Fluorescent triplet probes for measuring the rotational diffusion of membrane proteins

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We have previously described a method for measuring the rotational diffusion of membrane proteins by using fluorescent triplet probes [Johnson & Garland (1981) *FEBS Lett.* **135**, 252–256]. We now describe the criteria by which the suitability of such probes may be judged. In general, the greatest sensitivity is achievable with probes where the ratio of the quantum yields for prompt fluorescence (ϕ_f) and triplet formation (ϕ_t) are high, as with Rhodamine ($\phi_f/\phi_t \approx 10^3$). However, considerations of heat generation at the sample membrane, of time resolution of fast rotations and of irreversible bleaching of the fluorescent probe also apply. The immediate environment of a probe molecule at a membrane protein must also be important in determining the performance of a given probe. Nevertheless, we describe guidelines for evaluating the likely usefulness of fluorescent triplet probes in measurements of membrane protein rotation.

The rotational relaxation times of membrane proteins undergoing Brownian rotational diffusion are typically in the region of a few microseconds to several milliseconds (for review see Cherry, 1979). Such relaxation times can be measured by photo-selection methods, but only if the photoactivated state itself has a lifetime of comparable or longer duration. For this reason fluorescence depolarization methods are restricted to the measurement of rotational relaxation times of less than about $0.1 \mu\text{s}$, this limit being set by the short lifetime of the excited singlet state. However, the triplet state has a much longer lifetime, and can be exploited in photo-selection methods for measuring rotational diffusion of membrane proteins in the microsecond–millisecond time-scale (Naqvi *et al.*, 1973). For such measurements it is necessary to attach a suitable triplet-forming molecule, a so-called triplet probe, to the membrane protein(s) under study. Several optical methods have been used for detection of the triplet-state: triplet–triplet absorption (Naqvi *et al.*, 1973; Cherry *et al.*, 1976), ground-state depletion based on singlet–singlet absorption (Cherry *et al.*, 1976; Lavalette *et al.*, 1977), delayed fluorescence (Greinert *et al.*, 1979; Austin *et al.*, 1979; Garland & Moore, 1979) and phosphorescence (Austin *et al.*, 1979; Garland & Moore, 1979; Moore *et al.*, 1979). Of these, phosphorescence with the use of

Erythrosin (tetraiodoFluorescein) is the most sensitive, about 10^{-11} mol (Garland & Moore, 1979).

We have previously described a new method, called fluorescence depletion, for measuring the triplet state (Johnson & Garland, 1981). This method exploits the fact that triplet probes are generally fluorescent, so that in principle depletion of the ground state by triplet formation could be detected by depletion of fluorescence. Thus it is not the absorption of a measuring light-beam that is measured, but instead the prompt fluorescence that such absorption excites. The signal of interest is then the depletion of fluorescence that follows from depletion of the ground state. The fluorescence-depletion method overcomes certain drawbacks of the phosphorescence method, and improves the sensitivity by about 10^9 -fold. As a result of this much improved sensitivity it is now possible to make rotational diffusion measurements of proteins on the surface of a single cell (Johnson & Garland, 1981). The purpose of this present paper is to describe how we have assessed the suitability of probe molecules for measurement of their triplet state by fluorescence depletion.

Experimental

Materials

Fluorescein isothiocyanate, 2,7-dichloro-Fluorescein, 4,5-dibromoFluorescein, di-iodo-Fluorescein (substitution positions unspecified) and

Abbreviation used: NBD chloride, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole.

Rhodamine B isothiocyanate were from BDH Chemicals, Poole, Dorset, U.K. 4-Chloro-7-nitro-benz-2-oxa-1,3-diazole (NBD chloride) and tetramethylRhodamine isothiocyanate were from Cambridge Research Biochemicals, Huntingdon, Cambs., U.K. Alternatively, tetramethylRhodamine isothiocyanate was obtained from Research Organics, Cleveland, OH, U.S.A. Eosin 5-isothiocyanate was either from Molecular Probes, Plano, TX, U.S.A., or synthesized by Dr. C. H. Moore by the method of Cherry *et al.* (1976). Bovine plasma albumin (fraction V) was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. High-purity N₂ with an O₂ content of less than 0.001% was from Air Products, Crewe, Cheshire, U.K., and used as such. Affi-gel 10, a beaded agarose gel with added aliphatic arms 1.0 nm long terminated by carboxy-*N*-succinimide esters, was from Bio-Rad Laboratories, Watford, Herts., U.K. Ox liver catalase (EC 1.11.1.16) was from Boehringer Corporation, Lewes, Sussex, U.K. Glucose oxidase (EC 1.1.3.4; type V from *Aspergillus niger*) was from Sigma Chemical Co., Poole, Dorset, U.K.

