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Use of 1-Anilino-8-Naphthalene-Sulfonate as a Probe of Gastric Vesicle Transport

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Summary. The interaction of 1-anilino-8-naphthalene-sulfonate (ANS) with vesicles derived from hog fundic mucosa was studied in the presence of valinomycin and with the addition of ATP. Evidence was found for two classes of sites, those rapidly accessible to ANS with a K_D of 7.5 μM and those slowly accessible, but rapidly accessed in the presence of valinomycin with a K_D of 2.5 μM. ATP transiently increases the quantum yield of the latter ANS binding sites only in the presence of valinomycin, but does not alter the number of K_D of those sites. The time course of this increase correlates with H^+ uptake and Rb^+ extrusion by those vesicles and H^+ carriers such as tetrachlorsalicylanilide or nigericin abolish the ATP response. With ATP addition in the presence of $SC^{14}N$ and valinomycin there is transient uptake of SCN^-. It is concluded that ANS is acting as a probe of a structural change dependent on a potential and H^+ gradient.

The vesicular membrane fraction isolated from hog gastric mucosa has been characterized in some detail (Forte, Ganser, Beesley & Forte, 1975) and further subfractionated using ficoll-sucrose or ficoll-ficoll density gradients (Sachs, Rabon, Chang, Schackmann, Lewin & Saccomani, 1976). It has been shown in several species (Ganser & Forte, 1974*a*; Forte, Ganser & Tanisawa, 1974; Spenney, Strych, Price, Helander & Sachs, 1974*b*) that the membrane fractions isolated from gastric fundus contain a cation or K^+-stimulated ATPase which is insensitive to ouabain, but is also capable of forming a phosphorylated intermediate sensitive to K^+ (Tanisawa & Forte, 1971). The ATPase activity is also enhanced by valinomycin, gramicidin or nigericin (Ganser & Forte, 1973*b*) and more recently it has been claimed that a proton gradient is generated upon the addition of ATP (Lee, Simpson & Scholes, 1974) in a dog microsomal preparation.

The unique capacity of the gastric mucosa to generate a H^+ gradient of 10^6:1, across the membrane of the parietal cell in the mammal,

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combined with the observations outlined above, make this membrane fraction a particularly interesting model because of the apparent lack of the complex redox system associated with other H^+ transport vesicles. Since it has also recently been shown that these smooth membrane fractions are probably randomly oriented and are also, in the case of hog fractions, capable of absorbing H^+ and ejecting Rb^+ (Sachs, Saccomani, Rabon & Sarau, 1975; Sachs *et al.*, 1976) with the addition of ATP, it seems important to approach the problem of the membrane changes or energization induced by ATP. Some years ago, it was shown that ATP produced conformational changes in membrane fraction derived from dog mucosa by the use of corrected CD spectra (Masotti, Long, Sachs & Urry, 1972).

1-anilino-8-naphthalene-sulfonate (ANS) is a fluorescent membrane probe which has two major properties of interest for this study. First, fluorescent enhancement and a bathochromic shift occur with change of the environment from high to low dielectric constant which enables assessment of the change of hydrophobicity of the ANS environment (Stryer, 1965); and second, being negatively charged, the movement of ANS is sensitive to the alteration of potential gradients across a vesicular membrane such as mitochondria (Azzi, Chance, Radda & Lee, 1969*a*, Chance & Lee, 1969) everted submitochondrial particles (Azzi *et al.*, 1969*a*) or single layer liposomes (Bakker & Van Dam, 1974). It has also been suggested that site protonation may account for increased ANS binding (Chance, 1970).

In this paper we report significant effects on ANS fluorescence induced by ATP, that appear to be dependent on H^+ and potential gradients across the membrane and correlate these effects with the properties described elsewhere of H^+ and Rb^+ transport induced by ATP.

Materials and Methods

Smooth surface gastric vesicles (SGV) were prepared from hog gastric mucosa as described in detail elsewhere (Spenney, Saccomani, Spitzer, Tomana & Sachs, 1974*a*; Saccomani, Shah, Spenney & Sachs, 1975) but with the modification of teflon-glass homogenization. Using a 7–20% ficoll density step gradient (15 hr, SW 25, 23,000 rpm), the microsomal fraction was separated into three fractions, GI, GII and GIII (Saccomani, Stewart, Shaw, Lewin & Sachs, 1977), GI being the band obtained at the 7% ficoll interface. The rationale for using the GI fraction and the avoidance of sucrose in the gradient is that GI is more active in terms of H^+ transport (Sachs *et al.*, 1976) and Rb^+ efflux, and is also more active when prepared with pure ficoll gradients as compared to ficoll/sucrose gradients.

This GI fraction has been shown to contain a 20-fold enrichment of K^+ *p*-nitrophenyl phosphatase (*p*NPPase) or K^+-ATPase (Sachs *et al.*, 1976) and to contain less than 5% of the microsomal content of RNA, succinic dehydrogenase, monamine axidase or cytochrome *c* oxidase. Electronmicrographs showed it to be a smooth-surfaced vesicular fraction. It was stored in the refrigerator until use.

The fluorescence of ANS was measured in an Aminco Bowman spectrofluorimeter in a thermostatted cuvette at an excitation wavelength of 375 nm and an emission wavelength of 480 nm. Under each experimental condition repetitive scans were carried out to ensure stability of the scatter and emission peaks. Fluorescence was measured at a sensitivity setting of 30, and expressed as arbitrary units (FU).

