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PHOTOBLEACHING

A NOVEL FLUORESCENCE METHOD FOR DIFFUSION STUDIES IN LIPID SYSTEMS

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SUMMARY

(1) The fluorescent molecule 12(9-anthroyloxy)-stearic acid dimerises on irradiation with light of 366 nm wavelength.

(2) The dimer is nonfluorescent and can be reconverted to the parent compound by irradiation at 254 nm.

(3) Kinetic analysis suggests that the dimerisation proceeds by a diffusion-limited second order mechanism in many solvents.

(4) Anomalously high rates seen in other systems can be attributed to localised high concentration regions (clusters) of the fluorescent molecule.

(5) The analysis has been extended to oriented lipid bilayers and the results suggest that below the gel-liquid crystalline transition temperature the 12(9-anthroyloxy)-stearic acid is excluded by the lipid matrix and forms regions of localised high concentration.

(6) In fluid lipid the results suggest an isotropic distribution of the probe. Calculated diffusion coefficients correspond to those found by other techniques.

INTRODUCTION

Much interest has been expressed in the fluidity of lipid systems in recent years, since many biological problems concern possible modulation of membrane properties by fluidity changes consequent on interaction with small molecules, or with proteins.

Many studies of this sort have been carried out by spectroscopic methods using ESR [1], NMR [2] and fluorescence [3]. The usual fluorescence approaches have involved measurement of intensity, degree of polarisation, etc. of emission from small molecules introduced as "probes" to report on their environment by their spectroscopic properties. One such molecule which has found wide use is 12(9-anthroyloxy)-stearic acid (12-AS) which was first introduced as a lipid analogue by Stryer et al. [4].

We present here a novel method of observing the degree of aggregation of this

Abbreviation: 12-AS, 12(9-anthroyloxy)-stearic acid.

probe in lipid systems and a method possibly of considerable value in comparative studies of diffusion in such systems. The method has the advantages of simplicity, speed and high sensitivity, and is potentially applicable to many other fluorescent probes.

MATERIALS AND APPARATUS

Bleaching studies were carried out on a Wild type M20 microscope fitted with an HBO 200W mercury lamp as light source. The light from this was filtered to isolate the 366 nm ultraviolet emission and diffused with a ground glass filter to reduce the effects of arc wander on homogeneity of illumination of the sample. Fluorescence emission from the sample was isolated using two narrow bandwidth interference filters with a passband centred at 444 nm, in conjunction with a yellow ultraviolet filter provided with the microscope. Measurements were made on all samples under similar conditions of exciting light intensity as judged by the background count in absence of sample. All measurements compared were made on the same day within the space of several hours. Stability of lamp was monitored regularly, and all measurements are the average of several similar experiments taken at different times. The emission was detected by an RCA type 8850 photomultiplier in an Ortec base equipped with outputs connected both to the anode and to the final dynode. The dynode pulse was gated into an Ortec type 6620 multichannel analyser via a type 451 preamplifier and a type 420A single channel analyser which was set to pass only that range of pulse sizes which corresponded to true single photon events. The multichannel analyser was used in its multichannel scaling mode in which it merely counts pulses in a given channel and increments the channel number at a rate which is set by its internal time base. The analyser was directly interfaced to a DEC PDP 11-05 computer which was controlled via an ASR 33 teletype terminal. Data could thus be directly analysed and the results written in a vacant portion of the analyser memory for visual display. Data could also be plotted directly from the analyser via a Bryans type 26000 X-Y recorder fitted with a null detector for point plotting. Background count for stray light was measured, and subtracted by the computer program used to plot the second order data.

Lipid bilayers were prepared by mixing the appropriate amounts of lipid and probe in chloroform and evaporating in an N_2 stream on a microscope cover slip. The bilayers were allowed to hydrate overnight in a desiccator over a saturated NaCl solution before orientation. After hydration, bilayers were orientated by pressing onto them a top cover slip and applying slight shearing force and direct pressure while the cover slip was held in a stream of hot air at a temperature above the melting transition of the lipid concerned.

Lipids used were carefully purified samples kindly supplied by Dr. B. de Kruijff.

Samples of 12-AS in lauric acid glasses were prepared by solution of the probe and acid in chloroform, evaporation of solvent and finally melting the mixture between cover slips. Samples of probe in volatile solvents were prepared as thin films between cover slips, and the cover slips sealed with a ring of "Davcon" epoxy resin. Samples of probe in solvent were stored in sealed containers in the dark before use, and lipid bilayers were used immediately after preparation.

12-AS was synthesised as described before [5].

