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An Analysis of the Binding of Fluorescence Probes in Mitochondrial Systems

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Summary. Measurements of the binding of the fluorescent probes 8-anilinonaphthalene-1-sulfonate (ANS) and ethidium ions to whole and disrupted mitochondria and submitochondrial particles suggest that the inner mitochondrial membrane is freely permeable to the two probes. Equations relating the binding of permeant probes to the electro-chemical balance across the membrane of vesicular systems are derived and these equations used to analyze Scatchard plots of the binding of the two probes to energized and nonenergized mitochondria and EDTA particles.

Several hypotheses have been put forward to explain the changes in binding of the fluorescence probes ANS and ethidium observed on the energization of mitochondria or submitochondrial particles. They divide, as pointed out by Gains and Dawson (1975*a*) into three categories: those in which no assumptions regarding the permeability of the mitochondrial membrane to the probes are required, those which require that the membrane be permeable, and those which require it to be impermeable to the probes.

Measurements by Jasaitis, La Van Chu and Skulachev (1973), Layton, Symmons and Williams (1974) and Gains and Dawson (1975*a*) all indicate that ANS can penetrate the mitochondrial membrane, thus suggesting that the correct explanation of the binding changes must lie in one of the former two categories. Of the hypotheses so far put forward, the three that remain tenable if the probes are permeant ions are those of Skulachev (1970), involving a membrane potential-linked accumulation or extrusion of the probes, Radda and his co-workers (Barrett-Bee & Radda, 1972; Barrett-Bee, Radda & Thomas, 1972; Radda & Vanderkooi, 1972), involving energy-induced conformational changes in the mitochondrial membrane, and Gains and Dawson (1975*a*) involving a redistribution of bound probe between different sets of binding sites.

In the present paper, the earlier studies on probe penetrability are extended and the existence of a wide distribution of binding sites throughout the mitochondrion demonstrated. The general question of the binding of probes in vesicular systems is discussed and equations describing the binding of permeant probes in such systems are derived. These equations are then used to demonstrate that the magnitude as well as the direction of the changes in probe binding observed on energization of mitochondria and submitochondrial particles can be satisfactorily accounted for, in terms of the hypothesis put forward by Skulachev (1970).

Materials and Methods

Materials

Heavy beef heart mitochondria were prepared by the method outlined by Smith (1967), and EDTA particles by the method of Lee and Ernster (1967). The magnesium salt of ANS, obtained from Eastman Kodak, Ltd., was twice recrystallized from hot aqueous solution before use. Ethidium bromide, obtained from Sigma, Ltd. was used as purchased. All other chemicals were normal analytical grade laboratory materials.

Binding Studies

Probe binding to mitochondria and EDTA particles was measured using the centrifugation method of Azzi and Santato (1971) and a modified version of the fluorescence assay method of Brocklehurst, Freedman, Hancock and Radda (1970).

Fluorescence Measurements

Fluorescence measurements were carried out using a spectrofluorimeter constructed in this laboratory (Layton, 1973). The sample was contained in a 1-mm path length spectrophotometer cell. All measurements were made from the front surface of the cell. The slits of the monochromators were set at 2 mm, corresponding to a half-band width of 13.2 nm. Ethidium fluorescence was excited at 510 nm and measured at 620 nm; ANS fluorescence was excited at 395 nm and measured at 490 nm. Stray light in the exciting beam was reduced by the use of either a Corning CS 4–64 or a Corning CS 4–77 filter. Scattered light was reduced by the use of appropriate cut-off filters.

The fluorescence of ANS samples obtained from the centrifugation binding studies was enhanced by the addition of equal volumes of 3% v/v Triton X-100. All other fluorescence measurements were made on unenhanced samples.

Oxygen Consumption Measurements

Oxygen consumption by mitochondria was measured using a YSI 400 Clark Oxygen Probe (Yellow Springs Instruments Co.). Measurements were made using a simple cuvette system constructed in this laboratory. Reaction media details are provided in the relevant Figure caption.

Theory

The Scatchard equation (Scatchard, 1949), describing the interaction of small molecules with a system of homogenous macromolecules, has been widely adopted for the study of the binding of small molecules such as fluorescence probes in membrane systems. In this latter context it is commonly written as:

$$r = n - K_d \frac{r}{c} \tag{1}$$

where n is the number of μequiv of binding sites per gram of membrane protein, r the number of μequiv of the probe bound per gram of membrane protein, c the molar concentration of free probe and K_d the dissociation constant.

Eq. (1), as it stands, presupposes the existence of a single set of binding sites in equilibrium with a single uniform concentration of free probe. The possibility of the existence of multiple sets of binding sites can be allowed for by rewriting the equation in the form:

$$\sum_j r_j = \sum_j \left(n_j - K_{dj} \frac{r_j}{c} \right). \tag{2}$$

If we treat the mitochondrion, or EDTA particle, as a simple two-phase system consisting of an inner phase (the interior of the vesicle) and an outer phase (the suspension medium), then:

$$r = \sum_j r_{oj} + \sum_j r_{ij} \tag{3}$$

where the subscripts i and o refer to the inner and outer phases.

After making due allowance for the local free probe concentration in the inner and outer phases, c_i and c_o, the internal and external binding terms can be calculated using Eq. (2).

The equilibrium distribution of free probe between the two phases will be determined by the electro-chemical potential balance across the membrane. If, as suggested by Skulachev (1970), a membrane potential exists between the two phases, the distribution can be described by the Nernst equation:

$$E = \frac{RT}{ZF} \ln K \tag{4}$$

where E is the membrane potential, R the universal gas constant, Z the valence of the probe, F the Faraday and K the ratio of the activities of the free probe in the two phases. Assuming the activity coefficients in the two phases to be the same, K equals c_i/c_o and

$$r = \sum_j \left[n_j - \frac{K_{dj}}{K} \left(\frac{r_{ij} + K r_{oj}}{c_o} \right) \right]. \tag{5}$$

Results and Discussion

Scatchard Binding Plots

The binding of ANS to EDTA particles and to whole mitochondria was studied using both the centrifugation and the fluorescence assay methods. Typical sets of results obtained from parallel measurements made on the same stock suspensions are shown in Figs. 1 and 2.

