Membrane Potential and Surface Potential in Mitochondria

FLUORESCENCE AND BINDING OF 1-ANILINONAPHTHALENE-8-SULFONATE

(Received for publication, October 12, 1982)

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The effects of surface potential and transmembrane potential on the binding and fluorescence of 1-anilinonaphthalene-8-sulfonate (ANS) in suspensions of rat liver mitochondria was investigated. The binding of ANS is characterized by two classes of binding site: a high affinity ($K_d = 10-50 \, \mu M$), low capacity ($n = 3-8 \, \text{nmol/mg of protein}$) class in which bound ANS fluorescence strongly, and a low affinity (>500 \, \mu M), high capacity (>50 \, \text{nmol/mg of protein}) class with little fluorescence. The dissociation constant, $K_d$, of the high affinity site strongly depends on the surface potential of the external surface of the inner mitochondrial membrane. Hence, the binding and fluorescence of ANS can be used to estimate the surface potential. The dependence of ANS binding on the medium salt concentration is compatible with the Gouy-Chapman theory and allows accurate determination of the extent of uncharged ANS fluorescence. This decrease is the result of reduced ANS binding. However, the external surface potential as estimated from the charge screening effect of salt solutions is unchanged in energized membranes. The extent of decreased fluorescence correlates reasonably well with the magnitude of the transmembrane potential. The potential-induced quenching depends on pre-equilibration of ANS with the mitochondria, suggesting that the response is due to exclusion of ANS from the mitochondrial matrix. These findings do not support the suggestion that ANS quenching in energized mitochondria is due to an increase in the negative surface charge of the cytosolic surface of the inner membrane. The results are compatible with the suggestion that the response to energization is largely due to the formation of $\Delta \Psi$. However, because of the complex nature of the ANS response, it is concluded that neither the magnitude of surface potential nor the magnitude of membrane potential can be determined from the ANS response in energized mitochondria.

Substrate-induced “energization” of mitochondria induces the quenching of fluorescence of the anionic fluorescent probe ANS$^2$ (1). This discovery, over 13 years ago, was followed by a rapid spread of the use of ANS as a probe for energization in mitochondria and in many other membrane systems (for review, see Ref. 2). However, the mechanism as well as the direct cause of the quenching is still controversial (3). It is well recognized now that in coupled mitochondria substrate oxidation or ATP hydrolysis is associated with the formation of a large negative transmembrane potential (4). Consequently, it has been suggested that the negatively charged ANS responds to the formation of membrane potential by diffusing across the membrane to reach a new electrochemical equilibrium (5). This equilibration, it was suggested, led to quenching of the fluorescence in mitochondria as a result of the depletion of the matrix ANS. In submitochondrial particles, chromatophores, and other vesicles which generate positive membrane potential, ANS is believed to accumulate internally which leads to enhancement of the fluorescence. This interpretation is supported by the demonstration that ANS is a permeable anion (6) and by qualitative analysis of binding parameters of ANS to mitochondria and submitochondrial particles (7). An alternative model, first described by Azzi et al. (8) assumed that ANS only binds to the external membrane surface. The ANS binding, in this model, depends on the surface potential which modulates the apparent binding constant of ANS to the membrane. This interpretation is supported by the demonstration that the binding and the fluorescence of ANS in various membrane preparations are determined by the surface potential (9-11). Measurements of $\Delta \Psi$ potential in mitochondria appear to confirm this interpretation and suggest that energization is associated with an increase in the mitochondrial surface charge (12, 13). In this study, we attempt to resolve these contrasting interpretations by comparing the predictions of the two models in relation to ANS fluorescence both in energized and nonenergized mitochondria. The data are best fit by a model which includes the effects of both surface potential and transmembrane potential. It is demonstrated that while ANS binding and fluorescence strongly respond to modulation of surface potential, the energy-dependent quenching is largely due to the generation of $\Delta \Psi$ and can be accounted for by the movement of the anion across the membrane and from intramembrane sites in response to membrane potential. Hence, the ANS fluorescence quenching does not provide evidence to the claims that the mitochondrial surface charge becomes more negative when energized (12, 14-17).

EXPERIMENTAL PROCEDURES

Preparation of Mitochondria, Mitoplasts, and Submitochondrial Particles—Mitochondria from male Wistar albino rats, 150-250 g.

* This study was supported by National Institutes of Health Grants GM 28173 and AA 3442. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: ANS, 1-anilinonaphthalene-8-sulfonate; CCCP, carbonylcyanide m-chlorophenylhydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 4-morpholineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid.
were prepared by differential centrifugation as previously described (18). Mitoplasts were prepared according to Greenswalt (19) and submitochondrial particles by the method of Lindsay et al. (20). A Yeda press was used in both preparations. Bulk membrane potential (ΔΨ) was determined by the distribution of ⁸⁶Rb in the presence of valinomycin as measured by rapid centrifugation (21).

Fluorescence Measurements—All measurements of ANS fluorescence were made in an Eppendorf 1101M photometer with a front surface fluorescence attachment. Measurements were made at a 35° angle normal to the excitation beam. Excitation light was provided by a mercury lamp with a 408-nm primary interference filter for excitation. A 430-470-nm secondary window was used for emission. All measurements are expressed in arbitrary fluorescence units.

