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# The Behavior of the Fluorescence Lifetime and Polarization of Oxonol Potential-Sensitive Extrinsic Probes in Solution and in Beef Heart Submitochondrial Particles

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**Summary.** The fluorescence polarization and lifetime of the extrinsic potential-sensitive probes oxonols V and VI have been investigated both for the dyes free in aqueous and ethanol solutions and in the presence of beef heart submitochondrial particles under resting and energy-transducing conditions. The emission lifetime of the dyes appears to be inversely related to the solvent dielectric constant and increases as the solvent is changed from an aqueous medium to ethanol to the biological membrane. The fluorescence decay curve becomes biphasic in the presence of the membrane preparation and consists of a faster decaying component, the lifetime of which is the same as that of the probe in aqueous solution and of a slower decaying component. The longer lived component suffers an uncoupler-sensitive decrease in lifetime when ATP is added to the medium. The decrease in lifetime of the longer lived species is accompanied by large depolarizations of the dye fluorescence. These observations are consistent with a redistribution-type mechanism for the energy-dependent spectral changes involving the movement of probe from the aqueous phase to the membrane vesicles. The rotational relaxation time of oxonols V and VI is increased by over an order of magnitude when these dyes associate with the membrane. This observation is consistent with a previously developed model for the location of the dyes in the bilayer in which the side chains serve as anchors, preventing the rapid tumbling of the probe in the membrane.

**Key words.** Biomembranes, bioenergetics, mitochondria, energy transduction, extrinsic molecular probes.

A number of fluorescent dyes of the polyene class have proved to be useful indicators of charge separation across biological membranes in a variety of preparations, ranging from phospholipid lyposomes to intact tissue and organ level investigations. These dyes are often members of the cyanine, merocyanine, and oxonol classes. The cyanines and oxonols bear a positive and negative charge, respectively, that is delocalized over the conjugated carbon chain and ring systems characteristic of these classes of compounds. The merocyanines that have been employed in investigations of electical activity in biological preparations bear a localized negative charge. Since the wavelength of maximum absorbance in the visible spectral region increases with the number of carbon atoms in the polyene chain, it has been possible to design probes the absorption spectra of which do not significantly overlap the intense absorption bands of the pigments intrinsic to biological membranes.

Using the giant axon from the squid *Loligo peali*, Cohen et al. (1974) and Ross et al. (1974, 1977) have evaluated a large number of dyes and have discovered a number of promising potential-sensitive probes that have been systematically improved by modifications to substituent groups that are not part of the optical chromophore. These modifications have led to a substantial increase in the signal-to-noise ratio in work in which a potential gradient is applied across the axon by the use of microelectrodes.

Cyanines have been used in a number of quantitative investigations of membrane potentials in other preparations. Laris, Bahr and Chaffee (1975) have measured the potential in mitochondria by using diS-$C_3$-(5); diffusion potentials were used to calibrate the probe. Hoffman and Laris (1974) and Sims et al. (1974) have evaluated the resting potential in the red cell using a similar approach. Renthal and Lanyi (1976) have obtained a value for the membrane poten-

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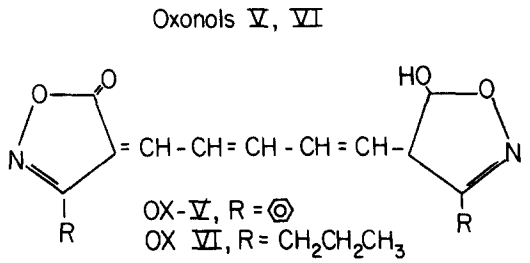


**Fig. 1.** The structure of the neutral form of oxonols V and VI. At the pH values employed in this work, these dyes exist as symmetrical anions. (*See* text)

tial in *Halobacterium halobium* using the diS-$C_3$-(5) probe.

A number of molecular probes also seem to be useful indicators of electrical activity in excitable tissues, notably the brains of small animals and in the perfused beating heart (Chance, Mayevsky & Smith, 1976; Bashford et al., 1979*a*). Neuronal activity in leach ganglia has also been recorded by Grinvald, Salzberg and Cohen (1977) and by Salzberg, Davila and Cohen (1973) using polyene dyes. Salama and Morad (1977, 1979) have also shown that the merocyanine M-540 is a faithful monitor of the time course of the action potential in the frog heart. Reviews of investigations employing extrinsic potential-extrinsic probes in a variety of systems have appeared by Waggoner (1976), Cohen and Salzberg (1978), and Bashford and Smith (1979).

The work to be described in this communication is concerned with a homologous series of oxonols developed in our laboratories from the original oxonol V[1] probe, the structural, spectral properties, and energy-dependent spectral changes of which have been described by Smith et al. (1976). Early work employing this dye has been presented by Chance (1975), Chance et al. (1974), and Chance and Baltscheffsky (1975). Oxonol V and its propyl derivative, oxonol VI, will be of primary concern in this communication. Additional work has been carried out using the methyl, oxonol VII, and the heptyl, oxonol VIII, derivatives of oxonol V and will be briefly described in the appropriate sections of the text. The structure of the neutral form of oxonols V and VI is illustrated in Fig. 1. Since the pK of the hydroxyl proton is approximately four (Smith et al., 1976), these dyes exist as symmetrical anions at the pH ranges employed in this work with the charge delocalized over the conjugated system of the molecules. Agents such as $NH_3$ or nigericin transiently increase the magnitude of the energy-dependent spectral changes of oxonol V (Smith & Chance, 1976) and oxonol VI (Bashford & Thayer, 1977), indicating that these probes are specifically sensitive to the potential gradient portion $\Delta\psi$ only of the electrochemical gradient since when $\Delta$pH is abolished by $NH_3$ or nigericin, $\Delta\psi$ is increased in order to maintain the proton motive force constant. Bashford and Thayer (1977) have used oxonol-VI and 9-aminoacridine in a double-probe experiment to estimate the value of the electrochemical gradient in submitochondrial particles. Smith and Chance (1979), Smith et al. (1979, 1980) and Bashford et al. (1979*b*) have investigated the kinetics of both passive and energy-dependent interaction of oxonol-VI with the submitochondrial particle membrane and the association of oxonol V with phospholipid vesicles.

In this communication, the behavior of the degree of polarization, $P$, and that of the dye fluorescence lifetime has been investigated both for the free dyes and when submitochondrial particles are present under resting and energy transducing conditions in an effort to further elucidate the mechanisms of the energy-dependent fluorescence yield changes that are exhibited by these probes.

## Materials and Methods

Well coupled submitochondrial particles were prepared according to the procedure developed by Hansen and Smith (1964) using heavy beef heart mitochondria. This procedure results in particles that retain an intact $F_1$ and exhibit respiratory control (Thayer, Yu & Hinkle, 1977). Sucrose, $MnCl_2$, $MgCl_2$, and glycerol were obtained from Baker Chemical Co. and were of reagent grade purity. HEPES buffer, ATP, and CCCP were purchased from Sigma Chemical Co., St. Louis, Mo. Oxonols V, VI, and VII were synthesized according to the procedures described by Smith et al. (1976). Oxonol VIII was custom synthesized by Nippon Kankoh Shikiso Kenkyusho, Okayama, Japan. Experimental conditions are given in the appropriate figure legends and tables.

