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OXONOL DYES AS MONITORS OF MEMBRANE POTENTIAL

THEIR BEHAVIOR IN PHOTOSYNTHETIC BACTERIA

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Summary

The responses of oxonol dyes to single and multiple single turnovers of the photosynthetic apparatus of photosynthetic bacteria have been studied, and compared with the responses of the endogenous carotenoid pigments. The absorbance changes of the oxonols can be conveniently measured at 587 nm, because this is an isosbestic point in the 'light-minus-dark' difference spectrum of the chromatophores.

The oxonols appear to respond to the light-induced 'energization' by shifting their absorption maxima. In the presence of K^+, valinomycin abolished and nigericin enhanced such shifts, suggesting that the dyes respond to the light-induced membrane potential. Since the dyes are anions at neutral pH values, they probably distribute across the membrane in accordance with the potential, which is positive inside the chromatophores. The accumulation of dye, which is indicated by a decrease in the carotenoid bandshift, poises the dye-membrane equilibrium in favor of increased dye binding and this might be the cause of the spectral shift.

The dye response has an apparent second-order rate constant of approx. $2 \cdot 10^6\ M^{-1} \cdot s^{-1}$ and so is always slower than the carotenoid bandshift. Thus the dyes cannot be used to monitor membrane potential on submillisecond timescales. Nevertheless, on a timescale of seconds the logarithm of the absorbance change at 587 nm is linear with respect to the membrane potential calibrated with the carotenoid bandshift. This suggests that under appropriate conditions the dyes can be used with confidence as indicators of membrane potential in energy-transducing membranes that do not possess intrinsic probes of potential.

Introduction

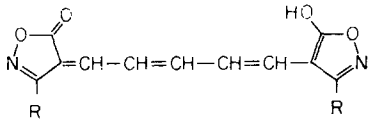
Extrinsic probes of transmembrane potential have been used extensively in studies of energy-transducing membranes [1—4], although the mechanisms by which such probes monitor membrane potential are not completely understood. Electrochromism [5] provides a physical basis for the mechanism by which some probes may respond to membrane potential [6], but this phenomenon is not widespread. Many of the more widely used extrinsic probes, such as cyanine and oxonol dyes [7] and 1-anilino-8-naphthalene sulphonate [8], appear to permeate membranes freely in their ionized forms, and distribute across the membrane in accordance with the membrane potential. This asymmetric distribution is then responsible for the observed spectral changes. Other mechanisms, such as probe aggregation [9], may also contribute to the probe response.

A full understanding of the behavior of an extrinsic probe of membrane potential depends on an independent evaluation of both the probe response and the membrane potential. Oxonol and cyanine dyes have been studied extensively in both black lipid [7,10] and nerve membranes [11] where their optical properties can be correlated with electrical activity monitored with electrodes.

Chromatophores from photosynthetic bacteria provide an energy-transducing system particularly suited for the evaluation of the properties of probes of membrane potential: electron flow is initiated photochemically, and short, saturating flashes of light can be used which cause the components of the electron transfer chain to turn over only once [12]. Furthermore, many photosynthetic bacteria possess carotenoids which shift their absorption spectra to the red in response to 'energy-linked' events (e.g. see refs. 13—15 for recent work on this topic). While there is considerable controversy over the mechanism of the carotenoid bandshift (e.g. refs. 13—15), there is general agreement that the carotenoids are responding to some form of transmembrane electrical potential. Indeed, when membrane potentials are induced with valinomycin-KCl pulses, the response of the carotenoid bandshift has been shown to be remarkably linear with respect to the magnitude of the induced potential [16—19,14,15]. Both oxonol [6] and cyanine [20] dyes exhibit 'energy-linked' spectral responses in chromatophores, so in the present study we have compared the behavior of the endogenous carotenoids with the response of a class of oxonol dyes which appear to be useful indicators of membrane potential in a wide variety of energy-transducing membranes [21,22].

Materials and Methods

Chromatophores were prepared from *Rhodopseudomonas sphaeroides* Ga, *Rhodospirillum rubrum* S-1 and *Chromatium vinosum* D as described previously [23]. The oxonol dyes OX-V, OX-VI, and OX-VII were synthesized in this laboratory [21]; the dyes OX-VIII and OX-IX were purchased from Nippon Kankoh-Shikiso Kenkyusho Co. Ltd., 2-3, Shimoishii 1 Chome, Okayama-shi, Okayama 700, Japan and were used without further purification. The structures of the oxonol dyes are illustrated in Fig. 1.



