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ASYMMETRIC MEMBRANE EXPANSION AND MODIFICATION OF ACTIVE AND PASSIVE CATION PERMEABILITY OF HUMAN RED CELLS BY THE FLUORESCENT PROBE 1-ANILINO-8-NAPHTHALENE SULFONATE

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SUMMARY

1. The membrane perturbations induced by the interaction of the fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS) with human red blood cells were studied.

2. ANS below 0.5 mM inhibits partially (20% maximum) the ouabain-insensitive Na^+ and K^+ influx and efflux. Above 0.5 mM ANS increases both Na^+ and K^+ leak fluxes. The increased cation leaks are larger for Na^+ than K^+.

3. The $(Na^+ + K^+)$-ATPase and ouabain-sensitive Na^+ and K^+ fluxes are inhibited by ANS. Ouabain-insensitive, Mg^{2+}-dependent ATPase activity of ghosts is stimulated by [ANS] < 0.3 mM and inhibited by [ANS] > 0.3 mM

4. ANS also inhibits the Na^+-dependent, ouabain-insensitive K^+ influx that is inhibited by ethacrynic acid and furosemide.

5. Red cells become crenated with [ANS] < 1 mM and sphere at [ANS] > 1 mM In the former conditions hypotonic hemolysis is decreased whereas the latter increase osmotic fragility.

6 It is suggested that ANS expands the membrane asymmetrically by binding preferentially to the external membrane surface.

7 It is concluded that ANS is a general inhibitor of ion transport, particularly of those processes thought to involve facilitated-diffusion mechanisms. The increased cation leaks observed at high ANS concentrations may be related to prehemolytic membrane disruption

8. The membrane perturbations caused by ANS are compared to those caused by other reversible inhibitors of anion exchange in red blood cells. Their possible modes of action are discussed.

Abbreviations ANS, 1-anilino-8-naphthalene sulfonate, EGTA, ethyleneglycol-bis-(β-aminoethylester)-*N,N'*-tetraacetic acid

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INTRODUCTION

The fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS) inhibits anion transport in human red cells [1] Its ability to cross the membrane via the same pathways as Cl^- and SO_4^{2-} [1] and its inhibitory effects suggest a rather specific interaction between the probe and the anion transport mechanism, which appears to reside mainly in a 95 000 mol wt protein [2] However, ns fluorescence spectroscopy of ANS in red cell ghosts has shown that ANS is ubiquitously distributed in both phospholipid and protein regions of the membrane [3–5]. ANS intercalation at the membrane-water interface depends on the local surface charge density and creates a negative surface potential [3, 4] which has been estimated to be of sufficient magnitude to account for at least part of its effect on anion transport because of electrostatic repulsion of anions from the permeation sites [1] This predicts that ANS should stimulate cation transport, by increasing the concentration of cations at the membrane surface, if the electrostatic field produced by the bound probe extends to the cation permeation regions

Furthermore, ANS has been used as a "conformational probe" of the $(Na^+ + K^+)$-ATPase [6] and has been found to induce crenation in red cells [7] in the concentration range that inhibits anion transport

It was therefore of interest to investigate the effect on ANS on passive cation transport, on the $Na^+ + K^+$ pump and the relationship between its effects on cell shape and ion permeability

In the present paper, the effect of ANS on Na^+ and K^+ fluxes in human red blood cells and $(Na^+ + K^+)$-activated ATPase activity in ghosts was investigated The sodium pump activity was determined as the ouabain-sensitive component of Na^+ efflux, K^+ influx and ATPase activity The ouabain-insensitive K^+ influx in human red cells appears to consist of at least two components, a flux that increases linearly with concentration, the "leak" flux [8, 9] and a saturable component observed when K^+ influx is measured as a function of $[K^+]_0$ in media containing Na^+ and ouabain [10]. This second component is kinetically similar to the ouabain-insensitive Na^+ efflux which is inhibited by ethacrynic acid and furosemide [11–14], and may be related to the Na^+ pump [15]. The experiments were designed so that the effect of ANS on each of the components of the ouabain-insensitive fluxes could be distinguished

The possibility that ANS expands the membrane and its consequences on cell morphology and stability were investigated with scanning electron microscopy and measurements of osmotic fragility

MATERIALS AND METHODS

Blood from healthy human adults was drawn into heparin and washed red cells were isolated by centrifugation ($3000 \times g/5$ min) and resuspension ($3–6\times$) in 40 volumes of 107 mM $MgCl_2$, 10 mM Tris · HCl pH 7.6 at 20 °C.

Measurement of Na^+ and K^+ fluxes $^{24}Na^+$ and $^{42}K^+$ influx and efflux were determined in triplicate by established methods [8, 10, 16] Where no standard errors are given, the scatter was within the size of the point in the figure The detailed composition of the incubation media is given in the figure legends

ATPase activity determination. Fragmented hemoglobin-free ghosts were pre-

pared by hypotonic lysis, stored frozen and assayed for ATPase activity as previously described [17]. Since ANS was used mainly as the Mg^{2+} salt, varying amounts of $MgCl_2$ were added to the assay medium to maintain $[Mg^{2+}]$ constant The composition of the incubation media was: NaCl : 120 mM, KCl : 10 mM, Na_4 ATP · 2 mM, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetracetic acid (EGTA) : 2 mM, $MgCl_2$ +magnesium ANS . 6 mM, Tris · HCl : 10 mM, pH 7.7 at 37 °C.

Hemolysis protection experiments. Washed red cells at a hematocrit of 1–2 % were incubated at room temperature in 150 mM NaCl, 20 mM Tris · HCl pH 7.2 containing various concentrations of NH_4 ANS After 5–10 min the suspensions were diluted with 20 mM Tris · HCl pH 7.2 and the corresponding NH_4 ANS concentration to give 42 % of the initial NaCl concentration. After 5–15 min the suspensions were centrifuged and the hemoglobin concentration in the supernatant was determined from absorbance at 546 nm. Under these conditions hemolysis was 30–60 % in the absence of ANS. In some experiments (e g. lower curve in Fig. 9) the red cells were added at 1 % final hematocrit to 63 mM NaCl, 20 mM Tris · HCl pH 7.2 and the indicated NH_4 ANS concentration, and centrifuged after 5 min at room temperature Under these conditions hemolysis in the absence of ANS was 10 %.

