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Fluorescent Probe Studies of Biological Membranes and Model Systems

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The high sensitivity of fluorescence spectroscopy has been responsible for the increasing number of applications of fluorescence techniques in studies of biological systems. This article is an attempt to highlight some recent applications of fluorescence probe techniques in studies of biological membranes and model systems. While reference is made largely to studies on lipids and membranes, a few applications to studies of proteins and simpler systems are included to illustrate the applicability of some aspects of the method. This review is not intended to be a comprehensive summary of the literature in this area and the reader is referred to the many excellent reviews that have already appeared[1-4]. The background to biochemical fluorescence has been summarized in a recent publication[5] and details of many practical aspects of fluorescence techniques are also available[6]. Applications of fluorescence techniques in membrane research are based primarily on the use of extrinsic non-covalent fluorescent probes, which have been defined by Edelman and McClure[7] as "small molecules which undergo changes in one or more of their fluorescence parameters, as a result of non-covalent interactions with a protein or other macromolecule". This definition may be enlarged to include molecules that exhibit changes in fluorescence parameters as a function of the microviscosity of the medium, thereby serving as 'fluidity probes'. Covalently bound dyes and native chromophores like tryptophan and tyrosine residues of proteins also yield useful information. However, few studies involving the use of intrinsic membrane fluorescence have been reported, although a large body of work has been carried out on the intrinsic fluorescence of soluble proteins[8,9]. The present article is confined primarily to applications involving the use of extrinsic fluorescent probes. A brief and qualitative background to the relevant fluorescence parameters used in these investigations is provided below.

Fluorescence Parameters

The usefulness of fluorescence techniques arises from the fact that the lifetime of the excited state is sufficiently long (nanoseconds) for a variety of chemical and physical interactions to take place. Rotational motion, solvent re-orientation, complex formation and energy transfer between two chromophores are some of the processes that can occur prior to emission. The experimentally observable parameters which yield information about the system under investigation are listed below.

(i) *Emission wavelength* (λ_{em})—Many fluorophores exhibit a pronounced sensitivity of the emission wavelength to solvent polarity. Arylaminonaphthalenes which have been used extensively in membrane research show a marked blue shift of λ_{em} with decreasing solvent polarity. For example, the emission maximum of N-phenyl-1-naphthylamine varies from 460 nm in water to 419 nm in ethanol[4].In most membranes, λ_{em} is around 405-415 nm. As a consequence, attempts have been made to correlate the polarity (dielectric constant) of membrane binding sites with emission wavelength[10]. While most studies reported in the literature draw qualitative conclusions about binding site polarities on the basis of observed emission wavelengths, it should be stressed that such interpretations may often be misleading. Electronic excitation of molecules results in 'Franck-Condon' states[11] that are non-equilibrium with respect to nuclear geometry and solvation. Further, these states are vibrationally excited. Three processes may then occur: (a) vibrational relaxation by interaction with the solvent, (b) attainment of an equilibrium excited state, and (c) solvent reorientation stabilizing the equilibrium excited state[4].

It is likely that the environmental viscosity can affect the above processes, thereby altering the observed emission wavelength. The term 'microviscosity' has been used to describe orientational constraints on the solvent in the immediate vicinity of the fluorescent probe. This parameter may be determined by fluorescence polarisation measurements, as described later. An excellent treatment of solvent effects on fluorescence spectra is provided in the review by Radda[4].

(ii) *Fluorescence intensities/quantum yields*—The quantum yield q is defined as : q = quanta emitted/quanta absorbed. The maximum value is 1, but this is

seldom reached because of competing deactivation processes from the excited state. A major internal process is inter-system crossing from the singlet to triplet states. Collisional quenching, chemical reactions leading to non-fluorescent products and radiationless decay through the vibrational states of the two electronic levels also lead to losses[5]. The quantum yield reflects the environment of the fluorescent probe and is dependent on the excited state lifetime. In most studies, the parameters reported are fluorescence intensities, which are determined routinely. For qualitative purposes, intensities are referenced with respect to standards. Most measurements of probe binding parameters (dissociation constants and stoichiometries) use uncorrected emission intensities[3]. In general, increasing solvent viscosity and low solvent polarity lead to enhanced emission intensities in the case of a variety of fluorophores, like arylaminonaphthalenes and dimethylaminonaphthalene sulfonyl groups. Several attempts have been made to establish correlations between quantum yields and solvent viscosity and polarity[12-15].

(iii) *Excited state lifetimes* (τ)—This parameter is defined as the time necessary for the fluorescence intensity to fall to $1/e$ of its initial value. A first order process is generally assumed in a homogeneous system and the values are in nanoseconds. The complexity of instrumentation to measure fluorescence decay curves[16], coupled with the difficulties in the analysis of multiexponential decay curves, has so far restricted the range of applications of these measurements. However, fluorescence decay measurements of probes in lipid bilayers have been used to study lipid fluidity[17-21].

(iv) *Fluorescence polarisation*—Irradiation of randomly oriented fluorophores with plane polarised light results in a photoselection process, since it affects only those molecules whose transition dipoles are parallel to the electric vector of the incident light. Unless the dipoles reorient during the excited state lifetime, the emitted light has the same plane of polarisation as the exciting light. The orientations of the dipoles are randomized by rotational diffusion. This randomization is dependent on the excited state lifetime, the viscosity of the microenvironment of the fluorophore and the flexibility of the macromolecule to which the probe is bound. The polarisation $p=\frac{I_{\parallel}-I_{\perp}}{I_{\parallel}+I_{\perp}}$ where $I_{\parallel}$ and $I_{\perp}$ are the intensities of light emitted with polarisation planes parallel and perpendicular to the exciting beam.

