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The Interaction of Fluorescent Probes with Anion Permeability Pathways of Human Red Cells

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Summary. The fluorescent probe ANS is a permeant anion in human red cells. The rate of ANS permeation is decreased by lyotropic anions and increased by low ionic strength, resembling the response of Cl and SO_4 transport to changes in the composition of the medium. ANS inhibits Cl and SO_4 exchange measured at 0 and 37 °C, respectively. The inhibitory potency of ANS isomers increases in the sequence 1,8 ANS <2,8 ANS < 1,4 ANS <2,6 TNS <5,2 ANS. The disulfonic stilbene derivative SITS inhibits Cl exchange 50%. Combinations of ANS and SITS result in additive inhibitory effects regardless of the ANS concentration. Combinations of dipyridamole and ANS show additive inhibitory effects only at low concentrations of the latter. The mechanisms of inhibition by ANS are discussed in terms of (1) interactions between the probe and an anion carrier and (2) modifications of the membrane surface charge by ANS. Assuming that ANS bound to the membrane surface produces a negative surface charge, ANS-dependent surface potentials of magnitude sufficient to account for the observed inhibition can be calculated using double layer theory. It is suggested that anionic amphiphiles inhibit anion and increase cation permeability through modifications of surface charge that alter the ion concentrations at the permeability barriers and a second step, affected by SITS, is involved in anion permeation.

In recent years, evidence has accumulated indicating that the transport of inorganic anions across the red cell membrane is a complex process involving a mechanism that mediates an obligatory anion exchange which is responsible for the high anion permeability of the membrane and may be unrelated to ionic diffusion pathways.

Microelectrode measurements of membrane potential and resistance in *Amphiuma* red cells (Hoffman & Lassen, 1971; Lassen, 1972) showed that Cl conductance is several orders of magnitude lower than that estimated from isotope fluxes (Tosteson, 1959). This finding was in agreement with

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the suggestion that ionophore-induced K loss in human red cells is limited by the ability of Cl to move through the membrane as a negative charge (Harris & Pressman, 1967; Henderson, McGivan & Chappell, 1969; Scarpa, Ceccheto & Azzone, 1970), and indicated that only a very small fraction of the Cl crosses the membrane by ionic diffusion. Hunter (1971) and Tosteson, Gunn and Wieth (1973), using valinomycin-treated human and sheep red cells, respectively, have estimated that Cl conductance is only about 10^2 times greater than K conductance, instead of 10^6 to 10^7 times greater if all the Cl crossed the membrane by ionic diffusion. Thus, a pathway that mediates an obligatory exchange of anions across the membrane, which may be separate from the ionic diffusion pathway, is responsible for the high anion permeability of the erythrocyte. This exchange pathway shows saturation kinetics, pH maxima, high activation energies, anion discrimination and is inhibited by a variety of compounds (Passow, 1969; Deuticke, 1970; Wood & Passow, 1971; Dalmark, 1972; Dalmark & Wieth, 1972; Gunn, Dalmark, Tosteson & Wieth, 1973) suggesting the existence of an anion carrier. Gunn (1972, 1973) has proposed a model of a titratable anion carrier to account for these observations.

Since anion permeability has been studied mainly with kinetic approaches in which the membrane mechanism cannot be specified, little is known on the nature and properties of the membrane regions involved in anion transport. Although the participation of membrane protein in anion permeability has been suggested by the effect of amino reagents (Passow & Schnell, 1969; Knauf & Rothstein, 1971; Poensgen & Passow, 1971) and proteolytic enzymes (Passow, 1971), many of the characteristics of the exchange pathway are also exhibited by phospholipid bilayer membranes (Bangham, Standish & Watkins, 1965; Pagano & Thompson, 1968; Ehrenspeck & Passow, 1973).

Attempting to obtain information on the membrane parameters related to anion permeability from fluorescence studies, we have searched for suitable fluorescent molecules that interact with the functional regions.

1-anilino-8-naphthalene sulfonate (ANS) seemed a potentially useful probe to study anion permeability owing to the large changes in its fluorescence parameters in different environments (Stryer, 1968), its ability to interact with red cell membranes (Rubalcava, Martinez de Muñoz & Gitler, 1969) and its negative charge.

Our initial studies (Fortes & Hoffman, 1971) showed that ANS interacts as an anion with ghosts and competes for membrane binding sites with lyotropic anions that inhibit anion permeability. This suggested that ANS may interact with the anion-permeable regions of the membrane.

In this paper, ANS permeability and the effect of ANS on Cl and SO_4 permeability were investigated in order to characterize functionally the membrane regions for ANS interaction or adsorption. Both the properties of the membrane domains where ANS adsorbs and the ANS-membrane equilibria were studied with nanosecond fluorescence spectroscopy and will be reported separately[1,2]. Parts of this work are included in a thesis (Fortes, 1972) and preliminary accounts have been previously presented (Fortes, Yguerabide & Hoffman, 1972*a*, *b*; Fortes & Hoffman, 1973).

Materials and Methods

Blood from human adults was drawn into heparin and washed red cells were isolated by centrifugation and resuspension (3 to 6×) in the same medium to be used in the experiment, usually 150 mM NaCl, 20 mM Tris-Cl, pH 7.4.

ANS Uptake

ANS uptake was studied by measuring the decrease in ANS concentration in the medium as a function of time after the addition of red cells. Usually, 1 ml of packed cells was added to 4 ml of the indicated incubation media containing ANS. Duplicate tubes were used for each condition. At appropriate times, 0.4-ml aliquots of the suspension transferred to plastic tubes were centrifuged in a Beckman Microfuge which accelerates to maximum speed in a few seconds, sedimenting the cells in about half a minute. The sample times correspond to the beginning of the centrifugation. The fastest sampling time was 15 to 20 sec. In some experiments, the uptake was started by adding ANS to the cell suspension. The ANS concentration in the supernatant was measured by fluorescence. Since ANS is not fluorescent in aqueous solutions, aliquots of the supernatant (20 μliters) were added to dioxane (2 ml) in which ANS is highly fluorescent. The fluorescence was measured in a Farrand fluorometer using Corning filters CS 7–37 for excitation and CS 3–72 for emission. The fluorescence was a linear function of ANS concentration, as evidenced by appropriate standard curves.

