J. Membrane Biol. 46, 255–282 (1979)

Kinetics of the Potential-Sensitive Extrinsic Probe Oxonol VI in Beef Heart Submitochondrial Particles

J.C. Smith and Britton Chance

Johnson Research Foundation, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

Received 6 November 1978

Summary. The interaction of the potential-sensitive extrinsic probe oxonol VI with beef heart submitochondrial particles has been investigated under time resolved and equilibrium conditions. The time course of the probe absorption spectrum red shift induced by ATP or NADH injection into a suspension of submitochondrial particles in a dye solution is biphasic, consisting of a faster process described by a second-order rate law with $k_2 \sim 3 \times 10^5$ M^{-1} sec^{-1}. For the ATP pulse experiments, the slower process follows first-order kinetics with $k_1 \sim 0.3$ sec^{-1}. In oxygen pulse experiments to an anaerobic dye-particle system, the slower process is not significantly developed due to rapid depletion of the oxygen, but the faster process follows second-order kinetics with the same rate constant as for the ATP and NADH cases. Evidence for permeation of the submitochondrial particle membrane by oxonol VI has been obtained; the slower process is interpretable as describing the permeation of the membrane bilayer. The results of the time-resolved work are consistent with a mechanism involving a redistribution of the dye from the bulk phase to the particle membrane. The value of the second-order rate constant for passive binding of the dye to submitochondrial particles is not compatible with a mechanism proposed to explain the microsecond probe response times in bilayer and excitable membrane experiments nor are such rapid signals observed in the oxonol VI-submitochondrial particle system.

The use of extrinsic probes in the study of biological membrane phenomena has received considerable impetus from the discovery of several related classes of dyes that are potential sensitive. These probes usually fall into the cyanine, merocyanine, or oxonol class of dyes. Dyes of the preceding three types consist of two ring structures joined by a conjugated carbon chain. Since the wavelength of maximum absorption increases with the length of the carbon chain, it has been possible to synthesize probes the spectral absorption and emission spectra of which do not significantly overlap the principle bands of intrinsic pigments of biological membranes. The visible absorption band of these dyes is

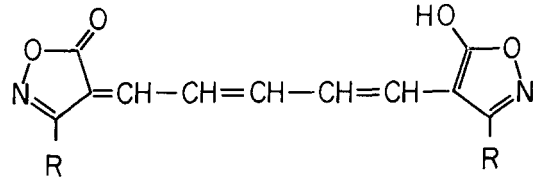
0022-2631/79/0046-0255 $05.60

usually quite intense, enabling the use of micromolar concentrations of these probes in membrane studies, thereby minimally perturbing the system under investigation. Most but not all of these probes are fluorescent which, in addition to providing additional sensitivity to conventional membrane suspension work, makes possible work on nontransmitting samples, such as intact organs by the use of surface fluorimetry (Chance, Mayevsky & Smith, 1976).

It is often possible to markedly alter the magnitude of the spectral responses of these probes to membrane energization by modifying substituent groups of the molecule which are not part of the optical chromophore but which control such factors as charge and membrane binding affinity. Using the giant axon from the squid, *Loligo peali*, Cohen *et al.* (1974) and Ross *et al.* (1977) have screened a large number of dyes and have identified a number of promising potential-sensitive probes which have been systematically improved based on the criterion of increased signal to noise ratio in experiments involving the application of potential gradients across the axon membrane by means of microelectrodes.

In a number of cases it has been possible to calibrate the spectral response of certain cyanine dyes such as diS-C_3-5 using valinomycin-induced diffusion potentials in the system under investigation. Using this approach, Laris, Bahr and Chaffee (1975) have measured the membrane potential in mitochondria, Hoffman and Laris (1974) and Sims *et al.* (1974) have measured the resting potential in the red cell, and Renthal and Lanyi (1976) have obtained the potential in *Halobacterium halobium*. Reviews on the design and use of extrinsic probes of membrane potential in a number of systems have appeared by Waggoner (1976) and by Cohen and Salzberg (1978). Waggoner, Wang and Tolles (1977) have also investigated the mechanism of the rapid response of certain cyanines and oxonols to a train of voltage pulses applied across a black lipid membrane. The dye merocyanine 540 has been employed by Salama and Morad (1976) as a probe of the action potential in the heart.

