Phosphorescence of Protein-Bound Eosin and Erythrosin

A POSSIBLE PROBE FOR MEASUREMENTS OF SLOW ROTATIONAL MOBILITY

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We used a pulsed dye laser working at 540 nm to excite triplet-state formation of eosin and erythrosin, either bound or unbound to bovine serum albumin, in aqueous solution anaerobically at pH 8 and 20–22°C. Delayed emission from radiative transitions of the triplet state was readily detectable, both as delayed fluorescence and as red phosphorescence. Detection of the triplet state by measurement of phosphorescence at 645 nm upwards was at least 100-fold more sensitive than by absorbance measurements of ground-state depletion at 500 nm. When immobilized in poly(methyl methacrylate), the phosphorescence of eosin and erythrosin was polarized with an anisotropy parameter [Jablonski (1961) *Z. Naturforsch.* A16, 1–4] of about 0.25. The phosphorescence of erythrosin is sufficiently intense to be distinguishable from the long-wavelength end of fluorescence under conditions of continuous rather than pulsed excitation. Our observations suggest that phosphorescence depolarization of eosin or erythrosin probes could be used as a highly sensitive method of measuring rotational relaxation times in the region from 10^{-5} to 10^{-3} s, such as those of the uniaxial rotation of membrane proteins.

Fluorescence depolarization of attached dyes has been widely used to measure rotational diffusion of proteins and the flexibility of parts of proteins occurring in the time scales of the order of the fluorescence lifetimes of the dyes, about 10^{-9} – 10^{-7} s (Weber, 1953; Brand & Witholt, 1967; Wahl, 1975). Slower molecular movements cannot be measured in this way, and available optical methods for measuring rotational relaxation times of the order of 10^{-5} – 10^{-3} s, such as those of some membrane proteins, have depended on photoinduced linear dichroism of either endogenous or added chromophores, as reviewed by Cherry (1975). The measurement of such dichroism is relatively insensitive, for the usual reasons relating to absorbance measurements, namely, that it is necessary to measure a small difference between two much larger signals each of which carries light-source noise and photomultiplier shot noise. Unfortunately there is a general need for higher sensitivity for application to membranes, because the concentration of any one protein in a membrane is rarely high. Attempts to improve sensitivity by the use of higher concentrations of membranes or by longer light paths are self-defeating, because the increased depolarization arising from particle scattering of the initially polarized exciting or measuring beams degrades the signal of interest (Junge & De Vault, 1975).

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Despite its limitations, photoinduced linear dichroism remains an attractive and relatively simple method for the measurement of slow rotational diffusion, particularly as the range of proteins open to study this way is in principle greatly extended by the development of eosin isothiocyanate as an exogenous optical probe for those many proteins that lack a native one (Cherry & Schneider, 1976; Cherry *et al.*, 1976*a,b*). In this method a dye laser flash of 1–2 μ s duration and about 50 mJ at 540 nm is used to excite about 25% of protein-bound eosin to its triplet state which, in the absence of oxygen quenching, has a lifetime of about 1 ms. If the laser emission is plane polarized photoselection of the eosin occurs, and therefore the extinction signal arising from either ground-state depletion or triplet-state population also becomes polarized when viewed with the appropriate geometry (Albrecht, 1961, 1970). Theoretically the anisotropy parameter, r , for the absorbance signal A , defined as (Jablonski, 1961):

$$r = \frac{A_{(\parallel)} - A_{(\perp)}}{A_{(\parallel)} + 2A_{(\perp)}} \quad (1)$$

where $(\parallel)$ and $(\perp)$ refer to the measuring beams polarized either parallel $(\parallel)$ or perpendicular $(\perp)$ to the plane of laser polarization, would have a value of 0.4 for an immobilized sample provided that there is zero angle between the absorption oscillators for excitation and measurement. For molecules that are rotating isotropically, the value of r at a time t after

excitation is given by (Jablonski, 1961):

$$r_t = r_0 \exp(3t/\sigma) \quad (2)$$

where r_0 is the value immediately after excitation before significant movement has occurred, and σ is the rotational relaxation time of the molecule, defined as the time taken for the molecule to rotate an angle θ° , where $\cos \theta^\circ = e^{-1}$, from its original position (Weber, 1953). Thus high sensitivity in studying rotation by depolarization requires not only sensitive detection of the chromophore, but also the highest possible value for r_0 , which, with a two-step photoselection procedure, can range anywhere from -0.33 to $+0.4$ according to the molecule (Albrecht, 1961, 1970).

For a variety of reasons, some of which we have discussed above, the insensitivity of the eosin-probe method in the absence of signal averaging limits its application to membranes to those proteins present at a concentration of about 0.5 nmol or more per mg of membrane protein. This excludes most membrane proteins. We have therefore sought to improve the sensitivity of the method for detection of the triplet state of eosin by measuring either its phosphorescence or its fluorescence (Parker & Hatchard, 1961). We should explain that the triplet state of eosin can return to the ground state not only by non-radiative transitions but also by radiative transitions. The direct radiative transition from the triplet to the ground state results in a red phosphorescence. Alternatively, intersystem crossing from the triplet to the excited singlet state, followed by a radiative transition of the singlet to the ground state, results in delayed fluorescence. The lifetimes for phosphorescence, delayed fluorescence and the triplet state of eosin in dilute solution are all identical, about 1 ms at room temperature. By contrast, the lifetime of prompt fluorescence of eosin is 0.9 ns (Porter *et al.*, 1974), although its emission spectrum is as for delayed fluorescence.