Measurement of K_d and n from ANS by Fluorescence Emission—The approach used to determine the dissociation constant, K_d, and n, the number of binding sites, from fluorescence yield was based upon the formalism of the Lineweaver-Burk derivation as outlined in detail by Wang and Edelman (22). Briefly, K_d may be determined from a titration of particles with ANS and a treatment of the data using the relationship

\[ \frac{1}{f} = \frac{1}{f_{\text{max}}} + \frac{K_d}{f_{\text{max}}} \times \frac{1}{(\text{ANS})} \]  

where f is fluorescence in arbitrary units, \( f_{\text{max}} \) is the fluorescence of excess ANS and (ANS) is the free ANS concentration. Plotting \( \frac{1}{f} \) versus \( \frac{1}{(\text{ANS})} \) allows determination of \( f_{\text{max}} \) from the y intercept and \( K_d \) from the slope. Free ANS concentration is determined by the procedure outlined below. To determine the number of ANS-binding sites (n), a series of titrations is performed at fixed (ANS) with varying particle concentration (C_p). Using the previously determined value of \( K_d \), substituting into the law of mass action and rearranging gives,

\[ \frac{\text{(ANS)}}{f} = \frac{1}{q} + \frac{K_d}{qnC_p} \]  

where q is a proportionality constant related to the quantum efficiency and determined from the y intercept. n may be estimated from a plot of (ANS)/f versus 1/(C_p).

Values for all slopes and intercepts were estimated using a regression curve-fit program on a Hewlett-Packard HP-85 personal computer. Values for the correlation coefficient in all cases were greater than 0.98.

\[
1/f = 1/f_{\text{max}} + K_d/f_{\text{max}} 	imes 1/(\text{ANS})
\]

\[
(\text{ANS})/f = 1/q + K_d/qnC_p
\]

RESULTS

Fluorescence and Binding of ANS in Nonenergized Mitochondria—The fluorescence yield of 1,8-ANS in water is very low and exhibits an emission maximum at 520 nm. Binding to protein or lipids results in a large increase in the quantum yield and a blue shift in the emission (2). It is apparent, however, that the relationship between probe binding and
fluorescence yield in mitochondrial membranes is a complex one.

Fig. 1A shows the binding and the fluorescence of ANS as function of the free ANS concentration. Unlike previous studies with mitochondria, the free ANS concentration was calculated from the amount of total bound ANS (see "Experimental Procedures") and not from the fluorescence of bound ANS. As the figure clearly shows, only at very low ANS concentration is there a correspondence between bound ANS and ANS fluorescence. At high concentration, there is extensive binding of ANS which does not contribute measurably to the fluorescence at short wavelength (<470 nm). Plotting the fluorescence intensity versus free ANS concentration in a double reciprocal plot (as in Fig. 2A) yields an estimate of the apparent dissociation constant ($K_d$) of the site with a high quantum yield. The $K_d$ estimated by this method lies in the range of 25–50 μM. This value is considerably lower than previous estimates (7). We attribute this difference to the fact that other investigators calculate the free ANS from the fluorescence of bound ANS, whereas we have corrected for total bound ANS.

By measuring the fluorescence of a fixed quantity of ANS as a function of mitochondrial content, it is possible to obtain an estimate of the number of binding sites (n) which have a high quantum yield. The value for n falls within a range of 3-8 nmol/mg of protein. The data shown in Fig. 1B, a double reciprocal treatment of the fluorescence data obtained in a typical titration, give a value for n of 6.6 nmol/mg of protein. This value is 1 order of magnitude smaller than previous estimates (7) which do not distinguish between fluorescent and nonfluorescent bound ANS. The combination of the two titrations allows a quantitative estimate of the binding parameters which relate to ANS fluorescence in mitochondria.

The results of the direct binding determination are plotted in a Scatchard analysis as shown in Fig. 1C. The curve obtained in C may be resolved into two straight lines by an iteration procedure which relates the total amount of probe bound to two classes of binding sites of apparent dissociation constants $K_d$ and $K'_d$ and capacities $n_1$ and $n_2$ (23). The resulting lines are shown in Fig. 1C. The class of "high affinity" sites resolved by this procedure are characterized by an apparent $K_d$ of 25 μM and a value for n of 4 nmol/mg of protein.

A "low affinity" class has a $K_d$ of 550 μM and a value for n of 68 nmol/mg of protein. For comparison, the parameters derived from the fluorescence data in Fig. 1, A and B, were used to construct a binding curve which agrees within experimental error with the curve relating to the high affinity class of sites as determined from direct binding. Clearly, previous investigators failed to distinguish between the high affinity site, which has high quantum yield and is blue-shifted and the low affinity site which has lower quantum yield. This may be the source of the early controversy regarding the characteristic of ANS binding to mitochondria and submitochondrial particles (see "Discussion"). ANS fluorescence in biological membranes is affected by the ionic composition of the surrounding medium (10–12). The addition of salts to suspensions of mitochondria and ANS increases fluorescence in a concentration-dependent manner. The enhancement can be shown to be the result of a decrease of the apparent dissociation constant ($K_d$) for ANS. Fig. 2A is a double reciprocal plot of fluorescence as a function of free ANS concentration in a low salt medium and in a medium containing 0.25 mM CaC12. From the slope and intercept, a value for $K_d$ of 27 μM was obtained for ANS in low salt medium. Addition of CaCl2 caused a drop in the value of $K_d$ to 16 μM, indicating a higher affinity for ANS. The number of available sites for fluorescent ANS in low and high salt medium (Fig. 2B) is constant within experimental error.

