PERTURBATION OF LIPID STRUCTURES BY FLUORESCENT PROBES

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Received 30 October 1975

1. Introduction

The equilibrium fluorescent probe 1-anilino-8-naphthalene sulfonate*, has found extensive use in studies of membrane structure. In an entertainingly written review, Radda and Vanderkooi have posed the question, 'Can fluorescent probes tell us anything about membranes?' [1]. A great deal of useful information has been derived from such probe studies. However the important question, 'Do fluorescent probes perturb biomembranes?' has not been conclusively answered. Indeed it has been suggested that probes can act as 'molecular tumours' and that unless proved otherwise a probe must be suspect of acting as a perturbant of the region it is monitoring [2]. In this communication, we report the use of the fluorescent hydrocarbon pyrene, to monitor structural changes, in lipids caused by ANS binding.

2. Experimental procedures

ANS and dipalmitoyl lecithin were obtained from Sigma Chemicals, USA. Pyrene was of analytical purity. ANS was used as the ammonium salt and was recrystallised from water, before use. Whole brain lipids were extracted from monkey and rat brains by the procedure of Folch et al. [3]. Lipid dispersions were prepared by sonicating a dispersion of the lipid in 10 mM Tris–HCl (pH 7.4) for 1–2 min with a Branson sonifier (20 kHz, 75 W/sq.inch). Pyrene was incorporated into lipids by addition of a 4.2 mM solution in ethyl alcohol to the aqueous dispersion such that the final pyrene concentration was 1.8 μM. The final alcohol concentration did not exceed 0.4% (v/v). For fluorescence experiments the lipid concentrations were fixed at 500 μg/ml, for optimum detection of excimer emission. Fluorescence spectra are uncorrected and were recorded on a Perkin-Elmer MP 203 spectrofluorometer. 1 cm matched quartz cuvettes with an initial vol of 2.5 ml were used. The pyrene excitation wavelength was 335 nm. Emission of dimer and monomer were monitored at 470 nm and 390 nm, respectively.

3. Results and discussion

Pyrene forms excited state dimers (excimers) in a diffusion controlled reaction and can function as an indicator of mobility, in the hydrocarbon phase of biomembranes [4,5]. The long wavelength, 470 nm, excimer emission is clearly distinguishable from the monomer fluorescence peak at 390 nm. The ratio of the dimer (D) fluorescence to that of the monomer (M) can then be related to the ease of diffusion of pyrene. The quantum yield of pyrene in lipids is much greater than that of ANS in lipids. Quantum yields of 0.6 have been reported for pyrene in cyclohexane [6], while the value for ANS in lecithin is 0.015–0.020 [7]. This difference in quantum efficiencies allows the use of pyrene, to monitor mobility changes in lipid dispersions in the presence of ANS. Fig.1 shows the emission spectra of pyrene incorporated into dipalmitoyl lecithin dispersions at ANS concentrations varying from 2 to 50 μg/ml. The intensity of the emission peak at 470 nm increases steadily with ANS concentration and there is a corresponding

* *Abbreviations:* ANS 1-anilino-8-naphthalene sulfonate. TNS 2-p-toluidinyl-6-naphthalene sulphonate.