In principle, measurements of the polarization of delayed fluorescence or of phosphorescence could be used to study slow rotational diffusion, as has been suggested by others (Parker, 1968; Albrecht, 1970). In practice such an approach has been considered difficult to use at around room temperature, where the quantum yields for delayed emission are very much lower than at the usual 77 K favoured by photochemists. However, even if the quantum yield for phosphorescence (ϕ_p) of eosin at 22°C was only 0.001 , the excitation and experimental conditions used by Cherry *et al.* (1976a) in studying photo-induced linear dichroism of erythrocyte 'ghosts' would have resulted in a laser-induced sample phosphorescence of several mW peak value. Such an emission would be easily detectable and yield a high sensitivity.

The usual effect of substituting a fluorescent aromatic compound with halogens of increasing atomic weight is to increase intersystem crossing from the excited singlet to the triplet state, to increase ϕ_p at the expense of ϕ_f (the quantum yield for prompt fluorescence) and to shorten the lifetime of the radiative transition from the triplet state to the ground state (Cox & Kemp, 1971; Kasha, 1960; McClure, 1949; Zander, 1968). We have therefore studied the laser-excited delayed emission at $20\text{--}22^\circ \text{C}$ not only of eosin (2',4',5',7'-tetrabromofluorescein) but also of erythrosin (2',4',5',7'-tetraiodofluorescein), both free in aqueous solution and bound non-covalently to bovine serum albumin. We find that measurement of the triplet state of eosin or erythrosin by phosphorescence is two orders of magnitude more sensitive than by absorbance measurements of ground-state depletion. The phosphorescence of eosin or erythrosin immobilized in poly(methyl methacrylate) exhibits an r_0 value of about 0.25 . Phosphorescence-depolarization measurements therefore have the potential even at room temperature in aqueous solution to provide a highly sensitive method of studying rotational relaxation times in the $10^{-6}\text{--}10^{-3} \text{ s}$ time range. We describe here the relevant photochemical behaviour of eosin and erythrosin.

Experimental

Instrumentation

Emission spectra with continuous excitation were measured by using a fluorimeter designed by Dr. J. C. Kernohan of this Department. The excitation source was a 50 W high-pressure mercury-vapour arc lamp, which illuminated an $f/4$ grating monochromator set to a spectral bandwidth of 20 nm . The output of this monochromator illuminated a $1 \text{ cm} \times 1 \text{ cm}$ -cross-section fluorimeter cuvette, whose emission was viewed at 90° to the excitation axis by a second but otherwise similar monochromator (Spex Minimate grating monochromator; Spex Industries, Box 798, Metuchen, NJ 08840, U.S.A.). The emitted light was detected with a red-sensitive side-window photomultiplier tube, type 9785B, from EMI Electron Tube Division, Ruislip, Middx, U.K. Polarization studies were done by using sheet-type polarizers type 03FPG003 from Melles Griot B.V., Nieuwe Kade 10, Arnhem, The Netherlands. One such polarizer was placed at the exit slit of the excitation monochromator, and another at the entrance slit of the emission monochromator. External levers permitted independent rotation of the polarization axes of the filters into vertical and horizontal laboratory axes. At any given wavelength, four measurements of emission intensity were made. Firstly, with the excitation plane vertical, measurements were made with the analyser plane vertical ($\parallel$) or horizontal ($\perp$). Secondly, with the excitation

plane horizontal, measurements were made with the analyser plane horizontal ($\parallel$)' or vertical ($\perp$)'. The ratio of these latter two readings $\{(\perp)/(\parallel)\}$ would be 1.0 for an ideal instrument free of polarization artefacts; the actual ratio was used as a correction factor to multiply the ratio of the first pair of readings $[(\parallel)/(\perp)]$ to give a polarization ratio corrected for instrumental error.

Measurements of laser-flash-induced ground-state depletion of eosin or erythrosin were made with a 90° angle between the excitation and measuring axes. This geometry is shown in Fig. 1. The laser was a flash-lamp-pumped dye laser yielding a $1\text{--}2\mu\text{s}$ pulse (model 43 dye laser from Electrophotonics, 1 The Cutts, Dunmurry, Belfast BT17 9HN, Northern Ireland, U.K.). The laser dye was 10^{-4}M -Coumarin-6 in methanol, and the emission (540nm for untuned operation) was polarized with an intra-cavity Glan-Taylor prism. The prism could be rotated to put the plane of polarization in the vertical or horizontal laboratory axis. The sample in a $1\text{cm} \times 1\text{cm}$ -section fluorimeter cuvette was illuminated with