Anthracene-9-carboxylic acid ethyl ester was prepared by reaction of the carboxylic acid with excess trifluoroacetic anhydride in the cold and followed by immediate addition of a large excess of ethanol. After vacuum evaporation the sample was purified by two recrystallisations from aqueous ethanol and gave a single spot on thin-layer chromatography using an eluant of chloroform/hexane/*n*-butanol (10 : 10 : 1, by vol.) on Camlab type Sil N HR UV_{254} precoated plates. This demonstrated freedom from contamination with the carboxylic acid and its anhydride. The ethyl ester melted between 102 and 104 °C uncorrected. The literature value is 102 °C. Both the carboxylic acid and the trifluoroacetic anhydride were purchased from R. N. Emanuel Ltd.

Fluorescence measurements were made using a Hitachi MPF 2A fluorimeter, and low temperature measurements using a Hitachi phosphorescence attachment in this instrument. Coolants used were either liquid N_2 or solid CO_2/methanol with the sample in a quartz tube in a Dewar flask.

THE METHOD

One of the greatest difficulties associated with fluorescence microscopy of biological materials under ultraviolet illumination has been the rapid decrease in intensity of emission of the dye used to stain the sample as a consequence of the high illumination intensities needed to excite the fluorescence. Attempts have been made to reduce this problem by the use of photon-counting techniques [6], where the light intensity is reduced and signal to noise ratio maintained by longer integrating times.

We present evidence in this paper that in certain cases this time-dependent decrease in intensity, which we have called "bleaching", can be used to advantage since it reflects the time course of a photochemical reaction occurring in the sample used.

It has been known for some time that long wavelength ultraviolet illumination of saturated solutions of anthracene and many of its compounds in several solvents give a precipitate of a dimer which is photochemically produced by reaction of an excited state anthracene molecule with a similar molecule in its ground state [7]. In the presence of oxygen a monomeric bridged peroxide can also be formed by a triplet state reaction with molecular oxygen. Such peroxide formation is favoured by some solvents, especially CS_2 [8].

Both the monomeric peroxide and the dimer are relatively stable at room temperature, but they decompose on heating to give anthracene. The dimer can be dissociated to anthracene by illumination with short wavelength light such as the 254 nm mercury line. This dissociation has been used to demonstrate excimer fluorescence from anthracene by 254 nm irradiation of dianthracene in a rigid matrix at 77 °K [9]. In this case a broad structureless band is observed at longer wavelength than the structured anthracene fluorescence. The quantum yield of the excimer is lower than that of anthracene, and the emission disappears if the sample is allowed to warm to room temperature and is then refrozen.

If a solution of 12-AS is irradiated with light of wavelength around 366 nm, its fluorescence also rapidly decreases with time. 12-AS dissolved in hexane was

irradiated in a glass vessel with the unfiltered light from a mercury arc. Under these conditions the shorter wavelengths in the ultraviolet region will be excluded by the glass. After several hours the solution was examined by thin-layer chromatography using the conditions mentioned for the ethyl ester of anthracene-9-carboxylic acid in the Materials section. It was found that the intensity of the fluorescent spot characteristic of 12-AS had decreased relative to a sample taken before irradiation and kept in a light-tight container. On 254 nm irradiation a new spot appeared below that due to 12-AS and this now fluoresced under 366 nm irradiation just as the 12-AS had done, although before the short wavelength irradiation no fluorescence excitable at this wavelength could be seen. Accordingly, a sample of the photolysed material was placed in a quartz cuvette and irradiated at 254 nm for several minutes before applying to the thin-layer chromatography plate. This sample showed no subsidiary spot and the spot corresponding to 12-AS was much more intense than was the control which had not been exposed to the 254 nm radiation. The fluorescence spectrum of the sample that had been irradiated at 254 nm was identical to that of 12-AS itself. The conclusion is either that a peroxide of 12-AS photolysable to the starting material, or else that a dimer had been formed by the 366 nm irradiation. In order to exclude the possibility of peroxide formation, a new sample was irradiated in light petroleum (B. Pt. 100–120 °C) which had been distilled three times under a current of dry O_2/free N_2, further deoxygenated by passage through a saturated alkaline pyrogallol solution. The 12-AS to which it was added was dissolved in chloroform and the chloroform removed by evaporation in a current of this dry purified N_2, to remove trapped air. The 12-AS was then dissolved in the hot light petroleum under an atmosphere of N_2 and irradiated in a sealed flask overnight. Thin-layer chromatography of the product after irradiation showed the behaviour previously described, and indicated that a comparable amount of product had been produced. The R_F values for 12-AS and dimer are 0.8 and 0.45, respectively, using the thin-layer chromatography system described in the Materials section. Thus it seems unlikely that peroxide formation competes significantly with the dimerisation in this system. This is in accord with the findings of Bowen [10] for anthracene in hexane. The product was found to be unstable to heat, as expected.