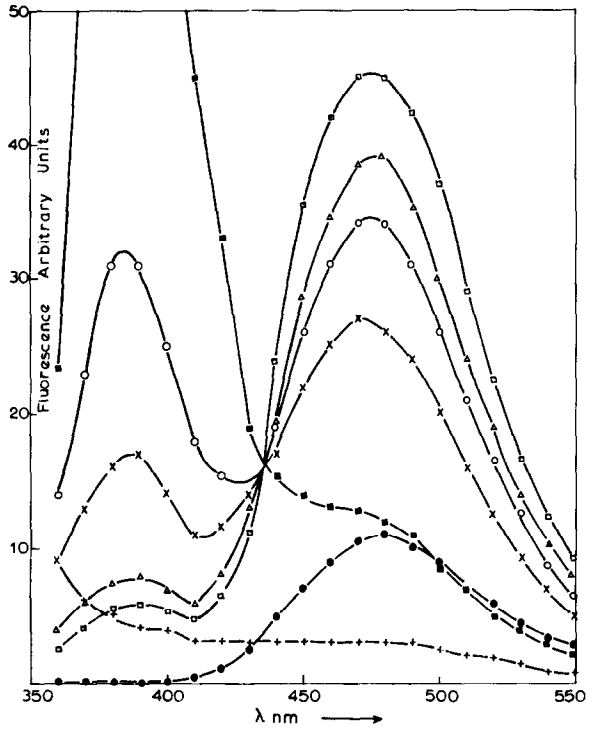

Fig.1. Uncorrected emission spectra of pyrene (1.8 μM) in dipalmitoyl lecithin dispersions (500 μg/ml) at varying ANS concentrations. Excitation 335 nm. ANS concentrations: (■) No ANS, (○) 2 μg/ml, (×) 5 μg/ml, (△) 10 μg/ml, (□) 15 μg/ml, (+) 2 μg/ml ANS control (no pyrene) (●), 50 μg/ml ANS control.

decrease in monomer fluorescence at 390 nm. An isoemissive points is observed at 435 nm. The control experiments using ANS-lipid dispersions, without pyrene are also shown to account for the background fluorescence due to ANS, by excitation at 335 nm.

Fig.2 shows the fluorescence of pyrene dimer, monomer and the dimer/monomer ratio as a function of ANS concentration. At ANS levels greater than 20 μg/ml there is a quenching of dimer fluorescence. The ANS excitation peak at 390 nm and pyrene monomer emission at 380–390 nm overlap strongly. It is conceivable that the fluorescence at 470 nm may also result from trivial reabsorption or Forster energy transfer from pyrene to ANS [8]. The observed fluorescence yields would require very high transfer