a measuring beam of white light from a 55W quartz/iodide/tungsten filament lamp that focused beyond the sample at the entrance slit of a monochromator (Monospek 600 grating monochromator from Rank-Hilger, Westwood, Margate, Kent CT9 4JL, U.K.). The monochromator slits were set for a 4nm spectral bandwidth, and a wavelength of 500 or 510nm was usually used for measurement. The light emerging from the exit slit was divided into ($\parallel$) and ($\perp$) components with a polarizing beam splitter (type BC 6P, Barr and Stroud, Anniesland, Glasgow G13 1HZ, Scotland, U.K.). Light-detection was with two end-window photomultiplier tubes, EMI type nos. 9798B or 9698B. Each tube viewed the polarizing-beam splitter through a sheet-type polarizing filter oriented with its polarizing axis vertical or horizontal according to its relation to the beam splitter. The photomultiplier gains were matched by adjustment of their separate high-voltage supplies. Conventional operational amplifier circuits provided linear conversion of the anode current into a voltage, zero offsets, and a time constant of $2\text{--}3\mu\text{s}$. Measure-

Fig. 1. Apparatus for detection of laser-induced triplet states either by absorbance measurements of ground-state depletion or by delayed emission

Full details are given in the text. (a) is a 55W quartz/iodide/tungsten-filament lamp, (b) a lens, (c) the sample cuvette, (d) the dye laser beam, (e) and (f) chopper wheels, (g) the monochromator, (h) a polarizing beam-splitting cube, (i) a pair of polaroid sheet filters, (j) and (k) end-window photomultiplier tubes and (l) is a pyroelectric joulemeter. Light beams polarized normal to the plane of the page are shown as ----, and beams polarized in the plane of the page as Photomultipliers (j) and (k) detect light polarized parallel ($\parallel$) and perpendicular ($\perp$) to the laser polarization respectively. Each photomultiplier had an independent high-voltage supply (EHT). When polarization was not being measured, the beam splitter (h) was removed, and only photomultiplier (k) was used, without sheet filter (i). The plane of polarization of the dye laser beam could be rotated through 90° to be in the plane of the page (laboratory horizontal axis) for control experiments. For absorbance measurements the chopper wheels were 'parked' in their open position. For delayed-emission measurements the lens (b) was placed to focus an image of the cuvette (c) at the entrance slit of the monochromator.

ments were made as % transmission (T), not absorbance, and were recorded in the range 95–105% T with zero suppression of the 100%- T signal. These signals were recorded with transient recorders, displayed on an oscilloscope, and plotted on an xy recorder. We used either a model-OS4000 dual-channel digital-storage oscilloscope from Gould Advance, Hainault, Essex IG6 3UE, U.K., or a model-512A single-channel transient recorder from Bryan's Southern Instruments, Mitcham, Surrey CR4 4UL, U.K.

Measurements of delayed-emission spectra were made with essentially the same apparatus as that used for ground-state depletion, except that the tungsten-filament lamp was not used other than as convenient and variable source of unpolarized light with which the monochromator entrance slit could be illuminated in order to match the gains of the ($\parallel$) and ($\perp$) photomultipliers when polarization was measured. Otherwise the polarizing filters and cube were removed, and only one photomultiplier was used. In addition, two wheel choppers were placed at the exit of the laser and the entrance of the monochromator (Fig. 1). Each chopper (type VFCZ from Sir Howard Grubb Parsons and Co., Newcastle upon Tyne NE6 2YB, U.K.) ran independently of the other, but at about the same speed, giving equal open and shut periods of 1.25 ms each with about 0.20 ms to switch between fully open to fully shut. No attempt was made to synchronize the choppers; only a single laser pulse was needed for any one measurement at a given wavelength, and this was timed with a logic circuit triggered by reference signals from the choppers. By this means the laser was fired just before one chopper blocked the laser and the other unblocked the entrance slit of the monochromator.

More sensitive measurements of laser-induced phosphorescence were made by viewing the cuvette with an end-window photomultiplier through a red-pass filter [3 or 6 mm of Schott glass RG645 (from HV Skan, Shirley, Solihull, West Midlands B9U 4AE, U.K.)]. This arrangement is shown in Fig. 2. When polarization of phosphorescence was measured, the gains of the photomultipliers were matched by setting them to the same sensitivity in response to non-polarized red light sampled from a 55 W tungsten-filament lamp through a 10 nm band-pass interference filter centered at 680 nm (type 03FIV 063 from Melles Griot). In all experiments involving use of the dye laser, a pyroelectric joulemeter (model J305 from Molelectron Corporation, Sunnyvale, CA 94086, U.S.A.) placed beyond the sample position downstream from the dye laser was used to measure laser energies.

Absorption spectra of samples were measured with the wavelength-scanning spectrophotometer described by Haddock & Garland (1971).

