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A Kinetic Analysis of the Changes in Fluorescence on the Interaction of 8-Anilinonaphthalene-1-sulphonate with Submitochondrial Particles

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A comparison of the fluorescence change on the addition of 8-anilinonaphthalene-1-sulphonate to succinate-energized submitochondrial particles with that on the addition of succinate to submitochondrial particles incubated with 8-anilinonaphthalene-1-sulphonate shows that these changes in fluorescence may be explained solely in terms of 8-anilinonaphthalene-1-sulphonate binding. This comparison does not support the proposal of an 8-anilinonaphthalene-1-sulphonate-monitored change in the conformation of submitochondrial-particle membranes [Brocklehurst, Freedman, Hancock & Radda (1970) *Biochem. J.* **116**, 721–731]. The biphasic nature of the decrease in fluorescence, which was found to follow the addition of uncoupler to submitochondrial particles incubated with ATP or succinate, or of antimycin A to submitochondrial particles incubated with succinate, does not support the existence of 'aplectic' and 'symplectic' states of the mitochondrial membrane [Barrett-Bee & Radda (1972) *Biochim. Biophys. Acta* **267**, 211–215].

Whether the energy-dependent changes in the fluorescence of 8-anilinonaphthalene-1-sulphonate bound to submitochondrial particles are caused entirely by changes in binding (Azzi, 1969; Azzi & Santato, 1971, 1972*a*; Azzi & Vainio, 1969) or partly by changes in binding and partly by changes in membrane conformation, leading to a change in the quantum yield of the already bound 8-anilinonaphthalene-1-sulphonate (Freedman *et al.*, 1969; Brocklehurst *et al.*, 1970; Barrett-Bee & Radda, 1972), is unresolved. Radda and his co-workers (Freedman *et al.*, 1969; Brocklehurst *et al.*, 1970) found that energization by succinate of submitochondrial particles incubated with 8-anilinonaphthalene-1-sulphonate resulted in a biphasic increase in fluorescence. Subsequent addition of uncoupler caused a biphasic decrease in fluorescence. The half-times that they found for the fluorescence increase were 3s and 20s and for the decrease 3s and 12s. They attributed the faster of these changes in fluorescence to a change in the quantum yield of the already bound probe, this resulting from an energy-dependent change in the conformation of the submitochondrial-particle membrane. The slower change was attributed to changes in 8-anilinonaphthalene-1-sulphonate binding, which followed the energy state of the membrane. These attributions are partly based on the observation that the maximum fluorescence value in plots of reciprocal fluorescence against reciprocal submitochondrial-particle concentration, at a fixed 8-anilinonaphthalene-1-sulphonate concentration, is increased on energization by succinate, and that a change in this maximum fluorescence value shows that part of the energy-dependent change in 8-anilinonaphthalene-1-sulphonate fluorescence monitors a change in membrane conformation (Brocklehurst *et al.*, 1970; Radda, 1971). But it is also possible that a change in the maximum fluorescence value found from this plot can result from a redistribution of 8-anilinonaphthalene-1-sulphonate between binding sites, with different quantum-yield values for bound 8-anilinonaphthalene-1-sulphonate (Gains & Dawson, 1975*a*).

In subsequent papers Radda and his co-workers (Barrett-Bee & Radda, 1972; Barrett-Bee *et al.*, 1972; Radda & Vanderkooi, 1972; Radda, 1975) have proposed that the energy-dependent changes in 8-anilinonaphthalene-1-sulphonate fluorescence can monitor the changes between four membrane states, aplectic or symplectic and unenergized or energized.

The kinetics of the interaction of 8-anilinonaphthalene-1-sulphonate with submitochondrial particles have been investigated with special reference to the possible occurrence of an energy-dependent 8-anilinonaphthalene-1-sulphonate-monitored transition in submitochondrial-particle membrane conformation.

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Materials and Methods

Ox heart mitochondria were prepared in an isolation medium containing 250mM-sucrose buffered at pH 7.6 with 10mM-Tris/HCl. The isolation of the mitochondria was based on the methods of Blair (1967) and Smith (1967). The isolated mitochondria were stored at −16°C, in a medium containing 250mM-sucrose buffered at pH 7.55 with 5mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid]/KOH until required for the preparation of the submitochondrial particles.

Ox heart submitochondrial particles were prepared as follows. The frozen mitochondria were thawed and washed once in a medium containing 250mM-sucrose buffered at pH 7.55 with 5mM-Hepes/KOH by centrifugation at 40000*g* for 15min. The pellet was resuspended at about 10mg of protein/ml and sonicated at 1.5A at 20000Hz for 60s in a 60W MSE Ultrasonic Disintegrator. The sonication vessel was cooled in a bath containing an ice/ethanol mixture at −10°C. The sonicated suspension was centrifuged at 40000*g* for 15min. The supernatant from this was centrifuged at 105000*g* for 60min. The pellet was resuspended, with the aid of a glass-on-glass homogenizer, in a medium containing 250mM-sucrose buffered at pH 7.55 with Hepes/KOH, to a concentration of 40mg of protein/ml. If the KCl concentration in the sonication medium was increased by 10mM or if 20mM-Tris/HCl or 15mM-$MgCl_2$ was added, then the yield of submitochondrial particles was decreased. As the energy-dependent changes in 8-anilinonaphthalene-1-sulphonate fluorescence were unaffected by the composition of the sonication medium, that producing the largest yield was used. Apart from the composition of the sonication medium, this preparation follows those of Beyer (1967) and Hansen & Smith (1964).

Protein content of the mitochondrial and submitochondrial-particle suspensions was determined, after clarification with 0.2% Triton X-100, by using biuret reagent (Gornal *et al.*, 1949), with bovine serum albumin as a standard.

