Membrane Studies with Polarity-Dependent and Excimer-Forming Fluorescent Probes

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1. The interaction of electron-transporting particles from heavy mitochondria of ox heart with several fluorescent probes was examined. 2. 1-Anilidonaphthalene-8-sulphonate and 2-(*N*-methylanilino)naphthalene-6-sulphonate both show an energy-dependent response. 3. Energy transfer between the electron-transporting particles and the dyes and the kinetics of the dye-particle interaction were studied in order to locate the binding regions in the membrane. 4. The energy-dependent probe responses were shown to be a result of changes in the quantum yield of fluorescence of the bound dyes together with increased binding of the dyes to the energized membrane. 5. Fluorescence lifetime measurements were also used to observe changes on energization. 6. A new type of probe was found in pyrene-3-sulphonate, which may be regarded as a 'volume indicator' for the internal membrane binding region, since it shows a concentration-dependent excimer fluorescence. 7. By comparing the responses of all these dyes when energized particles are uncoupled, a membrane transition with a time-constant of 2-3 s is inferred.

Non-covalently adsorbed fluorescent molecules can yield information about protein or lipoprotein structural changes through the effect of the protein environment on the probe fluorescence. Such applications of fluorescent dyes were first proposed by Weber & Laurence (1954), who noted that the fluorescence of ANS* was strongly affected on binding to bovine serum albumin. It was demonstrated that the quantum yield and emission spectrum of this molecule was sensitive to solvent polarity (Stryer, 1965; Turner & Brand, 1968). This probe has subsequently been used for structural studies on a number of proteins (Stryer, 1965; Weber & Young, 1964; Anderson & Weber, 1966), for detection of conformational changes in proteins (Dodd & Radda, 1967; McClure & Edelman, 1967; Cheung & Morales, 1969) and for following the rate of a protein-conformational change (Dodd & Radda, 1969).

The interaction of ANS with submitochondrial particles and the fluorescence of the complex have been studied (Azzi, Chance, Radda & Lee, 1969; Freedman, Hancock & Radda, 1969; Chance, Radda & Lee, 1969). ANS interacts with the membrane fragments to give a typical enhancement and blue-shift of fluorescence. Addition of oxidizable

substrates or ATP leads to a further increase in fluorescence that is reversed by uncouplers. This increase is therefore dependent on coupled electron transport, and may be said to reflect the 'energy state' of the membrane. Using oxygen pulse experiments, Azzi *et al.* (1969) demonstrated that the time-course of the changes in ANS fluorescence lagged behind those of oxidation and reduction of the cytochrome chain. It was deduced that the ANS changes reflect a change in conformation of membrane structural components. We have also studied the interaction of ANS with erythrocyte stroma, as another membrane with which to compare the phenomena observed in mitochondrial fragments (Freedman & Radda, 1969; Freedman *et al.* 1969). The effect of ions on the fluorescence of ANS-stroma complexes has also been reported (Rubalcava, de Munoz & Gitler, 1969).

In this study we examine further the nature of the changes in probe fluorescence with energy state of the particles in order to characterize more fully the nature of the probe sites and of the environmental change that the probes detect. We have done this by resolving the fluorescence change into changes in quantum yield and changes in amount of bound dye, by examining the kinetics of the ANS-particle interaction, by studying singlet energy transfer from tryptophan groups of the particles to the bound dye and by fluorescence lifetime measurements. In addition, we have studied other probe molecules in

* Abbreviations: ANS, 1-anilidonaphthalene-8-sulphonate; MNS, 2-(*N*-methylanilino)naphthalene-6-sulphonate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide; ETPH, electron-transporting particles (from heavy mitochondria of ox heart).

order to determine the essential structural and optical properties for sensitivity to membrane energy state. In particular, we have studied several environmentally sensitive analogues of ANS, and investigated a new type of probe whose fluorescence-emission spectrum is strongly dependent on its local concentration because of the occurrence of fluorescence from excited-state dimers (excimers) (Döller, 1962).

MATERIALS AND METHODS

Mitochondria were prepared from bullock hearts, collected from freshly slaughtered animals, by the method described by Smith (1967). The heavy mitochondria were stored frozen in a medium containing sucrose (0.25M), tris-HCl buffer, pH 7.8 (10mM), sodium succinate (1mM), ATP (1mM) and MgCl₂ (1mM). Coupled electron-transporting particles (ETPH) were prepared by sonication of the mitochondria in the presence of MgCl₂, MnCl₂, ATP and sodium succinate as described by Beyer (1967). The particles were resuspended and stored in a solution containing mannitol (225mM), sucrose (75mM) and tris-HCl buffer, pH 7.4 (20mM). Erythrocyte stroma was prepared by the method of Dodge, Mitchell & Hanahan (1963). Protein concentrations were determined by the Folin-Lowry method (Lowry, Rosebrough, Farr & Randall, 1951), with crystalline bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as the standard.