Fig. 2. Apparatus for measurement of polarized phosphorescence at high sensitivity

Full details are given in the text. (a) is a 55 W quartz/iodide filament lamp used at low powers as a non-polarized light source for calibration, (b) is an interference filter of 10 nm band-pass centred at 680 nm, (c) is the sample cuvette, (d) the dye laser beam, (e) is a 3 mm thickness of a red-pass glass filter (Schott RG645), (f) is a polarizing beam splitter, (g) is a pair of polarizing sheet filters, (h) and (i) are end-window photomultiplier tubes and (j) is a pyroelectric joulemeter. With the laser beam polarized normal to the plane of the page (i.e. in the laboratory vertical axis), photomultipliers (h) and (i) measured the ($\parallel$) and ($\perp$) phosphorescence signals respectively. Each photomultiplier had an independent high-voltage supply (EHT).

Reagents

Eosin was obtained from Fluka A.G. (Buchs, Switzerland) and used without further purification. Erythrosin B was obtained from BDH, Poole, Dorset, U.K., and was purified by dissolving it in ethanol and precipitating impurities by addition of acetone. Bovine serum albumin and Sepharose 6B were supplied by Sigma, Poole, Dorset, U.K. Eosin, and presumably erythrosin, adsorb strongly to albumin in 1:1 molar ratio (Youtsey & Grossweiner, 1967); such conjugates were made by mixing equimolar amounts of dye and albumin solution in 50 mM-potassium phosphate, pH 8.0. Albumin-dye complex (100 nmol) was conjugated to CNBr-activated Sepharose 6B in 0.3 M-NaHCO₃ at 4°C for 12 h (Porath & Axén, 1976). The Sepharose conjugate was then washed with 50 mM-phosphate buffer, pH 8.0, and suspended in 5 ml of approx. 75% (v/v)

glycerol in phosphate buffers. The glycerol prevents the Sepharose from settling during the course of an experiment.

Methyl methacrylate was obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K., and washed free of stabilizer with 5% (w/v) NaOH, then 20% (w/v) NaCl, then water, until the washes were neutral. The methyl methacrylate was dried with anhydrous CaCl_2 and distilled under N_2 . To make solid solutions of eosin or erythrosin, the dye (usually 50 nmol) in ethanol (25 μl) was added to methyl methacrylate (10 ml) containing 2,2'-azobis-(2-methyl-propanenitrile) (0.5 mg) in a glass reaction tube. The solution was deoxygenated by bubbling with argon for 10–20 min. The tube was then sealed and left overnight at 60°C to polymerize the methyl methacrylate. Subsequently the tube glass was broken and the polymerized sample machined to a bar 1.25 cm $\times$ 1.25 cm $\times$ 4 cm, polished, and stored under argon. The argon (Research Grade) was purchased from Air Products, Crewe, Cheshire, U.K. The oxygen content of the argon is specified by the makers at less than 1 p.p.m.

Results

Delayed-emission spectra of eosin and erythrosin

The delayed-emission spectra of eosin and erythrosin, both in free solution and bound to bovine serum albumin at a 1:1 molar ratio, are shown in Fig. 3. The emission intensities were those measured 0.25 ms after the laser flash, this being the time taken for the choppers to block off the laser axis and unblock the measuring axis. These choppers served two purposes: firstly, to protect the photomultiplier against the intense flash of prompt fluorescence when measuring in that spectral region; and secondly, to remove any suspicion that any long-lived emission from the laser-pumping flashlamp was masquerading as delayed emission from the sample. The delayed-emission spectra showed two distinct peaks, one in the 550–560 nm region and another in the 'red' region at about 680 nm. These peaks are uncorrected for photomultiplier sensitivity, which according to the maker's specifications is approx. 3-fold lower at 680 than at 550 nm. Nor are the peaks corrected for self-absorption by the 5 μM -dye solutions, but this effect is small at 550 nm and effectively absent at 680 nm. The shorter-wavelength peaks are identical in position with those of prompt fluorescence, and are therefore attributed to delayed fluorescence. The longer-wavelength peaks are attributed to phosphorescence. The delayed emission was abolished if deoxygenation of the samples by bubbling with argon gas was omitted; this is in keeping with the rapid quenching of the triplet state by oxygen.

Fig. 3. *Delayed-emission spectra of eosin and erythrosin*. Excitation was with a 30 mJ dye laser flash at 540 nm, and emission intensity was measured by using the apparatus shown in Fig. 1. The transient recorder readings at each wavelength were plotted on to an *xy* recorder, and the emission intensity 0.25 ms after the laser flash was used for the present Figure. In (a), the dyes were 5 μM in 50 mM-potassium phosphate pH 8.0. In (b), 5 μM -bovine serum albumin was also present. The samples were bubbled with argon for 10–15 min before sealing. In each case the solutions of eosin (■) or erythrosin (●) were at 20–22°C. The emission intensities are plotted as arbitrary units, which in fact correspond to the linear amplifier output in mV. The emission intensities of (a) and (b) correspond to identical instrumental sensitivities.