Scanning electron microscopy. Washed red cells were suspended at 1 % hematocrit in isotonic (120 mM) sodium phosphate pH 7.4 and the indicated concentrations of ANS (Mg salt). After the shape change was confirmed by Nomarski differential interference microscopy, using plastic coverslips to avoid the "glass effect", the suspensions were centrifuged, 95 % of the supernatant was removed and the cells were resuspended. The concentrated cell suspension was added dropwise, while swirling, to a 1 % glutaraldehyde solution otherwise identical in composition to the preincubation medium Final hematocrit was about 0 25 %. The cell suspensions were cooled in an ice bath, incubated 1 h, and washed with 120 mM sodium phosphate pH 7.4 (2×) and distilled water (2×). The fixed cells were dehydrated sequentially with ethanol and freon 13, and dried in a freon critical point dryer. The dried samples were coated with gold-palladium and examined in a Cambridge S4 scanning electron microscope. Examination of the cells with the Nomarski microscope showed no shape changes during fixation and dehydration.

Materials. 1,8 ANS, Na^+ salt from K and K laboratories was twice recrystallized from $MgCl_2$ solutions. In some experiments magnesium ANS obtained from the Eastman Organic Chemical Company and NH_4 ANS obtained from the Pierce Chemical Company were used. No significant differences were observed between the various ANS samples. ATP and ouabain were obtained from the Sigma Chemical Company and choline chloride was recrystallized from hot ethanol. All other chemicals were reagent grade.

RESULTS

I Effect of ANS on Na^+ and K^+ passive fluxes

Fig. 1 shows the effect of varying the concentration of ANS in the medium on Na^+ and K^+ fluxes in the presence of ouabain. Below 0.5 mM, ANS causes a 5–20 % decrease in Na^+ efflux (Fig. 1A), Na^+ influx (Fig. 1B) and K^+ influx (Fig. 1C). The relatively small decrease in cation fluxes at these ANS concentrations is considered significant since it was consistently observed in all experiments (3 Na^+ and 6 K^+

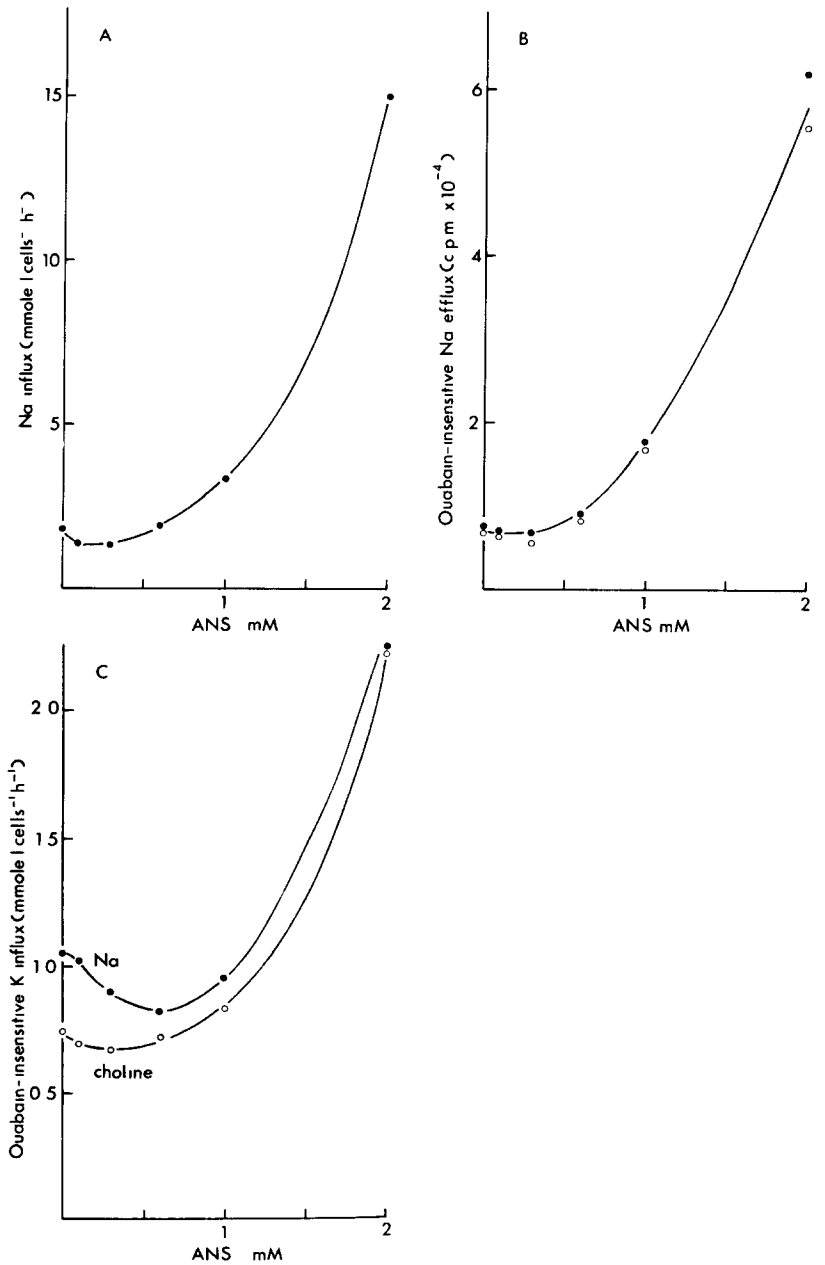


Fig 1 Effect of ANS on ouabain-insensitive Na^+ and K^+ fluxes In this and the following figures, $^{24}Na^+$ and $^{42}K^+$ fluxes were measured in triplicate tubes during 1 h incubation at 37 °C In all experiments the incubation media contained 10 mM Tris–HCl pH 7 5 at 37 °C, 1 1 10^{-4} M ouabain and the indicated concentrations of ANS (as the magnesium salt) In experiments on (B) and (C) the media contained 5 mM glucose (A) Na^+ influx The medium contained 140 mM NaCl, 10 mM KCl (B) Na^+ efflux in media with (●) and without (○) K^+, the medium contained 140 mM NaCl and 10 mM KCl (●) or 150 mM NaCl (○) (C) K^+ influx in high (●) and low (○) Na^+ media, the medium contained 5 9 mM KCl and either 120 mM NaCl, 30 mM choline chloride (●) or 5 mM NaCl and 145 mM choline chloride (○) The effect of ANS on K^+ efflux was similar to that on K^+ influx in choline medium