Binding of fluorescent dyes to albumin

NBD chloride and dyes available as isothiocyanates were covalently linked to bovine serum albumin as follows. The dye was dissolved in 0.1 M-sodium phosphate buffer, pH 8.0 (in some cases it was necessary first to dissolve the dye in a small amount of methanol or buffer, pH 9.5) to achieve a final concentration of approx. 3 mM. To this was added bovine serum albumin to a final concentration of 3.3 mM. The mixture was left for 2 h at room temperature or overnight at 4°C, then passed through a column of Sephadex G-25 to remove unbound dye. The dihalogenated Fluoresceins were not commercially available as isothiocyanates, but were bound non-covalently to albumin by merely substituting for the isothiocyanates in the procedure just described. Concentrations of dyes were measured spectrophotometrically, by taking the following millimolar absorption coefficients (mm⁻¹.cm⁻¹) for the visible-region absorption maxima: NBD chloride, 11.0 (Cantley & Hammes, 1975); Fluorescein, 88; dihalogen derivatives of Fluorescein, 95; Eosin, 99 (Bowers & Porter, 1967); Rhodamines, 80 (Chadwick *et al.*, 1958). These values were used for both bound and unbound dye solutions, and the absorption maxima were determined with a wavelength-scanning spectrophotometer.

Immobilization of dye-albumin complexes on agarose beads

Affi-gel 10 (0.1 g of slurry) previously washed free of storage solvent with distilled water and then 0.1 M-sodium phosphate buffer, pH 7.5, was added

to 0.2 ml of approx. 1 mM-dye-albumin complex in the same buffer. The mixture was left for 2 h at room temperature, then overnight at 4°C, then washed three times with several volumes of buffer. Sites that had not reacted were not blocked.

Preparation of anaerobic samples

To 50 µl of the dye-albumin solution was added 50 µl of a buffer solution (0.1 M-sodium phosphate, pH 7.5) containing D-glucose (83 mM), catalase (0.58 mg/ml) and glucose oxidase (0.83 mg/ml). These last three reagents were added to act as an O₂-scavenging system. Glycerol (0.9 ml) was added, and the solutions were mixed and gassed with N₂ for 5–10 min before being placed in an anaerobic glove box. The final concentration of glycerol was approx. 92% (w/w), giving a viscosity at 25°C of 222 cP (Segur, 1953). A small volume (2–5 µl) of the dye-albumin complex in glycerol with O₂-scavenging system was placed on a microscope slide, and spread under the weight of a cover slip, which was then sealed in place with quick-setting epoxy resin. The microscope-slide sample was kept in the anaerobic box until required. A similar procedure was used for preparing microscope-slide samples of dye-albumin complex immobilized on agarose beads. However, glycerol was omitted, and to prepare the sample for the slide 0.1 ml of N₂-flushed gel slurry carrying dye-albumin complex was suspended in 0.1 ml of a buffer solution containing D-glucose (200 mM), glucose oxidase (10 mg/ml) and catalase (7 mg/ml).

For the sensitivity experiments (see below), the dye-albumin complexes were placed on a 20 µm-thick slide (Helba Bacteria Chamber with Thoma ruling; Hawksley and Sons, Lancing, Sussex, U.K.) and the cover slip was sealed in place with silicone grease. Grease rather than epoxy resin was used to allow the slide to be used again.