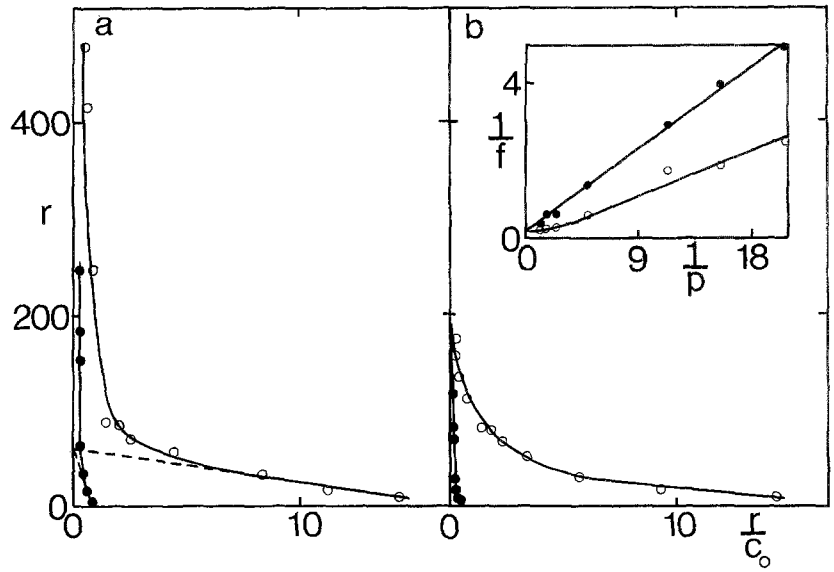

Fig. 1. Scatchard plots for 8-anilinonaphthalene-1-sulfonate binding to EDTA particles. Plots for the energized (○) and nonenergized (●) binding of 8-anilinonaphthalene-1-sulfonate to EDTA particles obtained using (*a*) the centrifugation method, or (*b*) the fluorescence assay method. The samples contained 0.45 mg · ml^{-1} mitochondrial protein suspended in 0.17 M sucrose, 10 mM Tris-acetate, pH 7.5, 2 μM rotenone, 1 μg · ml^{-1} oligomycin, 5 mM succinate. The nonenergized samples also contained 1.7 mM KCN. The range of probe concentrations was 6 to 1000 μequiv · liter^{-1}. The fluorescence assay results were normalized so as to match the centrifugation results in the high r/c_o region. The corresponding double reciprocal plot, extrapolated on the basis of this normalization, of the fluorescence yield f (expressed in relative units) of 16 μequiv · liter^{-1} of probe in the presence of varying concentrations of protein p (expressed in mg · ml^{-1}), is shown in the inset to the Figure

The fluorescence assay method is normally calibrated by determining the intercept value at infinite protein concentration of a double reciprocal plot of fluorescence yield against protein concentration for a fixed amount of probe (Brocklehurst *et al.*, 1970). In practice, the estimation of the correct intercept value is extremely difficult (Harris, 1971; Layton *et al.*, 1974). The problem is exacerbated by the fact that the values of r/c_o at low probe concentrations are particularly sensitive to the choice of intercept. Depending on whether a linear or a smooth curve extrapolation is employed, r/c_o values for energized EDTA particles can differ by up to 10-fold (*see* Layton *et al.*, 1974).

In view of these problems the alternative procedure of estimating the calibration value from a comparison of the results of the centrifugation and fluorescence assay methods was adopted in the present study. The intercept values in the double reciprocal plots were calculated from the

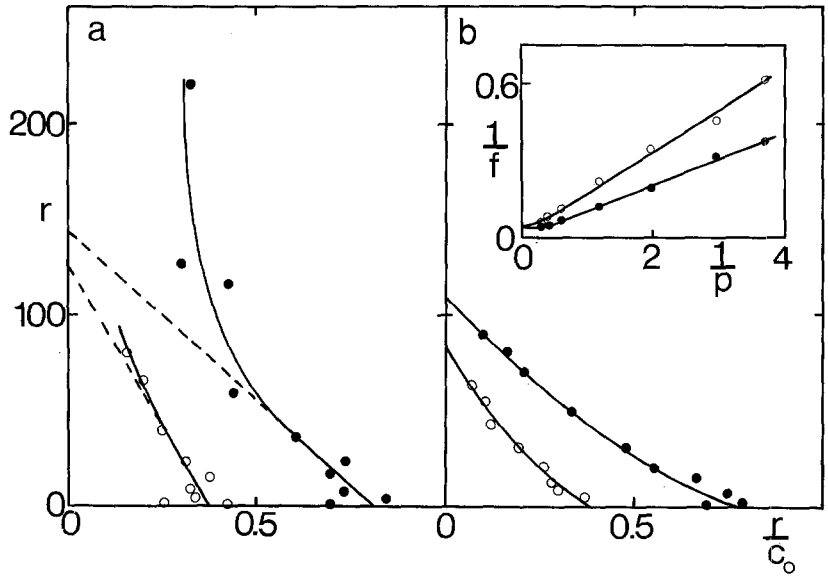

Fig. 2. Scatchard plots for 8-anilinonaphthalene-1-sulfonate binding to whole mitochondria. Plots for the energized (○) and nonenergized (●) binding of 8-anilinonaphthalene-1-sulfonate to whole mitochondria obtained using (*a*) the centrifugation, or (*b*) the fluorescence assay method. The samples contained 1.1 mg · ml^{-1} mitochondrial protein suspended in 0.25 M sucrose, 5 mM Tris-HCl, pH 7.4, 2 μM rotenone, 5 mM succinate. In addition, the nonenergized samples contained 1.7 mM KCN. The range of probe concentrations was 4 to 1000 μequiv · liter^{-1}. The fluorescence assay results were normalized so as to match the centrifugation results in the high r/c_o region. The corresponding double reciprocal plot is shown in the inset to the Figure