flux determinations). ANS concentrations greater than 0.5 mM progressively increase both Na^+ and K^+ influx and efflux, The response of K^+ efflux (not shown) follows the same pattern as K^+ influx in choline medium small decrease below 0.5 mM ANS and 1–2-fold increase above 1 mM ANS (Fig. 1C). At concentrations above 2 mM, ANS causes hemolysis, therefore no flux measurements were carried out at these higher concentrations. The increase in cation fluxes at [ANS] higher than 0 5 mM is greater for Na^+ than K^+ (Fig. 1). 2 mM ANS increases Na^+ influx and efflux 8- to 9-fold whereas the effect on K^+ fluxes is only 2–3-fold. The effect of ANS on passive cation fluxes, i.e inhibition at low concentrations and stimulation at high concentrations, parallels the effect of ANS on hypotonic hemolysis· osmotic resistance at low concentrations and increased osmotic fragility at high concentrations (see below).

It was of interest to distinguish the effect of ANS on the K^+ leak, i.e. the component of K^+ influx that increases linearly with concentration [7, 8], from the saturable K^+ influx observed in Na^+ media containing ouabain [9] since these components may represent different K^+ transport systems. Thus, the effect of ANS on K^+ influx was investigated by varying $[K^+]_0$ at a fixed ANS concentration in different media containing ouabain. Fig. 2 shows the passive K^+ influx as a function of $[K^+]_0$ in Mg^{2+} and Na^+ media In Mg^{2+} media the flux is linear with K^+ concentration (Fig. 2A), whereas in Na^+ media it is approximately hyperbolic at $[K^+]_0 <$ 10 mM and becomes linear at $[K^+]_0 > 10$ mM (Fig 2B, ref 18). Surprisingly, ANS inhibits K^+ influx in Na^+ media at low $[K^+]_0$ and increases the flux at high $[K^+]_0$ (Fig. 2B). In Mg^{2+} media, ANS increases the flux at any $[K^+]_0$ and the increase is

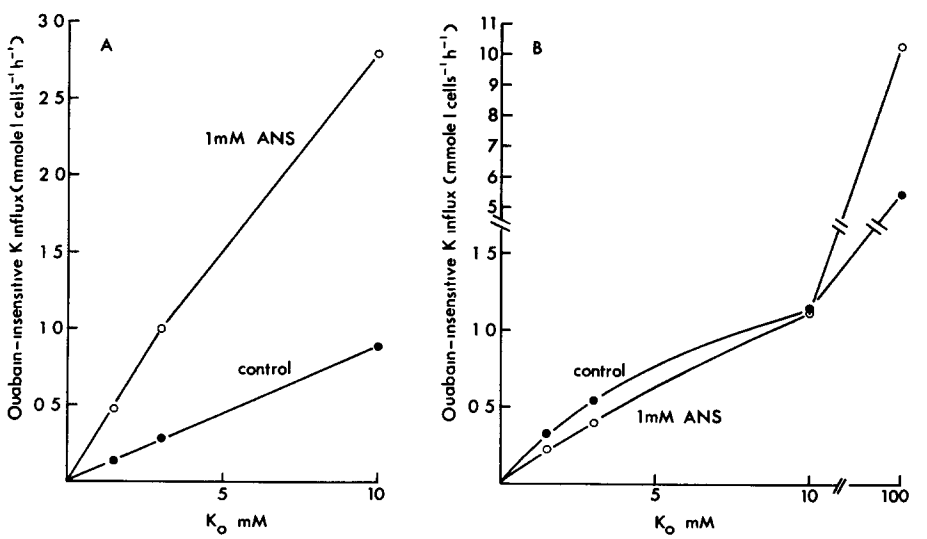


Fig 2. Effect of ANS on ouabain-insensitive K^+ influx as a function of $[K^+]_0$ in Mg^{2+} and Na^+ media Experimental conditions all tubes contained 20 mM Tris · HCl, pH 7 5 at 37 °C, 5 mM glucose and 1 1 10^{-4} M ouabain, hematocrit was 5 % and 1 mM ANS (magnesium salt) was present where indicated The Mg^{2+} medium (A) contained 1 5, 3 or 10 mM KCl and 105, 104 or 100 mM $MgCl_2$, respectively The Na^+ medium (B) contained 1 5, 3, 10 or 100 mM KCl and 150, 148, 140 or 50 mM NaCl, respectively

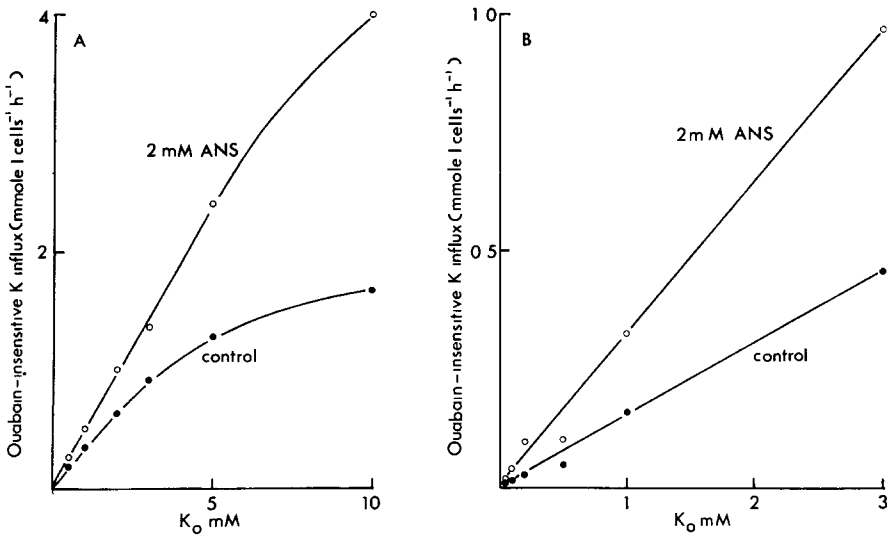


Fig 3 Effect of ANS on ouabain-insensitive K^+ influx as a function of $[K^+]_0$ in Na^+ and choline media Experimental conditions all tubes contained 20 mM Tris HCl, pH 7 5 at 37 °C, 5 mM glucose and 1 1 10^{-4} M ouabain, hematocrit was 5 % and 2 mM ANS (magnesium salt) was present where indicated Ion composition was varied so that [KCl] + [NaCl] = 150 mM in (A) and [KCl] + [choline chloride] = 150 mM in (B)