Sulfate Exchange

Sulfate exchange was assayed following the method of Gardos, Hoffman and Passow (1969). Red cells were loaded and equilibrated with $^{35}SO_4$ by incubation at 10% hematocrit for 2 to 3 hr at 37 °C in a medium containing about 1 μC $^{35}SO_4$. The composition of the incubation media is given in the figure legends. After incubation they were washed three to five times in ice-cold nonradioactive medium of the same composition as in the incubation. The flux was started by adding the loaded cells to prewarmed nonradioactive media of identical composition as the loading medium, but

1 Fortes, P. A. G., Yguerabide, J., Hoffman, J. F. 1974*a*. The nature of the red cell membrane binding sites of ANS as studied with nanosecond fluorescence spectroscopy. (*In preparation.*)

2 Fortes, P. A. G., Yguerabide, J., Hoffman, J. F. 1974*b*. Surface potential and the binding of ANS to the red cell membrane. (*In preparation.*)

containing ANS at the indicated concentrations, and incubated at 37 °C in a shaker bath. Duplicate tubes were used for each condition. Aliquots were centrifuged at different times and 0.2 ml of the supernatant added to 10 ml of Bray's solution for determination of radioactivity. No detectable hemolysis nor pH changes were observed in the experiments presented here.

Chloride Exchange

Chloride exchange was assayed following the method described by Dalmark and Wieth (1972). All the manipulations were done in the cold room (4 °C) and all the suspensions kept in ice. Loading for 30 min to 2 hr was done in the same media to be used for the efflux, including inhibitors when present and ^{36}Cl, at 65 to 70% hematocrit. At the end of the loading period the cell suspensions were concentrated by centrifugation in a Beckman Microfuge and the supernatants removed. Approximately 50 μliters of cells were drawn into a polyethylene tube and squirted into a vessel containing 20 ml of nonradioactive medium of identical composition to the loading medium with a syringe containing 10 ml of medium at ~0 °C. The vessel was kept at 0 °C with a circulating H_2O-methanol bath and the solution was vigorously stirred with a magnetic flea. The time of addition of the cells was time zero and rapid sampling was accomplished by drawing 2 to 3 ml of suspension through a 3-μ pore diameter Swinnex millipore filter mounted between a needle and a syringe. Each filtered medium was collected in a syringe within 1 sec and sampling could be done as fast as every 5 sec. The radioactivity in the filtrate was measured by liquid scintillation.

Exposure to SITS

Red cells were treated with 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) according to the method of Knauf and Rothstein (1971), by adding 0.3 mg SITS/ml cells to a 20% hematocrit suspension in 166 mM NaCl, 20 mM Na phosphate, pH 7.4. After incubating 30 min at room temperature, the suspension was centrifuged and the cells were washed once in the NaCl-phosphate medium and three times in 150 mM NaCl, 20 mM Tris-Cl, pH 7.4. This was a maximum dose of SITS, since exposure to 10-fold higher SITS concentrations did not increase the inhibition.

Calculations

Under the conditions used in this work, SO_4 and Cl fluxes followed first order kinetics (Gardos *et al.*, 1969). The rate constants were obtained as the slope from plots of the fraction of radioactivity remaining in the cells *versus* time according to the equation:

$$\ln\left(1-\frac{Y_t}{Y_\infty}\right)=-kt \tag{1}$$

where Y_t = radioactivity at time t, Y_∞ = radioactivity at isotopic equilibrium, and k = the rate constant.

Chemicals

1,8 ANS was obtained from K & K Laboratories and recrystallized twice as the Mg salt from hot concentrated $MgCl_2$ solutions in H_2O. 2,8 ANS, 1,4 ANS and 5,2 ANS were gifts from Dr. L. Stryer. 2-*p*-Toluidinyl-6-naphthalene sulfonate (TNS) was obtained from Sigma, SITS from Nutritional Biochemicals and dipyridamole from Geigy. All other chemicals were reagent grade.

Results

ANS as a Permeant Anion

When red cells are added to an ANS solution the concentration of ANS in the medium decreases with time. Fig. 1 shows the fraction of ANS remaining in the medium after mixing red cells with solutions of increasing ANS concentration. Medium ANS decreases rapidly in the first 15 to 30 sec after adding the cells followed by a slower decrease until equilibrium is reached. This slower process has a half time of about 8 min, independent of the ANS concentration (Fig. 1), in contrast with the rate of interaction of ANS with ghosts under similar conditions where equilibrium is reached within a few seconds (Freedman & Radda, 1969; Fortes & Hoffman, 1971). The initial fast process does resemble the interaction of ANS with ghosts in the amount of ANS taken up and its kinetics. Between 10^6 and 10^7 ANS molecules/cell are taken up with fast kinetics, as calculated by extrapolating the slow uptake to time zero. These values are of the same order of magnitude as the amount of ANS bound to ghosts under similar conditions as found by Rubalcava *et al.* (1969).

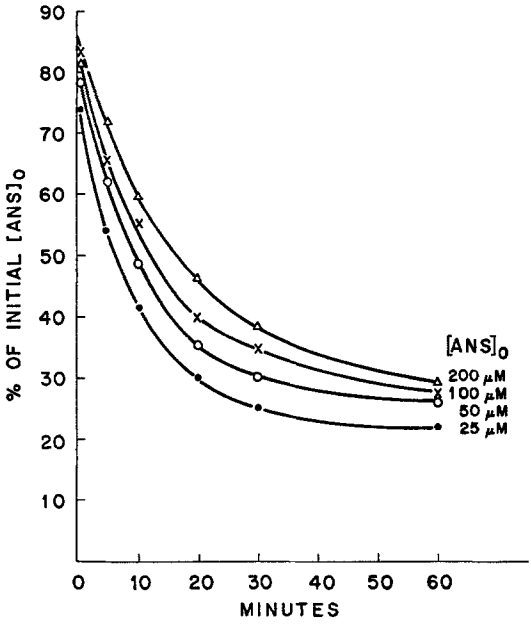

Fig. 1. Dependence of ANS uptake on ANS concentration. The disappearance of ANS from the medium is measured as a function of time after addition of red cells. Medium: 150 mM NaCl, 20 mM Tris-Cl, pH 7.5, and the indicated ANS concentrations. Temp. 23 °C. In this and the following figures, the uptake was started by adding 1 ml of packed red cells to 4 ml of solution (20% hematocrit). Ordinate: per cent of the ANS concentration in the medium before adding the cells. The half time of ANS uptake is about 8 min

