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ON THE NATURE OF THE ENERGISED STATE OF SUBMITOCHONDRIAL PARTICLES; INVESTIGATIONS WITH *N*-ARYL NAPHTHALENE SULPHONATE PROBES

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SUMMARY

1. A further investigation has been made of the way in which the fluorescent probes 1-anilino-naphthalene-8-sulphonate and 2-(*N*-methyl-anilino)naphthalene-6-sulphonate report on the energised state of bovine heart submitochondrial particles.

2. A comparison of the probe responses to energisation with ATP or to a potassium diffusion potential has been made. The fluorescence enhancements seen in these two cases have different characteristics, and in view of this it is questioned whether a substrate generated energised state of a submitochondrial particle can be equated with a trans-membrane potassium diffusion potential.

3. Substitution of ITP for ATP reduces the rate at which either of the probes respond to energisation. In contrast reducing the ATPase activity of the particles by treatment with the covalent ATPase inhibitors 4-chloro-7-nitrobenzofurazan or *N,N'*-dicyclohexyl-carbodiimide has no effect on this rate. This finding that the rate of the fluorescence changes is directly sensitive to events at the level of the ATPase, but not to the total ATPase activity, suggests that this rate may not be controlled by a delocalised energised state. Reduction of ATPase activity decreases the extent of the fluorescence enhancement and a relationship between the change in probe fluorescence and ATPase activity is given.

4. The results in this paper are discussed in the context of the mechanisms which have been proposed to account for the fluorescence enhancements of *N*-aryl naphthalene sulphonate probes upon energisation of submitochondrial particles.

INTRODUCTION

It is well established that the fluorescent probes 1-anilino-naphthalene-8-

Abbreviations: ANS, 1-anilino-naphthalene-8-sulphonate; DCCD, *N,N'*-dicyclohexyl-carbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MNS 2-(*N*-methyl-anilino) napthalene-6-sulphonate; Nbf-Cl, 4-chloro-7-nitrobenzofurazan (this compound has been named 7-chloro-4-nitrobenzo-2-oxa-1,3 diazole and abbreviated NBD-chloride elsewhere; the name and abbreviation used here follow CBN recommendations).

sulphonate (ANS) and 2-(*N*-methylanilino)naphthalene-6-sulphonate (MNS) bind to bovine heart submitochondrial particles, and that this interaction can be followed by a blue shift and enhancement of the fluorescence emission spectrum [1, 2, 3]. Addition of respiratory chain substrates or magnesium and ATP to the particles results in an increased probe fluorescence, which reflects an energised state of the membrane since addition of an uncoupler reverses this effect [1, 2, 3]. The origin of these fluorescence changes upon energisation has been shown to be an increase in the amount of probe bound to the membrane, although with respiratory chain substrates part of the effect can be attributed to an increased quantum yield of the bound probe molecules [4].

An explanation of these changes in probe fluorescence is important because they are probably closely related to the nature of the energy transduction process in submitochondrial particles. One interpretation relates the probe responses to the changes in membrane potential which are required by the chemiosmotic hypothesis to occur when a submitochondrial particle is energised [5]. Important for this view is the observation that the generation of a potassium diffusion gradient across submitochondrial particles has been shown to produce an uncoupler sensitive increase in ANS fluorescence [6, 7]. Other aspects of proposals for the mechanisms by which ANS responds to energisation have been reviewed elsewhere [8].

In this paper we wish to examine further the premise that the ANS fluorescence responses are direct indications of membrane potential changes. The rate at which the probes respond to energisation is a feature which has previously been largely neglected, and it is on this aspect that we wish to focus attention here. In addition a re-examination of the basis of the potassium diffusion potential supported ANS response is reported, following the work of Bakker and Van Dam [9] on artificial liposomes.

MATERIALS AND METHODS

Mg-ATP submitochondrial particles were prepared as described by Beyer [10] and were either used immediately after preparation or were stored at −20 °C in 20 mM Tris-HCl buffer, pH 7.4, containing 225 mM mannitol and 75 mM sucrose. The ATPase activity of these particles was hardly stimulated (15 % or less) by the addition of the uncoupler FCCP (carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone) at a concentration of 1 μM. Fluorescence measurements were made at 25 °C on a Hitachi Perkin-Elmer MPF-2A fluorimeter operating in the ratio mode. For ANS an excitation wavelength of 380 nm and an emission wavelength of 480 nm were employed and for MNS the corresponding wavelengths were 340 nm and 420 nm. ANS was used as the recrystallised half magnesium salt and MNS was from a sample prepared previously [2]. ATPase activity was determined as previously described [11] and where a correlation with fluorescence changes was required the same buffer and temperature was used for both types of measurement. 4-Chloro-7-nitrobenzofurazan was purchased from Serva (Heidelberg, G.F.R.), *N,N'* dicyclohexylcarbodiimide from British Drug Houses and valinomycin from Calbiochem. ATP was obtained from Boehringer and ITP (Grade 1) from Sigma; all other reagents were of highest grade commercially available. Protein was determined by the biuret method [12].