Binding of the dyes to albumin shifted their delayed-emission peaks by about 5 nm towards the 'red' region. The apparent enhancements of delayed-emission intensity on binding were not greatly different from those calculated from the increased absorbance at 540 nm (the laser excitation wavelength) of the protein-bound dyes (see below). However, even when differences of absorbance at 540 nm are taken

into account, the intensity for erythrosin measured 0.25ms after excitation was approx. 5-fold greater than that for eosin, irrespective of whether the dyes were bound to albumin or not.

Absorption spectra of eosin and erythrosin

Because excitation was carried out at a fixed wavelength, it was necessary to know the relative absorbance of the dye solutions at that wavelength in order to compare emission intensities, as just discussed. Fig. 4 gives the visible absorption spectra of eosin and erythrosin bound or unbound to bovine serum albumin. As noted by others, binding gave a 'red shift' of the absorption maximum of about 10nm, but only small changes in peak height. At 540nm the relative magnitudes of the absorbance of 5 μ M solutions of erythrosin+albumin, erythrosin, eosin+albumin and eosin were 4.7, 2.8, 4.0 and 1.0 respectively.

We noted during the present work that 'solid solutions' of erythrosin or eosin set in poly(methyl

methacrylate) appeared to have absorption coefficients some 50% lower than those for aqueous solution. This lowering of the absorption coefficient occurred as soon as the dye was dissolved in methyl methacrylate, before polymerization, and was independent of the amount of dye added. We have assumed therefore that the concentration of dye in our solid poly(methyl methacrylate) solutions is best calculated on the basis of absorption coefficients one-half of those taken for aqueous solutions. The peaks of the solid-solution absorption spectra are red-shifted to 540nm for eosin and 545 nm for erythrosin. We have assumed that ϵ_{mM} for eosin in aqueous solution at pH8.0 at 522nm is 9.9×10^4 (Kasche & Lindqvist, 1965), and have taken the same value for erythrosin at 530nm.

Triplet yields

Youtsey & Grossweiner (1967) observed that the triplet yield of eosin bound to albumin was approximately halved when the dye was bound to the albumin at a molar ratio of unity. The recordings of Fig. 5 confirm this observation and extend it to erythrosin.

Lifetimes of the triplet state and of phosphorescence

Semilogarithmic plots of the decay of the triplet state measured by ground-state depletion or by phosphorescence gave straight lines. For any given sample the lifetimes of phosphorescence and of the ground-state-depletion signal were identical. For solid solutions, the triplet lifetimes were 3.6ms for eosin and 0.65ms for erythrosin. For dye bound to

Fig. 4. Absorption spectra of eosin and erythrosin

The samples were (a) 3.3 μ M-eosin in 50mM-potassium phosphate, pH8.0; (b), as (a) plus 3.3 μ M-bovine serum albumin; (c) 3.1 μ M-erythrosin in 50mM-potassium phosphate, pH8.0; (d), as (c) plus 3.1 μ M-bovine serum albumin; (e) 4.4 μ M-eosin in poly(methyl methacrylate); (f) 3.1 μ M-erythrosin in poly(methyl methacrylate). In all cases the sample light-path was 1.0cm and the temperature 20–23°C. The aqueous solutions were not deoxygenated; the solid solutions were.

Fig. 5. Comparison of the triplet-state yield for eosin and erythrosin

Triplet yield was estimated from absorbance measurements of ground-state depletion at 500nm. Trace (a) is for 5 μ M-eosin in 50mM-potassium phosphate, pH8.0; (b) is as (a) plus 5 μ M-bovine serum albumin; (c) is for 5 μ M-erythrosin in 50mM-potassium-phosphate, pH8.0; and (d) is as (c) plus 5 μ M-bovine serum albumin. The samples were anaerobic and at 20–22°C, with a 1 cm light-path. The laser was fired at the moments indicated in the Figure by unlabelled arrows, and delivered 110mJ at 540nm.

albumin at a 1:1 molar ratio, in potassium phosphate buffer at pH 8.0 and 22°C, the triplet lifetimes were 0.6ms for eosin and 0.35ms for erythrosin (Figs. 6 and 7).

Sensitivity of triplet-state detection by phosphorescence compared with ground-state-depletion measurements

We found that detection of the triplet state measured by phosphorescence is very much more sensitive than by absorbance changes due to ground-state depletion. One advantage of measuring emission rather than absorption of light is that, in the former, a broad spectral band can be viewed with a large solid angle. Accordingly we used the simple but effective apparatus of Fig. 2 for measuring phosphorescence at high sensitivity. In Fig. 8 we show recordings of phosphorescence measurements of albumin-bound eosin and erythrosin at concentrations of 500nM, 50nM and 6.25nM. We also show measurements of laser-induced A_{500} changes for the highest of the dye concentrations, 500nM. It is obvious that the signal-to-noise ratio for the absorbance measurements was problematical even at the highest dye concentration. This performance is limited by photomultiplier shot noise. By contrast, the phos-

phorescence measurements did not show significant shot noise even at the lowest concentration of dye, 6.25nM.

