FLUORESCENCE STUDIES OF DRUG AND CATION INTERACTIONS WITH MICROSOMAL MEMBRANES

Hilary C. HAWKINS and R.B. FREEDMAN
Biological Laboratories, University of Kent at Canterbury, England

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1. Introduction

The value of non-covalently bound fluorescent molecules as extrinsic probes of membrane structure and function is now well established [1]. In the case of the inner mitochondrial membrane, analysis of the responses of fluorescent probes to changes in the state of the membrane [2] has led to some understanding of the physical changes accompanying energy transduction [3–6]. A similar analysis has been applied to chromatophore membranes [7]. In many other systems, such as the erythrocyte membrane [8], sarcoplasmic reticulum [9], brain microsomes [10] and numerous artificial membranes, the binding of the most popular probe ANS (1-anilino-naphthalene-8-sulphonate) has been studied; in general, these studies have not been particularly revealing about the major functions of the membrane systems.

Microsomal membranes of mammalian liver are complex systems whose major biological functions include i) the metabolism of drugs and other foreign compounds and ii) the binding of ribosomes and organisation of the synthesis of serum proteins. We have attempted to use a fluorescent probe to investigate these functions; it was found that the increase in probe fluorescence produced by certain drugs is a function of the ionisation state of the drugs and not a specific property of the drug–microsome interaction, and that fluorescent probes cannot detect differences in cation affinity between smooth microsomes and degranulated rough microsomes which might account for their different abilities to bind ribosomes.

2. Materials and methods

Egg-yolk L-α-lecithin, obtained from Sigma as a solution in hexane, was evaporated to dryness in a stream of N_2, suspended in 0.1 M KCl and dispersed by a MSE Ultrasonicator in bursts of less than 30 sec. Phospholipid concentration was estimated by the phosphate assay of King [11]. Human erythrocyte membrane fragments were prepared by the method of Dodge et al. [12] from aged blood supplied by the Kent and Canterbury Hospital. Rough and smooth microsomes were prepared from the livers of 200 g male hooded Lister rats by differential centrifugation of the post-mitochondrial supernatant on a discontinuous sucrose gradient [13]. EDTA treatment of part of the rough fraction to yield degranulated rough microsomes was by the procedure of Blyth et al. [13]. The protein concentration of membrane fractions was determined by the method of Lowry et al. [14] using crystalline BSA as a standard. Ribosomal content of the microsomal fractions was estimated from their RNA content determined by the method of Schmidt and Thannhauser [15] using the extinction data of Munro and Fleck [16]. RNA:protein ratios were 0.03–0.07 for smooth membranes, 0.06–0.12 for degranulated rough membranes and 0.15–0.30 for rough membranes.

ANS (1-anilinonaphthalene-8-sulphonate) was obtained from Kodak Ltd. as the magnesium salt. Aniline hydrochloride, Fisons SLR grade, was recrystallized twice from hot distilled water. Benzphetamine (α-*N*-methyl-*N*-benzyl-β-phenylisopropylamine) was obtained from Upjohn Ltd., and SKF 525A (β-diethylaminoethyl-diphenylpropylacetate) was

