Pyrene. A Probe of Lateral Diffusion in the Hydrophobic Region of Membranes†

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ABSTRACT: Pyrene is solubilized by phospholipid dispersions and biological membranes and appears to be located in the hydrocarbon core of the membrane. Pyrene forms excited state dimers (excimers) in a diffusion-limited reaction which depends upon membrane composition, temperature, and pyrene concentration. A critical study of the kinetics of the reaction by quantum yield and lifetime measurements indicates deviations from predictions based upon a steady-state model for the diffusion gradient. The data can be better described using the Smoluchowski diffusion theory in which the time dependence of diffusion is taken into account. These findings have general implications for the description of diffusion-controlled reactions in the hydrophobic regions of membranes.

The recently hypothesized fluid mosaic model of membrane structure (Gitler, 1972; Singer and Nicolson, 1972) emphasizes the need for a detailed understanding of the temporal relationship between membrane components. Consequently, development of quantitative procedures to measure the dynamic properties of membranes is of paramount importance in defining structure-function relationships. A number of physicochemical techniques have been applied to the measurement of such relevant quantities as diffusion coefficients of membrane components or of "probes" bound to them.

In this report, we have measured the lateral diffusion coefficients of the fluorescent aromatic hydrocarbon, pyrene, in a series of artificial and natural membrane systems. We have utilized the fact that, during its lifetime, a photoexcited molecule of pyrene may, *via* translational diffusion, approach close enough to an unexcited pyrene molecule to form a collisional complex called an "excimer." The broad structureless blue-green excimer fluorescence is readily distinguished from the highly structured violet monomer fluorescence. The kinetics of excimer formation have been studied in great detail, and it is now well established that excimer formation is a diffusion-limited process in a variety of solvents (Birks *et al.*, 1964). Pyrene is the molecule of choice for these studies since (a) it is the most comprehensively studied of the excimer forming dyes, (b) it is almost totally insoluble in water, so that interference from unbound pyrene is negligible, and (c) the fluorescence lifetime is very long so that excimer formation takes place with the lowest possible concentrations. A preliminary report of our findings has appeared (Vanderkooi *et al.*, 1974a).

Materials and Methods

Chemicals. Pyrene, obtained from the Eastman Chemical Co. (Rochester, N. Y.), was graciously donated by Dr. R. Cooperman (University of Pennsylvania) and was twice recrystallized from ethanol and water. Pfaltz and Bauer, Inc. (Flushing, N. Y.) supplied 3,4-benzpyrene. L-α-Dimyristoyllecithin was obtained from NBC Research (Cleveland, Ohio). Sigma Chemical Co. supplied L-α-lecithin and cholesterol, and egg lecithin was a gift from Dr. T. Gulik-Krzywicki, Gif-sur-Yvette, France. All other reagents were of the highest purity commercially available. Twice glass-distilled water was used throughout.

As a criteria of purity of the fluorescent dyes, the decay of fluorescence was measured in ethanol. Exponential decay over 5 log units indicates that the fluorescent dye was free of fluorescent impurities.

Membrane Preparations. Mitochondria were obtained from pigeon heart according to Chance and Hagihara (1963) and inner and outer mitochondrial membranes were purified by the method of Parsons *et al.* (1966). Fragmented sarcoplasmic reticulum membranes were prepared according to McFarland and Inesi (1971) and the procedure of Dodge *et al.* (1963) was used for preparation of erythrocyte plasma membranes from human blood. Artificial phospholipid membranes were prepared by sonicating the lecithin in a buffered solution at around 45° for 1–3 min with a Branson sonifier. Membrane protein concentration was determined by the biuret method (Gornall *et al.*, 1949).

Fluorescence Measurements. Steady-state fluorescence measurements were performed at 90° to the exciting beam, using a Hitachi MPF-2A fluorescence spectrometer, except where noted. The sample compartment was maintained at a constant temperature with the use of circulating water through the cell holder. Relative fluorescence quantum yield of the monomer was calculated by comparing the fluorescence intensity at 386 nm with that of a dilute solution which exhibited no excimer fluorescence. Dimer:monomer ratios were calculated by comparing the fluorescence intensity at 480 nm to that at 386 nm, using 342 nm as the excitation wavelength.

Time dependence of fluorescence emission was measured with an Ortec photon-counting fluorescence lifetime instrument, equiped with an Ortec 6220 multichannel analyzer and an RCA 8850 photomultiplier. The temperature was maintained constant by circulating water through the sample cell holder. A Corning glass filter (760) was used to isolate the wavelength region for excitation. The monomer emission was isolated using an ultraviolet interference filter with band pass at 390 nm, and the dimer emission was isolated using a 470-nm interference filter. Data from the multichannel analyzer were

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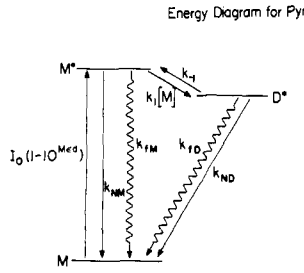

FIGURE 1: Energy diagram for monomer and excimer fluorescence emission of pyrene. Pyrene in its ground state, excited state, and excimer form is indicated by M, M*, and D*, respectively. The rate of conversion of M to M* is given by Beers law, where I_o is the light intensity, ϵ is the extinction coefficient, and d is the pathlength. Nonradiative decay of M* and D* is given by k_{NM} and k_{ND}, respectively. Radiative decay of M* and D* is given by k_{fM} and k_{fD}, respectively. The rate of reaction between M* and M is given by k_1 and dissociation of D* to M* is given by k_{-1}.

transferred by Cassette magnetic tape to a PDP-10 computer and plotted with a Calcomp recorder.

