BIOLOGICAL MEMBRANE MODELING WITH A LIQUID/LIQUID INTERFACE

Probing Mobility and Environment with Total Internal Reflection Excited Fluorescence

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ABSTRACT Total internal reflection of exciting light, in combination with fluorescence intensity and polarization measurements, was used to selectively study fluorescent compounds adsorbed to the interface region between two immiscible liquids. A fluorometer was constructed which provided excitation at variable angles of incidence and allowed sensitive detection of polarized fluorescence emitted from the interface. The compound 4,4'-bis-1-phenylamino-8-naphthalenesulfonate (bis-ANS) was examined at a decalin/water interface and was found to possess remarkable affinity for the interface region with the bulk of the adsorbed molecule residing in the decalin phase. The adsorbed fluorophore displayed an apparent hindered rotation in the plane of the interface with a rotational diffusion coefficient 3- to 12-fold lower than that expected for bis-ANS in solution. While other dyes examined were not found to be significantly surface active, the addition of cationic surfactant sufficed to induce adsorption of the anionic fluorophore 1-aminonaphthalene-3,6,8-trisulfonic acid. This fluoropore was found to reside in an aqueous environment when bound to the interface, and it also exhibited hindered rotation in the plane of the interface. As the concentrations of the dyes were increased, both adsorbed dyes exhibited polarization reductions consistent with excitation energy transfer. Adsorption of bis-ANS was reversed by addition of bovine serum albumin. The membrane protein cytochrome b_5 was found not to bind at the decalin/water interface, indicating that interaction with lipid is required for its adherence to biological membranes.

INTRODUCTION

The study of biological membranes has benefited from experimentation with less complicated model systems. The membrane models in general are composed of micelles, monolayers, vesicles, and lipid bilayer membranes (1–3). In these systems, however, it is often difficult to distinguish a subpopulation of molecules undergoing interactions with the membrane from molecules in the bulk solution. We have chosen to examine a membrane model that offers the advantage of allowing orientation of the membrane surface with respect to the laboratory frame. When combined with the spectroscopic technique of total internal reflection excited fluorescence (TIR-excited fluorescence or TIRF), molecules near the surface of the membrane may be selectively studied. This model is composed of a planar liquid/liquid interface. Aqueous buffer constitutes one liquid phase, while an immiscible hydrocarbon solvent serves as the second liquid phase. The water/hydrocarbon solvent interface is the simplest representation of a biological membrane surface which separates the aqueous cytosol or external medium from the apolar central region of a bilayer membrane. Lipids or other surfactant molecules may be added to the model systems to improve the resemblance of the interface to a biological membrane surface.

Total internal reflection of excitation light at the liquid/liquid interface can be used to selectively excite luminescent compounds that are located near that interface. When light traveling in the liquid of higher refractive index, n_1, is incident upon the interface at an angle greater than the critical angle, ϕ_c, transmission of light through the liquid of lower refractive index, n_2, does not occur and the light is completely reflected. The critical angle is determined by the refractive indices of the liquids as given in Eq. 1, where n_{21} denotes the quantity n_2/n_1:

$$\phi_c = \sin^{-1}(n_{21}). \quad (1)$$

Although the light undergoes total reflection, there is some penetration of light into the medium of lower refractive index in the form of an evanescent wave. The properties of this evanescent wave have been described (4, 5). The

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electric field amplitude, E, of the wave decreases exponentially with distance from the interface, x, according to Eq. 2, where d_p is the penetration depth normal to the interface and E_o is the electric field amplitude at the interface in the liquid of lower refractive index:

$$E = E_o e^{(-x/d_p)}. \quad (2)$$

The penetration depth is a function of the angle of incidence, ϕ, and the wavelength of the light in the liquid of greater refractive index, λ, as shown by Eq. 3:

$$d_p = \lambda/2\pi(\sin^2\phi - n_{21}^2)^{1/2}. \quad (3)$$

For typical experiments the penetration depth is on the order of 1,000 Å. If the luminescent compound is soluble only in the liquid of lower refractive index, then excitation of the compound can occur only via absorption of the evanescent light, or via absorption of the incident light if the compound protrudes through the interface into the liquid of higher refractive index. The latter case would apply to some amphiphilic molecules that adsorb to the interface region.

TIR excited fluorescence has previously been applied to the study of biological systems and has been reviewed recently (6). These applications have used total internal reflection at solid/liquid or solid/air interfaces, the solid generally being a glass substrate. The study of protein adsorption from aqueous buffer onto glass surfaces has been reported using intrinsic protein fluorescence (7, 8) and fluorescence from proteins labeled with reporter groups (9–12). Concentration quenching in chlorophyll *a* monolayers was examined at the interface between aqueous solvent and air (13). TIR-excited fluorescence has also been used to probe cell contact regions (14, 15), perform immunoassays (16), and classify viruses (17). The absence of TIR-excited fluorescence studies at liquid/liquid interfaces is understandable in view of the difficulty in forming a reproducible interface. The interface will alter with movement and requires special handling procedures. The liquid/liquid interface is more amenable to study by classical techniques such as surface tension measurements. Surface tension and potential jump measurements were used to examine the interaction between nucleotides and a water/hydrocarbon solvent interface in the presence of cationic detergent (18, 19). In addition, the partitioning of proteins between two liquid phases has been used to model the transfer of membrane proteins into membranes (20, 21). Total internal reflection studies at a liquid/liquid interface were reported in several Raman scattering studies of the binding of anionic dyes to a water/chloroform interface in the presence of cationic detergent (22–24). The polarization of Raman scattered light was used to determine the orientation of dye molecules at the liquid/liquid interface.

Fluorescence polarization measurements can provide valuable information about the rotational mobility of molecules in addition to information about orientation. Fluorescence polarization measurements at solid/liquid interfaces were reported recently for adsorbed labeled protein (25) and for fluorescent lipid distributed in a phospholipid monolayer (26). In the present work we have used fluorescence polarization measurements in addition to measurements of fluorescence intensity to characterize the binding of fluorescent molecules at a liquid/liquid interface. Polarized light that undergoes total internal reflection can produce polarized components along three axes in the evanescent wave. The inset in Fig. 1 shows the coordinate system that will be used in further discussion. The interface is contained within the YZ-plane and the path of the excitation light, L, is contained within the XY-plane. The excitation light strikes the interface at the origin of the coordinate system making an incident angle of ϕ. Incident light polarized with the electric field vector parallel to the Z-axis produces an evanescent wave in which the electric field vector is also polarized along the Z-axis. The electric field amplitude of the evanescent wave at the interface, E_{zo}, is related to the electric field amplitude of the polarized