RESULTS

Parameters of the ATP supported ANS response

Fig. 1 (Trace A) shows the time course of ANS fluorescence enhancement when magnesium and ATP are added to submitochondrial particles in the presence of ANS. This response follows first order kinetics; the rate constant is given in Table I. If the particles are energised with magnesium and ATP before adding ANS then the kinetics of the resulting fluorescence enhancement are not simply first order. (Fig. 2). Immediately after addition of ANS there is a very rapid fluorescence increase which is followed by a second phase which has the same rate constant as is observed when the

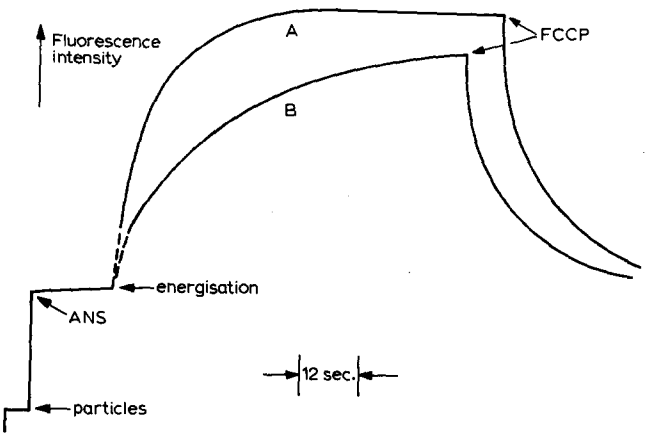

Fig. 1. Time course of ANS fluorescence intensity upon energising magnesium-ATP submitochondrial particles. Trace A: ATP as energy source. To 2 ml of 20 mM Tris-HCl buffer, pH 7.4, containing 225 mM mannitol and 75 mM sucrose, 0.5 mg particles and then 5 μM ANS were added, followed by 2 mM magnesium chloride and ATP. The effect of subsequently adding 1 μM FCCP as uncoupler is also shown. Trace B: ITP as energy source. Conditions as for Trace A except that 2 mM magnesium chloride and ITP were used for energisation. Variation of the ANS to particle ratio had no effect on these time courses.

TABLE I

FIRST ORDER RATE CONSTANTS FOR THE UPTAKE OF ANS AND MNS INTO SUBMITOCHONDRIAL PARTICLES ENERGISED BY ATP OR ITP, WITH OR WITHOUT PARTIAL INHIBITION OF ATPase BY Nbf-Cl

Rate constants were measured on a single preparation of submitochondrial particles. The particles which had been treated with Nbf-Cl had an ATPase activity which was 50 % of the control particles. Other conditions were as described in the legends to Figs. 1, 4 and 5.

Probe used	Control submitochondrial particles			Submitochondrial particles treated with Nbf-Cl.		
	k_{ATP} (s^{-1})	k_{ITP} (s^{-1})	$\frac{k_{ATP}}{k_{ITP}}$	k_{ATP} (s^{-1})	k_{ITP} (s^{-1})	$\frac{k_{ATP}}{k_{ITP}}$
ANS	0.132	0.07	1.9	0.15	0.08	1.85
MNS	0.015	0.007	2.0	0.0015	0.0075	2.0

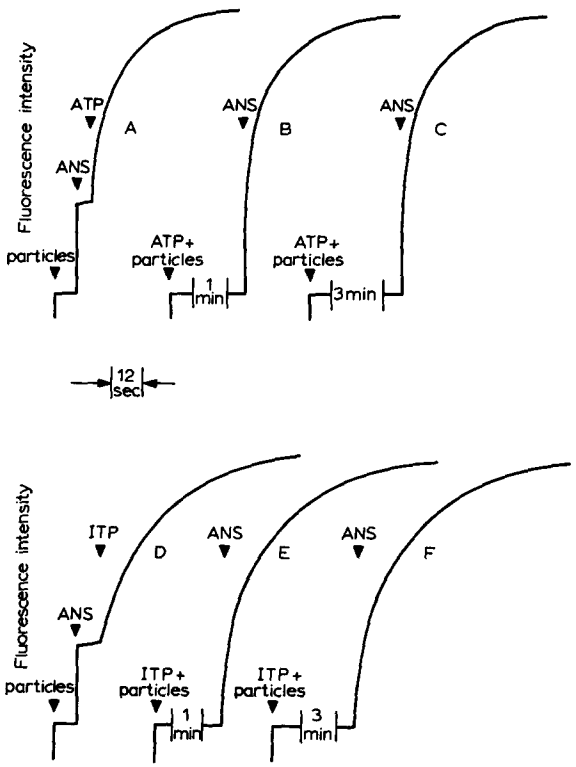

Fig. 2. Effect on the time course of ANS fluorescence intensity of energising submitochondrial particles before addition of ANS. Conditions were as described for Fig. 1 except that where indicated the particles were energised, either with ITP or ATP as shown, before addition of ANS. Trace A: ANS added before energisation by ATP. Trace B: ANS added 1 minute after energisation by ATP. Trace C: ANS added 3 min after energisation by ATP. Trace D: ANS added before energisation by ITP. Trace E: ANS added 1 min after energisation by ITP. Trace F: ANS added 3 min after energisation by ITP.