Fluorescence was measured in a front-faced fluorimeter constructed in this laboratory. The light source was from a Bausch and Lomb 40W quartz iodide lamp passed through a Wratten 18a filter. The maximum output was at 380nm. The emitted light was analysed at 480nm with a band-width of 10nm. The contents of the 15mm-square cuvette were continuously stirred and maintained at 30°C. The combined half-times of mixing and recorder response, as judged by the increase in fluorescence on the addition of 8-anilinonaphthalene-1-sulphonate to 70% (v/v) ethanol or to a 0.1% solution of Triton X-100, were between 100 and 200ms.

In analysing the experimental data it has been assumed that the interaction of 8-anilinonaphthalene-1-sulphonate is pseudo-first-order and that it is valid to use a plot of log [fluorescence change (total less partial)] against time (Azzi *et al.*, 1969; Azzi & Santato, 1972*b*; Brocklehurst *et al.*, 1970; Barrett-Bee & Radda, 1972; Ferguson *et al.*, 1976). The analysed data are an average of at least three reaction curves. The data are not corrected for the combined mixing and response times of the apparatus. This was not thought to be justifiable, as the experimental errors are probably in excess of the correction. Experimental errors are not given, as these are hard to assess, but the repeatability of the analysed half-times is within ±20%, except for the dissociation of 8-anilinonaphthalene-1-sulphonate on the dilution of membrane/fluorochrome mixtures. The half-times given in the text are averages of those at the different 8-anilinonaphthalene-1-sulphonate concentrations given in the corresponding Tables.

Reagents were obtained from the following sources. 8-Anilinonaphthalene-1-sulphonate was from Eastman Kodak Co., Rochester, NY, U.S.A. Antimycin A and ATP were from Boehringer, Mannheim, West Germany. Bovine serum albumin was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Hepes was from Hopkin and Williams, Chadwell Heath, Essex, U.K. Oligomycin, rotenone, succinate and Tris were from Sigma Chemical Co., St. Louis, MO, U.S.A. Valinomycin was from Calbiochem, Los Angeles, CA, U.S.A. Compound 1799, which is a chemical adduct formed from two equivalents of hexafluoroacetone to one of acetone (Racker, 1972), was a gift from Professor R. B. Beechey, Shell Research Ltd., Woodstock Agricultural Research Centre, Sittingbourne, Kent, U.K. The molecular weight of compound 1799 was taken to be 390, which is the sum of the molecular weights of the adducts given by Professor E. Racker. All other reagents were of analytical grade.

Results

Comparison of the fluorescence increase on the addition of 8-anilinonaphthalene-1-sulphonate to submitochondrial particles preincubated with succinate with that on the addition of succinate to submitochondrial particles preincubated with 8-anilinonaphthalene-1-sulphonate

The interaction of 8-anilinonaphthalene-1-sulphonate with submitochondrial particles was studied in a 20mM-Tris/HCl medium and in a 15mM-KCl medium. Figs. 1(*a*) and 1(*b*) are copies of single experimental traces showing the change in fluorescence on the addition of the fluorochrome, substrate and uncoupler or inhibitor against time. The data from at least three such experimental traces were averaged and then analysed by means of a plot of the log of the fluorescence change (total less partial) against time (Fig. 2*a*).

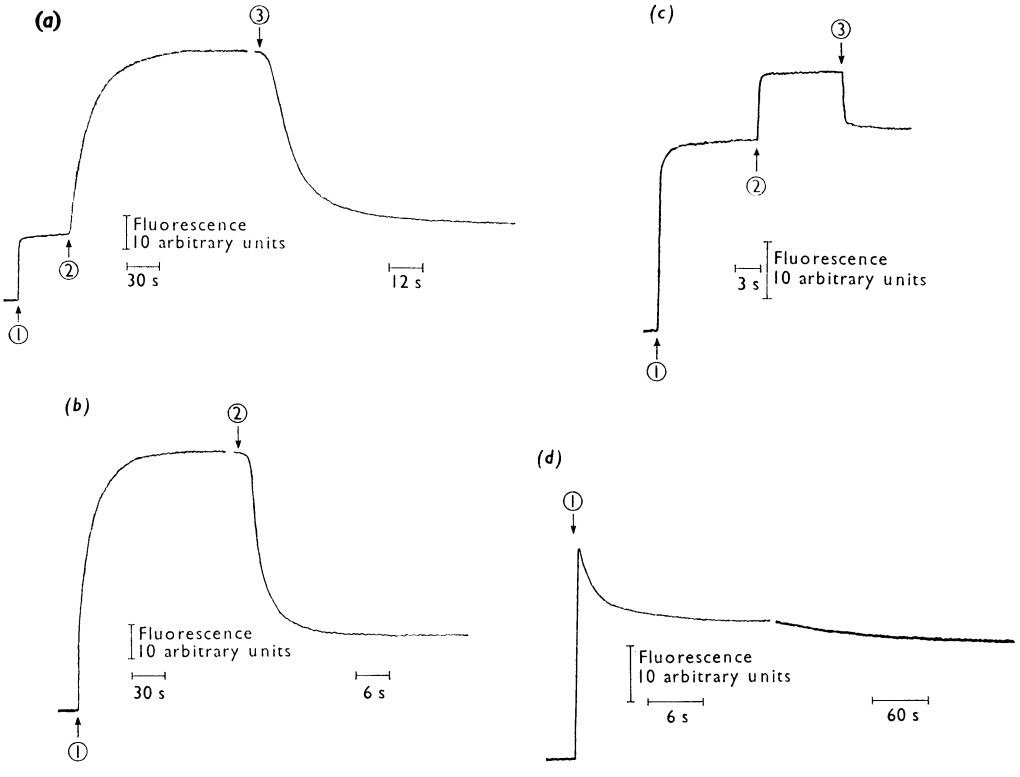

Fig. 1. *Traces of fluorescence change against time showing the effect of (a) adding 8-anilinonaphthalene-1-sulphonate, succinate and antimycin A to submitochondrial particles, (b) adding 8-anilinonaphthalene-1-sulphonate and compound 1799 to submitochondrial particles preincubated with succinate, (c) adding 8-anilinonaphthalene-1-sulphonate, $MgCl_2$ and EDTA to submitochondrial particles and (d) diluting by 100-fold a mixture of 8-anilinonaphthalene-1-sulphonate and submitochondrial particles*