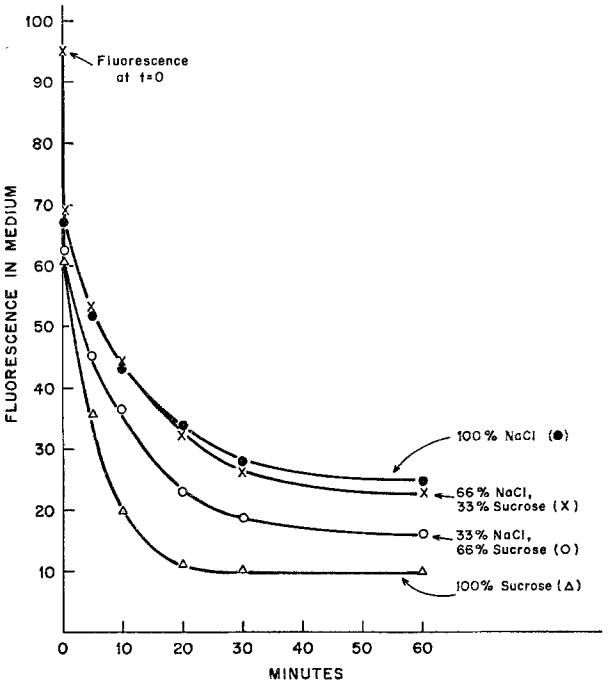

Fig. 2. Effect of ionic strength on ANS uptake by RBC. The incubation media were made by mixing 150 mM NaCl and 300 mM sucrose in the indicated proportions. In addition, all media contained 20 mM Tris-Cl, pH 7.5, 20 μM ANS. Temp. 23 °C

At equilibrium, intact red cells take up much more ANS than ghosts. The total amount of ANS taken up depends on the number of cells present and the initial ANS concentration. At 20% hematocrit, over an almost 10-fold concentration range (25 to 200 μM), a constant fraction (60 to 75%) of the ANS in the medium is taken up by the cells (Fig. 1) with no evidence of saturation, whereas ANS binding by ghosts appears to be saturated at ANS concentrations above 100 μM (Freedman & Radda, 1969; Rubalcava *et al.*, 1969; Fortes & Hoffman, 1971).

These observations lead to the conclusion that the initial fast uptake reflects the binding of ANS to the membrane, whereas the slow uptake represents the entry of ANS into the intracellular compartment and can be used as a measure of ANS permeability.

Since ANS is an anion, its distribution in the intra- and extracellular compartment should follow a Donnan equilibrium. Evidence suggesting that this is the case is shown in Fig. 2. The rate and extent of ANS uptake is increased when the electrolyte in the medium is substituted by equiosmolar amounts of sucrose. The differences in the equilibrium values with ionic

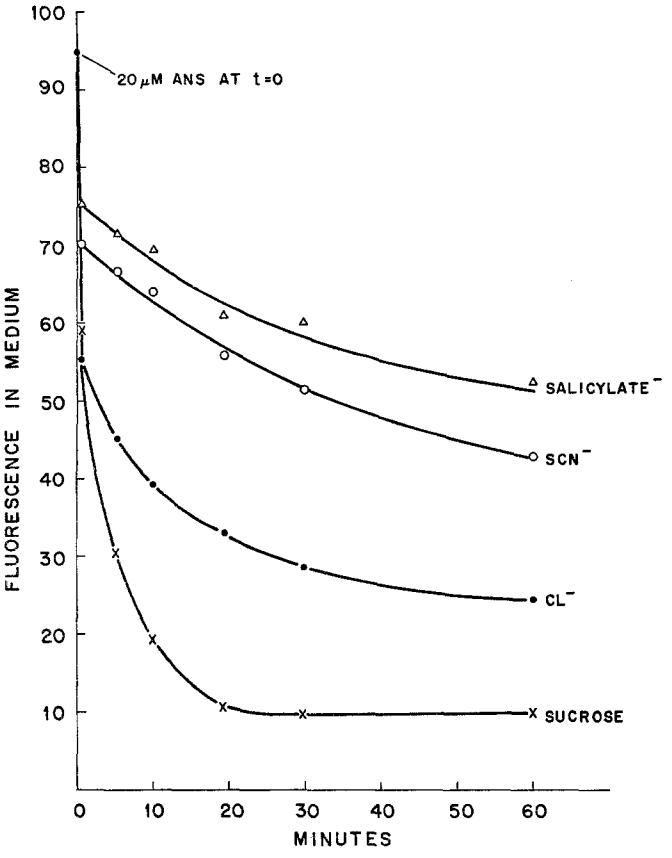

Fig. 3. Effect of lyotropic anions and low ionic strength on ANS uptake by RBC. All media contained 20 mM Tris-Cl, pH 7.5, 20 μM ANS. 300 mM sucrose or 150 mM Na salts were present where indicated. Temp. 23 °C

strength (Fig. 2) suggest that ANS does follow, at least qualitatively, a Donnan equilibrium. $[ANS]_i/[ANS]_0$ ratios calculated assuming that all the ANS taken up is in the cellular compartment and has an activity coefficient of unity are more than 10-fold higher than the Cl ratios, suggesting that the intracellular activity of ANS is low, probably owing to its adsorption to intracellular proteins.

The increased ANS uptake rate in sucrose media (Fig. 2) resembles the dependence of SO_4 permeability on ionic strength (Lepke & Passow, 1971) and may reflect a competition between ANS and Cl for permeability sites in the membrane. Also, a pH effect on the rate of ANS uptake is possible since the medium pH decreased about 0.5 unit upon addition of cells to the sucrose medium owing to the shift in the Donnan equilibrium in the

low Cl media. Whether ANS uptake occurs by exchange with intracellular anions or by ionic diffusion remains to be tested.

In view of the finding that ANS is a permeant anion in red cells it was of interest to see if ANS permeability could be altered by inhibitors known to affect anion permeability (Passow & Schnell, 1969; Wieth, 1970). Fig. 3 shows the time course of ANS uptake into red cells suspended in media that increase (sucrose) or decrease (SCN and salicylate) anion permeability compared to NaCl. The lyotropic anions decrease the extent of the initial fast uptake and the rate of ANS entry into the cell. The former is consistent with the effect of these anions on ANS binding to ghosts (Fortes & Hoffman, 1971), whereas the latter indicates that ANS permeability is affected by the composition of the medium in the same way as that of other anions.