### *Fluorescence Polarization Measurements*

Measurements of the degree of polarization made by hand are based on intensity measurements using a Hitachi-Perkin Elmer MPF-2A fluorimeter. Scanned emission spectra such as those shown in Fig. 6 were obtained using this instrument with the polarizers removed. Under conditions where rotation of the dye is negligible during the excited state lifetime, the degree of polarization is a function of the angle $\theta$ between the transition moment of an electronic transition of interest and that of some selected reference transition [Eq. (1)]. In the work to be described in this communication, the polarization of absorption moments relative to that of the fluorescence emission will be considered. Since the fluorescence emission spectra of the dyes used in this work were found to be constant and independent of excitation conditions, it may be assumed that only a single excited state is involved. The limiting cases are those in which the values of $P$ are $+{}^1/_2$ and $-{}^1/_3$ which

[1] *Abbreviations used:* ANS: 1-anilino-8-naphthalene sulfonate; ATP: adenosine 5′-triphosphate, disodium salt from equine muscle; CCCP: carbonyl cyanine *m*-chlorphenyl hydrazone; HEPES: N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; NADH: $\beta$-nicotinamideadenine dinucleotide; Oxonol V: bis[3-phenyl-5-oxo-isoxazol-4-yl]pentamethineoxonol; Oxonol VI: bis[3-propyl-5-oxo-isoxazol-4-yl]pentamethineoxonol; SMP: ATP-$Mg^{++}$ or ATP-$Mg^{++}$-$Mn^{++}$ beef heart submitochondrial particles.

correspond to $\theta$ being zero and 90 degrees, respectively. The $P$ values for intermediate orientations of the absorption and emission transition moments fall within the range defined by the values given above.

The degree of polarization, $P$, was calculated from the following relationship (Azumi & McGlynn, 1962):

$$P=\frac{I_{\parallel}-I_{\perp}(I'_{\perp}/I'_{\parallel})}{I_{\parallel}+I_{\perp}(I'_{\perp}/I'_{\parallel})}=\frac{3\cos^2\theta-1}{\cos^2\theta+3}. \quad (1)$$

The unprimed intensities are those for which the electric vector of the exciting light beam is perpendicular to the plane defined by the emitted and exciting light beams. $I_{\parallel}$ refers to the emission intensity when the optic axes of the emission and excitation polarizers are both perpendicular to this plane. $I_{\perp}$ is the intensity observed when the optic axis of the emission polarizer lies in this plane. The primed intensities are obtained by rotating the polarizer in the exciting light beam 90 degrees. The ratio $I'_{\parallel}/I'_{\perp}$ is a correction factor for the polarization inherent to the instrument optics; ideally it is unity. The four emission intensities were measured at selected fixed wavelengths and the degree of polarization calculated from Eq. (1).

Energy dependent changes in $P$ were followed by using a time sharing fluorescence polarization detector illustrated schematically in Fig. 2. The details of this device have been described by Smith, Graham and Chance (1978). The detector is based on a rotating polarizer mounted in the emitted light beam. The fluorescence intensity is monitored when the optic axes of the polarizers in the exciting and emitted light beams are parallel and perpendicular by means of a synchronization device based on a reflectance transducer. Since the detector can monitor only two intensities for a selected orientation of the polarizer in the exciting light beam, a correction capability was included in the electrical circuit such that the relative magnitudes of the two signals could be altered.

The polarizer in the exciting light beam was set so that the primed intensities were being observed. Under these conditions, an apparent degree of polarization $P'$ is being obtained from the detector and is described by the relationship:

$$P'=\frac{I'_{\parallel}-I'_{\perp}}{I'_{\parallel}+I'_{\perp}}. \quad (2)$$

The relative magnitudes of $I_{\parallel}$ and $I_{\perp}$ were adjusted electrically until $P'$ was zero by using the instrument polarization correction feature described above. The polarization correction factor in Eq. (1) then becomes unity. The polarizer in the exciting light beam was then rotated by 90 degrees. The corrected $P$ values were calculated by the detector circuit and made available on a digital display and were also fed to a strip chart recorder. Typical results are illustrated in Figs. 3 and 4.

## *Fluorescence Lifetime Measurements*

Fluorescence decay curves were measured using an apparatus developed by Hallidy and Topp (1977). The sample fluorescence excited by either second or third harmonic pulses derived from a mode-locked Nd:YAG laser was mixed with the fundamental frequency of the laser output using type II frequency conversion in a KDP crystal. The sum frequency signal was passed through a Spex monochromator to a photomultiplier, the output from which was fed to a Hewlett-Packard strip chart recorder. Approximately 10% of the exciting light was reflected to a separate photomultiplier; the resulting signal displayed on a strip chart recorder was used to monitor the laser stability. In some applications, the sum frequency signal was divided by this reference signal using

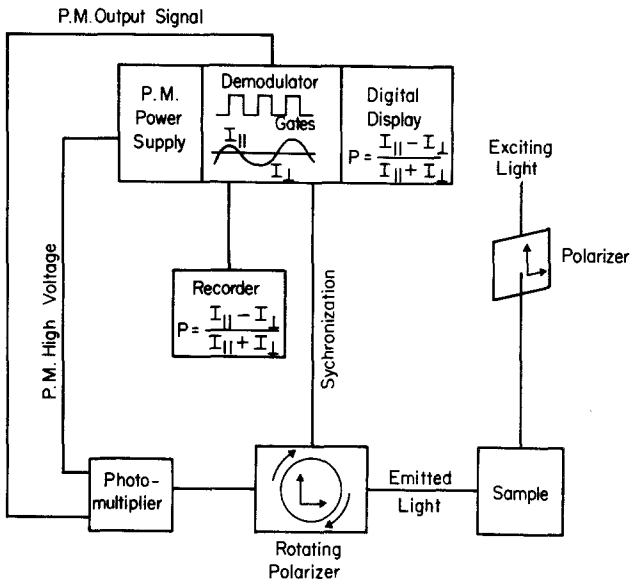


**Fig. 2.** A schematic illustration of the time-sharing fluorescence polarization detector used to follow energy-dependent changes in the degree of polarization. The details of the device have been described by Smith et al. (1978) (reproduced from the *Journal of Scientific Instrumentation* by permission). A time constant of 10 sec was used in measurements of $P$ that employed this device

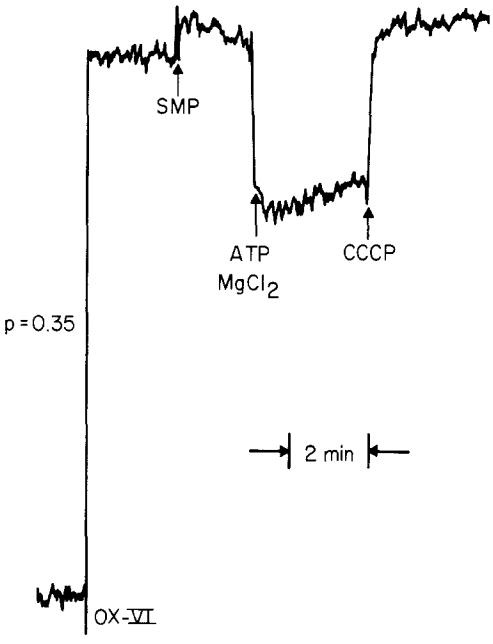


**Fig. 3.** The behavior of oxonol VI fluorescence polarization in submitochondrial particles. The value given for $P$ refers to the free dye and is relative to the baseline established prior to the addition of the probe. Temperature: 23 °C; medium: 5 mM Na-HEPES, pH = 7.5; 0.25 M sucrose; 1 mM $MgCl_2$. Concentrations: 0.2 mg/ml ATP-$Mg^{++}$ submitochondrial particle protein, 0.99 mM ATP-$MgCl_2$, 2 μM oxonol VI, 5 μM CCCP. Excitation wavelength: 580 nm; emission wavelength: 640 nm. The same wavelengths were used in obtaining the data illustrated in Figs. 4, 5, and 7. The degree of polarization, $P$, is plotted on the ordinate and time, in minutes, along the abscissa