Instrumentation

The fluorescence-depletion method for measuring the triplet state requires laser beams of two intensities differing by about 10³–10⁴-fold. The more-intense beam is used briefly (2–60 µs) to deplete the ground state of the dye by populating the triplet state. The less-intense beam indirectly measures the ground-state concentration by continuously exciting fluorescence therefrom. Our instrument obtains both beams from a single continuous-wave argon ion laser, with an acousto-optic modulator to alter the beam intensity between bleaching (i.e. ground-state-depleting) and measuring levels. In general, continuous-wave gas lasers are not of sufficient intensity to give significant triplet formation in a few microseconds, and it is necessary to use a lens to focus the laser beam to a small area on the sample. Focusing to a higher intensity was previously

described by Lessing *et al.* (1976), who used a mode-locked argon ion laser to study the kinetics of ground-state depletion of xanthene dyes on a nanosecond time-scale. Our own instrument (Johnson & Garland, 1981) is based on a laser-microscope combination (Garland, 1981) designed initially for measurement of lateral diffusion of membrane proteins by the fluorescence recovery after photobleaching method (Axelrod *et al.*, 1976; Koppel *et al.*, 1976).

A brief description of our apparatus is given elsewhere (Johnson & Garland, 1981). In greater detail, we used a fluorescence microscope (model 41; Vickers Instruments, York, U.K.) equipped with an epi-illuminator and dichroic mirror (Ploem, 1967). An argon ion laser of modest power (Lexel model 85-1 with an all-lines power of 1 W; from Lambda Photometrics, Harpenden, Herts., U.K.) provided the bleaching and measuring beam. The laser output was acousto-optically modulated (model 304A modulator; Coherent U.K. Ltd., Cambridge, U.K.) over a continuous range of 10^3 – 10^4 with a rise or fall time of less than 1 μ s. It should be noted that the precursor of this instrument (Garland, 1981) used a digital (Coherent model 304D) rather than analogue (model 304A) modulator. The digital version yields only two laser beam powers, either unattenuated or maximally attenuated. These two modulators differ in their electronic drive units, but not in the optical device attached to the laser output. Thus the optical component of the modulator can be used in either analogue or digital mode according to the drive unit. The modulator was adjusted for maximal first-order diffraction at 488 or 514.5 nm. The diffracted beam was isolated with a 2 mm-diameter pinhole, and then passed through a spatial filter consisting of a 9 mm-focal-length input lens (i.e. a 20 $\times$ microscope objective), a 10 μ m-diameter pinhole and an 80 mm-focal-length collimating lens (model 1526 beam expander with spatial filter; Oriel Scientific, Kingston-upon-Thames, Surrey, U.K.). The spatial filter attenuated higher spatial frequencies superimposed on the otherwise Gaussian profile beam, and it improved the contrast ratio (i.e. ratio of the unattenuated to fully attenuated intensities) of the analogue modulated beam from 10^3 to 10^4 . The collimating lens was held on a micrometer mount that was remotely adjustable with an electric motor and control (Motormike model Series B; Ardel Kinematic, College Point, NY, U.S.A.). With this arrangement the focus of the laser beam in the region of the back focal plane of the microscope objective, and thence on to the sample, could be finely adjusted while the sample was being observed through the microscope eyepiece. The microscope and laser were mounted on a 1.22 m $\times$ 0.91 m (48 in $\times$ 36 in) optical table (Oriel 1220), and extensive use was made of optical rails (Oriel standard

rails) and adjustable mirror mounts (Oriel model 1450) to direct and align the laser beam into the 2 mm-diameter pinhole, the spatial filter, the collimating lens and the epi-illuminator. The use of this adaptable optical rail system greatly simplified the design and construction of the instrument.

The unmodified version of our fluorescence microscope has an assembly of reflecting prisms positioned above the dichroic mirror and barrier filter. Normally one of these prisms is selected with a slide mechanism to reflect rays from the objective into either the eyepiece or the camera optics. We modified this prism assembly to provide a position in which the objective rays passed uninterruptedly upwards, first to form a real image in the plane of a pinhole mounted on an x – y -micrometer, and then to reach the photocathode of a photomultiplier tube mounted vertically above the microscope. This design follows that of Koppel *et al.* (1976), and discriminates against fluorescence emanating from other than the focused laser spot at the sample. The barrier filter was a 3 mm thickness of Schott KV550 non-fluorescent plastic laminate (H. V. Skan, Solihull, Birmingham, U.K.). For the experiments described below, the collimating lens of the laser-beam expander was adjusted so that the fluorescent spot excited at the sample appeared to just fill the circle of calibrated eyepiece graticule, corresponding to a laser spot radius of 11 μ m for a 20 $\times$ objective and 5.5 μ m for a 40 $\times$ objective. The pinhole radius was 0.5 mm.