OX-V, R = ⬡
OX-VI, R = $CH_2CH_2CH_3$
OX-VII, R = CH_3
OX-VIII, R = C_7H_{15}
OX-IX, R = C_9H_{19}

Fig. 1. The structures of the oxonol dyes OX-V, -VI, -VII, -VIII and -IX.

In all the experiments reported here, the ambient oxidation-reduction (redox) potential of the suspension was maintained at approx. 150 mV by the addition of small quantities of solid sodium ascorbate and/or succinate. The use of redox mediators in conjunction with $Na_2S_2O_4$ led to an irreversible bleaching of the oxonol dyes. Absorption spectra were recorded with a Johnson Foundation Scanning Double Beam spectrophotometer equipped with a digital memory [6]. The analysis of rapid flash-induced absorbancy changes in a dual-wavelength spectrophotometer has been described previously [23]. Dye absorbance changes were measured with the spectrophotometer operating in a single-beam mode.

Results

Oxonol dyes added to chromatophores exhibit light-induced shifts of their absorption spectra [6], and the response of the propyl-substituted dye, OX-VI, is shown in Fig. 2. It is characterized by a shift of approx. 20 nm to longer wavelengths. The shift was completely reversible and the original spectrum was restored within a minute of the cessation of illumination. Fig. 3 shows the 'light-minus-dark' difference spectrum of the response; the red shift of the probe provides the maximum at 625 nm and the minimum at 590 nm. In the absence of dye this region is relatively featureless at the sensitivities employed here. At shorter wavelengths the light-induced carotenoid bandshift is the major component of the spectrum.

The kinetics of the carotenoid bandshift and OX-VI response after a saturating single turnover flash are shown in Fig. 4. The response of OX-VI was measured at 587 nm, an isosbestic point in the 'light-minus-dark' difference spectrum of *Rps. sphaeroides* chromatophores; carotenoid changes were followed at 490—475 nm. The rate of the carotenoid changes greatly exceeded those of the dye; indeed, the change of dye absorbance correlated exactly with the initial decay of the carotenoid response (Fig. 4B). Chance and Baltscheffsky [6] have reported a Mg^{2+} dependence of OX-V fluorescence in *R. rubrum* chromatophores; no significant effect of Mg^{2+} (in the range 0—10 mM) on the absorbance changes of OX-VI was found in the present experiments, and the Mg^{2+} concentration was routinely maintained at 1 mM.

The response of OX-VI and the carotenoids to a train of saturating (more than 90%) flashes is shown in Fig. 5; the carotenoid absorbance change was

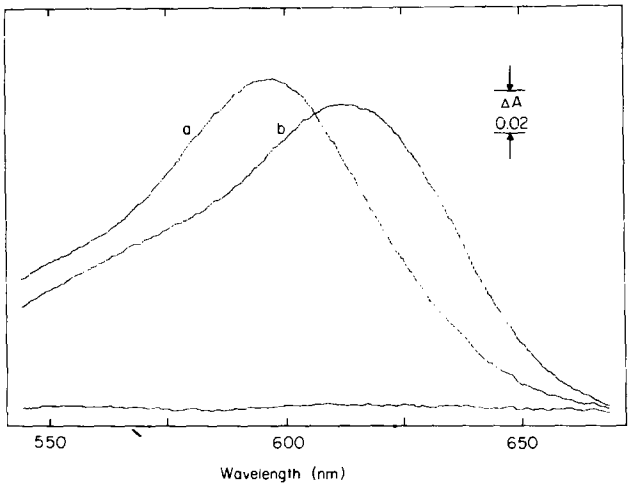


Fig. 2. The response of OX-VI to illumination of *Rps. sphaeroides* chromatophores. Chromatophores (21 μM bacteriochlorophyll) were suspended in 100 mM KCl, 20 mM MOPS, 1 mM $MgCl_2$ and 0.5 mM ascorbate, pH 6.9, at 23°C. The absorbances of chromatophores in the light and in the dark were stored in the digital memory of a Johnson Foundation Scanning double beam spectrophotometer with a reference wavelength of 670 nm. OX-VI was added to give a final concentration of 1.5 μM and spectrum a was recorded in the dark and spectrum b in the light. Illumination was provided by a 15-W tungsten filament lamp filtered through a Kodak Wratten Gelatin filter, number 88 A.

maximal after the fifth or sixth flash, whereas the dye response took 15—20 flashes to reach maximum levels. This observation is consistent with the finding that the dye response exceeds that of the carotenoids during steady-state illumination (Fig. 3), but is much smaller after a single turnover (Fig. 4).

