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Transmembrane Electrophoresis of 8-Anilino-1-Naphthalenesulfonate through Egg Lecithin Liposome Membranes

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Summary. Valinomycin has been shown to increase the amount of 8-anilino-1-naphthalenesulfonate (ANS) bound to egg lecithin liposomes and also to increase the maximum fluorescence value, as derived from double reciprocal plots. The assay conditions were such that addition of valinomycin would not produce a transmembrane potential. The formation of a valinomycin potassium ANS complex in the micelle membrane is proposed. This could account for the increase in the maximum fluorescence value and, by acting as an ANS transporter, could also account for the increase in ANS bound.

Tributylamine was also shown to increase the binding and maximum fluorescence of ANS. In assay conditions where the addition of valinomycin would produce a transmembrane potential negative inside, the tributylamine-induced fluorescence was reversed. The fluorescence decrease is interpreted as transmembrane electrophoresis of ANS in response to a transmembrane potential.

The impermeance to ANS of black films made of soy bean phospholipids has been shown by Conti and Malerba (1972). It is usually assumed that phospholipid membranes in general are impermeable to ANS (Chance, 1970; Flanagan & Hesketh, 1973), unless artificially induced by compounds such as phenyldicarbaundecaborane (Skulachev, 1971). However, recently Barker, Barrett-Bee, Berden, McCall and Radda (1974) have shown, using the nuclear magnetic resonance of the N-methyl groups in liposomes consisting of lecithin and phosphatidic acid (1:1), that ANS is a permeant anion of these liposomes. The permeation takes about an hour to go to completion. If this is assumed to be, say, four to six half times, then a half time of about 10 to 15 min is indicated for the overall reaction.

The possibility that ANS permeation may be induced or increased by a valinomycin potassium complex is suggested by the observation that this complex may be associated with anions (Davis & Tosteson,

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1971; Blok, De Gier & Van Deenen, 1974) and, more specifically, with ANS in water and chloroform (Feinstein & Felsenfeld, 1971) and possibly in phospholipid membranes (Haynes, 1972). Further, Feinstein and Felsenfeld (1971) have shown that the presence of valinomycin and potassium chloride increases the partition of ANS from water into chloroform. If a valinomycin potassium ANS complex can exist in both the apolar hydrocarbon and the polar head group regions of the membrane, then it may be expected to act as a transporter of ANS as well as of potassium through phospholipid membranes. This would mean that under appropriate conditions ANS could be induced to permeate phospholipid membranes in a uniport process, or with potassium in a charge neutral symport process.

Materials and Methods

Lecithin liposomes were made, essentially after the method of Johnson and Bangham (1969), by evaporating to dryness, under reduced pressure, a solution of lecithin in chloroform. The lecithin residue was shaken with a few glass beads in a solution containing 150 mM potassium chloride, 5 mM Hepes[1], and potassium hydroxide to pH 7.55, to give a suspension at 10 mg ml^{-1}. Sonication was carried out in a 60 W MSE Ultrasonic Disintegrator (No. 3000), at the maximum output (about 1.5 amps). The sonication vessel was cooled by an ice ethanol mixture at −10 °C. Unless otherwise stated, the liposomes were sonicated for 35 min.

Fluorescence was measured in a front face fluorimeter constructed in this laboratory. The contents of the cuvette were continuously stirred and maintained at 30 °C. The light source was a Bausch and Lomb 40 W quartz iodide lamp. An image of the lamp element was focused through a Wratten 18A filter so that it occupied the whole of the front face. The maximum intensity of the exciting light was at 380 nm. The emitted light was analyzed by a Bausch and Lomb monochromator (No. 33-86-02) at 480 nm with a bandwidth of 10 nm.

Two assay media were used. One was the same as the sonication medium. The other contained 250 mM sucrose, 5 mM Hepes and potassium hydroxide to pH 7.55.

The following reagents were used. Lecithin was a gift from Dr. E.J.A. Lee and had been purified by a modification of the procedure used by Singleton, Gray, Brown and White (1965). Valinomycin was from Boehringer Corp. Ltd. (London). Tributylamine was from Ralph N. Emanuel Ltd, Alperton, Middlesex. ANS magnesium salt was from Eastman Kodak Co., Rochester, New York. Hepes was from Hopkin and Williams, Chadwell Heath, Essex. Other reagents were of analar grade.