If a dimer had indeed been produced then it would be expected that excimer fluorescence might be seen from this in a rigid matrix after irradiation at 254 nm as for anthracene. Fig. 1 shows the fluorescence spectrum of the photoproduct before and after irradiation at 254 nm in an ethanol glass at the temperature of liquid N_2. The structured band at shorter wavelength is characteristic of 12-AS and reflects the presence of unreacted material. After the 254 nm irradiation a broad structureless band centred around 480 nm appears. This band disappears if the sample is warmed and refrozen, and its loss is accompanied by an increase in the structured component at shorter wavelength.

An attempt was made to observe excimer formation in liquid paraffin solutions under similar conditions. A solution of 12-AS in liquid paraffin was bleached with 366 nm light and cooled in solid CO_2/methanol. It was exposed to 254 nm radiation at this temperature and the spectrum measured. There was, however, no evidence of excimer formation in this case and the spectrum was characteristic of 12-AS in liquid paraffin at the same temperature. The experiment was repeated, but cooling to the temperature of liquid N_2. Here there was some evidence of excimer formation, but

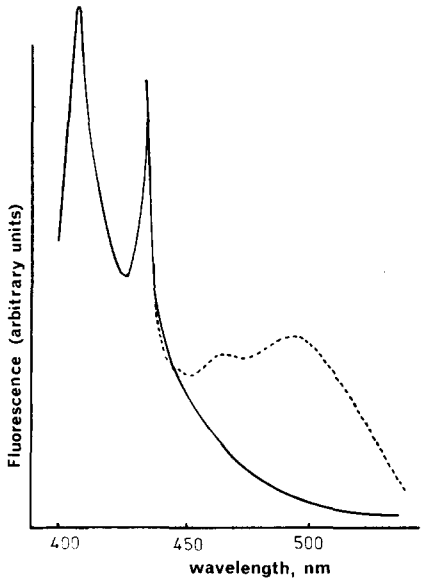

Fig. 1. Fluorescence spectrum of partially bleached 12-AS sample in ethanol at 77 °K. Broken line indicated new peak appearing after 254 nm irradiation. The peaks at short wavelength are characteristic of 12-AS under these conditions. Exciting wavelength is 366 nm. Spectra are normalised to equal intensities for the 12-AS component before and after irradiation at 254 nm.

this was not marked. The 12-AS emission in this solvent lacked the structure and resolution characteristic of its emission in ethanol.

To summarise, the evidence strongly supports the production of a photodimer of 12-AS when solutions of this compound are irradiated.

It was thought that the reaction might provide a useful method for comparing rates of diffusion in liquids if it proved to have a rate-limiting step which was diffusion controlled. This is not unlikely for a bimolecular reaction in a viscous solvent, and studies on excimer fluorescence have used this principle for similar measurements [11]. If the reaction were indeed diffusion limited and second order in probe, then plots of $1/I_t - 1/I_0$ as a function of time should be linear with a slope characteristic of the rate constant for diffusion, although an additional constant relating rate to reaction probability would also appear in the rate constant, The following section describes the results of such measurements in a variety of systems for 12-AS and for the compound anthracene-9-carboxylic acid ethyl ester, which is also expected to undergo bimolecular reactions as is known for many anthracene derivatives.

12-AS IN SOLVENTS

The rate of bleaching of 12-AS was measured in a variety of solvents at room temperature. The solvents were chosen to represent similar chemical structure but differing viscosities. It was found that, in a homologous series of alcohols, linear plots were obtained in all cases if the data were analysed as a bimolecular reaction. If the concentration of excited state is low relative to the ground state concentration it is resonable to assume that the rate of bleaching is proportional to the square of

the ground state concentration. The data can then be plotted in the form $1/I_t - 1/I_0 = kt$, where I_0 and I_t are the measured intensities of fluorescence at time zero and t, respectively. The slope of the plot, k, should then be independent of the initial concentration of 12-AS used. When the data for 12-AS bleaching in alcohols were so analysed it was found that this was the case and that the slope of the plots varied with solvent. If the reaction between ground and excited state were diffusion limited, it is expected that the fluorescence lifetime of the molecule in its excited singlet state would influence the rate of bleaching with all other factors constant. The relative intensities of initial fluorescence were therefore measured for the alcohols in the fluorimeter and the slopes normalised to constant fluorescence intensity, which was taken to be proportional to lifetime. When these normalised slopes are plotted as a function of the reciprocal viscosity of solvent a straight line plot results. The reciprocal viscosity is related to the rate of diffusion by the Debye equation [12], and the rate of diffusion of the molecule in the solvents can thus be plotted against normalised second order rate of bleaching (Fig. 2). The plot passes through the origin within experimental error. The Debye equation assumes a spherical molecule, an assumption almost certainly incorrect for 12-AS. However, the error thus introduced is expected to be constant in similar solvents.