The decay kinetics of the OX-VI and carotenoid changes after a train of

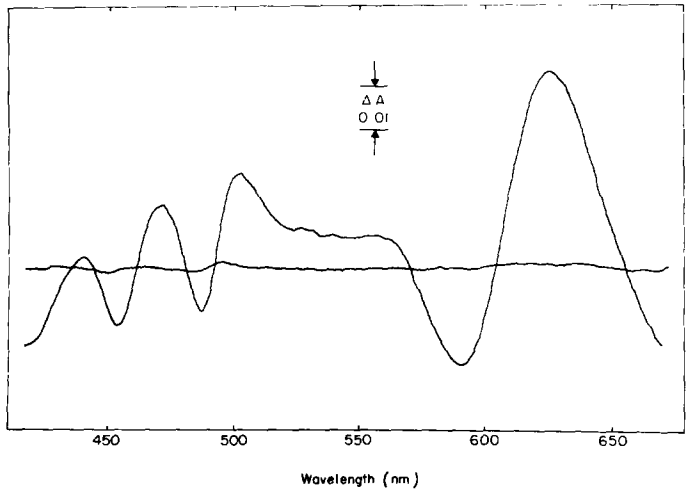


Fig. 3. The light-dark difference spectrum of *Rps. sphaeroides* chromatophores in the presence of OX-VI. Experimental conditions were as described in the legend to Fig. 2. The baseline stored in the computer memory was of chromatophores plus OX-VI.

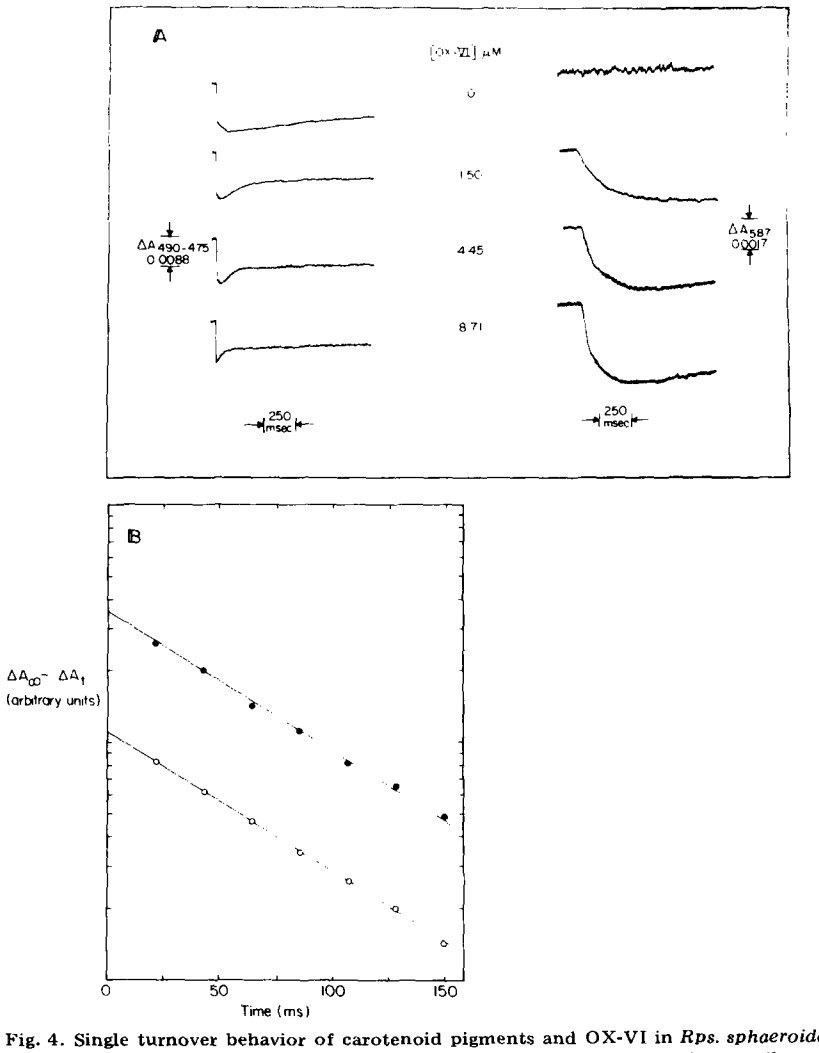


Fig. 4. Single turnover behavior of carotenoid pigments and OX-VI in *Rps. sphaeroides* chromatophores. Chromatophores (24 μM bacteriochlorophyll) were suspended in a medium similar to that described for Fig. 2 and with the OX-VI concentrations indicated. Carotenoid absorbance was monitored at 490—475 nm and OX-VI absorbance at 587 nm. (A) Experimental traces. (B) Semilogarithmic plots of the time courses of the initial decay of the carotenoid bandshift (●———●) and the rise of the OX-VI response (○———○) at an OX-VI concentration of 8.7 μM.