Results

The time course of the interaction of ANS with lecithin liposomes was found to have more than one phase (Fig. 1). This has been reported previously by Bakker and Van Dam (1974). The fast phase of the interaction is complete within the mixing time of the apparatus, which is less

1 N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid.

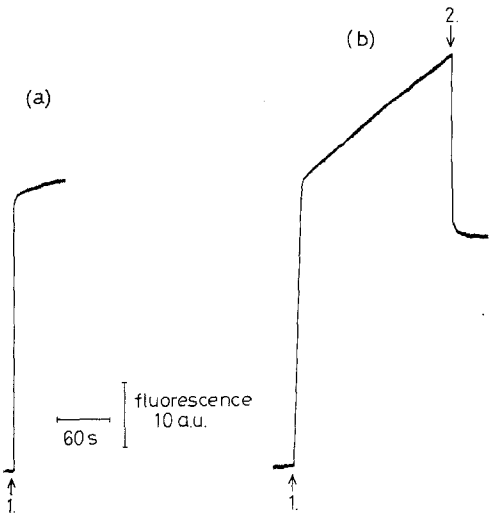

Fig. 1. Effect of ionic strength on the rate of ANS binding to lecithin liposomes. The assay medium contained (*a*) lecithin at 0.1 mg ml^{-1} in 150 mM potassium chloride and 5 mM $Hepes^-/K^+$ at pH 7.55 (when added, ANS at 2 μM); (*b*) lecithin at 0.1 mg ml^{-1} in 250 mM sucrose and 5 mM $Hepes^-/K^+$ at pH 7.55. Additions: first, ANS at 2 μM; second, valinomycin at 5 μg ml^{-1}

than 500 msec. The slower interaction is polyphasic. The half times of these slower phases cannot be estimated accurately as there appear to be several of them, and as the end point of the interaction is difficult to determine. For ANS binding to lecithin liposomes suspended in a 250 mM sucrose medium containing 5 mM $Hepes^-/K^+$ at pH 7.55 (Fig. 1 *b*), the interaction goes to completion in about one hour, giving a half time for the overall reaction of between 10 and 15 min. Inclusion of 150 mM potassium chloride in the assay medium (Fig. 1 *a*) slows down the rate of interaction of ANS with the liposomes. This interaction takes about two hours to go to completion; giving a half time for the overall reaction of between 20 and 30 min. It has been reported by Fortes and Hoffman (1974) that increasing the ionic concentration decreases the rate at which ANS interacts with red blood cells. The slow phase under appropriate conditions (low external potassium) is reversible by the addition of valinomycin (Fig. 1 b).

A plot of reciprocal fluorescence against reciprocal lecithin concentration is shown in Fig. 2. The upper line represents the fast phase of ANS interaction. The addition of valinomycin (lower line) to lecithin liposomes plus ANS increases the maximum fluorescence value and the number and/or affinity of the sites. As the same 150 mM potassium chloride

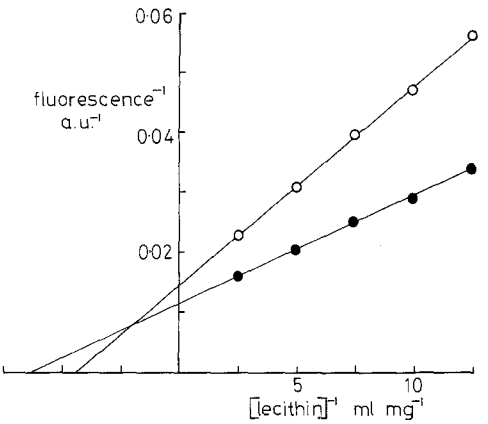

Fig. 2. Double reciprocal plot showing the effect of valinomycin on the fluorescence of ANS bound to lecithin liposomes sonicated and assayed in the same 150 mM potassium chloride medium. The assay medium contained lecithin liposomes in 150 mM potassium chloride and 5 mM $Hepes^{-}/K^{+}$ at pH 7.55. ○ plus ANS at 2 μM, ● plus ANS at 2 μM and valinomycin at 5 μg ml^{-1}

medium was used in both the assay and the sonication then the change in fluorescence cannot be caused by a valinomycin-induced transmembrane potential.