Incorporation of pyrene into the membranes was accomplished by adding a concentrated solution of pyrene in alcohol to the aqueous suspension of membranes. Distribution of pyrene into the membranes occurred within the mixing time. The final alcohol concentration did not exceed 0.4% v/v.

Interpretation of Data

We shall begin by adopting the reaction scheme for excimer formation which has been found adequate to explain all previous measurements of quantum yields and lifetimes in isotropic solutions (Birks *et al.*, 1964). This reaction scheme is illustrated in Figure 1 where the relevant rate processes are defined. The following differential equations govern the time dependence of the excited state monomer and excimer populations, where k_f is the sum of k_{fM} and k_{NM} and other notations

$$d[M^*]/dt = I_0(1 - 10^{-\epsilon Md}) - (k_f^* + k_1[M])[M^*] \quad (1)$$

$$d[D^*]/dt = k_1[M][M^*] - k_{fD}[D^*] \quad (2)$$

are given in Figure 1. In deriving these equations, we have assumed that the exciting light is weak enough so that the ground state population is a constant; thus, the rate of excimer formation is proportional to the concentration of M. We have also assumed that the rate of dissociation of the excimer (given by k_{-1} in Figure 1) is negligible at room temperature; this assumption has been verified for a variety of solvents (Birks *et al.*, 1964). To proceed further, it is customary to use equations such as (1) and (2) to derive quantum yields and lifetimes as functions of the rate constants of the system. A detailed description of the method as applied to excimer formation is given by Birks. However, Birks has generally assumed that the diffusion-controlled rate, k_1, is independent of time, *i.e.*, has the familiar form of the Einstein–Smoluchowski expression for a diffusion-limited reaction, *i.e.*

$$k_1 = 4\pi N'RD \quad (3)$$

where R is the sum of the interaction radii, D is twice the diffusion coefficient of pyrene, and N' is Avagodro's number per millimole. It was first pointed out by Smoluchowski (1917) and later discussed at length by others (Noyes, 1961; Yguerabide *et al.*, 1964; Ware and Richter, 1968; Schurr, 1970) that k_1 is, under general conditions, time dependent. The exact form of the rate constant within the assumptions of diffusion theory is

$$k_1 = 4\pi N'RD(1 + [R/(\pi Dt)^{1/2}]) \quad (4)$$

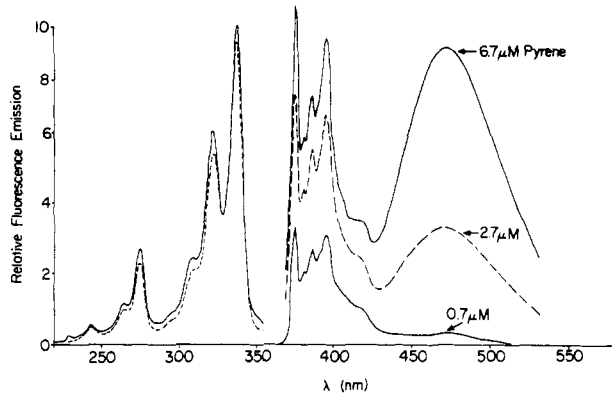

FIGURE 2: Uncorrected excitation and emission spectra of pyrene in L-α-dipalmitoyllecithin vesicles. The sample consisted of 0.3 mg of dipalmitoyllecithin/ml (in 10 mM PO_4, pH 7.4). Excitation and emission slits were 2.5 nm: (left) excitation spectra of 6.7 μM pyrene using 480 nm (—) or 372 nm (- - -) as respective emission wavelengths; (right) emission spectrum using 340 nm as excitation wavelength. The sample temperature was 25°.

In this expression, the rate constant is time dependent, but asymptotically approaches the time-independent expression (3) with increasing time.

$$I_{fM}(t) = I_{fM}(0) \exp - \left[k_f^* + 4\pi RN'D\left(1 + \frac{R}{(\pi Dt)^{1/2}}\right)M\right]t \quad (5)$$

Physically, the origin of the time dependence can be understood as follows: at time $t = 0$, there is a finite probability of two pyrene molecules being separated by a distance comparable to their interaction radii. If one of the pair is excited, excimer formation will be essentially instantaneous. After these close pairs are extinguished, the system will then come into a "steady state" where the relative distribution of ground state pyrenes around an excited one ("the diffusion gradient") is independent of time. For liquids of low viscosity like that of water, the transient term is significant only for times of less than 100 psec and may be safely neglected. In the case of viscous fluids such as the hydrophobic interior of membranes, the transient term is of paramount importance.

Yguerabide *et al.* (1964) have considered the theory of diffusion-controlled fluorescence quenching reactions. These authors have derived expressions for the rate of decay and fluorescence quantum yield of excited molecules which take into account the time needed to establish a steady-state diffusion gradient. It is easy to show that the kinetic scheme for fluorescence quenching used by Yguerabide *et al.* is identical with the kinetics of quenching of the excited monomer of pyrene. Thus, the equations governing the fluorescence yields and lifetimes for fluorescence quenching are applicable to our system. Following Yguerabide, the decay time of the monomer is, in our notation, described by eq 5, where $I_{fM}(t)$ is time-dependent fluorescence intensity of the excited monomer following δ function excitation. The relative quantum yield of monomer fluorescence, $\Phi_{fM(M)}$, under conditions of steady-state irradiation is

$$\Phi_{fM}(0)/\Phi_M(M) = [1 + (4\pi RN'DM)k_f^*] \times [1 - b(\pi/a)^{1/2} \exp(b^2/a)\mathrm{erfc}(b/a^{1/2})]^{-1} \quad (6)$$

where $a = k_f^* + 4\pi RN'DM$ and $b = 4R^2N'(\pi D)^{1/2}M$.