ANS was purified as described by Dodd & Radda (1969). MNS was synthesized by the method of Cory, Becker, Rosenbluth & Isenberg (1968). Pyrene-3-sulphonic acid sodium salt (K & K Laboratories Inc., Plainview, N.Y., U.S.A.) was purified by recrystallization from water. ATP disodium salt was obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). FCCP was a generous gift from Dr P. B. Garland. Ethanol was supplied by J. Burrough Ltd. (London S.E.11, U.K.). All other compounds were obtained from British Drug Houses Ltd. (Poole, Dorset, U.K.) and were AnalaR grade where available.

pH was determined with an E.I.L. direct-reading pH-meter. The u.v. and visible spectra were recorded on a Cary 14 recording spectrophotometer. Extinction readings were made in a Unicam SP.500 Mk 2 spectrophotometer.

Unless otherwise mentioned, 20mM-tris-HCl buffer, pH 7.4, containing 225mM-mannitol and 75mM-sucrose was used throughout.

Fluorescence studies were performed on a Zeiss spectrofluorimeter or on a more sensitive instrument developed in this laboratory (Dodd & Radda, 1969). Fluorescence decay curves and lifetime determinations were obtained with a TRW 75A decay-time fluorimeter coupled to a type 556 Tektronix dual-beam oscilloscope. The fast pulses were followed by a Type ISI sampling unit, which has provisions for converting the oscilloscope trace into a conventional recorder input. Decay curves were either photographed and simulated by an analogue circuit or recorded on a Servoscribe recorder and analysed from the charts.

Derivation of R_0 . The theory of singlet resonance

energy transfer has been developed by Förster (1959). Based on this theory R_0 , the interchromophore distance at which the rate of transfer is equal to the spontaneous rate of fluorescence decay, can be calculated from the equation (Brand & Wittholt, 1967):

$$(R_0)^6 = \frac{9 \times 10^6 (\ln 10)^2 k^2 c \tau_s}{16 \pi^4 n^2 N^2 \bar{\nu}_0^2} J_{\bar{\nu}}$$

$$\text{where } J_{\bar{\nu}} = \int_0^{\infty} \epsilon^D (2 \bar{\nu}_0 - \bar{\nu}_s) \epsilon^A(\bar{\nu}) d\bar{\nu}$$

τ_s is the donor lifetime in the absence of acceptor, $\bar{\nu}_0$ is the wavenumber of the 0,0 transition of the donor, k is an orientation factor (taken to be $\sqrt{2/3}$ as for rapidly rotating systems in random orientation, in the absence of precise geometrical information), $J_{\bar{\nu}}$ is the overlap integral of donor fluorescence and acceptor absorbance, n is the refractive index of the medium at the wavelength of emission, and c , π and N have their usual significance. Thus the evaluation of R_0 requires knowledge of n , τ_s , $\bar{\nu}_0$ and $J_{\bar{\nu}}$. By multiplying out the constants we get:

$$R_0 = \sqrt[6]{\left(\frac{1.69 \times 10^{-33} \tau_s J_{\bar{\nu}}}{n^2 \bar{\nu}_0^2} \right)}$$

The refractive index commonly taken for proteins is 1.6 (Stryer, 1959) and this value was assumed here. $\bar{\nu}_0$ is the mean of the wavenumbers of the absorption and corrected emission maxima of the donor. For tryptophan in ETPH and erythrocyte stroma the emission maxima (corrected by comparison with the absolute emission spectrum of tryptophan; Teale & Weber, 1957) was at 337nm ($2.97 \times 10^4 \text{ cm}^{-1}$) and the absorption maximum was at 280nm ($3.57 \times 10^4 \text{ cm}^{-1}$), so that $\bar{\nu}_0 = 3.27 \times 10^4 \text{ cm}^{-1}$. τ_s for membrane fluorescence measured directly, by using the nanosecond fluorimeter, was $2.5 \pm 0.5 \text{ ns}$. [A deuterium lamp was used for excitation together with an interference filter: Optics Technology Inc. 4308 (obtained via Avo International, London, U.K.) with maximal transmission at 270nm. A glass filter was used at the emission side.]

$J_{\bar{\nu}}$ is defined as the integral over all wavenumbers of the product of the donor emission intensity and acceptor absorption. The donor emission may be simulated by reflecting the donor absorption spectrum through the 0,0 transition wavenumber. Thus if both absorption spectra are determined as extinction coefficients ($\text{cm}^2 \text{ mm}^{-1}$) against wavenumber and the donor absorption is reflected through $\bar{\nu}_0$, the product of donor and acceptor spectral intensities at a given wavenumber can be determined, and hence the integral can be evaluated from the area of a plot of this function against wavenumber.