An aliquot of the lecithin suspension was taken before sonication and further aliquots were taken during sonication. Fig. 3 shows a plot of fluorescence on addition of 2 μM ANS to liposomes (0.1 mg ml^{-1}) in the 150 mM potassium chloride medium, against time of sonication. The curve represents the fast phase of the interaction. The lower of the two straight lines represents the final fluorescence level after incubation for four hours. The upper straight line represents the final fluorescence level after the addition of valinomycin at 5 μg ml^{-1}. Both of these final fluorescence levels were stable, and it can be seen from Fig. 2 that neither is caused by a limiting concentration of ANS. It can be seen from Fig. 3 that the final fluorescence levels are independent of the time for which the liposomes were sonicated, that the extent of the initial rapid fluorescence increase increases with the time of sonication, and therefore that the extent of the subsequent slower phases of the fluorescence increase decreases with the time of sonication. These data are consistent with sonication decreasing the average liposome size with a consequent increase in the total surface area (Chapman, Fluck, Penkett & Shipley, 1968; Johnson & Bangham, 1969), provided that the fast phase of the interaction is with the outside surfaces of the liposomes and the slow phase of the interaction, limited by the rate at which ANS permeates the liposomes,

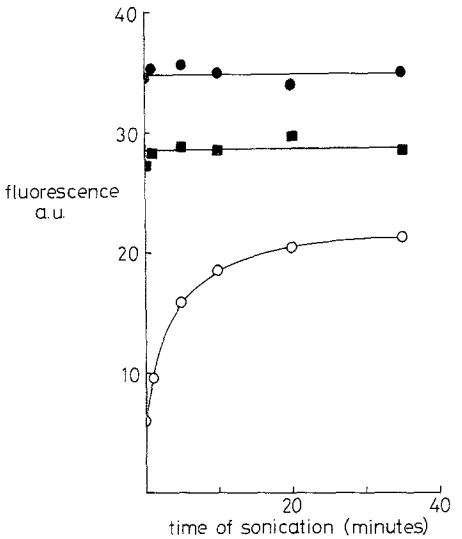

Fig. 3. Effect of the length of time of sonication of lecithin liposomes on the fluorescence level of ANS bound to them. Both the sonication and the assay media contained 150 mM potassium chloride and 5 mM Hepes$^-$/K$^+$ at pH 7.55. The assay medium contained lecithin liposomes at 0.1 mg ml^{-1}, ○ slow phase of the fluorescence increase on the addition of 2 μM ANS, ■ total fluorescence increase 4 hr after the addition of 2 μM ANS, ● total fluorescence increase after the addition of 2 μM ANS and of valinomycin at 5 μg ml^{-1}

is with the inside surfaces of the liposomes. The greater extent of the final fluorescence level after the addition of valinomycin may be accounted for by the valinomycin-induced increase in the maximum fluorescence value shown in Fig. 2. The valinomycin-induced increase shown in Fig. 3 is not caused by a transmembrane potential as the liposomes were sonicated and assayed in the same 150 mM potassium chloride medium.

The concentration of valinomycin used in these experiments is higher than that used by Bakker and Van Dam (1974). This was made necessary by the otherwise slow rate of increase in the ANS fluorescence. One possible explanation of these results is that this concentration of valinomycin has a chaotropic effect on the liposomes. This is overruled by the observation that, under appropriate conditions, the same concentration of valinomycin will decrease the ANS fluorescence; this is shown in Figs. 1*b* and 5.

Fig. 4 shows the rate of the fluorescence increase on the addition of valinomycin to lecithin liposomes plus ANS. The data in this Figure with that in Fig. 3 indicate that the valinomycin-induced increase in the rate of the fluorescence increase is caused by a valinomycin-induced in-

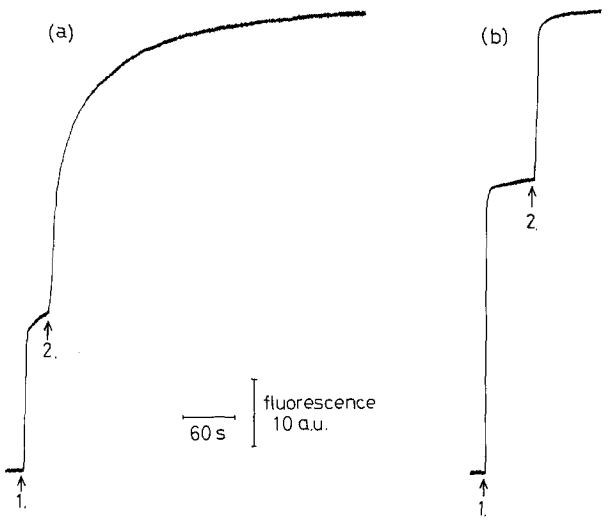

Fig. 4. Effect of valinomycin and length of time of sonication of lecithin liposomes on the rate at which ANS binds to them. The assay medium contained 150 mM potassium chloride and 5 mM Hepes$^-$/K$^+$ at pH 7.55. Additions: first, ANS at 2 μM; second, valinomycin at 5 μg ml^{-1}. (*a*) Liposomes sonicated for 1 min; (*b*) liposomes sonicated for 35 min