i.e. 3 nmol/mg of protein. The increase in ANS fluorescence intensity in the case of Ca2+ addition, therefore, seems to be due entirely to an increase in the affinity of the membrane for the probe. A similar analysis was carried out with inverted submitochondrial particles to determine binding parameters for ANS in association with the inner membrane. As shown in Fig. 2, C and D, the binding of ANS is similar to that in mitochondria in low salt and in medium containing 0.25 mM CaCl2. The values for $K_d$ and n in low salt were 25 μM and 13 nmol/mg of protein, respectively, while in 0.25 mM CaCl2 the apparent binding constant was 17 μM and n was 6 nmol/mg of protein. The increase in the value of n can be fully explained by the loss of soluble protein and outer membranes in the preparation and the enrichment of inner membranes.
as reflected by a 2- to 3-fold increase in cytochrome oxidase content of these particles.

The fluorescence of an ANS mitochondrial suspension is enhanced by the addition of all salts. This enhancement depends on the cation concentration and valence and is saturated at high salt concentration. The results of typical titrations with salts of monovalent and divalent cations are shown in Fig. 3A. Titrations with salts of monovalent cations such as NaCl or KCl show fluorescence saturation behavior at concentrations 2 orders of magnitude higher than MgSO₄ or CaHPO₄. Mg²⁺ salts enhance the fluorescence little more than monovalent cations but Ca²⁺ enhancement is three times as large. It is well known that the binding of ions to phospholipids or biological membranes depends upon the surface potential of the membrane (24). The dependence of the surface potential ($\psi$) on the membrane surface charge and the ionic composition of the medium is described by the Guoy-Chapman theory (24, 25). The binding of a charged molecule like ANS to a negatively charged mitochondrial membrane is determined by two factors: the affinity of ligand for the binding site and the concentration of ligand in the double layer which is determined by the surface potential. Thus, the binding constant is an apparent constant which depends upon the surface potential.

$$K_0 = K_0 \exp(-F\psi/RT)$$  \hspace{1cm} (3)

At low probe concentration ([ANS] $\ll K_0$), the fluorescence is proportional to the binding constant, hence (see Appendix I)

$$\Delta \psi = 59 \log F/F_0 \text{ (mV)}$$  \hspace{1cm} (4)

where $\Delta \psi$, is the difference in surface potential between two membrane states with fluorescence $F$ and $F_0$. In general, this method only gives the difference of $\psi$ between two states. However, if we know $F_0$, where $\psi = 0$ we can calculate the absolute values of $\psi$. We have developed a procedure to determine $F_0$ which is based on the fact that increased ionic strength reduces $\psi$. This is shown in Fig. 3B where the effect of KCl on ANS fluorescence in a suspension of rat liver mitochondria is shown. Thus, as the KCl concentration increases, $\psi$ approaches zero. Fig. 3A indicates that in addition to its charge screening effect Ca⁺ binds to the mitochondrial surface. When mitochondria strongly bind cations to the surface, the surface charge can become positive. In this case, the ANS fluorescence, which is initially very high, would decrease with increased salt concentration, because the net positive charge is also screened at high ionic strength. As shown in Fig. 3B, mitochondria were first allowed to bind calcium, which increased ANS fluorescence almost 2-fold. However, the addition of KCl (or any other salt) decreases the fluorescence. Therefore, the asymptotic approach of the two lines ($\pm$Ca⁺) represents the fluorescence, $F^*$, where $\psi = 0$, and allows a calculation of the absolute value of the surface potential.

![Fig. 3. Salts enhancement of ANS fluorescence in rat liver mitochondria. A, fluorescence was determined in 3 ml of medium containing 0.25 M sucrose, 10 mM Tris, pH 7.2, 8.3 μM ANS, 2 μM rotenone, 1.9 mg of protein/ml, and the indicated concentration of salt. Salt titrations were performed by additions of small aliquots of concentrated salt solution. The steady state fluorescence level was measured after each sequential addition of NaCl (O), KCl (●), MgSO₄ (△), or CaHPO₄ (●). B, null point salt titration for $\psi$ determination. Mitochondrial ANS suspensions were titrated with NaCl before (O) and after (●) addition of 0.25 mM CaCl₂. C, effect of cations on the mitochondrial surface potential. Values for $\psi$, calculated as described in the text (Equation 2) were plotted from a titration of the fluorescence as a function of cation concentration as in A. The value for $\psi_0$ was calculated by a null point fluorescence titration and this value was used as a reference for calculation of $\psi$, values after incremental salt additions. The value of $\psi$, for the particular mitochondrial preparation used was -11 mV. Titrations of 3A, were shown for La³⁺ (△), Ca³⁺ (●), Mg²⁺ (△), Na⁺ (O), and K⁺ (●). The theoretical change in $\psi$ with cation concentration was derived from the Guoy-Chapman theory as outlined in Appendix II. In each case, the initial concentration of divalent salt was assumed to be 10 μM and monovalent salt was 3 mM.]