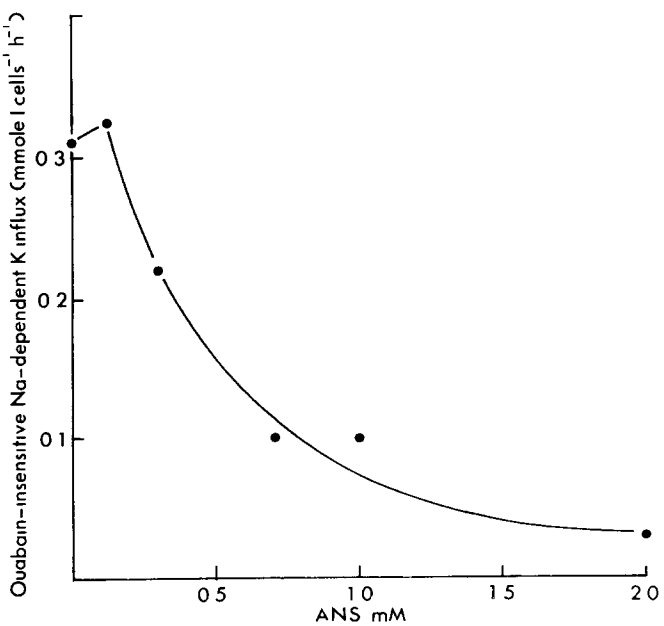


Fig 4 Effect of ANS on the Na^+-stimulated, ouabain-insensitive K^+ influx Experimental conditions as in Fig 1C The points represent the difference between K^+ influx in Na^+ and choline media

larger than in Na^+ media (Fig. 2A). These observations suggest that K^+ flux in the presence of ouabain consists of two components in Na^+ media. a saturable component inhibited by ANS and a linear component increased by ANS. Thus, the total flux will be decreased or increased depending on the relative magnitudes of these components. At low $[K^+]_0$, the saturable component is the major fraction of the flux and therefore less K^+ enters the cell, whereas the linear component is the main fraction of the flux at high $[K^+]_0$ where ANS increases K^+ uptake. This interpretation is supported by the experiment in Fig 3 where a higher ANS concentration (2 mM) increases K^+ influx at any $[K^+]_0$ in both high Na^+ (Fig 3A) and low Na^+ (Fig. 3B) media. In this case the increased K^+ leak is large enough to result in increased K^+ uptake despite the inhibition of the saturable component.

The saturable component, defined as the K^+ influx that depends on Na^+ in the medium, appears to be inhibited at lower ANS concentrations than those that increase the leak. Fig 4 shows the effect of various ANS concentrations on the saturable component of K^+ influx, measured as the difference in K^+ influx in Na^+ and choline media The concentration of ANS necessary for 50 % inhibition is less than 0 5 mM.

I. Effect of ANS on the Na^+ and K^+ pump

Fig 5 shows the effect of ANS on the ouabain-sensitive Na^+ efflux into K^+ containing and K^+ free media ($Na^+ : K^+$ and $Na^+ : Na^+$ exchange, respectively) [18, 19] and ouabain-sensitive K^+ influx in high and low Na^+ media (Fig. 5B). The inhibition of the pump is between 40 and 75 % with 2 mM ANS in different experiments. The apparent difference between ANS inhibition of $Na^+ : Na^+$ and $Na^+ : K^+$

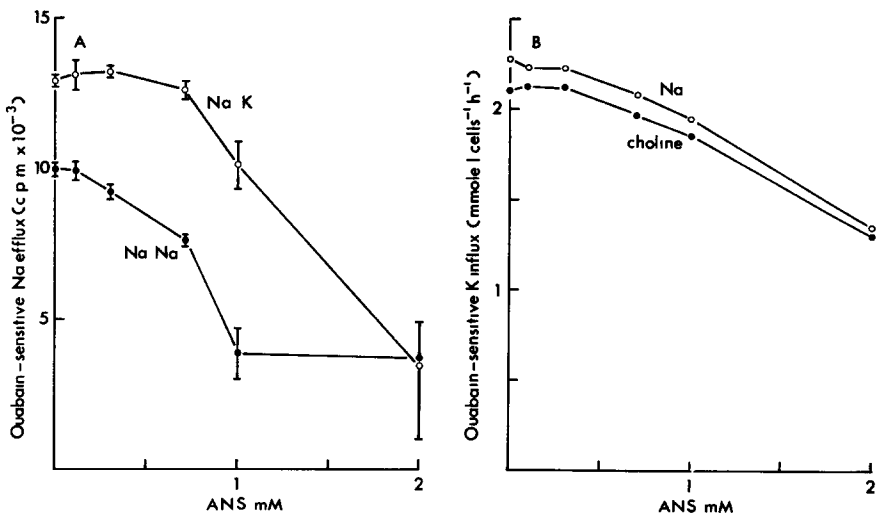


Fig. 5 Effect of ANS on ouabain-sensitive Na^+ and K^+ fluxes (A) Ouabain-sensitive Na^+ efflux Incubation medium 5 mM glucose, 10 mM Tris HCl pH 7 5 at 37 °C, the indicated ANS (magnesium salt) concentrations and 150 mM NaCl (●) or 140 mM NaCl+10 mM KCl (○) The bars show ±1 S E (B) Ouabain-sensitive K^+ influx Incubation medium 5 mM glucose, 10 mM Tris HCl, pH 7 5 at 37 °C, 5 9 mM KCl, the indicated ANS concentrations and either 120 mM NaCl, 30 mM choline chloride (●) or 5 mM NaCl, 145 mM choline chloride (○) In (A) and (B), each point is the difference in the flux in the presence and absence of 10^{-4} M ouabain, hematocrit was 5 %