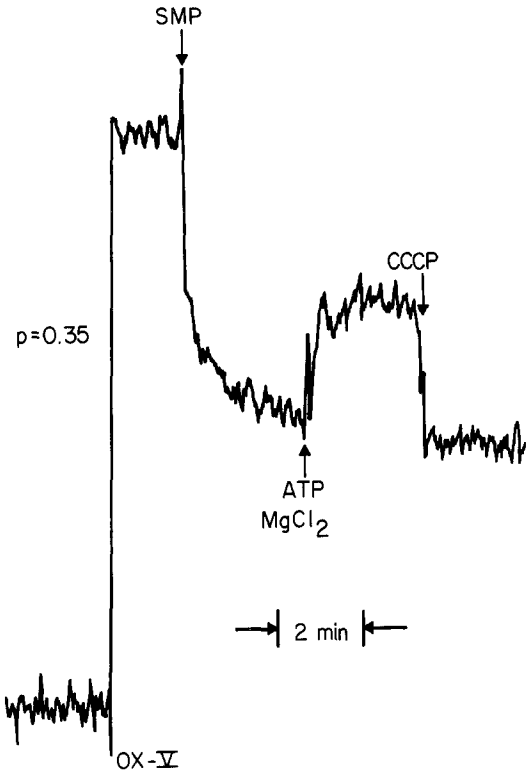


**Fig. 4.** The behavior of oxonol V fluorescence polarization in a submitochondrial particle suspension. Temperature: 23 °C. Medium: 5 mM Na-HEPES, pH = 7.5; 0.25 M sucrose; 1 mM $MgCl_2$. Concentrations: 0.1 mg/ml ATP-$Mg^{++}$ submitochondrial particle protein, 0.99 mM ATP-$MgCl_2$, 6 μM oxonol V, 5 μM CCCP. The value given for *P* is for the free dye relative to the base line established prior to addition of the probe to the medium. Time is the variable plotted on the abscissa and *P* on the ordinate

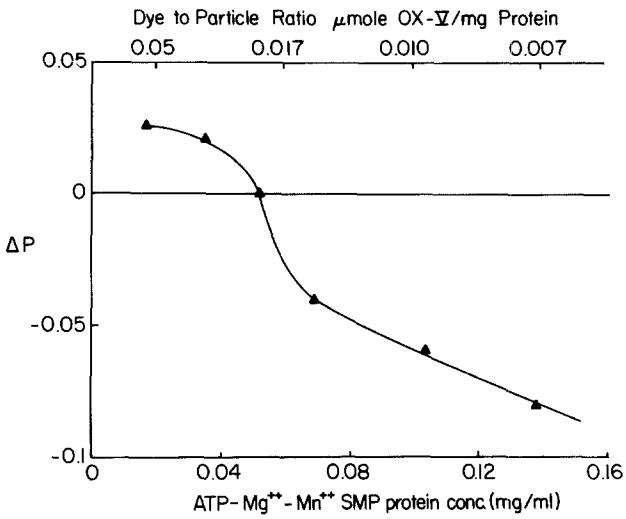


**Fig. 5.** The oxonol V degree of polarization change induced by ATP consumption in beef heart submitochondrial particles as a function of the dye to membrane protein concentration ratio. Temperature: 23 °C. Medium: 5 mM Na-HEPES, pH = 7.5; 0.25 M sucrose, 0.83 mM $MgCl_2$, 1 μM oxonol V

a ratiometer in order to minimize noise in the decay curves due to laser intensity fluctuations. Time resolution was achieved by a form of coincidence spectroscopy dependent on a slowly moving track-mounted prism that reflects the 1.06 μm laser output. The time at which the decaying fluorescence signal is mixed with the fundamental laser radiation is determined by the optical transit time to and from the reflecting prism. The effective time resolution was less than 50 psec. The decay curves illustrated in Figs. 8 and 9 were obtained by signal averaging with a time constant of either 10 or 30 sec.

An effort was made to perform the fluorescence lifetime measurements at the same dye and submitochondrial particle protein concentrations as used in the corresponding polarization experiments. When the latter concentrations were not feasible for lifetime measurements, the same dye to membrane protein concentration ratios were usually maintained for both experiments.

## Results and Data Analyses

### *Fluorescence Polarization Measurements*

Results from polarization measurements are illustrated in Figs. 3 and 4 for oxonols VI and V, respectively, in submitochondrial particle suspensions. Under the conditions of Fig. 3, the addition of submitochondrial particles to the free oxonol VI solution caused a slight increase in the degree of polarization; addition of ATP and $MgCl_2$ caused a massive depolarization which could be readily reversed by the uncoupler CCCP. The addition of submitochondrial particles to oxonol V solutions over the entire range of dye to membrane protein concentration ratios shown in Fig. 5 caused a depolarization of the dye fluorescence as illustrated in Fig. 4. The latter figure also illustrates the increase in the degree of polarization resulting from ATP addition to the dye-particle suspension at dye to membrane protein concentration ratios larger than 0.017 μmol oxonol V/mg protein. For values of this ratio below that cited above, the ATP-induced change in *P* became negative (Fig. 5) and at the upper limit of the range of the submitochondrial particle titration, the magnitude of $\Delta P$ approached that observed with oxonol VI in submitochondrial particle suspensions. When the oxonol V dye to particle ratio was the same as that for the experiment with oxonol VI shown in Fig. 3, 0.01 μmol dye/mg protein, the change in the fluorescence polarization was negative in both cases.

The energy-dependent changes in oxonol fluorescence polarization observed in submitochondrial particle suspensions are accompanied by a loss of fluorescence yield as illustrated in Fig. 6 for oxonol V. In this case, an 80% loss of intensity is observed on ATP addition. Substantially smaller energy-dependent decreases in fluorescence yield have been observed for oxonols VI, VII, and VIII in submitochondrial particle suspensions. (*See* Smith & Powers, 1980.)