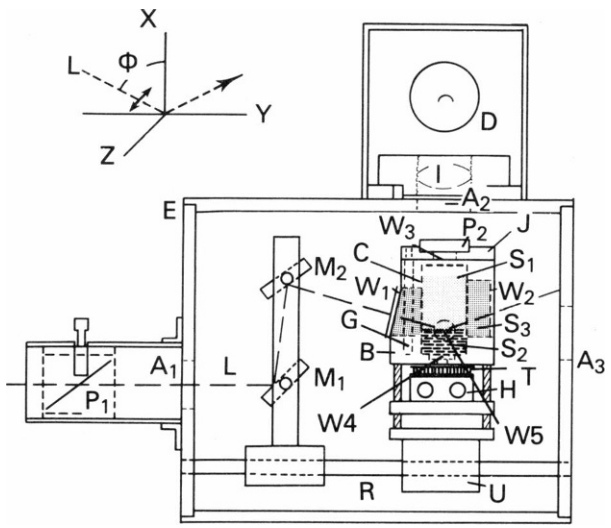

FIGURE 1 Drawing of the fluorometer sample compartment including the placement of excitation beam mirrors (M_1 and M_2), sample cell holder (B), and photomultiplier tube detector (D). Other components depicted are the excitation polarizer (P_1), excitation beam aperture (A_1), excitation light path (L), cell holder and mirror holder mounting rails (R), cell holder base (U), sample cell (C), upper solvent phase of sample (S_1), lower solvent phase of sample (S_2), index matching solvent external to sample cell (S_3), excitation beam entrance window (W_1), excitation beam exit window and Y-axis emission window (W_2), upper X-axis emission window (W_3), lower X-axis emission window (W_4), Z-axis emission window (W_5), emission polarizer assembly (P_2), sample compartment lid (J), X-axis emission aperture (A_2), Y-axis emission aperture (A_3), emission collection and focussing lens (I), thermoelectric module (T), and heat exchanger (H). The inset shows the coordinate system applicable to the sample geometry. The liquid/liquid interface is contained within the YZ-plane. The path of the excitation light is contained within the XY-plane and is incident upon the interface at an angle of ϕ degrees.

incident light, E_{zi}, by Eq. 4 (4):

$$E_{zo} = 2E_{zi} \cos \phi/(1 - n_{21})^{1/2}. \quad (4)$$

Incident light polarized with the electric field vector contained within the XY-plane produces evanescent light with electric field components in both the X and Y directions. For this reason we have chosen to use incident light with the electric field polarized parallel to the Z-axis for the present work. Also, to avoid problems with viewing emission along the edge of the interface, we have measured fluorescence from above the interface along the X-axis. In this system the fluorescence polarization, P, is determined by the relative intensities of fluorescence polarized parallel, I_z, and perpendicular, I_y, to the excitation light, as defined by Eq. 5:

$$P = (I_z - I_y)/(I_z + I_y) = 3r/(2 + r), \quad (5)$$

where r is the fluorescence anisotropy. The measured anisotropy or polarization is related to molecular rotation of a spherical molecule in an isotropic environment by the Perrin equation (27) given by Eq. 6:

$$r_o/r = 1 + 6R_d\tau = 1 + RT\tau/\eta V, \quad (6)$$

where R_d is the rotational diffusion coefficient, R is the gas constant, τ is the lifetime of the excited state, η is the viscosity of the medium, T is the absolute temperature, and V is the molar volume of the rotating unit which includes the fluorophore and any associated species such as solvent molecules rotating with the fluorophore. The limiting anisotropy, r_o, is the anisotropy observed in vitrified solution where rotational motion within the lifetime of the excited state is negligible. This value is determined by the angle, α, between the absorption and emission dipoles as described by Eq. 7 (28):

$$r_o = (3 \cos^2 \alpha - 1)/5. \quad (7)$$

It has been demonstrated that the rotational diffusion coefficient may be identified with a rate constant, k, for the transport of the excited state oscillator component from one axis to another (29). While in dilute solution k is related to molecular rotation, in concentrated viscous solution k corresponds to excitation energy transfer between molecules. The interpretation of the polarization values, therefore, is dependent upon the environment of the fluorophore.

Molecules adsorbed at an interface, however, are not in an isotropic environment and will be oriented relative to the plane of the interface. The absorption dipoles are expected to be randomly distributed only about the X-axis and rotational rates in the plane of the interface and out of the plane of the interface are expected to differ. The Perrin equation would only apply in the limit of equal rotational mobility in and out of the interface. A better description of polarized fluorescence observed from above a planar interface is afforded by a two-dimensional analysis of rotational motion within the plane of the interface. In the two-dimensional system polarization and anisotropy are both equal to $(I_z - I_y)/(I_z + I_y)$ since no X directional component exists. The equivalent of the Perrin equation is then given by Eq. 8:

$$P_o/P = r_o/r = 1 + 4k\tau \quad (8)$$

and limiting polarization is given by Eq. 9:

$$P_o = r_o = \cos^2 \alpha - 1/2 \quad (9)$$

A derivation of the two-dimensional equations is presented in the Appendix. This analysis strictly applies if the transition oscillators of the fluorophore are restricted to lie within the plane of the interface. Even if this is not true, the analysis is valid for the components of the oscillators projected onto the plane of the interface.

In work presented here, we have performed fluorescence intensity and polarization measurements on fluorophores excited at a decalin/water interface by total internally reflected light. Construction of a fluorometer was required to provide excitation at variable angles of incidence and allow sensitive detection of polarized fluorescence emitted from the interface. The initial experiments were intended to explore the potential of TIR-excited fluorescence measurements at liquid/liquid interfaces and therefore employed simple fluorescent compounds. In particular, the compound 4,4'-bis-1-phenylamino-8-naphthalenesulfonate (bis-ANS) (37) was found to be a useful example because of its high affinity for the interface and the large fluorescence shift observed upon binding. As a further study of the liquid/liquid interface the nonfluorescent surfactant cetyltrimethylammonium bromide (CTAB) was included in the two phase system to simulate lipid molecules in a biological membrane and to induce the binding of anionic fluorophores. Finally, the affinity of the membrane protein cytochrome b_5 for the decalin/water interface was briefly examined.

MATERIALS AND METHODS

Chemicals

Bis-ANS was synthesized as previously described (30). Spectro Grade decalin was obtained from Kodak Laboratory and Specialty Chemicals (Eastman Kodak Co., Rochester, NY) and CTAB, purum, was purchased from Fluka Chemical Corp. (Hauppauge, NY). Bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (St. Louis, MO). Cytochrome b_5 labeled with 5-dimethylamino-1-naphthalenesulfonyl chloride (dansyl chloride) was provided by Drs. Reid Gilmore and Michael Glaser (31). All other reagents were of reagent grade or better.

Fluorometer for TIR-excited Fluorescence Measurements

The fluorometer constructed for TIRF measurements at a liquid/liquid interface was composed of a source of tunable and collimated excitation light, a sample compartment, and the associated detectors and electronics

required to measure the interfacial fluorescence. Light pulses of ~10-ns width and 130-kW peak power were generated by a nitrogen laser constructed in a manner similar to that described by Bryant (32, 33). The output of the nitrogen laser was used to pump a DL-14 tunable dye laser (Molectron Corp., Sunnyvale, CA). A small fraction of the dye laser output was reflected onto a reference photomultiplier tube to allow normalization of fluorescence intensities to excitation pulse intensities. The remainder of the dye laser output was attenuated by neutral density filters before entering the sample compartment. The laser beam intensity was generally reduced to the point that less than 6 μJ/pulse reached the sample.