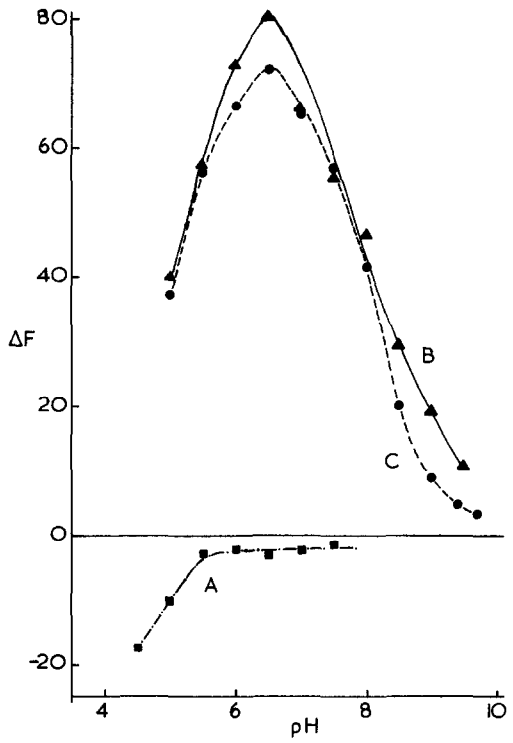

Fig. 1. The pH-dependence of drug effects on the fluorescence of ANS/microsomes. Incubation mixtures contained smooth microsomes (1 mg protein/ml) and ANS (100 μM) in buffers (I = 0.05) of various pH. The change in fluorescence ΔF (arbitrary units) was measured on addition of a concentrated drug solution to give A(■···■···■) 10 mM aniline; B(▲—▲—▲) 10 mM benzphetamine; C(●··●··●) 0.25 mM SKF 525A. Such additions caused a change in volume of less than 5%, and a change in pH of less than ± 0.05.

obtained from Smith, Klyne and French Laboratories Ltd; both were supplied as the hydrochloride salt. Fluorescence was measured on a Perkin–Elmer MPF-3 spectrofluorimeter with the cuvette block thermostatted at 25°, using excitation at 380 nm and emission at 480 nm. The excitation and emission beams were isolated by slits whose band pass never exceeded 10 nm. Drug and aniline enhancement of ANS/microsome fluorescence was investigated over the pH range 4.5–9.5 using sodium acetate/acetic acid buffers at pH 4.5–6.5, and Tris-HCl buffers at pH 7.0–9.5. Mg^{2+} enhancement of ANS/microsome fluorescence was investigated using an incubation mixture containing microsomes at a concentration of 1 mg protein/ml, 100 μM ANS and 25 mM KCl in 50 mM Tris-HCl buffer pH 7.5. Dilution of the stock microsome suspension containing 25 mg protein/ml and 5 mM $MgCl_2$ reduced residual Mg^{2+} in the incubation mixture to 0.2 mM. Mg^{2+} was titrated up to 10 mM by addition of a concentrated solution, the final increase in volume being less than 5%.

The pK_a of benzphetamine was determined by titration on a Pye 290 pH meter at room temp. using the expanded scale to give an accuracy of ± 0.005 pH units.

3. Results and discussion

3.1. *pH-dependence of drug effects on ANS/microsome fluorescence*

Diaugustine et al. [17, 18] showed that some drugs, which interact with liver microsomes or are metabolized by them, cause an increase in the fluorescence of ANS in the presence of microsomes at pH 7.4; other drugs have no such effect. Since the drugs showing the effect, such as benzphetamine and SKF 525A, are tertiary aliphatic amines, while the inactive category includes anilines, it seemed possible that the difference was simply a consequence of one class of drugs being charged at neutral pH while the others are uncharged. It is well established that simple salts, local anaesthetics and other charged species cause an increase of ANS/membrane fluorescence [8, 10, 19, 20]. The effect on ANS/smooth microsome fluorescence of aniline, benzphetamine and SKF 525A was studied as a function of pH. Fig. 1 shows that the increase in ANS fluorescence produced by benzphetamine and SKF 525A is rapidly reduced as the solution becomes alkaline. In both cases the increase in maximal at pH 6.5, and there is a sharp decline in the drug-induced fluorescence increase at lower pH values. (In the absence of the drugs the pH-dependence of ANS/microsome fluorescence is similar to that reported for other membranes (see e.g. [21]).) Near neutrality, aniline has little effect on ANS/microsome fluorescence, but a decrease in fluorescence is observed in its presence at pH < 6.

The decline in drug-induced increase in ANS/microsome fluorescence in the pH range > 6.5 is due to deprotonation of the drug; only the cationic forms cause an increase in ANS/microsome fluorescence.