The standard conditions were the addition successively of 100 µl of GI suspension (containing 5 mg protein/ml) to a quartz cuvette containing 2 ml of a solution of 150 mM, KCl, 50 mM, Tris acetate buffer, pH 6.1 at 27 °C. ANS was added in 10 µl methanol to achieve a final concentration of 2.5×10^{-6}M, followed by valinomycin (10 µl solution to give a final concentration of 5×10^{-6}M) and Mg ATP (10 µl solution to give a final concentration of Mg ATP of 5×10^{-5}M). In some experiments the concentration of each of the constituents was varied independently as was the pH of the suspending medium. When the protein concentration was varied at a fixed ANS concentration (Fig. 7), the fluorescence calculated at infinite protein concentration was assumed to be the fluorescence of the total quantity of ANS added. This allowed calculations of the quantity of ANS bound under conditions of varying ANS concentration and hence the free ANS from the difference between total added and amount bound. "Aging" of the SGV preparation was carried out at refrigerator temperature for a period of five days. Other additions such as nigericin (1 µg/ml), *m*-chlorocyanocarbonylphenylhydrazone (CCCP) (10^{-5}M), tetrachlorsalicylanilide (TCS) (10^{-6}M) were dissolved in 10 µl methanol before addition to the cuvette. Ten µl methanol alone was without any action. Analogs of ATP were used at the same final concentration as ATP and dicyclohexylcarbodiimide (DCCD) was added at a concentration of 10^{-3}M also in 10 µl of methanol.

With ethidium bromide, a positively charged fluorescent marker, all the conditions were the same, except that 10^{-6}M ethidium bromide was used and 530 nm was the excitation and 590 nm was the emission wavelength. No effect of K^+, valinomycin, or ATP was observed.

The generation of a proton gradient was measured as described elsewhere (Sachs *et al.*, 1975).

The distribution of SCN^- between vesicles and medium was assessed by adding 200 µl of vesicle suspension to 200 µl of a solution containing 150 mM KCl, 80 mM tris-acetate pH 6.1, 2 mM $MgCl_2$ and 1.82 mM $S^{14}CN$ (4 µCi) at room temperature. At the start of the experiment to one set of tubes in a final volume of 400 µl, valinomycin was added in 4 µl methanol at 10^{-6}M.

After incubation and sampling for 15 min, ATP was added to both sets of tubes and sampling (20 µl) continued. The samples taken at various times before and after ATP addition were injected into 1 ml of a 120 mM Li_2SO_4 solution at pH 6.1 and 0 °C. The mixture was filtered on a Millipore HAWP 0.45 microfilter, washed once with 4 ml Li_2SO_4 solution, the filters were dried and counted in an LKB 81,000 scintillation counter. In other experiments $^{36}Cl^-$ was used instead of SCN^-.

All chemicals were the highest purity available; nigericin was a gift from Eli Lilly Company. ANS was obtained from Eastman Kodak. Hog stomachs were obtained fresh at the slaughterhouse through the courtesy of Lumberjack Meat Company and the fractions prepared on the day of the slaughter.

Results

Initial Observations

Prior to ANS addition, the SGV suspension had negligible emission at 480 nm (Fig. 1). The addition of ANS at a final concentration of 2.5×10^{-6}M resulted in an increase of 12 FU rapidly ($t_{1/2}$ too small to measure with our technique) followed by a second slow phase which reached a plateau after 20 min (Fig. 1) at 22 FU. Omission of membranes gave, with addition of ANS, an emission at 575 nm.

Valinomycin added at 1 min after the addition of membranes resulted in an increase of fluorescence to 29 FU (Fig. 1). Without membranes at the standard concentration of valinomycin (5×10^{-6}M) no change in FU was observed, but at higher concentrations (5×10^{-5}M) a change of 5 FU was found, as has previously been described for several ionophores such as valinomycin or nigericin (Feinstein & Felsenfeld, 1971).

Subsequent addition of ATP produced a large transient increase in fluorescence (Fig. 1), 69 ± 2 FU, $n = 8$ being the mean $\pm$ SEM for a given

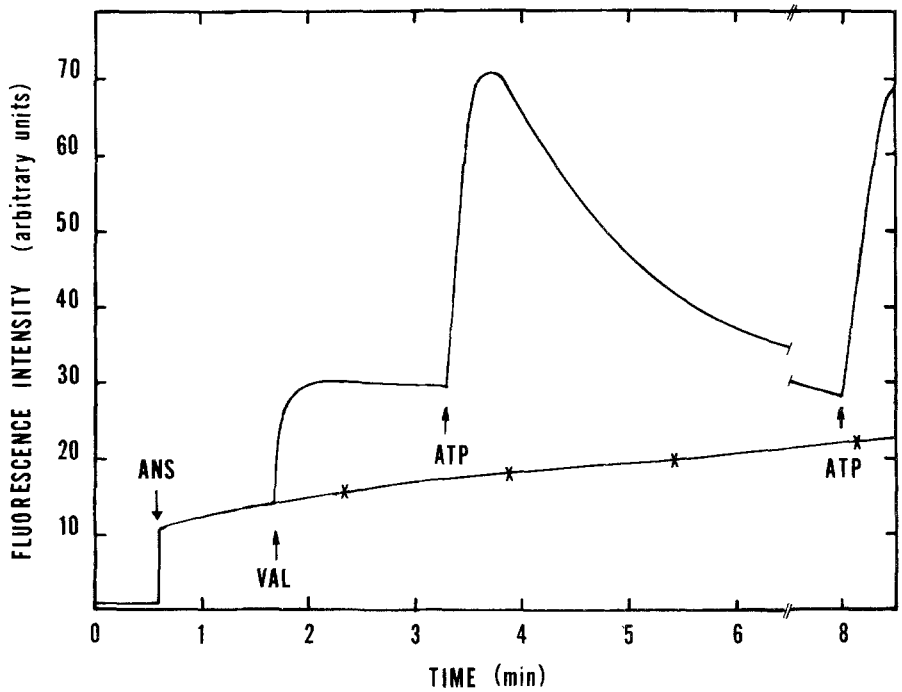

Fig. 1. The effect of addition of ANS to gastric vesicles on fluorescence at 480 nm showing an initial rapid rise followed by a slow increment. The addition of valinomycin prior to the slow rise results in rapid attainment of the final level. Addition of ATP only in the presence of valinomycin results in a transient fluorescent enhancement