The design of the sample compartment is presented in Fig. 1. The coordinate system relevant to this arrangement is shown as an inset to this figure. Light from the dye laser (L) traveling along the Y-axis entered the sample compartment (E) through a port that contained a Glan-Taylor prism polarizer for selection of light polarized parallel to the Z-axis. The light was then directed by two mirrors (M_1 and M_2) onto the center of the interface between the decalin phase (S_1) and the aqueous solution (S_2) contained in the sample cell (C). The sample cell was composed of a flat bottom pyrex cylinder, 44-mm high and 22-mm in diameter. The cell contained a hole near the bottom into which a piece of stainless steel tubing was cemented, which permitted the addition of reagents and removal of solution from the lower (aqueous) phase during experiments. The cell resided within a brass cell holder (B), which served to thermostat the sample cell, align the cell with the detectors, and minimize the amount of scattered light reaching the lower aqueous phase. The cell holder lid (J) protected the sample from dust particles and served to position the emission polarizer. Temperature control was provided with a thermoelectric module (T), model 940-31 (Borg Warner Thermoelectrics, Des Plaines, IL), sandwiched between the base of the cell holder and a brass heat exchanger (H) through which tap water was circulated. Precision thermistors (model 44008; Yellow Springs Instrument Co., Yellow Springs, OH) were cemented into holes in the side of the cell holder to provide for temperature measurement. Six quartz windows were cemented over openings in the bottom, top, and sides of the cell holder. Excitation light entered through a window (W_1) which was inclined at an angle of 11° to the vertical to lower the minimum allowable angle of incidence at the decalin/water interface. A second window (W_2) covered a similar but vertical opening in the rear of the cell holder through which the excitation light could exit the holder. A third window covered a square opening on the side of the cell holder facing the access door to the sample compartment and was used for visual alignment of the laser beam. Emission from the liquid/liquid interface in the X direction was viewed through window W_3 in the lid of the cell holder. Provisions were also made to view the interfacial fluorescence from the bottom of the cell (W4), along the Y direction (W2), and along the Z-direction (W5). To reduce the amount of stray and scattered light reaching the lower aqueous phase, a brass shield (G) containing a small aperture was positioned within a vertical slot in front of the sample cell within the cell holder. The shield was adjusted vertically to permit passage of the laser beam through the aperture while blocking light scattered at the entrance window of the cell holder. Scatter produced by the excitation light striking the sample cell wall was reduced by using decalin as an index matching solvent (S_3), which filled the interior of the sample holder and bathed the outside surface of the sample cell and inner surfaces of the sample holder windows.

Light emitted from the decalin/water interface was detected by a photomultiplier tube (D) which was mounted on the external wall of the sample compartment. A fused silica lens (I) served to focus light emitted from the interface onto the detector. Under the experimental conditions, this lens collected light emitted from the center of the interface within an angle of 7° to the X-axis. Consideration of the angular distributions of light emitted from a dipole located on the interface and oriented in either the X, Y, or Z directions (41) showed that the solid angle of light collection was suitable for emission polarization measurements. The small numerical aperture ensured that essentially no emission from a dipole oriented in the Z direction was detected by the photomultiplier tube. Fluorescence observed in the X direction was polarized by passage through a polarizer assembly (P_2) positioned in a slot located in the top of the cell holder lid. During an experiment the polarizer was rotated to alternately transmit emission polarized in the Z and Y directions. The polarizer material was a Polaroid HNPIB type technical film dichroic. Interfacial fluorescence was filtered by placing glass filters directly over the detection aperture (A_1). Alternatively, emission spectra were recorded by collecting light emitted in the X direction using a 3.2-mm diam glas fiber optic light guide (Ealing Corp., S. Naticks, MA). One end of the light guide was located above the interface and the other end of the light guide was located at the input port of a Jarrel Ash model 82-410 0.25-m Ebert monochromator (Fisher Scientific Co., Waltham, MA). A 2.5-mm slit (3.3 nm/mm dispersion) was used on the emission side of the monochromator while the end of the fiber optic bundle replaced the entrance slit. A photomultiplier tube detector was placed immediately after the monochromator exit slit. Model R928 side-on photomultiplier tubes (Hamamatsu Corp., Middlesex, NJ) were used for detecting fluorescence and a model 931A side-on photomultiplier tube (RCA, Lancaster, PA), was employed to monitor the laser pulse. The dynode chains of these detectors were wired for fast response according to Harris et al. (34).

Each photomultiplier tube anode was connected to an integrate-and-hold amplifier composed of a 50-pf integration capacitor and an LF 356 operational amplifier (National Semiconductor, Santa Clara, CA). The amplifier input was gated using two D-MOS FET switches (model SD5000; Signetics Corp., Sunnyvale, CA) to sequentially ground, close, and open the connection between anode and amplifier. Anode currents were integrated from several microseconds before the initial fluorescence rise until several microseconds after the initial rise. Timing of the gate was accomplished using two fast comparators (model AM686; Advanced Micro Devices, Inc., Sunnyvale, CA) buffered with high speed gate drivers. After the integration of each fluorescence pulse, the amplifier outputs were digitized with an ADC80 analog-to-digital converter (Burr-Brown Research Corp, Tucson, AZ) and read by a microcomputer constructed from an SDK-8085A System Design Kit (Intel Corp., Santa Clara, CA). A second integration was then performed, and the value obtained from this integration was subtracted from the first to correct for background and amplifier drift. Each fluorescence measurement was the sum of 500 pulses normalized to the laser pulse intensity. A mean and standard deviation was calculated generally from four or more measurements at each polarizer orientation. These values were then used to calculate the total fluorescence intensity and the emission polarization. Error bars in Fig. 2 and Figs. 7–10 represent one standard deviation of the propagated errors.

Sample Preparation and Experimental Procedure

Care was taken to ensure that samples contained no particulate matter that could scatter incident excitation light into the bulk aqueous phase during an experiment. Solutions of aqueous buffer and decalin were filtered and added to the sample cell while contained in a glove bag (Instruments for Research and Industry, Cheltenham, PA) purged with filtered nitrogen gas. The cell was placed in the cell holder and the lid attached before removal from the glove bag. The lower phase in the sample cell consisted of 3.0 ml of 50 mM sodium phosphate buffer at pH 7.0. The upper phase consisted of 6.0 ml of decalin. Cylindrical glass cells were judged to provide the least distortion of the interface between the two immiscible liquids. A flat interface was obtained by using cells treated for 7 s with 1% dichlorodimethylsilane in chloroform. The coating was removed after each experiment by washing the cells in alkaline detergent and soaking in nitric acid overnight. Fluorophores and detergents were added to the sample cell during the course of the experiment through the stainless steel tubing connected to the base of the cell. Interfacial fluorescence was monitored until stable with time before determining the total and polarized fluorescence intensities. 12-h equilibration periods were required at nanomolar and lower fluorophore

concentrations, while one to several hour equilibration periods were sufficient at higher concentrations.

At the end of each experiment, a correction factor was determined which was used to compensate for the different sensitivities of detection for parallel and perpendicular polarized light. To obtain this correction factor the incident angle of the laser beam was reduced below the critical angle and concentrated fluorophore was added to the aqueous phase until the absorbance of the aqueous phase was greater than 20 at the wavelength of the laser. This ensured that 99% of the refracted excitation light was absorbed within 1 mm beneath the interface, thereby providing an illuminated region with practically the same spatial characteristics as the TIR-excited fluorescence. The known polarization of the concentrated fluorophore was then used to compute the correction factor. To minimize any error due to a wavelength dependence of the correction factor, the polarization standard was either the same fluorophore used in the experiment or another decalin-insoluble fluorophore which possessed a similar emission spectrum. The correction never resulted in greater than a 5% change in the intensity of one polarized component relative to the other.