For (*a*) the assay medium contained 250mM-sucrose, 5mM-Hepes, 20mM-Tris/HCl, pH 7.55, rotenone at 2μg/ml, oligomycin at 2μg/ml and protein at 0.28mg/ml. The additions (arrows) were (1) 5μM-8-anilinonaphthalene-1-sulphonate, (2) 5mM-succinate and (3) antimycin A at 0.1μg/ml. For (*b*) the assay medium contained in addition 5mM-succinate. The additions (arrows) were (1) 5μM-8-anilinonaphthalene-1-sulphonate and (2) 30μM-compound 1799. For (*c*) the assay medium was the same as for (*a*). The additions (arrows) were (1) 5μM-anilinonaphthalene-1-sulphonate, (2) 1mM-$MgCl_2$ and (3) 2.5mM-EDTA. For (*d*) the assay medium, apart from the initial omission of the submitochondrial particles, was the same as for (*a*). The addition (arrow) was a mixture of 8-anilinonaphthalene-1-sulphonate and submitochondrial particles, which gave after a 100-fold dilution concentrations of 5μM and 0.28mg/ml respectively.

As demonstrated by the increase in fluorescence, the rate at which 8-anilinonaphthalene-1-sulphonate binds to energized and to unenergized submitochondrial particles is polyphasic. At least three phases may be distinguished, one or more of which are too fast to be measured by the apparatus used (see, however, Chance *et al.*, 1969) and two slower phases. For the fluorochrome binding to unenergized submitochondrial particles the half-times of the slower phases are 1.1s and 6.2s in the Tris/HCl medium (Table 1*a*) and 0.7s and 4.8s in the KCl medium (Table 2*a*). Subsequent addition of succinate causes a biphasic increase in fluorescence with half-times of 6.3s and 20s in the Tris/HCl medium (Figs. 1*b* and 2*a* and Table 1*a*) and 3.8s and 9.0s in the KCl medium (Table 2*a*). Half-times similar to these have been reported by Radda and his co-workers (Freedman *et al.*, 1969; Brocklehurst *et al.*, 1970). Although they attributed the faster of these fluorescence changes to a transition in membrane conformation monitored by the already bound fluorochrome and the slower phase to an increase in the amount of fluorochrome bound, they did not completely eliminate the possibility that both fluorescence increases might be caused by an increase in the amount bound. If Radda and his co-workers are correct, the faster of these two fluorescence