These equations show that in viscous solutions, the pyrene monomer is expected to decay nonexponentially. Also, plots of the inverse monomer fluorescence as a function of monomer concentration (Stern–Volmer plots) exhibit positive deviations.

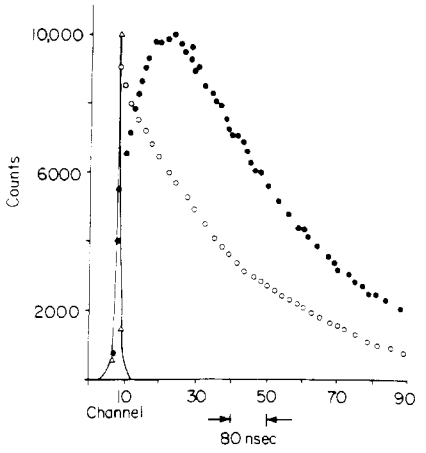

FIGURE 3: Monomer and excimer response to excitation light pulse. Conditions are described under Experimental Procedures. The sample contained 0.1 mg of egg lecithin/ml, 10 μM pyrene and 10 mM PO_4 buffer (pH 7.2) at 25°: (O) monomer emission; (●) dimer emission; (Δ) lamp. Some data points have been omitted for illustration purposes.

These nonlinear plots are an advantage to us in seeking diffusion coefficients. This is because in a complex biological membrane, the effective concentration, C, of pyrene is unknown. Since we can employ the parameters C, R, and D to fit the curves, we can obtain values of the diffusion coefficient without making assumptions about the interaction radius, or the effective concentration.

Results

Fluorescence Emission Properties of Pyrene in Membranes. Evidence of pyrene excimer formation in biological membranes and phospholipid vesicles is obtained from the excitation and emission spectra (Figure 2). The fluorescence spectra are characterized by: (a) a highly resolved monomer emission whose quantum yield is quenched at high pyrene concentrations, (b) a broad, structureless emission band due to the excimer which is observed only at high pyrene concentrations, and (c) identical excitation spectra for the two species. These are the characteristics which led Förster and coworkers to postulate the existence of an excited state complex (Förster and Kasper, 1955; Döller and Förster, (1962).

Further evidence of pyrene excimer formation in membranes is provided by the fluorescence decay curves of monomer and dimer (Figure 3). The monomer exhibits an instantaneous rise in fluorescence followed by a monotonic decay. In contrast, the dimer emission exhibits both a rise and a decay time. The spectral characteristics presented in Figures 2 and 3 are in qualitative agreement with the reaction scheme presented in Figure 1.

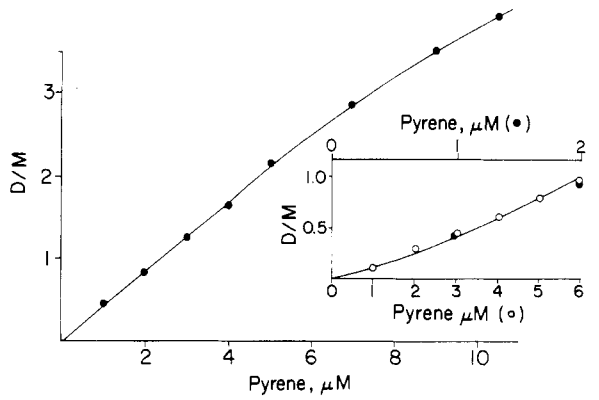

FIGURE 4: Dependence of dimer–monomer ratios and monomer quantum yield on pyrene concentration in egg lecithin. Pyrene in concentrations indicated in the figure was added to a sample of 0.33 mg/ml of egg lecithin (●) or 0.10 mg/ml of egg lecithin (O) suspended in 10 mM PO_4. The temperature was 24°. The dimer:monomer ratio (D:M) was measured by comparing fluorescence intensity at 480:386 nm.