These observations raise the possibility that ANS crosses the membrane through the same pathways as say Cl or SO_4. As suggested previously (Fortes & Hoffman, 1971) the interaction of ANS with anion permeability pathways should inhibit anion translocation.

ANS as an Inhibitor

Figs. 4 and 5 show the effect of increasing ANS concentration in the medium on ^{36}Cl and $^{35}SO_4$ efflux (measured at 0 and 37 °C, respectively) from preloaded cells. Increasing the ANS in the medium progressively inhibits the permeability of both the fast permeant Cl and the slow permeant SO_4. The observed rates are shown in Fig. 6. There is appreciable *inhibition even at ANS concentrations used in fluorescence work* which should be considered when using ANS as a probe. ANS concentrations higher than 500 μM inhibit Cl permeability more than 99% (Fig. 6). The inhibition is reversed by washing the cells. Note that the inhibitory effect increases at ANS concentrations higher than those at which ANS binding to ghosts

Fig. 4. Effect of ANS on Cl exchange. Medium: 150 mM NaCl, 20 mM Tris-Cl, pH 7.4, hematocrit ~0.3%. 1,8 ANS at the indicated concentrations was present during both equilibration and flux periods. Flux was started by adding ^{36}Cl-preloaded cells to nonradioactive medium. Temp. 0 °C. Rate constants, calculated according to Eq. (1), are shown in Fig. 6

Fig. 5. Effect of 1,8 ANS on SO_4 exchange. Medium: 0.18 M sucrose, 40 mM NaCl, 20 mM Na_2SO_4, 20 mM Tris-Cl, pH 7.5. Hematocrit, 10%. $^{35}SO_4$-preloaded cells were added to nonradioactive media containing the indicated 1,8 ANS concentrations. Temp. 37 °C

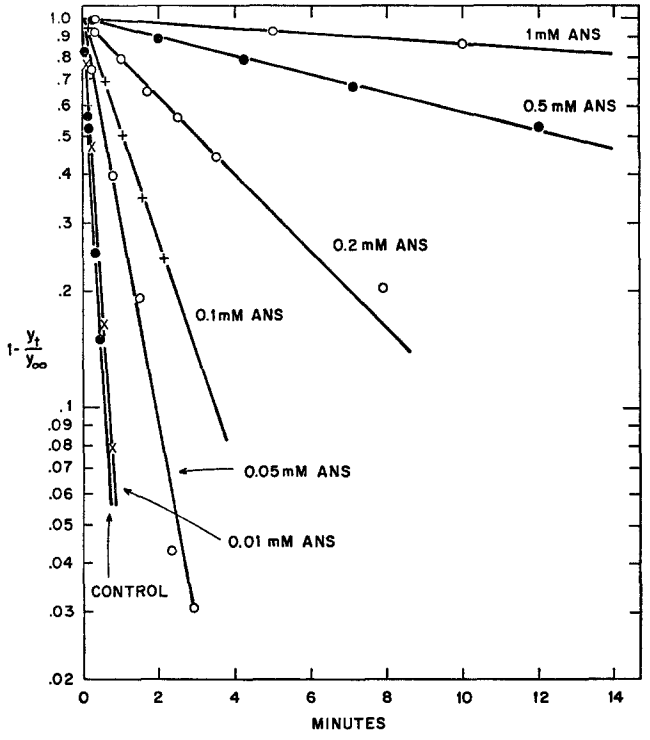
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
.09
.08
.07
.06
.05
.04
.03
.02
1- $\frac{y_t}{y_\infty}$
1mM ANS
0.5 mM ANS
0.2 mM ANS
0.1mM ANS
0.05mM ANS
0.01 mM ANS
CONTROL
0
2
4
6
8
10
12
14
MINUTES

Fig. 4

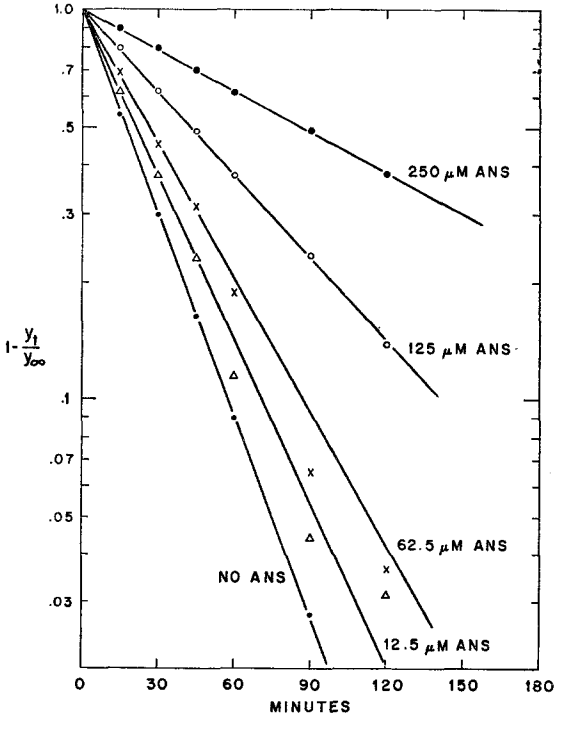
1.0
.7
.5
.3
.1
.07
.05
.03
1- $\frac{y_t}{y_\infty}$
250 μM ANS
125 μM ANS
62.5 μM ANS
12.5 μM ANS
NO ANS
0
30
60
90
120
150
180
MINUTES