However, at higher sensitivities a fast-decaying component appeared in the first 20–50 μ s of the phosphorescence signal following the laser flash. This component is more prominent with eosin than with erythrosin (Fig. 8). Its decay is too slow to be accounted for by the response time of the amplifiers (approx. 2 μ s), and we think that it is caused by the tail of the laser and flashlamp pump continuing to excite emission from the cuvette, buffer and sample. Detector overload and delayed recovery may also contribute. Despite this interference, which is open to correction by modification to the instrument, the sensitivity of the phosphorescence measurements for triplet detection is two orders of magnitude better than that achieved by absorbance measurements.

Polarization of phosphorescence

Phosphorescence polarization was measured in solid solutions of dye in poly(methyl methacrylate). Cherry & Schneider (1976) reported that the anisotropy parameter, r , for ground-state depletion of

Fig. 6. Lifetimes of the triplet state and phosphorescence of eosin

Ground-state depletion was measured at 500nm, phosphorescence at 645nm upwards. The same samples of solid solution of eosin (10 μ M) or 5 μ M-eosin-albumin in 50mM-potassium phosphate were used for both phosphorescence (solid solution, ■; aqueous solution, ▲) and ground-state depletion measurements (solid solution, ●; aqueous solution, ▼). The signals, either absorbance or phosphorescence, are given in arbitrary units.

Fig. 7. Lifetimes of the triplet state and phosphorescence of erythrosin

Experimental conditions were as for Fig. 6, except that erythrosin replaced eosin. The decay curves are for solid solution (phosphorescence, ■; absorbance, ●) and aqueous solution (phosphorescence, ▲; absorbance, ▼).

solid eosin solution excited with a polarized dye laser flash was 0.27. Our own instrument gave a slightly higher value of 0.36. For solid solutions of erythrosin we consistently observed lower values of r_0 than for eosin, typically about 0.25–0.28, when calculated

Fig. 8. A comparison of the sensitivity of triplet detection by measurement of ground-state depletion or phosphorescence

Absorbance measurements were made at 500 nm with the instrument shown in Fig. 1, whereas phosphorescence measurements were made at 645 nm upwards with the instrument shown in Fig. 2. The left-hand column of traces [(a)–(d)] refers to eosin, the right-hand column [(e)–(h)] to erythrosin. The top pair of traces [(a) and (e)] are absorbance recordings, all others are phosphorescence. The buffer used was 50 mM-potassium phosphate, pH 8.0, and the molar ratio of dye to bovine serum albumin was 1:1. The concentrations of dye were: (a), (b), (e) and (f), 0.5 μM ; (c) and (g) 0.05 μM ; (d) and (h), 6.25 nM. The same photomultiplier gain was used for measuring the phosphorescence at any one concentration of dye, but was increased as the concentration of dye fell. Thus (b) may be compared with (f), (c) with (g), and (d) with (h). The interrupted traces were recorded after the sample had been equilibrated with air. In all readings the laser energy was approx. 110 mJ per shot, and was delivered at the moment indicated by an unlabelled arrow in trace (a). The vertical bar by each phosphorescence trace indicates the recorder sensitivity.

from measurements of ground-state depletion (Fig. 9). The phosphorescence of solid solutions of eosin and erythrosin was also polarized, the r_0 values being around 0.30 and 0.25 respectively (Fig. 9). It is noteworthy that the laser energy was attenuated 200-fold for the phosphorescence measurements. For 3 μM -erythrosin bound to bovine serum albumin covalently linked to Sepharose 6B, we obtained an r_0 value of 0.15.

Fig. 9. Anisotropy of laser-induced triplet state of solid solutions of eosin and erythrosin in poly(methyl methacrylate)

In a trace (a), the triplet state of 10 μM -erythrosin was measured by absorbance changes (at 500 nm) of ground-state depletion. The laser energy was 80 mJ. In trace (b) the triplet state of 5 μM -erythrosin and in trace (c) that of 5 μM -eosin was measured by phosphorescence at 645 nm upwards, by using a laser energy of 0.4 mJ. Attenuation of the laser energy was done with neutral-density filters. In each case the laser was polarized in the vertical laboratory axis, and measurements at 90° geometry of absorbance or phosphorescence were made parallel (||) and perpendicular (⊥) to that axis. The differences between (||) and (⊥) measurements were abolished by rotating the plane of polarization of the laser by 90° into the laboratory horizontal axis.

To measure the wavelength-dependence of the phosphorescence of a solid solution of erythrosin, the instrument shown in Fig. 1 was used. We found that the anisotropy parameter, r , was, within experimental limits, constant across the emission spectrum from 650 to 730nm, averaging about 0.2. This is a lower value than that obtained by measuring the whole emission from 645nm upwards, but less certain, owing the poor signal-to-noise ratio obtained when the detection geometry of the Fig. 1 rather than the Fig. 2 instrument is used.