RESULTS

Partitioning Experiments

The initial study of liquid/liquid interfaces was performed with simple fluorescent molecules to define the capabilities of the TIR-excited fluorescence measurements. Fluorophores in these experiments were selected for very low solubility in non-aqueous solvents. Partitioning of minute amounts of fluorophore into the non-aqueous phase would produce large amounts of background fluorescence due to excitation of the fluorophore by the incident and reflected excitation light. Fluorophores were screened by partitioning relatively high concentrations of the fluorophores (3 mM to 0.2 M) between equal volumes of aqueous buffer and any of several organic solvents. The results of these experiments are presented in Table I. The detection of any fluorescence or absorbance of the fluorophore in the organic solvent is indicated by a plus sign in the table. A minus sign indicates no detectable partitioning and was a requirement for use of the fluorophore in the interface studies. Each of the organic solvents listed in Table I is suitable for use as the incident phase in TIR-excited fluorescence studies since each solvent is immiscible with water, possesses a refractive index greater than that of water, and is less dense than water. A density less than water was required since the fluorometer was designed for the upper phase being the incident medium. Decalin was chosen for the TIR-excited fluorescence experiments due to its availability in high spectral purity (absorbance < 0.01 at 270 nm) and its hydrocarbon character. A hydrocarbon solvent was assumed to provide a better approximation of the interior of a biological membrane since the apolar regions of phospholipids are primarily hydrocarbons.

TIR-excited Fluorescence Experiments: Adsorption of Bis-ANS

The adsorption of fluorescent compounds at the decalin/water interface was studied by titrating the fluorophores into the aqueous phase and exciting any interface-bound species with horizontally polarized light incident upon the interface at an angle of 72° ($\phi_c = 65°$). Fluorophores examined in this manner were aminoanaphthalenetrisulfonic acid, bis-ANS, riboflavin, perylenetetracarboxylic acid, and dansylaspartic acid. Of these compounds, only bis-ANS showed appreciable surface activity at aqueous phase concentrations of 0.1 mM and lower. The fluorescence recorded for bis-ANS excited at the decalin/water interface is plotted in Fig. 2 as a function of the amount of bis-ANS added to the aqueous phase. The total fluorescence intensity, $I_z + I_y$, shown as circles, was found to increase gradually as the bis-ANS concentration was increased from below 0.1 nM to 10 nM. A sharp increase in the fluorescence was observed between 10 and 50 nM followed by a reduction of intensity from 50 nM to 10 μM. The polarization of the emission, represented by diamonds in Fig. 2, remained fairly constant at a relatively high value of ~0.22 over the concentration range from below 0.1 nM to 50 nM. A reduction in polarization was then observed which coincided with the reduction in total fluorescence.

These data indicate a high affinity of bis-ANS for binding at the decalin/water interface. The sharp fluorescence increase between 10 and 50 nM bis-ANS is suggestive of monolayer formation. The decrease in emission polarization at concentrations greater than 50 nm suggests continued formation of the monolayer despite the decrease in total fluorescence intensity. As the monolayer forms, depolarization due to excitation energy transfer should occur as a result of decreased intermolecular distances and concentration quenching of bis-ANS becomes a possibility. Some contribution to fluorescence changes may also result from reorientation of the bis-ANS molecules at the interface in response to increased crowding of adsorbed molecules. Continued monolayer formation at concentrations

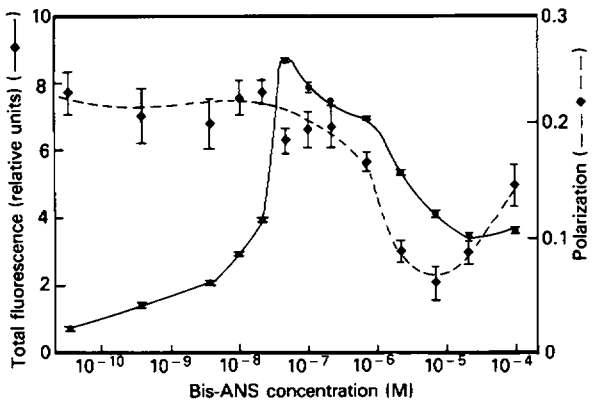

FIGURE 2 TIR-excited fluorescence intensity (*circles*) and emission polarization (*diamonds*) from a decalin/water interface at various total concentrations of bis-ANS. The aqueous phase contained 50 mM sodium phosphate at pH 7.0 and the sample cell was maintained at 20.0°C. Excitation light pulses were horizontally polarized at 385 nM with a 5-Hz repetition rate. Emission was measured through a 2 M sodium nitrite liquid prefilter (3-mm thick) and Corning No. 3-73 glass color filter (Corning Glass Works, Corning, NY).

TABLE I
PARTITIONING OF FLUOROPHORES BETWEEN AQUEOUS BUFFER AND IMMISCIBLE SOLVENTS

Aqueous phase: fluorophore and other reagents*	Organic solvent phase‡			
	o-Xylene $n = 1.5055$ $d = 0.8801$	Cyclooctane $n = 1.4586$ $d = 0.8349$	Dodecane $n = 1.4216$ $d = 0.7487$	Decalin $n = 1.4745$ $d = 0.833$
1-Amino-3, 6, 8-naphthalenetrisulfonate	–	–	–	–
+ 10 μM CTAB	–			–
ϵ-Dansyl-L-lysine	+	+	+	
Dansyl-L-aspartate	–	–	–	
+ 10 μM CTAB	–			–
ANS§	–			–
Bis-ANS				–
+ 0.1 mM CTAB				–
1-Amino-2-naphthalenesulfonate	+			+
4-Amino-1,5-naphthalenedisulfonate	–			–
+ 10 μM CTAB	–			–
Fluorescein				
pH 7.0	–			–
pH 11.0	–			–
Rhodamine B	+			+
N-Methylacridinium chloride§				–
3,4,9,10-Perylenetetracarboxylate				–
Riboflavin				–

*The aqueous phase initially contained between 0.2 and 3 mM fluorophore, depending upon the experiment. Buffer consisted of 0.05 M potassium phosphate, pH 7.0 except for the sample indicted at pH 11.0 which contained 0.05 M sodium bicarbonate.

‡Solvent indices of refraction and densities are literature or manufacturer values (35, 36) determined at 20°C (D line of sodium for refraction values). Detection of any amount of fluorophore partitioning from the aqueous to the organic phase is indicated by a +. Lack of partitioning is indicated by a –. Partitioning was conducted in unstirred solutions over periods ranging from 14 to 24 h.

§These fluorophores contained UV and/or blue emitting impurities which partitioned. The impurity fluorescence was well removed from the fluorophore emission.

above 50 nM bis-ANS is expected since a closely packed monolayer of bis-ANS would require a total bis-ANS concentration of at least 0.1 μM, assuming a cross-sectional area of 140 $Å^2$ for the bis-ANS molecule and assuming that all the bis-ANS binds at the interface. As will be seen in a later experiment, a large fraction of the bis-ANS was not adsorbed at concentrations below 0.1 μM.