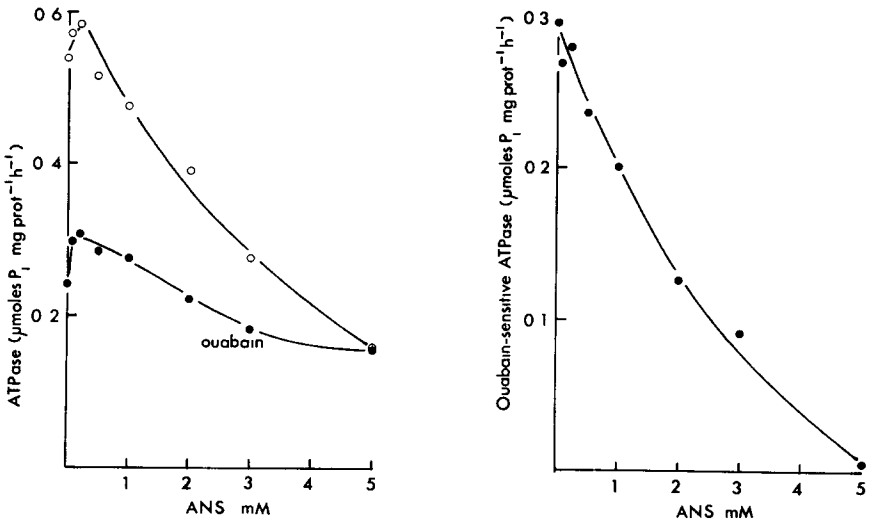


Fig 6 Effect of ANS on ATPase activity of red cell ghosts Experimental conditions 120 mM NaCl, 10 mM KCl, 2 mM Na_4ATP, 2 mM EGTA, 10 mM Tris HCl, pH 7 7 at 37 °C, $MgCl_2$ and magnesium ANS were mixed to give 6 mM Mg^{2+} and the indicated ANS concentrations The plot on the left shows ATPase activity in the absence (○) and presence (●) of 10^{-4} M ouabain The plot on the right shows the ouabain-sensitive ATPase activity

exchange is probably not significant, from considerations of the standard errors given

The inhibitory effect of ANS on the $Na^+ + K^+$ pump was further investigated with ATPase determinations in ghosts. Fig 6 shows ATPase activity as a function of ANS concentration Below 0 3 mM, ANS increases the ouabain-insensitive ATPase activity, but inhibits the ouabain-sensitive component Above 0 3 mM ANS both components are inhibited. The concentration dependence of the inhibition of the ouabain-sensitive component is of the same order as its effectiveness on Na^+ and K^+ pump fluxes in intact cells. The ATPase assays were carried out in the presence of 2

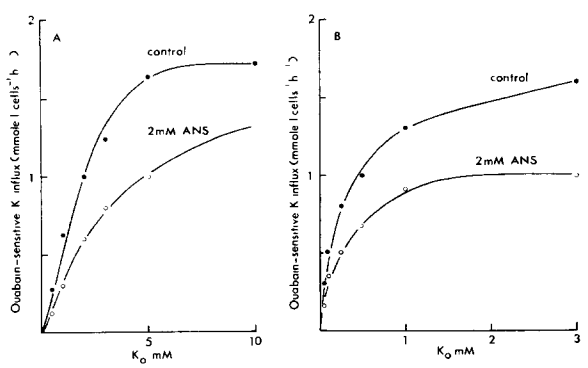


Fig 7 Effect of ANS on ouabain-sensitive K^+ influx as a function of $[K^+]_o$ in Na^+ and choline media Experimental conditions as in Fig 3 A, Na^+ medium, B, choline medium The points represent the difference in K^+ influx in the absence and presence of 1 1 10^{-4} M ouabain

mM EGTA and at constant [Mg] to eliminate any effects of either a calcium contamination in the ANS or changing [Mg] as the [magnesium ANS] increases. The stimulation of total ATPase activity at [ANS] < 0.3 mM was a consistent finding with three different samples of ANS including the NH_4 ANS salt. That the stimulation of ATPase activity was not due to contaminating Ca^{2+} in the ANS was confirmed by atomic absorption spectroscopy which yielded no detectable Ca^{2+} in a 10 mM magnesium ANS solution

The negative surface potential produced by ANS [3] might increase the K^+ concentration available to the outside surface of the pump and thus decrease the apparent K_m for K^+ influx through the pump. This was tested by measuring ouabain-sensitive K^+ influx as a function of external K^+ in Na^+ and choline media (Fig. 7). Clearly, no significant decrease in the K^+ concentration for half-maximal activation of the pump is observed in the presence of ANS. This indicates that the electrostatic field produced by the bound ANS ions is sufficiently low at the K^+ transport sites of the pump that alterations of the K^+ concentration at those sites are negligible. The kinetic behavior suggests that the V decreases owing to either a decreased number of functional pump sites or a decreased turnover of the pump when ANS is in the membrane, although modification of other substrate sites (i.e. Na^+, Mg^{2+}, ATP sites) cannot be excluded.