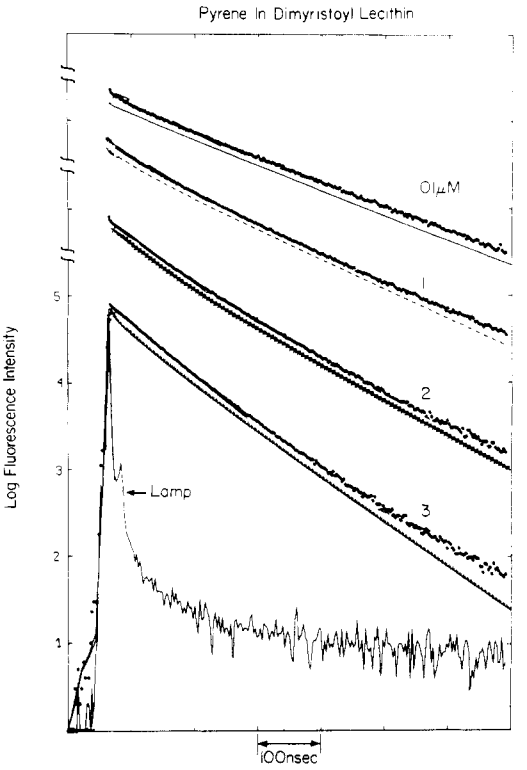

FIGURE 5: Fluorescence decay of pyrene monomer in dimyristoyllecithin membranes. Experimental points (●) for fluorescent decay of pyrene monomer in a sample containing 0.1 mg of dimyristoyllecithin/ml, 10 mM PO_4 buffer (pH 7.2), and pyrene in concentrations indicated in the figure. The temperature was 30°. Computer-drawn lines below the experimental lines refer to theoretical decay, calculated according to eq 5, using a fluorescent lifetime of 135 nsec, interaction radius of 10 Å, and 0 mM (—), 25 mM (- - -), 50 mM (×), and 75 mM (+) pyrene, and a diffusion constant of 3.0×10^{-8} cm^2/sec. The value of fluorescent lifetime (135 nsec) was experimentally determined in the absence of dimers in a sample the same as above and containing 0.1 μM pyrene.

In a membrane suspension, dimer formation is independent of total pyrene concentration in the sample, but is dependent upon the membrane lipid:pyrene ratio, thereby excluding the possibility that a significant concentration of pyrene is located in the aqueous phase. In our study, we assume that lateral diffusion of pyrene is confined to the fluid hydrocarbon region of the membrane. It is difficult to ascertain how good this approximation is; however, trypsin digestion of the mitochondrial inner membrane resulted in digestion of about 20% of the protein with no change in efficiency of dimer formation. Furthermore, although pyrene binds to bovine serum albumin in aqueous solution with increased fluorescence, dimers are not formed. Presumably, pyrene will bind to macromolecules such as proteins and DNA by interchelation. The pyrene thus immobilized would be unable to form excimers.

Analysis of Pyrene Diffusion in Membranes. Membrane composition, as well as pyrene concentration in the membrane and temperature, affect the efficiency of excited state dimer formation. In all membranes tested, the dimer:monomer (D:M) ratios and quantum yield of monomer deviate significantly from the linear dependence on concentration predicted from the simple diffusion theory (eq 3), positive deviation occurring

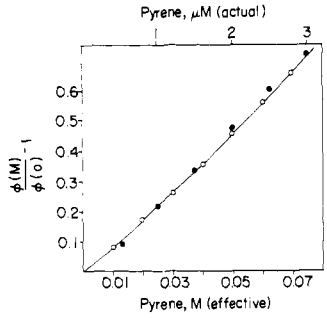

FIGURE 6: Fluorescence yield of pyrene monomer in dimyristoyllecithin vesicles: (●) experimental points using conditions given in Figure 5; (○) calculated values according to eq 6, using values given in Figure 5.

at low concentration and negative deviation at high concentration. An example of this behavior is given for pyrene excimer formation in egg lecithin vesicles (Figure 4). We shall attempt to show that dimer formation at low and intermediate pyrene concentrations can be analyzed using the more general approach to diffusion as formulated by Smoluchowski. Negative deviation which occurs at a high concentration of pyrene is not predicted by either the time-dependent or simple diffusion theory and may be related to an alteration in membrane caused by insertion of many pyrene molecules.

The decay of pyrene monomer fluorescence in dimyristoyllecithin is given in Figure 5. Increasing the pyrene concentration causes an increase in the rate of fluorescence decay; however, it is apparent that the decay of fluorescence is not exponential in the concentration range where excimers are formed. Given directly below the experimental decay curves in Figure 5 are computer-drawn plots calculated according to eq 5, assuming a diffusion constant of $D = 3.0 \times 10^{-8}$ cm^2/sec, a fluorescent lifetime of 135 nsec in the absence of dimer, an appropriate pyrene concentration, and an interaction radius of 10 Å. The fluorescent lifetime was experimentally determined, and other values were varied until a good fit was obtained between the calculated and experimental decay curves. The results of the experiment were tested for internal consistency by using the interaction radius and value for diffusion obtained by analysis of the decay curve to calculate the steady-state quantum efficiency of pyrene monomer fluorescence (eq 6). Comparison of the experimental values of monomer quantum yield with the theoretically calculated values closely agree (Figure 6).

Typical examples of the decay of pyrene monomer fluorescence and its quantum yield in biological membranes are given in Figure 7 and the steady-state quantum yield is given in Figure 8. For red blood cell membranes, the decay of pyrene monomer fluorescence was nonexponential, and the Stern–Volmer plot of quantum yield of monomer was nonlinear. As in the case of the artificial membrane system, good agreement was obtained between the experimental data points and calculated values, assuming $D = 2.8 \times 10^{-8}$ cm^2/sec for red blood cell membranes at 25 ° (not shown).

