaCl	100
+3.0 mosm $CaCl_2$	87.5
Triton X-100[c]	40.8
Cetyltrimethylammonium bromide[c]	35.0
Sodium dodecyl sulfate[c]	~0

[a] The 1-anilinonaphthalene-8-sulfonate concentration was 2.0×10^{-5} M. [b] Hemoglobin-free erythrocyte membranes in 20 mosm Tris-HCl buffer (pH 7.4). Protein concentration varied 0.46–1.73 mg. [c] Amphiphile (1–16 mM) dissolved in 10 mM Tris-HCl buffer (pH 7.4).

8-sulfonate does not fluoresce in aqueous solutions, the appearance of fluorescence on addition of hemoglobin-free erythrocyte membranes indicates the interaction of the dye molecules with the membranes. Figure 2 shows the change in fluorescence with increasing dye concentration of hemoglobin-free erythrocyte membranes in 20 mosm Tris-HCl (pH 7.4) and in the presence of NaCl to bring the concentration to isotonicity with the intact erythrocyte. A markedly enhanced fluorescence is observed in the presence of NaCl. A similar increase is obtained when 3.0 mosm $CaCl_2$ is added. This increase in fluorescence could be due either to an increase in the quantum yield of the molecules bound to the membranes in the presence of the added salt, or alternatively, to an increase in the number of molecules bound to the membrane, the quantum yield remaining unchanged. These alternatives were tested by determining the fluorescence of a fixed concentration of dye under conditions where all the dye molecules are bound to the membranes. This is difficult to obtain experimentally but may be derived from a double-reciprocal plot (Weber and Young, 1964) as shown in Figure 3. It can be seen that within experimental error, the relative fluorescence intensities of 1-anilinonaphthalene-8-sulfonate bound to hemoglobin-free erythrocyte membranes in the absence and presence of NaCl are essentially equal. A slight but consistent decrease is observed in the presence of $CaCl_2$. The derived values are presented in Table I, together with similar determinations with detergent micelles.

Klotz (1947) has shown that the binding of dyes to proteins can be described by the following equation

$$\frac{P_0}{xD_0} = \frac{1}{n}\left(1 + \frac{\bar{K}_{app}}{(1-x)D_0}\right) \quad (1)$$

where P_0 and D_0 are the total protein and dye concentrations, respectively; x is the fraction of the dye bound and can be obtained from the quotient of the observed fluorescence to that

TABLE II: Effect of Added Salts on the Binding of 1-Anilinonaphthalene-8-sulfonate to Hemoglobin-Free Erythrocyte Membranes.[a]

Additions to Hemoglobin-Free Erythrocyte Membranes in 20 mosm Tris-HCl Buffer (pH 7.4)	n (10^{-8} mole mg^{-1})	$\bar{K}_{app}$ (M $\times 10^{-6}$)
None	1.52	4.30
300 mosm NaCl	4.55	3.45
3.0 mosm $CaCl_2$	4.10	2.70

[a] Conditions as in Figure 4. Derived parameters are from Figure 4 and eq 1.

derived in Figure 3 when all the dye is bound; n is the number of binding sites and $\bar{K}_{app}$, the statistical average apparent dissociation constant for the binding of the dye to the nth sites. Figure 4 shows that the data of Figure 2 when plotted in this manner give good agreement with eq 1. The derived parameters for the equation are shown in Table II. It is apparent that increasing the NaCl or $CaCl_2$ concentration leads to a nearly threefold increase in the number of molecules of 1-anilinonaphthalene-8-sulfonate bound to the membranes. This is associated with a decrease in the apparent dissociation constant $\bar{K}_{app}$ which is greater for $CaCl_2$ than for NaCl. Data not shown indicate that addition of KCl leads to binding undistinguishable from that in the presence of NaCl. Figure 5 shows the changes in fluorescence of 1-anilinonaphthalene-8-sulfonate as the concentrations of NaCl or $CaCl_2$ are increased above that of 10 mosm Tris-HCl (pH 7.4). The data are plotted as a function of mosm ion concentration (Figure 5A) and of the square root of the mosm ion concentration (Figure 5B). In plotting the values for the effect of added NaCl (Figure 5B), 10 mosm Tris-HCl was assumed to elicit a response equivalent to 10 mosm NaCl. In the same figure, the plotted values for the fluorescence in the presence of added $CaCl_2$ extrapolate in the ordinate to the fluorescence of the hemoglobin-free erythrocyte membranes in 10 mosm Tris-HCl. The rate of binding of 1-anilinonaphthalene-8-sulfonate to the hemoglobin-free erythrocyte membranes with salt concentration is greatest in the region of 10–144 mosm NaCl and 0.15–1.69 mosm $CaCl_2$ and the response is linear when plotted as a function of the square root of the ionic concentration. This linear response would appear to indicate that we are dealing with a primary salt effect which could be visualized as resulting from an increased shielding of the electrostatic repulsion of some ionic groups of the membrane components by the ion atmosphere as the concentration of electrolyte is increased. The decreased slope above 144 mosm NaCl and 1.69 mosm $CaCl_2$ would be indicative of saturation of the sites or a decreased effectiveness of the ionic effect above this breakpoint. Approximately 100-fold less $CaCl_2$ than NaCl is required to elicit an equivalent response.