Another parameter used is the fluorescence anisotropy

$$r=\frac{I_{\parallel}-I_{\perp}}{I_{\parallel}+2I_{\perp}}.$$

The utility of polarisation measurements in biological studies results from the relatively long rotational reorientation times for macromolecules ($\sim 10^{-8}$ to 10^{-9} sec). As a consequence, the observed polarisation (p) can be related to the rotational mobility of the fluorescent probe by the Perrin equation:

$$P-\frac{1}{3}=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\frac{3\tau}{\rho}\right)$$

$$P_0=\frac{3\cos^2\alpha-1}{3+\cos^2\alpha}$$

where α is the angle between the absorption and emission transition moments; τ, the measured fluorescence lifetime; and ρ, the rotational relaxation time of the fluorescent probe. For a spherical molecule

$$\rho=\frac{3V\eta_{\text{micro}}}{RT}$$

where η_{micro} is the microscopic viscosity; and V, the effective molar volume of the solute.

The relatively simple experimental arrangement[4] for the determination of fluorescence polarisation has been responsible for the wide application of these measurements. A comprehensive review of polarisation techniques and their use in determining lipid fluidity parameters has been provided by Shinitzky and Barenholz[22].

(v) *Fluorescence energy transfer*—Electronic excitation energy can be transferred over distances as large as 70 Å given a suitable pair of chromophores that function as donor and acceptor. This phenomenon allows the estimation of spatial distributions and interactions within a biological system. The rate of this dipole-dipole energy transfer is dependent on the inverse sixth power of the distance between the donor and the acceptor[23,24]. According to the theory developed by Förster, the rate of energy transfer, k_T, and the efficiency of transfer, E, are given by

$$k_T=r^{-6}K^2Jn^{-4}k_F\times 8.71\times 10^{23}\ \text{sec}^{-1}\ \text{and}$$

$$E=r^{-6}/(r^{-6}+R_0^{-6})$$

where R_0, the distance at which transfer efficiency is 50%, is given by

$$R_0=(JK^2\ Q_0\ n^{-4})^{1/6}\times 9.7\times 10^3\text{Å}$$

r is the distance between the donor and the acceptor; K^2, the orientation factor; J, the spectral overlap integral, determined by the overlap of the donor emission and acceptor absorption spectra; n, the refractive index of the medium; k_F, the rate constant for donor emission; and Q_0, the quantum yield of the donor in the absence of the acceptor. The transfer efficiency, E, is given by the equation

$$E=1-Q_T/Q_0$$

where Q_T and Q_0 are the quantum yields in the presence

and absence of transfer. A review of energy transfer techniques has appeared recently[25].

The uses of fluorescent probes have been reviewed extensively[1-4,26-31]. A large number of fluorescent probes are commercially available or are synthesized easily. One of the most important steps in the study of a biological system by fluorescence techniques is the choice of the probe. Static interactions are best studied by the use of environmentally sensitive probes that undergo large changes in their spectra, quantum yields and lifetimes on binding. The motional characteristics of membrane molecules can be investigated using probes with constant emission wavelengths and quantum yields. Polarisation studies then yield information about molecular rotation alone, with non-interference from other perturbations. Similarly, meaningful measurements of excimer emission, dynamic quenching and excitation energy transfer can be made if other properties remain unaltered. Interference by the probe must also be borne in mind while interpreting the results. Here, the low concentrations at which fluorescence experiments are carried out generally preclude major perturbations of the biological system[4]. However, there are reports of perturbation of lipid structures by hydrophobic probes even at concentrations as low as 2 μg/ml[32]. The structures of some of the probes discussed in the subsequent sections are presented in Figs 1-5. The probes are identified in the text by the numbers in parentheses.

Arylaminonaphthalene Probes

The naphthalene derivatives, 1-anilino-8-naphthalene sulfonate, ANS (1), 2-*p*-toluidinylnaphthalene-6-sulfonate, TNS (2), and N-phenylnaphthylamine, NPN (3), have been used extensively in studies of membranes lipids and proteins[1,4]. Weber and Laurence[33] were the first to observe that the quantum yields of N-phenyl substituted naphthylamine dyes are enhanced in heat denatured proteins. An early report by Newton described the sensitivity of TNS fluorescence to polymixin induced changes in cell membranes[34]. A spate of studies using these probes have appeared subsequently in the literature; only some of the more recent studies are mentioned here. Since the pioneering work of Chance and his collaborators on the application of fluorescence spectroscopy to the study of mitochondria[35-37], several studies have been reported. The binding of probes to submitochondrial particles has been analysed and alternative interpretations suggested[38]. A comparison of ANS and ethidium bromide binding to submitochondrial particles, whole and disrupted mitochondria, established that the inner membrane is freely permeable to the two probes[39]. The fluorescence of ANS and TNS has been shown to be sensitive to changes in the surface potential of mitochondrial membranes. Energization of the electron transport particles from *Mycobacterium phlei* results in enhancement of ANS fluorescence[40]. Phase transitions in *E. coli* membranes[41] and the effect of colicin E_1 addition[42] have been monitored by the use of ANS and NPN. ANS fluorescence has been shown to be sensitive to H^+ uptake and Rb^+ extrusion by gastric vesicles[43]. These probes have also been used as indicators of bacterial thermosensitivity[44] and to monitor protein release during sea urchin egg fertilization[45]. In intact metabolically active human spermatozoa at neutral