Fluorescence intensity was measured with a photon-counting system with an EMI 9813B photomultiplier (EMI, Ruislip, Middlesex, U.K.) and a pre-amplifier followed by an amplifier-discriminator (models 9301 and 5C1 respectively; E.G. and G. Brookdeal, Bracknell, Berks., U.K.). Photon counts from repetitive sweeps each comprising a cycle of measure, bleach and measure were accumulated in a multichannel scaler (model 1170 signal averager; Nicolet Instruments, Warwick, U.K.). The address period was 10 μ s, which, with 1024 addresses in the memory, gave a sweep time of 10.24 ms. Approximately the first 1 ms of each sweep was used for recording the undepleted (unbleached) fluorescence. The photon counter was gated out during the high laser intensity of the bleach. A laboratory-constructed hybrid analogue and digital circuit interfaced the multichannel scaler, the photon counter and the drive to the acousto-optic modulator. A Pockels cell and half-wave plate used for polarization studies (Johnson & Garland, 1981) were omitted for the experiments described in the present paper. Laser powers were measured by placing a silicon diode photometer (model G101; Macam Photometrics, Livingstone, Scotland, U.K.) between the collimating lens and microscope; the power at this point was equated to the power

incident on the sample. Either the 488nm or 514.5nm laser line was used, according to the dye under study.

Results and discussion

Fluorescent triplet probes

With the triplet probes used previously, the emphasis has been on designing probes with heavy-atom substituents that increase the quantum yield for triplet formation, ϕ_t . Thus of the series Fluorescein ($\phi_t = 0.05$), dibromoFluorescein ($\phi_t = 0.49$) and Eosin ($\phi_t = 0.71$; Bowers & Porter, 1967) only the last two have been successfully used as triplet probes (Lavalette *et al.*, 1977; Naqvi *et al.*, 1973; Cherry & Schneider, 1976). The effects of heavy-atom substitution were also exploited in the development of a phosphorescent triplet probe, Erythrosin (tetraiodo-Fluorescein) by Garland & Moore (1979).

Different considerations apply to the choice of a probe when the triplet state is to be detected by fluorescence depletion. A high value of ϕ_t is still desirable, but in addition a high value for the fluorescence quantum yield (ϕ_f) is required to maximize the sensitivity of fluorescence detection. However, ϕ_t and ϕ_f are interdependent, an increase in one being at the expense of the other (Bowers & Porter, 1967). Accordingly there is a trade-off

between ease of triplet formation on the one hand and sensitivity of fluorescence detection on the other. There are limits to how far this compromise can be overcome by increasing the laser intensity at the sample. The bleaching pulse must be brief compared with the rotational relaxation times under study, and its intensity has an upper limit set by the rise in temperature that results from light-absorption by the sample (Axelrod, 1977). In addition, the measuring beam intensity must not be so high that it causes significant further and unacceptable triplet formation. It was therefore necessary to study several fluorescent dyes to determine how in practice the competing demands for high values of ϕ_t and ϕ_f might best be resolved.

Fluorescence depletion as a measure of triplet formation

Some general features of fluorescence-depletion measurements are shown in Fig. 1. We wish to make the following points. Firstly, the depletion of fluorescence following the bleaching pulse was transient, relaxing to the undepleted state within a few milliseconds. Secondly, the relaxation of fluorescence depletion was greatly accelerated by the presence of O_2 in the sample, as was to be expected from the known ability of O_2 to quench the triplet

Fig. 1. Fluorescence depletion of dyes bound to albumin in glycerol

Eosin and Rhodamine isothiocyanate were attached to albumin and prepared in glycerol on microscope slides as described in the Experimental section. Recordings: (a) Eosin, anaerobic; (b) Eosin, aerobic; (c) Rhodamine, anaerobic; (d) Rhodamine, aerobic. Repetitive sweeps (2048 for each recording) were made at an address time of $10\mu s$. The bleach pulse duration was $20\mu s$ for Eosin and $60\mu s$ for Rhodamine. Fluorescence photon counts memorized in the multichannel scaler were plotted on a pen recorder after digital-into-analogue conversion. The unbleached fluorescence intensity at the start of each recording is referred to as 100%, and the vertical bars indicate a 10% change in intensity. The bleach pulse was delivered at the points marked with arrows.