The value derived for membrane tryptophan to ANS transfer was $1.00 \times 10^{11} \text{ cm}^3 \text{ mm}^{-2}$. R_0 , the characteristic distance for membrane tryptophan to ANS transfer is then 23.3 Å.

By identical procedures, $J_{\bar{\nu}}$ for membrane tryptophan to MNS was found to be $1.96 \times 10^{11} \text{ cm}^3 \text{ mm}^{-2}$, so that for this pair of chromophores $R_0 = 25.5 \text{ Å}$.

RESULTS

Polarity-dependent probes

Time-course of enhancement. On addition of ETPH to a solution of ANS or MNS in water a rapid

rise in fluorescence is seen followed by a very small time-dependent increase. As for the interaction of ANS with sonicated erythrocyte membranes (Freedman & Radda, 1969), nearly all the enhancement is too rapid to be observed conventionally and the half-life of the fast phase is less than 5ms as measured in a rapid-flow apparatus (B. Chance & G. K. Radda, unpublished work). The rate of the slow phase depends on the structure of the probe, but varies little with the concentration of the probe. Thus the half-life is 60s for MNS and 15s for ANS with a probe concentration in the range 10–50 μM and ETPH concentration 0.2mg/ml.

Effect of succinate. As reported by Azzi *et al.* (1969), the addition of succinate to a mixture of ANS and ETPH leads to a slow enhancement of the ANS fluorescence with no instantaneous phase. We consider that this further enhancement indicates 'energization' of the particles.

A similar effect is observed with MNS as the probe (Fig. 1), but the measured rate is lower. In contrast, none of the uncharged derivatives of MNS shows any response to succinate. The half-lives for the succinate-dependent MNS response depend on the total concentration of MNS (Fig. 2); the rate increases with increasing MNS concentration. If particles are allowed to equilibrate with succinate and the probe is then added, both a rapid and a slow phase of enhancement are seen. Fig. 1 shows that all the additional MNS enhancement is in the slow phase. The same is true for ANS, with which we have compared the rapid enhancement with resting particles, the rapid enhancement with 'energized' particles and the total enhancement with both states of the particles as a function of ANS concentration (Fig. 3). The half-time of the fluorescence enhancement on addition of succinate to ANS-ETPH is about 30s, whereas the half-time of the slow phase of enhancement on addition of ANS to succinate + ETPH is 15–20s.

Fig. 1. Time-course of enhancement of MNS fluorescence. The concentration of ETPH was 0.43mg/ml and that of MNS was 25 μM ; excitation was at 360nm and emission at 440nm. Curve 1, MNS added to ETPH previously energized by 10mM-succinate; curve 2, MNS added to ETPH, then 10mM-succinate added.

Binding parameters. The substrate-induced enhancement of probe fluorescence could result from an increase in the number of dye molecules bound, which could either arise from the creation of new sites or from tighter binding. Alternatively, a change

Fig. 2. Dependence of rate of probe response to succinate on MNS concentration. The concentration of ETPH was 0.12mg/ml and that of succinate was 10mM. Half-times were derived from semi-logarithmic plots of the fluorescence enhancements after the addition of succinate.

Fig. 3. Titration of fast and total enhancements of energized and non-energized particles. The particles used (O-ESMP) were prepared by Dr C. P. Lee and behaved comparably with ETPH (Azzi *et al.* 1969); they were used at a concentration of 0.16mg/ml. Curves 1 and 2, fast and total enhancements respectively of ANS fluorescence on addition to non-energized particles; curves 3 and 4, fast and total enhancements respectively on addition of ANS to energized (with 10mM-succinate) particles.

Fig. 4. Double-reciprocal plots for titrations of probes with ETPH. ○, Energized ETPH; ●, unenergized ETPH. (a) With ANS (6.67 μM); excitation was at 380nm and emission at 480nm. (b) With MNS (10 μM); excitation was at 365nm and emission at 440nm.

Fig. 5. Double-reciprocal plots for titrations of ANS with ETPH. The concentration of ANS was 5 μM ; excitation was at 380nm and emission at 480nm. Curve 1, ETPH in standard buffer; curve 2, NaCl added to 0.5M.

a fixed value of total probe and extrapolating the reciprocal plot to infinite concentration, it is possible to estimate the dye fluorescence in the limit where all the dye is bound. Fig. 4 shows that these extrapolated intercepts differ for energized and non-energized particles, so that a change in quantum yield is involved. The intrinsic fluorescence at 480nm of ANS bound to energized particles is 2.5-fold that of ANS bound to resting particles, and the corresponding ratio for MNS at 440nm is 2.3. The error in the extrapolated ratio of intensities is not more than 10% on the basis of a number of titrations. This effect is in contrast with the enhancement brought about by increasing the ionic strength, where no change in the quantum yield is observed (Fig. 5). The fluorescence of dye-particle complexes is also enhanced by lowering the pH, and at pH 3 both quantum yield and binding changes contribute to the increased fluorescence (Table 1).