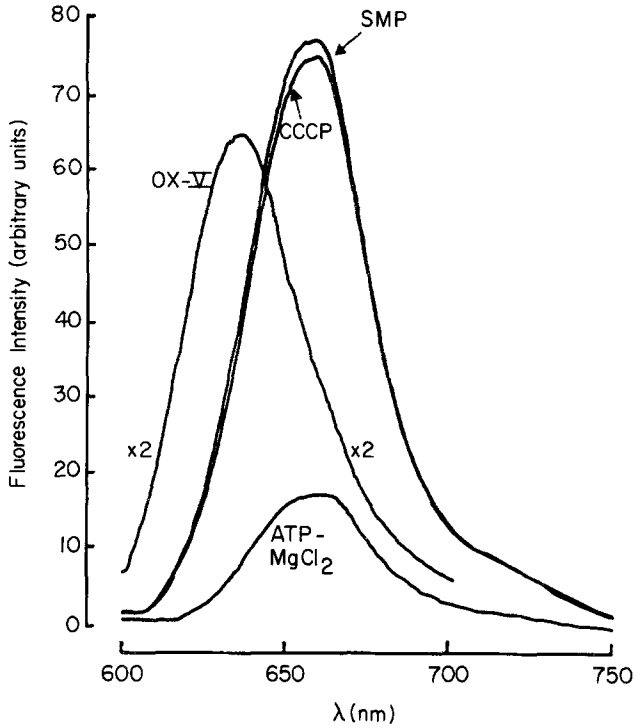


**Fig. 6.** The oxonol V emission spectrum for the free dye, after addition of submitochondrial particles and after ATP and CCCP addition. Note that the vertical scale has been multiplied by two in the case of the free dye spectrum. Approximately 80% of the fluorescence intensity is lost upon ATP addition to the dye-submitochondrial particle suspension but can be virtually completely recovered by uncoupler addition. Temperature: 23 °C. Medium: 5 mM Na-HEPES, pH = 7.5, 0.25 M sucrose; 1 mM $MgCl_2$. Concentrations: 0.1 mg/ml ATP-$Mg^{++}$ submitochondrial particle protein, 0.99 mM ATP-$MgCl_2$, 2 μM oxonol V, 5 μM CCCP. Excitation wavelength: 580 nm

**Table 1.** Fluorescence polarization values for dyes in solution

| Dye | *R* | *P* | |
|---|---|---|---|
| | | Glycerol | 5 mM Na-HEPES (pH = 7.5) 0.25 M sucrose |
| Oxonol VII | $CH_3$ | 0.43 | 0.35 |
| Oxonol VI | $CH_3CH_2$ | 0.43 | 0.35 |
| Oxonol V | Phenyl | 0.42 | 0.35 |
| Oxonol VIII | $C_7H_{15}$ | 0.43 | 0.35 |

Dye concentrations: ~1 μM; temperature: 23 °C.

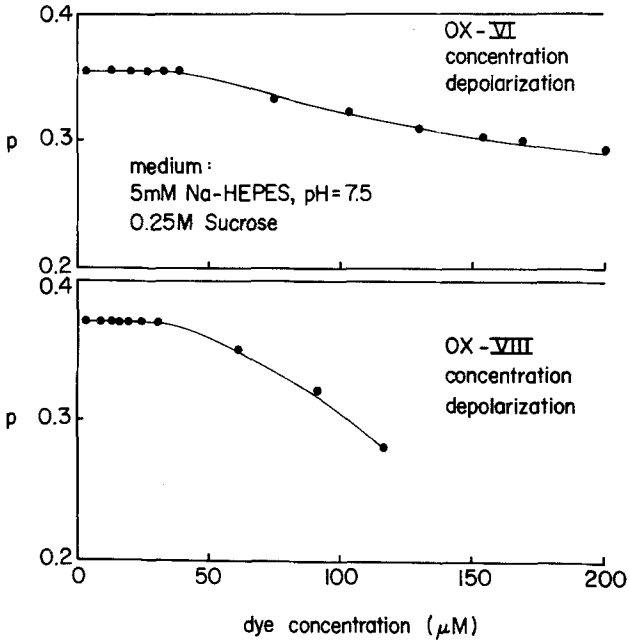


**Fig. 7.** The dependence of the degree of polarization for oxonols VI and VIII on the dye concentration in an aqueous medium. Concentration depolarization is evident at oxonol concentrations above approximately 20 μM. Medium: 5 mM Na-HEPES, pH = 7.5; 0.25 M sucrose. Temperature: 23 °C

It was possible to demonstrate an increase in the degree of polarization when the viscosity of the solvent of free dye solutions was increased. Table 1 is a summary of results for the oxonols used in this investigation. The degree of polarization approaches the theoretical limit of 0.5 when the dyes are dissolved in glycerol. The *P* value in the aqueous medium, however, is significantly lower for each dye. It is thus possible to mimic the increase in *P* when oxonols VI and VII bind to the submitochondrial particle membrane by an increase in medium viscosity. In aqueous solutions of the free oxonol dyes, concentration depolarization could be observed as the dye concentration was increased. Typical data are presented in Fig. 7 for oxonols VI and VIII.

*Fluorescence Lifetime Measurements*

Emission decays were obtained for essentially three cases: (i) the free dye in ethanol, (ii) the free decay in an aqueous medium, and (iii) the dye in a submitochondrial particle suspension. The third case can be further subdivided. Measurements were made on the dye-membrane suspension at equilibrium. A fresh dye-submitochondrial particle suspension was then prepared with the medium supplemented by ATP and the dye fluorescence decay curve measured. Finally the uncoupler CCCP was added to the mixture and the emission decay curve recorded. ATP was chosen as an energy source so that potential problems due to the onset of anaerobiosis could be avoided in the sometimes lengthy periods required to obtain a decay curve. Measurements on the oxonols were usually completed in less than one hour.

The decay curves were fitted to a function of the form

$$A(t) = \sum_{n=1}^{2} A_n \exp(-k_n t) \qquad (3)$$

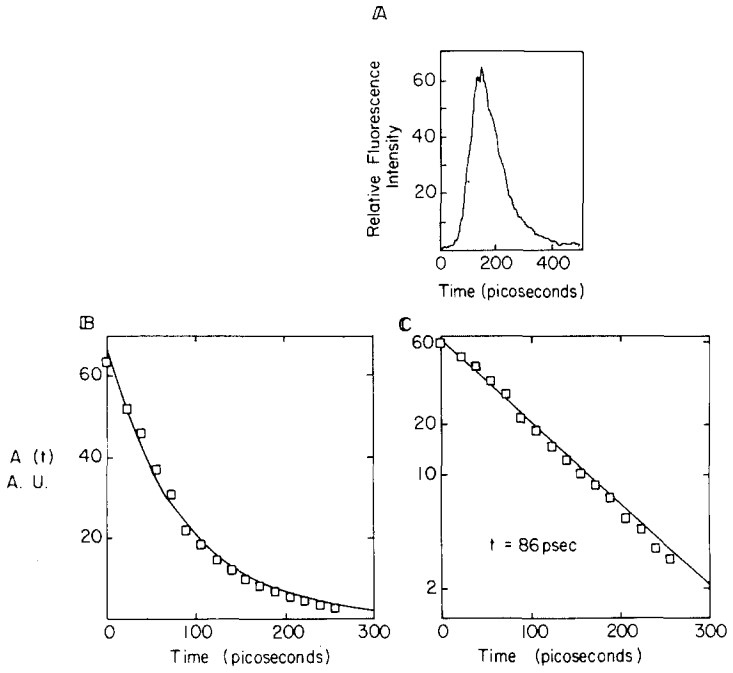


**Fig. 8.** Typical lifetime data for oxonol V in an aqueous medium. (*A*): The experimental decay trace. (*B*): A computer fit to the data; the squares are experimental data points which lie on a theoretical curve generated from the single exponential form of Eq. (3). (*C*): A computer generated log plot; the rate constant $k$ (Table 3) is obtained from the negative of the slope of the straight line. Again the squares are experimental data points and the solid line the theoretical fit to the data obtained from Eq. (3). The lifetime value obtained from this data is 86 psec. Temperature: 23 °C; excitation wavelength: 530 nm. Medium: 5 mM Na-HEPES, pH = 7.5; 0.25 M sucrose; 0.83 mM $MgCl_2$; 2 μM oxonol V. The amplifier time constant was 10 sec. In analyzing the fluorescence decay curves, the zero for time was taken at the onset of the decay process; this practice accounts for the difference in the time spans for the computer fits and the experimental traces