Fig. 5

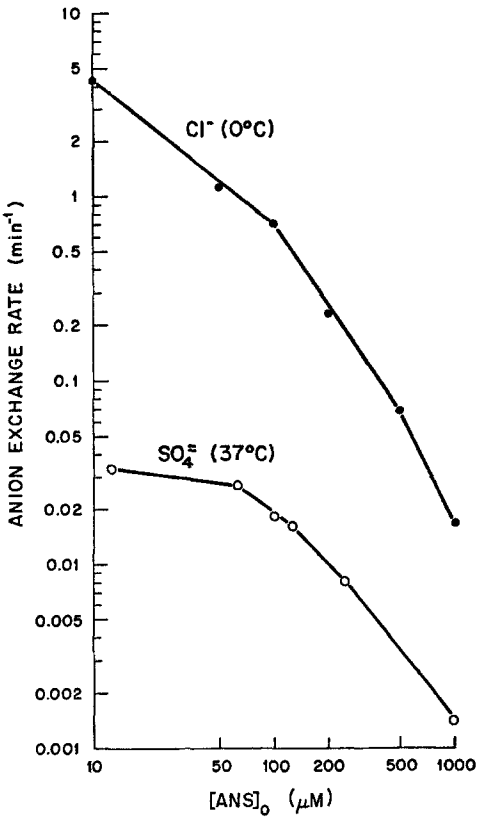

Fig. 6. Effect of 1,8 ANS on the rate constants of Cl and SO_4 exchange. Conditions as in Figs. 4 and 5

appears saturated. It is possible that the curvature in the ANS binding isotherms does not represent a real saturation (*see* footnote 2).

Comparing the effect of ANS on Cl permeability with that on SO_4 suggests that ANS is more potent in inhibiting the former. Although this may be so, the Cl and SO_4 experiments are not comparable quantitatively because of the differences in ANS binding at 0 and 37 °C (Fortes, 1972) and ANS uptake by the cells in the sulfate experiments where the ANS concentration in the medium at equilibrium could be less than half of the initial (*see* Figs. 2 and 3). Thus, to compare quantitatively the potency of ANS as an inhibitor of Cl and SO_4 permeability, fluxes should be measured at the same temperature and under the same medium conditions.

The high affinity of ANS for membranes probably depends on its amphipathic character which promotes ANS binding at the membrane

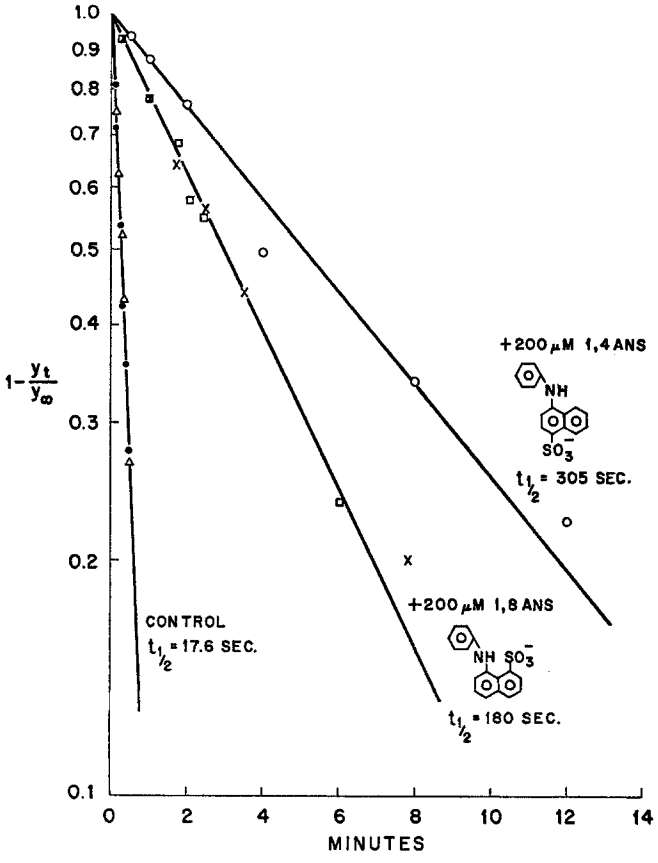

Fig. 7. Effect of molecular geometry on ANS inhibition of Cl exchange. Medium: 160 mM NaCl, 17 mM Tris-Cl, pH 7.4. The control and 1,8 ANS curves show two separate experiments (different symbols) done consecutively. Temp. 0 °C. The rate constant $k = 0.693/t_{1/2}$

surface (Rubalcava *et al.*, 1969; Lesslauer, Cain & Blasie, 1971; *see also* footnote 1). We tested ANS isomers that differ in the relative positions of the anilino and SO_3 groups with respect to the naphthalene ring to see if the molecular geometry is related to the inhibitory effect of ANS.

In 1,4 ANS the sulfonate is separated from the anilino group by the short axis of the ring, compared with 1,8 ANS where these groups are adjacent. This results in a greater inhibitory effect of 1,4 ANS on Cl permeability (Fig. 7). The different geometry of 1,4 ANS appears to increase its affinity and promotes a higher membrane concentration at equal medium concentration of the two isomers.

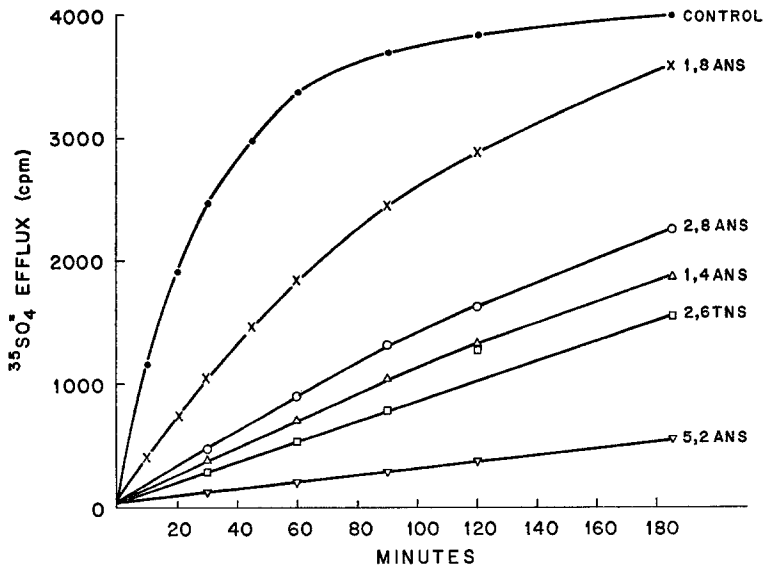

Fig. 8. Effect of ANS isomers on SO_4 efflux. Medium: 0.13 M sucrose, 40 mM NaCl, 20 mM Na_2SO_4, 20 mM Tris-Cl, pH 7.5. Hematocrit, 10%. Temp. 37 °C. Pre-equilibrated cells in $^{35}SO_4$ medium were added to nonradioactive media containing 0.2 mM ANS isomer where indicated