Studies (Chance *et al.*, 1974; Chance & Baltscheffsky, 1975) in our laboratory have been focused on a homologous series of oxonol dyes derived from the oxonol V probe, the structural, spectral properties, and energy-linked spectral changes of which have been described by Smith *et al.* (1976). Transient increases in the probe response are induced by agents such as NH_3 (Smith & Chance, 1976) and nigericin (Bashford & Thayer, 1977) which are known to abolish the ΔpH component of the electrochemical gradient. These findings indicate that these oxonols



OX-VI, R = $CH_2CH_2CH_3$

Fig. 1. The structure of the neutral form of oxonol VI. At pH values used in this work, the dye is a symmetrical anion since the pK of the hydroxyl proton is approximately 4

are sensitive to $\Delta\Psi$ only, which is increased as ΔpH is abolished in order to maintain the electrochemical gradient constant.

The derivative of oxonol V in which the phenyl groups bound to the isoxazolone rings have been replaced by propyl sidechains, oxonol VI [1], has proved to give much larger spectral shifts than any of the other members of our series in membrane preparations, such as photosynthetic bacteria chromatophores and submitochondrial particles in which the internal volume of the vesicle is positive with respect to the external phase. Oxonol VI has been used by Bashford and Thayer (1977) in a double probe experiment in which 9-aminoacridine was used as the probe of ΔpH, to obtain the value of the electrochemical gradient under equilibrium or steady-state conditions in submitochondrial particles.

In this communication, the kinetics of oxonol VI spectral changes in submitochondrial particles have been studied, and the implications of the findings for the mechanism by which the energy-linked spectral changes occur in this system are considered. The structure of the neutral form of oxonol VI is shown in Fig. 1. It should be borne in mind, however, that the dye is a symmetrical anion at physiological pH since the pK for the dissociation of the hydroxyl proton is approximately four (Smith *et al.*, 1976), and the resulting charge is delocalized over the conjugated system of the molecule.

Materials and Methods

Submitochondrial particles were prepared by the procedure described by Hansen and Smith (1964) which is only briefly outlined here. Heavy beef heart mitochondria were

1 *Abbreviations used:* ANS: 1-analino-8-naphthalene sulfonate; ATP: adenosine 5′-triphosphate, disodium salt, from equine muscle; CCCP: carbonyl cyanide *m*-chlorophenyl hydrazone; HEPES: N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; NADH: β-nicotinamideadenine dinucleotide; Oxonol VI: bis [3-propyl-5-oxoisoxazol-4-yl]pentamethineoxonol.

suspended at approximately 20 mg/ml protein in an incubation mixture consisting of 0.25 M sucrose, 10 mM K-HEPES at pH 7.5, 1 mM $MgCl_2$, 1 mM ATP, and 1 mM succinate. The suspension was stored frozen overnight and then thawed, and the mitochondria were isolated by centrifugation. The mitochondria were then resuspended at approximately 20 mg/ml protein in the medium described above with the following exceptions: the $MgCl_2$ concentration was increased to 5 mM, and 10 mM $MnCl_2$ was included in the mixture. The resulting mitochondrial suspension was sonicated at approximately 50 W by using a model W 185 Heat Systems Ultrasonics, Inc., cell disrupter for 35–40 sec. The submitochondrial particles were pelleted by ultracentrifugation of the supernatant obtained from an initial centrifugation to remove mitochondrial debris. The resulting preparation exhibits respiratory control.

The oxonol VI dye was synthesized by suitably modifying the procedure described by Smith *et al.* (1976) for the oxonol V probe. Relevant analytical data supporting the structure shown in Fig. 1 for the dye are given by Bashford *et al.* (1978b).

Sucrose, $MgCl_2$, $MnCl_2$, and K_2SO_4 were obtained from J.T. Baker Chemical Co. and were of reagent grade purity. All other compounds were purchased from Sigma Chemical Co. Experimental conditions are described in the appropriate figure captions.