The appearance of fluorescence upon addition of 1-anilinonaphthalene-8-sulfonate to hemoglobin-free erythrocyte membranes was instantaneous and changed only slightly with time.

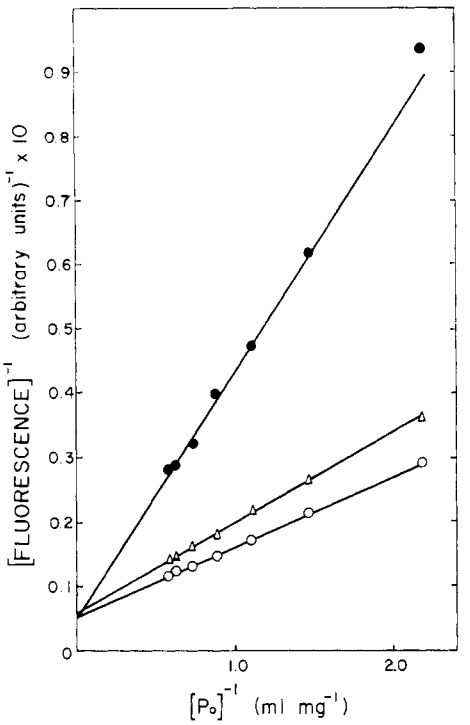

FIGURE 3: The binding of 1-anilinonaphthalene-8-sulfonate to hemoglobin-free erythrocyte membranes as a function of membrane protein concentration. Membranes in 20 mosm Tris-HCl buffer (pH 7.4) (●); with added 300 mosm NaCl (○); with added 3.0 mosm $CaCl_2$ (△). Total protein varied from 0.46 to 1.73 mg. 1-Anilinonaphthalene-8-sulfonate concentration was 2.0×10^{-5} M.

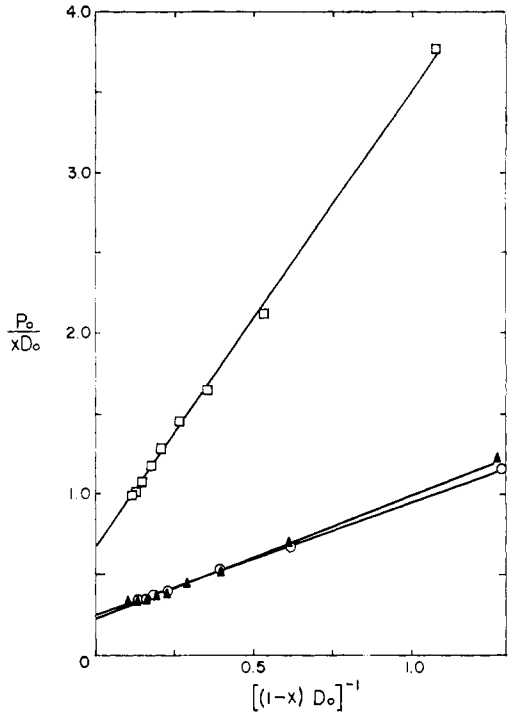

FIGURE 4: The binding of 1-anilinonaphthalene-8-sulfonate to hemoglobin-free erythrocyte membranes as a function of 1-anilinonaphthalene-8-sulfonate concentration. Data of Figure 2 plotted according to eq 1. Membranes in 20 mosm Tris-HCl buffer (pH 7.4) (□); with added 300 mosm NaCl (○); with added 3.0 mosm $CaCl_2$ (△). The units of the ordinate and abscissa are 10^8 mole^{-1} mg and 10^4 M^{-1}, respectively.