centrifugation results on the assumption that the two assay methods should, in the absence of artifacts, yield identical results. The value for the EDTA particles (inset to Fig. 1*b*) was calculated from the low probe concentration region of the centrifugation results for energized particles and the value for whole mitochondria (inset to Fig. 2*b*) from the corresponding results for nonenergized mitochondria. The low probe concentration region was chosen so as to avoid artifacts affecting the fluorescence assay plot (*see* discussion below). The choice of the energized plot for EDTA particles and the nonenergized plot for mitochondria was dictated by the fact that binding was highest in these states and that errors arising from the scatter of data points, a particular problem in the case of the mitochondria, could thus be minimized.

The fact that the resulting intercept values were both consistent with a reasonable extrapolation of the double reciprocal plots and that the

resulting fluorescence assay curves matched the centrifugation plots in both the energized and nonenergized states strongly supports the suggestion of Azzi, Gherardini and Santato (1971) that the fluorescence changes accompanying energization can be largely accounted for in terms of binding changes. While this test is insufficiently sensitive to entirely preclude the possibility of a simultaneous change in quantum yield of the bound probe of the type discussed by Radda and Vanderkooi (1972), it does suggest that any such changes could only be of secondary importance.

We have previously reported (Layton *et al.*, 1974) that Scatchard plots of ANS binding to EDTA particles obtained by the centrifugation method are characterized by an asymptotic section at low r/c_o values that is absent in plots determined by the fluorescence assay method (*cf.* Figs. 1*a* and 1*b*). A similar but even more pronounced effect was found for the binding of this probe to whole mitochondria (Figs. 2*a* and 2*b*). The effect appears more marked for mitochondria as energization results in a decrease in probe binding rather than the increase observed for EDTA particles. In the earlier paper the differences between the results obtained by the two methods were attributed to the fact that the centrifugation method did not distinguish between free probe trapped within, and probe bound to, the vesicles. Calculation of the free probe correction for mitochondria, equal to Kv_i [*see* Layton *et al.*, 1974, Eq. (5)], indicates, however, that it is too small to account for the discrepancy between the two methods. If the specific internal volume v_i, of beef-heart mitochondria is assumed to be similar to that for rat-liver mitochondria (i.e. about 0.001 liter $\cdot g^{-1}$ in the nonenergized state, Harris & Van Dam, 1968), this explanation would make K equal 100–200. An accumulation of free probe by nonenergized mitochondria of this magnitude is clearly unrealistic.

A much more reasonable explanation of the differences between the results obtained by the two methods is that the fluorescence values in the higher concentration region are lowered either by inner-filter effects or by concentration quenching of the fluorescence of bound probe. This view is supported by the fact that the ratio of the fluorescence yield value of ANS bound to whole mitochondria or EDTA particles (measured by the fluorescence assay method) to the amount of bound probe (measured by the centrifugation method) remains constant below about $r = 20$ μequiv $\cdot g^{-1}$ but falls with increasing values of r (Fig. 3). The fact that the lowering of the fluorescence yield is proportional to r rather than c_o indicates that the lowering is not due to inner-filter effects arising from free probe in the suspension medium. The two possibilities of an inner-filter effect

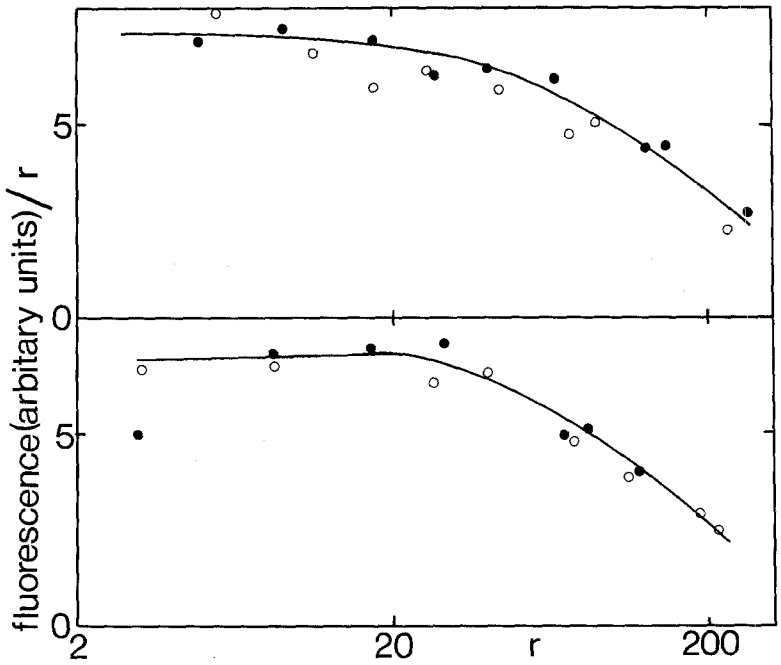

Fig. 3. Concentration dependence of the fluorescence yield of bound 8-anilinonaphthalene-1-sulfonate. Plots of the ratio of the fluorescence yield of bound probe to the actual amount of probe bound, r, as a function of r for (*a*) EDTA particles and (*b*) whole mitochondria for the energized (○) and nonenergized states (●). The values in this Figure were calculated on the basis of the results shown in Figs. 1 and 2

within the individual particles and a concentration quenching of fluorescence are, however, much more difficult to distinguish.