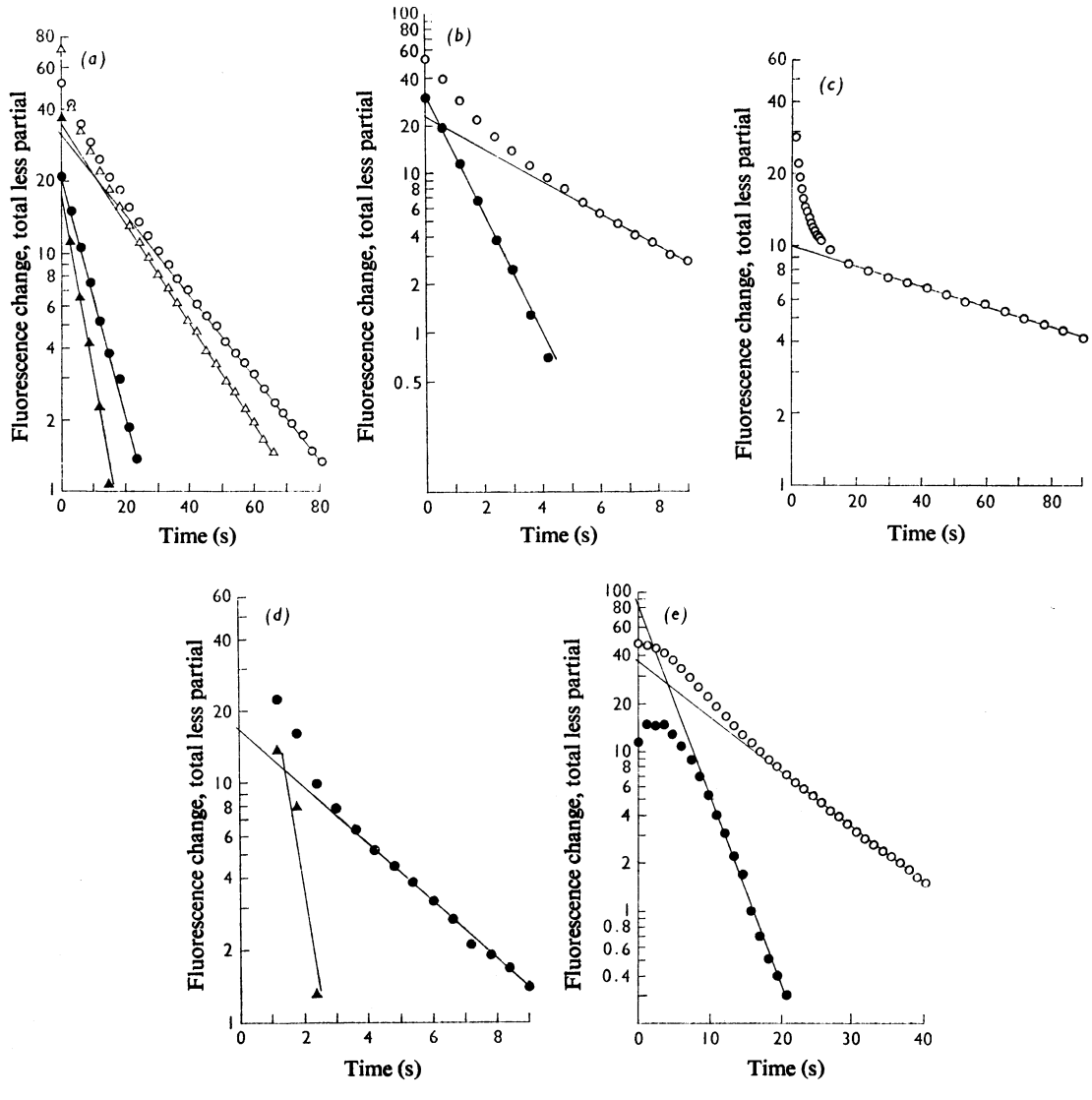

Fig. 2. *Plots of fluorescence change (total less partial) against time, (a) showing the independence of the fluorescence increase from the order of addition of succinate and 8-anilinonaphthalene-1-sulphonate, (b) the fluorescence decrease on the addition of compound 1799 to submitochondrial particles preincubated with 8-anilinonaphthalene-1-sulphonate and succinate, (c) and (d) the decrease on the dilution of a mixture of 8-anilinonaphthalene-1-sulphonate and submitochondrial particles and (e) the fluorescence decrease on the addition of antimycin A to submitochondrial particles preincubated with succinate and 8-anilinonaphthalene-1-sulphonate*

For (*a*) the assay medium was the same as for Fig. 1(*a*). △, ▲, 5 μM-8-anilinonaphthalene-1-sulphonate added to submitochondrial particles preincubated with 5 mM-succinate; ○, ●, 5 mM-succinate added to submitochondrial particles preincubated with 5 μM-8-anilinonaphthalene-1-sulphonate; ○, △, analysis of the slow rate; ●, ▲, analysis of the fast rate. For (*b*) the assay medium was the same as for Fig. 1(*a*), except for the addition of 5 mM-succinate and 5 μM-8-anilinonaphthalene-1-sulphonate. An analysis of the slow rate (○) and of the fast rate (●) is shown. For (*c*) and (*d*) the assay medium was the same, except for the initial omission of the submitochondrial particles as for Fig. 1(*a*). An analysis is shown of (*c*) the slow rate (○) and of (*d*) the medium (●) and the fast (▲) rates of the fluorescence decrease after the 100-fold dilution of a mixture of 8-anilinonaphthalene-1-sulphonate and submitochondrial particles; after dilution the concentrations were 5 μM and 0.28 mg/ml respectively. For (*e*) the assay medium was the same as for Fig. 1(*a*), except for the additions of 5 mM-succinate and 5 μM-8-anilinonaphthalene-1-sulphonate. An analysis of the slow rate (○) and of the fast rate (●) on the addition of antimycin A at 0.1 μg/ml is shown.

Table 1. *Kinetic analysis of the interaction of 8-anilinonaphthalene-1-sulphonate with submitochondrial particles in a Tris/HCl medium*

The assay medium was the same as for Fig. 1. Extent is measured in arbitrary units. The change in fluorescence is that induced by the last addition in each list.

(*a*) Binding of 8-anilinonaphthalene-1-sulphonate to unenergized and to succinate-energized submitochondrial particles
When present, succinate was at 5mM

Concn. (μM) of fluorochrome ...	0.5		5		50	
	$t_{\frac{1}{2}}$ (s)	Extent	$t_{\frac{1}{2}}$ (s)	Extent	$t_{\frac{1}{2}}$ (s)	Extent
Particles plus fluorochrome					—	94.5
					1.1	9.2
					6.2	11.6
Particles and succinate plus fluorochrome	—	2.3	—	19	—	114
	3.6	1.3	4.0	19	2.6	74
	12.6	7.6	14.0	35	15.6	34
Particles and fluorochrome plus succinate	6.5	3.7	6.0	21	3.0	76
	21.0	6.6	18.0	31	24.0	38

(*b*) Decrease in fluorescence after the de-energization of submitochondrial particles incubated with succinate and 8-anilinonaphthalene-1-sulphonate
Succinate was at 5mM; when added, antimycin A was at 100ng/ml and compound 1799 was at 30μM

Concn. (μM) of fluorochrome ...	0.5		5		50	
	$t_{\frac{1}{2}}$ (s)	Extent	$t_{\frac{1}{2}}$ (s)	Extent	$t_{\frac{1}{2}}$ (s)	Extent
Particles, succinate and fluorochrome	2.4	2.9	2.6	34	2.8	54
plus antimycin A	7.8	5.2	8.4	14	11.3	27
Particles, succinate and fluorochrome	1.2	4.1	0.8	28	1.0	67
plus compound 1799	5.0	6.8	3.0	23	4.5	29

(*c*) Increase in fluorescence on the addition of ATP and the subsequent decrease in fluorescence on the addition of compound 1799 to submitochondrial particles incubated with 8-anilinonaphthalene-1-sulphonate (2μM)
$MgCl_2$ (5mM) was added to the basic assay medium. When present, ATP was at 0.5mM and compound 1799 was at 30μM

	$t_{\frac{1}{2}}$ (s)	Extent
Particles and fluorochrome plus ATP	9.3	3.8
Particles, fluorochrome and ATP plus compound 1799	0.7	1.4
	3.2	2.8

increases, the one that they propose is characteristic of a change in membrane conformation, should not be present in the fluorescence increase after the addition of 8-anilinonaphthalene-1-sulphonate to submitochondrial particles preincubated with succinate. Under these conditions the membrane transition should have occurred before the addition of the fluorochrome and the increase in fluorescence should be caused entirely by an increase in the fluorochrome binding.

