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Synthesis, Structure Determination, Spectral Properties, and Energy-Linked Spectral Responses of the Extrinsic Probe Oxonol V in Membranes†

J. C. Smith,‡ P. Russ, B. S. Cooperman,* and B. Chance*

ABSTRACT: The direct synthesis of bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol, which is shown to be the fluorescent probe OX-V (formerly MC-V), is described. The emission lifetime (0.9 ± 0.1 ns) and the spectral properties of this dye in a number of systems are presented as well as the relative polarizations associated with the transition moments of the observable electronic transitions. The structure of OX-V was determined using elemental analysis and infrared and ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The use of the contact shift reagent, Eu(fod)$_3$-d_{27}, greatly facilitated the interpretation of the NMR results. In aqueous media, the anionic form of OX-V is present virtually exclusively due to the low solubility of the neutral species; formation of the latter species occurs when ethanol or methanol solutions of OX-V are acidiied. Both neutral and anionic dye forms can be detected in chloroform–ethanol solvents. The fluorescence intensity from excitation of the neutral species is an order of magnitude weaker than that from excitation of the anionic form and may result from the formation of excited anions due to the loss of a proton by the neutral species in the excited state. Polarization results indicate that the visible absorption of the dye is due to a single electronic transition. OX-V has been employed as a probe primarily in beef heart submitochondrial particles, reconstituted ATPase vesicles, and pigeon heart mitochondria. The energy-linked spectral changes of the probe in these preparations are described and mechanisms proposed for the spectral effects.

Dyes of the merocyanine and related classes have proved to be useful as probes of membrane-related phenomena. Cohen et al. (1974) have evaluated a large number of dyes as probes of changes associated with the action potential in squid giant axons. The merocyanine MC-I (also known as M-540) proved to be among the most sensitive probes of membrane changes occurring during the action potential. Ross et al. (1974) have also demonstrated that a large change in the absorption spectrum of MC-I occurs during the action potential in giant axons. Tasaki et al. (1976) have recently studied crab nerve membranes labeled with several fluorescent probes including M-540. The dyes MC-I, MC-II, and OX-V[1] (formerly MC-V) have also been used extensively as probes (Chance, 1975). A red shift in the absorption spectrum of these dyes is observed when submitochondrial particles stained with these dyes are energized. In particular, the dye OX-V has been given extensive use. Changes in the absorption and fluorescence spectrum of this dye have been demonstrated when *R. rubrum* chromatophores are energized with 860-nm light (Chance and Baltscheffsky, 1975). Cohen et al. (see Chance, 1975) have also demonstrated that the fluorescence of OX-V undergoes changes similar to those observed with MC-I in squid axons, but the former dye is not as sensitive in this system as the latter

† From the Department of Chemistry (P.R. and B.S.C.) and the Johnson Research Foundation (J.C.S. and B.C.), University of Pennsylvania, Philadelphia, Pennsylvania 19174. *Received November 14, 1975.* This work was supported in part by United States Public Health Service Grants GM-12202, NCHD 0-6274, and NINDS-10939.

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* Authors to whom correspondence should be addressed. B.S.C. is an Alfred P. Sloan Fellow (1974–1976).

[1] Abbreviations used: NMR, nuclear magnetic resonance; OX-V, (formerly MC-V), bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol; Eu(fod)$_3$-d_{27}, tris(heptofluorodimethyloctanedionato)-d_{27}-europium; TLC, thin-layer chromatography; ir, infrared; uv, ultraviolet; SMP, submitochondrial particles; EDTA, ethylenediaminetetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; PHM, pigeon heart mitochondria; Mopes, 3-(*N*-morpholino)propanesulfonic acid.

one. Chance (1975) has reviewed the response of OX-V as well as other probes in a number of systems.

Although OX-V has been used extensively as a probe, the structure and basic spectral properties of this dye have remained uncertain. OX-V was originally isolated as an impurity in the R6 (Chance et al., 1974) obtained using a procedure given by Brooker et al. (1951). It has been suggested that the structure of OX-V is similar to that of R6 (Chance and Baltscheffsky, 1975). Tentative structures of OX-V which have proven to be incorrect have appeared in the following papers: Chance et al. (1974), Chance and Baltscheffsky (1975) and Azzi (1975). In this paper we report the direct synthesis of bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol, which is shown to be the probe OX-V (formerly MC-V). The emission lifetime and the spectral properties of this dye in a number of systems are presented as well as the relative polarizations associated with the transition moments of the several electronic transitions experimentally observable. Energy-linked spectral responses of OX-V in mitochondria, submitochondrial particles, and reconstituted ATPase vesicles are also described.

Materials and Methods

The following materials were purchased from commercial sources: ethylbenzoyl acetate (97%) (Aldrich), glutaconaldehyde dianilide hydrochloride (Fairmount Chemical Co.), chloroform-*d* (99.8%), methanol-*d* (99%), and tris(heptofluorodimethyloctanedionato)europium-d_{27} [Eu(fod)$_3$-d_{27}] (Stohler). Soy bean lecithin, purified by the method of Kagawa and Racker (1971), was a gift of Dr. P. Chein. Pigeon heart mitochondria were isolated according to the procedure of Chance and Hagihara (1963). The reconstituted ATPase vesicles were prepared by P. Chein and Y. Ching; the F_1 complex portion of the mitochondrial ATPase was incorporated into the asolecithin vesicles by the method of Kagawa and Racker (1971). Beef heart submitochondrial particles were prepared by the procedure given by Lee and Ernster (1967). Phospholipid vesicles were prepared by dissolving lecithin plus 15% by weight cholesterol in chloroform and evaporating the solution to dryness. Sonication of a suspension of this mixture in water (5 mg/ml) was carried out at 0 °C for 3 min at 30-s intervals at 50–80 W using a Heat Systems-Ultrasonics, Inc., Model W185 cell disrupter. Electron micrographs revealed vesicles 250–500 Å in diameter.

Ascending thin-layer chromatography (TLC) was carried out on Machery-Nagel (Brinkmann Instruments) silica gel plates. Paper electrophoresis was performed using a Savant flat plate apparatus Model FP-22A on Whatman No. 3MM paper.

^{1}H NMR spectra were taken on Varian A-60 and Jeolco PS100 spectrometers, ir spectra were taken on a Perkin-Elmer 521 double grating spectrometer, uv and visible absorption spectra were taken on a Cary 15 spectrophotometer or on the scanning double-beam spectrophotometer described by Chance and Baltscheffsky (1975), and uncorrected excitation and emission spectra were taken on a Perkin-Elmer MPF4 fluorescence spectrophotometer. Polarized excitation and emission spectra were obtained using a Hitachi Model MPF-2A spectrofluorimeter equipped with a Hamamatsu R636 photomultiplier tube. The degree of polarization (P) was calculated from the equation given by Azumi and McGlynn (1962), which contains a correction factor for the inherent polarization of the instrument. For the case of parallel transition moments, $P = 0.5$ whereas, for perpendicular moments, $P = -1/3$. The precision of the values obtained from the photoselection measurements was ±0.02. OX-V absorption changes in submitochondrial particles, reconstituted ATPase vesicles, and pigeon heart mitochondria were recorded at fixed wavelengths using a Johnson Foundation time shared instrument operating as a fluorometer and double-beam spectrometer.

Preparation of 3-Phenyl-5-(4H)-isoxazolone. The preparation of 3-phenyl-5-(4*H*)-isoxazolone was based on the work of Dains and Griffin (1913). Hydroxylamine hydrochloride (30.0 g, 0.432 mol) and ethylbenzoyl acetate (49.5 ml, 0.287 mol) were refluxed for 5 min in 183 ml of methanol. The resulting clear bright yellow solution was filtered and cooled. The crystals were collected and washed with three 100-ml portions of water. Two recrystallizations from benzene gave white needle-like crystals in 24% yield, mp 152–153 °C dec (lit. 151–153 °C dec.; Boulton and Katritzky, 1961).

Preparation of Bis[3-phenyl-5-oxoisoxazol-4-yl]-pentamethineoxonol. To prepare bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol (Figure 1c), 3-phenyl-5-(4*H*)-isoxazolone (9.0 g, 56 nmol) was added to 63 ml of methanol in a round-bottomed flask. Glutaconaldehyde dianilide hydrochloride (5.3 g, 18.6 nmol) was added to the mixture followed by triethylamine (5.0 ml, 36 nmol). After refluxing for 15 min, the resulting dark blue solution was allowed to cool to room temperature. The reaction mixture was transferred to a 3-l. Erlenmeyer flask. The blue solution was then diluted with 1500 ml of methanol. With stirring, 650 ml of 0.1 N HCl was added dropwise to precipitate the dye. The product was collected, washed with 100 ml of water, and purified by twice redissolving in 200 ml of triethylamine–methanol (0.0018:1.00, v/v) per gram of product and reprecipitating with 0.1 N HCl (30.7 ml/200 ml of triethylamine–methanol), giving bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol as a dark green powder in 44.5% yield, mp 200.5–201.5 °C decomp. Anal. Calcd for $C_{23}H_{16}N_2O_4$: C, 71.87; H, 4.20; N, 7.29. Found (Galbraith): C, 71.53; H, 4.13; N, 7.27. The ir, ^{1}H NMR, and uv spectra are in accord with the assigned structure and are discussed in the Results section. On TLC, the purified material had R_f values of 0.60 (methanol–water, 1:1, v/v), of 0.18 (acetone–benzene, 7.3, v/v), and of 0.57 (methanol–acetone–benzene, 1:1:1, v/v/v). Chromatograms of some samples show trace amounts of pink and brown impurities.