Within a range of probe concentrations it is possible to obtain a meaningful titration curve by keeping the particle concentration constant. Such titration curves can be analysed by Scatchard's method, by using the values for fluorescence of bound material derived from the double-reciprocal plots in Fig. 4. The results in Fig. 6 show that energization does increase the affinity of the membrane for the dye, but does not alter the number of

in the quantum yield of the bound dye could be responsible for the enhancement. By performing a titration in which protein concentration is varied at

Table 1. *Dye-binding parameters*

Membrane	Range of probe concentration in titrations (μM)	Limiting enhancement (at 480nm)	K_d (μM)	Number of sites ($\mu\text{mol/g}$)
ETPH (0.1mg/ml)	ANS 0-40	104	35	80
ETPH (0.142mg/ml)+succinate (10mm)	ANS 0-40	251	20	80
ETPH (0.262mg/ml)	MNS 0-50	20 (at 440nm)	96	260
ETPH (0.262mg/ml)+succinate (10mm)	MNS 0-50	45 (at 440nm)	16	200
ETPH (pH3.1) (0.112mg/ml)	ANS 0-40	419	4.9	95
Stroma* (0.068mg/ml)	ANS 0-70	167	41	23
Stroma* (0.02mg/ml)	ANS 0-60	740	9	44
Stroma (M-NaCl, pH7.0) (0.071mg/ml)	ANS 0-40	167	48	105
Stroma after neuraminidase treatment (0.03mg/ml) in iso-osmotic phosphate buffer, pH7.4	ANS 0-50	208	17	39

* From Freedman & Radda (1969).

Fig. 6. Scatchard plots for ANS binding to ETPH. The concentration of ETPH was 0.63mg/ml. Fluorescence was observed as indicated in Fig. 5. Curve 1, non-energized ETPH titrated with ANS (0-40 μM); curve 2, ETPH+succinate (5mm) titrated with ANS (0-40 μM). The value of the intercept depends critically on the concentration of the particles for a given ANS concentration range. Similar Scatchard plots were obtained for several particle concentrations (cf. Table 1). The extrapolated intercepts in Fig. 5 give the fluorescence intensity per totally bound dye. From this the amounts of bound and free dyes at any point on the titration curve were calculated.

binding sites. The binding parameters for both ANS and MNS to mitochondrial membranes are summarized in Table 1, and for comparison some similar data for erythrocyte stroma are also included there. Binding to both the 'fast' and the 'slow' sites is included in the derivation of the binding parameters. If a titration is performed in which various quantities of ANS or MNS are added to a fixed quantity of energized particles, it is possible to

Table 2. *Proportion of 'fast' sites as a function of probe concentration*

Membrane	[Probe] (μM)	Fast enhancement (% of total)
ETPH (0.2mg/ml) +succinate (10mm)	ANS 4	50
	8	64
	12	72
	16	69
	20	78
ETPH (0.31mg/ml) +succinate (10mm)	MNS 10	49
	19	54
	28	52
	38	61
	47	59

obtain titrations of the rapid, slow and total enhancements. The results show that the proportion of the total enhancement that is fast increases with the concentration of the dye (Table 2), suggesting that the sites responsible for the slow enhancement may be saturated at lower dye concentrations than are the 'fast' sites.

It is clear that in a system as complex as a membrane there must be a large number of binding sites with different affinities and fluorescence-enhancement properties towards a dye such as ANS. Nevertheless, within a limited range of membrane and probe concentrations, where the double-reciprocal and Scatchard plots are linear, it is meaningful to speak of binding constants, enhancements and number of sites that represent an average property of sites of similar affinities. This is the reason why Table 1 contains the probe concentration range over which the binding parameters are applicable.

Within the same range the amount of free dye can also be measured by a method suggested to us by Professor Britton Chance. This involves the rapid addition of excess of bovine serum albumin after the probe-membrane equilibrium has been established. The fluorescence of free ANS is rapidly enhanced on binding to the protein, and this is followed by a further slow enhancement as a result of competition by the protein for bound ANS. The conclusions reached by this method are very similar to those obtained on the basis of binding studies by our method (B. Chance & G. K. Radda, unpublished work).