Scatchard plots of ethidium binding to EDTA particles and mitochondria are shown in Fig. 4. They show a similar reversal in the extent of probe binding on energization to that for ANS binding with the exception that in this case probe binding decreases on energization of particles and increases on energization of whole mitochondria. The other major difference between the plots for the two probes is that the ethidium plots, in contrast to the ANS plots, show little or no sign of an asymptotic section in the low r/c_o region.

Scatchard binding plots of the type shown in Figs. 1, 2 and 4 are based on the measurements of the amounts of probe bound to a fixed amount of mitochondria, or EDTA particles, made over a large range of probe concentrations. If any analysis of these plots is to be valid it is important to know whether or not the probes themselves interfere with the energization process. In order to check this, the rates of coupled and uncoupled

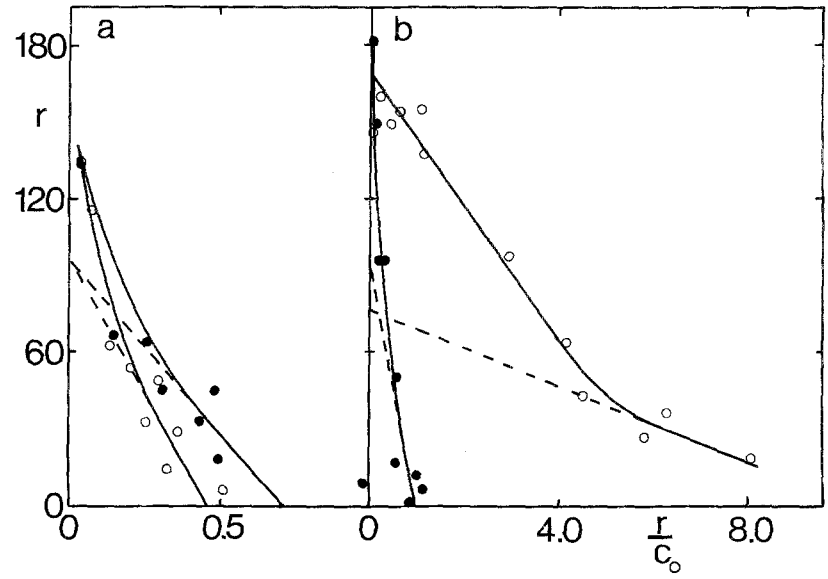

Fig. 4. Scatchard binding plots for ethidium bound to mitochondria and EDTA particles. Plots are shown for (*a*) EDTA particles and (*b*) mitochondria in the energized (○) and non-energized (●) states. The suspension media were as detailed in the legend to Fig. 1. The protein concentrations were 0.79 mg · ml^{-1} for the EDTA particle samples and 1.1 mg · ml^{-1} for the mitochondria samples. The range of ethidium concentrations used was from 15 to 3000 μequiv · liter^{-1} for the EDTA particle and from 20 to 5000 μequiv · liter^{-1} for the mitochondria samples

succinate driven O_2 consumption by mitochondria were measured in the presence of varying concentrations of the probes.

The results, shown in Fig. 5, indicate that while neither probe appears to act as an uncoupler, they both act as efficient inhibitors of respiration above a concentration of about 100 μequiv · liter^{-1}. The range of probe concentrations used in the binding plots extended up to 1000 μequiv · liter^{-1} in the case of ANS and 5000 μequiv · liter^{-1} for ethidium. It is likely that the degree of energization of the mitochondria is affected, to some extent at least, by the inhibiting action of the probes. Care must therefore be taken in the interpretation of the significance of the energized plots in the high probe concentration region.

The values of n and K_d for the four systems quoted in Table 1 have consequently been restricted to the low concentration range. These values, calculated on the basis of Eq. (1) from the extrapolations shown by dashed lines in Figs. 1, 2 and 4, are in good agreement with earlier published values for binding studies carried out using the centrifugation method (*cf.* Table 1 for earlier values). The agreement with values obtained by