state (Kasche & Lindqvist, 1964). The smaller effect of O_2 observed with Eosin than with tetramethyl-Rhodamine bound to albumin can perhaps be attributed to the possibility that Eosin, but not tetramethylRhodamine, occupied a site on the protein that provided protection from O_2 . Thirdly, the intensity and duration of the bleaching pulse required to obtain comparable fluorescence depletions was much less with Eosin than with Rhodamine. This difference was as expected from the high ϕ_t value for Eosin (0.71) compared with that for Rhodamine, which is 0.05 or less (Lessing & von Jena, 1979). Fourthly, if fluorescence depletion had been irreversible and its decay had occurred as a result of lateral diffusion, as in fluorescence-photo-bleaching-recovery experiments (Axelrod *et al.*, 1976), then it can be calculated that the resulting recovery time would have been some 1000-fold greater than that observed. Fifthly, the decay time for the depletion signals in the absence of O_2 quenching (Eosin, 1–2 ms; Rhodamine, 3–4 ms) are typical for triplet lifetimes. For these reasons we conclude that the fluorescence-depletion measurements such as those of Fig. 1 are measurements of ground-state depletion resulting from reversible population of the triplet state.

Laser energy required for triplet formation

We summarize in Table 1 the laser energies required to obtain some 20–30% fluorescence depletion for a range of dyes bound to albumin. We have excluded Erythrosin from the Table because the high quantum yield for triplet formation ($\phi_t \approx 1.0$) so weakens its fluorescence ($\phi_f \approx 0.02$; Bowers & Porter, 1967; Lessing *et al.*, 1976) as to make Erythrosin unsuitable for use in fluorescence-

depletion measurements unless much lower sensitivity can be tolerated. The bleaching intensities given in Table 1 are not directly comparable with each other, because the absorption coefficients of the dyes generally differ at any given laser line. Thus dichloroFluorescein and dibromoFluorescein were excited close to their absorption maxima with the 514.5 nm line, whereas Rhodamine and tetramethyl-Rhodamine were not. Nevertheless the relative laser intensities required to obtain comparable degrees of fluorescence depletion were much as expected. An approximate comparison of the relative ease of fluorescence depletion can be made by referring the various bleaching intensities to that for Eosin. Very roughly, Fluorescein required a 10-fold and the Rhodamines a 100-fold greater laser intensity than did Eosin to achieve 20–30% fluorescence depletion. After correcting for the relative absorption coefficients of Eosin and the Rhodamines at 514.5 nm, and assuming that ϕ_t for albumin-bound Eosin is not greatly different than for free Eosin (0.71), then we calculate that ϕ_t for the albumin-bound Rhodamines was 0.01 or less.

The transverse energy mode of the laser beam in our apparatus is Gaussian (Garland, 1981), and the visual determination of the laser spot radius by superimposition of a graticule overestimates the area where most of the laser energy falls. Thus the true laser intensities in the central regions of the spot may be 2–3-fold higher than those recorded in Table 1. In conventional ground-state-depletion measurements (Garland & Moore, 1979) with a flash-lamp-pumped dye laser to excite at 540 nm, we required a bleach pulse intensity of about $60 \text{ mJ} \cdot \text{cm}^{-2}$ ($0.6 \text{ nJ} \cdot \mu\text{m}^{-2}$) to obtain 30% ground-state depletion of albumin-bound Eosin, whereas the value given in Table 1 for

Table 1. *Laser energies required for conversion of fluorescent dyes into the triplet state*

Measurements were made on thin anaerobic films of albumin-bound dye in 92% (w/w) glycerol at room temperature. The dye concentrations were in the range 50–100 μM . The following microscope objectives and laser spot radii were used: Fluorescein and derivatives, 20 $\times$ and 11 μm ; Rhodamine and tetramethylRhodamine, 40 $\times$ and 5.5 μm ; NBD chloride, 40 $\times$ and approx. 1.4 μm . The fluorescence depletion given in the Table refers to that immediately after bleaching, and the laser power listed is the unattenuated power used for the bleach pulse. The length of the bleach pulse was taken from an oscilloscope display of the waveform used to drive the acousto-optic modulator. The depletion half-time is the time for half-recovery of the fluorescence to its undepleted level.