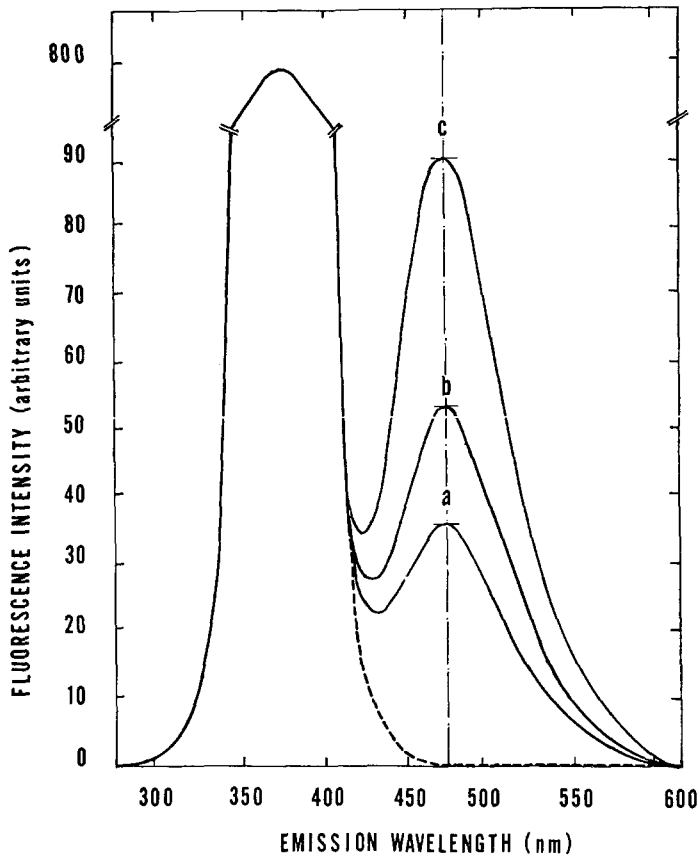

Fig. 2. An emission scan of fluorescence of gastric membranes (*a*) following ANS addition, (*b*) following valinomycin addition, (*c*) with the addition of ATP. Concentrations as in text

GI preparation and ranged from 45 to 90 with different preparations. The maximum fluorescence was reached in 30 sec, the mean $t_{1/2}$ being 7 sec $\pm$ 1.2 ($n=8$). The maximum is followed by a slow decrease, expressible as a simple exponential, to the basal value, the mean $t_{1/2}$ being 100 sec. The fluorescence enhancement can be obtained again by the readdition of ATP following the decay, but a progressive decrease is found with successive additions of ATP.

Under these conditions, no change in emission wavelength or in intensity of the scatter peak was found (Fig. 2).

K^+ *Valinomycin Requirement*

The ΔFU obtained with the addition of ATP was absolutely dependent on the presence of valinomycin, of the ionophores tested and on

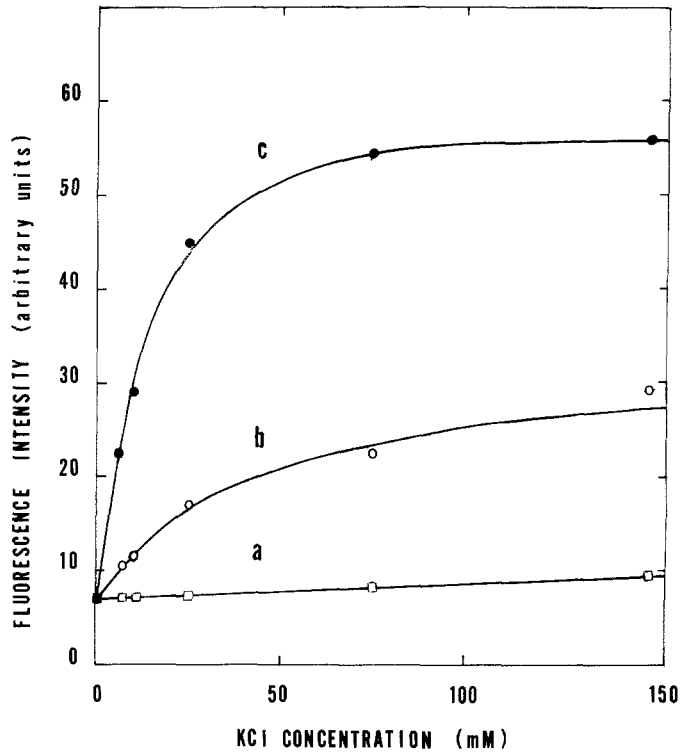

Fig. 3. The effect of varying the KCl concentration on (*a*) the fluorescence induced by ANS, (*b*) ANS + valinomycin, and (*c*) ANS-valinomycin + ATP

alkali cation. Thus with valinomycin in choline media no effect was detected. Other cations substituted for K^+ in the sequence K^+ (100) > Rb (90) > Cs (30) > Na, Li (1). The lipophilic cation triphenylmethylphosphonium ($TPMP^+$) was able to partially (50%) substitute for valinomycin, in terms of the valinomycin dependent ANS fluorescence enhancement, but did not substitute in the ATP effect where valinomycin was still required. Preincubation of the vesicles in KCl did not remove the requirement for valinomycin, and other ionophores such as nigericin or gramicidin also were unable to substitute for valinomycin.