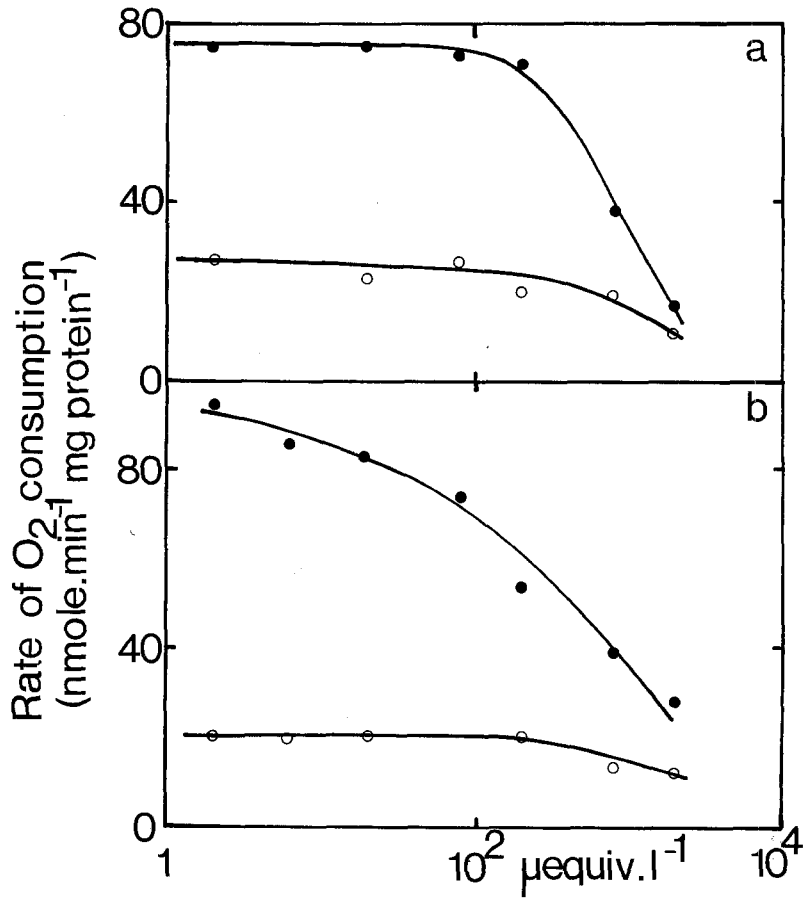

Fig. 5. Concentration dependence of inhibition of respiration by 8-anilinonaphthalene-1-sulfonate and ethidium. The rate of oxygen uptake by coupled (○) and uncoupled (●) mitochondria was plotted as a function of (*a*) 8-anilinonaphthalene-1-sulfonate and (*b*) ethidium concentrations. The samples contained 1 mg · ml^{-1} protein and the reaction medium was 0.25 M sucrose, 5 mM Tris-HCl, pH 7.4, 2 μM rotenone, 2 mM succinate plus the appropriate concentration of probe. The samples were uncoupled by the addition of dinitrophenol to a final concentration of 500 μM

direct measurements of the fluorescence of the bound probe is not so good. The discrepancy between the results obtained by the two methods can, however, as we have discussed above, largely be attributed to the difficulties involved in calibrating the fluorescence assay method.

Effect of Disruption on Probe Binding

To determine the appropriate form of the binding equation describing the interaction of the probes with the mitochondrial system, it is essential

Table 1. Collected values of the apparent binding constants for 8-anilinonaphthalene-1-sulfonate and ethidium binding to mitochondria and EDTA particles[a]

System	Energized		Nonenergized	
	K_d (μequiv · liter^{-1})	n (μequiv · g^{-1})	K_d (μequiv · liter^{-1})	n (μequiv · g^{-1})
8-anilinonaphthalene-1-sulfonate and mitochondria	340	125	175	143
8-anilinonaphthalene-1-sulfonate and EDTA particles	3.7[b]	62[b]	72[c]	62[c]
Ethidium and mitochondria	7.5[d]	78[d]	97[e]	93[e]
Ethidium and EDTA particles	210	96	160	96

[a] The values quoted in the Table were obtained from the Scatchard plots in Figs. 1, 2 and 4 on the basis of Eq. (1).

Compares with earlier values: ($K_d=3$, $n=25$)[b] and ($K_d=100$, $n=70$)[c], Nordenbrand & Ernster (1971); ($K_d=4$, $n=44$)[b] and ($K_d=134$, $n=114$)[c], Layton *et al.* (1974); ($K_d=6.8$, $n=120$)[d] and ($K_d=60$, $n=120$)[e], Azzi & Santato (1971).

to know whether or not the probes can penetrate the mitochondrial membrane. With this in mind, the earlier observations of ourselves (Layton *et al.*, 1974) and of Gains and Dawson (1975*a*) on the effect of disruption of EDTA particles and mitochondria on probe binding were extended by studying the effects of sonication on ANS binding both to whole mitochondria and to EDTA particles and of osmotic shock on the binding of the two probes to whole mitochondria.

Sonication measurements. Stock suspensions of mitochondria and EDTA particles were divided into two fractions. One fraction was sonicated in the presence of ANS. The other was sonicated in the absence of the probe and the probe added after sonication. The latter fraction served as a control to ensure that in the event of probe being nonpermeant any changes in binding were not merely reflections of the increased external surface area of the sonicated vesicles. The sonication conditions were those employed in the preparation of EDTA particles. The fluorescence yield of samples taken from the unsonicated stock and from the two sonicated fractions are set out in Table 2. Details of the reaction media are given in the Table caption.

The fluorescence yield values confirm that the ANS binding to non-energized mitochondria and EDTA particles is unaltered by sonication, independent of whether sonication is carried out in the presence or absence

Table 2. 8-Anilinonaphthalene-1-sulfonate fluorescence yields for mitochondria and EDTA particles sonicated in the presence and absence of probe

Sample	Fluorescence yield (relative units)
Mitochondria	
Unsonicated	17.0
Sonicated with probe	18.5
Sonicated without probe	18.5
EDTA particles	
Unsonicated	27.0
Sonicated with probe	29.5
Sonicated without probe	28.0

[a] The mitochondria samples contained 1.0 $mg \cdot ml^{-1}$ protein in 5 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 2 μM rotenone. The EDTA particle samples contained 0.84 $mg \cdot ml^{-1}$ protein in 10 mM Tris-acetate, pH 7.5, 0.17 M sucrose, 2 μM rotene. All EDTA particle samples and the sonicated mitochondria samples also contained 1 $\mu g \cdot ml^{-1}$ oligomycin. All samples contained 10 $\mu equiv \cdot liter^{-1}$ 8-anilinonaphthalene-1-sulfonate.

of the probe. This suggests either that the number of accessible binding sites is effectively unchanged by sonication or that if new sites previously inaccessible to the probes are exposed by sonication that their exposure must be accompanied by a decrease in binding to the original sites. The latter possibility seems extremely unlikely.

The existence of internal binding sites can be inferred from the fact that the probes bind to both mitochondria and submitochondrial particles. The distribution of such sites within the mitochondrion were investigated. A stock suspension of aged (uncoupled) mitochondria were sonicated and EDTA particles isolated in the usual way. Seven samples were taken at different stages in the isolation procedure (*see* Fig. 6). The protein content of each sample was measured, adjusted to 1 $mg \cdot ml^{-1}$ and ANS added. The fluorescence yields of the samples, measured under non-energized conditions, are listed in Table 3. The values are all of similar magnitude, suggesting that the binding capacities of whole mitochondria (sample 1), EDTA particles and mitochondrial debris (sample 4), matrix protein (sample 5) and purified EDTA particles (sample 7) are all rather similar.