A copy of an experimental trace showing the fluorescence increase on the addition of 8-anilinonaphthalene-1-sulphonate to submitochondrial particles preincubated with succinate is given in Fig. 1(*b*). Averaged values of these and similar data are shown in Fig. 2(*a*) in the form of a semi-log plot of fluorescence against time. Such an analysis shows that the addition of the fluorochrome to submitochondrial particles preincubated with succinate results in a polyphasic increase in fluorescence. In both the Tris/HCl and the KCl media, this may be resolved into one or more phases, the half-times of which are too fast to measure and the extents of which correspond to those of the fluorochrome interacting with unenergized submitochondrial particles. In addition, two slower components of the fluorescence increase may be resolved, with half-times of 3.4s and 14s in the Tris/HCl medium and of 3.5s and 9s in the KCl medium (Tables 1*a* and 2*a*). The half-times of these phases in the KCl medium are the same as, and in the Tris/HCl medium similar to, those caused by the addition of succinate to submitochondrial particles preincubated with 8-anilinonaphthalene-1-sulphonate. The independence of the existence of the

Table 2. *Kinetic analysis of the interaction of 8-anilinonaphthalene-1-sulphonate with submitochondrial particles in a KCl medium*

The assay medium was the same as that for Fig. 1 except that the 20mM-Tris/HCl was replaced by 12.5mM-KCl and the pH was adjusted to 7.55 by the addition of KOH. Extent is measured in arbitrary units. The change in fluorescence is that induced by the last addition in each list.

(*a*) Binding of 8-anilinonaphthalene-1-sulphonate to unenergized and to succinate-energized submitochondrial particles
When present, succinate was at 5mM

Concn. (μM) of fluorochrome ...	0.5		5	
	$t_{\frac{1}{2}}$ (s)	Extent	$t_{\frac{1}{2}}$ (s)	Extent
Particles plus fluorochrome			—	11.4
			0.7	2.2
			4.8	1.3
Particles and succinate plus fluorochrome	—	1.9	—	13.7
	3.1	1.2	3.8	11.0
	8.9	3.4	9.0	16.0
Particles and fluorochrome plus succinate	4.1	1.5	3.4	9.8
	8.9	3.2	9.0	19.6

(*b*) Decrease in fluorescence after the de-energization of submitochondrial particles incubated with succinate and 8-anilinonaphthalene-1-sulphonate
The succinate was at 5mM; when added, the antimycin A was at 100ng/ml and the valinomycin was at 100ng/ml

Concn. (μM) of fluorochrome ...	0.5		5	
	$t_{\frac{1}{2}}$ (s)	Extent	$t_{\frac{1}{2}}$ (s)	Extent
Particles, succinate and fluorochrome plus antimycin A	1.6	1.6	1.4	8.5
	4.1	2.5	5.3	14.6
Particles, succinate and fluorochrome plus valinomycin	1.0	4.2	0.7	24.0

(*c*) Binding of 8-anilinonaphthalene-1-sulphonate (50μM) to antimycin A-inhibited submitochondrial particles
The antimycin A was at 100ng/ml; when added, succinate was at 0.5mM and compound 1799 was at 30μM

	$t_{\frac{1}{2}}$ (s)	Extent
Particles and antimycin A plus fluorochrome	—	63
	1.1	11
	5.2	8
Particles, succinate and antimycin A plus fluorochrome	—	66
	1.1	12
	5.3	10
Particles, antimycin A and fluorochrome plus succinate	3.0	5.8
Particles, antimycin A, fluorochrome and succinate plus compound 1799	0.3	5.2

faster increase in fluorescence, half-time 3–7s, from the order of addition of succinate and the fluorochrome is consistent with the fluorescence increase being caused by the energy-dependent binding of the fluorochrome to submitochondrial particles. It would also seem to eliminate the possibility of an 8-anilinonaphthalene-1-sulphonate-monitored energy-dependent change in membrane conformation.

Kinetics of the fluorescence decrease on the uncoupling of submitochondrial particles incubated with succinate and 8-anilinonaphthalene-1-sulphonate

Radda and his co-workers (Freedman *et al.*, 1969; Brocklehurst *et al.*, 1970) have reported that the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to submitochondrial particles pre-incubated with succinate and 8-anilinonaphthalene-1-sulphonate results in a biphasic decrease in fluorescence. An analysis of the data in Fig. 1(*b*) (see Fig. 2*b* and Table 1*b*) shows that the fluorescence decrease on uncoupling succinate-energized submitochondrial particles with compound 1799 is biphasic, with half-times of 1.0s and 4.0s. These half-times are slower than those, 3.0s and 12s, found by Radda and his co-workers (Freedman *et al.*, 1969; Brocklehurst *et al.*, 1970), who attributed the faster of these changes in fluorescence to a fluorochrome-monitored change in membrane con-