![Fig. 4. Mitochondrial binding of ANS and fluorescence as function of pH. The medium was 0.25 M sucrose, 2 μM rotenone, and 10 mM buffer. Buffers were 10 mM MOPS, pH 5.5-6.0, 10 mM PIPES, pH 6.6-7.0, 10 mM HEPES, pH 7.3-7.5, 10 mM Tris, pH 7.8-8.3. The mitochondrial protein concentration in each experiment was 1.4 mg/ml. Binding was measured as described under "Experimental Procedures." O, fluorescence measured in low salt medium. ●, fluorescence in high salt medium (150 mM KCl).]
charge, \( \psi_c \). We have employed this approach throughout the remainder of this investigation to estimate \( \psi_c \) under various experimental conditions.

Fig. 3C is a plot of cation concentration versus \( \psi_c \), calculated from the null point titration as described above. In the preparation used for titration, the membrane surface potential, \( \psi_m \), in 10 mM Tris, 0.25 M sucrose, pH 7.2, was \(-10.6 \text{ mV}\). Addition of NaCl, KCl, or MgSO\(_4\) brings about a decrease in the charge. In separate experiments, a complete analysis of ANS-binding parameters as a function of pH, utilizing the fluorescence behavior indicates the presence of one or more types of ionizable group on the membrane surface. The decrease in fluorescence at low and high salt as a function of pH. The value of \( \psi_c \), calculated from a null point titration is zero near a pH value of 5.5. As the pH is increased, the value of \( \psi_c \) becomes negative and at pH 8.25, \( \psi_c = -19.1 \text{ mV}\). This behavior indicates the presence of one or more types of ionizable group on the membrane surface. The decrease in binding between pH 7 and 8 is mostly due to increased surface charge density. In separate experiments, a complete analysis of ANS-binding parameters as a function of pH, utilizing the fluorescence dependence on pH and mitochondria concentration, shows that, as the membrane surface becomes more negative valence, the surface potential of the mitochondria indicated by an increase in the fluorescence of ANS. The asymptotic rise to a maximum fluorescence intensity is reflected in an approach to a \( \psi_c \) value of zero. Addition of CaCl\(_2\) or LaCl\(_3\) results in a reversal of the surface potential to positive values. The effect of Ca\(^{2+}\) and La\(^{3+}\) is the result of the strong binding of these cations to mitochondria (25). Binding of positive ions to the membrane surface rather than accumulation in the diffuse double layer, as in the cases of Mg\(^{2+}\), Na\(^{+}\), and K\(^{+}\), should result in a change of the net charge of the membrane.

![Fig. 3C](http://www.jbc.org/)

**TABLE I**

**Fluorescence binding versus medium pH**

<table>
<thead>
<tr>
<th>pH</th>
<th>( K_a )</th>
<th>n</th>
<th>nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>22</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>47</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>7.8</td>
<td>58</td>
<td>7.3</td>
<td></td>
</tr>
</tbody>
</table>

As might be expected, the surface potential of the mitochondrial membrane is dependent upon the pH of the surrounding medium. Fig. 4 shows the binding of ANS and the fluorescence at low and high salt as a function of pH. The value of \( \psi_c \), calculated from a null point titration is zero near a pH value of 5.5. As the pH is increased, the value of \( \psi_c \) becomes negative and at pH 8.25, \( \psi_c = -19.1 \text{ mV}\). This behavior indicates the presence of one or more types of ionizable group on the membrane surface. The decrease in binding between pH 7 and 8 is mostly due to increased surface charge. In separate experiments, a complete analysis of ANS-binding parameters as a function of pH, utilizing the fluorescence dependence on pH and mitochondria concentration, shows that, as the membrane surface becomes more negative
Fluorescence in Mitochondria

FIG. 7. The effect of MgCl₂ on fluorescence during substrate oxidation and ATP hydrolysis. A, titration of fluorescence with Mg²⁺ before (○) and after addition of succinate, 10 mM (▲), or ATP, 3 mM (●). The medium was 0.25 M sucrose, 10 mM Tris, pH 7.8, 2 μM rotenone, 8.3 μM ANS, and 1.5 mg of protein/ml. B, titration of fluorescence with (●) or without (○) an artificially imposed potassium ion diffusion potential. All experiments were performed at 10 °C. The media consisted of 0.25 M sucrose, 10 mM Tris, pH 7.8, 2 μM rotenone, 8.3 μM ANS, and 1.5 mg/ml mitochondria. Valinomycin (10⁻⁷ M) was added to induce potassium diffusion potential.

at higher pH, the apparent binding constant increases from a value of 22 μM at pH 6.1 to a value of 55 μM at pH 7.8 (Table I). At the same time, the value of n is constant with pH. Substitution of the values for Kₐ at pH 7.8 and 6.1 into Equation 3 gives values of ψ, which are similar to the values obtained by the null point titration.