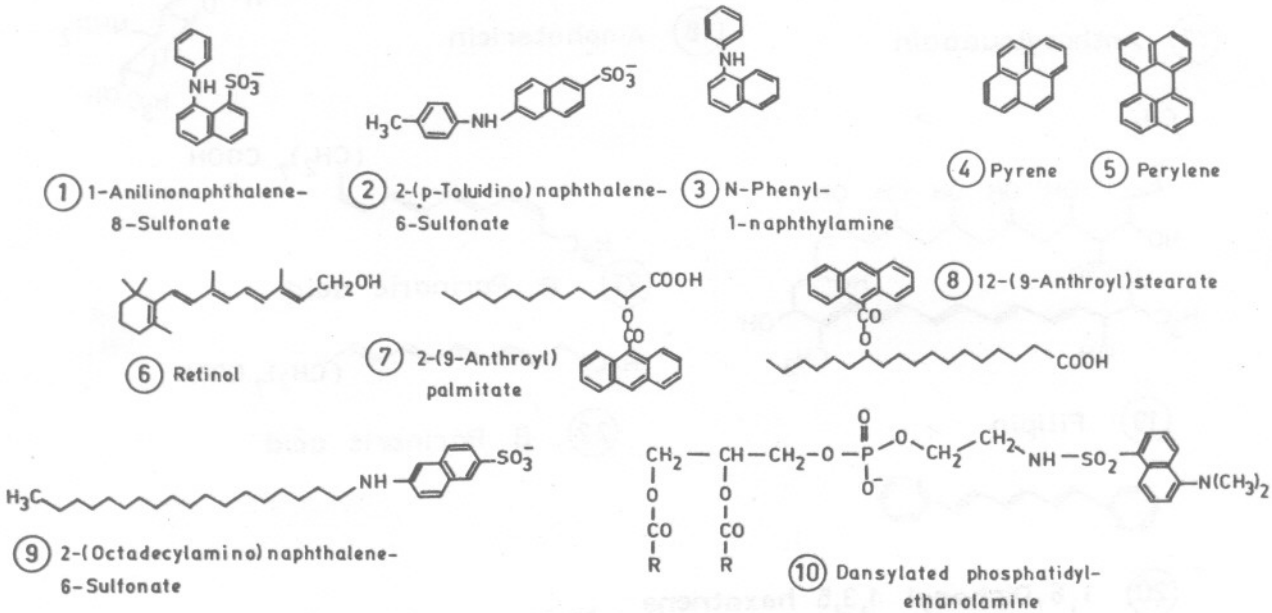
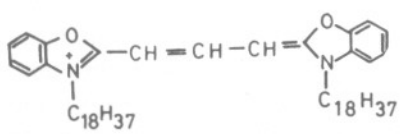
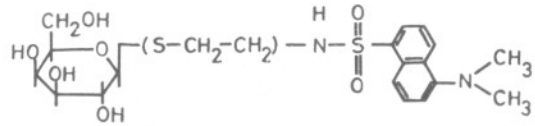


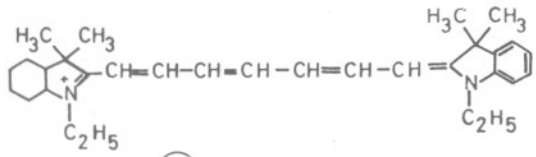
Fig. 1



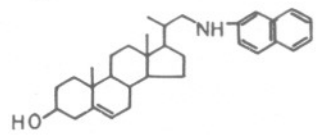
(11) N, N′ — Di (Octadecyl) Oxacarbocyanine



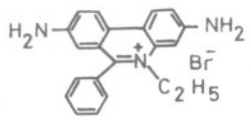
(12) Dansyl galactoside



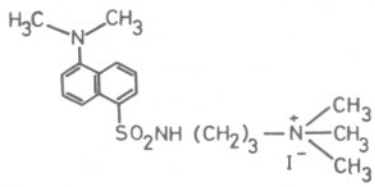
(13) Merocyanine



(14) N-(2-Naphthyl)-23,24-dinor-5-Cholesten 22 amin-3 β-ol

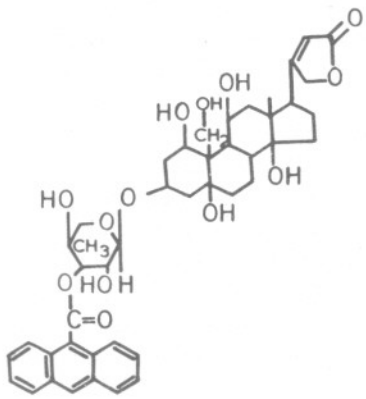


(15) Ethidium bromide

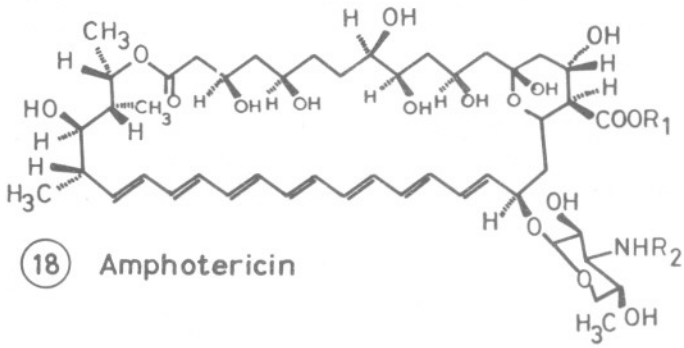


(16) 1-(5-Dimethylaminonaphthalene-1-sulfonamido) propane-3-trimethylammonium iodide