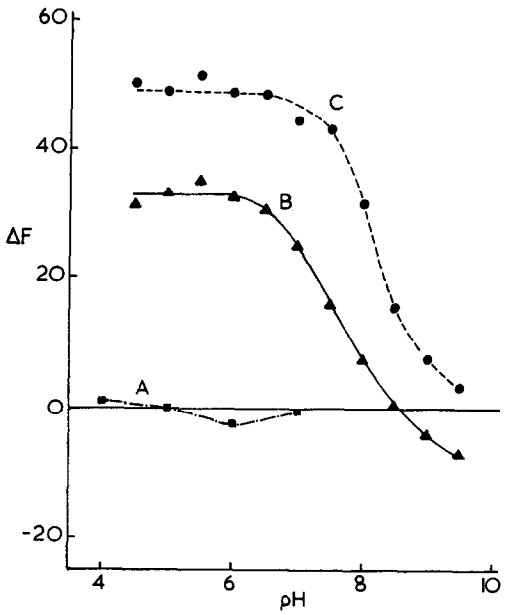

Fig. 2. The pH-dependence of drug effects on the fluorescence of ANS/lecithin dispersions. As for fig. 1, but microsomes were replaced by a lecithin dispersion (0.15 mg phospholipid/ml).

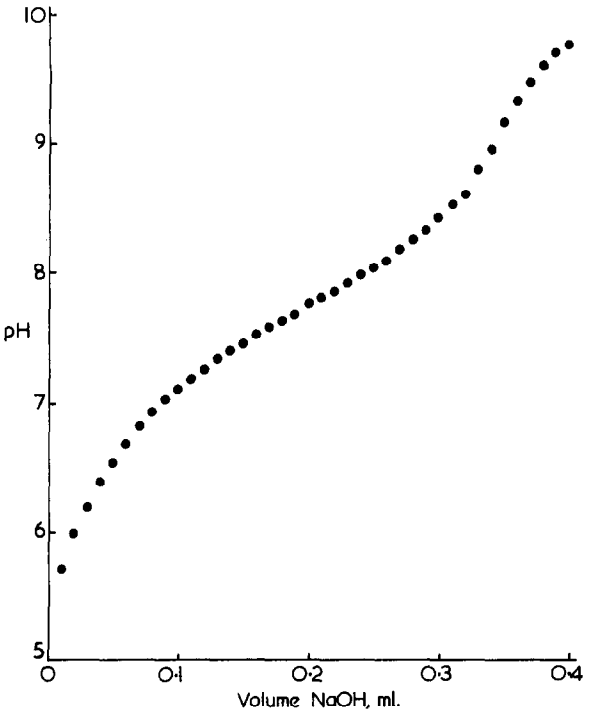

Fig. 3. Titration curve for benzphetamine hydrochloride. 50 ml of 1 mM benzphetamine hydrochloride were titrated against 10 μl aliquots of 0.1 M NaOH at room temp. with constant stirring.

The evidence for this is as follows:
i) At neutral pH benzphetamine and SKF 525A cause an increase in the fluorescence of ANS in the presence of a lecithin dispersion [18]. Aniline has no effect. The increase is pH-dependent, and in the range 6.5–9.5 it shows a similar titration to that found with microsomes (fig. 2).
ii) In the case of the lecithin dispersion, the drug-induced fluorescence increase reaches a plateau at pH values from 6.5 to 4, and hence it is possible to obtain an apparent pK_a for the process responsible for the increase. From fig. 2, a value of 8.2–8.3 is found for SKF 525A, and 7.6–7.7 for benzphetamine. A direct determination of the pK_a of benzphetamine by titration is shown in fig. 3 – the pK_a is 7.7. It was not possible to determine a corresponding value for SKF 525A because of its low solubility.