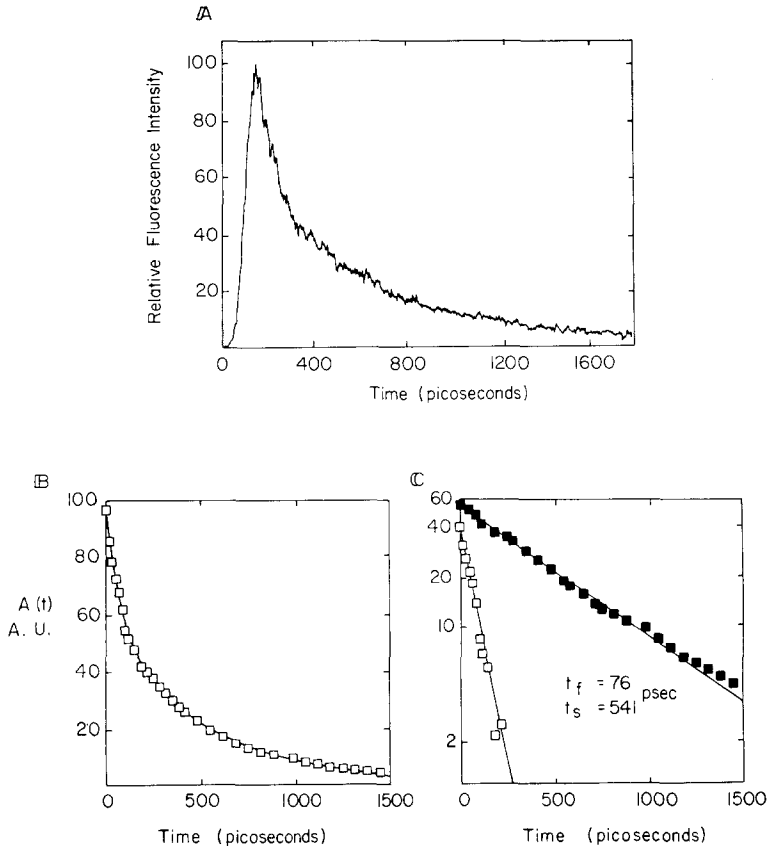


**Fig. 9.** Lifetime data for oxonol V in a submitochondrial particle suspension to which ATP has been added. (*A*): The experimental trace from a strip chart recorder. (*B*): The theoretical fit of the data to the double exponential from of Eq. (3). The squares are data points and the solid line the theoretical decay curve derived from the fitting procedure. (*C*): The decomposed log plots for the two exponential terms of Eq. (3). The lifetimes corresponding to the faster and slower decaying components obtained from this data are 76 and 541 psec, respectively. Temperature: 23 °C; exciting light wavelength: 530 nm. Medium: 5 mM Na-HEPES; 0.25 M sucrose, 0.83 mM $MgCl_2$, 33 mM ATP. The medium contained 2 μM oxonol V and 0.2 mg/ml ATP-$Mg^{++}$ submitochondrial particle protein. Amplifier time constants: 10 sec

by a nonlinear regression. The decay of the free dyes could be fitted satisfactorily to a single exponential function in each case. Typical experimental results are shown in Figs. 8 and 9. Table 2 contains a summary of the decay rate constants and lifetimes under a variety of conditions. The values shown in parentheses in this table are fitting errors. The lifetimes reported for the dyes free in either ethanol or aqueous media as well as those for the shorter lived components in the dye-membrane suspensions were repro-

**Table 2.** Summary of the fluorescence decay rate constants and lifetimes of oxonols V and VI

| Dye | Concentration (μM) | Medium | $k$ (psec$^{-1}$) | $\tau$ (psec) | Remarks |
|---|---|---|---|---|---|
| Oxonol V | 1 | Ethanol | 0.00677 (±0.00027) | 148 | Excitation wavelength: 353 nm |
| Oxonol VI | 1 | Ethanol | 0.00101 (±0.00035) | 99 | Excitation wavelength: 353 nm |
| Oxonol V | 2 | Aqueous | 0.01161 (±0.00082) | 86 | |
| Oxonol V+SMP | 2 | Aqueous | 0.01078 (±0.00278)<br>0.00159 (±0.00017) | 93<br>629 | 0.2 mg/ml submitochondrial particle protein |
| Oxonol V+SMP+ATP | 2 | Aqueous | 0.01321 (±0.00154)<br>0.00185 (±0.00010) | 76<br>541 | +33 mM ATP |
| Oxonol V+SMP +ATP+CCCP | 2 | Aqueous | 0.00531 (±0.00016)<br>0.00143 (±0.00027) | 188<br>699 | +6.7 μM CCCP |
| Oxonol VI | 2 | Aqueous | 0.01243 (±0.00097) | 80 | |
| Oxonol VI+SMP | 2 | Aqueous | 0.00872 (±0.00066) | 115 | 0.2 mg/ml submitochondrial particle protein |
| Oxonol VI+SMP+ATP | 2 | Aqueous | 0.01034 (±0.00074) | 97 | +17 mM ATP |
| Oxonol VI | 10 | Ethanol | 0.00888 (±0.00073) | 113 | Excitation wavelength: 353 nm |
| Oxonol VI | 10 | Ethanol | 0.00989 (±0.00041) | 101 | Excitation wavelength: 530 nm |

Temperature for all measurements: 23 °C. Unless otherwise indicated, the excitation wavelength was 530 nm for all measurements. The figures in the parentheses near the decay rate constants define an uncertainty range in which the probability of $k$ being in this range is 95%. Aqueous medium: 5 mM Na-HEPES, pH=7.5; 0.25 M sucrose; 0.83 mM $MgCl_2$.

ducible to within approximately ±5%. The lifetimes associated with the longer lived components exhibited considerably more variation, up to nearly ±10%. The latter variation, however, may reflect small differences in the amount of probe associated with the membrane. The longer lived component of oxonol V, for example, was found to depend somewhat on the dye to membrane concentration ratio (Fig. 12). The lifetime of the dyes in aqueous medium was found to be substantially shorter than that characterizing the probes in ethanol. The lifetime for oxonols V and VI in the aqueous medium was found to be approximately 85 psec whereas in ethanol it increased to over 100 psec. This difference is statistically significant in that the 95% probability ranges for the decay rate constants do not overlap for the oxonols in ethanol and aqueous solutions. The oxonols in ethanol were usually excited at 353 nm; the lifetime of these dyes, however, did not change, within the fitting error, when the excitation wavelength was increased to 530 nm. Typical results are given for oxonol VI in Table 2. All other experiments were carried out using 530 nm for the exciting light wavelength. It should be noted that the lifetimes reported in Table 2 have not been corrected for the finite decay time of the exciting laser pulse, so the values for the oxonols in aqueous solution especially should be regarded as upper limits to the dye decay times.