III. Expansion of the membrane by ANS

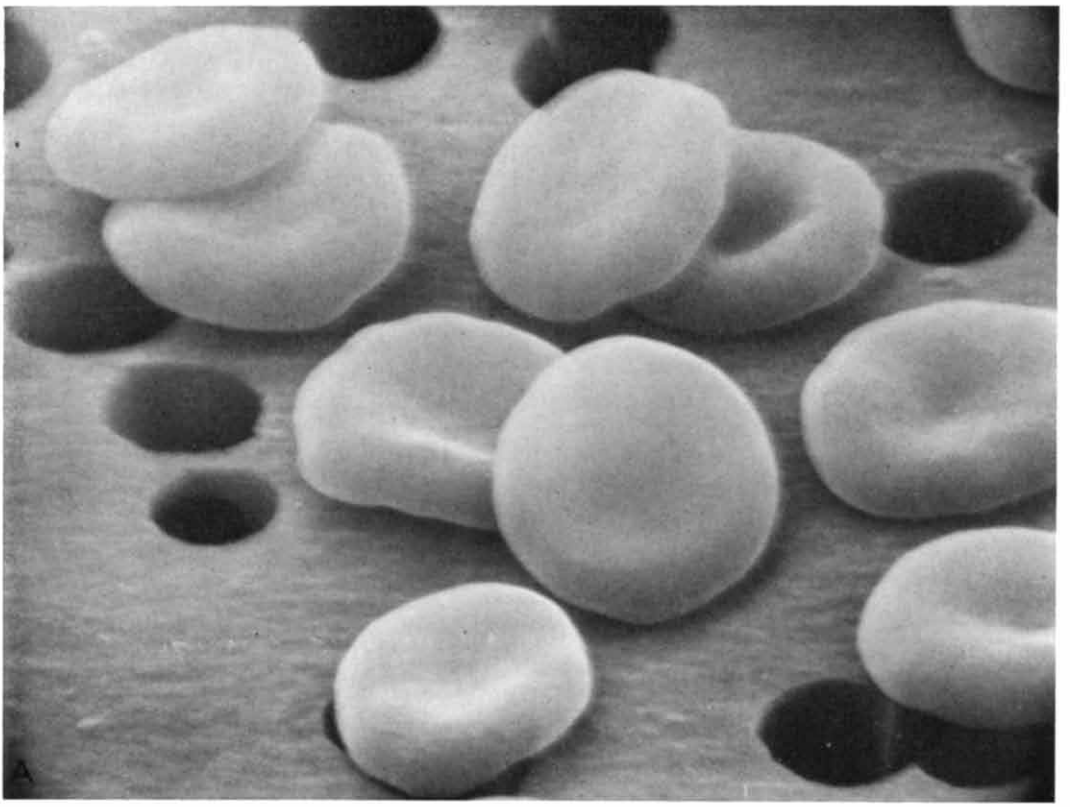
Under the conditions in which the above experiments were carried out, ANS alters the shape of the cells. As the concentration of ANS increases the cell changes from a discocyte to a discoechinocyte, to a spheroechinocyte, reversibly (Fig, 8, ref. 7) Sheetz and Singer [20] have proposed that this type of shape change can result from an asymmetric expansion of the outer half of the membrane by anionic amphipatic molecules which are repelled from the inner surface by the negatively charged phospholipids. This hypothesis is consistent with the finding that ANS in ghosts binds to only a fraction of the available membrane surface presumably owing to a high negative surface charge density [3].

In order to test the hypothesis that ANS expands the membrane, the effect of ANS on hypotonic hemolysis was investigated (Fig. 9). In the upper curve about 60 % of the cells hemolyzed in the absence of ANS, while in the lower curve hemolysis with no ANS was 10 %. Increasing ANS concentrations protect from hemolysis with maximum protection at about 0.5 mM ANS. Above this concentration hemolysis protection decreases and high concentrations become hemolytic. The degree of protection depends on the degree of hemolysis, as shown in the figure. The hemolysis protection curve in Fig. 9 is typical of those obtained with anesthetics and is interpreted to be due to expansion of the membrane which allows larger increases in cell volume before lysis [20, 21].

It is interesting to note that hemolysis protection by ANS occurs at roughly the same concentrations that inhibit ion permeability, whereas the increased cation leaks become apparent at concentrations where osmotic fragility is increased.