An alternative method of analysis gives the diffusion coefficient directly. The molar volume of the probe molecule can be calculated using well defined procedures and with this value an apparent diffusion coefficient can be calculated for the molecule in a given solvent from a published nomogram [13]. If the normalised second order rate in that solvent is taken to be proportional to the calculated diffusion coefficient, then the constant of proportionality can be used to obtain diffusion coefficients for other solvents from the normalised second order rates. This procedure makes the assumption that the calculated molar volume is realistic in the solvents used, and this again is probably not correct. The error thus introduced is, however, likely to be constant and relatively small. The value of diffusion coefficient may be plotted against the normalised second order rate as before and a similar straight line plot is found. The results indicate that in the alcohols the reaction is almost certainly diffusion controlled and that with assumptions it is possible to use the second order rate of bleaching to calculate diffusion coefficients.

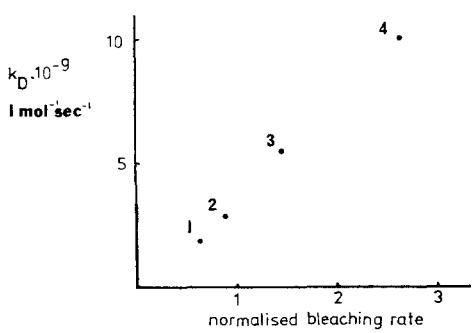

Fig. 2. The rate of bleaching normalised to a constant quantum yield is plotted against the rate of diffusion calculated from the Debye equation. Samples were constant thickness films between araldited cover slips. Concentration was 1 mM 12-AS in all cases. 1, *n*-butanol; 2, *n*-propanol; 3, ethanol; 4, methanol.

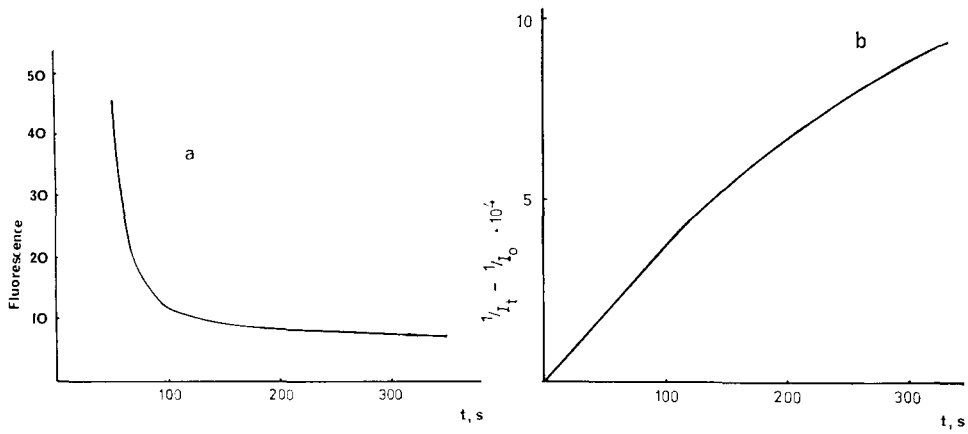

Fig. 3. Data (a) and resulting second order plot (b) for 1 mM 12-AS in dodecane solution. Fluorescence intensity is in counts/$\times 10^{-3}$. Similar data are obtained for liquid paraffin solutions. Temperature is 23 °C. Excitation at 366 nm.

The rate of bleaching was also measured in other solvents where the assumption of isotropic distribution of 12-AS cannot be made. Fig. 3 shows the data for dodecane and the resulting second order plot. It is clear that the rate of bleaching in this solvent is much greater than that found for the polar solvents, and the second order plot is non-linear. Similar results are found for liquid paraffin and hexane.

A model compound, anthracene-9-carboxylic acid ethyl ester, was used in similar bleaching studies both in the alcohols and in the hydrocarbon solvents. For this compound it was found that in all cases the data gave rise to second order plots with slow rates of bleaching in hydrocarbon solvents. The assumption of molar volume and spherical nature of the molecule are likely to be much better approximations for this compound, and no specific association is to be expected here. With 12-AS, however, it is possible that because of its amphilic properties aggregation might take place.