TABLE II

RATE CONSTANTS FOR THE RATE OF ANS AND MNS FLUORESCENCE INTENSITY INCREASE UPON ADDING THE PROBES TO ENERGISED SUBMITOCHONDRIAL PARTICLES

Rate constants were measured on a single batch of submitochondrial particles. Conditions were as described in the legends to Figs. 1 and 4.

Probe used	Particles energised after addition of probe		Particles energised 3 min before addition of probe	
	$k_{ATP}(s^{-1})$	$k_{ITP}(s^{-1})$	$k_{ATP}(s^{-1})$	$k_{ITP}(s^{-1})$
ANS	0.14	0.077	0.12	0.06
MNS	0.015	0.007	0.014	0.006

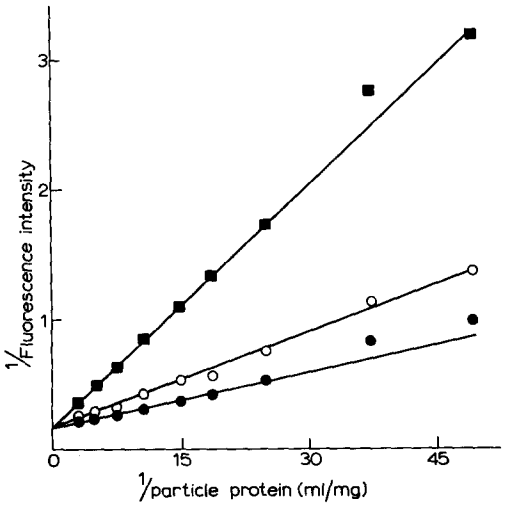

Fig. 3. Double-reciprocal plots for titrations of ANS with submitochondrial particles. The ANS concentration was 5 μM and the buffer as described in the legend to Fig. 1. ■—■, unenergised particles; ○—○, particles energised by 2 mM ITP, 2 mM magnesium chloride; ●—●, particles energised by 2 mM ATP, 2 mM magnesium chloride.

ANS is added before the substrate (Table II). The rapid increase in ANS fluorescence is too fast to measure with our instrument but corresponds to the rapid fluorescence increase which is seen when ANS is added to submitochondrial particles which are not energised. A half time for this process has been estimated as 5 ms [2]. Fig. 2 and Table II both demonstrate that the extent of the delay between adding magnesium-ATP and ANS does not affect the kinetics of the ANS fluorescence increase.

As mentioned in the introduction, the ANS fluorescence change observed when energy is supplied to submitochondrial particles by ATP is thought to be a consequence of more ANS binding to the particles, rather than an increase in the intrinsic fluorescence of the bound ANS. One of the methods used to reach this con-

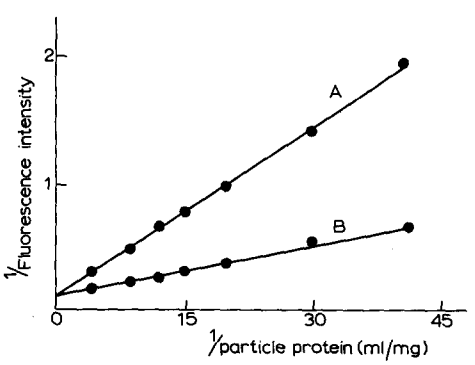

Fig. 4. Double-reciprocal plots for titrations of MNS with submitochondrial particles. The MNS concentration was 5 μM and the buffer as described in the legend to Fig. 1. Line A; unenergised particles. Line B; particles energised by 2 mM ATP, 2 mM magnesium chloride.

clusion has been the construction of a double-reciprocal plot of (ANS fluorescence)$^{-1}$ against (particle concentration)$^{-1}$. Although such a plot has been referred to previously [4], the data have not been presented. Fig. 3 shows such a plot which confirms that the response to energisation is a result of increased probe binding. Supporting evidence for this has come from preliminary measurements of the lifetime of ANS when bound to submitochondrial particles. A heterogeneous population of lifetimes was observed and upon energisation with ATP no change in this population could be detected (Ferguson, S. J., Lloyd, W. J. and Radda, G. K., unpublished observations).