Dye	λ_{max} (nm)	Laser line (nm)	Laser power (mW)	Bleach time (μs)	Bleach energy at focus ($\text{nJ} \cdot \mu\text{m}^{-2}$)	Power intensity at focus ($\text{nW} \cdot \mu\text{m}^{-2}$)	Fluorescence depletion (%)	Depletion half-time (ms)
Fluorescein	502	488	17	40	1.79	0.044	20	1
DichloroFluorescein	517	514.5	14	20	0.74	0.037	28	2
DibromoFluorescein	514	514.5	6	2	0.03	0.016	31	0.9
Di-iodoFluorescein	526	514.5	10	20	0.53	0.026	26	0.5
Eosin	533	514.5	6	10	0.16	0.016	29	1–2
Rhodamine	559	514.5	41	60	25.9	0.43	22	3–5
TetramethylRhodamine	559	514.5	39	40	16.4	0.41	21	1–3
NBD chloride	448	488	5.3	30	161	5.3	24	3

the fluorescence-depletion method was nearly 4-fold less at $0.16 \text{ nJ} \cdot \mu\text{m}^{-2}$. In view of the uncertainties involved, we consider this to be a reasonable agreement. It can be noted in passing that a conventional laser-flash-photolysis apparatus (e.g. Cherry, 1978; Garland & Moore, 1979), not using a lens to focus the laser beam to a small area at the sample, would need laser-flash energies of several joules to achieve 20–30% ground-state depletion of Rhodamine dyes. Such energies are possible, but only at increased cost and lower repetition rates. It is therefore not surprising that Rhodamines have not previously been used as triplet probes, despite their satisfactory triplet lifetimes (Fig. 1).

Temperature increases at the sample

Axelrod (1977) calculated that if a laser beam of 1 mW power and Gaussian profile was focused to a spot with a $1/e^2$ radius of $1 \mu\text{m}$ on a biological membrane carrying tetramethylRhodamine label at a density of 5×10^3 molecules/ μm^2 and with an absorption coefficient of $5 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at the laser wavelength, then the resulting steady-state temperature increase would be 0.03°C and be established within a few microseconds. The calculation was made for a worst-case situation in which all absorbed photon energy would be converted into heat (i.e. $\phi_f = 0$). In practice, if ϕ_f approached 1.0 then the only absorbed energy appearing as heat would originate from the Stokes shift, whereby the fluorescence is of longer wavelength and therefore lower energy than the excitation. Thus for the Rhodamines ($\phi_f \approx 1.0$, excite at 514 nm, emit at 560–660 nm), the actual temperature increase could be 5–10 times lower than that calculated for $\phi_f = 0$. In Axelrod's (1977) calculation a 1 mW laser power focused to a $1/e^2$ radius of $1 \mu\text{m}$ would give an average intensity of $0.28 \text{ mW} \cdot \mu\text{m}^{-2}$. The steady-state temperature rise T_s at the laser spot is related

to the laser beam power P , the dye absorption coefficient ϵ at the laser wavelength, the number of dye molecules n per μm^2 of membrane and the $1/e^2$ laser-beam radius ω by the following equation (Axelrod, 1977):

$$T_s = \frac{K P \epsilon n}{\omega} \quad (1)$$

where K is a constant that includes the mass density, specific heat and thermal diffusivity of the aqueous medium adjacent to the membrane. In Table 2 we list the laser-beam powers that, on the basis of the data of Table 1, would be required to achieve 20–30% fluorescence depletion in $10 \mu\text{s}$ if focused to a spot of either $1 \mu\text{m}$ or $5 \mu\text{m}$ radius. We also list the calculated T_s values for a dye intensity of 5×10^3 molecules/ μm^2 assuming that T_s had been reached and that $\phi_f = 0$. It is evident from Table 2 that only negligible temperature increases are calculated for Fluorescein, dibromoFluorescein and Eosin. In these cases the duration of the bleach pulse could be decreased to 1–2 μs with a higher laser power, improving the time resolution without causing unacceptable heating ($T_s < 1^\circ\text{C}$). The calculated temperature increases for the Rhodamines are larger, and would possibly be unacceptable if the laser-beam power was increased to allow a shorter bleach pulse, particularly with the larger ($5 \mu\text{m}$) spot radius. Nevertheless a $10 \mu\text{s}$ bleach pulse would be sufficiently fast to resolve the rotational relaxation of many membrane proteins (Cherry, 1979).