Since there were two phases of ANS-induced fluorescence, the possibility that the addition of valinomycin served to accelerate the slow phase of ANS binding was examined by adding valinomycin after 20 min following ANS addition. Indeed this resulted in a 90% reduction in the effect of valinomycin, but the ionophore was still required for the ATP effect.

The effect of variation of KCl concentration was examined in some detail. The effect of valinomycin was sensitive to KCl concentration.

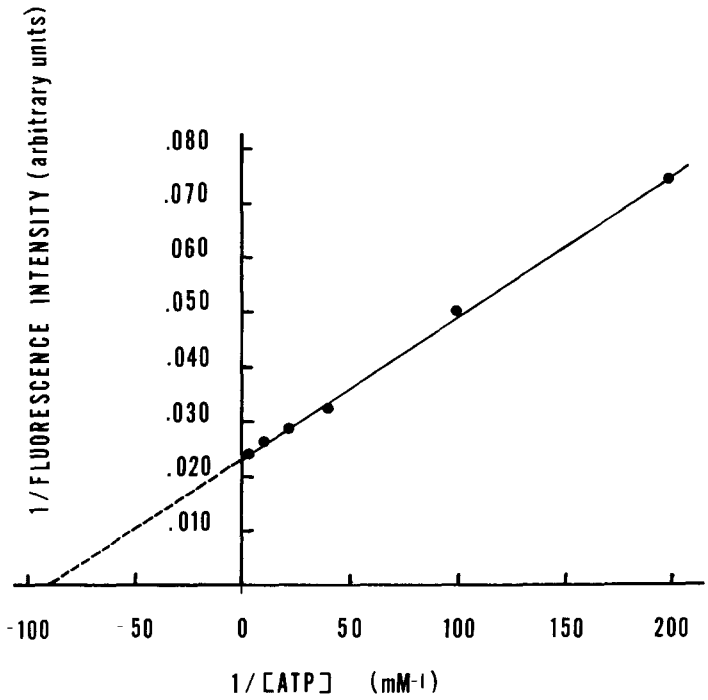

Fig. 4. A Lineweaver Burk plot of the effect of varying the ATP concentration of the maximal fluorescence obtained

However, varying the K^+ gradient by preincubating the vesicles in KCl and adding the vesicles to equimolar KCl or choline chloride instead of KCl did not alter the fluorescence obtained when KCl was present initially only on the exterior of the vesicles. The apparent K_A for valinomycin-induced fluorescence was 5×10^{-2}M (Fig. 3).

Variation of KCl also affected the ATP-induced ANS fluorescence enhancement. Two effects were noted. The maximal fluorescence as plotted in Fig. 3 gave a good fit to Michaelis-Menten kinetics ($r=0.996$) with an apparent K_A of 1.5×10^{-2}M. However, K^+ also affected the rate, decreasing the $t_{1/2}$ of excitation and decay. If the initial rate of fluorescence change was used the data did not conform to Michaelis-Menten kinetics but instead the change in fluorescence was linearly related to the log of the K^+ concentration ($r=0.992$) and the zero fluorescence intercept corresponded to 1 mM K^+. The slope corresponded to 8.4 FU for a 10-fold change in K^+ concentration. In the ATP situation, the direction of the initial gradient had slight, variable effects on the fluorescence obtained.

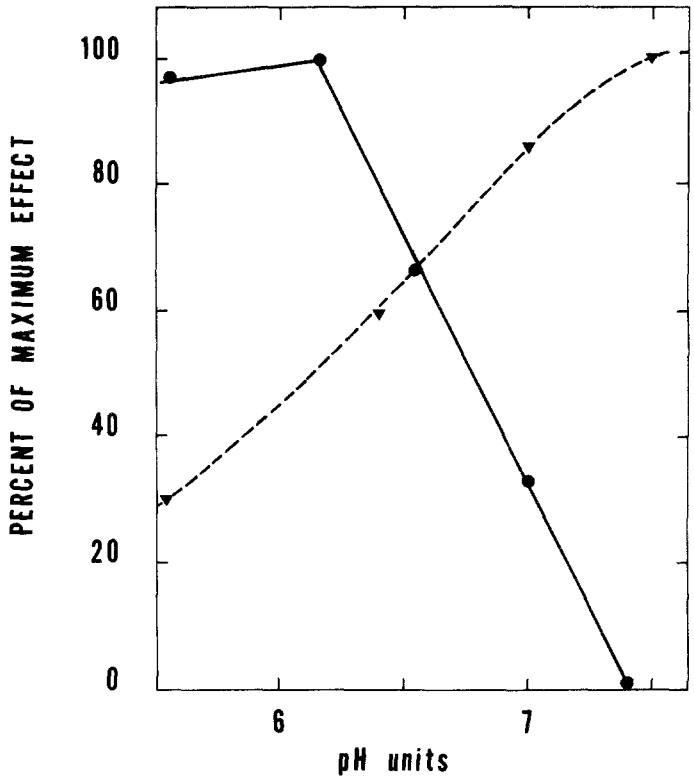

Fig. 5. The variation of maximal fluorescence obtained with ATP addition as a function of medium pH (●——●). Superimposed is the K^+-ATPase activity as a function of pH under identical conditions

Substrate Specificity

At various concentrations GTP, UPT, ITP, TTP, ADP, acetylphosphate, and *p*NPP were all shown to be ineffective in promoting a fluorescence enhancement.