Results

The Structure of Bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol. We have synthesized bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol via the two-step sequence shown below:

ethylbenzoyl acetate + hydroxylamine hydrochloride

$$\xrightarrow[\Delta]{\text{MeOH}} \text{3-phenyl-5-(4}H\text{)-isoxazolone} \quad (1)$$

3-phenyl-5-(4*H*)-isoxazolone

+ glutaconaldehyde dianilide hydrochloride $\xrightarrow[\Delta]{\text{Et}_3\text{N, MeOH}}$

bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol (2)

The structures are shown in Figure 1. Reaction 1 had been previously described by Dains and Griffin (1913). Reaction 2 is new, but is quite similar to other reactions reported in the literature for the condensation of keto methylenes with aldehydes and anilides (Donleavy and Gilbert, 1937; Dains and Griffin, 1913; Brooker et al., 1951), particularly Hamer's (1964) synthesis of 4,4′-tricarbocyanine from lepidine ethio-

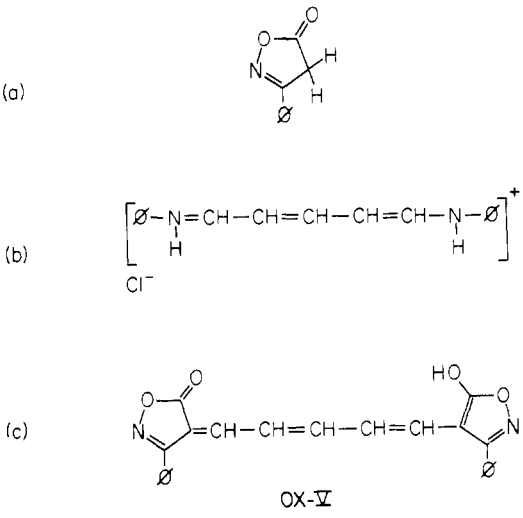


FIGURE 1: The structures of (a) 3-phenyl-5(4*H*)-isoxazolone, (b) glutaconaldehyde dianilide hydrochloride, and (c) bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol (OX-V).

dide and glutaconaldehyde dianilide. In addition to the reasonable synthetic route and the correct elemental analysis (see Materials and Methods), our structural assignment is strongly supported by the ^{1}H NMR spectrum in the presence of the pseudo-contract-shift reagent (Rondeau and Sievers, 1971), Eu(fod)$_3$-d_{27}. As can be seen in Figure 2c, in the absence of shift reagent the spectrum is very difficult to interpret, due to the overlap of phenyl and pentamethine protons. However, analyses of the spectra obtained at several concentrations of shift reagent show them to be clearly consistent with the proposed structure, as will be discussed below. In these spectra the chemical shift of the residual $CHCl_3$ in the solvent was found to be independent of added shift reagent (δ 7.29 ± .01) and provided a convenient internal reference. The spectrum of product in the presence of 30 mM shift reagent (Figure 2d) clearly contains a pattern of peaks (marked by x's) which is very similar to what is seen for the phenyl protons in 3-phenyl-5-(4*H*)-isoxazolone (Figure 2a), if the large peak at δ 7.57 in Figure 2d is aligned with the large peak at δ 7.55 in Figure 2a. The peak at δ 7.52 in Figure 2d is higher than the corresponding peak at δ 7.50 in Figure 2a, because of an overlapping methine resonance (see below). The polymethine chain in glutaconaldehyde dianilide hydrochloride (Figure 2b) shows a 2 H doublet at δ 8.4 (J, 11.2 ± 0.5 Hz, carbons 1 and 5) a 2 H triplet at δ 6.3 (J = 12.1 ± 0.7 Hz, carbons 2 and 4) and a 1 H triplet at δ 7.9 (J = 12.7 ± 0.5 Hz, carbon 3). In Figure 2d one can make out the middle (δ 7.95) and downfield (δ 8.17) peaks of one triplet and the middle (δ 7.20) and upfield (δ 7.00) peaks of another triplet. These triplets are more clearly seen at higher shift reagent concentrations in Figure 2e (arrows) at δ 8.22 (J = 12.9 ± 0.2 Hz, carbons 2 and 4) and at δ 7.25 (J = 12 Hz, carbon 3). Although the doublet corresponding to the hydrogens on carbons 1 and 5 of the polymethine chain could never be fully resolved, its existence may clearly be inferred by the following considerations. In Figure 2e, the phenyl resonance has split into a characteristic two major region pattern with the meta and para hydrogens in the larger region, centered at δ 7.57, and the ortho hydrogens in the smaller major region, centered at δ 7.74. However, integration of these peaks gives, in place of the expected 3:2 ratio, a 2:1 ratio. This ratio could be explained if the missing doublet fell entirely in the δ 7.57 region. Consistent with this interpretation are the findings, in spectra not shown, that at 66 mM shift reagent the observed ratio was 7:5, implying half of the doublet in each region, and that at 100 mM shift reagent the ratio was 1:1, implying that the entire doublet was in the δ 7.74 region. Thus it appears that the doublet is subject to a large downfield shift, in the same direction but smaller in magnitude than the 2 H triplet shift discussed earlier. Hints for the presence of the doublet also come from careful examination of the spectra. For example, in spectrum 2c there is a large peak at δ 7.25 which is believed to be the upfield half of the doublet since it is not present in 2d, and in 2d there is an anamolously high peak at δ 7.52, suggesting the presence in part of an underlying doublet. In summary then the ^{1}H NMR spectra in the presence of varying amounts of EU(fod)$_3$ provide clear evidence for the presence of the phenylisoxazolone and pentamethine moieties in the appropriate ratio of 2:1.

The ir spectrum (KBr pellet) of the product shows a weak, broad absorption in the region 3120–2480 cm^{-1}, characteristic of intramolecular hydrogen binding (Nakanishi, 1962), a strong carbonyl absorption at 1745 cm^{-1}, a peak at 950 cm^{-1} characteristic of trans–cis conjugation (Lunde and Zechmeister, 1954) not present in glutaconaldehyde dianilide HCl [which is known to have an all-trans conformation (Marvel et al., 1967)], and two peaks at 790 and 732 cm^{-1}, characteristic of cis double bonds and also not present in glutaconaldehyde anilide hydrochloride. The carbonyl absorption in the product is 58 cm^{-1} lower than in the 3-phenyl-(5-(4*H*)-isoxazolone) starting material, and this drop is of the magnitude seen in structures where there is intramolecular hydrogen bonding (Nakanishi, 1962). These results, taken together, lead to a suggested product structure (allowed by Dreiding models) in which one of the double bonds in the polymethine chain is in the cis conformation, and there is a hydrogen bond between the carbonyl and enol oxygens of the isoxazolone rings. It should be pointed out that the ir spectrum is of the protonated, neutral product, while in neutral or basic solution the product is present as the anion (as shown by its migration toward the anode in paper electrophoresis experiments at pH 6.5 and 7.5) so that the intramolecular hydrogen bond is lost.

Bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol Is OX-V. OX-V (formerly MC-V), isolated as an impurity of R6, was found to be identical with purified oxonol by the following criteria.

1. R_f values are identical in six different TLC systems.
2. ^{1}H NMR, ir, and uv and visible spectra are identical.

In subsequent discussion, we will refer to the compound as OX-V. The chemical properties of OX-V and the optical properties to be established subsequently appear to be identical with those of the dye NK 2047 (Nippon Kankoh-Shikiso Kenkyusho, Ltd., Okayama, Japan; A. Waggoner, personal communication).

Spectral Properties. In aqueous media, the absorption spectrum of OX-V in the visible region has a maximum near 600 nm, corresponding to the anionic form (see above). In order to detect the neutral form of the dye, the effect of pH on the absorption and emission spectra of OX-V was observed using Tris-acetate buffers in the range pH 9.6 to 3.1. A progressive decrease in the absorbance as the pH is lowered is observed, indicating that the protonated form of the dye has low solubility in aqueous medium, the decrease in absorbance being due to precipitation of the neutral form. Consequently, the anionic form of the dye dominates the absorption properties under these conditions; no shift in the 600-nm absorption maximum was observed as the pH was decreased.