Sensitivity of fluorescence-depletion measurements

In principal detection of the triplet state by fluorescence depletion becomes more sensitive as the ratio ϕ_f/ϕ_i increases. This is because the measuring beam intensity can be increased without causing significant further population of the triplet state if ϕ_i is low. There are also instrumental considerations,

Table 2. *Calculated temperature increases at the laser spot on a membrane*

The temperature increase T_s is that calculated for a laser-beam power sufficient to cause 20–30% fluorescence depletion in $10 \mu\text{s}$, by using the data of Table 1 and the calculation methods of Axelrod (1977). It is assumed that the energy required to achieve a given fluorescence depletion was independent of the bleach pulse duration when, as was the case, the pulse duration was much shorter than the triplet lifetime. Absorption coefficients at the laser lines were calculated from absorption spectra of albumin-bound dyes and from published absorption coefficients at the absorption maxima for the free dyes in aqueous solution. The calculated values are therefore approximate.

Dye bound to albumin	Laser line (nm)	ϵ_{mM} at laser line ($\text{mM}^{-1} \cdot \text{cm}^{-1}$)	Required laser intensity at focus ($\text{mW} \cdot \mu\text{m}^{-2}$)	T_s ($^\circ\text{C}$)	
				Radius $1 \mu\text{m}$	Radius $5 \mu\text{m}$
Fluorescein	448	60	0.18	0.02	0.1
DibromoFluorescein	514.5	95	0.003	<0.001	0.003
Eosin	514.5	46	0.012	0.002	0.01
Rhodamine	514.5	30	2.58	0.17	0.85
TetramethylRhodamine	514.5	30	1.52	0.10	0.5
NBD chloride	488	30	5.3	0.34	1.7

such as the efficiency of the photomultiplier photocathode response at the fluorescence wavelengths concerned. However, an essential feature of the high sensitivity of the fluorescence-depletion method for triplet detection is the ability of the apparatus to collect repetitive sweeps quite rapidly (e.g. 100 Hz) until sufficient photon counts have been collected in each address to provide the desired signal-to-noise ratio. However, if during the repetitive sweeps of triplet formation and decay there is also irreversible loss of probe by whatever means, then sensitivity cannot be increased indefinitely just by increasing the number of sweeps. This problem becomes particularly relevant when the probe molecules are attached to membrane proteins and cannot necessarily be replaced by lateral diffusion of fresh molecules into the area under the laser spot. Thus the rate of lateral diffusion could be too slow, or the supply of new molecules finite. To model this situation we immobilized albumin-bound dyes on agarose beads, and determined how far the fluorescence of the dyes declined after many repetitive sweeps of fluorescence-depletion measurements. Some results from this approach are given in Fig. 2, which shows the fluorescence-depletion signals obtained from sequential sets of sweeps made on the same surface spot of a fluorescent agarose bead.

Each of the fluorescent dyes used in the experiments shown in Fig. 2 became irreversibly bleached by the fluorescence-depletion measurements. Fluorescein became irreversibly bleached more rapidly than did Eosin or tetramethylRhodamine. The irreversible bleaching of dye was not complete, and in the case of Eosin and tetramethylRhodamine appeared almost to cease once 30–40% of the original fluorescence intensity had been lost. The mechanism for anaerobic photochemical destruction of the fluorescent dyes is not known, but is presumably influenced by the immediate chemical environment of the dye.

A comparison of the relative sensitivities of detection for various dyes was made by making fluorescence-depletion measurements on films of known thickness ($20\mu\text{m}$; see the Experimental section). Albumin-bound dye was used, and the experimental conditions were chosen to give fluorescence depletion of 10–30% and a total measuring time of 5–10 ms. The number of dye molecules in the path of the laser beam was calculated from the path length ($20\mu\text{m}$), beam radius and dye concentration. We then calculated the number of photons per $10\mu\text{s}$ address collected in 1024 sweeps for 10^3 dye molecules in the laser path. The values of this number relative to that for Eosin were: tetramethylRhodamine, 140; Rhodamine, 120; NBD chloride, 55; Fluorescein, 5; dichloro-Fluorescein, 3; di-iodoFluorescein, 0.75.