The probe MNS can sense energisation of submitochondrial particles in a similar manner to ANS [2, 3] and differs only in having a slower response time to energisation by ATP as shown in Table I. As with ANS the fluorescence enhancement can also be shown to originate in an increased binding of MNS by the particles (Fig. 4). Table II shows that adding MNS to energised submitochondrial particles results in a very similar rate of fluorescence enhancement as occurs when the probe is added first.

Effect of reducing the rate of energy input on the parameters of the ANS and MNS responses.

The data presented in the previous section support the view that the rates at which the two fluorescence probes respond to energisation by ATP do not correspond to a rate at which the particles become fully energised, but rather reflect the speed with which the probes can increase their binding to the energised membranes. In this case these rates may be sensitive to the extent to which submitochondrial particles are energised. This proposition was examined by arranging conditions where the energisation was reduced. Two methods were used, the first of which took advantage of the fact that the mitochondrial ATPase hydrolyses ITP more slowly than ATP [13]. The second method involved partial inactivation of the ATPase with either 4-chloro-7-nitrobenzofurazan (Nbf-Cl) [11, 14] or *N,N'*-dicyclohexylcarbodiimide (DCCD) [15].

When ITP was used as an energy source for submitochondrial particles both ANS and MNS fluorescence was enhanced at approximately half the rate observed with ATP (Fig. 1, Table I). Fig. 2 and Table II demonstrate that this slower rate of probe response was also observed if the probe was added at various times after energising the particles. Fig. 3 reveals that the ANS response was again due to an increased binding of the probe. The particles used in these experiments hydrolysed ITP at approximately 40 % of the rate at which ATP was converted to ADP, and showed a similar difference in the rates at which the two nucleoside triphosphates were able to support the energy-linked transhydrogenase reaction. This was in agreement with previous reports [16]. Thus reduction in the rate of probe response when ITP was used instead of ATP closely followed the decrease in nucleoside triphosphatase activity. ITP supported a final fluorescence level with both probes which had usually about 80 % of the intensity seen with ATP. 5 μM ANS or MNS had no effect on the rates of ATP or ITP hydrolysis. Exactly the same rates of ANS response to ATP energisation were observed at 10 μM and 20 μM ANS. Therefore the ATPase is not inhibited by the two probes.

In contrast, when the rate of nucleoside triphosphate hydrolysis was reduced by partial covalent inhibition of the ATPase activity with Nbf-Cl, no change in the

TABLE III

FIRST ORDER RATE CONSTANTS FOR ANS FLUORESCENCE INCREASE WITH SUB-MITOCHONDRIAL PARTICLES ENERGISED BY ATP AFTER REDUCTION OF ATPase ACTIVITY BY TREATMENT WITH Nbf-Cl.

Conditions were as described in the legend to Fig. 5. For comparison a rate constant of 0.05 s^{-1} was measured for an ITP energised fluorescence enhancement with the preparation of particles used in these experiments.

ATPase activity %	$k(s^{-1})$
100	0.11
73	0.12
61	0.12
43	0.11
35	0.10
18	0.12

rate at which either ANS or MNS responded to energisation by ATP or ITP was detected (Table I). Table III summarises rate constants observed for ANS at various levels of inhibition. When MNS was used the extent of the fluorescence enhancement was rapidly reduced on treating the particles with Nbf-Cl. This was almost certainly due to the non-specific reaction of Nbf-Cl with membrane thiol groups forming the thiol-Nbf chromophore that absorbs at 420 nm, and so probably quenches the MNS

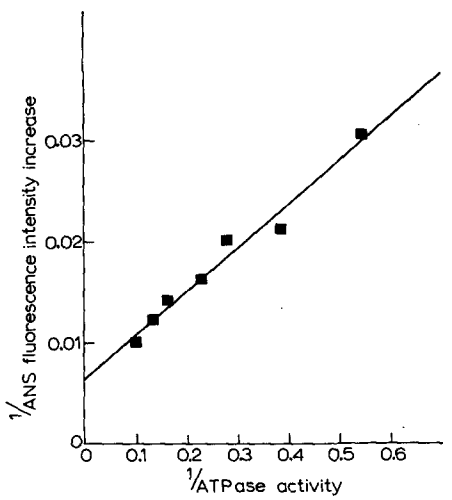

Fig. 5. Double-reciprocal relationship between energy linked ANS fluorescence enhancement and ATPase activity of submitochondrial particles which have been reacted with Nbf-Cl. Submitochondrial particles 22 mg/ml were incubated in 20 mM Tris-HCl, pH 7.7, 225 mM mannitol, 75 mM sucrose at 0 °C with 0.2 mM Nbf-Cl. At appropriate times samples of particles were taken and tested for ATPase activity and ability to support an ATP driven ANS fluorescence enhancement. The data are normalised so that control particles, not treated with Nbf-Cl, gave a fluorescence increase of 100 arbitrary units and had an ATPase activity of 10 arbitrary units. This activity was 0.3 μmol $P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$ when assayed in the buffer used for fluorescence measurements, at 25 °C with 2 mM magnesium chloride and 2 mM ATP.

fluorescence by an energy transfer mechanism. A consequence of this quenching was that the rate of MNS response could be examined only over a limited range of ATPase activity when the fluorescence enhancement was sufficient to make accurate measurements of rates. Fortunately the ANS fluorescence is not appreciably affected in this way, and in a previous publication we showed that reactivation of ATPase activ- by dithiothreitol led to a substantial restoration of ANS fluorescence enhancement [14].