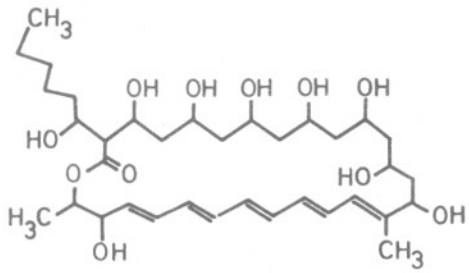
Fig. 2



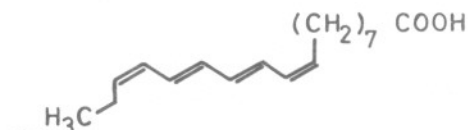
(17) Anthroylouabain



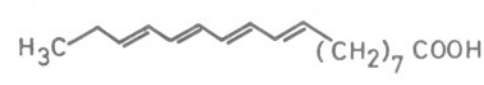
(18) Amphotericin



(19) Filipin



(21) α Parinaric acid



(22) β Parinaric acid

(20) 1,6,Diphenyl 1,3,5 hexatriene

Fig. 3

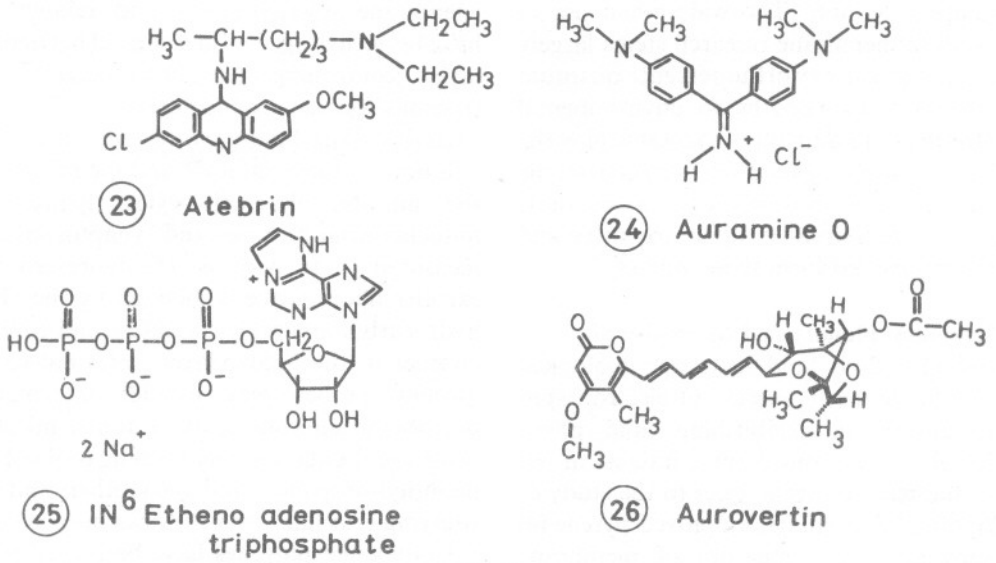


Fig. 4

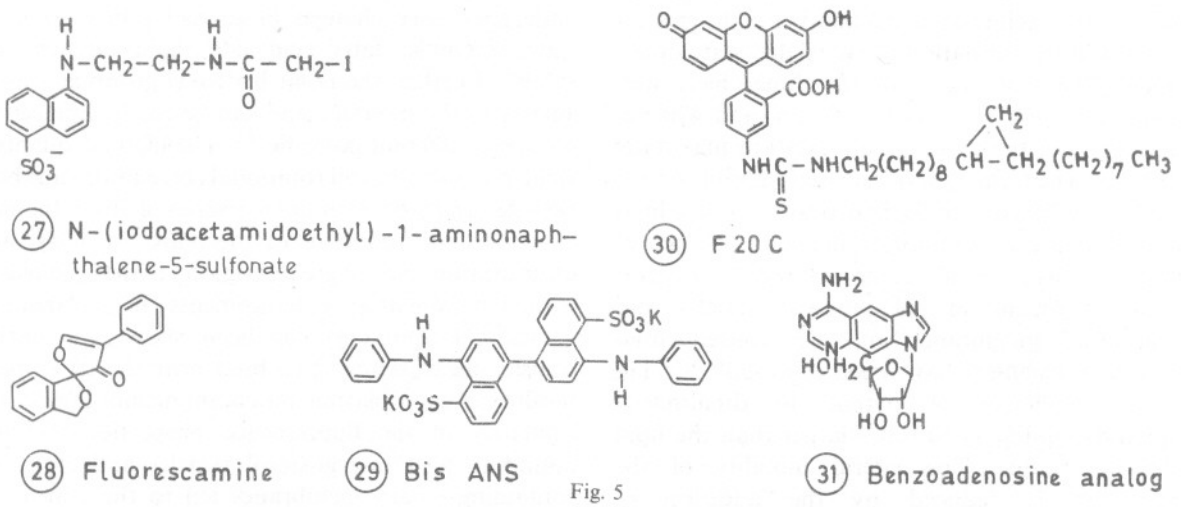


Fig. 5

*p*H, ANS binds to the plasma membrane, with some preferential binding to the midpiece, while at low *p*H, interaction with the acrosome is observed[46]. The fluorescence of ANS bound to sperm cells is sensitive to the metabolic state[46] and to the binding of prostaglandins[47]. Conformational changes associated with the addition of salts and sugars to rabbit intestinal brush border membranes have been monitored by ANS fluorescence[48]. The strong interaction of ANS with phospholipids is well established[49-52]. The binding of ANS to hydrophobic sites on proteins has been documented by the early studies on apohemoglobin and apomyoglobin[53], cholinesterases[54] and serum albumins[55]. The ease with which ANS and related molecules interact with diverse systems is a reflection of the lack of binding specificity of the probes. Detailed interpretations of ANS binding data and the sensitivity of probe fluorescence to cation concentrations and *p*H are likely to lead to ambiguous and uncertain conclusions[56]. The conventional manner of deriving binding data by Scatchard analysis[3] has been questioned recently and alternative techniques have been suggested[57]. Recent applications include the use of pulsed fluorometry to study the interactions of ANS and NPN with *E. coli* membrane[58]. Single photon counting has been used to measure the anisotropic decay of fluorescence polarisation of myosin rods labelled with ANS and also dansyl chloride, leading to an understanding of the movements of the rods[59]. An interesting application of TNS fluorescence to detect conformational changes in oligopeptide monolayers involved the use of intensity measurements as a function of the degree of polymerisation. Discontinuities were taken to represent conformational changes[60]. Myelin basic protein, proteolipid and myelin membrane have been studied using ANS and TNS as probes[61,62]. An intriguing approach to the characterization of β-structures in polypeptides using TNS was reported by Lynn and Fasman[63], but no extensions to proteins or

membranes appear feasible. The widespread use of probes like ANS in membrane research stems largely from the enormous sensitivity of fluorescence quantum yields and emission wavelengths to environmental conditions. However, the difficulties in establishing the nature of the probe binding sites often detract from the utility of these studies. Nevertheless, considerable information about cell surfaces, lipid structures and proteins has been derived from these studies.

Probes of Membrane Fluidity and Microviscosity