flashes are shown in Fig. 6. In the presence of dye there is a rapid decay of the carotenoid reponse which is not resolved on a timescale of seconds; this is followed by a much slower decay which is the only phase observed in the absence of dye. The decay of the OX-VI response correlated with the slow phase of the carotenoid decay. The presence of 1 μM valinomycin in the presence of potassium completely abolished the dye response and left a small transient carotenoid response (Fig. 5). The addition of NH_4Cl (5 mM) or nigericin (10 nM) increased the dye response to a single turnover by less than 15%, but enhanced the steady-state OX-VI response by approx. 40%; there were similar effects on the magnitude of the carotenoid bandshifts.

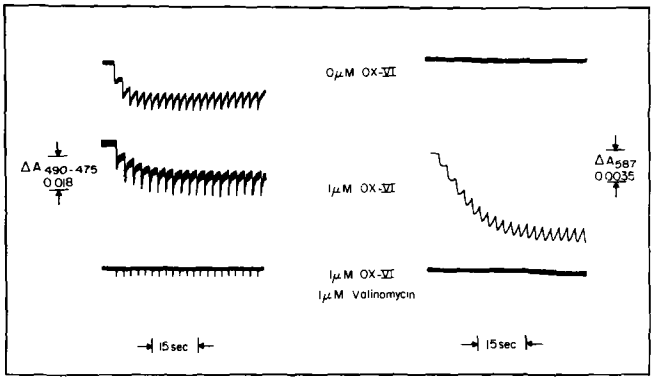


Fig. 5. The response of OX-VI and carotenoids to a train of single turnover flashes in *Rps. sphaeroides* chromatophores. Chromatophores (24 μM bacteriochlorophyll) were suspended in a medium similar to that described for Fig. 2. Additions of OX-VI and valinomycin were made as indicated. Carotenoid and OX-VI absorbance were measured as described for Fig. 4.

The extent and the halftime of the OX-VI response to a single turnover depend on the concentration of dye (Figs. 4 and 7), with both parameters approaching limiting values at high dye concentrations. When the concentration of chromatophores is in a large excess, so that all the dye might be expected to interact with the membrane, the extent of the dye response at 587 nm approaches $4.5 \cdot 10^4\ M^{-1} \cdot cm^{-1}$. This is the change observed when OX-VI is completely bound by phospholipid vesicles [24] and so the value can be used as an 'extinction coefficient' to calculate the concentrations of bound and free dye.

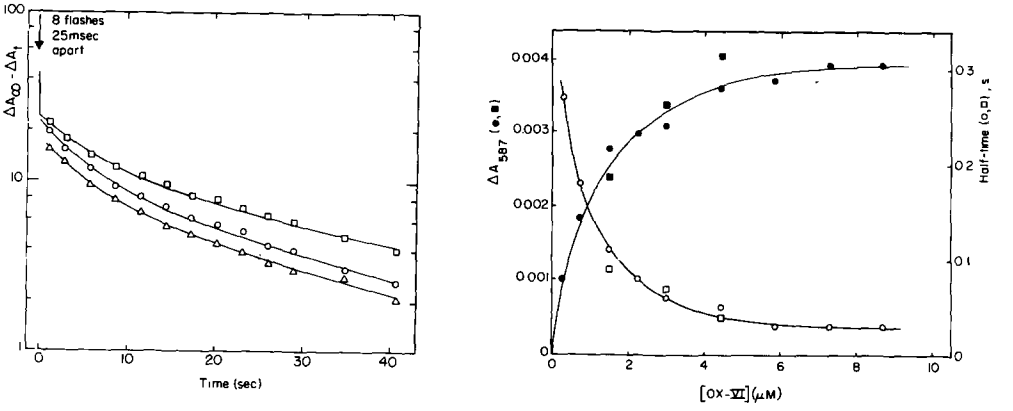


Fig. 6. Semilogarithmic plots of the decay of the flash-induced absorbance changes of OX-VI and carotenoids in *Rps. sphaeroides* chromatophores. Chromatophores (24 μM bacteriochlorophyll) were suspended in a medium similar to that described for Fig. 2. Carotenoid and OX-VI absorbance changes were measured as described for Fig. 2 after a train of eight single turnover flashes separated by 25 ms. □———□, carotenoid decay in the presence of 1 μM OX-VI. ○———○, carotenoid decay in the absence of dye. △———△, OX-VI (1 μM) decay.