Kinetic studies. At most probe concentrations the increase in intensity on energization is the result both of changes in quantum yield of fluorescence of bound dye and of further binding of the dye. The rate of enhancement on energization is thus fairly complex, and is further complicated when succinate is used because of the slow utilization of succinate by the particles. Some of these complications may be eliminated by observing the transition in the opposite direction. When energized particles with ANS or MNS are rapidly treated with FCCP ($2.5\mu\text{M}$) the decline in fluorescence can be analysed as a sum of two exponential decays by plotting $\log (F_0 - F_\infty)/(F - F_\infty)$ against time (Fig. 7). The half-life for the slow phase is $12 \pm 1\text{ s}$ when ANS is used and $60 \pm 10\text{ s}$ with MNS as the probe. The rapid phase of the fluorescence decrease gives a half-time of $2.5 \pm 0.5\text{ s}$ with either MNS or ANS as the probe. Similar curves were obtained by using pyrene-3-sulphonate as the probe (see below). For comparison, the rate of decline in fluorescence when a probe-particle mixture is rapidly diluted was also determined. Small quantities ($20\mu\text{l}$) of ETPH (15 mg/ml) and probe (1 mM) were first equilibrated and then rapidly mixed into a cuvette containing buffer. The rate of fluorescence change showed only one phase with a half-time of $12 \pm 2\text{ s}$ for ANS.

Fluorescence lifetimes. The lifetime of free ANS is too short to be measured accurately with our apparatus, but when the dye interacts with particles a longer lifetime is observed. For both ANS and MNS complexes the decay curves could not be fitted by a single exponential, so that the bound probe molecules are heterogeneous with respect to fluorescence lifetimes. Fig. 8 shows decay curves for the ANS complex and the shape of the lamp pulse. The early part of the curve for MNS could be fitted with a simulated decay of 3 ns , whereas the latter part showed a lifetime of 6 ns when unenergized particles were used. Because of the small separation of the time-constants for the two lifetimes and the possibility of several lifetimes the actual values must be regarded as covering a range of lifetimes. However, on energization the lifetimes for MNS increased to 4 and 8 ns , reflecting the change in quantum yield. For ANS

Fig. 7. Kinetics of probe fluorescence changes on uncoupling of energized ETPH. The concentration of ETPH, energized with 25 mM -succinate, was 0.5 mg/ml ; probe concentrations were $10\mu\text{M}$, and uncoupling was carried out with FCCP ($2.5\mu\text{M}$). Curve 1, with ANS, half-times 12 and 2.5 s ; curve 2, with pyrene-3-sulphonate, excited at 350 nm and observed at 500 nm , half-times 30 and 2 s ; curve 3, with MNS, half-times 60 and 2.5 s . The determination of half-times for the fast phases requires correction of the observed fast rate by the contribution of the slow phase.

the lifetimes were 5 and 9 ns with a small increase on energization. Because of the difficulty of separating the two or more lifetimes we have attempted to assign the observed total fluorescence changes in energization to species with different lifetimes by another procedure. For instance, when the decay curve for the particle-ANS complex is photographed at intervals after energization or uncoupling (Fig. 8) the percentage change in intensity of the signal close to the maximum of the lamp flash is less than that at later stages of the decay (e.g. $20\text{--}30\text{ ns}$ after the flash). This indicates that the decay curve in the energized system contains a greater proportion of longer-lived components, so that energization leads to an increase in the proportion of bound molecules with longer lifetimes. By using the sampling oscillo-

Fig. 8. Fluorescence decay curves for ANS-ETPH at various time-intervals after uncoupling by FCCP. Curve 1, N_2 -lamp shape; curve 2, with ANS ($5\mu M$) and ETPH (0.15 mg/ml) energized with 10 mM -succinate; curves 3, 4 and 5, 8s, 27s and 52s respectively after the addition of $2.5\mu M$ -FCCP. The decay curves are traced from a single photograph with multiple exposures of the oscilloscope screen. Excitation was through a Corning 7-60 filter and emission through a Corning 3-72 filter.

Fig. 9. Kinetics of uncoupling of the fluorescence decay curve observed at fixed delay times. Conditions were as described for Fig. 8. The sampling unit was set to monitor continuously at a given point of the decay curve (arrows at 4 and 36 ns marked on Fig. 8). The signal on the oscilloscope was fed to a conventional recorder.

scope in the appropriate manner it is also possible to select manually any part of the nanosecond decay curve, and the fluorescence intensity at that particular point can be converted into a recorder output. In this case we can follow the kinetics of energization or uncoupling at fixed time-intervals after the nanosecond flash. Fig. 9 shows the kinetics of uncoupling, by FCCP followed by ANS as the probe, measured at 4 and 36 ns delays after the lamp flash on the decay curves. These curves show that the percentage change is larger when the uncoupling is followed at the 36 ns point than when it is followed at the 4 ns part of the curve.