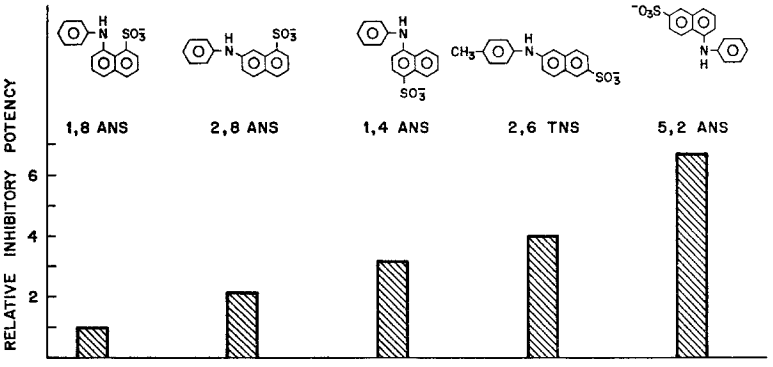

Fig. 9. Relative inhibitory potency of ANS isomers on SO_4 permeability. SO_4 exchange at Donnan equilibrium (37 °C) was assayed in the presence of 0.2 mM of the indicated isomers, as in Fig. 8. Inhibitory potency of 1,8 ANS = 1

A further increase in potency is observed with 5,2 ANS and 2,6 TNS that have the anilino and sulfonate groups separated by the long axis of the ring. Fig. 8 shows the effect of these isomers on SO_4 efflux. The relative potency of the different ANS isomers (Fig. 9) appears to depend on the

separation between the anilino and sulfonate groups. This is probably related to increased hydrophobic interactions of the more potent ANS isomers, whose apolar segments will be embedded deeper into the membrane phase. A similar sequence is observed in the affinity of these isomers for ghosts as measured by fluorescence titrations (Fortes, *unpublished observations*).

Synergism Between ANS and Other Inhibitors

Although the ability of a compound to inhibit anion permeability seems to depend on its being an anion with high affinity for hydrophobic regions, such as ANS, trinitrocresolate (TNC) and certain lyotropic anions (Passow & Schnell, 1969; Wieth, 1970; Gunn & Tosteson, 1971), there are other compounds that may inhibit anion permeability by different mechanisms. We tested other inhibitors in combination with ANS to see if the inhibitory action of ANS is altered by them, attempting to isolate different steps in anion permeation.

The disulfonic stilbene derivative SITS, although anionic, differs from the inhibitor anions mentioned above in that it only acts on the outer cell surface (Maddy, 1964) and it appears to inhibit through binding to a limited number of specific sites (Knauf & Rothstein, 1971; Cabantchik & Rothstein, 1972) modifying anion permeability without appreciable effects on cation permeability.

Cl permeability is about 50% lower in cells that have been exposed to SITS and washed according to Knauf and Rothstein (1971) as shown in Fig. 10. This was a maximum effect of SITS since pretreatment with a 10-fold higher concentration did not increase the effect. The inhibitory effect of SITS is equivalent to that of 50 μM ANS, which also inhibits Cl permeability about 50%. SITS does not appear to alter the inhibition by ANS, since the addition of 50 μM ANS to SITS-treated cells also inhibits about 50% (Fig. 10), so that the combination of the inhibitors results in an additive effect. However, when high concentrations of ANS (0.5 mM) are used the SITS-treated cells still have an approximately 50% lower rate than the untreated cells even if the permeability is about 60 times lower than in the absence of ANS (Fig. 10). Thus, SITS decreases Cl permeability to one-half of its value, whether this value is normal or has been substantially decreased by ANS. This suggests that ANS and SITS act on different steps in the Cl translocation process and that these steps are in series with each other. Otherwise, the relatively small inhibition by SITS would be obscured by the large inhibition with 0.5 mM ANS.

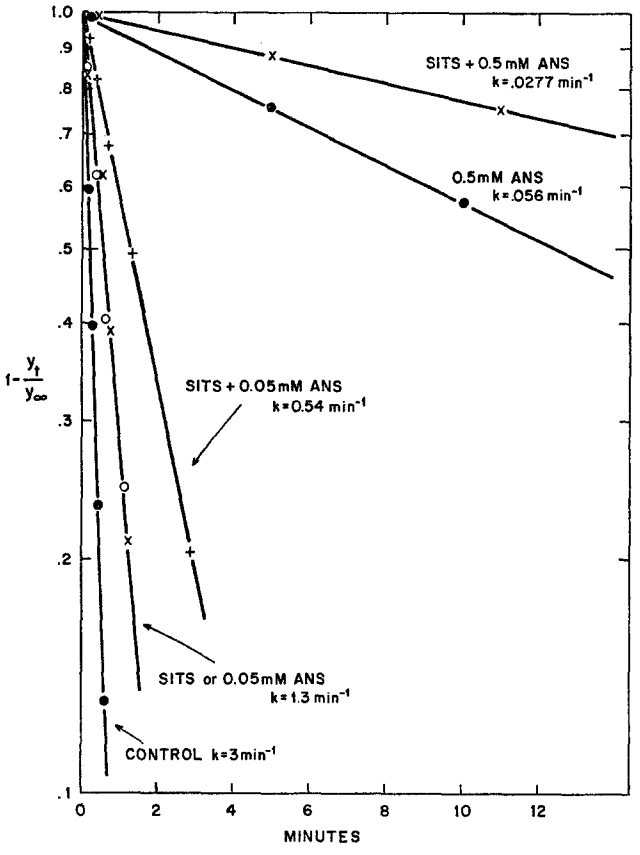

Fig. 10. Effect of SITS and ANS on Cl exchange. Experimental conditions as described in legend of Fig. 4. Control (circles) and SITS-treated cells (crosses) were preloaded in and added to media containing 0.05 or 0.5 mM ANS where indicated. Increasing the SITS concentration (0.3 mg/ml cells) 10-fold did not increase the inhibition