Progressive inhibition of ATPase activity by Nbf-Cl is paralleled by a decrease in the extent of the ANS fluorescence enhancement upon energisation [14]. The relationship between ATPase activity and fluorescence intensity is brought out by plotting the reciprocal of ATPase activity against the reciprocal of the fluorescence increase produced by energisation. Fig. 5 illustrates the linear nature of such a plot. As the particle concentration is constant, the fluorescence level before energisation is also constant so that a plot of (total ANS fluorescence)$^{-1}$ against (ATPase activity)$^{-1}$ is linear as well. A plausible interpretation of this relationship is that reducing ATPase activity is formally equivalent to decreasing the concentration of submitochondrial particles. A titration of a fixed ANS concentration by particles (e.g. Fig. 3) gives a linear double-reciprocal plot because the only variable is the particle concentration, as the number of ANS binding sites per mg protein and the various binding constants which describe these sites remain constant [17]. When the ATPase activity is reduced at a fixed particle concentration as in Fig. 5 then the variable can be either the number of sites or the binding constants.

When ITP is used to energise particles which have been partially inhibited with Nbf-Cl the slower rate of fluorescence enhancement relative to that with ATP is preserved with both ANS and MNS (Table I).

The use of DCCD rather than Nbf-Cl as the ATPase inhibitor produced similar changes in the ANS response. Again the rate of fluorescence enhancement was not altered over a wide range of ATPase activities (Table IV), while a plot of (ATPase activity)$^{-1}$ against (ANS fluorescence increase)$^{-1}$ was also linear (Fig. 6). An advantage of using DCCD is that interaction with the membrane is extremely specific [15, 18] and unlike Nbf-Cl it does not change the optical properties of the membranes.

TABLE IV

FIRST ORDER RATE CONSTANTS FOR ANS FLUORESCENCE INCREASE WITH SUBMITOCHONDRIAL PARTICLES ENERGISED BY ATP AFTER REDUCTION OF ATPase ACTIVITY BY TREATMENT WITH DCCD.

Conditions were as described in the legend to Fig. 6. For comparison a rate constant of 0.043 s^{-1} was measured for an ITP energised fluorescence enhancement with the preparation of particles used in these experiments, which gave slightly slower ANS response, than the particles used in the experiments reported in Table I and Table III.

ATPase activity %	$k(s^{-1})$
100	0.084
45	0.089
31	0.089
25	0.093
18	0.104

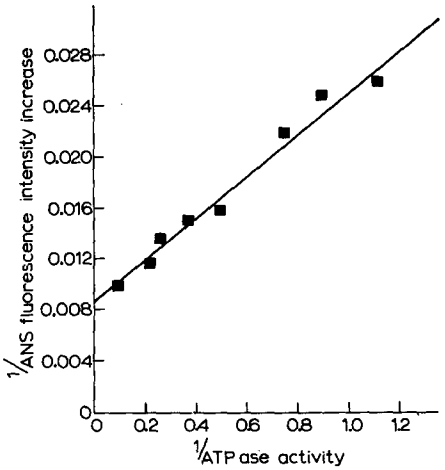

Fig. 6. Double-reciprocal relationship between energy linked ANS fluorescence enhancement and ATPase activity of submitochondrial particles which have been treated with DCCD. 5.6 mg of submitochondrial particles were incubated at 0 °C with 4 μg DCCD in 0.2 ml of 20 mM Tris-HCl, pH 7.4, 225 mM mannitol. 75 mM sucrose. At appropriate times samples of particles were taken and tested for ATPase activity and ability to support an ATP driven ANS fluorescence enhancement. The data are normalised so that control particles, not treated with DCCD, gave a fluorescence increase of 100 arbitrary units and had an ATPase activity of 10 arbitrary units. This activity was 0.2 μmol · min^{-1} · (mg protein)$^{-1}$ when assayed in the buffer used for fluorescence measurements, at 25 °C, with 2 mM magnesium chloride and 2 mM ATP.