Osmotic shock measurements. Eight identical samples of freshly prepared well-coupled mitochondria (ratio of uncoupled/coupled electron transport > 2.5) were suspended in 0.35 M sucrose, 5 mM Tris-HCl, pH 7.4,

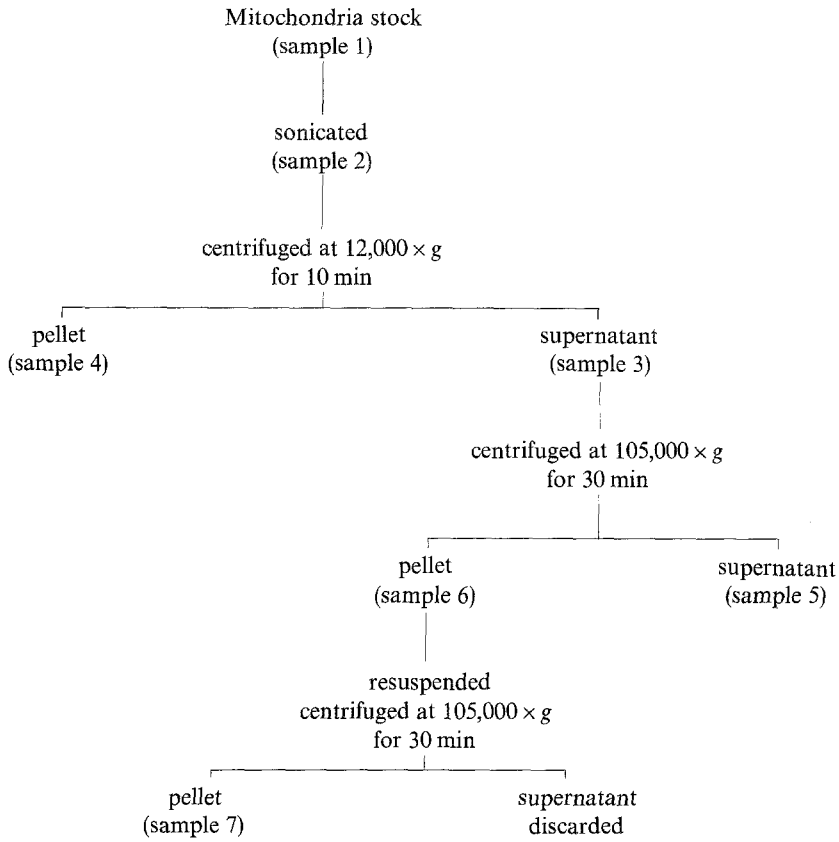

Fig. 6. Experimental procedure adopted in the isolation of different mitochondrial and submitochondrial fractions. The normal procedure for isolating EDTA particles was followed and samples taken at the stages indicated in the Figure

Table 3. 8-Anilinonaphthalene-1-sulfonate fluorescence yields of mitochondria and submitochondrial fractions[a]

Sample number	Fluorescence yield (relative units)
1	20.2
2	17.4
3	16.6
4	19.1
5	12.4
6	17.4
7	16.2

[a] Details of sample isolation and coding are given in Fig. 6. The reaction media were those appropriate to the stage of isolation. All samples contained 1 mg · ml^{-1} of protein and 20 μequiv · liter^{-1} of 8-anilinonaphthalene-1-sulfonate. Samples 2 – 7 also contained 1 μg · ml^{-1} of oligomycin.

Table 4. Effect of osmotic shock on 8-anilinonaphthalene-1-sulfonate and ethidium binding to mitochondria[a]

Sample	Probe	Amount bound (μequiv · liter^{-1})
		Whole mitochondria
A	8-anilinonaphthalene-1-sulfonate	14.3
B	8-anilinonaphthalene-1-sulfonate	14.0
C	ethidium	38.0
D	ethidium	42.5
		Broken mitochondria
E	8-anilinonaphthalene-1-sulfonate	12.8
F	8-anilinonaphthalene-1-sulfonate	11.6
G	ethidium	36.7
H	ethidium	34.2

[a] Each sample contained 1 mg · ml^{-1} of protein. The final suspension media was 5 mM Tris HCl, pH 7.4, 0.23 M sucrose, 2 μM rotenone plus 100 μequiv · liter^{-1} of either 8-anilinonaphthalene-1-sulfonate or ethidium. Samples A, B, C, D were initially suspended in 0.35 M-buffered sucrose and E, F, G, H in unbuffered sucrose. The probe was added to samples A, C, E, G at the initial suspension stage and to B, D, F, H after final suspension in 0.23 M sucrose.

and centrifuged to yield eight pellets. Four of the pellets were resuspended in 0.35 M sucrose (an adequate osmotic support) and the other four in buffer containing no sucrose (an inadequate osmotic support, promoting lysis of the mitochondria). Ethidium and ANS were included in four of the resuspending media but omitted from the other four. All suspensions were then made up to the same final sucrose concentration by the dropwise addition of appropriate volumes of either 0.175 or 0.35 M buffered sucrose. Probe was introduced at this stage to the four samples in which it was previously absent. The suspensions were then centrifuged and the amount of probe bound to the pellets calculated in the usual way.

The final results, together with details of the suspension media, are given in Table 4. Again, no appreciable increase in binding appears to take place on disruption of the mitochondria, either in the presence or absence of the probes, suggesting that the mitochondrial membrane is freely permeable to both probes.