Another inhibitor of anion permeability, dipyridamole (Deuticke, 1970), is not an anion and its mechanism of action may be different to that of ANS. Fig. 11 shows an experiment similar to that of Fig. 10 but using dipyridamole instead of SITS. In the presence of 5 μM dipyridamole, Cl exchange does not follow a single exponential; the curvature in the semi-logarithmic plots is small, but consistently found in all experiments and may reflect a variable sensitivity to low doses of the drug of different cell populations in the sample. The inhibition by these concentrations of dipyridamole is 40 to 60% and the effect is additive with low concentrations (50 μM) of ANS (Fig. 11*A*). However, unlike SITS, the small inhibition by dipyridamole is obscured by the large inhibition caused by 0.5 mM ANS,

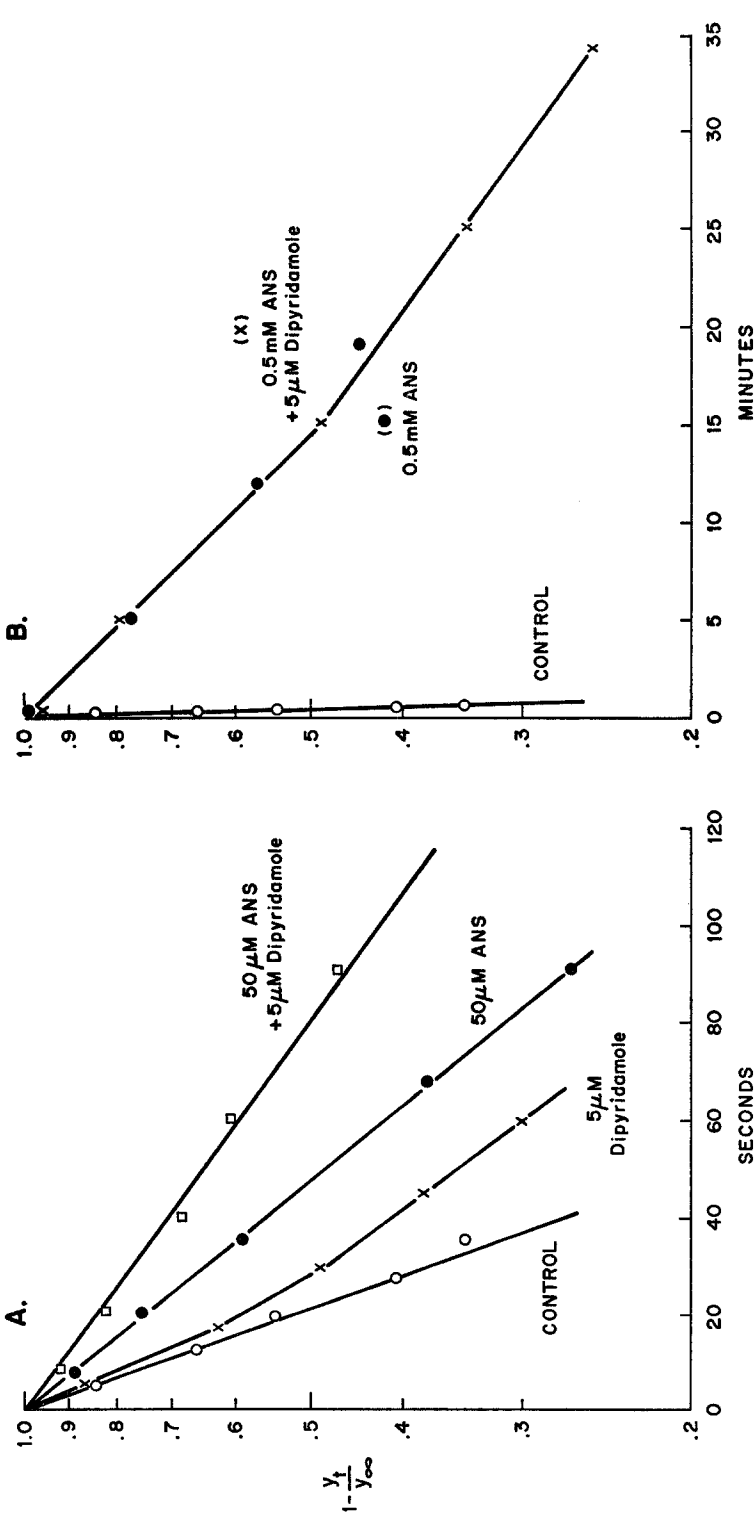

Fig. 11. Effect of dipyridamole and ANS on Cl exchange. Experimental conditions as in Fig. 4, 5 μM dipyridamole and/or 50 μM (*A*) or 0.5 mM (*B*) ANS were present where indicated. In *B*, (●) refers to ANS alone and (×) to ANS+dipyridamole. The curvature of the plots in the presence of these concentrations of dipyridamole is a consistent finding

since no significant difference in the rate of Cl exchange in 0.5 mM ANS is observed in the presence and absence of dipyridamole (Fig. 11*B*). This suggests that ANS and dipyridamole act on the same step in Cl translocation.

Discussion

The main findings of this paper are that ANS is a permeant anion and a potent inhibitor of anion permeability in red cells. Regardless of the actual mechanisms involved, the inhibitory effects indicate that ANS and related compounds are not inert in their interactions with membranes. Since ANS is a widely used probe, care must be exercised when interpreting observations done in the presence of a probe when the probe itself alters the characteristics of the membrane.

As mentioned in the Introduction, anion translocation in red cells appears to involve at least two different mechanisms: ionic diffusion and facilitated exchange diffusion. At present, there is no direct information regarding the effect of ANS on the ionic diffusion pathway. Under the conditions used in the present study the measured anion fluxes are mostly through the exchange pathway. ANS could inhibit anion permeability by preventing either anion binding to the carrier or diffusion of the carrier-anion complex across the membrane. In addition, the inhibitory action of ANS could result from electrostatic effects produced by the binding of the negatively charged molecule to the membrane, which would decrease the availability of anions to both pathways. Insight into these mechanisms may be gained by correlating information on the location and nature of the ANS binding sites in the membrane obtained from fluorescence studies and on the electrostatic effects of ANS adsorbed to ghosts, phospholipid bilayers and monolayers.