The fluorescence lifetime of oxonol V in ethanol was found to be a function of dye concentration. The inverse of the probe lifetime is linearly related

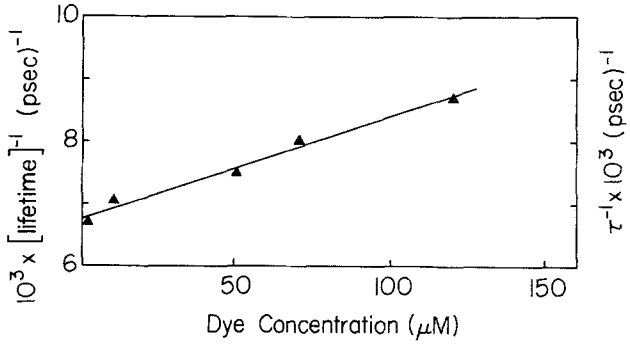


**Fig. 10.** The concentration dependence of the fluorescence lifetime of oxonol V in ethanol. The inverse dependence of the lifetime on dye concentration can be rationalized by the Stern-Volmer equation. (*See* text.) Temperature: 23 °C. Excitation wavelength: 530 nm

to the concentration as illustrated in Fig. 10. This behavior is readily explained by the Stern-Volmer relationship (Parker, 1968):

$$(\Phi_f^0/\Phi_f) - 1 = k_0 \tau^0 [Q] \tag{4}$$

where

$$\Phi_f = \tau/\tau_r \tag{5}$$

is the fluorescence efficiency and $\tau_r$ is the radiative lifetime. The superscript refers to the case in which the quencher concentration $[Q]$ is zero. By substituting Eq. (5) into Eq. (4), the following result is obtained

$$\frac{\tau^0}{\tau_r^0}\frac{\tau_r}{\tau}=1+k_0\tau^0[Q]. \quad (6)$$

If the radiative lifetime is unaffected by the quencher then $\tau_r=\tau_r^0$ and Eq. (6) reduces to

$$\frac{1}{\tau}=\frac{1}{\tau^0}+k_0[Q]. \quad (7)$$

The concentration dependence of the oxonol V fluorescence lifetime is decribed by Eq. (7) as shown in Fig. 10. The dye fluorescence thus appears to be self quenched as the concentration of the probe is increased.

In the presence of submitochondrial particles, the fluorescence decay curves of oxonol V could not be described by a single exponential function. However, the curves could be satisfactorily fitted using the two-exponential form of Eq. (3). Typical experimental data are shown in Fig. 9. The shorter lifetime of oxonol V in submitochondrial particle suspensions was, within the uncertainty of the fitting procedure, the same as that of the free dye in aqueous solution. The latter lifetime did not change when ATP was included in the medium. The longer lived component, however, suffers a statistically significant decrease in lifetime when ATP is in the medium containing oxonol V. Addition of uncoupler then increases the lifetime of the longer lived species. In the case of oxonol V, the longer lifetime measured after CCCP addition was the same as that of the dye-submitochondrial particle suspension before ATP addition. Thus a reversible energy-dependent change in the longer dye lifetime of oxonol V has been demonstrated.

The dye to membrane protein concentration ratio used in the oxonol V lifetime experiments with submitochondrial particles falls in the range in which $\Delta P$ is negative in Fig. 5. The shorter lifetime was also found to be increased slightly by the addition of CCCP to the oxonol V-submitochondrial particle suspensions (Table 2).

Since the affinity of oxonol VI for phospholipid membranes, is approximately an order of magnitude weaker than that of oxonol V (Bashford et al., 1979; Smith & Powers, 1980), the double exponential fitting procedure was not successful for the former dye. It was possible to fit the fluorescence decay curve of oxonol VI in the submitochondrial particle suspension to a single exponential function. The composite lifetime obtained from such a fit was found to decrease when the medium contained ATP as shown in Table 2.

By measuring the degree of polarization and fluorescence lifetime of oxonols V and VI as a function of submitochondrial particle protein concentration, it has been possible to estimate the limiting $P$ values

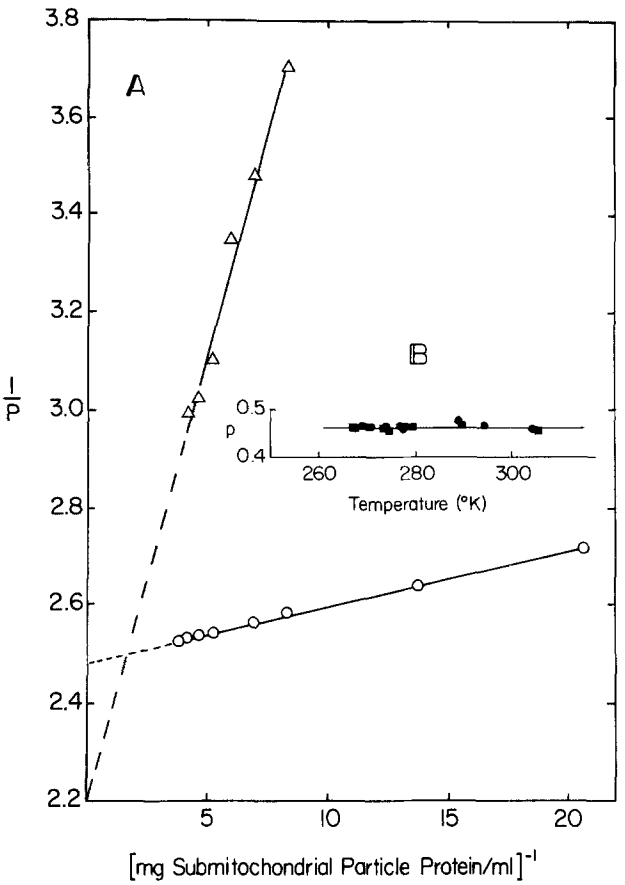


**Fig. 11.** (*A*): Double reciprocal plots of $P^{-1}$ *vs.* submitochondrial particle protein concentration$^{-1}$ obtained in a titration of fixed quantities of oxonol V (—△—) and oxonol VI (—○—) with the membrane preparation. The limiting values of $P$ in which all dye is bound to the membrane are obtained from the intercepts of these plots. Temperature: 23 °C. Medium: 5 mM Na-HEPES, pH = 7.5; 0.25 M sucrose; 1 mM $MgCl_2$. Dye concentrations: oxonol V, 4 μM, oxonol VI, 2 μM. Exciting light wavelength: 580 nm; emission wavelength: 650 nm. (*B*): The inset is the degree of polarization of oxonol V (—●—) and oxonol VI (—■—) in glycerol at various temperatures. The $P$ value did not vary with temperature over the range indicated in the figure. The average value was used as $P_0$ in Eq. (8). Dye concentrations: 2 μM for both oxonols. The exciting and emission wavelengths were the same as in *A*

and lifetimes under conditions where all the probe is bound to the membrane. The limiting degree of polarization value was obtained from a double reciprocal plot as shown in Fig. 11 for oxonols V and VI. The limiting $P$ value is obtained from the intercept on the ordinate resulting from an extrapolation of the straight line to infinite submitochondrial particle protein concentration.