One problem in the interpretation of ANS fluorescence behavior involves the observation of a biphasic increase in the fluorescence of the probe when mitochondria or mitoplasts are added to a cuvette containing ANS. Normally a fast increase, about 80% of the total, occurs immediately followed by a slow increase until a maximum level is reached. The slow phase of the response is temperature dependent and occurs with a half-time of 10–15 min at 10 °C (Fig. 5). This biphasic response has been interpreted as evidence for the existence of two types of binding site for ANS (27). We have found that the duration of the slow increase is very dependent upon the cation concentration of the surrounding medium. For instance, as shown in Fig. 5, addition of 100 mM KCl to the

FIG. 8. The relationship between transmembrane potential and ANS fluorescence quenching. A, the effect of CCCP on ATP- or succinate-generated membrane potential. Membrane potential was measured by ⁴²⁷Rb distribution after addition of succinate (10 mM; ○) or ATP (3 mM; ▲) to mitochondrial suspensions in 0.25 M sucrose, 10 mM Tris, pH 7.2, 50 mM KCl, 1 mM MgCl₂, 2 μM rotenone, 1.5 mg of protein/ml, valinomycin (10⁻⁷ M) and ⁴²⁷Rb. After substrate addition, suspensions were incubated for 1 min. After CCCP addition, the suspension was mixed, incubated for 30 s, and centrifuged. ⁴²⁷Rb distributions and membrane potential values were determined as outlined by Rottenberg (21). B, the effect of CCCP on ANS fluorescence during succinate oxidation or ATP hydrolysis. The medium used was 0.25 M sucrose, 50 mM KCl, 10 mM Tris, 1 mM MgCl₂, pH 7.2, 2 μM rotenone, 1.5 mg of protein/ml, and 8.3 μM ANS. Succinate (10 mM; ○) or ATP (3 mM; ▲) was added and fluorescence was recorded. A titration with CCCP was performed over a concentration range of 0–500 pmol/mg of protein. C, relationship between Δψ and fluorescence quenching in energized mitochondria. Data are compiled from A and B for succinate (●) and ATP (▲). For the diffusion potential experiments (○), the medium was 0.2 M sucrose, 10 mM Tris, pH 7.2, 3 μM rotenone, 8.3 μM ANS, and 1.5 mg of mitochondrial protein/ml. Mixtures of NaCl-KCl (total concentration, 100 mM) were added to produce different values of potassium concentration gradient. The diffusion potential was initiated by the addition of 10⁻⁷ M valinomycin and was measured by ⁴²⁷Rb distribution (21) 30 s after the addition of valinomycin.
medium prior to mitochondrial addition ends the slow phase and results in a fluorescence value which is equal to that at 100 mM KCl when added near the end of the slow increase. This result led to the supposition that the slow change may in fact be due to a potassium diffusion potential which is generated by leaking potassium and which would eventually collapse. In fact, when membrane potential was estimated from triphenylmethylphosphonium distribution under parallel experimental conditions (Fig. 5), it was found that $\Delta \psi$ drops from a value of $-46$ mV immediately upon mitochondrial dilution to a value near $-20$ mV after 20 min. Addition of either 100 mM KCl or 0.2 mM CaCl₂ near the beginning of the experiment, i.e. soon after dilution, results in immediate collapse of the potential. If the addition of KCl is delayed for 30 min after the mitochondrial dilution the slow phase becomes much faster, equilibrating in several minutes (not shown). These data point to a simplified interpretation of the biphasic fluorescence event. The ion diffusion potential initiated upon dilution of the mitochondria inhibits the rate of transport of ANS across the membrane and thus reduces the binding of ANS inside the mitochondrial matrix.

Energy-dependent Quenching of ANS Fluorescence—Upon generation of a membrane potential in mitochondria, quenching of ANS fluorescence is observed. Using the double reciprocal treatment of fluorescence titration data (Fig. 6A), it is apparent that the addition of succinate to mitochondria reduces the apparent affinity of the mitochondrial membrane for ANS. In a nonenergized state, the apparent $K_a$ measured for ANS is 22 $\mu$M. Addition of succinate increased the apparent $K_a$ to 47 $\mu$M. An analysis of the number of binding sites at two different ANS concentrations is shown in Fig. 6B. The data suggest only a small decrease from 3.9 to 3.3 in the number of binding sites upon the generation of membrane potential.

To test the hypothesis that the increase in the apparent $K_a$ of the ANS-binding sites is due to an increase in surface potential (12, 16) we have investigated the effect of salts on ANS fluorescence in energized mitochondria. Fig. 7A shows a titration of ANS fluorescence with MgSO₄ before and after the addition of succinate or ATP. Fig. 7B shows a similar titration before and after generation of a diffusion potential. In all cases Mg²⁺ enhances the fluorescence by a similar magnitude in energized and nonenergized mitochondria. Hence, the surface charge if calculated for each case from the difference $\pm$ MgSO₄ is nearly identical in energized and nonenergized mitochondria. This indicates that the change in $K_a$ observed upon energization cannot be attributed to change in external surface potential but rather to a change in the apparent affinity or the number of binding sites for ANS.

It seems clear that a relationship exists between $\Delta \psi$, the membrane potential, and ANS fluorescence quenching in rat liver mitochondria. Using KCl to clamp the value of $\Delta \psi$ in a valinomycin-induced potassium diffusion potential or by titration with uncoupler of the ATP- and succinate-induced potentials, it is possible to correlate $\Delta \psi$ with quenching. Fig. 8 shows the results of an experiment where $\Delta \psi$ (as measured by ⁸⁶Rb distribution) was generated by ATP or succinate and collapsed by CCCP. Titration with the uncoupler CCCP reduces the membrane potential (Fig. 8A) and in parallel enhances ANS fluorescence (B). Since ATP addition enhances the fluorescence when ATP hydrolysis is blocked by oligomycin, the quenching is calculated from the difference $\pm$ oligomycin. The results of these experiments and the data of a similar experiment for potassium diffusion potentials are compared in Fig. 8C showing the relationship between fluorescence and $\Delta \psi$. While in each experiment the extent of fluorescence quenching is correlated with $\Delta \psi$, these relationships are not completely linear with succinate or diffusion-