The effect of pH on the dye emission spectrum (Figure 3) parallels that on the absorption. There is a progressive loss of

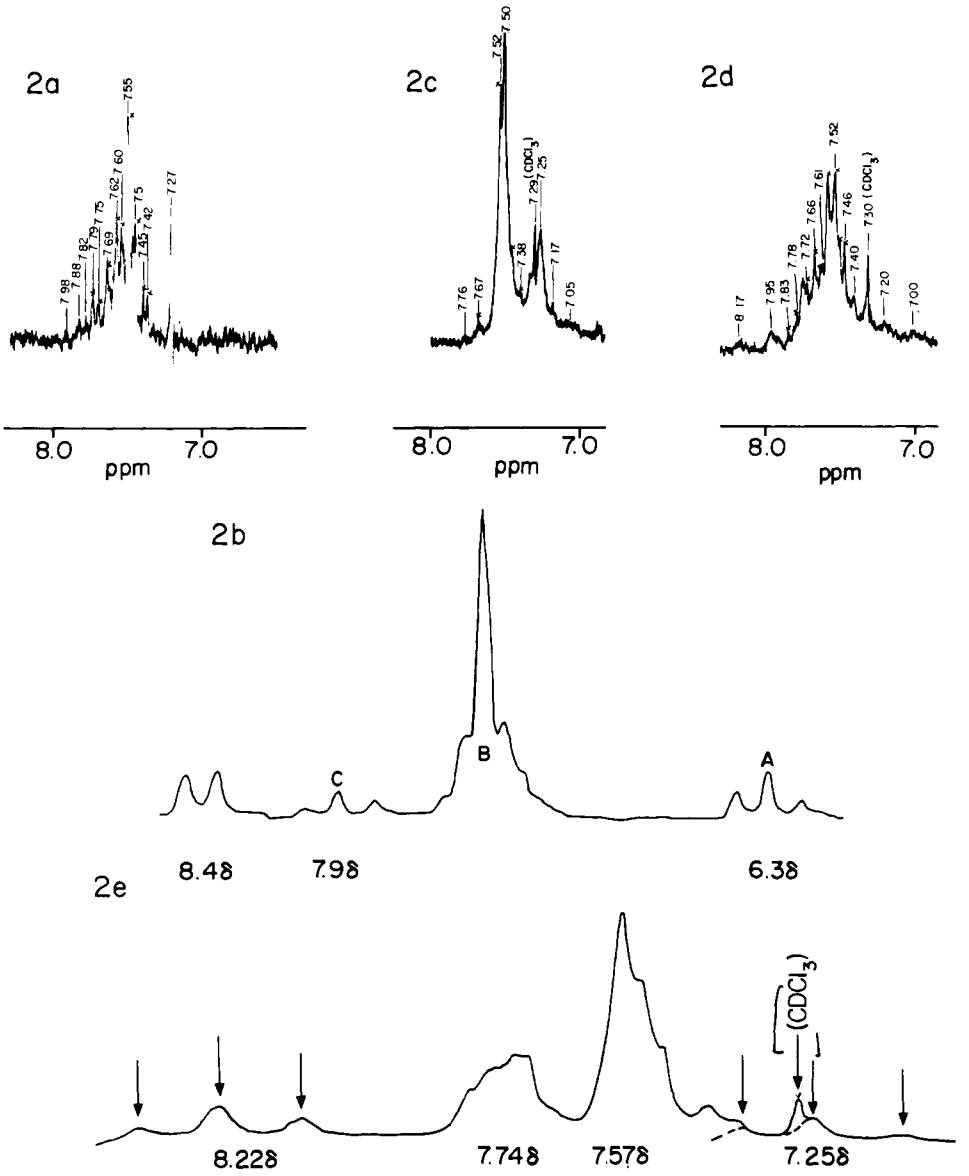


FIGURE 2: (a) ^{1}H NMR spectrum of 3-phenyl-5(4*H*)-isoxazolone in $CDCl_3$ (Varian A-60); (b) ^{1}H NMR spectrum of glutaconaldehyde dianilide hydrochloride in CD_3OD (Jeol FT-100, expanded scale); (c) ^{1}H NMR spectrum of OX-V, 0.1 M, in $CDCl_3$ containing 0.13 M triethylamine (Varian A-60); (d) same as c but containing 30 mM Eu(fod)$_3$-d_{27} (Varian A-60); (e) Same as c but containing 47 mM Eu(fod)$_3$-d_{27} (Joel FT-100, expanded scale).

fluorescence intensity as the pH is lowered, indicating the precipitation of the neutral species. No detectable shift in the emission maximum is observed.

Similar effects on the absorption spectrum of OX-V have been noted when K_2SO_4 solution is added. There is a progressive decrease in the absorbance as the amount of K_2SO_4 is increased, which suggests that the salt is causing precipitation of the dye. Tasaki et al. (1976) have also observed that the solubility of the merocyanine, M-540, is markedly reduced by salt.

It was, however, possible to obtain spectral data on the neutral form of OX-V in methanol or ethanol solution. The spectrum of the dye in methanol shows a 10-nm red shift of the absorption maximum compared with an aqueous medium. The addition of sodium hydroxide solution causes no additional shift in the absorption maximum, suggesting that the dye is virtually entirely in the anionic form in this solvent; i.e., methanol is a strong enough base to accept a proton from the enol hydroxyl group. The addition of 1% by volume of 12 M hydrochloric acid, however, causes a large blue shift in the absorption spectrum; the 609-nm band is lost, and a band at 473 nm appears. There is a threefold reduction in the absorbance, but no precipitation could be detected visually. The 473-nm band is apparently due to the neutral form of the dye. The formation of the neutral species is shown more clearly in Figure 4 where the absorption spectrum of OX-V as a function of the hydrogen chloride concentration in the ethanol solvent is presented. There is a characteristic decrease in the 609-nm band associated with the anionic species and an increase in the absorbance of the 473-nm band associated with the neutral species as the hydrogen chloride concentration is increased. Furthermore, an isosbestic point is observed at 538 nm; the latter observation offers direct evidence for the presence of two and only two species in equilibrium under the conditions of this experiment.

Additional evidence for these two species is present in the absorption spectrum of OX-V in chloroform (Figure 5, curve 1). Absorption bands with maxima near 495 and 633 nm are

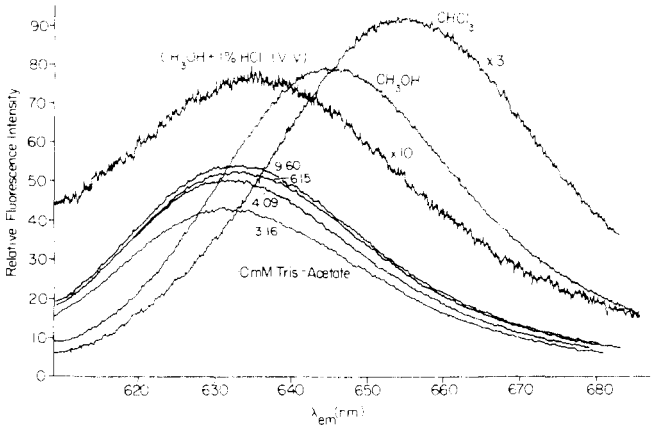


FIGURE 3: Emission spectra of OX-V in several solvents. Concentrations: methanol and methanol plus 1% 12 M HCl, 8.1 μM; Tris-acetate, 5.0 μM; chloroform, 12.1 μM. The numbers near the spectra of the dye in Tris-acetate refer to the pH of the cuvette contents. Excitation wavelengths: methanol, chloroform, and Tris-acetate spectra, 580 nm; methanol plus 12 M HCl, 475 nm. Temperature: 25 °C.

observed; the former maximum is near that of the protonated form in acidified methanol whereas the latter is near that of the anion in alcohol. The differences in the wavelengths at which the absorption maxima of the neutral and anionic species occur in chloroform and in the alcoholic solvents are probably due to a medium effect. The results of a titration of the dye in chloroform (curve 1) with ethanol are presented in curves 2–4. There is a progressive decrease in the absorbance of the band near 495 nm and an increase in that of the band near 623 nm as the fraction of ethanol is increased. This behavior can be explained on the basis of an increase in the anionic form of the dye at the expense of the neutral species as the fraction of ethanol, the more strongly basic solvent, is increased.

For comparison with the spectra obtained in chloroform and ethanol, the spectra of the dye bound to soy bean lecithin vesicles and to beef heart submitochondrial particles are shown in curves 5 and 6, respectively. It is apparent that, in the aqueous media used in recording the latter two spectra, the dye exists predominantly as an anion, the spectral maximum of which is red shifted from the free dye maximum of 600 nm (curve 7) when binding to hydrophobic membranes occurs.