Polarization of the emission of erythrosin under conditions of continuous excitation

The phosphorescence emission of erythrosin can be resolved from the red end of prompt fluorescence not only by its time-dependence (prompt fluorescence is effectively completed within the duration of the laser flash), but also by its wavelength-dependence under conditions of continuous as opposed to pulsed excitation. Fig. 10 shows the emission spectra of aerobic and anaerobic aqueous solutions of erythrosin ob-

tained by use of a conventional fluorimeter and excitation with the intense 546nm line of a high-pressure mercury-vapour arc lamp. We also show in Fig. 10 the polarization ratio of the emitted light, expressed as the ratio, $(\parallel)/(\perp)$, of the emission intensities polarized parallel ($\parallel$) and perpendicular ($\perp$) to the plane of polarization of the exciting light. The emission spectrum of aerobic erythrosin showed no unexpected features; there was a peak at 550nm and a long tail running out to 690nm (Fig. 10). The polarization ratio was quite high, 1.7 compared with the theoretical maximum for an immobile sample of 3.0; this polarization is consistent with the exceedingly short fluorescence lifetime of erythrosin, which is 0.11 ns (Porter *et al.*, 1974). The anaerobic spectrum showed three additional features, each of which can be attributed to the presence of the triplet state in the absence of oxygen quenching. Firstly, there was a 5–10% enhancement of the fluorescence peak at 550nm, presumably due to the additional contribution of delayed fluorescence. Secondly, there was a well-marked new peak at 670–680nm attributable to phosphorescence. Thirdly, the polariza-

Fig. 10. Polarization emission spectra of erythrosin by using continuous excitation. Erythrosin ($5\mu\text{M}$) in 0.1M-potassium phosphate buffer pH8.0 at 20–22°C was excited with 546nm light from a mercury-vapour arc lamp (see the Experimental section). The emission intensity is plotted in arbitrary units (in fact, mV of amplifier output), and the scale is expanded 20-fold at 620nm upwards. The sample was either aerobic ($\blacksquare$) or anaerobic ($\bullet$). The polarization ratio is defined as the ratio of the emission intensities measured parallel and perpendicular to the plane of polarization of the exciting light, after correction for instrumental bias (see the Experimental section). The photomultiplier and amplifier gain for the measurements of this Figure were identical with that used for Fig. 11, allowing direct comparison between emission intensities.

tion ratio started to decline above 630nm and decreased to 1.05 or less by 680nm, indicating that the lifetime of the phosphorescence is very much longer than the rotational relaxation time of unbound erythrosin in free solution, which it should be.

When the previous experiment (Fig. 10) was repeated in the presence of bovine serum albumin at a 1:1 molar ratio with respect to erythrosin, the effects of oxygen exclusion were in general much as described above for unbound erythrosin. However, additional effects attributable to binding of erythrosin to albumin were as follows (Fig. 11). Firstly, the peaks of fluorescence and phosphorescence were shifted slightly towards the red region. Secondly, the peaks of fluorescence and phosphorescence were enhanced about 5-fold, which is twice as great as that expected from the increased light absorption of erythrosin bound to albumin (see Fig. 4). Thirdly, the red 'tail' of the aerobic sample fell away less rapidly. For instance, the ratio of the emission intensities at 630nm to that at 680nm in the aerobic samples was 11.6 for unbound and 6.0 for bound erythrosin. This may be due to a significant contribution from triplet phosphorescence even in the aerobic state, when the erythrosin is partially protected from oxygen quenching by being protein-bound, as is the case with protein-bound eosin (Cherry & Schneider, 1976). In keeping with this conclusion is the observation, fourthly, that although albumin binding increased the polarization ratio of the emission of aerobic

erythrosin to about 2.5 at the fluorescence peak, the ratio fell with increasing wavelength to less than 1.10 at 700nm. Anaerobic conditions caused the polarization ratio to fall not only at the red end of the spectrum, as expected for phosphorescence, but to a lesser extent over the fluorescence peak (Fig. 11). Conceivably the contribution of delayed fluorescence to the total fluorescence is greater than that calculated solely on the basis of the small increase of fluorescence on going from aerobic to anaerobic conditions.

With eosin we were unable to detect any significant red enhancement attributable to phosphorescence on comparing aerobic and anaerobic emission spectra. The long-wavelength tail of eosin fluorescence dominates the red end of the emission spectrum, which is in keeping with the fact that the quantum yield for prompt fluorescence (ϕ_f) is 0.19 for eosin, whereas for erythrosin it is 0.02. (Porter *et al.*, 1974.)

Discussion

The delayed emission of eosin and erythrosin and its quantum yield

There can be little if any doubt that the laser-induced delayed emission exhibited by both eosin and erythrosin in aqueous solution at 20–22°C consists of delayed fluorescence and phosphorescence, as was previously shown for solutions of eosin in glycerol or ethanol at similar temperatures by Parker & Hatchard (1961),

Fig. 11. Polarization emission spectra of albumin-bound erythrosin, by using continuous excitation. The experimental conditions and instrumental settings were identical with those of Fig. 10, except that the erythrosin solution also contained 5 μ M-bovine serum albumin. The aerobic sample is shown as $\blacksquare$, the anaerobic as $\bullet$.