Fluorescence polarizations. The polarizations of fluorescence of ANS ($25\mu M$) or MNS ($25\mu M$) with ETPH (0.3 mg/ml) were measured for the unener-

Fig. 10. Absolute excitation spectra of ANS. Curve 1, ANS ($10\mu M$) in aq. 50% (v/v) ethanol observed at 510 nm ; curve 2, ANS ($25\mu M$) + ETPH (0.125 mg/ml) observed at 480 nm .

gized and energized particles. (Excitation was at 380 nm and emission was observed at 480 nm for ANS; for MNS the wavelengths chosen were 365 and 440 nm respectively.) For MNS the polarization values are 0.17 and 0.14 , and for ANS they are 0.22 and 0.18 , for the unenergized and energized particles respectively. In all these measurements polarized exciting light was used and corrections were made in the usual way (Dodd & Radda, 1969) for instrumental polarization. It is impossible to exclude entirely the occurrence of some depolarization due to scattering of the emitted light. An estimate of this depolarization was obtained by measuring the polarization of a 'dansylated' bovine serum albumin sample in the presence and absence of ETPH (0.3 mg/ml). Only a small change, from 0.2 to 0.17 , was observed on the addition of particles and no further change was found on energization of ETPH by succinate.

Energy transfer. Some information about the environment of the probe can be derived from observations of energy transfer between membrane components and the bound probe. The excitation spectrum (Fig. 10) of bound ANS shows a maximum at about 280 nm , which is absent for ANS in water or ethanol. This is indicative of energy transfer between tryptophan groups of the membrane and the bound probe. MNS also shows this effect. If the probe-particle complexes are excited at 290 nm and the emission spectra are observed, curves are obtained like those in Fig. 11. As probe concentration is increased, the membrane fluorescence is quenched (after allowing for the inner filter effect). The existence of an isoemissive point provides further evidence for energy transfer and supports the method

Fig. 11. Emission spectra of MNS-ETPH. The concentration of ETPH was 0.15 mg/ml; excitation was at 290 nm. Curves 1-4, 5 μ M-, 10 μ M-, 25 μ M- and 50 μ M-MNS respectively.

of calculation of bound dye concentration, as it implies that the quantum yield of the bound dye is independent of the number of sites occupied (Anderson & Weber, 1965). Since the membrane tryptophan emission does not change shape, the corrected signal at 290-335 nm is taken as a measure of the quantum yield of membrane tryptophan fluorescence. The transfer efficiency, T , is given by $T = 1 - Q_T/Q_0$ where Q_T is the quantum yield of donor in the presence of transfer and Q_0 is that in the absence of transfer. From the fluorescence of the ANS complexes at 380-480 nm the concentration of the probe in the membrane is derivable. At a given total concentration of membrane and probe, the transfer efficiency is greater in the energized complexes, but allowing for the greater concentration of bound dye no change in intrinsic transfer efficiency was observed on energization.

The time-courses of enhancement at 440 nm on addition of MNS to energized particles when exciting directly (365 nm) or in the energy-transfer mode (290 nm) were compared. The proportion of the enhancement that is fast is 50% when observed after direct excitation and 60% when excited through energy transfer (MNS 10 μ M, ETPH 0.32 mg/ml). Similar differences have been consistently observed at a variety of MNS concentrations.

Because of the better overlap between tryptophan emission and the dye absorption band for MNS than for ANS the transfer efficiencies are considerably higher for the former dye, but the derived distances are very close to those measured by transfer to ANS (Table 3).

Pyrene-3-sulphonate, an excimer-forming probe. Like pyrene and other pyrene derivatives, pyrene-3-sulphonate exhibits fluorescence properties that are concentration-dependent (Döller, 1962). Thus at low concentrations the emission maximum is at about 400 nm, whereas at higher concentrations first a shoulder and later a distinct emission peak can be observed at 500 nm (Fig. 12). The appearance of the long-wavelength peak is always accompanied

Table 3. *Energy-transfer parameters for probe-membrane complexes*

Membrane	[Probe] _{total} (μ M)	Transfer efficiency (T)	R (Å)
Stroma (0.07 mg/ml) iso-osmotic phosphate buffer, pH 7.4	4 ANS	0.06	36.9
	10	0.15	31.1
	20	0.28	27.3
	30	0.36	25.7
	40	0.40	24.9
Stroma (0.07 mg/ml) (0.15 M-NaCl adjusted to pH 3.1)	4 ANS	0.22	28.8
	10	0.51	23.1
	20	0.68	20.5
	30	0.75	19.4
	40	0.79	18.7
Stroma (0.07 mg/ml) (M-NaCl) (pH 7.0)	4 ANS	0.03	41.6
	10	0.15	31.1
	20	0.26	27.8
	30	0.29	27.1
	40	0.33	26.2
ETPH (0.15 mg/ml)	10 ANS	0.21	29.0
	24	0.34	26.1
	40	0.40	25.0
ETPH (0.14 mg/ml) + 10 mM-succinate	4 ANS	0.07	36.0
	10	0.22	28.8
	20	0.30	26.8
	30	0.44	24.3
	40	0.47	23.8
ETPH (0.11 mg/ml) (pH 3.1)	4 ANS	0.40	24.8
	6	0.51	23.1
	8	0.58	22.1
	10	0.62	21.5
	12	0.69	20.5
	24	0.81	18.3
	40	0.84	17.6
ETPH (0.107 mg/ml)	4.9 MNS	0.175	33.0
	9.8	0.26	30.3
	14.6	0.33	28.7
	10.4	0.38	27.7
	24.2	0.38	27.7
	33.5	0.45	26.4
	47.2	0.57	24.3
ETPH (0.107 mg/ml) + 10 mM-succinate	4.9 MNS	0.213	31.7
	9.8	0.33	28.7
	14.6	0.39	27.5
	19.4	0.44	26.5
	24.2	0.49	25.7
	33.5	0.48	25.8
	47.2	0.58	24.2