The decline in the drug-induced increase in ANS/microsome fluorescence in the range below pH 6 is due to an effect on the surface of the microsomal particles. The evidence for this is as follows: i) At pH values below 6 there is clumping of the microsomal suspension. At pH 5.5 this is fairly slow, at pH 5.0 it is quite rapid and at pH 4.5 it leads to extensive precipitation.
ii) In the ANS/lecithin dispersion system, there is no change in the drug effect on ANS fluorescence in the range of pH 4–6 (fig. 2) and no change in the physical state of the dispersion.
iii) Benzphetamine and SKF 525A cause an increase in the fluorescence of ANS/erythrocyte membrane mixtures, and this increase shows a pH-dependence similar to that of ANS/microsomes (fig. 4). In the case of the erythrocyte membranes, the decline in drug-induced fluorescence increase at low pH is also correlated with clumping of the membranes.
The evidence in this section suggests that the increase in the fluorescence of ANS/microsome mixtures promoted by drugs which interact with microsomal membranes, is not an indicator of a specific binding of the drug to the membrane. Similar increases are observed with a non-metabolising membrane and with a model membrane system, and the pH-dependence of the

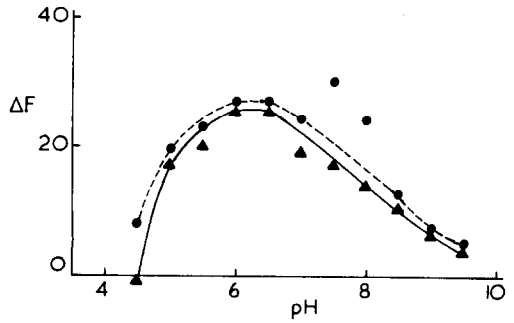

Fig. 4. The pH-dependence of drug effects on the fluorescence of ANS/erythrocyte membranes. As for fig. 1 but microsomes were replaced by erythrocyte membranes (also 1mg protein/ml).

fluorescence increase shows that it can be induced only by the protonated forms of the drugs. Since many inorganic and organic cations are known to increase ANS/membrane fluorescence, it is reasonable to conclude that the effects of benzphetamine and SKF 525A are not the result of any metabolically significant interaction.

3.2. *ANS as a probe of Mg^{2+} binding to microsomal membranes*

Smooth microsomes obtained by fractionation of total microsomes will not bind ribosomes except in the presence of steroid hormones [22]. However smooth membranes obtained by degranulation of rough microsomes by the use of EDTA (degranulated microsomes) will bind ribosomes readily, in the presence of the correct concentration of Mg^{2+}. The structural basis of this difference in behaviour is not known. One possibility is that there may be specific sites in degranulated microsomes which bind Mg^{2+} ions in such a way that they are able to form a bridge between the membranes and ribosomes. If this were the case, titration of these membranes with Mg^{2+} would reveal a class of binding sites not present in smooth microsomal membranes.

Gomperts et al. [10] have shown that the interaction of cations with membranes can conveniently be studied using ANS. The cations increase ANS/membrane fluorescence by increasing the number of sites on the membrane at which ANS can bind; this effect presumably arises because the bound cations neutralise negative charges at the membrane surface and