Variation of ATP concentration had significant effects on the time course and maximum fluorescence obtained. A double reciprocal plot of the effect of ATP on maximal fluorescence is shown in Fig. 4, giving an apparent K_M of 1.1×10^{-5}M and a calculated maximal FU of 46.5 for this preparation. A change of fluorescence could readily be detected with concentrations of ATP as low as 5×10^{-6}M.

Dependence on K^+-ATPase Activity

The effect of pH is shown in Fig. 5 where it can be seen that there is a linear decrease of ATP-induced ΔFU from pH 6.1 to pH 7.4, whereas

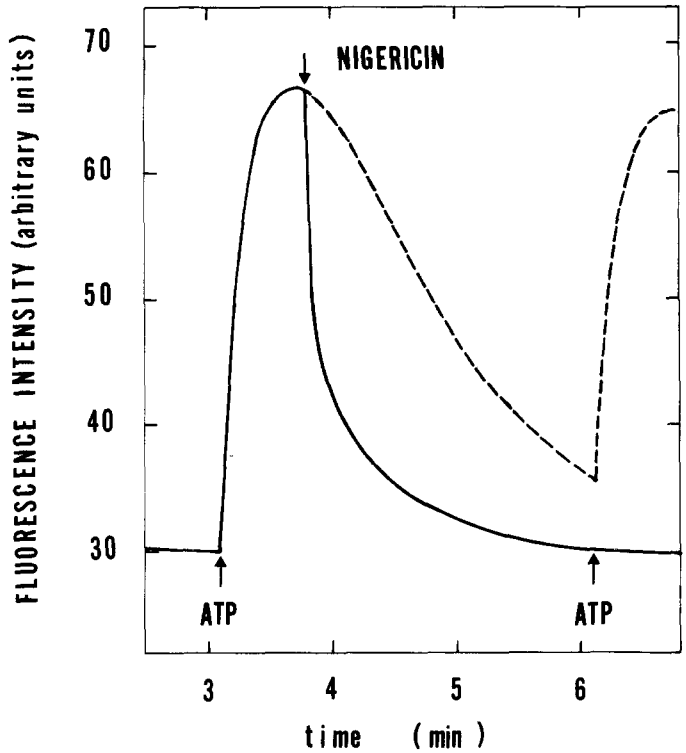

Fig. 6. The effect on fluorescence of the addition of ATP followed by the addition of nigericin at the peak of fluorescence, and also showing the lack of effect of a subsequent ATP addition. The dotted line shows the decay and reexcitation of fluorescence in an identical control preparation

ATPase activity increased over this range, being optimal at pH 7.4, where the ΔFU is not detected. β-γ methylene ATP, a nonhydrolyzable analog of ATP did not produce a ΔFU. DCCD blocked the ANS response and also inhibited the K^+-ATPase activity.

Effect of Ionophores

Fig. 6 shows the effect of the addition of nigericin which is similar to the effect of addition of CCCP (10^{-5}M) or TCS (10^{-6}M). Thus, prior addition of nigericin blocks the response to ATP, or addition of nigericin at the peak of fluorescence rapidly collapses the ΔFU observed (Fig. 6). Gramicidin also inhibited the fluorescence, due to the H^+/K^+ exchange characteristics of this ionophore.

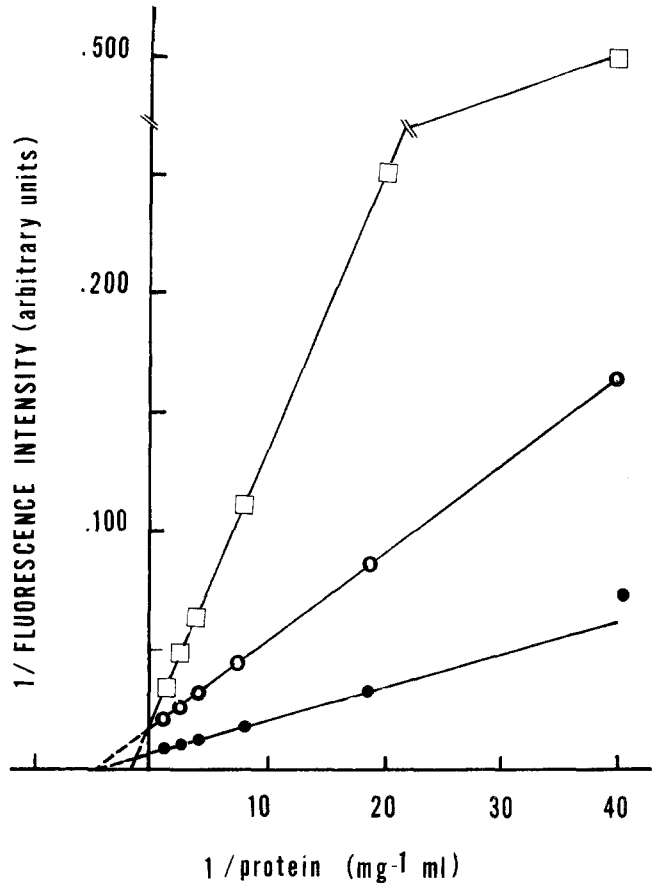

Fig. 7. A double reciprocal plot of the variation of fluorescence intensity at a fixed ANS concentration with alteration in membrane concentration: □—□ ANS alone; ○—○ ANS+valinomycin; ●—● ANS+valinomycin+ATP

Effect of Vesicle Integrity

Aging the vesicles by maintaining them at refrigerator temperature results in gradual loss of response to ATP, but does not significantly change the response to valinomycin. Two cycles of freeze thawing also sharply reduced the response to ATP, but again not the valinomycin effect.