12-AS IN LIPIDS

Rates of bleaching of 12-AS in oriented layers of lipids were measured at room temperature. In all cases the mol ratio of probe to lipid was 1 : 1000; it is quite possible that significantly lower concentrations could be used, since intensity of emission is high for such samples.

The lipids were chosen so as to represent differing degrees of fluidity but similar chemical structure. They were dipalmitoyl phosphatidylcholine, which is below its gel-liquid crystalline transition temperature at 23 °C, dimyristoyl phosphatidylcholine, dilauroyl phosphatidylcholine and dielaidoyl phosphatidylcholine. Dimyristoyl phosphatidylcholine is very close to its transition temperature under the experimental conditions used while dilauroyl phosphatidylcholine is certainly in a fluid state, since it has a characteristic phase transition at 2 °C. Dielaidoyl phosphatidylcholine was included to determine the effect of unsaturation. It too is fluid at room temperature.

It was found that under identical conditions of incident light intensity the fluid

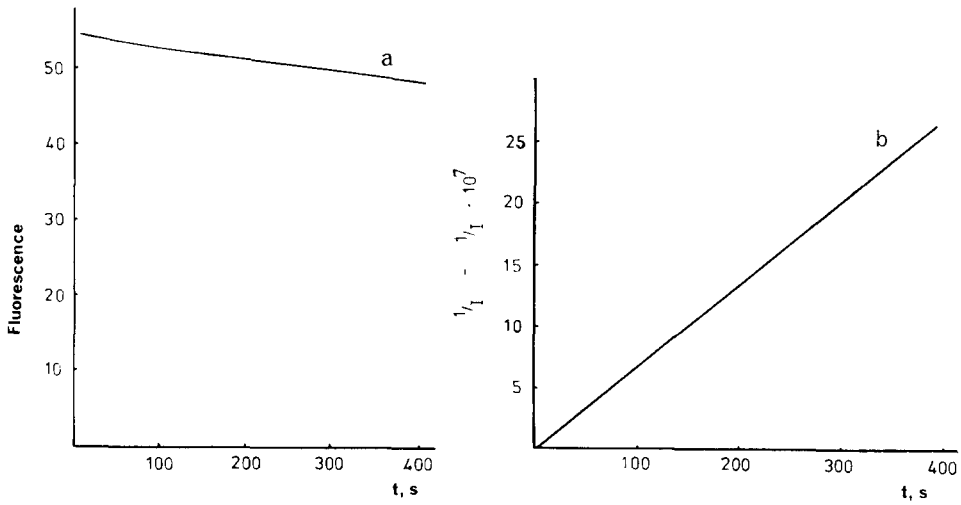

Fig. 4. (a) Decrease in intensity of fluorescence of 12-AS in dilauroyl phosphatidylcholine with time under constant illumination at 366 nm. (b) Second order plot resulting from this. Concentration of 12-AS was in mol ratio of 1:1000 probe:lipid. The sample was an oriented hydrated multilayer prepared between cover slips. Intensity units are counts/$\times 10^{-3}$. Temperature 23 °C.

phosphatidylcholines were bleached very slowly and the data for each gave a good linear second order plot (Fig. 4). In dielaidoyl phosphatidylcholine there was some suggestion of curvature in the initial part of the plot, but this was not marked. Rates are given in Table I. Dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine, however, behaved very differently. Dimyristoyl phosphatidylcholine was bleached much faster than the fluid phosphatidylcholines and the data for this did not give rise to a linear second order plot. The average rate was an order of magnitude larger than that for dilauroyl phosphatidylcholine, and the second order plot was clearly curved (Fig. 5). Photodimerisation in dipalmitoyl phosphatidylcholine was faster still, and the curvature of the second order plot was more marked than that for dimyristoyl phosphatidylcholine (Fig. 6). This behaviour was thought to be

TABLE I

Initial rates of bleaching of 12-AS in various media

INITIAL RATES OF BLEACHING OF 12-AS IN VARIOUS MEDIA

Bleaching rates are in arbitrary units. Diffusion coefficients are calculated assuming that the rates of bleaching are directly proportional to the rate of diffusion. D for dimyristoyl phosphatidylcholine from ESR studies is $3.0 \cdot 10^{-8}$ cm$^2 \cdot$ s^{-1} [14].