The partition of 1-anilinonaphthalene-8-sulfonate between octanol and water measured by the enhanced fluorescence of the dye in the octanol phase is invariant when the NaCl or $CaCl_2$ concentrations are increased in the aqueous phase through the range of concentrations which lead to the changes with the hemoglobin-free erythrocyte membranes noted above. Thus there seems to be no change in the lipophilic character of the dye after addition of cations.

Binding of 1-Anilinonaphthalene-8-sulfonate to Detergent Micelles. For the purpose of comparison, the same determinations have been performed for the binding of 1-anilinonaphthalene-8-sulfonate to micelles of either cationic (cetyltrimethylammonium bromide), neutral (Triton X-100), or anionic (sodium dodecyl sulfate) amphiphiles. All studies were performed above the critical micelle concentrations of the various detergents. Only micelles of positive or neutral charge bind 1-anilinonaphthalene-8-sulfonate as is evidenced by the absence of a fluorescence increase in the presence of sodium dodecyl sulfate (Table I and Figure 5). The data also conform to eq 1 substituting Ao, the total amphiphile concentration, for P_0. Thus good linearity is observed (Figure 6). The derived parameters indicate that 1 molecule of 1-anilinonaphthalene-8-sulfonate binds per 200 molecules of Triton X-100 and per 230 molecules of CTABr. The $\bar{K}_{app}$ values being 1.35×10^{-5} and 3.8×10^{-5} M for Triton X-100 and cetyltrimethylammonium bromide, respectively.

Literature values for the micellar association number are 169 for cetyltrimethylammonium bromide in 0.013 M KBr (Debye, 1949) and 82–321, with a reported average of 180, for Triton X-100 (Kushner and Hubbard, 1954). This would indicate that within the limits of these calculations, one molecule of 1-anilinonaphthalene-8-sulfonate binds per cetyltrimethylammonium bromide per Triton X-100 micelle.

In Figure 5 are also shown preliminary results which indicate that the binding of 1-anilinonaphthalene-8-sulfonate to sodium dodecyl sulfate micelles increases as the NaCl concentration is increased. No changes were observed within this range of NaCl concentration when cetyltrimethylammonium bromide or Triton X-100 was studied. When these values are plotted as a function of the square root of the NaCl concentration, a reasonably good straight line is observed.

Discussion

The constancy in the apparent partition coefficient of 1-anilinonaphthalene-8-sulfonate between octanol–water on addition of salts indicates that the lipophilic character of the dye is not increased by cations in the range studied. It would seem therefore that the enhanced fluorescence of 1-anilinonaphthalene-8-sulfonate on addition of salts to the hemoglobin-free erythrocyte membranes is due to changes in the membrane *per se*. These changes do not seem to lead to a modification in the hydrophobicity of the sites to which 1-anilinonaphthalene-8-sulfonate binds as judged by the constancy of the relative fluorescent intensities (Table I) and are due rather to an increase in the number of sites to which 1-anilinonaphthalene-8-sulfonate molecules can bind. The magnitude of this increase is emphasized by the following