The lifetime of the longer lived component of the oxonol V fluorescence was found to increase slightly with the submitochondrial particle concentration, apparently due to the reduction of interaction between membrane-bound dye molecules as the membrane concentration was increased. A semireciprocal plot

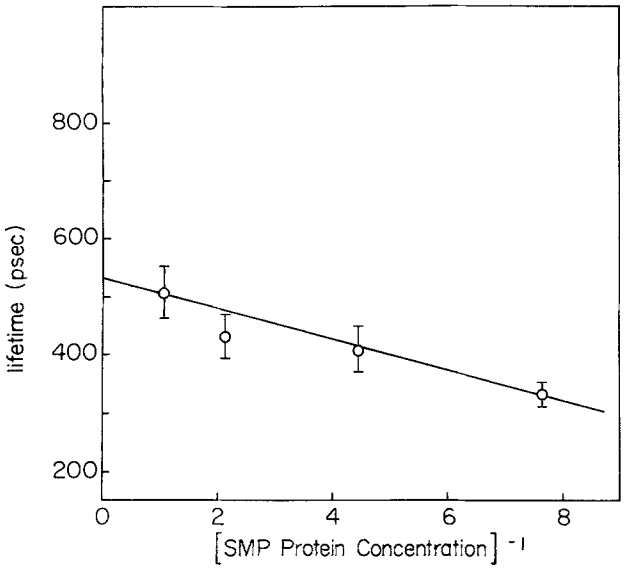


**Fig. 12.** The lifetime of oxonol V as a function of the submitochondrial particle membrane concentration$^{-1}$ in a titration of a fixed quantity of dye with the membrane preparation. The limiting lifetime value under conditions in which there is no dye-dye interaction is obtained from the intercept of the plot on the ordinate. Temperature: 23 °C. Medium: 5 mM Na-HEPES, pH=7.5; 0.25 M sucrose; 0.83 mM $MgCl_2$. Oxonol V concentration: 2 μM. Exciting light wavelength: 530 nm

was employed to obtain the value of the longer lived component at infinite membrane concentration and hence no dye-dye interaction as illustrated in Fig. 12. No dependence of the oxonol VI lifetime on submitochondrial particle concentration could be detected. Indeed, the membrane concentration had to be increased to more than 3 mg/ml protein before a long lived component could be detected in the decay curve. The lifetime obtained under the latter conditions has been employed in the calculation of the rotational relaxation time to be described next.

The degree of polarization $P$, the dye fluorescence lifetime $\tau$, and the rotational relaxation time $\rho$ are related according to the Perrin equation (Parker, 1968):

$$1/P - 1/3 = (1/P_o - 1/3)(1 + 3\tau/\rho) \quad (8)$$

where $P_0$ is the principal polarization, or the degree of polarization observed in the absence of depolarizing factors. Equation (8) is valid only for vertically polarized exciting light.

Since the limiting $P$ values and fluorescence lifetimes $\tau$ are known for oxonols V and VI, the rotational relaxation time $\rho$ can be calculated from Eq. (8). The values for $P$ used in this calculation are those obtained from the intercepts of the plots shown in Fig. 11. Likewise, the limiting lifetime for oxonol V was obtained from the intercept of the plot illustrated

**Table 3.** Summary of the calculation of the rotational relaxation times of oxonols V and VI

| Dye | Medium | $\tau$ (psec) | $P$ | $P_o$ | $\rho$ (sec) |
|---|---|---|---|---|---|
| Oxonol V | Submitochondrial particle membrane | 535 | 0.452 | 0.463 | $2\times10^{-8}$ |
| Oxonol V | Aqueous | 86 | 0.350 | 0.463 | $7\times10^{-10}$ |
| Oxonol VI | Submitochondrial particle membrane | 590 | 0.403 | 0.459 | $1\times10^{-8}$ |
| Oxonol VI | Aqueous | 100 | 0.350 | 0.459 | $8\times10^{-10}$ |

Temperature: 23 °C. Aqueous medium: 5 mM Na-HEPES, pH=7.5; 0.25 M sucrose. The values of $P$ for oxonols V and VI in the aqueous medium were taken from Table 1.

in Fig. 12. The value of the degree of polarization of oxonols V and VI in chilled dilute glycerol solutions was taken as $P_0$. The lifetime of these dyes is sufficiently short that the value of $P$ was independent of the temperature of the glycerol solutions over the range shown in Fig. 11 (inset). The values of the various parameters employed in these calculations and the results for $\rho$ are collected in Table 3. A calculation of $\rho$ for the oxonols free in aqueous solution is also included in this table. The results indicate that the rotational relaxation time increases by over an order of magnitude when the dyes associate with the submitochondrial particle membrane.

## Discussion

The fluorescence lifetime and hence the fluorescence efficiency of the oxonols are sensitive to the dielectric constant of the solvent. A systematic increase in the lifetime has been observed as the solvent is changed from an aqueous medium to ethanol to the biological membrane. Since the dielectric constant decreases with these solvent changes, the emission lifetimes appears to be inversely related to this quantity. This finding is often observed in lifetime work. N-arylaminonaphthalene sulfonates such as ANS exhibit a marked solvent "polarity" or dielectric constant dependence (Weber & Laurence, 1954). The mechanisms giving rise to these solvent effects have been studied in some detail by a number of workers (McClure & Edelman, 1966; Stryer, 1968; Turner & Brand, 1968; Seliskar & Brand, 1971; Kosower & Tanizama, 1972; Smith & Woody, 1976). In a number of models, the fluorescence efficiency of these compounds depends on the ability of the system to depopulate the first excited singlet state via a radiative mechanism *vs.* the intersystem crossing mechanisms which compete with the radiative process and lead to population of the first triplet state from which nonradiative path-

ways to the ground state are operative at room temperature. In these models, it is proposed that the intersystem crossing efficiency is inversely dependent on the singlet-triplet energy difference and that the latter difference is sensitive to the solvent dielectric constant due to the charge transfer character of the lowest energy transition in these compounds. Intersystem crossing rates can also depend on the hydrogen bonding ability of the solvent. It is not known, however, if similar mechanisms are relevant to the oxonols since no detailed molecular orbital calculations have been undertaken for these dyes. Semiquantitative calculations by Loew, Bonnevill and Surow (1978) indicate that the charge transfer character of the lowest electronic transition of the oxonols may be small.

The tendency of the oxonols to quench the fluorescence as the dye concentration is increased is consistent with the strongly overlapping emission and visible absorption envelopes of these dyes (Smith et al., 1976; Smith & Powers, 1980). Since a portion of the energy absorbed by the excited species is likely lost through radiationless processes competing with energy transfer, the fluorescence efficiency and hence the lifetime of the oxonols decreases with increasing dye concentration (Fig. 10).

The degree of polarization is dependent on both the emission lifetime and the rotational relaxation time which is related to the local viscosity that the fluorophore experiences. The changes observed in $P$ will thus depend on which of these parameters is dominant – lifetime or rotational effects – as described by the Perrin equation (8). An additional factor that must be considered in discussing changes in $P$ is concentration depolarization arising from energy transfer. The latter effect can be described by the following relationship (Weber & Laurence, 1954)

$$1/P - 1/3 = (1/P_0 - 1/3)\left(1 + \frac{4\pi N R_c \times 6 \times 10^3 c}{15(2a)^3}\right) \qquad (9)$$

where $N$ is Avogadro's number, $2a$ the molecular diameter, $R_c$ the critical distance at which the probability of energy transfer is equal to that of emission and $c$ the molar concentration. Equation (9) is valid only for vertically polarized light. The degree of polarization in this model is thus inversely related to dye concentration. It was possible to demonstrate energy transfer and a concomitant reduction in P as the oxonol concentration was increased beyond approximately 20 μM as shown in Fig. 7.