The increasing potency of the ANS isomers in inhibiting anion permeability (Figs. 7 to 9) probably reflects their higher affinity owing to increased hydrophobic interactions with the membrane. This is consistent with the location of bound ANS at a polar-apolar interface in both synthetic lipid membranes (Lesslauer *et al.*, 1971) and ghosts[1] which suggests that the "permeability sites" affected by ANS are located at the membrane-H_2O interface. Fluorescence lifetimes of ANS in ghosts obtained from nanosecond decay studies showed that the ANS binding sites in ghosts are of two different types, presumably constituted by phospholipid in a bilayer state and lipoprotein, respectively, (Fortes, 1972; *see also* footnote 1). Although evidence was obtained that some of these sites also bind other anion per-

meability inhibitors, a distinction between sites related to permeability and nonspecific ANS binding sites is difficult, particularly if the number of anion carriers per cell may be as small as 90 (Gunn *et al.*, 1973) whereas between 10^6 and 10^7 ANS molecules are bound per cell.

Electrostatic Factors in the Inhibition of Anion Permeability by ANS

Previous work has shown that ions with high affinity for membranes can change the membrane charges and affect the equilibrium of ANS (and other ions) with the membrane (Fortes & Hoffman, 1971). When ANS is adsorbed to the membrane a similar effect is to be expected. The nanosecond fluorescence studies showed that although ANS is distributed in two environmentally different regions of the membrane, its affinity for both regions depends on the same electrostatic forces. The ANS adsorption isotherms for both membrane regions and their dependence on the electrolyte concentration in the medium were described adequately by an electrochemical equilibrium model in which ANS acts as a negative surface charge that builds up as the amount adsorbed to the membrane increases. This predicted that ANS-dependent surface potentials in ghosts between -5 and -30 mV are produced in the presence of 10 to 50 μM ANS; the ANS-dependent surface potentials were calculated using the surface density of bound ANS and the Gouy-Chapman equation (Fortes, 1972; *see also* footnote 2). It is interesting that Haynes (1972), independently, has described a similar equilibrium for ANS in phospholipid vesicles.

Some preliminary observations support this interpretation: Direct measurements of surface potential indicate that ANS does indeed produce a negative surface charge when bound to a membrane. ANS added to the aqueous phase produces a substantial negative potential in monolayers of red cell lipids; neuraminidase-treated red cells and phosphatidyl choline vesicles (normally isoelectric) acquire a high mobility towards the anode in the presence of ANS, as measured by microelectrophoresis (Fortes, *unpublished observations*).

As mentioned above, the modified surface charge in the presence of ANS would alter the ion concentrations at the membrane-H_2O interface where the permeability barriers are located and could therefore decrease anion fluxes.

Such an effect has been observed in phospholipid bilayers where ANS increases nonactin-K conductance, presumably by increasing the K concentration at the membrane surface (McLaughlin, Szabo & Eisenman, 1971).

The surface potential produced by ANS was estimated by these authors from changes in conductance.

A similar approach was used attempting to estimate ANS-dependent surface potentials in red cells from Cl flux measurements (Fortes & Hoffman, 1973). Although the values of surface potential calculated with the latter method agree with those estimated from fluorescence measurements of ANS binding to ghosts, the former calculations were done assuming a linear relationship between Cl concentrations and Cl flux, which is only valid at low Cl concentrations (Wieth, Dalmark, Gunn & Tosteson, 1973).

It is therefore of interest to investigate the relationship between the surface potential produced by ANS and the inhibition of anion permeability by this compound. For this it is necessary to know the magnitude of the ANS surface potential under the conditions in which the flux is measured. Using the electrostatic model for ANS binding derived from fluorescence studies in ghosts (Fortes, 1972; Fortes *et al.*[2]) estimates of the expected ANS surface potentials in the flux experiments show that a substantial electrostatic potential is produced. We estimate that 1 mM ANS produces a surface potential of the order of -100 mV at 0 °C, which would decrease the Cl concentration at the membrane surface to about 1% of its concentration in the medium. Such electrostatic effects could be sufficient to account for the observed inhibition, although the estimated values are subject to uncertainties since the parameters used were derived from experiments in ghosts at 20 °C and the contribution of surface dipoles to the total charge has not been considered. ANS binding studies under the same conditions of the flux measurements will allow more accurate evaluation of the quantitative relationship between surface potential and anion permeability.

Nevertheless, the production of a negative surface charge by ANS suggests that the mechanism of at least part of its inhibitory effect on anion permeability is electrostatic, in addition to the possibility of direct interactions with an anion carrier.

Surface Charge and Ion Permeability

The hypothesis that ANS inhibits anion permeability through a surface charge mechanism is consistent with the effects of amphipathic and lyotropic anions such as trinitrocresolate (Gunn & Tosteson, 1971) and salicylate (Passow & Schnell, 1969; Wieth, 1970). The inhibitory activity of a variety of compounds, including uncharged molecules (Deuticke, 1970), whose common denominator is their high affinity for polar-apolar interfaces may be related to alterations of the electrical double layer. The inhibitor need

not bear a net charge to modify the surface potential. A high dipole moment of the inhibitor molecule and its appropriate orientation in the plane of the membrane as well as reorientation of membrane components upon binding the inhibitor will alter the potential arising from membrane dipoles (Davies & Rideal, 1963) which appears to contribute substantially to the ionic permeability of phospholipid bilayers (Szabo & Eisenman, 1973; Haydon & Myers, 1973) and possibly biological membranes.

The surface potential hypothesis is also consistent with the observation that most inhibitors of anion permeability increase cation permeability (Passow, 1969). However, much higher concentrations are necessary to increase cation permeability and the changes are much smaller than with anions. This behavior is also observed with ANS, which increases Na and K leaks at concentrations above 0.5 mM (Fortes & Ellory, 1974)[3]. The asymmetry of anion and cation permeability responses could indicate that the cation-permeable regions sense a smaller electric field because they are separated by some distance from the anion-permeable regions where the inhibitors bind since the electric field of a surface charge decreases with distance.

A surface charge mechanism is also consistent with the synergism between ANS and SITS (Fig. 10) which suggests the existence of at least two steps, in series, in anion permeation. It may be speculated that ANS decreases the concentration of permeant anions available to the membrane, while SITS decreases their diffusion across the membrane either by decreasing the number of "sites" or carriers, or their mobility.

Although more information is needed to evaluate the relative contributions of the different factors involved in anion permeability mentioned in this discussion, the approaches presented in this work suggest that the correlation between membrane properties studied directly with inhibitors that offer the advantages of ANS may help to "dissect" anion permeability stepwise, provided the properties of each compound used are known.

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