The emission spectrum of OX-V in methanol (Figure 3) has a maximum at 645 nm; upon acidification there is a blue shift of approximately 10 nm and a tenfold loss of intensity. The shift in the fluorescence emission maximum is much less than that observed in the maxima of the absorption and excitation spectra and will be considered further in the Discussion section. The fluorescence emission spectrum of OX-V in chloroform has a maximum near 655 nm, which is a 10-nm red shift relative to the emission maximum of the dye in methanol. This red shift is expected since there is a corresponding shift in the dye absorption maximum in these two solvents, 609 vs. 625 nm, and may be attributed to the greater stabilization of the anion excited state relative to the gound state in chloroform. The parallel shifts in the emission spectrum and in the long wavelength absorption maximum of the dye in chloroform relative to those in methanol point out the dominance of the anionic form in emission processes.

The excitation spectra of the dye are shown in Figure 6. In methanol, there are pronounced vibrational peaks at 565, 595, and 620 nm. Addition of 1% 12 M hydrochloric acid causes the expected blue shift and a tenfold loss of intensity as observed in the emission spectrum. Vibrational maxima are evident at 470, 484, and 495 nm in the excitation spectrum of the dye in the acidified medium. The presence of low intensity vibrational bands in the 470–500-nm region of the methanol spectrum indicates the presence of a small fraction of the neutral species. Although the absorbances of the 495- and the 623-nm bands of OX-V in chloroform (Figure 5, curve 1) are comparable, the excitation spectrum of the dye in this solvent is dominated by the 623-nm band which is associated with the anionic form. This behavior is to be expected since the fluorescence from excitation of the 462-nm band is an order of magnitude weaker than that from excitation of the 623-nm band. Indeed, a careful inspection of the chloroform excitation spectrum reveals the presence at barely detectable intensities of the vibrational maxima characteristic of the neutral form in the 470–500-nm region; these results again illustrate the dominance of the anionic species in emission.

The pK_a of OX-V in aqueous media cannot be determined because of the extreme insolubility of the neutral form of the dye. The neutral species is soluble in ethanol as demonstrated in Figure 4. The absorbance at 615 nm in this figure was used to obtain a value for pK_a. The difference in the absorbance at zero HCl, corrected for the 615-nm absorbance of the neutral form at 2.26 mM HCl, and the absorbance after successive additions of acidified ethanol was used to calculate the ratio of the neutral and ionized forms of OX-V. The pH was calculated assuming complete ionization of HCl at the indicated concentrations. The value of pK_a computed from $pK_a = pH + \log([DH]/[D^-])$ in ethanol is 4.2.

Polarization Properties. The polarization of the OX-V electronic transitions in the visible and ultraviolet regions was investigated using the method of photoselection; results are presented in Figure 7. The degree of polarization P of the emission spectrum of OX-V relative to the absorption maximum of the first electronic transition is constant over the spectral range spanned by the fluorescence. This result is expected since the emission is entirely from the first excited singlet state due to relaxation by internal conversion. Since the fluorescence transition moment is used as a reference for the measurement of polarization of the excitation spectrum, the polarization of the emission was checked to ensure that only one reference transition was involved in the emission; this condition is met since P is invariant over the emission envelope.

The polarization of the excitation spectrum of OX-V is constant over the 460–620-nm spectral region, indicating that a single electronic transition accounts for the dye absorption in the visible region. At 400 nm, however, the P value assumes a negative value of maximum absolute magnitude, suggesting that a second transition is located in the 380–400-nm region; note that a corresponding shoulder is present in the absorption spectrum in this region. The polarization then becomes positive at wavelengths less than 380 nm, indicating the presence of a third transition beyond the latter wavelength.

Since the P values for the visible absorption band of OX-V approach the theoretical maximum of 0.5, the transition moment of this band must be nearly parallel to that of the emission band. A minimum angle of 45° between the transition moments of the visible band and the band near 400 nm is predicted from the P values; if the transition moments of the first electronic transition and the emission are assumed to be parallel, the angle becomes 65°.

Concentration Dependence. The concentration dependence of the absorption spectrum of OX-V in water has been investigated over a 20-fold range of concentration. The absorbance at 602.5 and at 560 nm is linear with concentration up to 26

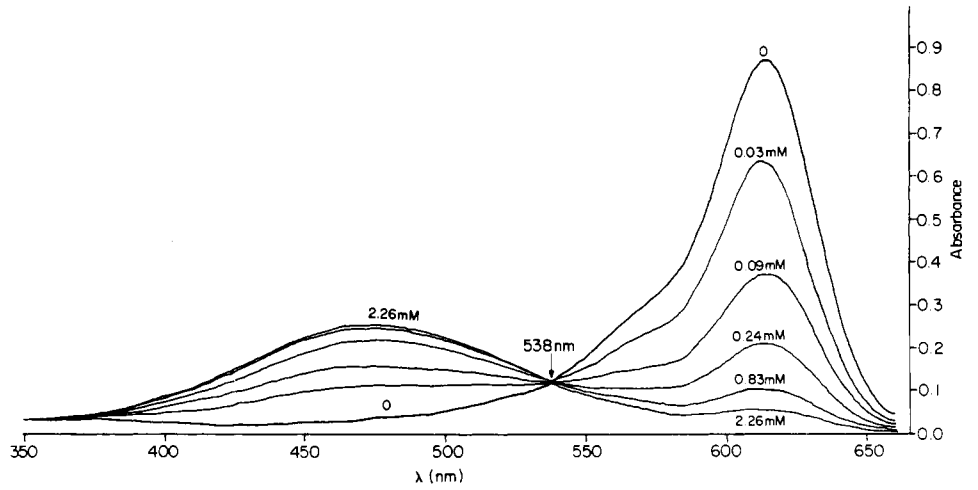


FIGURE 4: The absorption spectrum of OX-V as a function of hydrogen chloride concentration in the ethanol solvent. The numbers near the absorption maxima refer to the hydrogen chloride concentration in mM units. The dye concentration prior to addition of hydrogen chloride was 7.3 μM. The HCl gas concentration in the ethanol used in the titration was determined by diluting the ethanol solution 100 times with water and titrating with 0.1 M NaOH solution. Temperature: 22 °C.

μM, the upper limit of dye concentration employed. Because buffer solutions of moderate ionic strength have been used in the energy-linked OX-V spectral changes in several membrane systems, which are described later in this section, the concentration dependence of OX-V in 100 mM NaCl was also investigated since shielding of the charge on the OX-V anion by salt ions may allow formation of dye aggregates. The absorbance at 603 and at 560 nm is linear with dye concentration, however, up to at least 22 μM, the upper limit of the dye titration in this case. The 22 μM OX-V concentration limit exceeds the highest concentration value used in any of the membrane systems by a factor of three. These results suggest that the dye does not aggregate at least up to the concentration limits used in this study. An aggregation mechanism has been proposed to explain spectral changes of merocyanine dye M-540 (also known as MC-I) associated with the action potential in squid and crab axons (Ross et al., 1974). Sims et al. (1974) have also proposed an aggregation mechanism to account for the decrease of fluorescence intensity of the cyanine diS-C_3-(5) caused by valinomycin-induced K^+ efflux from the red cell and phospholipid vesicles. The latter authors have detected weak absorption bands beyond 700 nm; these absorption peaks are commonly referred to as J bands and are attributed to dye aggregate formation. No such long wavelength bands could be detected in the spectrum of OX-V at the maximum concentration used in the titration.

Fluorescence Lifetime. The lifetime of the OX-V emission as measured by modulation detection fluorometry is 0.9 ± 0.1 ns (T. Yoshida, personal communication).