The association of the weaker binding oxonols VI and VII with the submitochondrial particle membrane results in a modest increase in the degree of polarization, as illustrated in Fig. 3. The binding of oxonol VI is accompanied by an increase in the composite dye lifetime (Table 2). In terms of the Perrin equation (8), this observation suggests that the increase in the rotational relaxation time $\rho$ of the membrane-bound fraction of dye is the dominant effect in this case since the lifetime change alone would predict a decrease in $P$ contrary to experimental observation. Since the viscosity of the submitochondrial particle membrane is higher than that of the aqueous medium, an increase in $\rho$ is to be expected for the membrane-bound fraction of oxonol VI. The latter expectation has been verified as shown in Table 3 which contains a comparison of $\rho$ for the dye in aqueous medium and in the membrane.

Passive association of oxonol V with the submitochondrial particle membrane was observed to produce a depolarization of the dye fluorescence (Fig. 4) over the range of dye to membrane protein concentration ratios covered in the titration shown in Fig. 5. The binding of oxonol V to the membrane results in an increase in the fluorescence yield (Fig. 6) and in the composite emission lifetime. These findings are consistent since an increase in the lifetime of the membrane-bound dye fraction would lead to an increase in the fluorescence efficiency of this fraction (Parker, 1968). The fluorescence decay curve under these conditions can be described by the sum of two exponential processes, one of which has a lifetime substantially longer than that of the free dye in solution. The lifetime of the shorter lived species is not statistically different from that of the free dye in aqueous solution. These results suggest that the longer lived component contains contributions primarily from the membrane-bound fraction of dye whereas the shorter lived component is primarily due to the remaining free probe. The increase in the oxonol V lifetime and the depolarization that is observed on association of the dye with the membrane are consistent in terms of the Perrin equation (8). The increase in $\rho$ observed on oxonol binding to the submitochondrial particle membrane (Table 3), however, is not accounted for by the depolarization associated with the binding of the oxonol to the membrane unless it is assumed that the lifetime change is the dominant factor. An alternative explanation of the depolarization observed in the latter case is that the local concentration of the membrane-bound dye is sufficiently high that concentration depolarization due to energy transfer is occurring and that the lifetime change is due to the decrease of the solvent dielectric constant described at the beginning of this section. Although the latter model is supported by the stronger binding affinity of oxonol V than oxonol VI for the membrane vesicles, contributions from the former may also occur.

The addition of ATP to the oxonol VI-submitochondrial particle suspension results in a decrease in

the composite fluorescence lifetime of the dye (Table 2) and a massive depolarization as illustrated in Fig. 3. In the case of oxonol V, it was possible to measure ATP-dependent lifetime changes only in the dye to membrane protein concentration ratio regions where depolarization was observed (Fig. 5). A substantial decrease in the lifetime of the longer lived component in the decay curve was observed in the presence of ATP. The lifetime of the shorter lived component did not change in a statistically significant manner compared to either that of the free probe in aqueous medium or to the shorter lived component in the membrane-dye suspension prior to ATP addition (*see* Table 2). An energy-dependent loss of fluorescence yield is observed on ATP addition (Fig. 6); this loss can be explained by a decrease in the fluorescence efficiency which is proportional to the membrane-bound dye lifetime (Parker, 1968). The decrease in the fluorescence lifetime and degree of polarization of oxonols V and VI when ATP is supplied to the dye-submitochondrial particle suspensions cannot be explained in terms of Perrin behavior since the lifetumes changes would predict an increase in *P*. The results are, however, explicable in terms of a dominant energy transfer effect leading to concentration depolarization and the loss of emission through nonradiative processes competing with the energy transfer mechanisms. The energy-dependent changes in fluorescence polarization and lifetime of the oxonols are consistent with a redistribution type mechanism suggested by equilibrium and time-resolved investigations of energy-linked spectral changes exhibited by these probes. More oxonol V and VI are found to be associated with the submitochondrial particle membrane in the presence than in the absence of substrate (Smith et al., 1976; Bashford & Thayer 1977; Smith & Chance, 1979). The transfer of probe from the aqueous medium to the membrane would be expected to increase the local concentration of the membrane-bound fraction of dye. Energy transfer processes and concentration depolarization are favored under these circumstances, as has been demonstrated by the Stern-Volmer behavior of the oxonol V fluorescence lifetime at high dye concentration (Fig. 10) and by the marked tendency of the oxonols toward concentration depolarization as the dye concentration is increased even in aqueous solution (Fig. 7).

Fluorescence lifetime measurements dealing with both intrinsic and extrinsic optical indicators offers the advantage of being independent of the efficiency with which the fluorophores are excited. This property may thus be used in assessing the ability of a selected fluorophore to respond to experimentally induced perturbations such as axonia in the cerebral cortex of small animals, potential gradient formation in respiring preparations, etc. In complex tissue and organ level investigations, interference caused, for example, by an increase in blood volume in the cerebral cortex makes the interpretation of changes in fluorescence yield alone difficult to interpret. The decrease in oxonol V fluorescence yield observed in the exposed cerebral cortex of the gerbil or rat (Bashford et al., 1979*a*) upon the onset of anoxia due to $N_2$ inhalation is one example where the cause of the loss in fluorescence yield is ambiguous because of the possible screening effect of the increase in blood supply to the cortex in the anoxic state. Dye lifetime measurements would be helpful in determining if the loss in fluorescence yield is due to a screening effect or is indicative of emission quenching in this case.

The lifetime measurements have also removed an ambiguity present in steady-state fluorescence yield work with submitochondrial particle suspensions, namely that the energy-dependent reduction in oxonol fluorescence yield (Fig. 6) may be due to a decrease in the dye excitation efficiency that results from the energy-dependent red shift of the probe absorption spectra since the excitation wavelength is on the short wavelength side of the absorption maximum of the dye. The results of the present investigation, however, indicate that the loss of fluorescence yield is at least in part due to a decrease in the emission lifetime of the membrane-bound fraction of dye.

The ATP-induced decrease in the lifetime of the longer lived oxonol V species is in qualitative agreement with preliminary results reported by Chance et al. (1974). The oxonol V lifetimes given in this communication, however, are somewhat shorter than those reported by Chance et al. Possible reasons for this difference are that the submitochondrial particle preparations and substrates used in the two studies are different. An EDTA preparation was employed by Chance et al. with succinate as a substrate. The membrane preparations used in the lifetime work described in this communication retain an intact $F_1$ and also contain the manganous ion which is paramagnetic and may shorten the oxonol V lifetimes. The time resolution of the exciting laser and detector has also been substantially increased since the report by Chance et al.

The fluorescence polarization changes observed when the dye to membrane concentration ratio is greater than approximately 0.017 (Fig. 5) is difficult to interpret since lifetime changes could not be measured under these conditions due to the small amount of membrane present. It was possible to observe small energy-dependent losses in fluorescence yield over this dye to membrane protein concentration range, however. These observations suggest that the lifetime of the membrane-bound dye fraction has been decreased

in the presence of ATP and is consistent with the increase in the degree of polarization observed in this dye to membrane concentration ratio range. Concentration depolarization due to energy transfer can be ruled out.

The large increase in the rotational relaxation time for oxonols V and VI bound to the submitochondrial particle membrane compared to that for the dyes free in solution (Table 3) indicates that the motion of the fluorophore is much more restricted in the membrane than in aqueous solution. These results are consistent with but by no means prove a model described by Smith et al. (1979) in which the oxonol chromophore occupies a site near the onset of the hydrophobic portion of the bilayer with the two sidechains extending into the interior of the bilayer and thus serving as anchors preventing rapid tumbling of the dye in the membrane.

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