It is important for the experiments described here that the only effect of Nbf-Cl or DCCD should be to inhibit ATPase activity. Nbf-Cl has been shown to act on that part of the ATPase which can be released from the mitochondrial inner membrane as a water-soluble protein, often called F_1 [14]. A tyrosine residue which is probably on the surface of the enzyme is modified by Nbf-Cl and it seems unlikely that this could have any effect other than on ATPase activity. The non-specific reaction between Nbf-Cl and membrane thiol groups also only slightly perturbs the membrane since reactivation of the ATPase with dithiothreitol, which does not under our conditions remove the Nbf group from thiols, substantially restores the ANS fluorescence enhancement upon energisation with ATP [14]. DCCD does not inhibit the soluble ATPase (F_1) but acts on a low molecular weight protein which is firmly associated with the membrane [18]. The energy coupling properties of submitochondrial particles which are deficient in ATPase, for instance those which have been treated with alkali, Sephadex and urea, are improved by limited treatment with DCCD [19]. That this coupling effect of DCCD was not significant in our experiments was indicated by the observation that the ANS fluorescence enhancement produced by succinate oxidation was identical both in particles which had been treated with DCCD until ATPase was abolished and in those which were untreated. Furthermore if DCCD had the dual effect of inhibiting ATPase activity and increasing the coupling of the membrane, the relationship between decreasing ATPase activity and ANS fluorescence enhancement (Fig. 6) would not be expected. In summary there is no evidence that in the magnesium-ATP particles used by us there is any significant secondary effect of the ATPase inhibitors.

The nature of the ANS response produced on applying a potassium diffusion gradient to submitochondrial particles

In agreement with the earlier work of others [6, 7], Fig. 7 shows that addition of potassium chloride to a suspension of submitochondrial particles which have been treated with valinomycin gives rise to a very rapid increase in ANS fluorescence. This may be related to the generation under these conditions of a potassium diffusion potential, positive inside and negative outside the particles. The subsequent decrease of the ANS fluorescence probably parallels the decay of this potential. The ANS fluorescence enhancement is more rapid than the response time of our fluorimeter, but a reasonable estimate is that the ANS fluorescence increases upon application of a diffusion gradient at least ten times faster than when ATP is used to energise the particles.

Bakker and Van Dam [9] observed similar changes in ANS fluorescence on applying potassium diffusion gradients to phospholipid liposomes. In their experiments a double reciprocal plot of ANS fluorescence against lipid concentration indicated that the ANS fluorescence changes resulted from an increase in quantum yield upon imposing a diffusion potential. This result differs from that obtained in this laboratory for the nature of the potassium diffusion potential induced ANS response in submitochondrial particles [4]. In those experiments the origin of the enhancement was concluded to be an increased binding of the probe. The crucial difference between the experiments on liposomes and those on submitochondrial particles is that in the latter case a double-reciprocal plot was constructed by varying the particle concentration while keeping the valinomycin fixed whereas the data with liposomes was obtained with a constant liposome to valinomycin ratio. Maintenance of a constant particle to valinomycin ratio ensures that the same potential is produced for each particle as the concentration of submitochondrial particles is varied. When the experiments were done using these conditions the double reciprocal plot shown in

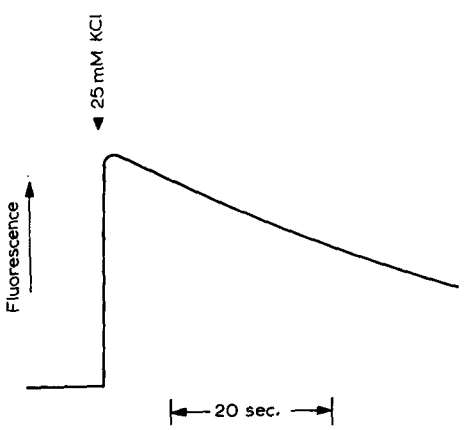

Fig. 7. The time course of the fluorescence intensity of ANS upon applying a potassium diffusion gradient to submitochondrial particles. 2 mg submitochondrial particles, washed free of potassium ions, were incubated with 0.7 μg valinomycin using 2 ml of the buffer described in Fig. 1. A pulse of potassium chloride sufficient to make the solution 25 mM in the salt was then added and the change in ANS fluorescence monitored. The final fluorescence level was the same as that observed if 25 mM potassium chloride was added in the absence of valinomycin.