Scanned absorption spectrum measurements were made using a Hitachi-Perkin Elmer model 356 spectrophotometer in the split beam mode. Rapid mixing experiments were carred out using a Johnson Foundation Model E (Chance, 1973) rapid mixing device with an 80:1 mixing ratio adapted to a Johnson Foundation 200 Hz time-sharing double beam spectrometer equipped with 500-mm Bausch and Lomb monochromators. The detector output was displayed on a type 564 Tektronix storage oscilloscope and a model 165 Perkin Elmer strip chart recorder as required. *See Results* for details. The titrations used in equilibrium binding investigations were monitored by a Beckman model UV 5270 spectrometer.

Results and Data Analyses

The observation of a red shift in the absorption spectrum of oxonol VI when either passive binding (Bashford *et al.*, 1978*b*) or substrate consumption by submitochondrial particles suspended in dye solution occurs (Fig. 2) was used as the basis for monitoring the time course of the spectral changes to be described in this section. Fluorescence changes could in principle have been used for this purpose; the sense as well as the magnitude of the emission intensity change, however, are functions of dye-to-particle ratio, which in the experiments to be described is an experimental variable. The changes in transmission are thus more easily interpreted. The apparent isosbestic point at 603 nm (Fig. 2) provided a convenient reference, while the large change at 630 nm was used as the measuring point in the double beam transmission experiments.

Two basic types of rapid mixing experiments are reported in this communication. The first and by far the simpler to analyze is the passive binding experiment in which the major syringe of the rapid mixing device contains dye solution mixed with submitochondrial particles via the minor syringe. The time course of the passive binding process was suffi-

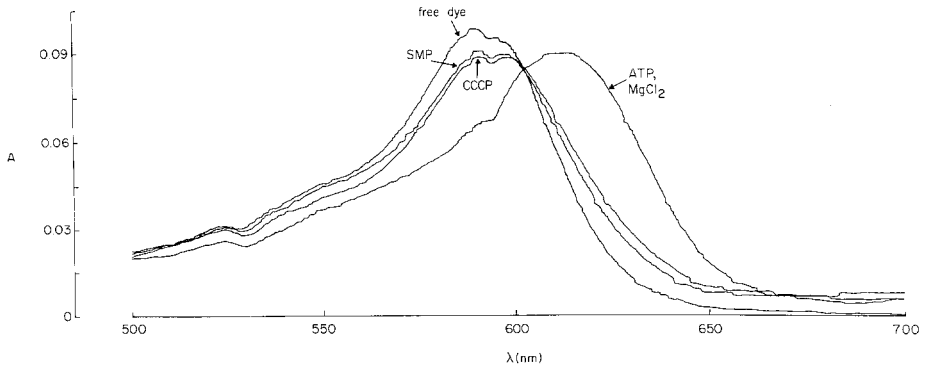


Fig. 2. The absorption spectrum of oxonol VI as the free dye, as slightly red shifted when the dye is bound to the submitochondrial particle membrane, the large red shift induced by ATP energization of the particles, and the complete reversal of the red shift by addition of the uncoupler CCCP. Medium: 0.25 M sucrose, 5 mM Na-HEPES at pH 7.5; concentrations: 1.2 μM oxonol VI, 0.25 mg/ml ATP-Mg^{++}-Mn^{++} submitochondrial particle protein, 1.7 mM ATP-$MgCl_2$, 5 μM CCCP