formation and the slower to a change in the amount of the fluorochrome bound. The latter is supported by their observation that the fluorescence decrease on the dilution of a mixture of 8-anilinonaphthalene-1-sulphonate with submitochondrial particles is monophasic, with a half-time of 12s (Brocklehurst *et al.*, 1970). However, the decrease in fluorescence, and presumably the dissociation of the fluorochrome from the submitochondrial particles, can be much faster than this. The decrease in fluorescence which follows the chelation of Mg^{2+} ions by EDTA has a half-time of 200ms or less (Fig. 1*c*). The rate at which 8-anilinonaphthalene-1-sulphonate dissociates from submitochondrial particles and from frozen–thawed mitochondria was also measured. A trace of the fluorescence decrease which follows the dilution of a mixture of the fluorochrome and submitochondrial particle is shown in Fig. 1(*d*). By way of a control it was attempted to monitor the fluorescence decrease that follows the dissociation of the fluorochrome from Triton X-100, phosphatidylcholine liposomes and bovine serum albumin. Either no decrease in fluorescence was observed, or the decrease in fluorescence was too small and too rapid to analyse. However, as subsequent doubling of any one of the reactants only slightly less than doubled the fluorescence, then all, or nearly all, of the dissociation reaction must have occurred within the combined mixing and response times of the apparatus. Figs. 2(*c*) and 2(*d*) show an analysis of the data in Fig. 1(*d*), the result of which is shown in Table 3. This analysis and those for the dissociation of the fluorochrome from submitochondrial particles and from frozen–thawed mitochondria on dilution in a KCl medium (Tables 4*a* and 4*b*) show that the dissociation is polyphasic. Apart from the dissociation that may occur during the mixing time, three phases may be distinguished, with half-times of 0.5–0.9s, 2.5–8.8s and 47–75s. Except for the slowest phase, the half-times of this decrease in fluorescence closely resemble those found on the addition of compound 1799 to succinate-energized submitochondrial particles incubated with the fluorochrome (Tables 1*b*, 3 and 4). This supports the proposal of Azzi and his co-workers (for example Azzi & Santato, 1972*b*) that the changes in fluorescence which occur after the addition of uncouplers to energized submitochondrial particles incubated with 8-anilinonaphthalene-1-sulphonate may be accounted for solely in terms of a decrease in fluorochrome binding.

Table 3. *Decrease in fluorescence after the dilution of a mixture of submitochondrial particles and* 8-*anilinonaphthalene*-1-*sulphonate in a Tris/HCl medium*

The assay medium was the same as for Fig. 1. The final submitochondrial-particle concentration was 0.28mg of protein/ml; that of the 8-anilinonaphthalene-1-sulphonate, after a 100-fold dilution, is given below.

Concn. (μM) of fluorochrome ...	0.5		5		50	
	$t_{\frac{1}{2}}$ (s)	Extent	$t_{\frac{1}{2}}$ (s)	Extent	$t_{\frac{1}{2}}$ (s)	Extent
	0.5	16	—	—	0.7	32
	3.4	0.8	2.5	17	4.4	53
	52	1.3	74	10	47	24

Table 4. *Decrease in fluorescence after the dilution of* (*a*) *a mixture of submitochondrial particles and* 8-*anilinonaphthalene*-1-*sulphonate in a KCl medium, and* (*b*) *a mixture of frozen–thawed washed mitochondrial membranes and* 8-*anilinonaphthalene*-1-*sulphonate*

In (*a*) the assay medium was the same as that for Fig. 1, except that the 20mM-Tris/HCl was replaced by 12.5mM-KCl and the pH was adjusted to 7.55 by the addition of KOH. The final submitochondrial-particle concentration was 0.28mg of protein/ml; that of the 8-anilinonaphthalene-1-sulphonate, after a 100-fold dilution, is given below.

Concn. (μM) of fluorochrome ...		5		50			50	
		$t_{\frac{1}{2}}$ (s)	Extent	$t_{\frac{1}{2}}$ (s)	Extent		$t_{\frac{1}{2}}$ (s)	Extent
	(*a*)	1.2	11	2.2	48	(*b*)	0.9	138
		7.2	3	6.8	36		4.9	24
		59	4	75	22		49	25

In (*b*) the assay conditions were the same as in (*a*), except that the final mitochondrial-membrane concentration was 0.5mg/ml.

Energy-dependent changes in the fluorescence of 8-anilinonaphthalene-1-sulphonate in relation to the proposed aplectic and symplectic states of submitochondrial-particle membranes

The existence of four membrane states, aplectic or symplectic and unenergized or energized, have been proposed by Radda and his co-workers (Barrett-Bee & Radda, 1972; Barrett-Bee *et al.*, 1972; Radda & Vanderkooi, 1972; Radda, 1975). The following summarizes this proposal. As isolated, submitochondrial particles are in the unenergized aplectic state. Addition of succinate causes a transition to the energized symplectic state. This transition is completely reversed by the addition of uncoupler and partially reversed by the addition of antimycin A. The latter causes a transition to the unenergized symplectic state. Energization of submitochondrial particles by ATP causes a transition to the energized aplectic state; subsequent addition of uncoupler reverses this. These changes in membrane state may be monitored by changes in the fluorescence of 8-anilinonaphthalene-1-sulphonate. The change in fluorescence which is characteristic of the transition between the aplectic and symplectic states has a half-time of 3s, and that for the transition between unenergized and energized states has a half-time of between 12s and 20s. From this it should follow that the addition of ATP to submitochondrial particles incubated with the fluorochrome should give rise to a monophasic increase in fluorescence with a half-time of 12–20s, and subsequent addition of uncoupler should result in a decrease in fluorescence with a similar half-time. Similarly, addition of succinate to submitochondrial particles incubated with the fluorochrome and antimycin A should result in a monophasic fluorescence increase with a half-time of 3s, and subsequent addition of uncoupler should result in a decrease in fluorescence with a similar half-time.