induced $\Delta \psi$. The quenching is linear at low potential but at high potential the slope increases. In contrast, ATP-induced potential is linear over a wider range of potentials but with more moderate slope and little response at low potential. These differences arise from the correction procedure since oligomycin does not block the ATPase completely and does not completely collapse $\Delta \psi$. Fig. 9 shows the results of an experiment conducted at 10 °C where succinate was added at different points along the time course of the slow fluorescence increase. When succinate is added immediately after mitochondrial addition, the fluorescence increases. Addition of succinate at a later time induces smaller increases in the fluorescence. After 10 min of incubation, the fluorescence has increased by 30% and subsequent succinate additions quench the fluorescence. Finally, when the fluorescence reaches a steady value, the addition of succinate induces the maximal fluorescence quenching as observed in previous experiments. If CCCP is added after the addition of succinate, there is a fast and large increase in intensity and a slow relaxation to the equilibrium value. The magnitude of the initial CCCP effect is the same at the beginning or the end of the incubation period. The slow phase of fluorescence enhancement is due to the slow decay of the residual diffusion potential (Fig. 5). However, the diffusion potential is relatively small compared to substrate-induced potential and further quenching should be observed by substrate addition if the response depends on external ANS. The fact that, initially, enhancement rather than quenching is observed suggests that substrate-induced fluorescence quenching is due to potential-induced efflux of ANS from the mitochondria. Since in low temperature, initially, there is no ANS inside the matrix, no quenching is observed. The enhancement by succinate may be attributed to the salt effect on the surface potential. The effect of CCCP in collapsing both the diffusion and the substrate-induced potential is to accelerate the uptake and internal binding of ANS. Initially, when protons collapse the diffusion potential, the internal pH becomes very low, which enhances the internal fluorescence (Fig. 4). As the potassium leaks out of the mitochondria, the pH equilibrates, leading to decreased fluorescence.

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1. H. Rottenberg, unpublished observations.
DISCUSSION

Analysis of Binding and Fluorescence in Nonenergized Mitochondria—Since ANS has very low quantum efficiency in a polar medium such as water (2, 27), there is little doubt that the enhanced fluorescence and the blue shift in the emission spectra which is observed on addition of membranes or proteins to ANS solution is due to ANS binding to proteins and/or lipids in the membrane. However, since in biological membranes there are possibly more than one class of binding sites with different binding parameters and quantum efficiencies and since these parameters may be dependent on the metabolic state of the system, the analysis of fluorescence changes may become quite difficult. Earlier attempts to characterize the binding parameters in mitochondria and submitochondrial particles have resulted in conflicting results (7, 27-29).

We believe that some of the earlier data reflect the presence of significant contamination of bis-ANS in most commercial preparations. A more important source of ambiguity in previous studies was the implicit assumption that all bound ANS has high (and equal) quantum efficiency. The binding of ANS was usually estimated by a fluorescence assay without independent measurement of total binding. In a recent study, Williams et al. (7) used the centrifugation assay to measure total binding independent of the fluorescence. However, even though their Scatchard plots indicate a low affinity, high capacity class of binding sites which do not appear in the fluorescence assay, they did not correct their estimated free concentration for this bound nonfluorescent fraction. As a result, theirs as well as previous estimates include both types of binding sites, hence grossly overestimating both the value of $K_d$ and of $n$ of the fluorescent site. As discussed in detail in previous sections, in all our analyses of binding data, the free ANS concentration was always determined, for each experimental point. In each case after reading of the fluorescence, the mitochondria were pelleted, and the total bound dye was determined. We believe that this procedure is necessary to get reliable estimates of binding parameters in mitochondria. The weak binding class with low fluorescence and large number of sites is probably due to nonspecific absorption to membrane and protein surfaces. Because of the strong effect of salts on the fluorescence both in mitochondria and in submitochondrial particles, it appears that the high affinity sites are distributed both on the matrix and cytosolic face of the membrane.

Mitochondrial Surface Charge and ANS Binding—The salt dependence of ANS binding and fluorescence in mitochondria suggests that, similar to other membrane systems (9-11, 26), the surface charge of the membrane determines the ANS concentration in the double layer adjacent to the membrane surface and, hence, the apparent dissociation constant. Our data fully support this explanation by the demonstration that salt affects the dissociation constant but not the number of binding sites. The dependence of the salt effect on the caton valence and concentration is in good agreement with Guoy-Chapman theory, provided that allowances are made for the binding of cations to the mitochondrial surface. The effect of pH is also due to an increased apparent dissociation constant and is reversed by salt, indicating that this is largely an effect on the surface charge of the mitochondrial membrane. These results justify the use of ANS fluorescence for the estimation of surface potential in nonenergized mitochondria. For accurate estimates of the value of surface potential, it was necessary to determine the fluorescence of ANS when the surface potential is zero. For that purpose, we have developed a null point fluorescence titration which approaches the zero value by screening the charges of both negative and positive (Ca$^{2+}$-treated) membranes. It must be stressed that for reliable estimates the ANS concentration must be well below the $K_d$, which we used routinely 8 µM. Our results indicate a surface potential of up to $-20$ mV at high pH and low salt. However, the binding of small amounts of Ca$^{2+}$ or other cations effectively reduces the potential and may even result in a positive surface potential.