Fig. 7. The dependence of OX-VI absorbance changes to a single turnover flash in *Rps. sphaeroides* chromatophores on dye concentration. Chromatophores (24 μM bacteriochlorophyll) were suspended in a medium similar to that described for Fig. 4. The extent (●, ■) and the half-time (○,□) of the OX-VI response were determined in two different chromatophore preparations.

TABLE I

THE BEHAVIOR OF OXONOL DYES IN *RPS. SPHAEROIDES* CHROMATOPHORES

Chromatophores (≈ 20 μM bacteriochlorophyll) were suspended in 100 mM KCl, 20 mM morpholinopropane sulfonate, 1 mM $MgCl_2$, 0.5 mM ascorbate and 1.5 μM oxonol, pH 7.0, at 23°C.

	OX-V	OX-VI	OX-VII	OX-VIII	OX-IX
Continuous illumination shift (nm)	4	20	5	−1	−2
$\Delta A_{587nm}/A_{587nm}$	−0.07	−0.41	−0.17	0.05	0.03
Single turnover $1 \cdot 10^3 \cdot \Delta A_{587nm}/A_{587nm}$	−0.65	−2.20	−0.85	1.10	0.54

The results of Fig. 7 are replotted in Fig. 8 as a Scatchard plot, which indicates that the dye binds with an apparent dissociation constant of 0.9 μM. Following a single turnover, a maximum of 0.4 dye molecule is bound per reaction center. At these saturating oxonol concentrations the dye-induced decay of the carotenoid bandshift (Fig. 4) is approx. 45%.

All of the oxonols illustrated in Fig. 1 exhibit light-dependent changes at 587 nm in *Rps. sphaeroides* chromatophores and Table I summarizes their behavior in steady-state and single turnover illumination. Oxonols V, VI and VII had qualitatively similar properties; a shift of their absorbance to the red and a loss of absorbance at 587 nm, although there were significant differences in the magnitude of the changes observed. On the other hand, OX-VIII and IX showed light-dependent blue-shifts of absorbance, with an increased absorbance at 587 nm. Nevertheless, the kinetic characteristics of their response resembled those of the other dyes (Fig. 9).

The effect of antimycin on the single turnover responses of carotenoid and oxonols is shown in Fig. 9. Antimycin specifically inhibits electron transfer in

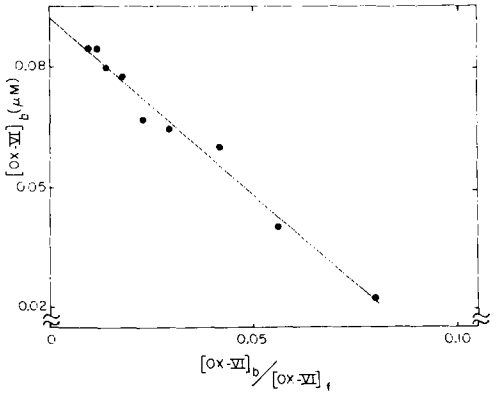


Fig. 8. The single turnover flash-dependent binding of OX-VI to *Rps. sphaeroides* chromatophores. The data presented in Fig. 7 are replotted in the form of a Scatchard plot. The concentrations of free and bound dye were calculated assuming a differential absorbance coefficient of $A_{587nm} = 0.045\ \mu M^{-1} \cdot cm^{-1}$. The slope of the line, −0.89 μM and the ordinate intercept 0.09 μM were determined by linear regression analysis with a correlation coefficient, $r^2 = 0.98$. The number of binding 'sites' per flash was 0.0038 per bacteriochlorophyll or approx. 0.4 per reaction center.