Medium	$k_{\text{bleaching}}$	Diffusion coefficient (cm$^2 \cdot$ s^{-1})
n-Butanol	$2.9 \cdot 10^{-7}$	$0.11 \cdot 10^{-5}$
Water	$2.0 \cdot 10^{-6}$	–
Dodecane	$3.7 \cdot 10^{-6}$	–
Lauric acid	$2.0 \cdot 10^{-6}$	–
Dilauroyl phosphatidylcholine	$6.8 \cdot 10^{-9}$	$2.6 \cdot 10^{-8}$
Dielaidoyl phosphatidylcholine	$1.25 \cdot 10^{-8}$	$5.3 \cdot 10^{-8}$

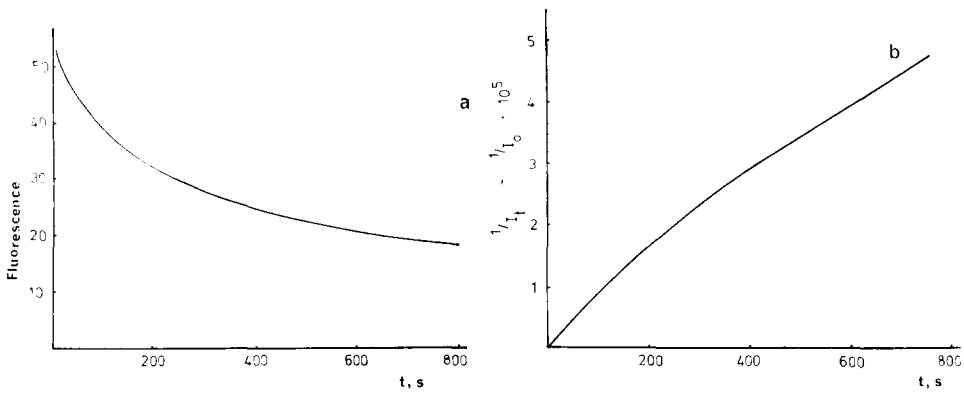

Fig. 5. Bleaching of 12-AS in dimyristoyl phosphatidylcholine at 23 °C: (a) Data and (b) resulting second order plot. Sample was an oriented multilayer with 12-AS to lipid mol ratio of 1:1000. Intensity is in counts/$\times 10^{-3}$. Excitation at 366 nm.

indicative of areas of local high probe concentration produced by some sort of clustering with the probe acting as an impurity which is excluded from the gel matrix. This sort of effect has been seen for spin labels [14] and suggested for fluorescent probes [15]. In order to test this idea a sample of 12-AS was sonicated into water to a concentration approx. 1 mM. 12-AS is known to form micelles in these circumstances from the blue shift in fluorescence emission characteristic of a nonpolar environment around the anthracene ring. The micelle solution was bleached with the same light intensity used for the lipid samples. Fig. 7 shows the data and corresponding second order plot. The plot is clearly biphasic, and if the initial rate is taken it is found to be of the same order as the rate characteristic of the fast phase in the dipalmitoyl phosphatidylcholine bilayer. As a test of the exclusion of the probe from a relatively rigid matrix, a sample was made up in a lauric acid melt as described in the Methods section. When this cooled between cover slips a transparent rigid glass was formed.

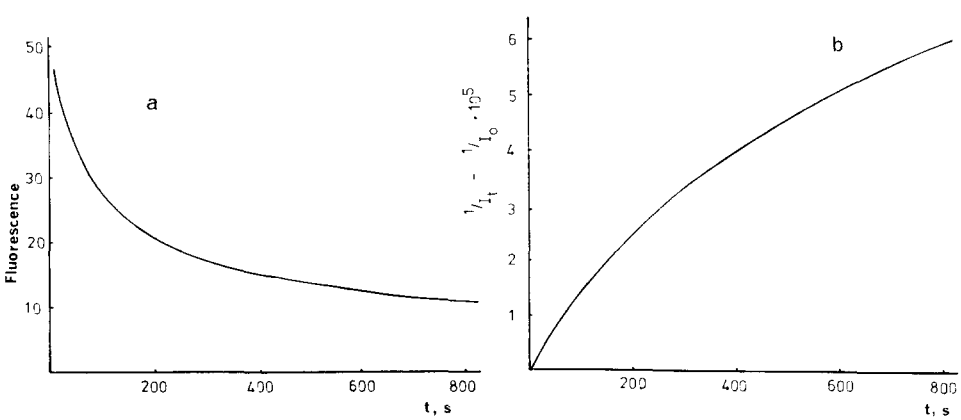

Fig. 6. Data (a) and second order plot (b) for 12-AS in dipalmitoyl phosphatidylcholine oriented multilayers. Probe to lipid mole ratio is 1:1000. Intensity is in counts/$\times 10^{-3}$. Temperature was 23 °C. Excitation was at 366 nm.