who were following up an even earlier report of delayed eosin emission by Boudin (1930). The absence of the emission in the presence of oxygen, the elimination of certain possibilities of instrumental error by the use of optical choppers, and the correlation between the lifetimes of phosphorescence and of the triplet state as measured by ground-state depletion, all reinforce this conclusion. We have made rough estimates of the quantum yield for phosphorescence (ϕ_p) for erythrosin in two ways. Firstly, we can assume that ϕ_t is 0.02, compare the areas under the fluorescence and phosphorescence peaks of an emission spectrum such as that of Fig. 10, assume that the sensitivity of the photomultiplier tube is three times greater in the spectral range of fluorescence than that of phosphorescence, and thereby calculate that ϕ_p for albumin-bound erythrosin is about 0.003. Alternatively, the peak transient light intensity emitted as phosphorescence and measured in the apparatus shown in Fig. 2 could be matched with a steady-state illumination from the tungsten-filament lamp selected with a red filter. Next the steady-state illumination could be measured with a calibrated photometer situated at the photomultiplier position. Given this information, ϕ_p can then be calculated from the energy of the exciting laser flash, the absorbance of the sample at the exciting wavelength, and the geometry of the sample and detector. We did all this, and calculated that ϕ_p for protein-bound erythrosin is about 0.002, in agreement with the result of the first method. These values are not far removed from the estimate of 0.0039 for ϕ_p of eosin in ethanol at 25°C given by Parker & Hatchard (1961). For protein-bound eosin, ϕ_p is apparently lower than that for erythrosin by a factor of 3–5-fold (Fig. 8).

Polarization of phosphorescence

The transition moments for phosphorescence of aromatic molecules are usually perpendicular to the plane of the molecule and therefore perpendicular to the transition moment for singlet–singlet light absorption. This means that the anisotropy parameter for phosphorescence of such a molecule when immobilized would be negative, with a limiting value of -0.25 (Zander, 1968). The value of r_0 for immobilized-fluorescein phosphorescence is zero (Pilipovich, 1961). The effect of halogen substitution of aromatic molecules, as typified by naphthalene, is to bring the transition moments for phosphorescence into the plane of the molecule and to increase r_0 to positive values towards the upper limit of 0.4 (El-Sayed & Pavlopoulos, 1963; Pavlopoulos & El-Sayed, 1964). This is clearly the case with immobilized eosin and erythrosin, where r_0 was found to be approx 0.3 and 0.25 respectively for solid solutions in poly(methyl methacrylate) (Fig. 9).

Possible use of eosin or erythrosin as probes for measurement of slow rotational diffusion by depolarization of phosphorescence

Both eosin and erythrosin fulfil the requirements for sensitive detection and high r_0 values. Eosin isothiocyanate has already been successfully used by Cherry *et al.* (1976a) to study rotational diffusion of the so-called 'band-3 protein' of erythrocyte 'ghosts', by using absorbance measurements of ground-state depletion. Recently we have synthesized erythrosin isothiocyanate (Moore & Garland, 1979). We have measured the blank phosphorescence signals from erythrocyte 'ghosts' and membrane fragments from *Escherichia coli* on exposure to a 540 nm dye laser flash, and find them no greater than a buffer blank (P. B. Garland & C. H. Moore, unpublished work). It seems, therefore, that the development of phosphorescence depolarization as a method for studying slow rotational diffusion at temperatures of biological interest is both feasible and desirable. There is no reason to suppose that eosin and erythrosin are the only suitable probes; the availability of intense pulsed laser sources emitting at a wide variety of wavelengths, including u.v., should enable phosphorescence of other potential probes to be explored. Some useful guidelines to the structural requirements for intense and positively polarized phosphorescence at 77 K are given by Zander (1968), and could be usefully explored at higher temperatures.

It remains to be seen whether the wavelength-resolved phosphorescence of erythrosin can be used as a basis for steady-state measurements of rotational diffusion analogous to fluorescence-depolarization methods (Perrin, 1926; Weber, 1953). Provided that changes in the triplet lifetime of a sample can be avoided, continuous measurement of phosphorescence depolarization might provide a sensitive means of detecting changes of rotational mobilities of membrane proteins as they undergo biologically interesting events.

Delayed fluorescence

Measurements of delayed fluorescence were technically difficult, for two reasons. Firstly, the photomultiplier could be overloaded and damaged by the intense light arising from scattering of the laser flash (540 nm) or from prompt fluorescence (peak at 555–560 nm). Secondly, any tail of emission from the laser flashlamp can excite laser dye emission which, even if it is below the threshold for laser action, can still fall on the sample cuvette and prolong prompt fluorescence beyond completion of the laser flash. Thus our only measurements of delayed emission were made with the use of mechanical choppers, introducing a dead time of 0.25 ms (Fig. 3). Despite these instrumental difficulties, which in principle can

be overcome, measurement of the depolarization of delayed fluorescence could be preferable to phosphorescence depolarization for determining rotational mobilities. This is because the polarization of delayed fluorescence of most aromatic dyes usually exhibits a large positive anisotropy parameter, which generally is not the case for phosphorescence (Pilipovich, 1961).

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