Effect of Protein Concentration

At a fixed concentration of ANS (5×10^{-6} M), increasing the protein concentration resulted in a fluorescence that was a hyperbolic function of protein concentration under all conditions studied: ANS, ANS+valinomycin and ANS+valinomycin+ATP. Fig. 7 shows this finding in

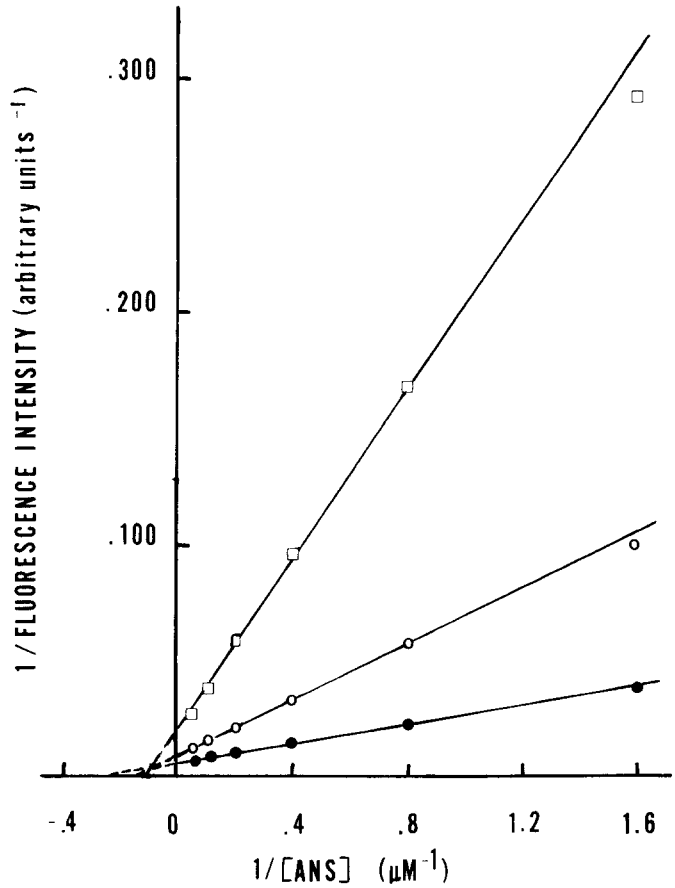

Fig. 8. A Lineweaver Burk plot of the variation in fluorescence produced by a variation in ANS concentration: □——□ ANS alone; ○——○ ANS + valinomycin; ●——● ANS + valinomycin + ATP

double reciprocal form, from which it can be calculated that at infinite protein concentration, since all the ANS can be assumed to be bound, ANS or ANS + valinomycin show the same maximal fluorescence (50 FU) but the addition of ATP increased the maximal fluorescence for the same quantity of ANS to 125 FU, i.e., increased the quantum yield for ANS by a factor of 2.5 (Brocklehurst, Freedman, Hancock & Radda, 1970).

Effect of ANS Concentration

The effect of varying the ANS concentration on the total fluorescence obtained with the membranes alone, the membranes with valinomycin and with, in addition, ATP is shown in double reciprocal form in Fig. 8.

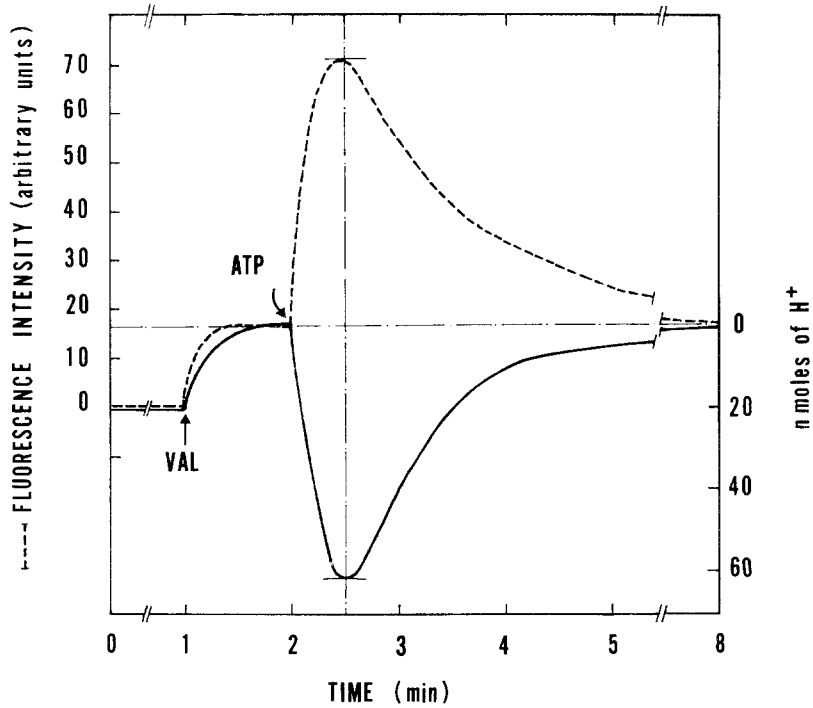

Fig. 9. The time course of development of the ANS response to ATP addition (dotted line) and the medium alkalinization developed in response to an identical ATP concentration (solid line) in the same preparation under otherwise identical conditions. The bar on the right corresponds to 10 nmoles of H^+

The raw data suggest that there is little effect of valinomycin on the affinity of the sites as compared to ANS alone, but there is an apparent increase in the number of sites. ATP apparently augments the number and affinity of these sites.