The results shown in Figs. 1(*a*), 1(*b*), 2(*a*) and 2(*b*) and in Tables 1 and 2, and which have been discussed above, support the hypothesis of Barrett-Bee & Radda (1972) in that they show that a biphasic increase in fluorescence follows the energization by succinate of submitochondrial particles incubated with 8-anilinonaphthalene-1-sulphonate, and that a biphasic decrease in fluorescence follows the subsequent addition of compound 1799. So too does the observation that the addition of ATP to submitochondrial particles incubated with the fluorochrome results in a monophasic increase in fluorescence (Table 1*c*). Although the subsequent addition of compound 1799 results in a biphasic decrease in fluorescence (Table 1*c*) this is not necessarily in contrast with the proposal of Barrett-Bee & Radda (1972) as the interaction of the uncoupler may not be a single process (Azzi & Santato, 1972*b*); this is discussed below. The decrease in fluorescence which results from the addition of uncoupler to submitochondrial particles incubated with ATP and the fluorochrome has previously been reported to be monophasic by J. A. Berden, G. K. Radda & E. C. Slater (unpublished work reported by Barrett-Bee & Radda, 1972) and to be biphasic by Azzi & Santato (1972*b*). However, the biphasic decrease in fluorescence which follows the addition of antimycin A to submitochondrial particles incubated with succinate and the fluorochrome (Figs. 1*a* and 2*e* and Tables 1*b* and 2*b*) is not only in contrast with the findings of Barrett-Bee & Radda (1972), who reported a monophasic decrease in fluorescence, but also does not support the proposed transition from the energized symplectic to the unenergized symplectic membrane state. Unless it is also proposed that the symplectic and aplectic states are not absolute but intergrade between one another.

The kinetics of the fluorescence increase on the addition of 8-anilinonaphthalene-1-sulphonate to submitochondrial particles incubated with succinate and antimycin A were compared with those on the addition of the fluorochrome to unenergized submitochondrial particles. No difference in the rates was found, but the extent of the fluorescence increase in the latter case was greater (Table 2*c*). Addition of succinate to submitochondrial particles incubated with antimycin A and the fluorochrome results in an increase in fluorescence with a half-time of 3s. Subsequent addition of compound 1799 reverses this increase in fluorescence. The half-time of this decrease is of the order of 0.3s, only slightly slower than the mixing/response half-time of the apparatus.

These results show that the two components of the biphasic increase in fluorescence found on the addition of succinate to submitochondrial particles incubated with 8-anilinonaphthalene-1-sulphonate may be resolved. Only the faster is present on the addition of succinate to submitochondrial particles incubated with antimycin A and the fluorochrome, and only the slower on the addition of ATP. This is superficially consistent with the proposal, by Barrett-Bee & Radda (1972), of aplectic or symplectic and unenergized or energized membrane states. However, their proposal is based on an 8-anilinonaphthalene-1-sulphonate-monitored transition in membrane conformation which, as has been indicated above, seems not to occur.

Interaction of valinomycin with submitochondrial particles incubated with 8-anilinonaphthalene-1-sulphonate and succinate

The addition of valinomycin to submitochondrial particles incubated with 8-anilinonaphthalene-1-sulphonate and succinate in a medium containing 15mM-KCl results in a fluorescence decrease with a half-time of about 1s (Table 2*b*). This decrease in fluorescence presumably follows the outward trans-

membrane electrophoresis of the fluorochrome, which would occur in response to the valinomycin-induced efflux of K^+ ions (Gains & Dawson, 1975*b*). If such an efflux of K^+ ions does occur, it would be of ions accumulated by some energy-dependent process, for the submitochondrial particles were sonicated in a medium containing only one-sixth of the K^+ ion concentration of the assay medium. A similar fluorescence decrease was also observed in a medium containing 100mM-KCl where the initial external to internal K^+ ion ratio was 40:1.

Permeability of mitochondrial membranes to 8-anilinonaphthalene-1-sulphonate

An analysis of the rates at which 8-anilinonaphthalene-1-sulphonate binds to unenergized, succinate-energized and frozen–thawed mitochondria is shown in Table 5. The fluorescence increase in all three cases is polyphasic, with one or more phases of which the half-times are too fast to measure, and two slower phases with half-times of 0.8s and 4.1s. These rates are similar to those of the fluorochrome binding to unenergized submitochondrial particles (Tables 1*a* and 2*a*). Although energization decreases the total fluorescence increase, it has little or no effect on the rates at which the fluorochrome binds.

A comparison of the rates and of the extents of the fluorescence increase that follows the addition of the fluorochrome to whole unenergized and to frozen–thawed mitochondria shows that they are very similar. This indicates that the fluorochrome is a permeant anion of mitochondrial membranes and that these membranes do not measurably, as far as this technique is concerned, slow the rate at which the fluorochrome penetrates the mitochondrial membrane and binds to the internally situated sites. This assumes that in mitochondria there are internally situated sites at which 8-anilinonaphthalene-1-sulphonate binds and fluoresces (Gains & Dawson, 1975*a*). Either the premise or the assumption is consistent with the widely published observation (for example, Azzi *et al.*, 1969) that the fluorochrome fluoresces when bound to sonicated submitochondrial particles.

Table 5. *Kinetic analysis of the interaction of 8-anilinonaphthalene-1-sulphonate with mitochondria*

The assay medium was the same as that for Fig. 1, except that the 20mM-Tris/HCl was omitted, that the pH was adjusted to 7.55 with KOH and that the rotenone concentration was 5μg/ml. The final 8-anilinonaphthalene-1-sulphonate concentration, after a 100-fold dilution, was 20μM. Extent is measured in arbitrary units. The increase in fluorescence is that induced by the last addition in each list.