Further insight into the behavior of bis-ANS at the decalin/water interface was provided by the emission spectra of adsorbed fluorophores. Figs. 3–6 present four technical emission spectra recorded at various total concentrations of bis-ANS. The spectra in Figs. 3–5 were recorded using total internal reflection of the exciting light at the decalin/water interface. The spectrum in Fig. 6, however, was recorded using excitation light incident upon the interface at an angle less than the critical angle resulting in the transmission of some light into the bulk aqueous solution. The contribution of fluorescence from interface-bound species can safely be assumed to be negligible in this situation, and therefore this spectrum represents fluorescence from bis-ANS in an isotropic aqueous environment. The fluorescence maximum for bis-ANS excited with the refracted light was ~540 nm, in agreement with the fluorescence spectrum of bis-ANS recorded in aqueous buffer using a conventional spectrofluorometer

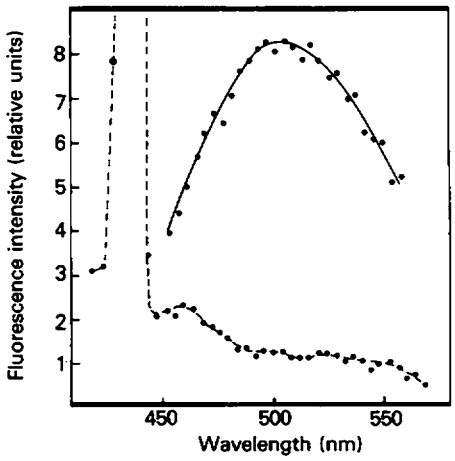

FIGURE 3 TIR-excited fluorescence emission spectrum of 1×10^{-10} M bis-ANS at a decalin/water interface (*solid line*) and emission spectrum of interface in the absence of bis-ANS (*dashed line*). Bis-ANS emission was measured with a total concentration of 0.1 nM bis-ANS originally in the aqueous phase containing 0.05 M sodium phosphate, pH 7.0, at 20.0°C. Excitation light was horizontally polarized at 385 nm with a 5-Hz repetition rate. Each point represents the sum of fluorescence from 500 consecutive pulses.

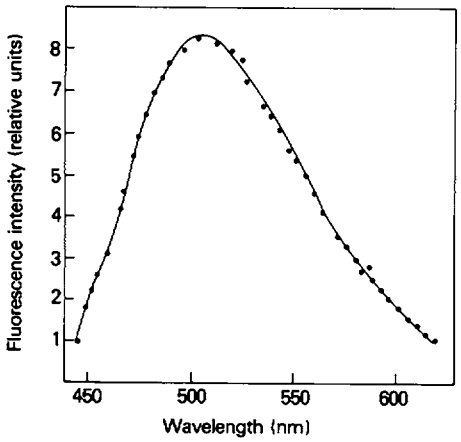

FIGURE 4 TIR-excited fluorescence emission spectrum of bis-ANS at a decalin/water interface. Experimental conditions are the same as in Fig. 3 except that a total concentration of 36.4 nM bis-ANS was originally in the aqueous phase. Each point represents the sum of fluorescence from 200 consecutive pulses.

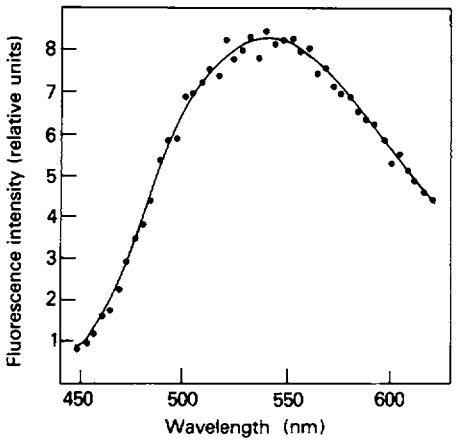

FIGURE 6 Fluorescence emission spectrum of bis-ANS at a decalin/water interface region excited by light refracted into the aqueous phase. Experimental conditions were otherwise the same as in Fig. 5.

equipped with the same model photomultiplier tube detector used in the TIR fluorometer. The spectrum in Fig. 3 is the interface fluorescence spectrum of a sample that contained 0.1 nM bis-ANS in the aqueous phase. This corresponds to a concentration at least three orders of magnitude below that required for monolayer formation. It is interesting to find that the emission maximum of adsorbed bis-ANS occurred at a wavelength 35 nm less than the peak emission of bis-ANS in the bulk solution. The emission maximum of the adsorbed fluorophore is similar to that found for bis-ANS bound to BSA or dissolved in a lower polarity solvent such as *n*-propanol (37). In addition, a 30 nm decrease in the bis-ANS fluorescence maximum was observed in aqueous solution when 0.1 mM CTAB was added, indicating incorporation of bis-ANS into CTAB micelles. The longer wavelength emission maximum of bis-ANS adsorbed to the interface, therefore, suggests that the bulk of the bis-ANS molecule

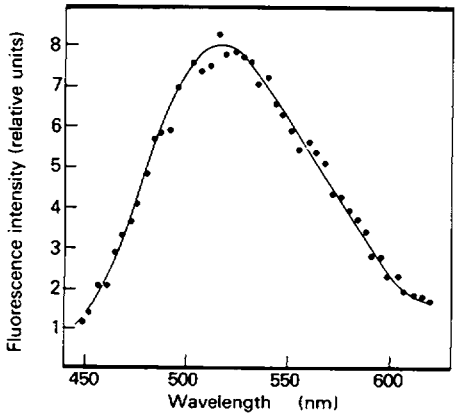

FIGURE 5 TIR-excited fluorescence emission spectrum of bis-ANS at a decalin/water interface. Experimental conditions are the same as in Fig. 3 except that a total concentration of 83.3 μM bis-ANS was originally in the aqueous phase. Each point represents the sum of fluorescence from 100 consecutive pulses.

resides in the apolar environment of the decalin phase. In view of the molecular structure, the sulfonic acid substituents must anchor the molecule to the interface while the naphthalene rings extend into the decalin phase. Also included in Fig. 3 is the background emission recorded in the absence of bis-ANS which demonstrates the sensitivity of the TIR fluorometer. The peak intensity of the Raman scattered light at 436 nm has a relative value of 35 in this figure and the background light intensity was 5.9-fold less than the bis-ANS emission at 505 nm.

The spectrum in Fig. 4 was recorded using a sample containing 36 nM bis-ANS. This corresponds to a concentration at which substantial monolayer formation has occurred but excitation energy transfer is just becoming noticeable in the polarization measurements (see Fig. 2). The emission spectrum is indistinguishable from that recorded at a bis-ANS concentation of 0.1 nM, indicating no appreciable change in the environment of the fluorophore. The spectrum recorded at 83 μM bis-ANS and presented in Fig. 5, however, does signify a change in bis-ANS environment. The fluorescence maximum has shifted to ~518 nm, indicating a more polar environment. The bis-ANS molecule has apparently adopted an orientation that allows greater interaction with the aqueous side of the interface. As will be discussed later, at high surfactant concentrations aggregation at the interface is possible and also could account for the spectral change since aggregates would scatter excitation light into the bulk aqueous phase where bis-ANS molecules emit at longer wavelengths.