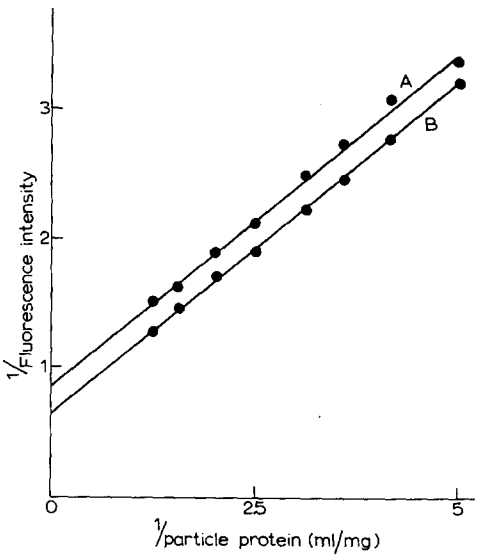

Fig. 8. Double-reciprocal plot of the fluorescence intensity of ANS as a function of the submitochondrial particle concentration, after addition of potassium chloride in the presence of valinomycin. The conditions were as described in the legend to Fig. 7. The ratio valinomycin/submitochondrial particles was 0.35 μg valinomycin per mg particles. Line A. ANS fluorescence with particles in the presence of 25 mM potassium chloride. No valinomycin. Line B. ANS fluorescence with particles in the presence of potassium chloride and valinomycin. The fluorescence intensity was measured 12 s after the addition of the potassium chloride. A least squares fit gave a gradient of 0.52 with standard deviation of 0.02 for line A, and a gradient of 0.50 with a standard deviation of 0.01 for line B.

Fig. 8 was obtained. A quantum yield change for ANS is evident from the distinct intercepts on the ordinate at infinite particle concentration for the particles in the presence and absence of the potential. It is appropriate to recognise at this point that the use of double-reciprocal plots, or in other words extrapolation to infinite particle concentration, may not always be a valid procedure. Nevertheless, since the plot for particles under the influence of a potential is virtually parallel to that for resting particles, it seems unlikely in this instance that any non-linearity in the plots at high protein concentration could alter the conclusion that a quantum yield change here is the main, if not the sole, cause of ANS fluorescence enhancement.

The finding that MNS responded more slowly than ANS to energisation by ATP prompted an examination of the way in which MNS reported on potassium diffusion potentials. However, in a number of experiments under conditions where ANS responded, no increase in MNS fluorescence could be observed.

DISCUSSION

A notable feature of the ATP supported ANS fluorescence enhancement in submitochondrial particles is that the rate of this response is independent of the number of active ATPase enzymes (Tables III and IV). As the extent of the fluorescence change decreases with increasing inhibition of ATPase activity (Figs. 5 and 6), it appears that the response rate is not dependent on the extent to which the particles

are energised. However, if the turnover rate of the ATPases is reduced by using ITP rather than ATP as the energy source, then an approximately proportional decrease in the rate of fluorescence enhancement does occur (Fig. 1, Table I). Apparently the kinetics of the ANS response are directly sensitive to events at the level of the ATPase. Exactly the same arguments are applicable when the probe MNS, which has a different characteristic response time, is used (Table I).

An interpretation of this data requires an understanding of factors which control the rates of ANS responses. The simplest view is that these rates represent the speed at which ANS (or MNS) is able to move to membrane binding sites which develop as a result of energisation. These binding sites are different from most of the sites in non energised submitochondrial particles to which ANS binding is extremely rapid, since if the effect of energisation were simply to increase the affinity of these sites, then the relatively slow rates of energy linked fluorescence enhancement would not be expected. The kinetics of ANS (or MNS) responses to energisation could well reflect the kinetic barrier to the dyes reaching the "slow" (= slowly accessible) sites. A rationale of the data reported in this paper could be that the ANS moves into these sites via regions of the membrane which are directly affected by an ATPase. Consequently the rate constant for the energy linked responses is not altered when a proportion of these ATPases are covalently inhibited, since in this situation the ANS would enter the membrane only via those areas that are directly associated with an active ATPase. However, when a more slowly hydrolysed substrate such as ITP is used, then the ANS can move to "slow" sites less quickly. Possible explanations for this are that either ITP maintains the membrane in a less energised conformation which presents a greater kinetic barrier to the ANS, or that the rate of charge separation within particular areas of the membrane may be crucial so that ITP supports a lower rate of entry than ATP. There can be no direct relationship between the ATP hydrolysed and the number of ANS molecules taken up. This is the obvious consequence of the fact that addition of various amounts of ANS to submitochondrial particles will, for the same amount of ATP hydrolysed, result in differing extents of ANS binding.

Any explanation of the origin of the ANS response should take into account the relationship between the ATPase activity and the extent of ANS fluorescence enhancement as shown in Figs. 5 and 6. Most studies on energy linked ANS fluorescence changes with submitochondrial particles have attributed most of the change to a decrease in the dissociation constants for the ANS membrane interaction [2, 20]. For the reasons outlined above we believe that the response involves movement of the probe to the "slow" sites. In this context three interpretations of Figs. 5 and 6 are considered here. Firstly it might be that there are regions of the membrane which are associated with an inactive ATPase and so can no longer contribute to the pool of sites which becomes available upon energisation. Implicit within this proposal is the idea that the membrane is locally energised. On the other hand if the extent of the ANS response reflects an overall energisation of submitochondrial particles, then decreasing the ATPase activity could reduce the number of ANS sites since the extent of energisation will also decline. If this is the appropriate description then the extent of overall energisation must follow rather closely the rate of energy input from ATP, and energy leaks must not become more significant at low ATPase activities. The third interpretation is essentially similar to the second except that the result of reduced energisation would be to increase the dissociation constants rather than decrease the

number of sites. It will be hard to distinguish between these possibilities but the observations on the rates of ANS (and MNS) responses reported in this paper do lend some support to the idea that the fluorescent probes may sample local energisation.