Energization of Mitochondria and ANS Binding and Fluorescence—There are currently three different interpretations for the effect of energization on ANS fluorescence and binding. According to the explanation advanced by Jassaitis et al. (5), the quenching in mitochondria is the result of ANS extrusion from the matrix driven by $\Delta\psi$. An alternative interpretation, first outlined by Azzi et al. (8) and recently advocated by Aiuchi et al. (12) and Wojtczak and Nalecz (17) is that energization results in an increase in the negative value of the surface charge, hence the reduction of external binding and fluorescence of ANS. The third hypothesis is that the energy-induced changes in fluorescence intensity are due to changes in quantum yield associated with the energized state of the membrane (50-52). The main purpose of the present study was to test the validity of the first two explanations. While the results are not equivocal, we feel that they do not support the surface charge hypothesis whereas they add further evidence to the membrane potential hypothesis. As for the membrane “state” hypothesis, our data indicate that the energy-induced quenching is mostly a result of decreased binding (of both fluorescent and nonfluorescent dye) as expressed in the change of the apparent $K_d$. While the analysis indicates a small change in the extrapolated $n$ value, which cannot be distinguished from decreased quantum efficiency, the major change is due to a decreased amount of bound ANS. If one does not distinguish between the two classes of binding sites, a decrease in binding of the low capacity site may appear as an overall decrease in quantum efficiency at high ANS concentrations.

The most striking evidence against the surface charge hypothesis is that the quenching due to energization is completely insensitive to salt effects. This is in contrast to the pH effect and to the general behavior of nonenergized membranes. Even in energized membranes, the salt response of the unquenched fluorescence is identical with that of the nonenergized system. Hence, if the quenching was induced by increased surface charge, salt should have enhanced the fluorescence to its original level. The other evidence against the surface charge hypothesis which at the same time supports the membrane potential hypothesis is the response at low temperature immediately after the addition of ANS. Succinate does not induce any quenching but CCCP causes normal enhancement. This observation suggests that the succinate-induced quenching and the subsequent enhancement by CCCP requires transport of ANS across the membrane. In this respect, we have also compared the energy-induced ANS response to the 6-p-toluidino-2-naphthalenesulfonic acid response. The results confirm earlier observations that while the magnitude of the response is the same the kinetics is much slower with 6-p-toluidino-2-naphthalenesulfonic acid (8). Since 6-p-toluidino-2-naphthalenesulfonic acid is known to be much less permeable than ANS (11), this difference in kinetics indicates that the response depends on ANS transport into and across the membrane. In addition, the correlation between ANS quenching and $\Delta\psi$ while not perfect suggests a close causal relationship between membrane potential and the ANS response. The only evidence that appears to support the surface charge hypothesis and is difficult to explain by the membrane potential hypothesis is the observation that the quenching is mostly the result of increased
changes in membrane potential, particularly in particles in
unequivocal. In summary, we believe that there is no une-
pertable evidence appears on closer examination to reflect the large
potential-induced activation of the enzymes. Finally, the
increase in that the
port, deformation of the organelles,
Tb"+ is not transported by mitochondria these results appear
are located exactly at the plane of the phospholipid head groups on
both sides of the membrane lipid bilayer. However, since it is likely
that the fluorescent ANS is bound to various proteins, it may
be located at various distances from the phospholipid head
group projecting into and out of the membranes. Hence, the
measured Kd probably represents a weighted average of these
binding sites, which are different from each other both in
their intrinsic Kd and the free ANS concentration in their
vicinity. The generation of membrane potential in energized
mitochondria would increase the apparent Kd values of sites in
the matrix and also in the membrane by driving the free
ANS out. Because of the composite nature of this parameter,
it would appear simply as higher average Kd.

Effect of Energization on Mitochondrial Surface Charge—
The evidence for a change in the surface charge due to energization of mitochondria depends on four experimental
findings: (i) the potential, as measured by the electrophoretic
mobility of the mitochondria becoming slightly more negative
in energized membrane (12, 13, 16); (ii) the apparent Kd for
various charged substrates in energized mitochondria is
different in nonenergized mitochondria (17); (iii) the free form
of a positively charged spin-probe decrease on energization
(34); and (iv) the binding of ANS is reduced. As discussed
above, the latter observation is not compatible with the surface
charge hypothesis and appears to result from the gener-
ation of Δψ. However, it is not possible to conclude from the
ANS results alone that there is no change in surface charge;
only that the effect of Δψ on ANS fluorescence would obscure
a change of surface charge if it indeed exists. We have recently
examined the binding of the positively charged spin-probe
4-(dodecyltrimethylammonium)-1-octyl-2,2,6,6-tetramethylpi-
peridine bromide (Cat,12) in mitochondria and concluded that
the disappearance of the external free form is due to potential
driven uptake and internal interaction of the probe.3 As for
the changes in the apparent Kd of membrane enzymes, these
may also result from potential-dependent transport or possibly
potential-induced activation of the enzymes. Finally, the
potential measurement may reflect both electrogenic transpor-
t, deformation of the organelles, or changes in orientation
and position of charge carrying groups. It should be stressed
that the potential change is also insensitive to the ionic
strength of the medium (12) suggesting that it is not due to
changes in surface charge. Recent studies on the binding
affinity of Tb"+ to mitochondrial membranes do not indicate
a significant change in surface charge on energization. Since
Tb"+ is not transported by mitochondria these results appear
unequivocal.4 In summary, we believe that there is no une-
quivalent evidence for an increase in surface charge on energ-
ization of mitochondrial membranes. Most of the quoted
evidence appears on closer examination to reflect the large
increase in Δψ in energized mitochondria.