The study of pyrene diffusion was extended to other artificial and biological membrane systems. A summary of the diffusion constants for pyrene, as well as other epr and fluorescent probes, is given in Table I. In examining the data presented in Table I, we note that the rate of diffusion of pyrene is similar in artificial and biological membranes. This finding is supportive of the idea of a continuum of lipid bilayer as a structural component of the biological membrane. Pyrene diffusion is significantly slower in erythrocyte plasma membranes, which contain cholesterol (Hagerman and Gould, 1951), than in sarcoplasmic reticulum membranes which are nearly cholesterol free

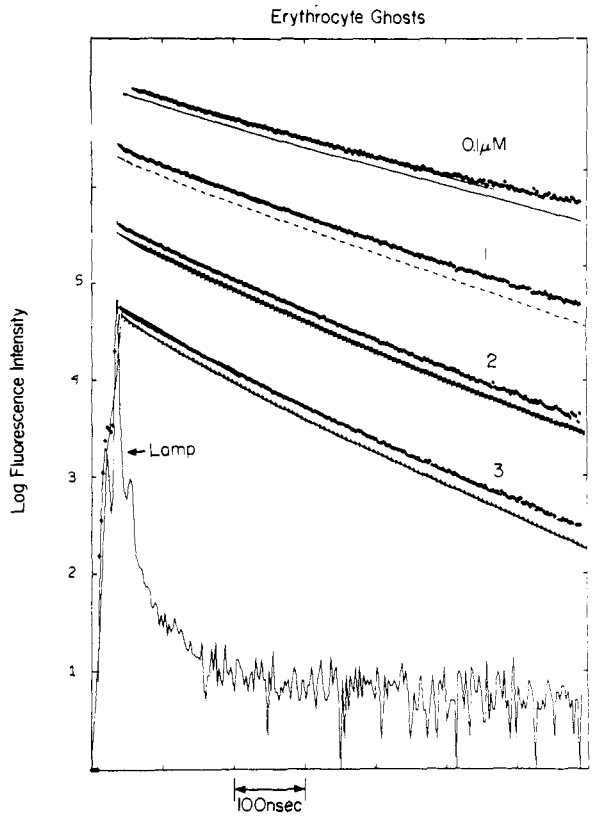

FIGURE 7: Fluorescence decay of pyrene monomer in red cell membranes. (●) Experimental points for fluorescent decay of pyrene monomer in a sample containing 0.2 mg of erythrocyte membrane protein/ml, 0.154 M NaCl, and pyrene in concentrations indicated. The temperature was 25°. Computer-drawn lines below the experimental lines refer to theoretical decay calculated according to eq 5, using fluorescence lifetime of 155 nsec, interaction radius of 10 Å, and 0 mM (—), 31 mM (- - -), 50 mM (×), and 75 mM (+) pyrene and a diffusion constant of 3.0×10^{-8} cm^2/sec. The fluorescent lifetime was experimentally determined in a sample containing 0.1 μM pyrene.

(Martonosi, 1969). Cholesterol has been hypothesized to introduce order in the hydrocarbon chain region (Chapman, 1973; Vanderkooi *et al.*, 1974b). Indeed, addition of cholesterol to dimyristoyllecithin dispersion resulted in a lowering of pyrene diffusion rates (Table I). Consistent with the kinetic nature of excimer formation, increasing the temperature results in an increase in excimer formation. The energies of activation for excimer formation in egg lecithin or sarcoplasmic reticulum membranes are about 5–6 kcal; such low energies of activation are characteristic of diffusion-limited processes.

Some attempt was made to fit the data with other kinetic models. For example, nonexponential decay would be obtained if pyrene diffusion is described by the simple diffusion theory

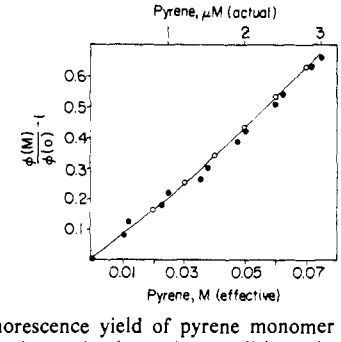

FIGURE 8: Fluorescence yield of pyrene monomer in red cell membranes: (●) experimental points using conditions given in Figure 7; (○) calculated values according to eq 6, using values given in Figure 7.

TABLE I: Diffusion in Biological Membranes.

Membrane	Diffusant	Technique	T (°C)	D (cm²/sec)	η (P)	ΔH	Ref
Egg lecithin	Pyrene	Fluorescent excimer	20	3.0×10^{-8}	0.572	6.9	*a*
	Spin-labeled phosphatidyl-choline	Epr	25	1.8×10^{-8}			*b*
	Phospholipids	Nmr	20	0.9×10^{-8}			*c*
	Spin-labeled androstane	Epr	20	1.0×10^{-8}			*d*
	Perylene	Fluorescence depolarization	37		0.73	7.3	*e*
Dimyristoyl-lecithin	Pyrene	Excimer	30	3.0×10^{-8}	0.592		*a*
	Benzpyrene	Excimer	32	3.2×10^{-8}	0.557		*a*
Dimyristoyl-lecithin–cholesterol (2:1)	Pyrene	Excimer	30	1.3×10^{-8}	1.36		*a*
Sarcoplasmic reticulum	Pyrene	Fluorescent excimer	20	5.8×10^{-8}	0.295	6.9	*a*
	Spin-labeled phospholipid	Epr	37	6×10^{-8}			*f*
Erythrocyte membrane	Pyrene	Fluorescent excimer	25	2.8×10^{-8}	0.620		*a*
	Perylene	Fluorescence depolarization	37		1.20	3.9	*g*
Submitochondrial particles	Perylene	Fluorescence depolarization	37		0.95	4.9	*g*

[a] This study. [b] Devaux and McConnell (1972). [c] Lee *et al.* (1973). [d] Träuble and Sackmann (1972). [e] Cogan *et al.* (1973). [f] Scandella *et al.* (1972). [g] Rudy and Gitler (1972).

and there is more than one pool of lipids, offering differing effective viscosities for pyrene diffusion. For a two-pool system, the decay of monomer fluorescence is expected to be described by two exponential decay rates; however, we found that decay of the monomer fluorescence is much better described by the Smoluchowski diffusion theory than by simple diffusion in two-pool models. Another question is whether the nonexponential decay of fluorescence is due to heterogeneous binding sites. However, a non-excimer-forming dye, anthracene, displays in membranes a single lifetime in the concentration range where pyrene decay is nonexponential, suggesting that the hydrophobic environment in which the hydrocarbon dyes are located is not so heterogeneous as to cause multiple lifetimes. Also, the frozen pyrene–membrane suspension shows exponential decay, again indicating that heterogeneous sites do not produce the nonexponential decay.