The concept of a fluid lipid matrix in biological membranes[64] and the early success of electron spin resonance techniques in establishing lipid phase fluidity[65] have generated considerable interest in the application of fluorescence techniques to the study of membrane fluidity. Early studies employed pyrene (4) excimer fluorescence as a measure of membrane fluidity. Collisional interactions between a pyrene molecule in the excited state and another in the ground state result in the formation of an excited state dimer (excimer). The monomer and the dimer have well separated emission maxima ($\sim$ 390 nm and 470 nm respectively) but have the same excitation maximum ($\sim$ 330 nm). The formation of excimers depends on the ease of lateral diffusion of the hydrocarbon in the lipid phase[66]. Pyrene excimer fluorescence in mitochondrial membranes has been demonstrated[67]. Lateral diffusion of pyrene in phospholipid vesicles and mitochondrial membranes and the consequential formation of excimers have been reported[66,68]. The diffusion coefficient of pyrene in dipalmitoyl phosphatidylcholine is 10 times larger than the lipid self-diffusion value. The lateral mobility of the hydrocarbon is reduced by the addition of cholesterol[69] and its quantum yield in solution is quenched by oxygen[70]. Differences in the temperature dependence of pyrene lifetime and quantum yield in the inner and outer membranes of *E. coli* have been noted[71]. Pyrene excimer fluorescence has been used in the study of *E. coli* membrane fluidity[72], and normal and virus transformed hamster kidney cells[73]. The presence of the protein component in a reconstituted plasma lipoprotein[74] and in an unsaturated fatty acid auxotroph of *E. coli* decreases the rate of pyrene diffusion[75]. The activity of lecithincholine acyl transferase depends on the physical state of lipids and has been studied using pyrene excimer emission[76]. The Ca^{2+} induced phase separation of 10-pyrenyl decanoic acid (PDA) in dipalmitoyl phosphatidylcholine[77] and PDA transfer from one liposome to the other by solubilization in the aqueous bulk have been reported[78]. The transfer of pyrene between human plasma high density lipoproteins is much faster than would be consistent with a fusion mechanism[79]. Membrane microviscosity and related parameters have been envisaged as mechanical barriers in the lipid layers, controlling transport processes[80] and signal transmission across membranes[81]. The microviscosities of various lipid mixtures, the effect of cholesterol addition on these values[82] and the microviscosities in the interior of erythrocytes, lymphocytes, submitochondrial particles and synaptosomes have been measured using perylene (5) fluorescence[83]. Pyrene excimer fluorescence is dictated by the ability of the hydrocarbon molecules to diffuse and collide with one another in the lipid bilayer. Intramolecular excimer forming probes may provide information about positioning and rotational freedom in micelles. The use of intramolecular excimer forming probes like 1,3-di-α-naphthyl-propane[84] and diaryl ethers and amines[85] in studying micellar viscosities has been described.