crease in the rate at which ANS permeates the liposomes. The assay medium was the same as the 150 mM potassium chloride medium in which the liposomes had been sonicated. The liposomes in Fig. 4*a* were sonicated for 1 min, those in Fig. 4*b* for 35 min. The time taken to reach maximum fluorescence is far shorter for the liposomes sonicated for the longer time, indicating that sonication reduces the number of ANS permeability barriers in the liposome. The rate of fluorescence increase on addition of valinomycin in the presence of ANS to lecithin liposomes sonicated for 1 min can be analyzed into three or more phases, and to lecithin liposomes sonicated for 35 min, into two phases. An accurate determination of the number of phases might, if they were sufficiently distinct, give an indication of the average number of ANS permeability barriers in the liposomes.

Fig. 5 shows the binding of ANS to liposomes sonicated in the 150 mM potassium chloride medium and assayed in a 250 mM sucrose medium (containing 4 mM K^+). Subsequent addition of 5 μM tributylamine causes a fluorescence increase. This is reversed by addition of valinomycin which will cause an efflux of potassium ions from the liposome. The double reciprocal plot in Fig. 6 shows that the tributylamine-induced increase in fluorescence is caused partly by an increase in the maximum fluorescence of bound ANS and partly by an increase in the amount bound. Further, this plot shows that the valinomycin-induced decrease in fluorescence

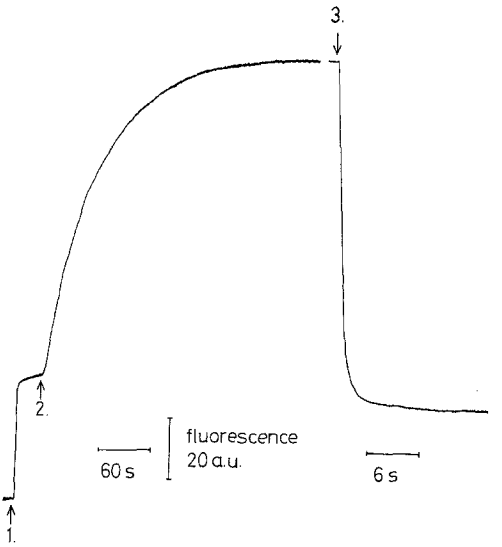

Fig. 5. The effect of valinomycin and tributylamine on the binding of ANS to lecithin liposomes sonicated in a 150 mM potassium chloride medium and assayed in a 250 mM sucrose medium. The assay medium contained lecithin liposomes at 0.1 mg ml^{-1} in 250 mM sucrose and 5 mM $Hepes^{-}/K^{+}$ at pH 7.55. Additions: first, 2 μM ANS; second, 5 μM tributylamine; third, valinomycin at 5 μg ml^{-1}

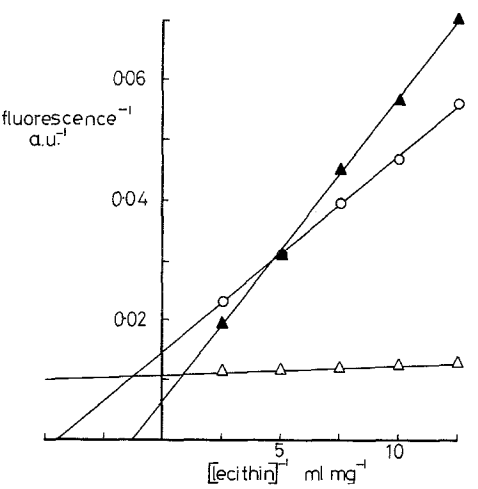

Fig. 6. Double reciprocal plot showing the effect of tributylamine and valinomycin on the fluorescence of ANS bound to lecithin liposomes sonicated in a 150 mM potassium chloride medium and assayed in a 250 mM sucrose medium. The assay medium contained lecithin liposomes in 250 mM sucrose and 5 mM $Hepes^{-}/K^{+}$ at pH 7.55, ○ plus 2 μM ANS, △ plus 2 μM ANS and 5 μM tributylamine, ▲ plus 2 μM ANS, 5 μM tributylamine and valinomycin at 5 μg ml^{-1}

shown in Fig. 5 is caused by a decrease in the amount of ANS bound. As is shown in Fig. 2 the addition of valinomycin causes an increase in the maximum fluorescence value of the bound ANS.