by a proportionate decrease in the emission at 400 nm. The ratio of the two intensities is also dependent on viscosity at high concentrations of pyrene-3-sulphonate; e.g. in water the 400 nm/500 nm ratio is 6 and in glycerol it is 80 for 1 mM-pyrene-3-sulphonate. Changes in solvent polarity did not effect the pyrene-3-sulphonate fluorescence. The lifetime of the pyrene-3-sulphonate emission (400 nm) is 50 ns and is not sensitive to oxygen.

When ETPH (0.3 mg/ml) is added to a solution

Fig. 12. Emission spectra of pyrene-3-sulphonate. Excitation was at 350nm. Curve 1, pyrene-3-sulphonate (1mM) in water; curve 2, pyrene-3-sulphonate (25 μ M) in water in the presence and absence of ETPH (0.3mg/ml); curve 3, pyrene-3-sulphonate (25 μ M) and ETPH (0.3mg/ml) energized with 25mM-succinate.

Fig. 13. Energy-linked fluorescence changes of pyrene-3-sulphonate in ETPH. The concentration of pyrene-3-sulphonate was 25 μ M and that of ETPH was 0.3mg/ml; excitation was at 350nm.

of pyrene-3-sulphonate (25 μ M) no significant change in the probe fluorescence occurs. On addition of succinate to this mixture a slow increase in the emission at 500nm and a decrease at 400nm were observed (Fig. 13). By recording the changes at these two wavelengths in a dual-beam spectrofluorimeter they can be observed simultaneously and were always found to respond in the opposite direction. Thus after about 15min with excess of succinate (25mM) the system goes anaerobic and a decrease in the probe fluorescence at 500nm and an increase at 400nm was observed. On the introduc-

tion of oxygen into this solution the changes were reversed (Fig. 13). Addition of the uncoupler FCCP to the energized particle-pyrene-3-sulphonate mixture again leads to a decrease in the pyrene-3-sulphonate fluorescence at 500nm and a corresponding increase at 400nm. These changes can again be analysed as a sum of two exponentials, the half-life of the first phase being 2.5s and that of the second phase 30s (Figs 7 and 13).

In the absence of energized particles the emission spectrum of 25 μ M-pyrene-3-sulphonate contains only the 400nm peak, but on addition of energized ETPH a distinct increase at 500nm was observed (Fig. 12). In contrast with the other probes, when pyrene-3-sulphonate is added to ETPH only a slow increase is observed at 500nm over that of the fluorescence of the free dye.

DISCUSSION

Azzi *et al.* (1969) have shown previously that the changes in ANS fluorescence brought about by substrates of the respiratory chain are energy-linked. MNS and the polarity-insensitive probe pyrene-3-sulphonate are also indicators of energy-linked changes.

For a full interpretation of these changes it is first necessary to gain some insight into the location of the probes in the membrane. Kinetically we have distinguished two rates of enhancement for ANS and MNS. The simplest interpretation of this is that the fast enhancement represents binding to easily accessible sites ('fast' sites) and that the slow enhancement represents interaction with sites that require penetration of the probe into the membrane ('slow' sites). The distances measured on the basis of energy-transfer efficiencies put the location of the two environmentally sensitive probes close to the protein. This conclusion is supported by the observation that when ANS binds to pure proteins the average distances between the tryptophan residues and the dye are also in the range 16–25 Å (Weber & Daniel, 1966; M. B. Taylor & G. K. Radda, unpublished work). The similarity of the derived distances for ANS-stroma and ANS-ETPH in spite of the differences in the lipid composition of the two membranes is also consistent with location of the probes close to the protein. The fact that the derived distances for ANS and MNS are in close agreement in spite of the large difference in the transfer efficiencies for the two dyes (Table 3), because of the different J_v values, gives further validity to the application of this technique to membranes. When the fluorescence increase on addition of MNS to energized particles is measured by direct excitation of the dye and by excitation in the energy-transfer mode, the proportion of the total

enhancement that is fast is slightly greater in the latter case. This means that energy transfer to MNS occurs in both types of sites.