Bis-ANS was caused to desorb from the decalin/water interface by the addition of protein to the aqueous phase. Fig. 7 presents the results of a titration in which increasing amounts of BSA were added to a sample containing a total bis-ANS concentration equal to 1 μM. The dependence of the fluorescence on BSA concentration is the reverse of that observed in Fig. 2 for the addition of bis-ANS. Since bis-ANS has a high affinity for BSA, the decreased adsorption of bis-ANS at the interface must result from

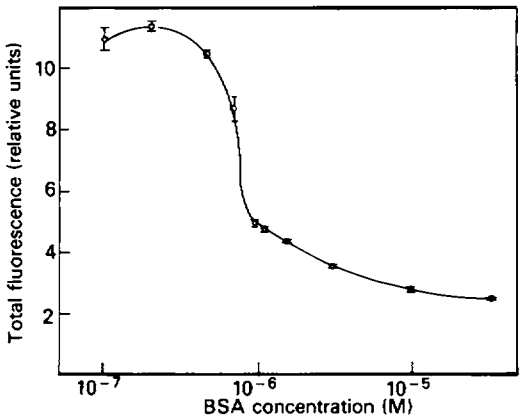

FIGURE 7 TIR-excited fluorescence intensity of bis-ANS at a decalin/water interface with the addition of various amounts of BSA to the aqueous phase. The aqueous phase contained 50 mM sodium phosphate, pH 7.0, and a total concentration of 1.0 μM bis-ANS. Other experimental conditions were identical to those described in Fig. 2.

the competitive binding of bis-ANS to the protein. The half point of the fluorescence decrease in Fig. 7 occurs with <1 μM BSA since the protein contains two high affinity binding sites for bis-ANS (37). Addition of cytochrome *c* caused a similar desorption of bis-ANS at the interface.

Interface Adsorption in the Presence of Charged Surfactants

As mentioned above, the formation of an adsorbed layer of bis-ANS at the decalin/water interface probably continues past the total bis-ANS concentration of 50 nM where the total fluorescence intensity is at a maximum. Further support of this conclusion was provided by the addition of CTAB to an aqueous phase which contained 0.1 nM bis-ANS. The interfacial fluorescence observed as the CTAB concentration was increased is plotted in Fig. 8. In this experiment, a large increase in total fluorescence was observed as the CTAB concentration was increased from 0.1 to 1 μM (*diamonds*). This increase reflects the adsorption of CTAB molecules to the interface since the binding

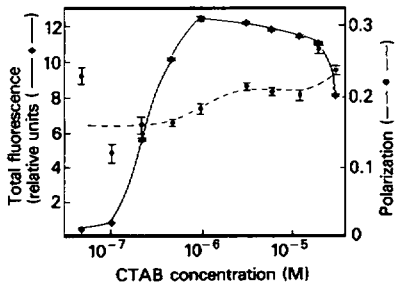

FIGURE 8 TIR-excited fluorescence intensity (*diamonds*) and emission polarization (*circles*) of bis-ANS at a decalin/water interface with the addition of various amounts of CTAB to the aqueous phase. The aqueous phase contained 50 mM sodium phosphate, pH 7.0, and a total concentration of 0.1 nM bis-ANS. Other experimental conditions were identical to those described in Fig. 2.

of the cationic detergent is expected to increase the adsorption of the anionic fluorophore. The fluorescence increase also indicates that the majority of bis-ANS molecules is not bound to the interface in the absence of CTAB. Therefore, the fluorescence maximum in Fig. 2 does not correspond to the completion of monolayer formation because nearly all the bis-ANS added to the aqueous phase would be required to bind at the interface to achieve even a loosely packed monolayer. The data in Fig. 8 also show that fluorescence depolarization does not accompany binding of bis-ANS to the interface when the concentration of bis-ANS is well below that necessary to cause the close packing of molecules in the adsorbed layer (*circles*).

Surface active molecules that mimic membrane lipids can be added to cause nonsurfactant molecules to adsorb to the interface region. Data are plotted in Fig. 9 for the titration of 0.1 mM aminonaphthalenetrisulfonic acid with CTAB. The total fluorescence for the decalin/water interface is represented by open circles, and changes in fluorescence intensity similar to those observed for the binding of bis-ANS to the interface are shown. However, unlike the titration experiment in which CTAB was added to a bis-ANS solution, the polarization was reduced by increased binding of aminonaphthalenetrisulfonate to the interface (Fig. 9, *solid circles*). Sufficient aminonaphthalenetrisulfonate was present in this experiment to cause the formation of a densely packed monolayer in which excitation energy transfer would be allowed. The emission spectrum of dye bound at the decalin/water interface was recorded for a 0.1 mM aminonaphthalenetrisulfonic acid solution containing 60 μM CTAB and is presented in Fig. 10. The fluorescence maximum was observed at 520 nm. Solution fluorescence spectra of aminonaphthalenetrisul-

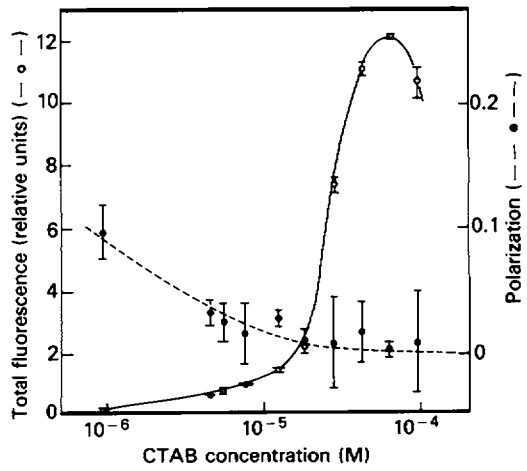

FIGURE 9 TIR-excited fluorescence intensity (*open circles*) and emission polarization (*solid circles*) of aminonaphthalenetrisulfonic acid at a decalin/water interface with the addition of various amounts of CTAB. The aqueous phase contained 50 mM sodium phosphate, pH 7.0, and a total aminonaphthalenetrisulfonic acid concentration of 97 μM at 20.0°C. Excitation light was horizontally polarized at 366 nm with a repetition rate of 5 Hz. Emission was measured through a 2 M sodium nitrite liquid prefilter and a Corning No. 3-73 glass color filter.

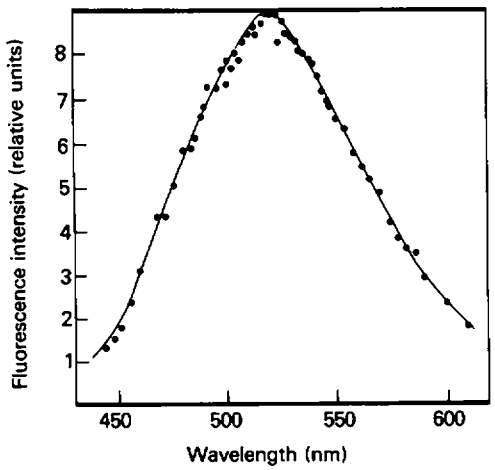

FIGURE 10 TIR-excited fluorescence emission spectrum of aminonaphthalenetrisulfonic acid at a decalin/water interface in the presence of CTAB. The aqueous phase contained 50 mM sodium phosphate, pH 7.0, 0.1 mM aminonaphthalenetrisulfonic acid, and 60 μM CTAB. Excitation light was horizontally polarized at 366 nM at a repetition rate of 5 Hz. Each point represents the sum of fluorescence from 500 consecutive pulses.

fonic acid dissolved in aqueous buffer at pH 7.0 and dissolved in ethanol showed fluorescence maxima at 520 and 490 nm, respectively. This demonstrates that the environment of aminonaphthalenetrisulfonate adsorbed at the decalin/water interface is highly polar and little interaction between the fluorophore and the decalin phase occurs.