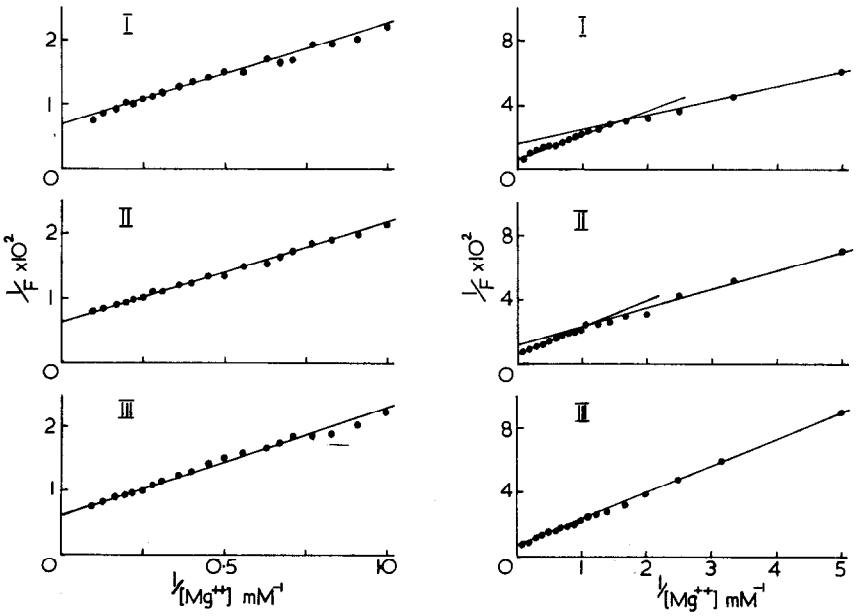

Fig. 5. Double reciprocal plot of ANS/microsome fluorescence as a function of Mg^{2+} concentration. I, smooth microsomes; II, degranulated rough microsomes; III, rough microsomes. Methods and conditions were as described in Materials and methods.

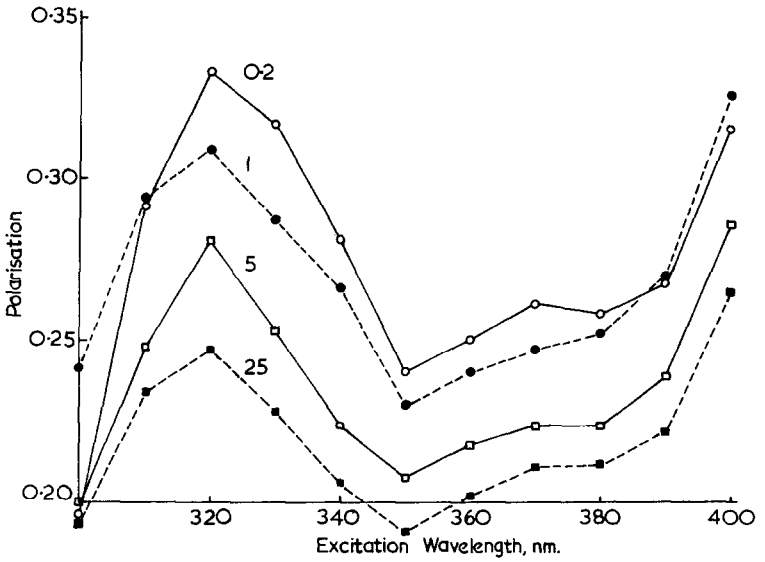

Fig. 6. Polarisation spectra at emission wavelength 480 nm of ANS/rough microsomes at different Mg^{2+} concentrations. 0.2 mM (○—○—○), 1 mM (●--●--●), 5 mM (□—□—□), 25 mM (■--■--■). The incubation mixtures contained microsomes (1 mg protein/ml), 10 μM ANS, 25 mM KCl and the stated concentration of $MgCl_2$ in 50 mM Tris-HCl, pH 7.5.

reduce the repulsion of ANS, which is itself anionic. If the increase in fluorescence is directly proportional to the number of cations bound, then a plot of 1/(fluorescence increase) *vs.* 1/(cation concentration) is a Klotz plot from which the cation binding parameters can be derived. Fig. 5 shows such plots for the interaction of Mg^{2+} with smooth, rough and degranulated microsomes in the presence of ANS. The plots show a discontinuity such as has been observed for the interaction of divalent cations with brain microsomes [10].

The association constants for Mg^{2+} are 1.8 mM^{-1} and 0.45 mM^{-1} with smooth microsomes, 0.8 mM^{-1} and 0.4 mM^{-1} with degranulated rough microsomes, and 0.4 mM^{-1} with rough microsomes. Thus both smooth and degranulated rough membranes possess high affinity sites for Mg^{2+} which are not present in rough membranes, the affinity of these sites being greater in smooth membranes. It seems likely either that there are no characteristic tight binding sites specific to degranulated rough membranes, or that binding of Mg^{2+} to such sites does not cause an increase of ANS fluorescence and is therefore not detected by the method.