It has been suggested, based on nanosecond decay studies, that saturation of membrane "sites" by ANS is only apparent, and that the saturation observed is due to electrostatic shielding preventing ANS access (Fortes, 1976). If correct, and also for the gastric vesicles, calculation of the number and affinity of the ANS "sites" based on the above data will underestimate the true values. However, one may assume that there are two types of sites—one type, superficial, binding ANS rapidly and the other type binding ANS slowly to which access is increased by valinomycin. Considering these as independent and noninteracting, it is possible to consider the fluorescence of each site independently by subtracting the basal ANS fluorescence from the valinomycin-induced fluorescence. If this is done, it can be concluded that the number of

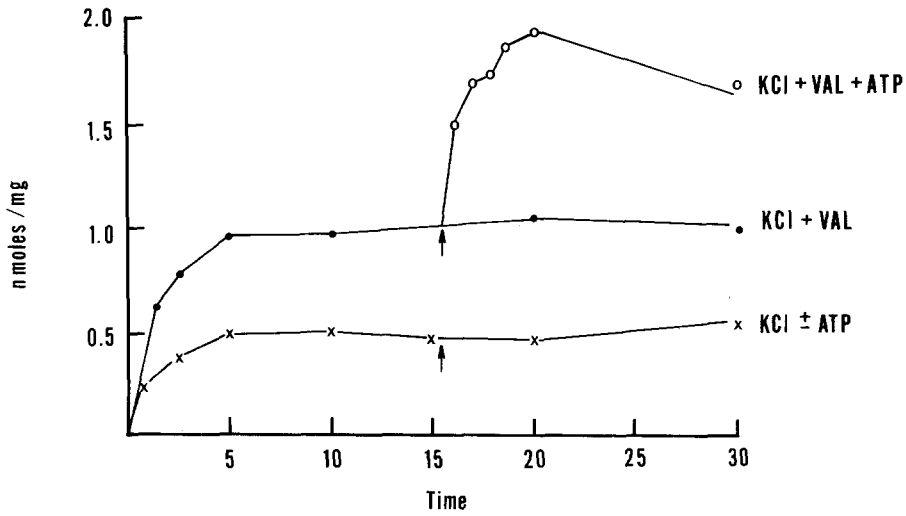

Fig. 10. The time course of SCN^- uptake by the gastric vesicles in the absence of valinomycin (lower curve) and the presence of valinomycin (upper curve). The addition of ATP enhances SCN^- uptake only in the presence of valinomycin (upper curve)

superficial sites is the same as the number of sites dependent on lipid permeable cation, such as K^+ valinomycin. The superficial sites have an apparent affinity of 7.5×10^{-6}M and the "slow" sites an affinity of 2.5×10^{-6}M.

Further, since the ATP effect is to increase the quantum yield of the ANS bound, and if the assumption is also made that ATP has no effect on the superficial sites, it is possible to calculate corrected values for the ATP-induced fluorescence. Such a plot is curvilinear (concave upwards) on the double reciprocal plot at high ANS concentrations, which may be due to ANS inhibition of the ATPase ($K_I = 75$ μM) or because ATP also affects the superficial sites. As a first approximation, however, this manipulation of the data suggests that ATP does not affect the number or affinity of the valinomycin-dependent sites but simply increases the quantum yield of the ANS bound at those sites.

Relation to H^+ Gradient

Where the uptake of H^+ was measured under identical conditions on the same preparation on the same day, and compared to the fluorescence change induced by ATP, the curves of Fig. 9 were obtained. It can be seen that the time course is very similar and that the decline of fluorescence corresponded to the decline of the H^+ gradient.

SCN^- Uptake

Fig. 10 shows the uptake of SCN^- by the gastric vesicles. It can be seen that valinomycin enhances uptake of SCN^- as compared to control conditions. The addition of ATP in the absence of valinomycin has no effect on the uptake of SCN^-, whereas in the presence of valinomycin there is an additional SCN^- uptake, increasing the uptake by 100%.

Discussion

The changes in ANS fluorescence following the primary binding reaction to hydrophobic groups have been interpreted generally in two ways, either due to changes in the ANS sites, or due to movement of ANS into the medium due to changes of an electrochemical gradient in the vicinity of those sites (Jasaitis, Kuliene & Skulachev, 1971). The data presented are best interpreted as due to the former, namely a character change in the binding site of the gastric membranes, an interpretation which is now more generally accepted for other systems as well.

As in other work (Friedman & Radda, 1969) there are two phases of ANS binding, most likely due to two classes of sites. The rapid binding sites are likely to be on the external face of the membrane, whereas the slower sites are either within the membrane, or located on the inner face of the membrane. At varying concentrations of ANS after prolonged incubation, the binding achieved was almost the same as that in the presence of valinomycin, and the effect of the ionophore was apparently to increase the rate of ANS binding. Calculation also showed that the affinity of the valinomycin-dependent sites was greater than the affinity of the initial ANS sites and that the affinity of the "slow" sites was similar to the valinomycin "sites". At high protein concentrations there was also an overshoot of fluorescence with the addition of valinomycin, which may be a function of the K^+ gradient present upon adding the vesicles.