In both CTAB titration experiments (Figs. 8 and 9), the total interfacial fluorescence began to decrease sharply at high surfactant concentration. One explanation for the fluorescence decrease is the formation of CTAB micelles in the bulk aqueous solution which compete for the binding of the fluorophore. Anomalous behavior at high surfactant concentration may also result from the formation of aggregates which alter the mobility and orientation of the surfactants as well as cause the scatter of excitation light. In one experiment, a visible film was observed on the interface at 90 μM CTAB. Aggregation at high concentrations of charged surfactants has been reported in the literature (38). For these reasons, data obtained near 0.1 mM surfactant concentration should be interpreted cautiously.

Adsorption of Cytochrome b_5

After the examination of simple fluorophore binding to liquid/liquid interfaces, the next step was to study the surface activity of biological molecules that interact with cell membranes. To this end, the binding of dansyl-labeled cytochrome b_5 at the decalin/water interface was briefly studied. Surface activity of this membrane protein is anticipated because it is composed of two protein domains, one of which is hydrophilic in character and one of which is hydrophobic in character and accounts for the ability of the protein to bind to membranes (31). Cytochrome b_5 was obtained labeled to an extent of 0.9 dansyl groups per protein with the label attached to the hydrophobic domain (31). The labeled protein was titrated into the aqueous phase of a decalin/water system to a maximum concentration of 11 μM. No evidence for binding of protein at the decalin/water interface was observed in this concentration range. Binding of cytochrome b_5 to biological membranes and lipid vesicles has been reported for protein concentrations within the range used in the present study (31, 39).

DISCUSSION

A liquid/liquid interface between water and an apolar solvent possesses properties in common with biological membranes. In particular, the liquid/liquid interface is analogous to the interface a lipid bilayer forms with water at either the inner or outer membrane surface. The liquid/liquid interface, in its simplest form, is a model for membranes in which the polar head groups are removed. This first approximation of a biological membrane cannot be modeled using bilayer membranes since the head groups are required for membrane formation. Further ordering and embellishing of the interface may be accomplished by the addition of surface active compounds to provide a second order approximation of the biological membrane. In work presented here, detergent molecules were added to the aqueous phase to examine surface binding of charged molecules. The detergent CTAB is similar to a biological lipid molecule in that both are composed of a long hydrocarbon segment with a polar region at one end. It is anticipated that biological processes that occur at membranes, such as transport, receptor function, electron transfer and energy transduction, and coupled enzymatic reactions may be studied at different levels of complexity by using liquid/liquid interfaces to which varying numbers of membrane components have been added. Total internally reflected light is particularly useful for probing the liquid/liquid interface as demonstrated by work presented in this paper. We have used measurements of fluorescence intensity, fluorescence polarization, and fluorescence emission spectra to determine the degree of interface adsorption, the rotational mobility, and the environment of fluorescent molecules. Total internally reflected light is capable of selectively exciting molecules within the vicinity of the membrane. This was demonstrated, for example, by the fluorescence emission spectrum of bis-ANS adsorbed at the decalin/water interface (Fig. 3). The adsorbed bis-ANS was shown to fluoresce with maximum intensity at 505 nm without interference from the 540-nm bulk aqueous phase emission. Even at 0.1 mM bis-ANS the 540-nm emission was small and may have been reflecting altered environments of bis-ANS at the interface instead of contributions from bulk phase fluorescence. The relative amounts of interface-bound bis-ANS and bulk phase bis-ANS excited by the internally reflected light may be estimated using the penetration depth calculated for the experimental conditions (920 Å) and assuming a tightly

packed monolayer of bis-ANS at the interface. The number of free bis-ANS molecules in a 0.1 mM solution which are located within the penetration depth of the interface is thereby estimated to be only 0.6% of the number of bis-ANS molecules contained in a monolayer.

The emission spectra were important in establishing the manner in which bis-ANS and aminonaphthalenetrisulfonate were adsorbed at the decalin/water interface. Bis-ANS was found to reside primarily on the decalin side of the interface, whereas aminonaphthalenetrisulfonate was adsorbed on the aqueous side of the interface. This is important not only for determining the location of a bound species but also for interpretation of the data since the fluorescence lifetimes and quantum efficiencies will differ greatly depending upon the environment of the fluorophores. In addition, the intensity, and in some situations the polarization, of the excitation light absorbed by a fluorophore will depend upon whether the fluorophore resides in the medium of greater refractive index or lower refractive index.

Emission polarization measurements were valuable as an indicator of both energy transfer and molecular motion. In Figs. 2 and 9, the increased accumulation of bis-ANS and aminonaphthalenetrisulfonate at the interface, and hence the decreased average distance separating adsorbed fluorophores, was demonstrated by the decreases in emission polarizations resulting from energy transfer. The decreasing fluorescence intensity in the bis-ANS titration (Fig. 2), which coincided with the decreasing polarization, can be explained by concentration quenching which may have accompanied the energy transfer. Transfer of energy among the adsorbed molecules could rapidly lead to transfer of the excitation energy to a few bis-ANS molecules on the aqueous side of the interface where the energy would be dissipated in a nonfluorescent manner (ϕ_{water} = 0.004, reference 37). Before significant monolayer formation, the average distance separating molecules was too large to allow significant energy transfer, and therefore polarization values provide a measure of the rotational motion of adsorbed molecules. Measurements made from above the plane of the interface, as performed in this work, reflect rotational mobility in the plane of the interface. Although it is expected that rotation perpendicular to this plane is slow due to required changes in solvation, it is not obvious how in-plane rotation is affected. Even though water and decalin have low viscosities individually, the apparent viscosity for a molecule adsorbed at the interface was relatively large as indicated by the polarization values of adsorbed bis-ANS. The rotational rate for bis-ANS in a two-dimensional system can be calculated using Eqs. 7–9. The limiting polarization of bis-ANS with 385-nm excitation was reported to equal 0.41 in solution (37). This corresponds to an angle between the absorption and emission oscillators of 21.8° (Eq. 7). Using this value in Eq. 9, the minimum limiting polarization in a two-dimensional system is determined to be 0.36. The observed polarization of interface fluorescence was 0.22 at low bis-ANS concentrations. This leads to a value of 0.16 for $k\tau$ upon substitution in Eq. 8. For a freely rotating molecule in decalin, the more viscous of the two solvents with η = 2.415 (36), k is predicted to equal 2.0×10^8 using the relationship between k (or R_d) and the molecular volume, solvent viscosity, and temperature found in Eq. 6. The lifetime of bis-ANS in hydrocarbon solvents is difficult to determine due to low solubility; however, reported values in other non-aqueous environments range from 2.0 ns for bis-ANS dissolved in formamide to 4.6 ns when it is dissolved in propylene glycol, to 9.4 ns when it is adsorbed to BSA (30, 37). These translate to $k\tau$ values ranging from 0.41 to 1.9 which are 2.6- to 12-fold larger than the $k\tau$ value observed for bis-ANS adsorbed at the decalin/water interface. A long lifetime for adsorbed bis-ANS is indicated by its blue-shifted emission which is similar to that observed for bis-ANS adsorbed to BSA (37). However, this conclusion may be regarded with some skepticism since it is not known how the environment at the interface may separately affect the lifetime and emission energy of an adsorbed molecule. The lower $k\tau$ value observed for interface-bound bis-ANS may reflect only a shorter lifetime (~0.8 ns) rather than reduced mobility.