Conjugated polyenes have been used to advantage as lipid probes. The fluorescent probe retinol (6) undergoes large changes in geometry in the excited state, resembles fatty acid side chains and is lipid soluble. Further, the polar hydroxyl group acts as an anchor at the interface and can be easily replaced by groups of different polarities[86]. The lifetime, quantum yield, polarisation and rotational correlation time have been determined for retinol in a series of lipids. Increase in cholesterol content or decrease in fatty acid unsaturation leads to greater rigidity of the lipid matrix with consequential enhancements in polarisation values[87]. Filipin (19) has been shown, by energy transfer measurements, to bind near the tryptophan residues of sarcoplasmic reticulum membranes[88]. The similarity in the fluorescence properties of filipin bound to lecithin-ergosterol vesicles and ergosterol containing ciliary membranes led to the conclusion that its interaction with phospholipid bilayers is relevant to its antibiotic action. The biological activity of antibiotics like filipin and amphotericin (18) is believed to result from their ability to alter the permeability of membranes of microorganisms[89]. The polyene antibiotic, lucensomycin, and cholesterol form a complex. The measurement of the fluorescence parameters of this complex in micelles and erythrocyte membranes suggests that the complex is virtually immobile within the membrane[90]. α- and β-Parinaric acids (21,22) have been used to study synthetic phospholipid membranes. Parinaroyl phosphatidylcholine has been used in one- and two-component lipid mixtures. Lipid phase transitions are marked by two- to four-fold changes in lifetime and quantum yield and a large change in the probe rotational diffusion coefficient[91]. Unsaturated fatty acid auxotrophs of *E. coli* have been grown under conditions permitting 3% incorporation of parinaric acid into the membrane phospholipid. Fluorescence changes then reveal phase

transitions that reflect the fatty acid composition of the cell[92]. Thermally induced changes in the structure of cultured animal cell membranes have been monitored by changes in parinaric acid fluorescence[93].

1,6-Diphenyl-1,3,5-hexatriene, DPH (20), is probably the most widely used probe of membrane fluidity. It has a stable all-*trans* configuration and an elongated rectangular shape[22]. It has a strong absorption maximum at 335 nm and the transition moment of the fluorescence and absorption bond are parallel to the long axis of the molecule. Consequently, fluorescence depolarisation reflects almost exclusively the angular displacement of this axis. Its efficient partitioning into lipid domains enhances its utility as a probe of lipid microviscosity. DPH polarisation has been used to show that the physical properties of phospholipid bilayers in single and multilamellar vesicles are different[94], and to monitor phase transitions in artificial phospholipid membranes[95] and in one- and two-component phosphatidylcholine liposomes[96,97]. Membrane changes resulting from the action of a peptide antibiotic on *E. coli*[98] and in transformed 3T3 cells[99] have been demonstrated by DPH polarisation. Increase in the cholesterol content of fibroblasts and lymphocytes[100] and erythrocyte membranes[101] leads to increased membrane microviscosity and a corresponding enhancement of DPH fluorescence anisotropy. A decrease in the cholesterol content of human platelets, which play an important role in blood clotting, is accompanied by a reduction in DPH rotational diffusion[102]. The conclusion that malignant transformation of normal lymphocytes[103] and fibroblasts[104] is accompanied by increase in surface membrane fluidity is derived from DPH studies. The development of the chick embryo heart membrane is accompanied by a progressive increase in unsaturated fatty acid content and a consequential decrease in membrane viscosity, as measured by DPH[105]. Other reports employing DPH polarisation include studies of inside-out and rightside-out plasma membrane vesicles[106], bovine serum high density lipoprotein[107] and LM cell membranes[108]. Nanosecond fluorescence techniques have been used to study the interaction of DPH with lipid bilayers[18], egg lecithin vesicles[109] and di(dihydrosterculoyl)-phosphatidylcholine liposomes with varying amounts of cholesterol[20]. Lipid fluidity in artificial bilayers of microsomal lipids from *Tetrahymena* has been monitored by time resolved fluorescence depolarisation of DPH[109]. The effect of temperature acclimatization on *Tetrahymena* membranes has been followed by these techniques. It has been shown that the physical properties of membrane lipids are dependent on the cellular growth temperature and that membrane fluidity decreases in the order : microsomes > pellicles > cilia, throughout a wide range of growth temperatures[110]. The addition of insulin (10^{-9} *M*) to a suspension of rat liver plasma membranes increases the overall lipid microviscosity by 10-20%, as measured by DPH. The specificity of the interaction has been established by the lack of change in membrane microviscosity on adding insulin analogues to liver plasma membranes and insulin to human erythrocyte membranes[111]. Other recent applications of DPH fluorescence include a differential polarised phase fluorometric study of lipid bilayers[112] and the study of micellar aggregation of bile salts in aqueous solution[113].