	$t_{\frac{1}{2}}$ (s)	Extent
Mitochondria plus fluorochrome	—	30
	0.8	20
	3.9	12
Mitochondria and succinate plus fluorochrome	—	20
	0.6	13
	4.2	4
Frozen-thawed mitochondria plus fluorochrome	—	30
	0.8	18
	4.1	13

Discussion

The results in Tables 1(*b*) and 2(*b*) show that the order of addition of 8-anilinonaphthalene-1-sulphonate and succinate to submitochondrial particles has no effect on the presence or the extent of the faster component of the energy-dependent fluorescence increase. This change in fluorescence cannot be monitoring a change in membrane conformation with a half-time of 3s, for when the fluorochrome is added to the submitochondrial particles after the succinate, such a change in membrane conformation would have occurred before the addition of the fluorochrome during the preincubation period. The increase in the fluorescence maximum found in plots of reciprocal fluorescence against reciprocal submitochondrial-particle concentration may be explained if after energization more of the fluorochrome is bound to sites with a higher quantum yield for the bound fluorochrome than before energization (Gains & Dawson, 1975*a*). This faster phase of the fluorescence increase is also present on the addition of succinate to submitochondrial particles incubated with antimycin A and the fluorochrome. The most obvious difference, but as far as the explanation of this result is concerned not necessarily the correct one, between antimycin A-inhibited submitochondrial particles before and after the addition of succinate would be the difference in the oxidoreduction state of the respiratory chain between succinate dehydrogenase and the point at which antimycin A inhibits. This could be the difference between the 8-anilinonaphthalene-1-sulphonate-monitored aplectic and symplectic states proposed by Barrett-Bee & Radda (1972) (see also Chance, 1969). Azzi *et al.* (1969) concluded that none of the energy-dependent changes in fluorescence could be responding directly to the oxidoreduction state of the respiratory chain. But whether or not this conclusion is justified from their observation that the fastest energy-dependent change in fluorescence is 4000 times slower than the slowest change in the oxidoreduction state of the respiratory chain depends on what is meant by 'directly'. In the sense 'without delay' their conclusion is valid, but in the sense 'as a consequence of' it is not necessarily valid. For it is possible that new fluorochrome-binding sites are created simultaneously with the reduction of the respiratory chain, but that the factor that

limits the increase in fluorescence is the rate at which the fluorochrome binds to these sites.

The slower of the energy-dependent increases in fluorescence is independent of the oxidoreduction state of the respiratory chain in that it is induced by ATP when the respiratory chain is oxidized and it is abolished by antimycin A when the respiratory chain is reduced. The results of Layton *et al.* (1974) and of Gains & Dawson (1975*a*) indicate that 8-anilinonaphthalene-1-sulphonate is a permeant anion of mitochondrial membranes. It might be expected that, if a transmembrane potential, positive inside the submitochondrial particles, is generated on energization, then part of the fluorescence increase would be caused by the fluorochrome binding to the internally situated sites as a consequence of its uptake and accumulation. This has been ruled out by Barker *et al.* (1974) on the basis that the half-time with which the fluorochrome penetrates phosphatidylcholine liposomes is much slower than the half-times of the energy-dependent fluorescent changes of the fluorochrome bound to submitochondrial particles. However, it has been shown by Tsong (1975*a*, *b*) that the rate at which the fluorochrome penetrates some phospholipid bilayers can be far more rapid than the rate found by Barker *et al.* (1974). As far as mitochondrial membranes are concerned, the results in Table 5, which show that freeze–thawing mitochondria does not increase the overall rate at which the fluorochrome binds, indicate that the rate at which the fluorochrome penetrates mitochondrial membranes is fast compared with the rate of the energy-dependent changes in fluorescence. If it is accepted on this evidence that the fluorochrome would be accumulated by the energy-dependent transmembrane potential, then the slower of the energy-dependent fluorescence changes must be explicable in terms of it. The rate of the fluorescence increase may follow the rate at which the fluorochrome binds to the internally situated sites rather than the rate at which it is accumulated. This observation is consistent with the observations of Ferguson *et al.* (1976), who found that the rate of the fluorescence increase after the addition of ATP was independent of the degree of inhibition by 4-chloro-7-nitrobenzofuran or *NN'*-dicyclohexylcarbodi-imide of the adenosine triphosphatase activity. However, it is not consistent with their observation that the rate of the fluorescence increase is dependent on whether ATP, ITP or KCl plus valinomycin are used to energize the submitochondrial membrane unless it is also proposed that the means by which the membrane is energized in some way affects the rate at which the fluorochrome binds. Alternatively it is possible that the rate at which 8-anilinonaphthalene-1-sulphonate penetrates energized submitochondrial particles cannot be inferred from the rate at which it penetrates unenergized mitochondria. In this case the rate at which the fluorochrome penetrates the submitochondrial-particle membrane, and the rate of the energy-dependent fluorescence increase, would be affected by the substrate used to energize the membrane.

The decrease in fluorescence on the addition of uncoupler to submitochondrial particles incubated with succinate and 8-anilinonaphthalene-1-sulphonate could be caused by one of several processes, but more likely by several of them. First, as a direct consequence of the uncoupler causing the oxidation of the respiratory chain, fluorochrome dissociation may occur. Secondly, the uncoupler may compete, as do chaotropic anions (Chance & Mukai, 1969; Chance *et al.*, 1970; Ernster *et al.*, 1971) with the fluorochrome either for the same binding sites or for some energy-dependent accumulation process. Thirdly, the uncoupler may cause the decrease in fluorescence by abolishing an energy-dependent accumulation process. The decrease in fluorescence on the addition of antimycin A to submitochondrial particles incubated with succinate and the fluorochrome or of uncoupler to submitochondrial particles incubated with ATP and the fluorochrome may also be explained by one or more of the above processes.

The results described show that there is no conclusive evidence for an 8-anilinonaphthalene-1-sulphonate-monitored transition in membrane conformation. They are consistent with part of the succinate-induced increase in fluorescence being caused by fluorochrome binding to sites, the existence of which is dependent on the oxidoreduction state of the respiratory chain, and part by fluorochrome binding to internally situated sites subsequent to its energy-dependent accumulation, provided that, in the latter case, the source of energization can modify either the rate at which the fluorochrome binds or the rate at which it penetrates the submitochondrial-particle membrane.

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