Correlation of ANS Quenching and Δψ—ANS fluorescence
has been used extensively as a quantitative estimate of changes
in membrane potential, particularly in particles in
which the potential is positive. In most cases, the signal is
calibrated by the imposition of a potassium diffusion poten-
tial. Fig. 8C summarizes the correlation between the potential
(measured by 40Rb distribution) and ANS quenching. Note
that even for the potential generated by potassium gradient
we actually measured the resulting potential rather than
estimate it from the potassium gradient. The results indicate
that in succinate-energized system and as a result of the
generation of a diffusion potential the signal is a linear
function of the potential at low potential but the sensitivity
of response to the potential decreases at very high potential.
This is expected from the model which assumes extrusion
of ANS as a basis for quenching since the fluorescence due to
internal binding would be already negligible at moderate
potential. This also explains why the ANS fluorescence
does not respond to the reduction of Δψ on transition from state 4
to state 3 since both potentials (−150 and −130 mV, respec-
tively) are above the responsive range. However, when ener-
gized by ATP, the fluorescence appears linear over almost the
entire range. This is mostly due to the lower potential gener-
ated by ATP. The small nonlinear portion at low potential is
probably due to an overcorrection of the direct ATP effect on
ANS fluorescence from using oligomycin as a reference point.
Considering the complexity of ANS binding and fluorescence
in mitochondria, such small discrepancies are not unexpected.
Even though ANS fluorescence in mitochondria appears to
correlate with Δψ, the lack of sensitivity of the response at
high potential together with the relatively small changes in
fluorescence and its response to surface potential prohibit the
use of ANS as a probe for Δψ in mitochondria.

APPENDIX 1

The Relation between ANS Fluorescence and the Surface
Potential—To derive the relation between ANS binding and
the surface potential, Δψ, we start from the Gibbs free energy
of the dissociation reaction for ions

Kd = exp(ΔG/R T)  (1)

where Kd is the dissociation constant, R the gas constant, and T
the absolute temperature. ΔG is the apparent Gibbs free energy
of the dissociation which is a function of the surface potential.
Hence

ΔG = ΔG° + zFΔψ,  (2)

where z is the charge of the ion and F is the Faraday constant.
Substitution of Equation 2 in 1 gives

Kd = exp(ΔG°/RT)exp(zFΔψ/RT)  (3)

and rearrangement

Kd/Kd° = exp(zFΔψ/RT)  (4)

where K" is the true dissociation constant. Thus, measurement
of Kd° and the apparent Kd allow calculation of Δψ from
Equation 4 since

Δψ = (RT/zF)ln(Kd/Kd°)  (5)

when concentration of the ligand is well below Kd the binding,
b, is proportional to Kd. In general,

b = n[c]/(Kd + [c])  (6)

where n is the binding capacity and [c] is the concentration
of the ligand. However, when [c] ≫ Kd, Equation 6 is reduced
to

b = n[c]/Kd  (7)

Therefore, when [c] ≫ Kd, the fluorescence of ANS is propor-
tional to the binding.
where \( q \) is the quantum efficiency of bound ANS. If we measure the fluorescence, of membrane-bound ANS, at the same ANS concentration in the presence and absence of surface charge, it follows from Equation 8 that

\[
F/F_0 = K_2/K_4
\]

hence from Equations 9 and 4 we get

\[
\Delta \psi = -(2.3RT/F) \log(F/F_0)
\]

### APPENDIX II

**Surface Potential as Function of Salt Concentration** — The relation between surface potential, \( \psi_s \), surface charge, \( q \), and the ionic strength, \( [c]^{0.75} \), is derived from the Poisson-Boltzmann expression (25). For \( z \)-\( z \) electrolytes, the integration yields the following relationship (at 25 °C) (35)

\[
q = 11.74[c]^{0.75} \sinh(ZF/51.7)
\]

when the solution contains both mono, \([C^+]\), and divalent, \([C^{2+}]\), salts the following quadratic equation was obtained

\[
2[C^{2+}] \cosh(F_F/RT) + [C] \cosh(F_F/RT)
\]

\[
- (2[C^+] + 2[C^{2+}] + q^2/2A^2) = 0
\]

where \( A = (\rho RT\lambda)/(2\pi) \) (\( \lambda \) is the permability of water). To solve

Equation 2 numerically, we define \( X = \cosh 0.0387 \psi_s \) (at 25 °C) and get the simplified expression

\[
2[C^{2+}] \times + [C^+] \times - (2[C^+] + [C^{2+}] + q^2/2A^2) = 0
\]

in order to fit the salt titration, we start by guessing a value for \( q \) that will satisfy Equation 3 when the initial measured value of \( \psi_s \) is inserted in the expression. This procedure is reiterated to arrive at the best guess. Then we solve Equation 3 for different salt concentrations to arrive at the calculated dependence of \( \psi_s \), on salt concentration.

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