While quantitative interpretations of lipid phase fluidity are best attempted using DPH or perylene, molecules with more complex geometries and varying lipid phase orientations have been used to provide valuable information. The use of lipid soluble probes like 2-(9-anthroyl) palmitate, AP (7), 12-(9-anthroyl) stearate, AS (8), 2-(octadecylamino) naphthalene-6-sulfonate, ONS (9) and dansyl phosphatidylethanolamine, DPE (10) has been reviewed[4]. In recent studies, the quenching of AS fluorescence in erythrocytes by energy transfer from haemoglobin has been used to study the binding of the protein to the membrane[114]. Fluorescence depolarisation and differential scanning calorimetry data for AS and two anthroyl palmitate probes have been reported[115]. The motional properties of AS and AP are sensitive to phase transitions of dimyristoyl and dipalmitoyl phosphatidylcholine vesicles[116]. Dansyl phosphatidyl ethanolamine has been used to study sarcoplasmic reticulum membranes[117], phospholipid vesicles[118], cardiolipin micelles[119] and reconstituted membranes of cytochrome b_5 and phospholipid vesicles[120].

Probes of Membrane Potential

Cyanine (11) and oxonol dyes respond to changes in trans-membrane electrical potential with corresponding changes in their light absorption properties[121]. The most extensively studied potential sensitive dyes are the cyanine, merocyanine (13) and styryl dyes[122,123]. 3,3′-Dipropyl thiadicarbocyanine has been used to monitor the resting potential across the plasma membrane of the ciliary protozoan, paramecium. Cations bring about rapid depolarisation of the plasma membrane, which is reflected in the dye fluorescence. Such changes in potential probably influence ciliary action[124]. Oxonol V has been used as a probe in beef and pigeon heart mitochondria and reconstituted ATPase vesicles[125]. Action potentials and voltage clamp steps in stained liquid axons are accompanied by changes in absorption, fluorescence and birefringence, these changes being linearly related to membrane potential[126]. Cyanine dyes have also been

used to study trans-membrane potentials of red cells[127], bacterial cells[128], synaptosomes[129] and purple membrane vesicles[130]. The emission intensity of 3,3'-dipentyloxo-carbocyanine iodide in boar spermatozoa varies with membrane potential, as established by studies in the presence of valinomycin or gramicidin and varying concentrations of external sodium and potassium[131]. In a recent study, the mechanism of the membrane potential sensitivity of merocyanine - 450 has also been examined[132].

Probes of Calcium Binding Sites

The importance of Ca^{2+} in the maintenance of membrane structural integrity and a variety of other physiological functions[133] has generated interest in the binding of Ca^{2+} to membranes. Fluorescent trivalent lanthanides have been used as inhibitors of Ca^{2+} binding and transport[134]. La^{3+} can replace Ca^{2+} in a metabolically significant manner[135]. Lanthanides have been used to identify and characterize the Ca^{2+} binding sites in lymphoid cells[136] and erythrocytes[137]. Studies on mitochondrial membranes[138] and acetylcholine receptor[139] have been carried out.

The tetracycline antibiotics form fluorescent chelates with Ca^{2+} and other divalent metal ions. The most widely used molecule is chlorotetracycline (CTC). Since the initial report by Caswell and Hutchinson[140] on the emission properties of metal-chlorotetracycline complexes, the fluorescent chelates have been employed in the visualization of membrane bound cations[141] and in studies of Ca^{2+} transport in sarcoplasmic reticulum membranes[142-144]. Studies of Ca^{2+} and Mg^{2+} movements in mitochondria have been carried out using CTC[145,146]. Calcium distribution in red cell membranes has been examined by CTC fluorescence leading to the conclusion that Ca^{2+} preferentially binds to the inner leaflet of the bilayer[147]. A comparison of radioactivity and CTC fluorescence data on Ca^{2+} migration in sarcoplasmic reticulum has been made. It is suggested that ADP is involved in additional binding of Ca^{2+} to the membranes[148]. Recent studies suggest that CTC fluorescence is extremely sensitive to *p*H in the range 7-8 and a new emission band at 430 nm (excitation 345 nm) is observed at *p*H values above 7.4. At lower *p*H, only the band at 520 nm (excitation 400 nm) is detected. The *p*Ka values of the 430 nm and 520 nm bands are 7.7 and 3.5 respectively, suggesting their assignment to the ring C-D and ring A chromophores. Divalent metal binding enhances the intensity of both the bands and causes energy transfer from the 430 to 520 nm bands, the transfer being greater for Mg^{2+} than Ca^{2+}. It is likely that this emission may be useful in studies on metal binding and cation translocation across membranes[198].

Miscellaneous Fluorescent Probes

Among the other fluorescent probes that have been used in the study of various biological systems are atebrin (23), aurovertin (26), antimycin and auramine 0 (24). Atebrin has been used in studies of the energized state in chloroplasts[149], bacterial membranes[150] and energized submitochondrial particles[151]. Aurovertin fluorescence has been employed in monitoring energy linked conformational changes in mitochondrial ATPases[152] and in studies of isolated F_1 mitochondrial ATPase[153] and its β-subunit[154]. Auromine 0 forms a complex with liver alcohol dehydrogenase[155] and has been used as a model for the interaction of cationic ligands with proteins[156].

Analogues and derivatives of physiologically important substances have been used as fluorescent probes. Anthroylouabain (17) is a specific probe for the cardiac glycoside binding site of Na^+-K^+ ATPase. The inhibition of enzymes from human red cells, eel electroplax and rabbit kidney cells has been reported[157]. A preliminary study on a fluorescent cholesterol analogue (14) has been carried out[158]. Dansyl galactosides (12) are specific probes for the β-galactoside carrier protein in bacterial membrane vesicles[159,160]. Membrane vesicles in which the lac transport system was absent did not show fluorescence changes on the addition of D-lactate. These results suggest that the dansyl galactosides bind to the lac carrier.