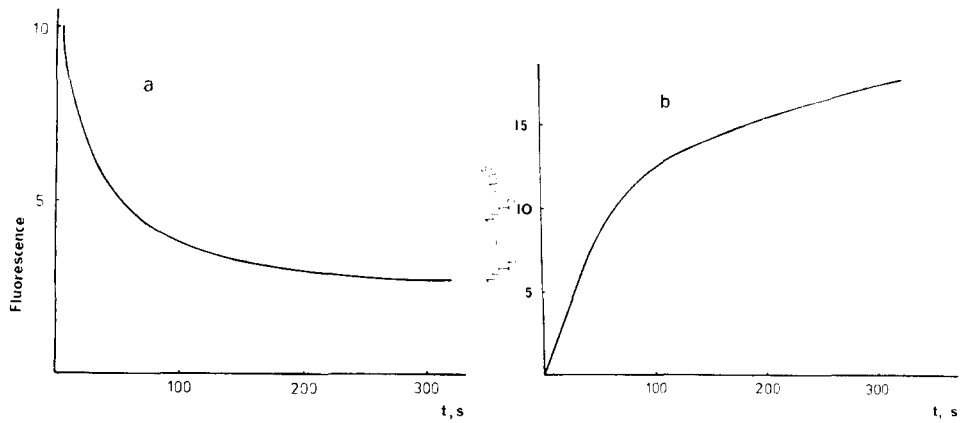

Fig. 7. Bleaching data (a) and second order plot (b) resulting for 12-AS in pH 7 aqueous phosphate buffer at 23 °C. Concentration was 1 mM. Suspension was sonicated until homogeneous and passed through an 8 μm Millipore filter before use. Intensity is in counts/ $\times 10^{-3}$. Excitation was at 366 nm.

Probe in this matrix exhibited exactly the behaviour seen for the micelles and for the dipalmitoyl phosphatidylcholine (Fig. 6). The later portion of the decay gave a rate which was at least two orders of magnitude slower than the initial rate, although it is difficult to measure accurately such fast initial rates. As a further test of the clustering hypothesis, the bleaching data for the lauric acid matrix was examined and the slow portion extrapolated back to the origin to give an estimate of the relative proportion of clustered and non-clustered probe molecules. A computer simulation of decay was produced by summing second order plots assuming rate constants appropriate to the fast phase of micelle bleaching and a value of the slow phase typical of diffusion in a solid. The amplitude of each component was weighted according to the relative amounts of clustered and non-clustered molecules obtained as above. The assumption is that there are two non-interacting sets of molecules, the bleaching rates of which

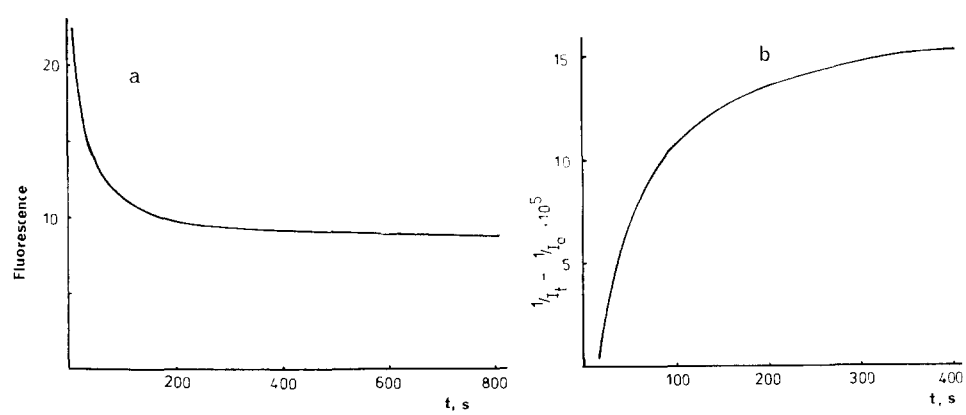

Fig. 8. Bleaching of 12-AS in dodecanoic acid (lauric acid). Probe was at mol ratio of 1 : 1000 in lauric acid: (a) data and (b) second order plot resulting. Temperature was 23 °C. The exciting light was at 366 nm.

obey second order kinetics in both cases. Both sets are assumed to emit with the same quantum yield. An excellent fit was obtained, suggesting that in this case the assumptions are reasonable.

DISCUSSION

The photodimerisation of 12-AS in different solvents shows two types of behaviour. In solvents (like alcohols) where isotropic probe distribution is expected the rate of photobleaching can be analysed as a linear second order reaction. In the different alcohols the rates of bleaching relate linearly to the rates of probe diffusion (calculated from the Debye equation). Thus, for this type of behaviour the rates are all of the right order for a diffusion-controlled reaction, as has been suggested for the dimerization of anthracene [16].