DISCUSSION

The main findings of this paper are: ANS up to 0.5 mM inhibits Na^+ and K^+ influx about 20 % maximum, and the Na^+-stimulated, ouabain-insensitive K^+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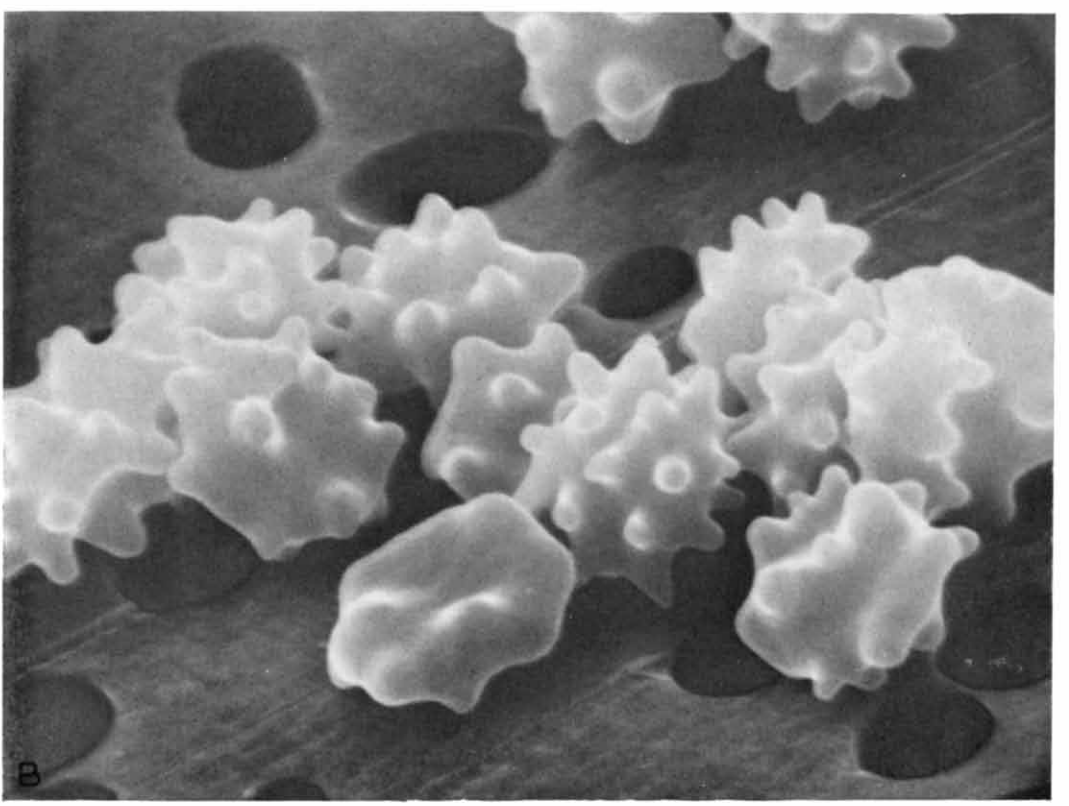
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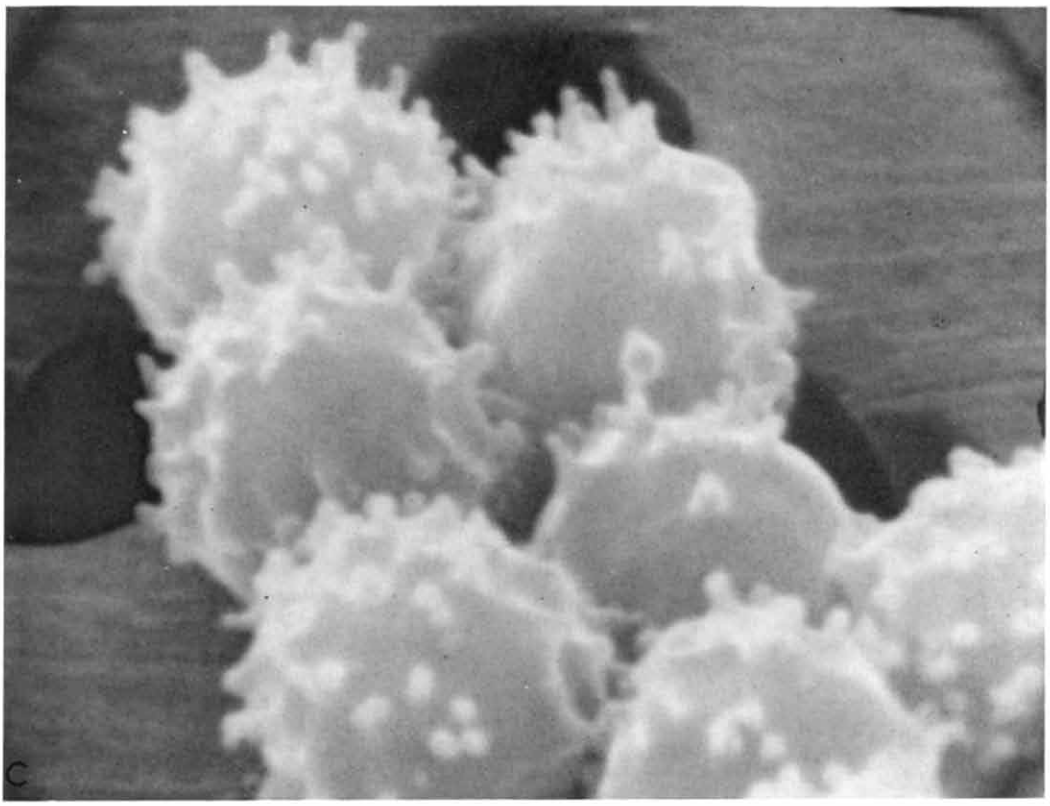