It is noteworthy that the binding constants for the ANS and MNS interaction are similar in both ETPH and erythrocyte stroma. This similarity is also apparent in the low polarization of fluorescence for the bound dyes in both systems. The value of about 0.2 is much lower than would be expected (cf. Dodd & Radda, 1969) if the dye-membrane complex were rigid, and suggests either flexibility in the membrane or some movement of the non-covalently linked dye. From the enhancement and shift of the dye fluorescence on binding, the region of interaction may be said to be hydrophobic, but it is not possible to assign a dielectric constant for the binding region because other factors (e.g. immobilized water) may also contribute to the modification of the excited state of the dye. Intuitively, then, one may place the probes at polar-non-polar interfaces.

From the various titrations with polarity-dependent probes (Figs. 4-6) it emerges that the enhancement of the probe fluorescence on energization is due partly to an increase in quantum yield of emission of the bound dye molecules, and partly to an increased affinity for dye, resulting in additional binding. This additional binding is also demonstrated by the observation that the rate of succinate-dependent probe response depends on the nature of the probe. The increase in the fluorescence lifetime of the probe is at the same time a clear demonstration of the change in quantum yield. All the energy-dependent responses of the probes occur at 'slow' sites (which we regard as sites inside the membrane) (Figs. 1 and 3).

The change in quantum yield and the movement of the probe can be more clearly separated by following the kinetics of the fluorescence changes on uncoupling the energized particles. The slow phase of the decline can be associated with the efflux of probe molecules due to looser binding. It occurs at a different rate for each probe, and the rate is comparable with that found by simply diluting the conjugates. The initial fast phase of the uncoupling change occurs with a halftime of 2-3 s and is independent of the nature of the probe (Fig. 7). This suggests that this fast phase may represent a change in the membrane structure. A value of 2-3 s for the half-time of the membrane transition has also been found by extrapolating the rate of enhancement of fluorescence on addition of NADH to infinite protein concentration, where there is no ANS flux (Freedman *et al.* 1969; Chance *et al.* 1969).

We are now in a position to compare the changes observed with pyrene-3-sulphonate as the probe and those observed with the polarity-dependent probes. The general features of the pyrene-3-

sulphonate response at 500 nm are similar to those of the other probes. In particular the response is energy-linked and shows an identical fast phase and a different slow phase on uncoupling. It is thus reasonable to suggest that, in terms of the structural transitions of the membrane, the same transitions are measured by this probe as by the naphthalene derivatives. However, the origins of the fluorescence changes are different; the emission of pyrene-3-sulphonate at 500 nm is due to the excimer that is formed by the interaction of a molecule in its excited state with one in its ground state:

Any change at 500 nm must be accompanied by a reciprocal change at 400 nm if it is due to excimer. Excimer formation requires sufficient freedom of movement of the dye molecules during the excited-state lifetime (50 ns). Since this emission is observed in the membrane, it implies mobility of the probe, as suggested on the basis of polarization of fluorescence measurements with the other probes. From the comparison of the uncoupling kinetics measured by the different probes we can conclude that the membrane transition with half-life 2-3 s leads to a decrease in the excimer fluorescence. This could be due to one of two causes: an increase in the viscosity of the microenvironment of the dye or an increase in the average separation of dye molecules brought about by an increase in the volume of the binding region. From the change in the polarization of fluorescence of MNS and ANS and also the change in the average lifetimes of these dyes in the two states of the membrane one can calculate any viscosity change from the relation:

$$\frac{p_1(p_0 - p_2)}{p_2(p_0 - p_1)} = \frac{\tau_2\eta_1}{\tau_1\eta_2}$$

where the subscripts 1 and 2 refer to the two states, and p , τ and η have their usual meaning. Within the accuracy of the lifetime measurements we find no change in viscosity. We regard pyrene-3-sulphonate as a 'volume indicator' for the internal binding region of the responsive probe molecules. This conclusion is also supported by the observation that pyrene-3-sulphonate excimer emission in the membrane is only observed in the 'slow' sites.

None of the fluorescence changes we observed can be associated with the redox changes in the electron-transport chain, as shown by the lack of fluorescence changes in the presence of uncouplers and by the rates of the changes, which are always lower than the changes in the redox state of cytochrome oxidase (Azzi *et al.* 1969). Thus we can conclude that

energization alters the environment of some of the 'slow' probe-binding sites so that their affinity for the charged probes increases, and the lifetime and quantum yield of the bound environmentally sensitive probes increase. Our present hypothesis is that the charged probes occupy a region in the membrane that contains structured water. This would account for the fluorescence enhancement in the case of ANS and would also allow sufficient mobility of the probe in a restricted volume to account for the excimer emission and low polarizations. Energization could then lead to the exclusion of water from the membrane (Williams, 1969), thus bringing the membrane to a state where energy coupling could occur.

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