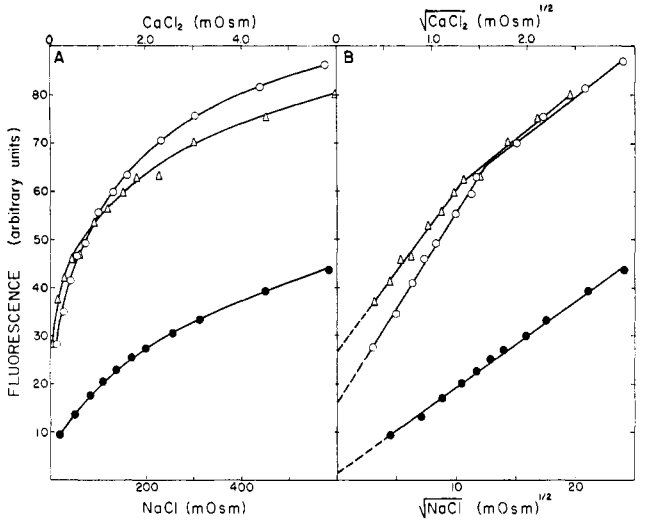

FIGURE 5: Binding of 1-anilinonaphthalene-8-sulfonate to hemoglobin-free erythrocyte membranes and sodium dodecyl sulfate micelles as a function of cation concentration. The membranes (0.77 mg of protein) in 10 mosm Tris-HCl buffer (pH 7.4) were titrated using an Agla micrometer syringe with NaCl (○) and with $CaCl_2$ (△). The sodium dodecyl sulfate (16 mM) in 20 mosm Tris-HCl buffer (pH 7.4) was similarly titrated using NaCl (●). The 1-anilinonaphthalene-8-sulfonate concentration in all cases was 2.0×10^{-5} M. Data are expressed as a function of cation concentration (A) and square root of the cation concentration (B).

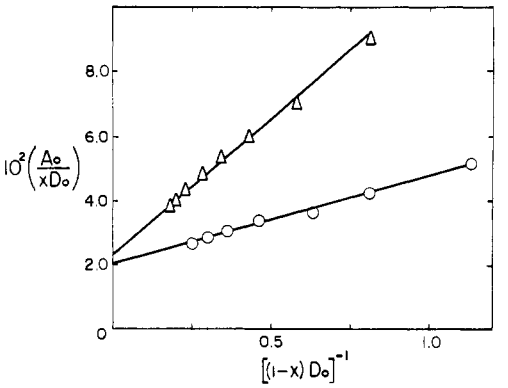

FIGURE 6: The binding of 1-anilinonaphthalene-8-sulfonate to micelles of cetyltrimethylammonium bromide (△) and Triton X-100 (○). The detergents (16 mM) in 20 mosm Tris-HCl buffer (pH 7.4) were titrated with 1-anilinonaphthalene-8-sulfonate using an Agla micrometer syringe. Units in the abscissa are 10^4 M^{-1}.

rough calculation. From the values shown in Table II for n (10^{-8} mole of 1-anilinonaphthalene-8-sulfonate bound/mg of membrane of protein) and assuming that each hemoglobin-free erythrocyte contains roughly 11.4×10^{-13} g of protein (Dodge *et al.*, 1963), one calculates that 1.05×10^7 molecules of 1-anilinonaphthalene-8-sulfonate are bound per cell in 20 mosm Tris-HCl buffer (pH 7.4) and that an increase to 3.14×10^7 and 2.83×10^7 molecules results from the presence of 300 mosm NaCl and 3.0 mosm $CaCl_2$, respectively.

The binding of 1-anilinonaphthalene-8-sulfonate to detergent micelles indicates that it behaves similarly to other dye molecules interacting only with neutral or oppositely charged micelles (Hartley, 1934). The fact that the binding is accompanied by an increased fluorescence indicates that the chromophore is located in a region of the micelle which is of lower dielectric constant than water. Above the critical micelle concentration, in the range studied, it is likely that we are dealing with spherical Hartley micelles in which the detergent molecules are oriented with the hydrocarbon chains directed away from the water in close van der Waals contact, while the polar groups are oriented toward the micellar surface to maximize their interactions with water. Since 1-anilinonaphthalene-8-sulfonate is an amphipathic molecule, it is likely that it interacts with the micelle in such a manner that the sulfonate group is located at the aqueous layer and the remaining portion of the molecule is directed toward the hydrophobic interior. It may be reasonably concluded from the interaction of 1-anilinonaphthalene-8-sulfonate with detergent micelles, that 1-anilinonaphthalene-8-sulfonate is a probe for an apolar–polar interface of neutral or cationic nature. (A similar conclusion may be drawn from the binding of 1-anilinonaphthalene-8-sulfonate to serum albumin; Laurence, 1952.) The model studies with the detergent micelles indicate also that the addition of cations to an anionic micelle (sodium dodecyl sulfate) leads to an increased binding of 1-anilinonaphthalene-8-sulfonate as a result of the shielding of the anionic charge by the ion atmosphere (Figure 5).