The finding that in the presence of ANS the application of a potassium diffusion gradient to submitochondrial particles results in an increased fluorescence from the probe, has been used as evidence for the substrate generated ANS response indicating membrane potentials [6, 7]. From the experiments presented in this paper, there appear to be two reasons why it may not be valid to equate the diffusion potential with the energised state produced by the hydrolysis of ATP. Firstly the rate at which ANS responds to energisation by ATP is markedly slower than its response to a diffusion potential, even when ample time is allowed for the ATP supported energised state to reach an equilibrium value (Table II). Under the conditions of the experiments described here the potassium chloride pulse in the presence of valinomycin should generate a membrane potential which may be estimated by the Nernst equation to be approximately 100 mV [7]. According to the chemiosmotic hypothesis this is the same order of magnitude as might be expected to result from ATP hydrolysis by a coupled submitochondrial particle, so that on this basis the fluorescence enhancements produced would be expected to have similar characteristics. It is also noteworthy that the extent of the ANS fluorescence increase produced by the diffusion potential is significantly less than that obtained by ATP hydrolysis, as has been previously reported by Azzi et al. [7].

The reason for the discrepancy between these rates of ANS response becomes clearer when the second important difference between these two types of ANS response is examined. Comparison of Fig. 4 and Fig. 8 provides a clear indication that the diffusion potential produces an increase in the ANS quantum yield while ATP simply increases the fraction of ANS which is bound to the particles. This difference explains why a diffusion potential elicits a much faster response from ANS than energisation by ATP. A further significant point is that MNS was not able to respond to a diffusion gradient in contrast to its ability to reflect energisation by ATP or respiratory chain substrates [2, 3]. Taken together these observations suggest that the physical basis for the diffusion potential supported ANS response is distinct from that supported by ATP. However, both types of response are collapsed by uncouplers which means that, if the two responses are different, then a dual role for uncouplers cannot be ruled out. In one case they collapse a trans membrane potential but in the other they dissipate a different but undefined energised state of the membrane.

At this point it is pertinent to refer to the work on probe responses to applied potentials across excitable and artificial membranes. Conti and his coworkers [21] have found that application of voltage pulses to nerves or bilayers which have been stained with various *N*-aryl-aminonaphthalene dyes produce proportional changes in the fluorescence of these molecules. Significantly no correlation was found between the charge on the dye and the nature of the fluorescence change (enhancement or quenching). By way of explanation it was suggested that the crucial effect leading to these changes was an interaction of the large electric dipoles of excited states of the probes with the applied electric field. An electrophoretic mechanism was not consistent with the data. In their experiments with liposomes Bakker and Van Dam [9] reached the conclusion that the imposition of a potassium diffusion potential pushed ANS into the membrane and increased its quantum yield but again excluded an

electrophoretic mechanism. In view of these findings it is not unreasonable that in submitochondrial particles the formation of a diffusion gradient should increase the intrinsic fluorescence of the bound ANS molecules rather than increase the probe binding. The work on nerves may also be relevant to understanding why MNS cannot report a potassium diffusion potential in submitochondrial particles.

To summarise, several experiments described in this paper lead to the conclusion that the fluorescence of ANS should not be used as an indicator of membrane potential in submitochondrial particles. In our opinion the phenomena which ANS probes remain to be established. Attempts to do this have perhaps been obscured by arguments centering on whether inner mitochondrial membranes are permeable to ANS. This is not necessarily crucial. It is worth noting though that the aryl sulphonate, diazobenzenesulphonate, has been used as a membrane impermeable reagent for studying inner mitochondrial membranes [22]. If our reservations about using ANS fluorescence as a membrane potential indicator with submitochondrial particles are valid, then the basis of other similar methods such as the uptake of synthetic anions like phenyldicarbaundecaborane [5] may be affected, although the properties of most of these ions are rather different from ANS. It would be of interest to know if the rate of uptake of these ions is sensitive to the substitution of ATP by ITP as energy source, but insensitive to a decrease in ATPase activity by covalent modification of the mitochondrial ATPase.

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Azzi and Santato [23] have also shown that the ANS fluorescence enhancement seen when submitochondrial particles are energised by ATP is due to an increased binding of the probe to the membranes.

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