Probe Permeability

The results of the measurements of probe binding in disrupted mitochondria and EDTA particles add further support to the conclusions of

earlier studies of this type (Layton *et al.*, 1974; Gains & Dawson, 1975*a*) that ANS type probes can freely penetrate the inner mitochondrial membrane. Jasaitis *et al.* (1973) reached the same conclusion on the basis of observations of the rate of titration of intra-mitochondrial buffer in the presence of ANS. Barker, Barrett-Bee, Berden, McCall and Radda (1974), however, have questioned this conclusion, on the basis of measurement of the rate of penetration of ANS into liposomes using a nuclear magnetic resonance technique. They found the rate of entry to be slow, taking up to an hour to reach completion. Gains and Dawson (1975*b*), while confirming the slow rate of penetration of ANS into liposomes, have pointed out the danger of drawing too close an analogy between experiments performed on such widely differing systems.

The Scatchard plots in Figs. 1, 2 and 4 indicate that the mitochondrial systems have a maximum occupancy of about 200 μequiv per gram of membrane protein for ethidium and about 200–400 μequiv per gram for ANS. The exact values, particularly in the case of ANS, are difficult to estimate. These values can be compared to the sort of maximum value that might be expected from studies in model systems. In the absence of specific binding data for mitochondrial lipids and protein such calculations must necessarily be approximate. However, assuming mitochondrial protein to have a binding capacity similar to bovine serum albumin (i.e. one equivalent/15,500 grams; Daniel & Weber, 1966) and mitochondrial lipid to have a capacity similar to lecithin (i.e. about one equivalent/3,200 grams; a value based on the work of Haynes & Staerk, 1974) and a protein to lipid ratio in mitochondria of about 3:1, the mitochondrial preparations might be expected to have a maximal occupancy of about 180 μequiv per gram of protein. The fact that this value is of the same order as the experimental value adds further support to the idea that the probes are widely bound throughout the mitochondrion. The values quoted above correspond to total mitochondrial protein. If the probes were nonpermeant, allowance would need to be made for the fact that binding could only take place on the external surface of the vesicles. It would be very difficult to account for the much higher occupancies, especially for whole mitochondria, predicted under such circumstances.

The very high binding capacities of the mitochondrial systems, the wide distribution of potential binding sites throughout the mitochondrion (Table 3) and the absence of any marked increase in the nonenergized fluorescence yield on disruption of the mitochondria or EDTA particles all strongly suggest that the inner mitochondrial membrane is freely permeable to the two probes. The necessity of obtaining a direct demonstra-

tion of this permeability to supplement the indirect demonstrations cited above before a final decision can be made on this point is, however, acknowledged.

Analysis of Binding Plots

If the inner mitochondrial membrane is indeed freely permeable to the probes, the direction of the binding changes observed on energization can readily be explained in terms of the hypothesis proposed by Skulachev (1970). Skulachev attributed the changes in probe binding to a displacement of the internal equilibrium between free and bound probe following the accumulation or exclusion of charged probes in response to the generation of a transmembrane potential. This hypothesis has received considerable support from observations of an uncoupler insensitive increase in ANS fluorescence on generation of a diffusion potential in submitochondrial particles by the addition of potassium ions in the presence of valinomycin (Azzi *et al.*, 1971; Jasaitis, Kuliene & Skulachev, 1971).

The relationship between these latter changes and the changes in ANS fluorescence accompanying energization has, however, recently been questioned by Ferguson, Lloyd and Radda (1976). They point out that the kinetics of the diffusion potential-linked changes are much faster than those associated with ATP energization. Their observations also suggest that the diffusion potential-linked changes, in contrast to the ATP-induced changes, are mainly reflections of a quantum yield change and not a change in binding. Bakker and Van Dam (1974) have also reported a potassium diffusion potential-linked change in the quantum yield of ANS bound to lecithin liposomes. They, however, were of the opinion that the corresponding change in submitochondrial particles was almost completely caused by an enhanced binding. Gains and Dawson (1975*b*) have reported that the addition of valinomycin to lecithin liposomes suspended in media containing potassium ions both accelerates the rate of penetration of ANS and leads to changes in ANS fluorescence yield even in the absence of a transmembrane potential. They suggest that these changes might be attributed to an association between ANS and a valinomycin potassium complex. If this is indeed the case and if a similar complex is formed in mitochondrial systems, the differences reported by Ferguson *et al.* (1976) may well be similarly explained.

The alternative explanations of the fluorescence yield changes accompanying energization put forward by Radda and his co-workers (Barrett-Bee & Radda, 1972; Radda & Vanderkooi, 1972) and by Gains

and Dawson (1975*a*) are less clear on the origin of the changes. Radda's explanation, while adequate for explaining ANS binding in submitochondrial particles, does not lend itself to easy generalization for the explanation of binding in the other systems: the hypothesis of Gains and Dawson offers no specific predictions on binding changes. This is not to say that the explanation offered by Skulachev (1970) is necessarily unique, nor that the change in electro-chemical balance across the membrane predicted in the Skulachev hypothesis will not be accompanied by changes of the type postulated in the other two hypotheses.

If, for the present, we accept the Skulachev hypothesis, the magnitude of the membrane potential change required to account for the observed probe binding changes can be estimated as follows. Treating the vesicles as simple two-phase systems, Eq. (3) reduces, as we have shown, to Eq. (5). In the normal Scatchard analysis based on Eq. (1) n and K_d are calculated from the intercept and slope of a plot of r against r/c_o. The corresponding analysis based on Eq. (5) would require plotting r against $(r_i + Kr_o)/c_o$. The intercept and slope of such a plot would then yield n and a function of the dissociation constants $f(K_{dj}/K)$. A comparison of the slopes of the energized and nonenergized plots would, using Eq. (4), provide a simple method of estimating the change in membrane potential, ΔE, accompanying energization.