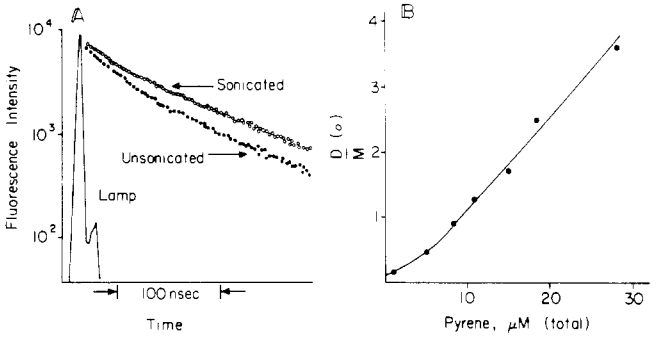

FIGURE 9: Pyrene monomer fluorescence decay and dimer:monomer ratio in dipalmitoyllecithin vesicles. (A) Sample contained 0.1 mg of dipalmitoyllecithin/ml sonicated in 10 mM PO_4 (pH 7.2). Pyrene (4 μM) was added and fluorescence decay was measured as described under Experimental Procedures before (○) and after (●) sonication. (B) The reaction mixture contained 0.3 mg of dipalmitoyllecithin/ml sonicated in 10 mM PO_4 buffer (pH 7.2) and pyrene in concentrations indicated. Sample was not sonicated after addition of pyrene.

Artificial Membrane Systems in Which Pyrene Diffusion Parameters Are Not Described by Either the Simple or General Diffusion Theory. In some model membrane systems, pyrene diffusion could not be fit by the analytical procedures outlined above. One such example is dipalmitoyl- and dimyristoyllecithin vesicles below the phase transition. Inspection of the fluorescence decay curves of pyrene in the presence of these lipids with theoretical decay curves such as those plotted in Figure 8 revealed deviation from exponentiality which is greater than that predicted by the Smoluchowski treatment (Figure 9). In addition, the fluorescence decay curves and the dimer:monomer ratios are affected by the previous treatment of the sample. For example, the decay of fluorescence is altered when the sample is sonicated and the temperature profile of the dimer:monomer ratios shows hysteresis (Vanderkooi *et al.*, 1974a). An assumption in the diffusion theory is that the distribution of pyrene molecules in the membrane is random. Nonrandom distribution of pyrene is likely in dipalmitoyllecithin artificial membranes below the phase transition, presumably since the insertion of a pyrene molecule into the crystalline hydrocarbon region introduces disorder, making it likely that the next pyrene molecule will be inserted into this area. Evidence

for such phase separations has been obtained using spin-label probes (Shimshick and McConnell, 1973). Local areas of high pyrene concentration can be the cause of the marked "cooperative" behavior of the dimer:monomer ratios.

Soap micelles solubilize pyrene (Dorrance and Hunter, 1972; Grätzel and Thomas, 1973; Pownall and Smith, 1973); however, the decay of pyrene monomer fluorescence at concentrations where excimers are formed is nonexponential. Deviation from the Stern–Volmer relationship has been reported for pyrene diffusion in $CetMe_3NBr$[1] micelles on the basis of steady-state measurements (Pownall and Smith, 1973) and was reproduced by us. An assumption in both diffusion theories is that each membrane unit (micelle or vesicle) contains an equal number of pyrene molecules. At 0.2 M pyrene and 10 mM $CetMe_3NBr$, there is an average of 1.7 molecules per micelle, taking the literature value of 85 $CetMe_3NBr$ molecules/micelle (Tarter, 1959). A distribution of molecules is expected, leading to micelles with several pyrene molecules in which the fluorescence is highly quenched, and micelles with only one molecule. Consequently, the decay of fluorescence is composed of a summation of fluorescence decay which ranges from unquenched to highly quenched. Such a problem does not arise in the phospholipid vesicles, since they contain about 8000 molecules/vesicle as minimum value (Huang, 1969), nor does it occur in the biological membranes which we tested, since again the membrane units are large enough to allow for an averaging of the distribution of pyrene.

In one biological membrane system, mitochondria, diffusion of pyrene was poorly fit by the Smoluchowski diffusion theory. Since intact mitochondria are composed of two membrane systems, the experiment was repeated using either isolated inner or outer mitochondrial membranes. For both membranes, evidence of nonexponentiality was seen even at low pyrene concentrations in the absence of excimers. Mitochondrial membranes contain many components which can interfere with the fluorescence emission of pyrene, and thus make calculation of diffusion difficult. Förster energy transfer from pyrene excited state to the heme proteins is one mechanism which results in quenching of pyrene fluorescence, and has been invoked to explain the quenching of fluorescence of anthroylstearic acid by cytochrome *c* (Vanderkooi *et al.*, 1973). In addition, the many paramagnetic centers, including nonheme iron, quinones, and flavines, will further cause a quenching of fluorescence, leading to nonexponential decay.