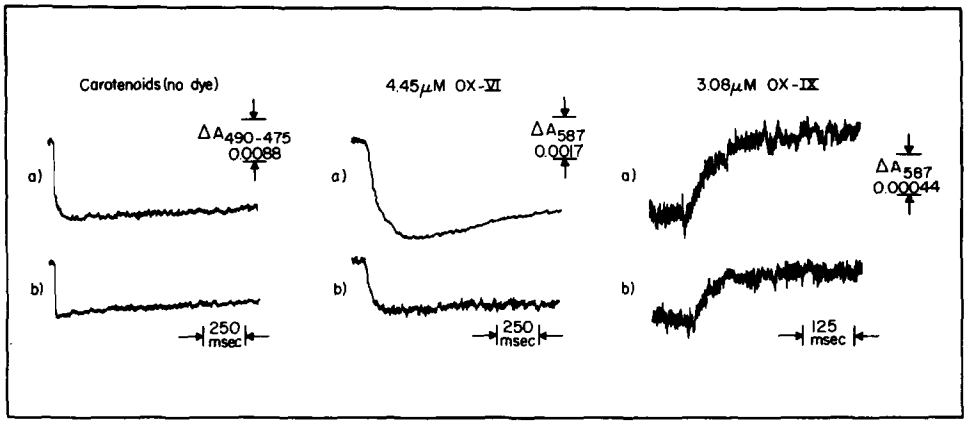


Fig. 9. The effect of antimycin on the single turnover flash behavior of OX-VI, OX-IX and carotenoids in *Rps. sphaeroides* chromatophores. Chromatophores (24 μM bacteriochlorophyll) were suspended in a medium similar to that described for Fig. 2. Oxonol and carotenoid absorbance changes were measured as described for Fig. 4. Conditions were as indicated, traces labelled a were in the absence and those labelled b in the presence of antimycin (1.4 μM).

the ubiquinone-cytochrome b-c_2 oxidoreductase, and reduces the flash generated carotenoid bandshift by approx. 30% at E_h = 150 mV (Fig. 9 and see ref. 25). The inhibitor has a similar effect on the responses of each of the oxonols, and this can be ascribed to a decreased binding of the dyes in the presence of antimycin.

The behavior of OX-VI was also studied in chromatophores prepared from *R. rubrum* and *C. vinosum*. In both cases the kinetics and the extent of the dye response to single turnover flashes were very similar to those found in the *Rps. sphaeroides* chromatophores described above (Fig. 10).

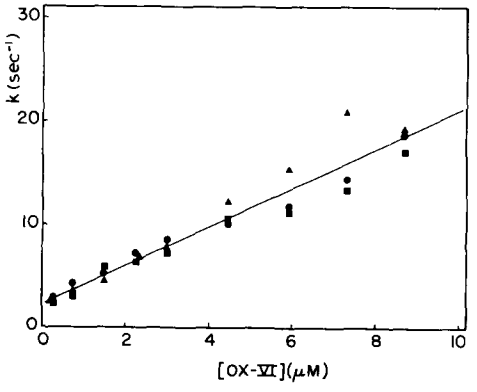


Fig. 10. The single turnover kinetics of the OX-VI absorbancy change in chromatophores. Chromatophores (20 μM bacteriochlorophyll) were suspended in a medium similar to that described for Fig. 2. The apparent first-order rate constant was calculated according to the relationship $k = \ln 2/t_{1/2}$ measured from experimental traces similar to those presented in Fig. 4. Three preparations of chromatophores were employed: *Rps. sphaeroides* (▲), *C. vinosum* (■), and *R. rubrum* (●). The line drawn was fitted by linear regression analysis, and has a slope of $1.88 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ with a correlation coefficient $r^2 = 0.97$ and an intercept of 2.20 s^{-1}.

Discussion

Oxonol dyes have been used to monitor changes of membrane potential in a wide variety of membrane systems [7,10,11,21] and the dyes appear to be valuable indicators of membrane potential in phosphorylating submitochondrial particles [22]. The experiments reported here were designed to evaluate some of the properties of the dyes that contribute to their spectral behavior in energy-transducing membranes. In particular, it was desirable to have the independent assessment of membrane potential afforded by the carotenoid pigments of photosynthetic bacteria [13—19].

When added to chromatophores, the response of OX-VI to illumination closely resembles that seen following the 'energization' of submitochondrial particles either by respiration or by ATP hydrolysis [22]. This observation reinforces the analogies between the two systems [12], and suggests a common mode of action for the dye. In both systems the 'energy-linked' oxonol response is abolished by valinomycin in the presence of K^+, circumstantial evidence that the dye monitors membrane potential. Furthermore, under conditions where energy is conserved almost entirely as membrane potential, for example in the presence of nigericin [26] or ammonium salts [27], the magnitudes of the dye responses are maximal [22].