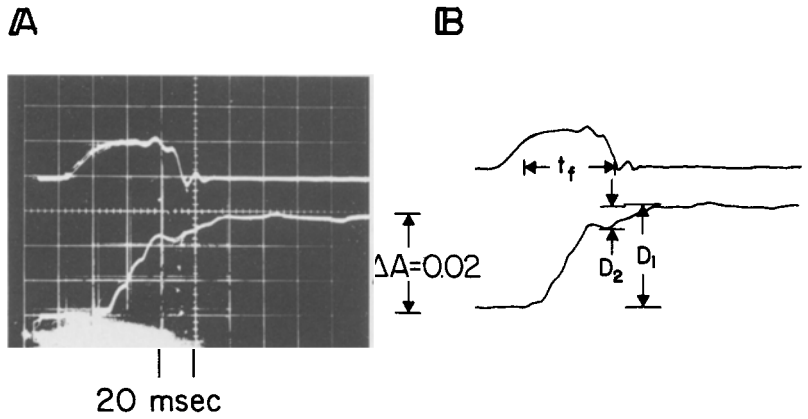


Fig. 3. (A): The time course of the red shift measured as the difference in transmission at 603–630 nm caused by passive binding of oxonol VI to submitochondrial particles. Medium: 25 mM K_2SO_4, 10 mM K-HEPES at pH 7.5, 0.25 M sucrose. Concentrations: 15 μM oxonol VI, 3 μM CCCP, 0.09 mg/ml submitochondrial particle protein; time constant: 3 msec. (B): A diagram illustrating the determination of the apparent first-order rate constant from the oscilloscope trace using Eqs. (1) and (2). In this experiment $k_{app} = 150\ sec^{-1}$. The flow velocity trace is in the upper portion of the diagram, and the lower trace is the time course of the optical signal. *See* text for details

ciently rapid that the results could be analyzed in terms of the continuous flow case (Chance, 1973) since the signal reached a plateau before the flow through the observation tube ceased. A typical photograph of an oscilloscope record of a passive binding experiment is presented in

Fig. 3*a*. The apparent first-order rate constant k_{app} is given by (Chance, 1973)

$$k_{app} = (2.3/t_m) \log (D_2/D_1). \quad (1)$$

The displacements D_1 and D_2 are defined in Fig. 3*b*; t_m is related to t_f which is obtained from the flow velocity trace (Fig. 3*b*) by

$$t_m = t_f (V/V_d) \quad (2)$$

where V is the volume from the point of observation to that of mixing and V_d is the volume discharged in time t_f. These characteristic volumes must be determined for the particular mixing device in use. Since the rate constant for the second-order binding process was to be determined, the submitochondrial particle concentration was adjusted such that the dye would be the reactant in excess. Under these conditions, the apparent first-order rate constant is related to the second-order rate constant by

$$k_{app} = k_{2nd} X_D \quad (3)$$

where X_D is the concentration of the reactant in excess, which in this case is the oxonol VI dye. A plot of k_{app} *vs.* X_D will thus be a straight

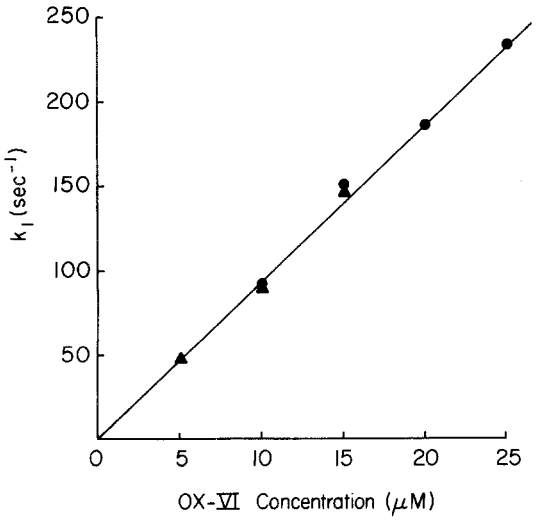


Fig. 4. A plot of the apparent first-order rate constant k_{app} *vs.* dye concentration for the passive binding of oxonol VI to submitochondrial particles with the dye in excess. The second-order rate constant is obtained from the slope of the line as described by Eq. (3) in the text. Experimental conditions are given in the caption to Fig. 3*a*. The value of the second-order rate constant is given in Table 1. Data taken in the absence (●) and presence of 3 μM CCCP (▲) fall on the same line, indicating the lack of endogenous substrate in the submitochondrial preparation

line the slope of which gives k_{2nd}. Such a plot for the passive binding experiment in which the submitochondrial particle concentration was held constant and that of the dye varied is presented in Fig. 4. The value of the second-order rate constant is presented in Table 1.

In the second type of rapid mixing experiment, the major syringe of the rapid mixing device contained submitochondrial particles suspended in oxonol VI dye solution in which the passive binding process had reached equilibrium. That the latter process was complete could be easily ascertained by simply discharging the contents of the major syringe through the observation tube with the minor syringe empty and the valve connecting the minor syringe to the mixing chamber closed. A flat line was obtained, indicating that no passive binding was in progress.