OX-V Energy-Linked Spectral Responses in Membranes. OX-V has been employed as an extrinsic probe primarily in mitochondria, submitochondrial particles (SMP), and reconstituted ATPase vesicles. Representative examples of energy-linked changes of this probe in biological preparations are illustrated in Figures 8–10. When succinate is added to a suspension of EDTA-type SMP, the absorption spectrum of OX-V is red shifted; the long wavelength portion of the scanned absorption trace is displaced about 4 nm (Chance, 1975). In Figure 8, the change in the dye fluorescence and absorbance at fixed wavelengths is illustrated. When NADH is provided to the particles, a fluorescence decrease (curve 1) and a change in the 620–650-nm differential absorbance (curve 2), due to the red shift of the dye absorption spectrum, are observed. The

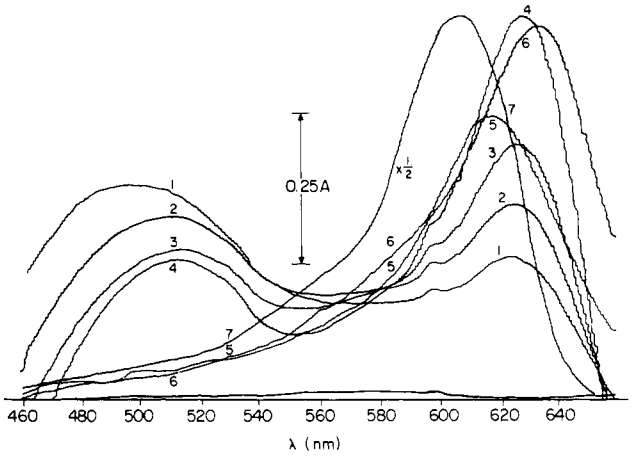


FIGURE 5: The absorption spectrum of OX-V under several conditions recorded using a scanning double beam spectrometer; reference wavelength, 650 nm. The numbered curves are as follows: (1) 52 μM in chloroform; (2) 50.2 μM in chloroform plus 3.4% ethanol by volume; (3) 48.5 μM in chloroform plus 6.7% ethanol; (4) 47 μM in chloroform plus 9.7% ethanol; (5) OX-V at 8.1 μM bound to soy bean lecithin vesicles containing 15% cholesterol by weight (0.46 mg/ml) in water at pH 6.3; (6) OX-V at 5.3 μM bound to beef heart submitochondrial particles (34 mg of protein/ml; medium, 0.25 M sucrose, 10 mM Tris-Cl at pH 7.2); (7) OX-V at 24 μM in 100 mM Tris-acetate at pH 8.85. The horizontal trace is the baseline read out from the memory circuit of the instrument. The apparent shoulder near 600 nm is an artifact due to an irregularity in the baseline.

NADH is consumed in about 1 min after which the fluorescence and absorbance levels revert to the values prior to substrate addition. The cycle can be repeated by successive addition of NADH. Oligomycin addition causes an enhancement of the spectral responses, which reflects a more tightly coupled system since the membrane is more tightly sealed by the oligomycin. The rate at which the NADH is consumed also decreases after oligomycin has been provided as expected from the increased coupling.

Succinate and ATP also induce similar OX-V spectral responses in SMP; the former substrate-indued response is slow compared with that caused by NADH. As in the NADH case, the succinate-induced spectral changes can be reversed by uncouplers such as CCCP or FCCP. The rates of the ATP-

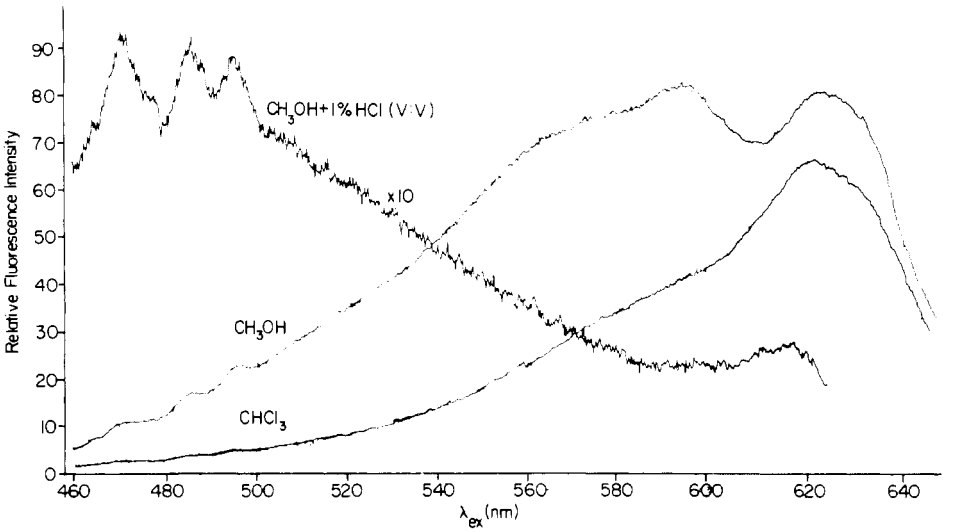


FIGURE 6: Excitation spectra of OX-V in several solvents. Concentrations: methanol and methanol plus 12 M HCl, 8.1 μM; chloroform, 12.1 μM. Emission wavelengths: methanol, 645 nm; methanol + HCl, 475 nm; chloroform, 655 nm. Temperature: 25 °C.

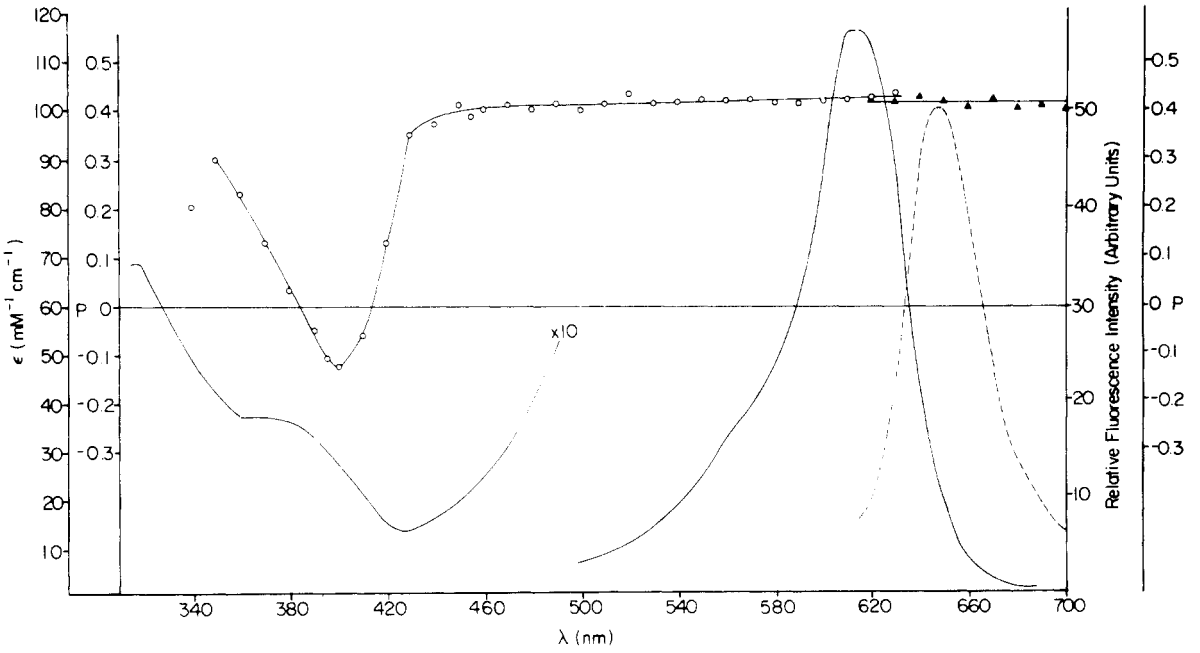


FIGURE 7: The absorption (—) and emission (- - -) spectrum of OX-V at 12.5 μM in glycerol at 0 °C (excitation wavelength, 580 nm). (○) The polarization of the excitation spectrum relative to the emission maximum, 645 nm (slits, ex = 5 nm, em = 10 nm); (▲) the polarization of the emission spectrum relative to the absorption maximum, 610 nm (slits, ex = 5 nm, em = 3.5 nm).

induced fluorescence decrease and absorption red shift fall between those of the succinate- and NADH-induced changes. The ATP-induced spectral effects can be reversed by uncouplers and by oligomycin.

The OX-V spectral response caused by ATP addition to the reconstituted ATPase vesicle system is illustrated in Figure 9. The substrate causes a fluorescence decrease (curve 1) and an absorption red shift (curve 2) as also observed in ATP-Mg-type SMP. The preceding spectral changes can be reversed by oligomycin as illustrated and are also reversed more rapidly by the uncoupler FCCP. In general, the reconstituted system behaves in a manner qualitatively identical with that of ATP-Mg SMP.