On the other hand, the quantum yield was not changed by the addition of valinomycin, hence it may be concluded that the action of valinomycin-K^+ complex is to allow a more rapid access of ANS to restricted, higher affinity sites located within the membrane or within the vesicle.

The effect of valinomycin may be due to several factors. The presence of a lipophilic cation, the valinomycin-K^+ complex, may facilitate the transport of the anionic ANS. Clearly, neither gramicidin nor nigericin could then substitute and this explanation is substantiated by the finding

that $TPMP^+$ can mimic the effect of valinomycin when added to the mixture of ANS and membranes. Moreover, a mixture of ANS and K^+ alone results in enhanced fluorescence when valinomycin is added, although quantitatively much less than the effect in the presence of membranes, and this shows the possible formation of a valinomycin-K^+ ANS complex (Feinstein & Felsenfeld, 1971). The increased binding of SCN^- in the presence of valinomycin is then formally similar to the effect of valinomycin on ANS.

An important point which has often been considered is the movement of ANS due to the development of a membrane potential. Under the standard conditions of our assay, the prevailing K^+ gradient would result in a potential such that the vesicle interior was positive with the important reservation that a K^+-selective conductance has to be present.

Since protonophores do not dissipate a preformed pH gradient nor induce a pH gradient in the presence of K^+ gradients it is reasonable to conclude that a cation-selective conductance is not present in the untreated gastric membranes. However, even in the presence of valinomycin, varying the direction of the K^+ gradient does not affect the valinomycin component of the fluorescence; hence this component is not dependent on potential. At 27 °C, in the presence of valinomycin, the initial inward K^+ gradient would dissipate rapidly ($t_{1/2}=2.5$ min) so that at the time of ATP addition only a small residual gradient would be present. We have shown (Sachs *et al.*, 1976) that addition of ATP under these conditions (i.e., cationic equilibrium) generates a gradient (medium to vesicle) of cations such as K^+ or Rb^+.

The cationic enhancement of ANS fluorescence has been described by many authors in other systems (Rubalcava, deMunoz & Gitler, 1969) and has been interpreted as being due to charge redistribution within membranes or hydrophobic regions. The effect is not restricted to the alkali metal cations, Ca^{2+} being more effective than K^+, for example (Rubalcava *et al.*, 1969). With the gastric membranes cation alone is not sufficient, but a lipophilic cation is required due to, perhaps, the restricted permeability of these particles. The fluorescent enhancement caused by valinomycin is a function of the K^+ concentrations (as shown in Fig. 3 with an apparent K_A of 50 mM) but not of the K^+ gradient.

Although, as discussed above, the stimulation of ANS fluorescence by valinomycin appears independent of the potential gradient, this is not the case with the ATP effect. Indeed both a potential gradient and an increased vesicular H^+ concentration appear to be required.

The rate of change of fluorescence with the addition of ATP is a

function of the log $[K^+]$, as would be expected of a Nernst diffusion potential due to the K^+ electrode characteristics of the valinomycin-treated vesicle and the appearance of a K^+ gradient due to ATP. Using radioactive SCN^-, the only evidence for development of a potential was obtained in the presence of both valinomycin and ATP. Studies of cation efflux (Sachs *et al.*, 1976) have shown that a cation gradient develops in these vesicles with ATP addition. Studies of dissipation of a preformed H^+ gradient have shown that these vesicles have a low K^+ conductance in the absence of valinomycin (Sachs *et al.*, 1976). It is reasonable, therefore, to consider that only in the presence of valinomycin would a potential develop across the vesicle membrane due to the ATP-induced K^+ gradient.

Since the fluorescence enhancement due to valinomycin alone does not seem to be sensitive to a potential an additional factor is required. The H^+ gradient generated by ATP would seem the most likely candidate. It can be calculated that a pH of less than 2 may be achieved inside the vesicle with the addition of ATP (Sachs *et al.*, 1976). Nigericin would dissipate a H^+ gradient without necessarily affecting the K^+ diffusion potential (Markin, Sokolov, Boguslavsky & Taguzhinsky, 1975) and nigericin inhibits the ATP effect. The fluorescence of ANS is increased by addition of H^+ to the vesicle in the presence of valinomycin.

Hence, it is proposed that the valinomycin and ATP-dependent ANS fluorescence enhancement is due to the establishment under these conditions of a simultaneous H^+ and potential gradient. Both of these are required for the fluorescent shift, and this results in an alteration in the ANS site giving an enhanced quantum yield which accounts for most of the fluorescence increase.

A similar change in ANS fluorescence is observed in mitochondria and submitochondrial particles (Azzi, Gherardini & Santato, 1971) which also appears to depend on at least the development of a change in potential across the membrane. On the other hand, a change in ANS fluorescence is also obtained with the addition of ATP to sarcoplasmic reticulum vesicles in the presence of Ca^{2+} (Vanderkooi & Martonosi, 1971). The anionic shunt conductance of these membranes is such that a development of a potential is unlikely. Hence, this latter phenomenon has been explained as due to increased binding of ANS by the internally accumulated Ca^{2+}.

The increase of quantum yield of ANS would imply a shift of binding to a more hydrophobic region. This could be due to a change in conformation dependent on the proton concentration at the inner surface as well as on the membrane potential.

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