Discussion

Fig. 4 shows that valinomycin increases the amount of ANS fluorescence when it is added to liposomes in conditions where the internal and external potassium concentrations are the same. Fig. 2 shows that this increase in fluorescence results from an increase in the amount of ANS bound as well as in the maximum fluorescence value. The increase in binding may be caused by either the potassium valinomycin complex increasing the ANS occupancy of the outer surface of each liposome or by allowing the ANS to permeate the whole of each liposome. In the former case the fast phase would presumably be caused by ANS interaction at the surface of the liposomes and the slower phase by the migration of ANS to a less accessible binding site located outside the permeability barrier of the outermost liposome membrane. In the second case the fast phase would presumably be caused by ANS interacting at the surface of the liposomes, but the slower phase by the migration of ANS through the membrane and its subsequent binding to the surfaces inside the liposomes. These explanations are applicable whether or not the rate of the slow phase of the ANS interaction is enhanced by the addition of valinomycin. In the absence of ANS permeation through the outermost of the liposome membranes it would be expected that sonication would cause not only an increase in the amount of ANS bound to the superficially located binding sites, but would also increase the amount of ANS bound to the less accessible binding sites; thus increasing the total ANS fluorescence. It may be seen from Fig. 3 that this does not occur. In contrast it shows that as the length of time for which the liposomes were sonicated is increased the resulting increase in the extent of the fast phase of the fluorescence increase is matched by a proportionate decrease in the extent of the slow phase; the total fluorescence increase remaining the same. Further, the slow phase of the interaction of ANS with liposomes is polyphasic. The number of these phases decreases as the length of time for which the liposomes were sonicated was increased. While these results do not show that ANS has actually reached the inner phase of the liposomes they are at least consistent with this proposal. In this case, the slower increase which is observed on the addition of ANS to lecithin liposomes would be monitoring the rate at which ANS binds to and penetrates the successive membrane layers within the liposomes. The possibility that the slower phase of this interaction is caused by ANS permeation into but not through the outermost of the liposome membranes is highly improbable as the predictions which this theory

requires, and which have been outlined above, are inconsistent with the observations in this paper. However ANS must, to permeate through the liposome membranes, occupy parts of the membrane other than the superficial binding sites. What proportion of the bound ANS occupies this part of the membrane is not known. It is possible that the increase in the maximum fluorescence value in Fig. 2 after the addition of valinomycin and in Fig. 6 after the addition of tributylamine is caused by an increase in the amount of ANS occupying sites other than the superficial ones. Other possible causes of the increase in the maximum fluorescence values of these plots are discussed below.

What factor most affects the quantum yield of ANS is uncertain (Flanagan & Ainsworth, 1968; Ainsworth & Flanagan, 1969; Radda, 1971; Penzer, 1972). It may be the polarity, the hydrophobicity or the planar conformation that the solvent or the binding site confers on the ANS molecules. Regardless of this uncertainty, the action of valinomycin or the valinomycin potassium complex may be to alter the environment of the polar and/or the apolar regions of the whole membrane, to induce the probe to move into the apolar region of the membrane, or to form a complex with ANS in the membrane. The last two possibilities, which are not necessarily mutually exclusive, involve the induction or creation of a second ANS binding site.

These results, with those that show the valinomycin potassium complex to be associated with anions (Davis & Tosteson, 1971) including ANS in water and in chloroform (Feinstein & Felsenfeld, 1971), strongly suggest that a valinomycin potassium ANS complex is formed in the phospholipid membrane (Haynes, 1972). The increase in the maximum fluorescence value, shown in Fig. 2, may be explained if the valinomycin potassium ANS complex is neutral (Feinstein & Felsenfeld, 1971) and occupies the apolar region of the membrane.

A valinomycin potassium ANS complex could facilitate potassium ANS symport or ANS uniport. The former would be electroneutral, the latter electrogenic. The type of transport would presumably vary with the conditions.