The OX-V spectral responses caused by succinate addition to a pigeon heart mitochondria (PHM) suspension are illustrated in Figure 10. The substrate causes a fluorescence decrease curve 1) and an apparent absorption blue shift (curve 2); that is, the absorbance change is of the opposite sense as in SMP and reconstituted ATPase vesicles. These spectral changes are readily reversed by the uncoupler CCCP. The same OX-V spectral changes can be induced on a qualitative basis by the addition of ATP and are blocked by oligomycin and by atractyloside which prevents the transport of ATP across the mitochondrial membrane. The rate at which the OX-V fluorescence decrease occurs is clearly slower than that of the absorbance change in Figure 10. The succinate-induced fluorescence decrease in SMP is also lower than the absorbance change, but the difference is not as pronounced as in the PHM case. Additional work on the details of the kinetics of the OX-V energy-linked spectral responses is in progress. The substrate-induced OX-V fluorescence decrease has also been observed in rat liver and beef heart mitochondria, but the ab-

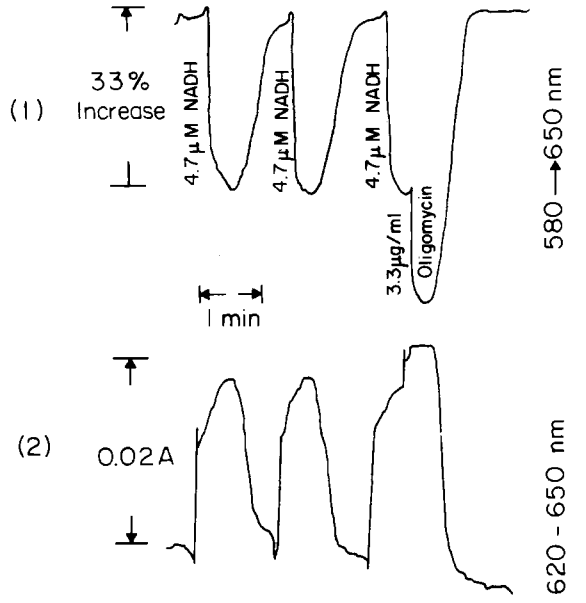


FIGURE 8: OX-V spectral changes induced by addition of NADH and oligomycin to EDTA-type submitochondrial particles, 0.1 mg of protein/ml. The OX-V concentration is 1.3 μM. Medium: 0.25 M sucrose and 10 mM Tris-Cl, pH 7.4. Temperature: 22 °C. The particles were prepared by Professor C. P. Lee. The percentage change calibrations for the fluorescence traces in this figure and in Figures 9 and 10 are relative to the total dye fluorescence intensity attained prior to substrate addition.

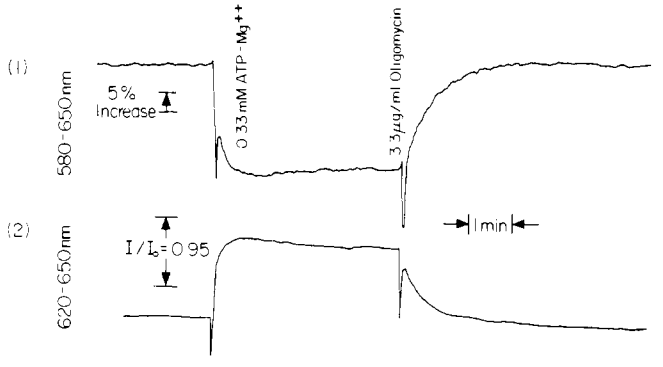


FIGURE 9: OX-V spectral responses caused by ATP-Mg^{2+} addition to reconstituted ATPase vesicles. The dye concentration is 6.7 μM. Temperature: 23 °C. The medium contains 0.25 M sucrose, 10 mM Tris-SO_4, pH 7.4, and 75 mM K_2SO_4. The reconstituted vesicles were prepared by Y. Ching and P. Chein and consist of the F_1 complex of the mitochondrial ATPase incorporated into asolecithin vesicles.

sorbance changes have not been detected. The magnitude of the energy-linked fluorescence change is smaller in the latter two systems than in PHM presumably because of the presence of more endogenous substrate in the beef heart and rat liver preparations. Rotenone or cyanide blocks were used when the mitochondria were energized with succinate or ATP, respectively.

The effect of OX-V on electron flow through site I was investigated in both ATP-MgSMP and PHM using NADH and glutamate–malate, respectively. At up to 3 μM concentrations of OX-V, the decrease in the rate of oxygen consumption, as measured by an oxygen electrode, caused by addition of an aliquot of the dye in ethanol, could be accounted for to within 5–10% or less by the addition of the corresponding quantity of ethanol alone. Also, no effect of OX-V was observed on the rate of oxygen consumption in SMP and PHM due to succinate oxidation. The dye, therefore, does not inhibit site I at the 1 μM

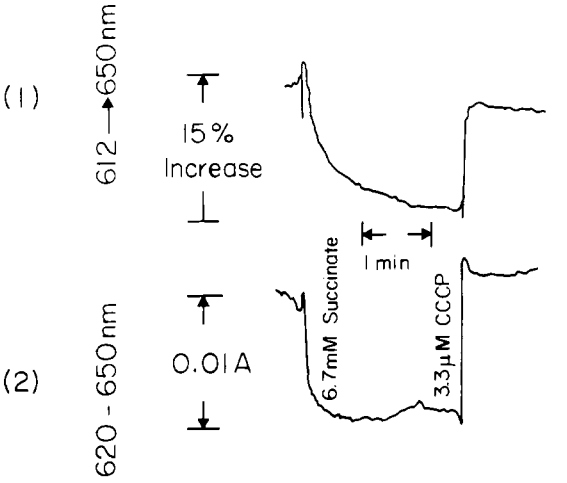


FIGURE 10: OX-V spectral changes due to succinate addition to pigeon heart mitochondria, 0.2 mg of protein/ml. Dye concentration is 1.3 μM. The medium contains 0.225 M mannitol, 0.075 M sucrose, 0.05 M Mopes, pH 6.0, and 3.7 μM rotenone. The mitochondria were prepared by Dr. I. Y. Lee. Temperature: 23 °C.

level which has been used in most of the experiments on energizable preparations described in this report.

Discussion

Evidence for the presence of the neutral and anionic species in methanol, chloroform, and mixed solvents has been presented. In aqueous media, however, the anionic form is present virtually exclusively due to low solubility of the neutral species. An increase in the ionic strength of the dye solution will likely decrease the solubility of the neutral species and thus lead to dye precipitation, and this would account for the decrease in absorbance at 600 nm caused by addition of K_2SO_4. There is no spectral shift associated with pH in the range 6 to 8 which is likely to be used in biomembrane studies. The solubility of the dye is somewhat dependent on pH in this range. OX-V, therefore, is not suitable for measuring pH changes in aqueous medium, although we have demonstrated that the dye is sensitive to proton and potassium ion flux across vesicle walls and to potassium transport by valinomycin in *R. rubrum* chromatophores (Baltscheffsky, 1967; Chance et al., 1974; see Table I).

Polarization results indicate that the visible absorption of the dye is due to a single electronic transition and that the absorption and emission transition moments are nearly parallel. A characteristic red shift of the absorption maximum is observed when OX-V binds to soy bean lecithin vesicles or beef heart submitochondrial particles.

The blue shift of the absorption, excitation, and emission spectra of OX-V observed when a methanol or ethanol solution is acidified apparently reflects the destabilization of the excited state due to the formation of a neutral species from a charged one in a polar medium. The proposed structure for the dye also suggests that protonation of the hydroxyl group would preclude resonance with the ring systems, thereby leading to additional destabilization.

The blue shift in the absorption maximum observed when the dye anion is protonated is predicted by the equation

$$E^2 = E_I^2 + (b_L - b_R)^2$$

derived by Platt (1956) to account for the transition energies E of a number of Brooker dyes. E_I is the energy of the dye under "isoenergetic" conditions at which the two resonance

TABLE I: Sign of Oxonol V (OX-V) Changes in Various Membrane Systems.[a]

	ATPase Vesicles		Lecithin Vesicles		Submitochondrial Particles		Chromatophores (R.R.)		Rat Brain Cortex	Squid Axon	Pigeon Heart Mitochondria	
	A	F	A	F	A	F	A	F	F	F	A	F
K^+ influx			↑	↑	↑	↑	↑	↑	↑			
K^+ efflux			↓	↓		↓	↓	↓	↓		↓	↓
Depolarization										↓		
Hyperpolarization										↑		
Energization	↑	↓			↑	↓	↑	↓			↓	↓

[a] Note: ↑ indicates a red shift in the absorption spectrum (A) or an increase in fluorescence (F), and ↓ an absorption blue shift or a fluorescence decrease.