Diffusion of Other Fluorescent Hydrocarbons in Membranes. Excimer formation resulting from diffusion-controlled collisions can be demonstrated for other fluorescent hydrocarbons. Excimer formation was observed for 1,2-benzpyrene and 3,4-benzpyrene. The diffusion constant for 3,4-benzpyrene in dimyristoyllecithin at 32° was $D = 3.2 \times 10^{-8}$ cm^2/sec, which is near to $D = 3.0 \times 10^{-8}$ cm^2/sec measured for pyrene under similar conditions.

Pyrenebutyric acid has been reported to form excimers in mitochondrial membranes (Brocklehurst *et al.*, 1970). We have observed excimers of pyrenebutyric acid and pyrenesulfonic acid in egg lecithin vesicles (not shown). The apparent efficiency of formation appears to be very much lower than the uncharged parent compound, which can be partly attributed to significant partitioning of the butyric acid derivative in the aqueous phase, and partly due to its likely location near the charged head group region.

[1] Abbreviation used is: $CetMe_3NBr$, cetyltrimethylammonium bromide.

Discussion

The hypothesis that membrane structure can be described as a viscous two-dimensional fluid is attractive from two points of view. First, it is consistent with data available on membrane structure. Second, it is a hypothesis for which experiments can be readily devised. We have used pyrene excimer formation as a model chemical reaction occurring in a membrane to examine the fluid properties of the hydrocarbon core. Pyrene is solubilized by both biological and model membrane systems and is localized in the hydrocarbon core of the membrane, as indicated from the emission spectra. Furthermore, pyrene molecules are capable of rapid lateral diffusion, since the formation of excimers can be clearly demonstrated. The quantum yields and lifetimes of both monomer and excimer fluorescence depend on concentration and temperature in a manner which reflects the composition and structure of the membrane. Qualitatively, these results are described by kinetic equations which have been successfully applied to excimer formation in isotropic and fluid solutions. Quantitatively, however, both yield and lifetime data show deviations from the previous theory, which must be interpreted to afford values of diffusion coefficients and microviscosities. We have compared the results for pyrene diffusion in membranes with those predicted by eq 5 using the previously obtained values of R and D, and the Smoluchowski diffusion theory does, in fact, fit the experimental points reasonably well in most membrane systems.

Under some conditions, however, excimer formation could not be described by the Smoluchowski diffusion theory. When the membrane unit is small, as with soap micelles, or below the phase transition, as with dipalmitoyllecithin, deviations occurred. Also, in mitochondria, the presence of colored and paramagnetic substances appears to interfere with the fluorescence assay of excimer formation. In addition, the observed saturation effects of dimer formation at high pyrene concentrations in both the biological and artificial membranes are not predicted either by the simple diffusion theory or by Smoluchowski's treatment. It is highly probable that there is a finite accumulation number for pyrene in the fluid part of the membrane. At higher concentrations, some pyrene molecules may be forced to occupy sites closer to the polar head groups of the lipids; molecules localized here would diffuse more slowly and form less excimers, thus producing a saturation effect. Another possibility is that the pyrene molecules induce a local ordering of the lipid chains in their immediate vicinity, leading to a "stiffening" of the bilayer, much as has been observed for cholesterol (Chapman, 1973). This stiffening could also produce a concentration-dependent diffusion coefficient, leading to saturation effects.

Oxygen quenches singlet fluorescence emission presumably by catalyzing singlet → triplet conversion. In our hands, deoxygenating the solution by bubbling with Ultra Pure nitrogen increased pyrene fluorescence by 2–6 times in both the biological membranes and in artificial membranes composed of phospholipids or cationic detergents, indicating that oxygen penetrates into the hydrocarbon interior of the membrane. This is in disagreement with claims in the literature that oxygen does not penetrate into cationic detergent micelles (Dorrance and Hunter, 1972). Although oxygen quenches pyrene fluorescence, the calculated diffusion constants for pyrene are independent of oxygen concentration; consequently, in most experiments, samples were not deoxygenated prior to measurement. Identical diffusion rates were obtained for degassed samples and air-saturated samples, using the quantum yield of monomer for calculation.

Diffusion parameters for pyrene and other electron para-

magnetic resonance (epr) and fluorescent probes are summarized in Table I. The diffusion of pyrene in membranes is consistently faster than the diffusion of spin-labeled phospholipids measured under similar conditions. Since each of the nuclear magnetic resonance (nmr) and epr techniques as well as the fluorescent techniques described here has a number of inherent theoretical assumptions and unique practical difficulties, it is useful to make a comparison between the various techniques. In addition, diffusion of molecules of differing size and charges can be expected to occur at different rates, as for example, a charged molecule located near the phosphate head group region, *vs.* a small uncharged lypophilic molecule. The diffusion coefficients calculated using spin label, fluorescent label, and nuclear magnetic resonance techniques range from 10^{-8} to 10^{-7} in a variety of membranes.

A systematic error in both the epr probe technique and our measurements using pyrene is likely to be in the calculation of "effective concentration." In our calculations, we consider both effective concentration, C, and interaction radius R as unknown, and fit the data to variable values for C and R. We find that the data can be best fit using an interaction radius of 10 Å. An X-ray study by Robertson and White (1947) has shown that the molecule is 9.2 Å in the longest dimension; thus, the collision radius and molecular dimensions are nearly the same.