ANS does not partition from water into hexane or chloroform, but will into a solution of dodecylamine in hexane (Flanagan & Ainsworth, 1968), and, in the presence of valinomycin and potassium ions into chloroform (Feinstein & Felsenfeld, 1971). Tributylamine increases the rate of the slow phases of ANS interaction with liposomes (Fig. 5). Fig. 6 shows that the tributylamine-induced increase in fluorescence is caused by an increase in binding as well as in the maximum fluorescence value. The

latter may be caused by ANS occupying the apolar region of the membrane either in the form of a charge neutral solution or complex. The increase in binding may be caused by an increase in affinity or by ANS permeating the whole of each liposome, or by both.

In conditions where ANS can permeate the whole liposome the creation of a transmembrane potential, negative inside, would be expected to result in a decrease in the amount of ANS bound. This can be seen in Fig. 5. Creation of a transmembrane potential in liposomes also reverses the slow phase of the ANS fluorescence increase (Fig. 1*b*). This suggests that the slow phase of the fluorescence increase in untreated lecithin liposomes may also be caused by ANS permeation, and is comparable in rate to that shown by Barker *et al.* (1974). The final fluorescence level after the addition of valinomycin (Figs. 1*b* and 5), is lower than the level of the initial fast phase. Fig. 6 shows this to be caused by a decrease in the amount of ANS bound. This would be expected if the membrane acts as a capacitor and the transmembrane potential, negative inside, induces a negative surface potential on the outermost surface of the liposomes. The creation of a transmembrane potential would seem to have two distinct effects: efflux of ANS from the interior of the liposomes in an electrophoretic manner, as has been proposed by Jasaitis, Kuliene and Skulachev (1971), and a decrease in binding caused by the surface on the outside of the liposome becoming negative in response to the transmembrane potential, as has been proposed by Azzi, Gherardini and Santato (1971).

Whether or not ANS is a permeant anion of mitochondrial membranes is in dispute. The work of Jasaitis, La Van Chu and Skulachev (1973), Layton, Symmons and Williams (1974), and Gains and Dawson (1975) indicates that it is. No direct evidence has been published which shows that it is not. Barker *et al.* 1974) interpret their data on liposomes as indicating that the relatively rapid energy-linked changes in the fluorescence of ANS incubated with submitochondrial particles cannot be caused by movement of the probe across the inner mitochondrial membrane. There are two reasons why such a close analogy between lecithin liposomes and submitochondrial particles should not be drawn. Firstly, the membrane composition differs grossly and this could well affect the rate at which ANS and similar molecules can permeate the two systems. That changes in the membrane composition can alter the rate of ANS entry into liposomes is shown in Fig. 5. Secondly, the conditions are different in that the permeation of ANS into liposomes is passive whereas that into energized submitochondrial particles may be driven by a transmembrane potential. Fig. 5 shows that the efflux of ANS from lecithin lipo-

somes in the presence of valinomycin and in conditions where a transmembrane potential is generated can be as fast as, if not faster than, those energy-dependent changes in ANS fluorescence reported for submitochondrial particles (Brocklehurst, Freedman, Hancock & Radda, 1970).

However, if it is assumed that ANS is a permeant anion of mitochondrial membranes and that a transmembrane potential is generated on energization, then any interpretation of the energy-dependent changes of ANS fluorescence must, apart from any proposed conformational changes of the mitochondrial membrane, take the following into account. The formation of a transmembrane potential by energization will lead to the exclusion of ANS from the interior of mitochondria and to its accumulation in submitochondrial particles (Mitchell, 1970; Jasaitis *et al.*, 1971; Layton *et al.*, 1974). It will also affect the surface potentials on both sides of the membrane (Azzi, 1969; Azzi *et al.*, 1971; McLaughlin, Szabo & Eisenman, 1971). This will lead to a decrease in the amount of ANS bound to the outside surface of submitochondrial particles. The energy-dependent changes in the maximum fluorescence value (Brocklehurst *et al.*, 1970) may be caused by a transmembrane potential acting on the membrane and affecting its "conformation", by acting on the dipole moment of the ANS molecule, slightly altering its orientation or position in the membrane (Conti & Malerba, 1972; Bakker & Van Dam, 1974), or by acting on the negative charge of the ANS molecule and causing it to migrate from one site to another with a different value for bound ANS. These possibilities may not be mutually exclusive.

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