The second type of behaviour is most clearly seen for 12-AS in water. In this solvent 12-AS forms micelles and consequently its photo-bleaching is faster than the diffusion-controlled rate. The time course of this reaction is best analysed in terms of two second order reactions, the first one being characteristic of locally segregated probe molecules. In hydrocarbons, too, the rates of photoreaction suggest that the probe is again "compartmented" in some way since the characteristic curved appearance of the second order plots is always associated with an initial rate which is an order of magnitude higher than that predicted for a diffusion controlled reaction. This is in agreement with the anomalous viscosity behaviour of these solvents [17] and fluorescence polarization studies on 12-AS in them [15].

In contrast, the ethyl ester of anthracene-9-carboxylic acid gives linear second order rates in all solvents examined.

The results obtained for 12-AS in phosphatidylcholines correlate well with the behaviour expected. In the fluid phosphatidylcholines, good second order plots are obtained. The rates of photodimerisation found for 12-AS in fluid phosphatidylcholine bilayers are very much smaller than in solvents such as the alcohols, as expected from the relative magnitudes of the diffusion coefficients. ESR data indicate a diffusion coefficient of $3 \cdot 10^{-8}\ cm^2 \cdot s^{-1}$ in phosphatidylcholines [14], whilst a diffusion coefficient of $1.1 \cdot 10^{-6}\ cm^2 \cdot s^{-1}$ is calculated for 12-AS in *n*-butanol from the nomogram of Othmer and Thakar [13]. The "diffusion coefficients" in phospholipids are calculated on the assumption that the rates of bleaching are directly proportional to the rate of diffusion. It is well known that two dimensional diffusion cannot be strictly treated this way [18]; however, we can still obtain an order of magnitude estimate for the absolute diffusion coefficients by our method. More importantly the relative values of the diffusion coefficients in different lipids and under different conditions are strictly comparable. We have not attempted to apply the more sophisticated diffusion treatment [18], since we have no easy estimate of the probability of reaction during an encounter. The degree of curvature of the second order plots increases on passing through the phase transition as seen for dipalmitoyl and dimyristoyl phosphatidylcholines. The fast reaction component may be analysed by extrapolation to give an indication of the relative numbers of molecules in clustered and non-clustered environments. The results can be checked by computer simulation of the bleaching curve.

Our data indicate that measurement of the rates of bleaching for anthracene

compounds provides a simple and direct indication of probe aggregation in model systems. The method is rapid and sensitive, and an indication of the relative amount of probe which is locally concentrated is readily obtained. Such local aggregation of probe is important in interpretation of results obtained by fluorescence methods in biological systems. For example, in energy transfer measurements it is frequently important to know whether an assumption of isotropic probe distribution is valid. Similarly, many studies are presently being conducted to determine the extent of assumed lateral phase separation in mixed lipid systems. Such an approach might be valuable as an aid to interpreting data in these cases.

In addition the method has a potential value in determination of diffusion rates for probes in systems where isotropic distribution is expected. Since the absolute magnitudes of the derived diffusion rates depend on the assumed molar volume of the probe, the method is more meaningful in comparative measurements. The absolute value of the bleaching rate also depends on the probability of reaction for a given collision frequency. If, as is assumed in the literature [16], the dimerisation of anthracene compounds proceeds by a singlet mechanism, then the lifetime of the excited singlet will influence the measured rates. In the systems studied here the lifetime of the probe was approximately constant within each group of solvents or lipids studied, as were the other spectral parameters.

Relative rates in lipids of similar composition, as used in this study, are directly comparable, since the variables mentioned above are common to all the systems.

The approach outlined in this paper could be extended to other fluorescent probes. Anthracene itself could be used in similar experiments. This measurement would be comparable to the estimation of diffusion rates from excimer fluorescence [11]. Many other fluorescent molecules undergo photoreactions: for example, coumarins are known to dimerise [19]. The resulting products need not be non-fluorescent so long as their spectral properties are sufficiently altered to allow the reaction to be followed easily.

Since bleaching provides a way of removing the fluorescence from a well defined area of a lipid system, measurement of the rate of return of fluorescence to this area by photon counting could be used to give diffusion rates in lipids. We have made preliminary measurements of this kind and these indicate that even for fluid lipids the time course of such return extends over many hours at room temperature and over days with lipids below their phase transition temperature. The experiments could be done in reverse by introducing the dimer into the lipid and locally regenerating the fluorescence by 254 nm irradiation. The long time course of such experiments makes them less attractive than the simpler method presented in this paper. The possibility of generating fluorescence locally, for example by a sensitised mechanism, has not so far been explored, but might find some future application.

ACKNOWLEDGEMENTS

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