After equilibrium was reached, various substrates or oxygen were mixed with the submitochondrial particle-oxonol VI suspension via the minor syringe under a number of conditions, some of them of a control nature, to be described subsequently. The time course of the spectral shift caused by substrate or oxygen addition was such that only a negligible optical change occurred during the time of flow, i.e., the stopped flow case was applicable for data analysis.

Initial substrate pulse experiments were carried out with NADH. The time course of the resulting signal for a typical experiment is shown in Fig. 5*a*. The analysis of these data are illustrated in Fig. 5*b*. Since on a log scale the data at long time was reasonably linear with time,

Table 1. Summary of rate constants characterizing the kinetics of oxonol VI in submitochondrial particles

Remarks	Rate constants
Passive binding, dye in excess	$9.53\ (\pm 0.35) \times 10^6\ \text{M}^{-1}\ \text{sec}^{-1}$
Oxygen pulse	$2.40\ (\pm 0.40) \times 10^5\ \text{M}^{-1}\ \text{sec}^{-1}$
Decay of oxygen pulse-induced dye response	$0.218\ (\pm 0.001)\ \text{sec}^{-1}$
NADH, faster process	$2.70\ (\pm 0.40) \times 10^5\ \text{M}^{-1}\ \text{sec}^{-1}$
ATP pulse, faster process	$3.10\ (\pm 0.20) \times 10^5\ \text{M}^{-1}\ \text{sec}^{-1}$
ATP pulse, slower process	$0.33\ (\pm 0.04)\ \text{sec}^{-1}$
ATP pulse, faster process, K_2SO_4 absent	$2.46\ (\pm 0.20) \times 10^5\ \text{M}^{-1}\ \text{sec}^{-1}$
ATP pulse, slower process, K_2SO_4 absent	$0.55\ (\pm 0.15)\ \text{sec}^{-1}$

Medium except as noted: 0.25 M sucrose, 25 mM K_2SO_4, 10 mM K-HEPES, pH 7.6. For the second order rate constants, the concentration unit refers to oxonol VI concentration; the numbers in parantheses are the standard errors in the values of the rate constants derived from fits by linear regression. The mean values and standard deviation are reported for the slower process observed in the ATP pulse experiments.