Unfortunately, the analysis cannot be carried out so easily in practice as the fluorescent probe technique does not distinguish between r_i and r_o. However, an approximation to this analysis can be made by splitting Eq. (5) into its two component parts:

$$r_i = \sum_j r_{ij} = \sum_j \left(n_{ij} - \frac{K_{dj} r_{ij}}{K c_o} \right) \tag{6}$$

and

$$r_o = \sum_j r_{oj} = \sum_j \left(n_{oj} - \frac{K_{dj} r_{oj}}{c_o} \right). \tag{7}$$

If either r_i or r_o can be shown to greatly outweigh the other, the system can be analyzed to a fair degree of accuracy by approximating the larger term to r. Thus, if $r_r \gg r_o$, the slope of a plot of r against c_o will lead to a slope close to $f(K_{dj})/K$ and if $r_o \gg r_i$ to a slope close to $f(K_{dj})$.

In the case of the plots for the energized systems, a decision on the relative importance of r_i and r_o is relatively straightforward. Energized accumulation of the probes involves both an increase in r_i and, as a consequence of decreases in c_o, a decrease in r_o. Conversely, energized extrusion

Table 5. Classification of probe binding plots in terms of the relative contributions from internal and external binding[a]

System	Energized	Nonenergized
8-Anilinonaphthalene-1-sulfonate and mitochondria	$r_o \gg r_i$	$r_i > r_o$
8-Anilinonaphthalene-1-sulfonate and EDTA particles	$r_i \gg r_o$	$r_i > r_o$
Ethidium and mitochondria	$r_i \gg r_o$	$r_o > r_i$
Ethidium and EDTA particles	$r_o \gg r_i$	$r_o > r_i$

[a] Full details of the classification and the criteria adopted in classifying the plots are given in the text.

involves a decrease in r_i and an increase in r_o. The decision is more difficult in the case of the nonenergized systems. However, the very much higher capacity of nonenergized EDTA particles and whole mitochondria to bind ANS than ethidium (as reflected in the presence or absence of the asymptotic sections of the Scatchard plots) suggests that the former probe may be accumulated, and the latter excluded, from the vesicles, even in the nonenergized state. Some limited support for this view can be taken from the reports of Tupper and Tedeschi (1969) and Harris and Pressman (1969). Tupper and Tedeschi, using a microelectrode technique, obtained a value of about 10 mV for the membrane potential of nonenergized *Drosophila* mitochondria. Harris and Pressman estimated a value of about 30 mV for rat liver mitochondria on the basis of anion distribution data. In both cases the potentials were interior positive. These values, however, must be set against a value of 33 mV, interior negative, reported by Mitchell and Moyle (1969) from K^+ distribution data. No information appears to be available regarding the possible membrane potential of nonenergized EDTA particles. The classifications we have assigned to the individual binding plots are set out in Table 5. For the purposes of calculation, the systems classified either as $r_i \gg r_o$ or $r_i > r_o$ were analyzed by Eq. (6) and the systems classified as $r_o \gg r_i$ or $r_o > r_i$ by Eq. (7).

Dealing first with EDTA particles, using the subscripts e and ne to refer to the energized and nonenergized states and the subscripts ANS and eth to refer to the two probes, the slopes of the ANS plots are, according to the above analysis, $f(K_{dj})_{\mathrm{ANS}}/K_e$ and $f(K_{dj})_{\mathrm{ANS}}/K_{ne}$. The slopes of the ethidium plots are both approximations to $f(K_{dj})_{\mathrm{eth}}$ and are, as would be expected, rather similar.

In the case of mitochondria, the slopes of ethidium plots are $f(K_{dj})_{\mathrm{eth}}/K_e$ and $f(K_{dj})_{\mathrm{eth}}$ and those of the ANS plots are $f(K_{dj})_{\mathrm{ANS}}/K_{ne}$ and $f(K_{dj})_{\mathrm{ANS}}$.

Table 6. Estimated values for the ratios of internal and external free probe concentrations membrane potentials and probe dissociation constants for mitochondria and EDTA particles[a]

Mitochondria			
K_{ne}	1.9	E_{ne}	+17 mV
K_e	0.036	E_e	−85 mV
K_{ne}/K_e	53	ΔE	−102 mV
EDTA Particles			
K_{ne}	4.7	E_{ne}	+40 mV
K_e	92	E_e	+115 mV
K_e/K_{ne}	19.5	ΔE	+75 mV
$f(K_{dj})_{ANS} = 340$ μequiv · liter^{-1} : $f(K_{dj})_{eth} = 210$ μequiv · liter^{-1}			

[a] The values in the Table were calculated according to the procedure outlined in the text.

If we assume the values of $f(K_{dj})_{eth}$ and $f(K_{dj})_{ANS}$ to be similar for mitochondria and EDTA particles, the individual values of K_e and K_{ne} and hence values for E_e and E_{ne} can be estimated for the two systems.

The slopes of the individual plots were taken to equal those quoted in Table 1. The values of K_e, K_{ne}, E_e, E_{ne} and ΔE calculated on the basis of this analysis, together with the values of $f(K_{dj})$ used in their calculation, are set out in Table 6. The values for K quoted in the Table are for convenience all expressed in terms of ANS concentration ratios.

It should be emphasized that these calculations are of necessity approximate. It is, however, interesting to note that the size of the membrane potential changes accompanying energization, calculated on the basis of this analysis, about 80–100 mV, although somewhat lower, are not in serious disagreement with experimental values quoted in the literature (Mitchell & Moyle, 1969; Nicholls, 1974; Laris, Bahr & Chaffee, 1975). It must, however, be acknowledged that other workers, in particular Tedeschi (1974), strongly dispute the existence of such changes.

The significance of the present results does not, however, lie in the absolute values of the potential changes, which in any case refer to the equilibrium "trapped" in the vesicles by cooling which may well differ from that existing under room-temperature conditions. Their significance lies rather in that they add further support to the explanation of probe binding offered by Skulachev (1970) by demonstrating that this hypothesis can provide an internally consistent basis for explaining the magnitude as well as the direction of the probe binding changes observed on energization of mitochondria and submitochondrial vesicles.

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