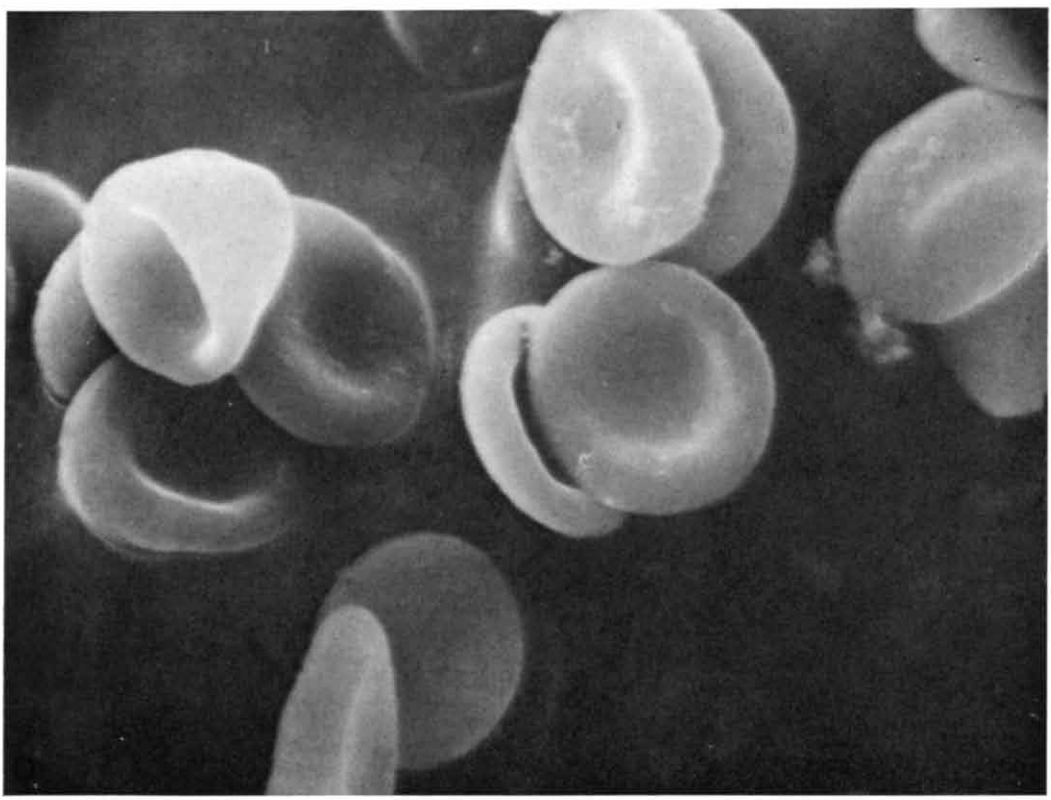

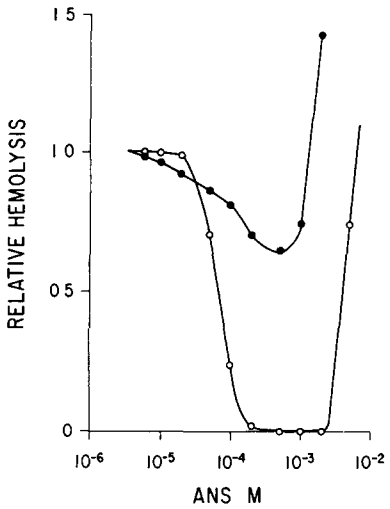

Fig 9 Effect of ANS on hypotonic hemolysis In the upper curve (●) red cells were preincubated 10 min at 23 °C at 1 % hematocrit in 150 mM NaCl, 20 mM Tris HCl pH 7 2 and the indicated ANS concentrations before dilution with 20 mM Tris HCl+ANS to give 42 % of the initial NaCl concentration Hemolysis in the absence of ANS was 54 % In the lower curve (○) red cells were added directly to 63 mM NaCl, 20 mM Tris HCl pH 7 2 and the indicated ANS concentration and centrifuged after 5 min at 23 °C Hemolysis in the absence of ANS was 9 5 %

influx At these concentrations ANS induces a disc-echinocyte transformation and protects maximally from hypotonic hemolysis, which indicate expansion of the membrane, preferentially on the outer half of the bilayer Above 0 5 mM ANS cation leaks increase exponentially with [ANS], the Na^+ stimulated ouabain-insensitive K^+ influx is completely inhibited and the $Na^+ + K^+$ pump fluxes as well as $(Na^+ + K^+)$-ATPase activity of ghosts are progressively inhibited Under these conditions hemolysis protection by ANS decreases and, above 2 mM, ANS becomes hemolytic The ouabain-insensitive ATPase activity is stimulated below 0 3 mM ANS and inhibited at higher concentrations

It is not possible, with the evidence available at the present, to speculate on the mechanism of ANS activation and inhibition of the ouabain-insensitive ATPase, or why ANS increases Na^+ leaks more than K^+ leaks Further studies on the physicochemical changes in the membrane and on the properties of the transport mechanisms may provide insight on these phenomena

Previous work has shown that ANS inhibits Cl^- and SO_4^{2-} exchange, with significant effects apparent at concentrations between 10^{-5}–10^{-4} M and more than 99 9 % inhibition at 1 mM [1] The present results indicate that the inhibitory effect of ANS is not specific for the anion transport system of red cells. With the exception of the increased cation leaks observed at high concentrations (Figs 1–3), all transport systems so far studied are inhibited by ANS, including anion exchange [1], cation diffusion (Fig. 1), Na^+-dependent ouabain-insensitive K^+ influx (Fig 4) and the $Na^+ + K^+$ pump (Figs 5–7)

Fig 8 Perturbation of red cell shape by ANS A, control cells, B, 50 μM ANS, C, 1 mM ANS, D, 1 mM ANS washed once in ANS-free medium The magnification in C is approximately twice the magnification in A, B and D

These effects, plus the expansion of the membrane by ANS indicated by the changes in cell shape and hemolysis protection, may be the functional consequence of the binding of ANS molecules to phospholipid regions and to proteins other than the anion carrier shown by the nanosecond fluorescence studies [3–5]

The similarity between the dose-response curves for inhibition of cation transport, crenation and hemolysis protection by ANS may indicate a sensitivity of these transport systems to expansion of the bilayer. However, some compounds that alter cell shape do not inhibit anion exchange [22].

It remains to be tested whether or not cation and nonelectrolyte transport are unaffected by other compounds that change the cell shape or expand the membrane.

Since the increased cation leaks are observed at ANS concentrations that increase the osmotic fragility of the cells (Figs 1–3, 9), it is possible that they represent prehemolytic membrane disruption. In addition, the charging of the membrane with ANS anions may contribute to the increased cation leaks. Analyses of the binding curves of ANS in ghosts are consistent with the creation of a significant negative surface charge by bound ANS [3–5]. The negative surface potential created by ANS has been measured directly in phospholipid monolayers and by electrophoresis of lecithin liposomes and neuraminidase-treated red cells in which ANS increases the mobility towards the anode (Fortes, unpublished observations). The ANS negative surface potential increases K^+ conductance in nonactin-treated phospholipid bilayers [23] and has been estimated to be of sufficient magnitude to account for the inhibition of anion transport[1] However, the present results indicate that the ANS surface potential has little or no effect on cation transport. No change in the K_m for K^+ in the pump is observed with 2 mM ANS (Fig. 7) and no increases in cation leaks are observed at concentrations (below 1 mM) that inhibit anion transport substantially, whereas a purely electrostatic mechanism predicts a substantial increase of cation concentration near the ANS binding sites, which would increase the cation leaks. This suggests that the cation permeation regions are at a sufficient distance from the ANS binding sites to allow for decay of the electric field. In the conditions of the present experiments, the Debye length is 5–10 Å, which sets a lower limit for this distance.

The fluorescence properties of ANS offered the possibility of a correlation between the functional perturbations caused by the probe and the characteristics of its binding sites as obtained from spectroscopic studies. However, it is important to note that the effects of ANS on anion permeability and cell shape are not specific for the fluorescent probe. All reversible inhibitors of anion exchange are also reversible crenators or cup-formers [22, 24] Also, these inhibitors characteristically increase cation leaks at high concentrations [25–28] and, when tested, have been found to inhibit other transport systems. Trinitrocresolate also inhibits the $Na^+ + K^+$ pump [28] and phloretin [29] alos inhibits the transport of glucose [30] and small nonelectrolytes like urea and glycerol [31]

These observations suggest that the perturbations of membrane function caused by these inhibitors may have a common denominator. Although they are unrelated structurally, these inhibitors are all amphipathic molecules that may bind preferentially to one of the halves of the bilayer, either because of their charge or their membrane impermeability [20].

Thus, it is possible that the perturbations caused by these compounds are a

consequence of their interaction with the bilayer, rather than or in addition to specific perturbations of the transport mechanisms. Whether or not the perturbation necessarily depends on an asymmetric interaction with the membrane, as that suggested by the effect of ANS and other inhibitors on cell shape and seen with phlorizin on anion exchange [32, 33], remains to be studied

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