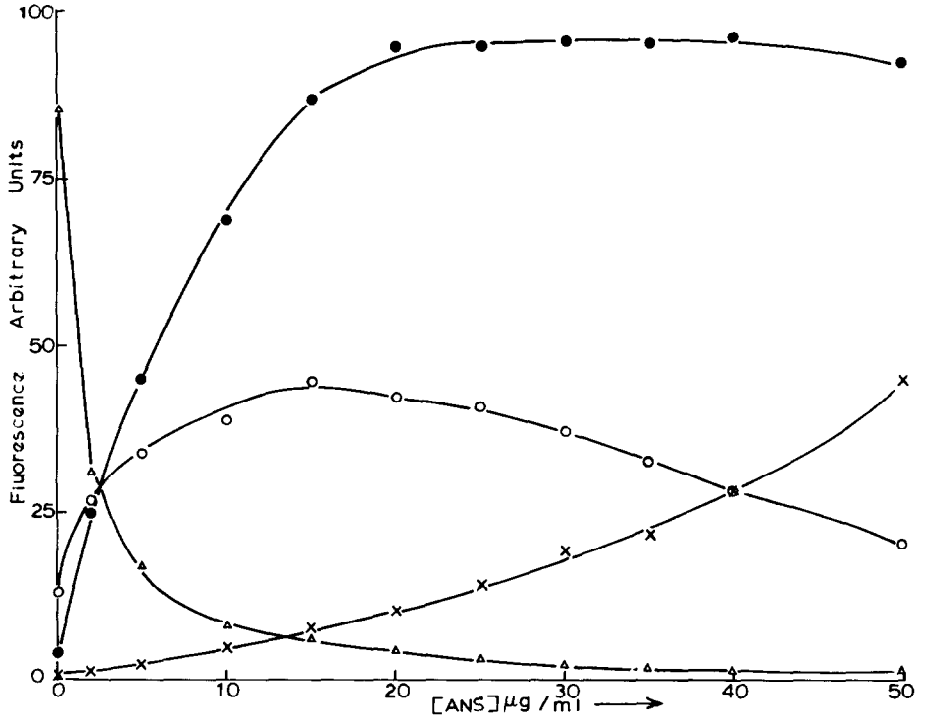

Fig.2

efficiencies. Further the quenching of the 470 nm emission, on excitation at 335 nm, at high ANS concentrations argues against significant ANS contribution to the 470 nm peak. If reabsorption or transfer were present an increase or levelling off would be expected for the emission intensity at 470 nm. Quenching by ANS self absorption, at these concentrations, is ruled out, since direct excitation of ANS at 390 nm does not show any dampening effect (fig.2). We therefore attribute the enhancement in emission at 470 nm to increased pyrene excimer formation. This in turn arises from an increase in the pyrene diffusion rate and is indicative of increased 'fluidity' in the hydrocarbon phase. It is likely that above a certain concentration ANS reduces the lifetime of the monomer excited state making excimer formation less probable. Complex formation and quenching of the pyrene excimer is less likely on geometric grounds. Similar experiments were carried out at high ionic strengths (1.6 M KCl) where ANS binding is greatly enhanced [9,10]. Here direct quenching of the pyrene monomer was observed and no increase in dimer emission was detectable. This is understandable since high levels of bound ANS will quench pyrene monomer by energy transfer processes. Also direct complexation may quench both monomer and dimer. These results, at high levels of bound ANS, further support our thesis that the increase in 470 nm emission at low levels of ANS, is a result of facile excimer formation. With lipids, extracted from whole brain, ANS induced pyrene excimer formation was less pronounced. We have observed that ANS binding to brain lipids is weaker than to lecithin, reflecting the lower percentage of positively charged head groups, in these dispersions (unpublished results).

Evidence for the binding of ANS near the choline head group in lecithin has been reported [11,12]. X-ray diffraction studies of lecithin dispersions in the presence of high ANS concentrations (ANS: fatty acid, 1 : 3) show a reduction of bilayer thickness from 62 Å to 38 Å. The naphthalene ring penetrates only a short distance between the fatty acid chains. The polar trimethyl ammonium groups are pushed apart in the plane of the bilayer by interaction with the anionic sulfonate moiety [12]. ANS has been used to monitor lipid phase transitions, in model systems and biomembranes [13], and has been shown to have no detectable effect on the transition temperature. However it is observed that the transition curve obtained below 40°C for lecithin is smeared out, compared to light scattering and volumetric measurements [14]. At high concentrations (0.1 M), ANS has been shown to disrupt low and high density human plasma lipoproteins and phospholipid dispersions [15]. Morphological changes leading to a disk to sphere transformation of red blood cells has been observed at ANS levels ranging from 0.03 to 3 mM [16]. The naphthalene sulfonates ANS and TNS completely immobilise mammalian sperm at 1 mM levels [17]. Binding to and perturbation of plasma membrane structure by these molecules is likely to be the cause of this dramatic change in mobility. These observations support the view that ANS causes structural changes in lipid and membrane structures. Our experiments show that these effects are present even at concentrations normally employed in fluorescence studies of biological systems. We would like to point out that the use of one fluorescent probe to monitor the perturbations caused by another, is in no way different from the general application of these probes as reporters of molecular interactions. NMR studies are likely to yield more detailed information about ANS-lipid interactions and are currently in progress.

Acknowledgements

We are grateful to Professor T. Ramasarma for use of his spectrofluorometer. K.S.K. thanks the U.G.C. for a fellowship.

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Fig.2. Variation of fluorescence intensities with ANS concentrations. (△) Emission at 390 nm, pyrene monomer. Excitation 335 nm. (○) Emission at 470 nm, pyrene excimer. Excitation 335 nm. (×) Dimer/monomer fluorescence ratio. (●) Fluorescence intensity at 470 nm on excitation at 390 nm. Direct ANS emission.

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