A charged molecule such as aminonaphthalenetrisulfonate, which is adsorbed at the interface due to interaction with charged surfactants, is expected to rotate slower due to the larger effective size of the fluorophore surfactant complex. This was demonstrated by the relatively large polarization value of 0.1 observed for aminonaphthalenetrisulfonic acid, at low concentration, adsorbed to the decalin/water interface in the presence of CTAB (Fig. 9). Since the emission spectrum of the adsorbed aminonaphthalenetrisulfonic acid indicated its location on the buffer side of the interface, the polarization value of the adsorbed fluorophore may be compared with its polarization value of 0.0027 determined in the bulk aqueous phase. The limiting polarizations of several aminonaphthalenesulfonates have been reported and show high values near 0.4 when excitation is within the longest wavelength absorption band, as expected for a planar aromatic molecule (40). Using P_o = 0.4, the value of $k\tau$ in an isotropic aqueous environment would equal ~30. Calculations using the two-dimensional measurements of polarization from fluorophore adsorbed at the interface provide a value of $k\tau$ = 0.62. The rotational diffusion coefficient, therefore, is apparently 50-fold smaller for rotations within the plane of the interface relative to rotations in the bulk aqueous phase.

Higher polarizations may also be expected if the adsorbed fluorophore is oriented such that a slow rotation axis is perpendicular to the interface. For a planar molecule, rotation around the axis perpendicular to the plane of the molecule is often considerably faster than rotation around an axis in the plane of the molecule (40). Polarization measurements made in isotropic solution reflect both rotational rates and a polarization value intermediate to

those expected for either rotation alone are observed. Polarization values measured for rotation in the plane of the interface, therefore, may be larger than those measured in solution if only the slow rotation occurs in the plane of the interface. With respect to bis-ANS, the two ANS halves of the molecule cannot adopt a coplanar configuration and it is not obvious that rotation around any particular axis should be appreciably larger than rotation around another molecular axis. In the case of the planar molecule aminonaphthalenetrisulfonate, the molecule could conceivably orient at the interface such that in-plane or out-of-plane rotations are selectively observed.

It should be pointed out that other factors may affect the observed intensity and polarization of fluorescence. For example, conformational changes in the bis-ANS molecule may occur upon binding to the interface, and further conformational changes may result from increased crowding of the interface as monolayer formation proceeds. Perhaps the most important change in conformation would result from rotation around the bond connecting the two anilinonaphthalenesulfonate halves of the molecule. The angle between the planes containing the two naphthalene rings was reported to be 78.5° from analysis of the crystal structure (30). A 90° angle would preclude electronic interaction between the two rings while interaction would increase with decreasing angle. Interaction of the two rings in bis-ANS should have a large effect upon the fluorescent lifetime of the molecule as well as alter the absorbance and fluorescence emission spectra. The orientation of a fluorophore at the interface will also be important in determining the intensity of fluorescence measured. An orientation that preferentially places the emission dipole in the plane of the interface will increase the intensity measured in the X direction. A decrease in observed fluorescence, therefore, may be a result of the reorientation of an adsorbed fluorophore to favor placement of the dipole along the X-axis.

As a better approximation of a biological membrane, dye binding to the decalin/water interface was examined in the presence of a charged detergent similar to lipids of natural origin. Addition of CTAB increased the affinity of bis-ANS for the interface and also caused a dye with little surface activity to bind at the interface. The attraction of aminonaphthalenetrisulfonate for the interface in this case was certainly an electrostatic interaction between the anionic fluorophore and the positive surface created by CTAB adsorption. Undoubtedly this form of interaction is important for many membrane-active biomolecules. As shown in the present work, the amphoteric environment at the decalin/water interface is not sufficient to cause the binding of the membrane protein cytochrome b_5. Specific interaction with lipid molecules is an apparent requirement since the protein binds readily to synthetic phospholipid vesicles (31). The lipid requirement for cytochrome b_5 binding could be examined by the addition of various natural lipids to the liquid/liquid interface system.

As a final note, biological molecules that do not directly interact with a membrane can be studied using the liquid/liquid interface. An example of this was presented in which bis-ANS was caused to desorb from the decalin/water interface due to competitive binding with BSA. The removal of bis-ANS from the interface may also have relevance to the apparent function of serum albumins as scavengers of toxic compounds. In addition, this experiment demonstrates the potential for studying coupled interactions, which are important in such processes as electron transfer and membrane-mediated transport.

APPENDIX

The two-dimensional fluorescence equations may be derived by a procedure analogous to that used by Spencer and Weber (29) for fluorescence in a three-dimensional system. For steady-state conditions, the problem is reduced to the solution of two simultaneous equations:

$$(\tau^{-1} + 2k)F_y - 2kF_z = \delta_y \tag{A1}$$

$$(\tau^{-1} + 2K)F_z - 2kF_y = \delta_z \tag{A2}$$

where F_y and F_z are fluorescence intensities from dipoles aligned along the Y-and Z-axes, respectively, and δ_y and δ_z are weighing factors dependent upon the distribution of excited molecules at the time of absorption and defined by Eq. A3 and A4, respectively:

$$\delta_y = F_{yo}/(F_{yo} + F_{zo}) \tag{A3}$$

$$\delta_z = F_{zo}/(F_{yo} + F_{zo}), \tag{A4}$$

where F_{yo} and F_{zo} are the fluorescence intensities from dipoles aligned along the respective axes in the absence of depolarizing phenomena. The limiting polarization is therefore given by Eq. A5.

$$P_o = (F_{zo} - F_{yo})/(F_{zo} + F_{yo}) = \delta_z - \delta_y. \tag{A5}$$

Combining the above equations provides Eq. 8, which is the two-dimensional equivalent of the Perrin equation if k is identified with the rotational diffusion coefficient.

The dependence of the limiting polarization on the angle between the absorption and emission dipoles is also derived in a manner analogous to that for the three-dimensional system (28). For colinear absorption and emission dipoles with excitation light polarized along the Z-axis, the fluorescence intensity observed along the X-axis through a polarizer oriented parallel to the Z-axis, I_z, or perpendicular to the Z-axis, I_y, is determined by the average angle the oscillators make with the Z-axis, θ, as described by Eqs. A6 and A7:

$$I_z = \overline{\cos^2\theta} \tag{A6}$$

$$I_y = \overline{\sin^2\theta}. \tag{A7}$$

Polarization is therefore given by Eq. A8:

$$P = 2\,\overline{\cos^2\theta} - 1. \tag{A8}$$

The average $\cos^2\theta$ is given by Eq. A9 where the number of molecules with dipoles oriented between θ and $d\theta$ is proportional to $d\theta$ instead of $\sin\theta d\theta$ as in the three-dimensional case:

$$\overline{\cos^2\theta} = \frac{\int_0^{\pi/2} \cos^2\theta \cos^2\theta\, d\theta}{\int_0^{\pi/2} \cos^2\theta\, d\theta} = 3/4. \tag{A9}$$

Colinear oscillators in the absence of motion therefore result in a polarization value equal to ½. Combining the limiting polarization for colinear dipoles with the angular dependence of Eq. A8, now representing the angle between absorption and emission dipoles, leads to the expression in Eq. 9. The limiting polarization may therefore take on values between ½ and $-½$ as compared with the limiting values in a three-dimensional system of ½ and $-⅓$.

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