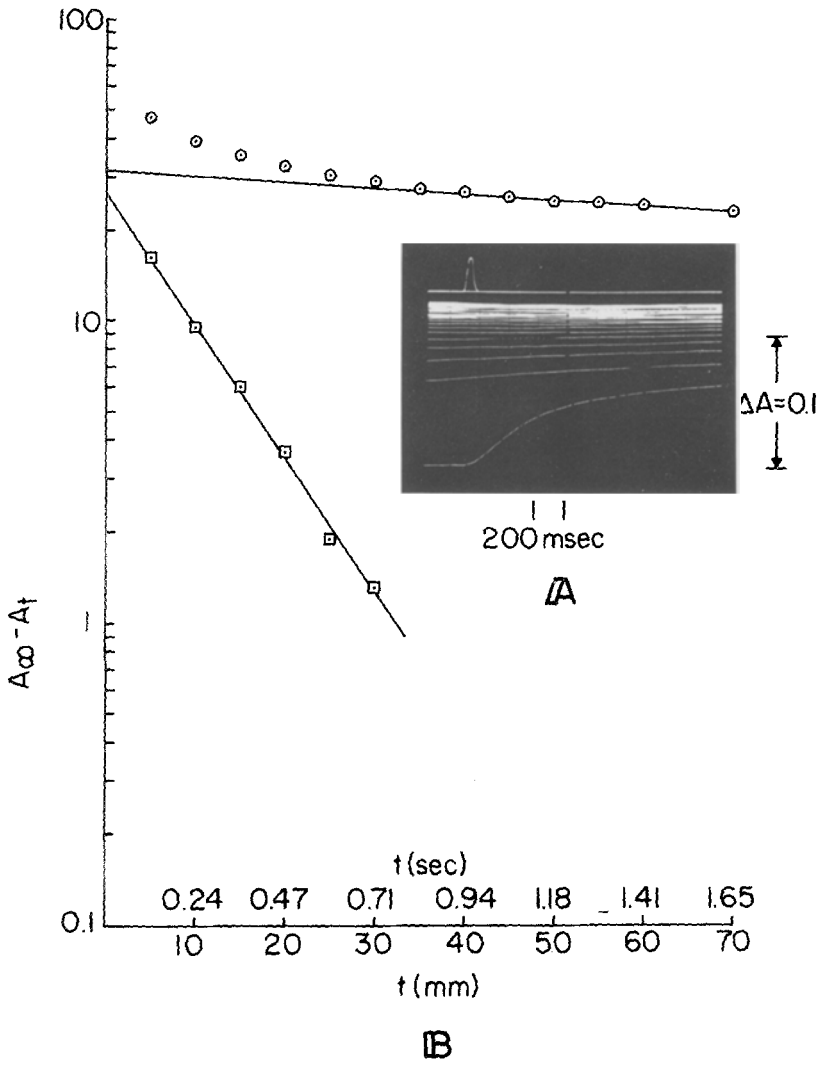


Fig. 5. (A): A photograph of an oscilloscope record of the time course of the oxonol VI red shift induced by a NADH pulse to submitochondrial particle suspension in the dye solution. The scope was allowed to sweep repeatedly until no further change was observed. The displacement at the latter time was taken as A_∞. Medium: 0.25 M sucrose, 10 mM K-HEPES at pH 7.6, 25 mM K_2SO_4. Concentrations: 15 μM oxonol VI, 0.3 mg/ml submitochondrial particle protein, 0.1 M NADH (initital concentration). (B): The graphical procedure used to extract the apparent first-order rate constant for the faster process by subtracting the estimated contribution of the slower process from the experimental data at selected time intervals. *See* text for details

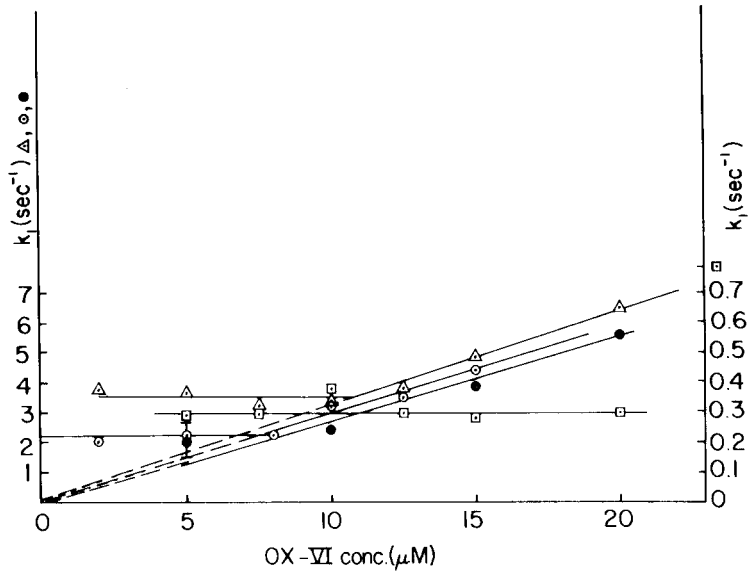


Fig. 6. Plots of the apparent first-order rate constants as a function of oxonol VI concentration: ⊙, NADH pulse faster process, 0.15 mg/ml protein; ●, oxygen pulse, faster process, 0.47 mg/ml protein; △, ATP pulse, faster process, ⊡, ATP pulse, slower process, 0.30 mg/ml submitochondrial particle protein. Values of the rate constants are collected in Table 1

the line through the latter points was extrapolated to the ordinate. The amplitude of the extrapolated line was subtracted from the experimentally observed amplitude at selected time intervals in which curvature was present in the plot of the data. The resulting differences are plotted as the line of steeper slope in Fig. 5*b*. The preceding procedure was adequate for extracting an apparent first-order rate constant for the faster process from the experimental data. The apparent first-order rate constant for the faster process was plotted as a function of dye concentration as shown in Fig. 6. The plot is linear with dye concentration over a range of concentration values, then the plot breaks and becomes independent of dye concentration. This behavior is consistent with a second-order process and will be discussed further in this section. The second-order rate constant value for the NADH pulse experiments is given in Table 1.