These results are somewhat different from those of Dallner and Azzi [20]. They do not show their primary data, but tabulate dissociation constants for Mg^{2+} derived from double reciprocal plots of fluorescence enhancement against ion concentration; they find a single association constant for Mg^{2+} of 4 mM^{-1} for each of their membrane fractions.

3.3. *Interpretation of changes in ANS/microsome fluorescence*

The observation that addition of Mg^{2+} causes an enhancement of fluorescence in ANS/microsome systems obviously requires some analysis. This enhancement could be due to an increase in the number of sites to which ANS can bind, or to an increase in the affinity of the membrane for ANS, or to an increase in the quantum yield of fluorescence of bound ANS molecules, or to a combination of these factors. These parameters can theoretically be determined from titrations in which ANS and membrane concentrations are varied separately (see e.g. [3]). We have determined these parameters for the interaction of ANS with smooth, rough and degranulated rough microsomes at four levels of Mg^{2+} concentration, 0.20 mM, 1 mM, 5 mM and 25 mM. With the exception of the data at 0.20 mM, all the binding data are consistent with the existence of 20–25 μmoles of binding sites for ANS per gram of membrane protein, with a dissociation constant of approx. 10 μM. At 0.20 mM Mg^{2+}, the data

suggested a smaller number of binding sites for Mg^{2+} ($\simeq$10 μmoles per gram of membrane protein), with a lower affinity (K_d = 25 μM). The enhancement of ANS/microsome fluorescence in raising the Mg^{2+} concentration from low values to 1 mM therefore seems to be the result of changes in binding parameters; however, the further enhancement in ANS fluorescence on raising the Mg^{2+} concentration to higher levels must be the result of changes in the intrinsic fluorescence of bound ANS. Relative to free ANS (=1) the fluorescence of bound ANS increases from $\simeq$140 to 220 on raising the Mg^{2+} concentration from 1 to 25 mM. There are no significant differences observed in the ANS binding and fluorescence parameters of the three microsomal preparations.

Polarization spectra of ANS were determined for each membrane preparation at each Mg^{2+} concentration. Fig. 6 shows that the degree of polarization of fluorescence decreased with increase in Mg^{2+} concentration in rough microsomes; similar results were obtained using smooth and degranulated rough microsomes.

The ANS binding and fluorescence parameters quoted do not agree entirely with those of Dallner and Azzi [20], who found differences in the ANS binding parameters of rough and smooth microsomal fractions, and interpreted the changes in ANS fluorescence following the addition of cations as due entirely to a change in the affinity of the membrane for ANS. They do not quote values for the dissociation constant of ANS but their data appear to support a value of 30–50 μM in the absence of Mg^{2+} and a value of 4–7 μM in the presence of 5 mM Mg^{2+}.

In part these differences may arise from differences in the preparation of microsomal fractions, and from the fact that the titrations of Dallner and Azzi were performed on unbuffered microsomes. However, we believe that the differences in analysis reveal intrinsic problems in the use of fluorescence probes. Scatchard plots are constructed on the basis of experimental values for the 'intrinsic enhancement' of fluorescence of the bound probe. These values are usually derived from double reciprocal plots of fluorescence enhancement data. Even if the data themselves are of good quality, this extrapolation may involve considerable uncertainty. Harris [6] has shown that although such plots may appear linear over a long range, a linear extrapolation to the abscissa may not be justified. In fact even if such an extrapolation is justified, it may lead to an intercept so close to the origin that a small absolute uncertainty in the value of the intercept may be a large uncertainty in relative terms. Since the derived fluorescence enhancement is the reciprocal of this intercept, the uncertainty in its value is further magnified. Such uncertainty will cast doubt on the binding parameters subsequently derived. The interpretation of such binding parameters in molecular terms may therefore be a procedure of doubtful validity. A characterisation of membrane binding sites for fluorescent probes requires investigation of further properties such as fluorescent lifetime, fluorescence polarization and energy transfer from membrane to probe [1].

Acknowledgement

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