The oxonols used in this study are anions at physiological pH values [21], and increase the conductance of black lipid membranes [7]. Indeed, when solutions of OX-VI are separated by a bilayer membrane, the potential across the membrane is accurately predicted by the Nernst equation (S. Krasne, personal communication), indicating that the anionic dye freely permeates the membrane. This permeability provides the simplest explanation for the response of anions to membrane potential [28]; the dyes distribute across the membrane in accordance with the Nernst relationship and therefore accumulate in the 'positive' aqueous phase. In illuminated chromatophores the internal aqueous phase is positive with respect to the bulk solution [14—19], so the dyes are accumulated in this phase. The accumulation of oxonol dyes by chromatophores would poise the dye-membrane equilibrium in the internal phase in favor of increased binding. Because the inner aqueous phase is much smaller than the bulk solution (the ratio is approx. 1 : 1000 [29]), the additional binding of the inner surface of the membrane is not completely compensated by decreased binding on the outer surface so the total amount of bound dye is increased. This probably provides the basis for the observed spectral shift since the passive binding of oxonol dyes to lipid membranes is accompanied by spectral changes very similar to those reported here [24].

The explanation given above accounts for the oxonol-dependent decay of the carotenoid bandshift (Figs. 4, 5, and 6), because the movement of the dye into the chromatophore would diminish the membrane potential. The observation that the initial, oxonol-dependent decay of the carotenoid response and the rise of the oxonol response have identical kinetics (Fig. 4B) supports this contention. Furthermore, oxonols stimulate the rate of electron transfer in the ubiquinone-cytochrome b-c_2 oxidoreductase in a manner entirely analogous to that of K^+ in the presence of valinomycin (see ref. 25).

The concentration dependence of the extent of the flash-induced oxonol

response (Fig. 7) has the appearance of a hyperbolic binding function. Assuming that the bound and free dye can be discriminated by their absorbance at 587 nm, it is possible to determine the apparent binding parameters (Fig. 8). In the presence of antimycin, which reduces the flash-induced membrane potential approx. 30% under these conditions (Fig. 9), less dye is taken up by the chromatophores. In contrast, much more dye is bound during uninhibited steady-state illumination (Fig. 5). These differences in dye response can be ascribed to changes in the apparent number of binding 'sites'.

The change in the rate of the oxonol response as the dye concentration is varied has the character of a second-order reaction. A plot of the apparent first-order rate constant ($\ln 2/t_{1/2}$) vs. oxonol concentration is linear with a slope of close to $2 \cdot 10^6\ M^{-1} \cdot s^{-1}$, although it is not clear why the plot does not pass through the origin (Fig. 10). Very similar apparent second-order rate constants were obtained with *C. vinosum* and *R. rubrum*. At the concentrations used in the experiments reported here (0—10 μM), the halftimes of the dye responses (20—140 ms) are much shorter than those of naphthalene sulfonate probes in mitochondrial membranes, where the halftime has been measured as 3 s [30]. However, the oxonols responded much more slowly than the carotenoids (Fig. 4), which can respond within 100 ns [16]. This means that the oxonol dyes should not be used to estimate fluctuations in membrane potential on submillisecond timescales. Nevertheless, both the carotenoid and oxonol responses have very similar kinetics when the membrane potential changes slowly (Fig. 6). Saphon et al. [31] have reported that chromatophore ATP synthesis is associated with a stimulation of the decay of the carotenoid bandshift on a timescale of seconds. It therefore seems likely that oxonols could prove useful indicators of membrane potential changes associated with phosphorylation.

Oxonols have been reported to respond to potential changes in the microsecond time domain in black lipid membranes and axons [10,11]. Waggoner et al. [10] have suggested that these rapid changes are dependent on a pre-existing asymmetric distribution of the oxonol across the membrane, but we have been unable to find any evidence for such rapid changes in chromatophores, even under conditions (50% saturating background illumination) which generate an anisotropic distribution of oxonol before the actinic flash. In the steady state the magnitudes of the oxonol response are large, $\Delta A/A$ up to 0.5, although under single turnover conditions $\Delta A/A$ was in the range $1 \cdot 10^{-3}$—$1 \cdot 10^{-4}$ (Table I). In axons and black lipid membranes, $\Delta A/A$ is in the range 10^{-4}—10^{-5}, so one possible explanation for our failure to see a fast phase is that it represents the change of only a very small fraction of the dye population, which is masked by larger changes in the remainder of the dye population. It is also possible that the rapid, dye-dependent responses of the axon and black lipid membrane systems represent changes in optical properties of these membranes [10,11], such as birefringence or refractive index, which would not be detected in an isotropic suspension of chromatophores.