Although the procedure described above was adequate for obtaining a rate constant for the faster process, the rate constants for the slower process could not be accurately determined primarily due to a lack of data at longer time. If, however, the time scale of the oscilloscope was compressed to accommodate the slower process, the details of the faster

one would be obscured. In order to gather data over extended periods of time without losing information on faster events, a strip chart recorder was connected to the detector system in addition to the usual storage oscilloscope. The recorder chart drive was switched on prior to mixing, and the substrate pulse experiments were repeated with ATP. Since the time scales of the oscilloscope trace and the recorder overlapped at time periods slightly longer than 1 sec, a scale correction factor could be obtained from the ratio of the measured amplitudes of the two traces at a convenient point in time. It was necessary to select a time longer than 1 sec since the latter value is the approximate rise time of the recorder. After the data was adjusted to a common amplitude scale, it was stored in the memory of a Digital Equipment Corporation PDP 10 computer and fitted to a function of the form

$$A(t) = \sum_{n=1-3} A_\infty [1 - \exp(-k_n t)]. \tag{4}$$

The data for the ATP pulse experiments were satisfactorily fitted using two exponential terms, $n=2$ in Eq (4). Extending the fit to three exponentials did not significantly alter the rate constants obtained from the two-exponential fit, indicating that the faster and slower processes were adequately resolved by the two-term fit. Furthermore, the rate constant k_3 varied in apparent random fashion and was some two to three orders of magnitude smaller than k_2 and hence is negligible. A typical oscilloscope trace, strip chart recorder trace, computer fit to the ATP pulse data, and a decomposed log plot are shown in Fig. 7*a–d*, respectively. The apparent first-order rate constant for the faster process was a linear function of the dye concentration.[2] The second-order rate constant obtained from the slope of the concentration dependence of the apparent first-order rate constant was essentially equal to that obtained from the NADH pulse experiments. The numerical value is given in Table 1 and the plot of k_{app} *vs.* dye concentration is included in Fig. 6.

The breaks in the plots of Fig. 6 at which the slope becomes zero are a consequence of the breakdown of the pseudo first-order condition

2 The apparent first-order rate constant for the faster process observed in NADH pulse work also varied linearly with membrane concentration under conditions in which the submitochondrial particles were the reactant in excess. The second-order rate constant was roughly 2 sec^{-1} (mg protein/ml)$^{-1}$. The binding stoichiometry for the faster process can be computed from the ratio of the second-order rate constants with the dye and membrane respectively in excess; the value is approximately 7 nmol oxonol VI/mg protein. Investigations of the time course of oxonol VI interaction with the particle membrane with the latter preparation in excess were not persued in detail because of the prohibitively large quantities of the membrane preparation required.