In analogy with these results, it is likely that 1-anilinonaphthalene-8-sulfonate binds to an apolar–polar interface of the membrane. As mentioned in the introduction such interfaces could be due to phospholipid aggregates or to hydrophobic regions of membrane proteins. The greater effectiveness of $CaCl_2$ as compared with NaCl in bringing about an enhanced binding of 1-anilinonaphthalene-8-sulfonate (Table II and Figure 5) would appear to favor interaction of the cations with a more polarizable phosphate group of the phospholipids, rather than with an ionized carboxyl group of proteins (Bungenberg de Jong, 1949). Also, preliminary results indicate that treatment of hemoglobin-free erythrocyte membranes with trypsin which reduced the protein content of the membrane by some 50% does not significantly alter the salt effect as measured by 1-anilinonaphthalene-8-sulfonate binding. On the other hand, treatment with phospholipase C which does not destroy the cellular morphology (Lenard and Singer, 1968) does lead to a reduction in the number of binding sites. Thus it is plausible to envisage at present the sites where 1-anilinonaphthalene-8-sulfonate binds as those resulting from phospholipid aggregates within the membrane. The enhanced binding of 1-anilinonaphthalene-8-sulfonate on addition of cations would then be viewed as resulting from the decreased coulombic repulsion of the ionic groups of the phospholipids due to the presence of counterions in the Stern layer. This shielding would facilitate the penetration of 1-anilinonaphthalene-8-sulfonate and also lead to closer packing of the molecules making the interface more hydrophobic. There is ample evidence that the addition of cations to phospholipid micelles or monolayers bring about such changes (Abramson *et al.*, 1964; Shah and Schulman, 1965; Cerbón, 1967; Luzzati and Spegt, 1967).

The osmotic responses observed in the present study follows closely the report of Weed *et al.* (1963) with hemoglobin-free human erythrocyte membranes and those of Theorell (1952) and Stein (1956) with partially hemolyzed cells. These results indicate that an increase in the tonicity of NaCl solutions added to hemoglobin-free erythrocyte membranes leads to re-

gions of linear volume decrements and thus to the conclusion that the membranes behave as osmometers (see discussion by Weed *et al.*, 1963, and Whittam, 1964). We are therefore faced with the problem of how a membrane that is permeable to a molecule as large as hemoglobin and other cytoplasmic enzymes under hypotonic conditions regains impermeability to ions when resuspended in saline solutions.

It is tempting to propose that the addition of low concentrations of NaCl to the hemoglobin-free erythrocyte membranes leads to changes in the membrane structure which make it impermeable to the diffusion of cations, so that further additions of NaCl now lead to an osmotic response. This is supported by the fact that the packed cell volume is not a continuous linear function of tonicity (Figure 1). Low NaCl concentrations below 110 mosm do not decrease the volume or only slowly while above this level the slope increases markedly. Conversely, the binding of 1-anilinonaphthalene-8-sulfonate to the hemoglobin-free erythrocyte membranes shows the greatest rate of increase with NaCl concentration below 144 mosm; a significant decrease in slope being observed above this level (Figure 5). The changes in the packing of the phospholipids due to charge neutralization by added cations which are thought to lead to the enhanced binding of 1-anilinonaphthalene-8-sulfonate would also account for the regain in the membrane's impermeability to cations.

Since hypotonic hemolysis is also associated with hypoionic conditions, it is conceivable that the changes in the membrane which lead to cation and protein leakage during hemolysis are the reverse of those studied here. Mainly that the reduction in the concentration of external cations leads to an increased coulombic repulsion of the polar groups of the phospholipid molecules and thus to a looser packing and to the formation of aqueous channels without actual rupture of a rigid structure. It can be argued that this conclusion is in opposition to the classical experiments of Wilbrandt (1941) in which sucrose is substituted for external ions without hemolysis being observed. This substitution is only possible if some cations are presented in the external solution. However, if erythrocytes are suspended in sucrose or lactose in the absence of cations, they become freely permeable to monovalents cations (Bolingbroke and Maizels, 1959, and references therein).

It would appear that fluorescent probes are important tools for the further study of membrane function. Thus, very low concentrations of the dyes are needed which should not alter significantly the system under study. Since only bound molecules fluoresce, it is not necessary to remove free dye and errors due to traping are reduced. The approximate relation between quantum yield of the fluorescence and microscopic polarity in the vicinity of the dye should allow estimates to be made of the dielectric constant within membranes or micelles. Transient phenomena may be approached through the changes in the fluorescence or in its polarization. While this manuscript was in preparation, a communication along this latter aspect involving nerve depolarization has appeared (Tasaki *et al.*, 1968).

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