While the anionic hydrophobic arylnaphthylamine probes like ANS and TNS have been used extensively in membrane studies, positively charged probes have been employed in relatively few studies. Ethidium bromide (15), a very useful probe of nucleic acid structure[161-164], has been applied to the study of rat hepatic microsomes[165], mitochondrial membranes[39] and erythrocyte membranes[166]. Fluorescent alkylamines with spacer alkyl chains of varying length between the fluorescent dansyl group and the amino head group have been introduced. Dansyl cadaverine has been shown to be a potential probe for anionic sites on membranes[167]. Applications of these probes to studies of bile salt aggregation[113], mixed phospholipid bile salt micelles[168] and membranes[169] suggest that probe binding is likely to be affected by surface charge and also lipid packing.

The major problem in fluorescent probe studies of biological systems is often the low specificity of the binding interaction. To further enhance the potential of probe methods, it has been suggested that specifically synthesized 'active-site directed' probes may prove useful in studies of biological interactions[170]. Cholinergic ligands like 16 (Fig. 2) have been used to study insect and horse serum cholinesterases[171]. These ligands have also been used

to explore the binding sites of cholinergic receptors[172,173]. Cholinergic fluorescent probes have been useful in studying receptor-rich membrane fragments from *Torpedo marmorata*[174]. Recently, a series of homologous cholinergic probes have been employed in the study of cholinesterases and extensions to membrane bound receptors and enzymes appear feasible[175]. An elegant demonstration of the use of specifically tailored fluorescent molecules is the use of UDP-MurNAc-Ala-D-Glu-Lys (N^{ϵ}-dansyl)-DAla-DAla in a study of cell wall biosynthesis. The fluorescent reporter group is introduced into the membrane environment. Although the dansylated analogue is not utilized as effectively as the undansylated pentapeptide, the method may permit monitoring of the intra-membranal translocation involved in peptidoglycan synthesis[176]. The extensive work of Leonard and coworkers[177–179] on fluorescent derivatives of nucleotide bases has resulted in the synthesis of many novel and interesting fluorescent probes (*see* for example 25, Fig. 4 and 31, Fig. 5). While few applications of fluorescent nucleotides in membrane research have been reported, a fluorescent photoaffinity label of 3′,5′-cyclic AMP receptor sites has been introduced and applied to the study of a crude beef heart preparation containing cyclic AMP dependent protein kinase[180]. A number of covalent probes for labelling sulphhydryl groups[181,182] and amino groups[183] that are currently being used extensively for proteins are finding applications in surface labelling. These include N-(iodoacetamidoethyl)-1-aminonaphthalene-5-sulfonate (27)[184], fluorescamine (28)[185] and dansyl aziridine[186]. New fluorescent probes of membrane mobility include F20C (30) whose utility in studies of malignant and mitogen transformed cell membranes has been demonstrated[187].

An ANS dimer (bis-ANS, 29) has a high affinity for nucleotide binding sites of proteins and its binding to glutamate dehydrogenase and myosin ATPase has been studied[188,189]. Future applications to membrane systems appear attractive.

Fluorescence Energy Transfer Studies

Energy transfer studies have great potential in estimating inter- or intra-molecular distances. A determination of the surface density of membranes has been reported recently[190]. The energy transfer technique has been reviewed comprehensively[25]. The examples cited here are, therefore, only illustrative. Several intrinsic and extrinsic fluorescent probes have been used as donor-acceptor pairs. These include tryptophan-ANS[191], dansyl-fluorescein isothiocyanate[192] and N-(iodoacetamidoethyl)-1-aminonaphthalene-5-sulfonic acid-fluorescein isothiocarbamido cystamine[193]. The expressions derived by Förster[23] have been verified experimentally using donor-acceptor pairs held in a rigid framework[194] and in structurally defined systems[195]. The dependence of transfer efficiencies on distance was determined in an early study using oligomers of proline as spacers and naphthalene and dansyl groups as the donor and acceptor, respectively[196]. An exhaustive review of the applications is available[25]. The availability of specific labelling agents has introduced a considerable degree of sophistication into these measurements. A recent report involves the study of the erythrocyte anion transport system by energy transfer techniques. Band-3 vesicles, prepared from human erythrocyte, were labelled at sulphhydryl groups by fluorescein mercuric acetate. Using fluorescence depolarisation to monitor energy transfer between identical fluorophores it has been suggested that the major polypeptide exists as a dimer in the membrane[197].

The enormous versatility of fluorescent probe studies is demonstrated by the flood of literature on the subject. A wide range of biological phenomena may be studied and illustrative examples have been mentioned. In addition to further instrumental sophistication and greater refinement in data analysis, the design of specific fluorescent reporters is likely to receive continued attention in the future.

Summary

Recent applications of the fluorescent probe methodology in studies of biological systems are reviewed. A brief introduction to fluorescence parameters and their use is provided. Applications are discussed under the headings arylaminonaphthalene probes, probes of membrane fluidity and microviscosity, probes of membrane potential, probes of calcium binding sites, miscellaneous fluorescent probes, and fluorescence energy transfer studies.

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