structures for the conjugated system have the same energies; $b_L - b_R$ is a measure of stabilization of one of the structures relative to the other depending on solvent conditions. Platt further demonstrated that the b's are related to the basicities of the groups on either end of the conjugated chain and hence are sensitive to solvent polarity. Since two identical resonance structures can be drawn for the anionic form of OX-V, b_L is equal to b_R, and transitions occur at the isoenergetic wavelength for a given solvent system. When protonation occurs, the groups at the ends of the carbon chain become distinguishable, i.e., b_L is no longer equal to b_R; because of the quadratic nature of the equation above, E can only increase. The red shift of the 600-nm band observed when the dye binds to a membrane can be explained on the basis of a decrease in E_1 plus a possible contribution from the $b_L - b_R$ term since the end groups may well be in different environments in the membrane system. The formation of the neutral species when an alcohol solution is acidified is accompanied by a tenfold reduction in the emission intensity, although the absorbance at the wavelengths used to excite the anionic and neutral species (475 and 580 nm, respectively) is approximately the same. Thus, a mechanism leading to a reduction in the emission intensity resulting from excitation of the neutral species appears to be operative. If protonation were to shift the entire vibrational manifold of the first excited state to the blue, then fluorescence from the lowest vibrational level should give an emission spectrum with a maximum slightly to the red of the 473-nm absorption maximum; such an emission spectrum is clearly not observed experimentally since there is only a 10-nm shift in the emission maximum (Figure 3) upon acidification of the solvent. The polarization of the excitation spectrum of OX-V (Figure 7) is constant in the 620–460-nm spectral region. The latter finding suggests that the effect of protonating the dye anion is to alter the overlap of the vibrational wavefunctions in the ground and excited states of OX-V such that absorption leads to a population of higher levels in the neutral species than in the anionic one. Vibrational relaxation in the neutral form would lead to the population of the lowest vibrational level of the first excited electronic state; emission from this vibrational level could give rise to an emission spectrum closely resembling that of the anion, assuming that protonation did not alter the energy differences between this level and those of the ground state significantly. An inspection of the absorption spectrum of the neutral species formed by addition of 1% 12 M hydrochloric acid by volume to an alcoholic solution of OX-V (not shown), i.e., complete conversion to the neutral dye species, indicates essentially zero overlap in the ground-state, nuclear configuration between the lowest vibrational level of the first excited state and the vibrational levels of the ground state since there is no absorption near the 635-nm emission maximum. Barring a major change in nuclear configuration in the dye excited state, one would expect the neutral form of the dye to be essentially nonfluorescent.

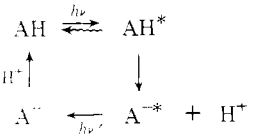
A mechanism which accounts for the observed emission when the neutral dye species is excited at the absorption maximum is an increase in the acidity of the neutral species in the excited state. Parker (1968a) has given the following expression for the difference in the values of pK in the ground and excited state (pK*):

$$pK^* - pK = -\frac{E_1 - E_2}{2.3RT} = -\frac{Nh(\Delta\nu)}{2.3RT} = -\frac{6.25 \times 10^3}{T}(\Delta\bar{\nu})$$

where K is the equilibrium constant for the reaction $AH \rightleftharpoons A^- + H^+$, $E_1 - E_2$ is the difference in the enthalpies of the excited and ground state, N is Avogadro's number, h is Planck's constant, and $\Delta\bar{\nu}$ is in μm^{-1}. R, T and $\Delta\nu$ have the usual meanings. The equation assumes that the reaction entropies of the ground and excited states are equal; this assumption is borne out in the results from a number of systems as compiled by Parker (1968a).

An approximation for $\Delta\bar{\nu}$ can be obtained from the absorption maximum of the neutral and anionic forms. The value of $\Delta\bar{\nu}$ in the case of OX-V is rendered somewhat uncertain since the absorption of the neutral form is rather broad. Using 473 and 609 nm for the maxima of the neutral and anion absorption spectra, respectively, one obtains $pK^* - pK = -9.9$ at 25 °C. This calculation then suggests a marked increase in the acidity of OX-V in the excited state leading to the formation of an appreciable fraction of excited anions which then fluoresce, giving the observed emission spectrum which is shifted only 10 nm relative to that observed before acidification of the dye solution. The latter shift is likely due to the change in solvent due to acid addition. The dye anion in the ground state would be rapidly reprotonated in the acidified medium due to the increase in pK relative to pK*. This model is summarized in Scheme I, where the symbol ⇝ indicates a radiationless transition.

Scheme I.



The disparity in the emission intensities observed in the acidified vs. alcoholic solvent system may be due to the short lifetime of the excited state (0.9 ∓ 0.1 ns); i.e., it is probable

that equilibrium between the neutral and anionic excited state species is not reached during this time period since the rate constant for vibrational relaxation to the ground state can be as high as $10^{12}\,s^{-1}$ (Parker, 1968b), while that for the proton should not exceed $10^{10}\,s^{-1}$ (Hammes and Amdur, 1966). Thus, only a fraction of the equilibrium concentration of excited anions should be reached, namely about one-tenth assuming that no change in the quantum yield of the anion occurs in the acidified vs. alcoholic medium.

Changes observed in the absorption and fluorescence spectra of the merocyanine M-540 in response to action potentials in the squid axon (Ross et al., 1974) and crab nerve (Tasaki et al., 1976) have been explained on the basis of a change in the monomer–dimer equilibrium of the dye associated with the membrane. Tasaki et al. (1976) have suggested that the shoulder on the short wavelength side of M-540 visible absorption is due to the dimer species. Since an analogous shoulder is observed in the absorption spectrum of OX-V (Figure 7), we have investigated the possibility of dimer formation by this dye. As stated in the Results section, the 560-nm absorbance is linear with concentration up to 26 μM, the concentration limit used. Furthermore the ratio $A_{602.5}/A_{560}$ does not change, 2.70 ± 0.18, over the indicated concentration range. In the presence of 100 mM NaCl, the A_{603}/A_{560} value was 2.52 ± 0.17. The latter ratios should be particularly sensitive to aggregate formation. These results indicate that, in aqueous or in 100 mM NaCl medium, no OX-V dimer formation occurs up to at least 26 μM. The polarization P value does not change through the 560-nm shoulder region; this result suggests that the shoulder is due to a transition to a higher vibrational level of the first excited state.

Care must be used in interpreting the polarization curve in Figure 7 with respect to dimer formation. If dye dimers were to make a contribution to the 560-nm shoulder, the dimers would be expected to be weakly or nonfluorescent species since transitions to the lower energy component of the split monomer state are expected to be forbidden since no evidence for another electronic transition to the red of the 609-nm maximum of the monomer is present. Since the lower energy state would be populated by internal conversion, little or no dimer emission would be detected. The photoselection experiment would, therefore, not detect the presence of dimers. The polarization properties are consistent with the spectral concentration dependence but do not offer direct evidence for the absence of dimers.

The preceding observations do not necessarily preclude an aggregation process as a mechanism of the OX-V fluorescence changes observed in squid axons by Cohen et al. (see Chance, 1975) or in the membrane systems studied in this report. Changes in the membrane structure during the action potential or substrate consumption or small lateral movement of the dye species may bring monomers into sufficient proximity that overlap of the dye orbitals occurs leading to an aggregate with altered absorption and fluorescence properties. Such aggregate formation cannot be excluded in regions of high local dye concentration in membranes particularly in such systems as *R. rubrum* chromatophores which have great affinity for the OX-V probe.

The energy-linked OX-V spectral changes in the biological preparations described in the preceding section are summarized in Table I, which contains information on a number of additional systems. An inspection of this table reveals that a red shift of the OX-V absorption spectrum occurs in SMP, ATPase vesicles, or *R. rubrum* chromatophores made positive inside by light or substrate-induced electron flow. Similar absorption red shifts in the SMP and chromatophore systems are caused by valinomycin-induced K^+ influx which generates a charge separation across the membrane which has the same polarity as that due to electron flow. In the case of PHM rendered negative inside by substrate oxidation, an apparent OX-V absorption blue shift is observed; that is, the absorption change is of the opposite sense as that in the preceding systems. Valinomycin-induced K^+ efflux from the PHM interior, which results in a charge separation with the PHM negative inside, produces an apparent absorption blue shift similar to that resulting from electron flow. In asolecithin vesicles, the absorbance changes caused by K^+ influx or efflux are of the same sense as those observed in SMP (and chromatophores) or PHM, respectively. The OX-V absorbance changes therefore correlate with the potentials developed either by illumination, substrate oxidation, or by valinomycin-induced K^+ fluxes in asolecithin vesicles, SMP, ATPase vesicles, *R. rubrum* chromatophores, and PHM.

In chromatophores, ATPase vesicles, or SMP, the electron-flow-induced absorption red shift caused a loss of OX-V absorbance at the 580-nm fluorescence excitation wavelength, which is accompanied by a decrease in the fluorescence intensity. There are, however, a number of exceptions to the correlation between loss of absorbance at the fluorescence excitation wavelength and the decrease in emission intensity. Valinomycin-induced K^+ influx into the internal volume of the chromatophores, SMP, and asolecithin vesicles causes an increase in the dye fluorescence intensity even though an absorption red shift results from the influx. In the PHM case, a fluorescence decrease results from either substrate oxidation or valinomycin-induced K^+ efflux from the mitochondria interior. To the extent that the OX-V absorption change in PHM induced by electron flow can be regarded as a pure blue shift, a fluorescence increase would be expected because of the increased absorbance at the excitation wavelength. Thus valinomycin-induced K^+ influx gives fluorescence changes of the opposite sense to membrane energization in SMP and chromatophores whereas a fluorescence decrease occurs in both cases in PHM.