Fig. 2. *Fluorescence depletion of immobilized dyes* Albumin-bound dyes, anaerobic, were immobilized as described in the Experimental section. The address time for photon counting was $40\mu\text{s}$. Repetitive sweeps (2048) were collected sequentially into successive quarters of the multichannel scaler. Thus the left-hand recording is of sweeps 1–2048, the next recording of sweeps 2049–4096, and so on. The bleach pulse durations and laser powers were: (a) Fluorescein, $60\mu\text{s}$ at 22 mW; (b) Eosin, $20\mu\text{s}$ at 6.8 mW; (c) tetramethylRhodamine, $60\mu\text{s}$ at 30 mW. The laser spot radii were $11\mu\text{m}$ in each case.

Selection of fluorescent dyes as triplet probes for measurement of rotational diffusion constants of membrane proteins by depolarization of fluorescence depletion

The experimental observations and considerations given above provide guidelines for the choice of fluorescent triplet probes for use in studying membrane protein rotation by depolarization of fluorescence-depletion methods. It is

known that ϕ_t and ϕ_f can be altered by binding to proteins (Youtsey & Grossweiner, 1967). It is also highly likely that the ability of the dyes to resist irreversible photochemical bleaching is also dependent on environment. Thus, although the probable performance of a dye can be predicted from measurements of ϕ_t , ϕ_f and photochemical stability in simple model systems, in practice the performance may differ somewhat when the probe is attached to a particular membrane protein. To date the best sensitivity in a membrane system has been achieved with Eosin attached to band-3 protein of human erythrocyte 'ghosts', where rotation was measured in a membrane area $2\mu\text{m}$ in diameter (Johnson & Garland, 1981). Assuming that the surface area of a 'ghost' is $130\mu\text{m}^2$ and that there are 1.1×10^6 molecules of band-3 protein per 'ghost' (Nigg *et al.*, 1979), then there are 2.7×10^4 molecules of band-3 protein in an area of membrane $2\mu\text{m}$ in diameter. The ratio ϕ_t/ϕ_f for Eosin is considerably less than for Rhodamines, and it is likely that the latter dyes will provide even greater sensitivity.

Apart from sensitivity and stability, it is necessary that the fluorescent triplet probe should have a linear transition dipole moment for light-absorption at the laser wavelength used, otherwise photoselection by plane-polarized light is less effective (Albrecht, 1961, 1970). Finally, derivatives of the dye capable of covalent attachment to proteins, drugs, hormones or other ligands of membrane proteins should be available or be readily synthesized. As with the fluorescent dyes used for measuring lateral diffusion by fluorescence photobleaching recovery (Peters *et al.*, 1974; Axelrod *et al.*, 1976; Koppel *et al.*, 1976; Jacobson *et al.*, 1976; Edidin *et al.*, 1976), it is preferable to use absorption bands well into the visible region, and away from the absorption of natural and fluorescent chromophores such as NADH or flavoproteins that may cause troublesome autofluorescence of cells (Aubin, 1979). In these respects Fluorescein, dibromoFluorescein, Eosin, Rhodamine and tetramethylRhodamine are satisfactory. NBD chloride is converted into the triplet state only at higher laser intensities, and may be less satisfactory.

Effects of rotational diffusion kinetics

In our experiments the bleaching pulse and measuring beam of the laser were linearly polarized in the same plane. Consequently the observed kinetics of the decay of fluorescence depletion were expected to be influenced not only by repopulation of the ground state but also by rotational diffusion. Rotation of the polarization plane by 54.7° between the bleaching pulse and measuring beam would have removed this complication (Lessing & von Jena, 1976). However, in practice this was not necessary for the purposes of the present investigation. In the

experiments with albumin-bound dyes free to diffuse in glycerol, the rotational relaxation times for the albumin molecule would have been less than $20\mu\text{s}$ (Moore *et al.*, 1979) and barely detectable at the time resolution used in the present study. In the experiments with albumin-bound dyes immobilized on agarose beads, rotational diffusion was presumably inhibited. In both instances fast ($\ll 1\mu\text{s}$) but restricted movement of the dye relative to albumin would have been undetected. Thus the observed decay kinetics of fluorescence depletion in the millisecond time range were governed by ground-state repopulation, without significant interference from rotational diffusion.

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