The final question which we shall address is whether it is possible to calibrate the oxonol response in terms of the membrane potential. Calibrations using potassium gradients in the presence of valinomycin have been very useful for the carotenoid bandshift [14—19], but are unreliable for permeant anions

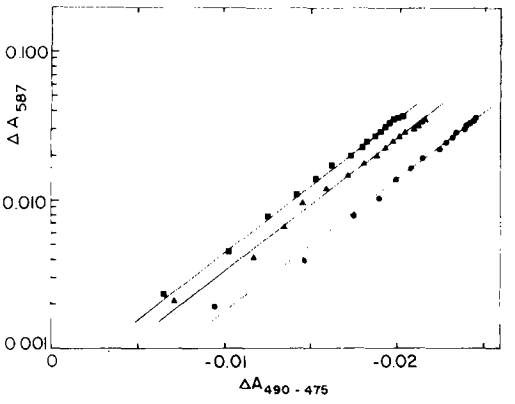


Fig. 11. The response of OX-VI and the carotenoid bandshift in *Rps. sphaeroides* chromatophores. Chromatophores (19.4 μM bacteriochlorophyll) were suspended in a medium similar to that described for Fig. 2. OX-VI was 3.0 μM (●), 5.9 μM (▲) or 8.7 μM (■). The oxonol response, measured at 587 nm, and the carotenoid bandshift, measured at 490—475 nm, were determined 135 ms after each of a train of saturating single turnover flashes.

which copermeate with K^+ [32,33]. However, the calibrated carotenoid bandshift can be used to estimate the membrane potential in the presence of the oxonols, allowing an indirect calibration of the dye response. In order to allow the oxonol to equilibrate with the membrane potential, the responses of both it and the carotenoid bandshift were measured 135 ms after each of a train of saturating single turnover flashes. Typical results of three such experiments are shown in Fig. 11, and it can be seen that the logarithm of the absorbance change at 587 nm is linear with respect to the carotenoid response at three different dye concentrations. If the absorbance change is used to calculate the concen-

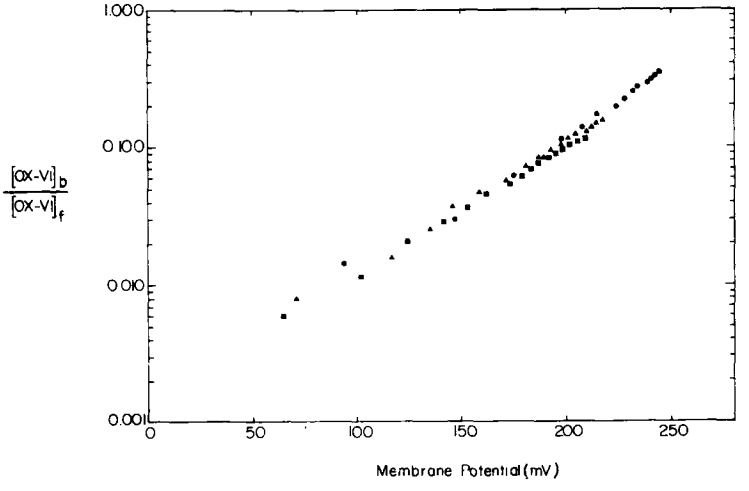


Fig. 12. The dependence of the OX-VI absorbance change on membrane potential in *Rps. sphaeroides* chromatophores. The data of Fig. 11 are replotted with the oxonol changes shown as the ratio of $[dye]_{bound}/[dye]_{free}$, and the carotenoid bandshift converted into membrane potential using the data of Takamiya and Dutton [19] who calibrated these chromatophores using valinomycin-KCl pulses.

trations of bound and free dye, the logarithm of the ratio $[dye]_{bound}/[dye]_{free}$ varies almost linearly with the carotenoid bandshift, and the three experiments in Fig. 11 fall on the same line. Takamiya and Dutton [19] have calibrated the carotenoid bandshift in the chromatophores used in this experiment, and Fig. 12 presents the data with the logarithm of $[dye]_{bound}/[dye]_{free}$ as a function of the membrane potential. The nearly linear response of oxonol behavior with membrane potential suggests that the dyes can be used with confidence as indicators of membrane potential in energy-transducing membranes which do not possess intrinsic probes of potential.

Acknowledgments

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