The electron-flow-induced absorption red shift and the accompanying fluorescence decrease observed in the ATPase vesicles, SMP, and chromatophores are consistent with the electrochromic effect as proposed by Platt (1961) and developed on a more rigorous theoretical basis by Reich and Schmidt (1972) and by Cheng (1975). Chance (1975) has also discussed the electrochromic effect in connection with carotenoid and extrinsic probe spectral changes in a number of systems. In terms of the theory of electrochromism, the spectral shift $h\Delta\nu$ is a direct consequence of the interaction of the electric field of strength $\vec{\mathbf{F}}$, due to a potential difference across the membrane, and the difference in the dipole moments $\Delta\vec{\mu}$ or polarizabilities $\Delta\alpha$ of the dye in the ground and excited states:

$$h\Delta\nu = -\Delta\vec{\mu} \cdot \vec{\mathbf{F}} - \tfrac{1}{2}\Delta\alpha\mathbf{F}^2 \qquad (1)$$

Equation 1 demonstrates that the shift $h\Delta\mu$ can vary with the field strength $\vec{\mathbf{F}}$ in both a linear and a quadratic manner. The sense of the spectral shift depends on the orientation of $\Delta\vec{\mu}$ relative to $\vec{\mathbf{F}}$ and on the relative magnitudes and signs of $\Delta\vec{\mu}$ and $\Delta\alpha$ as discussed by Cheng (1975). The time scale of the electrochromic effect is sufficiently fast that the time course of potential development in respiring or photosynthetic systems can be followed. There is some question, especially in the mitochondrial and SMP systems, as to whether the kinetics of the OX-V spectral changes are fast enough to be compatible with

the cytochrome turnover rates in the respiratory chain. Additional work on this question is in process.

An alternative explanation of the OX-V spectral changes observed in the preceding systems is based on the following observations. A red shift of the OX-V absorption spectrum occurs when the dye binds to membranes (Figure 5). At low probe to PHM and SMP membrane protein ratios, binding of the dye to membranes causes an increase in the fluorescence, but when the ratio exceeds approximately 20 nmol of dye/mg of protein, the fluorescence begins to decrease; that is, quenching occurs. In the case of *R. rubrum* chromatophores, Chance and Baltscheffsky (1975) have found that the OX-V fluorescence decreases on binding; the magnitude of the quenching increases as the chromatophore membrane concentration is increased at constant probe concentration or as the probe concentration is increased at fixed membrane concentration. Since a red shift of the OX-V absorption spectrum occurs when the dye binds to membranes, the red shift induced by electron flow or K^+ influx in asolecithin vesicles, SMP, ATPase vesicles, or chromatophores may be due to additional binding of the OX-V dye by these systems in the energized state. The fluorescence decrease observed when the membranes of these systems are energized can be explained on the basis of quenching of the emission due to the increment of local concentration of dye in the membrane. (The properties of OX-V summarized in Figure 7 strongly suggest that fluorescence quenching is to be expected. The extinction coefficient of the visible band is quite high, 120 mM^{-1} cm^{-1}; the absorption and fluorescence transition moments are nearly parallel and the absorption and emission envelopes overlap strongly. The inner filter effect and reabsorption processes are favored by these properties; indeed, the fluorescence intensity of OX-V in ethanol is not linear with dye concentration above 2 μM.) The fluorescence increase caused by K^+ influx in SMP or asolecithin vesicles may be due to a disparity in the magnitudes of the potentials developed by the charge separation on valinomycin-facilitated K^+ influx. If the latter potential is small compared with that developed by substrate consumption, then the amount of dye bound to the energized membrane would not exceed the 20 nmol/mg protein ratio necessary for fluorescence quenching to occur, and an increase in the emission would result. During respiration, however, the potential developed is apparently of sufficient magnitude to allow this ratio to be exceeded with the resulting quenching of the dye fluorescence.

In the PHM case, the apparent absorption blue shift and fluorescence decrease caused either by valinomycin-induced K^+ efflux or substrate oxidation can be explained on the basis of an ejection of the dye from the membrane. The OX-V blue shift would then be the reverse of the red shift caused by association with the PHM membrane, and the fluorescence decrease is essentially due to the loss of the emission intensity gained from the passive binding process since, as expected from the low dye/membrane ratio (6.5 nmol of OX-V/mg of PHM protein), the initial binding of OX-V to the PHM membrane enhances the dye emission intensity.

The OX-V fluorescence changes given for the rat brain cortex were obtained by A. Mayevsky in an investigation of spreading depression in the brain. The stimulation of the latter effect is known to cause a tenfold increase in the extracellular K^+ concentration due to efflux of this ion from the internal volume of the cells (Mayevsky et al., 1974). An OX-V fluorescence decrease is observed during K^+ efflux and an increase is found during K^+ influx after cessation of spreading depression. These fluorescence changes are of the same sense as those found for K^+ fluxes in asolecithin vesicles and illustrate a correlation of the dye fluorescence changes in a complex organ with those in the simplest model system.

The spectral changes of OX-V due to K^+ fluxes in *R. rubrum* chromatophores are more complex than those expected on the basis of either binding changes or the electrochromic effect. An absorption red shift and a fluorescence increase are observed during K^+ influx, and the opposite changes are found for K^+ efflux from the chromatophore internal volume. Since an absorption red causes a reduction in absorption at the fluorescence excitation wavelength, 580 nm, a decrease in the emission intensity would be expected, which is not experimentally observed. Similarly since the red shift observed with K^+ influx is indicative of enhanced binding of OX-V to the membrane and since passive binding of the dye in this system invariably quenches the emission, a decrease in OX-V fluorescence is expected in this model as well. The blue shift of the absorption spectrum caused by K^+ efflux from the chromatophores would be expected to give rise to a fluorescence enhancement by the reverse of the arguments offered above. Such enhancement is also not observed. The behavior of the OX-V emission in *R. rubrum* chromatophores is thus quite complex and may involve interactions with the carotenoid pigments in this membrane.

The fluorescence changes in the squid axon during hyperpolarization (inside negative) and depolarization (inside positive) are the opposite of those expected on the basis of the fluorescence changes produced by K^+ efflux and influx in the asolecithin vesicles. Although the fluorescence intensity decrease caused by depolarization of the axon does correlate with that induced by substrate addition to SMP, the fluorescence increase on hyperpolarization does not agree with the decrease observed in energized PHM. The OX-V fluorescence changes in the squid axon may therefore be of a different origin than those observed in the energizable systems.

The summary provided in Table I, however, demonstrates that a correlation between the OX-V absorption changes and membrane potential exists. A red shift is consistently observed in systems in which a membrane potential, which is positive in the interior of the system, is developed by K^+ influx, light, or substrate consumption whereas a blue shift is found in the case of PHM in which a potential which is negative inside is developed either by K^+ efflux or substrate consumption. The nature of the fluorescence changes, however, is strongly dependent on the membrane system and the method of generating the potential.

In contrast to a number of probes that give information on fixed properties of membranes such as volume or microviscosity, the oxonol probes give what we consider to be more interesting information, namely that related to function, as illustrated in Table I, for energy coupling in the mitochondrial membrane systems, action potential probe responses in the squid axon and for spreading depression in the brain. It is the latter class of probes from which we may expect the most interesting information in the future contrary to the suggestion of Metcalfe (1975) who appears to emphasize structural rather than functional response of probes. These fluorescence probes are much better indicators of function than are the spin-label and NMR probes, and are, in fact, sensitive enough to detect very subtle activations of energy coupling in mitochondrial membranes.

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A Functionally Active Tryptic Fragment of *Escherichia coli* Elongation Factor Tu†

Gary R. Jacobson‡ and Jurg P. Rosenbusch*

ABSTRACT: Mild tryptic hydrolysis of native elongation factor Tu from *Escherichia coli* yields a unique fragment which lacks some 65 residues at the amino-terminal end of the protein. This fragment retains the capacity to bind GDP, to form a ternary complex with GTP and aminoacyl-tRNA and to stimulate in vitro protein synthesis. Like the native protein, the fragment renatures from a random coil conformation to a fully functional form. These results show that the fragment is an independent structural and functional unit.

In a study of the apparent membrane association of a major protein from *Escherichia coli* (Jacobson et al., 1976), we noted that mild tryptic hydrolysis nearly quantitatively converted this polypeptide (molecular weight 44 000) to a species with an apparent mass of 37 000 daltons. More recently, we have identified this protein as elongation factor Tu (Jacobson and Rosenbusch, 1976). In its role in protein synthesis, EF-Tu[1] interacts with a number of other proteins and substrates (for

† From the Department of Microbiology, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland. *Received June 30, 1976.* This investigation was supported by Grant 3.513.75 from the Swiss National Foundation for Scientific Research.

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[1] Abbreviations used are: EF-Tu, EF-Ts, and EF-G, protein synthesis elongation factors Tu, Ts, and G, respectively; EF-Tu^t, the 37 000-dalton tryptic fragment of EF-Tu; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.