The lateral diffusion of membrane proteins has been demonstrated by the elegant work of Frye and Edidin (1970), and it is very well possible that lateral diffusion of membrane constituents is required for the enzymatic function of a variety of processes. This hypothesis can perhaps be tested in photosynthetic systems where light activation makes it possible to accurately resolve the kinetics of the reaction between, for example, coenzyme Q and the cytochrome electron donors. The microsomal hydroxylation systems found in kidney, liver, adrenal glands, etc. are other examples in which lateral diffusion in the membrane may be a requirement of the physiological function, and where experiments can be devised to measure the diffusion rates. Since the proteins and lipids involved in these reactions are very large, and the "solvent" (*i.e.*, the membrane) in which the reaction occurs is highly viscous, it can be expected that the kinetics of these reactions will deviate markedly from that of the simple diffusion theory, and can only be described by taking into account the finite time needed to establish a diffusion gradient, as described by Smoluchowski and illustrated in this paper.

It has come to our attention that pyrene has been used to measure lateral diffusion in artificial membranes (Galla and Sackmann, 1974). The results are in qualitative agreement with ours.

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References

Birks, J. B., Lumb, M. D., and Munro, I. H. (1964), *Proc. Roy. Soc., Ser. A 280,* 289.

Brocklehurst, J. R., Freedman, R. B., Hancock, D. J., and Radda, G. K. (1970), *Biochem. J. 116,* 721.

Bowen, E. J. (1954), *Trans. Faraday Soc. 50,* 97.

Chance, B., and Hagihara, B. (1963), *Proc. Congr. Biochem., 5th Moscow, 1961 5,* 31.

Chapman, D. (1973), *in* Biological Membranes, Vol. 2, Chapman, D., and Wallach, D. F. H., Ed., New York, N. Y., Academic Press, p 91.

Cogan, U., Shinitzky, M., Weber, G., and Nishida, T. (1973), *Biochemistry 12,* 521.

Devaux, P., and McConnell, H. M. (1972), *J. Amer. Chem. Soc. 94,* 4475.

Dodge, J. T., Mitchell, C., and Hanahan, D. J. (1963), *Arch. Biochem. Biophys. 100,* 119.

Döller, E., and Förster, Th. (1962), *Z. Phys. Chem. (Frankfurt am Main) 34,* 132.

Dorrance, R. C., and Hunter, T. F. (1972), *J. Chem. Soc., Faraday Trans. 168,* 1312.

Förster, T., and Kasper, K. (1955), *Z. Elektrochem. 59,* 976.

Frye, L. D., and Edidin, M. (1970), *J. Cell Sci. 7,* 319.

Galla, H., and Sackmann, E. (1974), *Biochim. Biophys. Acta 339,* 103.

Gitler, C. (1972), *Annu. Rev. Biophys. Bioeng. 1,* 51.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177,* 751.

Grätzel, M., and Thomas, J. K. (1973), *J. Amer. Chem. Soc. 95,* 6885.

Hagerman, J. S., and Gould, R. G. (1951), *Proc. Soc. Exp. Biol. Med. 78,* 329.

Huang, C. (1969), *Biochemistry 8,* 344.

Hubbell, W. L., and McConnell, H. M. (1971), *J. Amer. Chem. Soc. 93,* 314.

Lee, A. G. Birdsall, N. J. M., and Metcalfe, J. C. (1973), *Biochemistry 12,* 1650.

Martonosi, A. (1969), *Biochem. Biophys. Res. Commun. 36,* 1039.

McFarland, B. H., and Inesi, G. (1971), *Arch. Biochem. Biophys. 145,* 456.

Noyes, R. M. (1961), *Progr. React. Kinet. 1,* 129.

Parsons, D. F., Williams, G., and Chance, B. (1966), *Ann. N. Y. Acad. Sci. 137,* 643.

Pownall, H. J., and Smith, L. C. (1973), *J. Amer. Chem. Soc. 95,* 3136.

Robertson, J. M., and White, J. G. (1947), *J. Chem. Soc.,* 358.

Rudy, B., and Gitler, C. (1972), *Biochim. Biophys. Acta 288,* 231.

Scandella, C. J., Devaux, P., and McConnell, H. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 2056.

Schurr, J. M. (1970), *Biophys. J. 10,* 700.

Shimshick, E. J., and McConnell, H. M. (1973), *Biochemistry 12,* 2351.

Singer, S. J., and Nicolson, G. L. (1972), *Science 175,* 720.

Smoluchowski, M. (1917) *Z. Phys. Chem. (Leipzig),* 128.

Tarter, H. V. (1959), *J. Colloid Sci. 14,* 115.

Träuble, H., and Sackmann, E. (1972), *J. Amer. Chem. Soc. 94,* 4499.

Vanderkooi, J. M., Callis, J., and Chance, B. (1974a) *Histochem. J. 6,* 301.

Vanderkooi, J. M., Erecinska, M., and Chance, B. (1973), *Arch. Biochem. Biophys. 154,* 219.

Vanderkooi, J. M., Fischkopf, S., Chance, B., and Cooper, R. (1974b), *Biochemistry 13,* 1589.

Ware, W. R., and Richter, H. P. (1968), *J. Chem. Phys. 48,* 1595.

Yguerabide, J., Dillon, M. A., and Burton, M. (1964), *J. Chem. Phys. 40,* 3040.