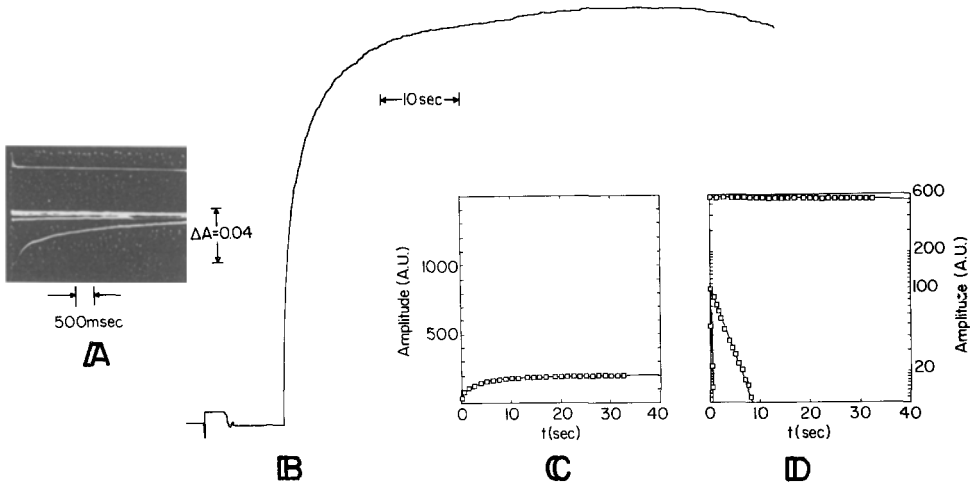


Fig. 7. Typical ATP pulse data. (A): An oscilloscope trace for recording primarily the faster process. (B): A strip-chart recorder trace for recording the corresponding slower process. (C): The computer fit to the data using the three-exponential form of Eq. (4). (D): The decomposed log plots from which the three apparent first-order rate constants are obtained. For the data illustrated in this figure, $k_1 = 3.90\ \text{sec}^{-1}$, $k_2 = 0.26\ \text{sec}^{-1}$, and $k_3 = 0.001\ \text{sec}^{-1}$. Thus k_3 is negligible compared to the remaining two rate constants. Medium: 0.25 M sucrose, 10 mM K-HEPES at pH 7.6, 25 mM K_2SO_4; concentrations: 12.5 μM oxonol VI, 0.30 mg/ml submitochondrial particle protein, 0.1 M ATP-$MgCl_2$ (initial concentration)

that the dye concentration greatly exceeds the number of membrane binding sites. As the dye concentration is lowered, a region is reached where this condition is no longer met; the concentration of binding sites equals or exceeds that of the dye. Since the membrane concentration is not a variable, the slope becomes zero. Note that the break in the plot derived from NADH pulse experiments occurs at a lower dye concentration than that for the plot resulting from the ATP pulse case. The latter difference is expected since the submitochondrial particle concentration for the ATP pulse experiments was twice that for the NADH pulse case.

The third type of pulse experiment was one in which a suspension of submitochondrial particles in an oxonol VI solution containing NADH was allowed to become anaerobic in the major syringe of the rapid mixing device. The onset of anaerobiosis could be followed by monitoring the blue spectral shift of the oxonol VI spectrum, i.e., the reversal of the red shift induced by energization with NADH. When anaerobiosis was complete, oxygenated buffer was mixed with the contents of the major syringe and the time course of the red shift recorded. Because

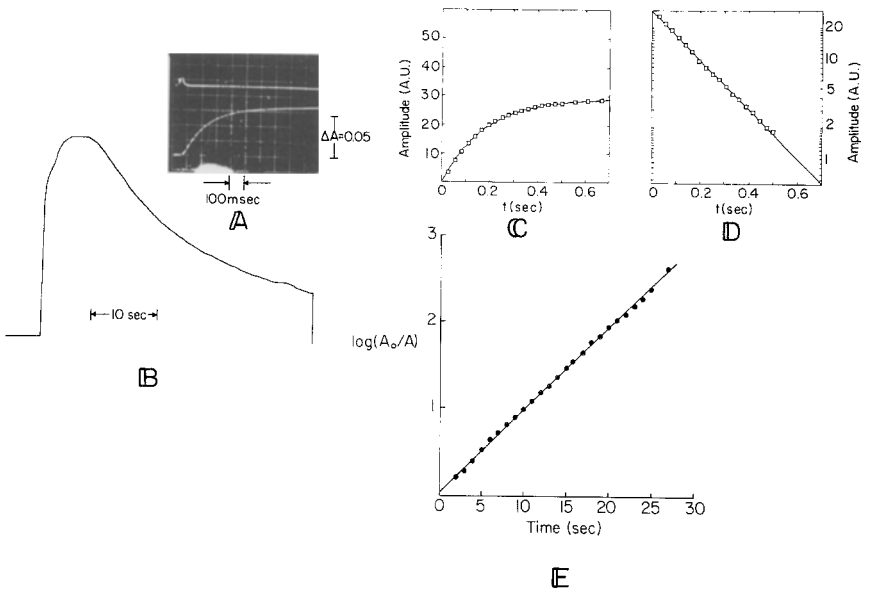


Fig. 8. Typical oxygen pulse data. (A): An oscilloscope trace for recording the faster second-order process. (B) The corresponding strip-chart recorder trace illustrating that the slower process is not significantly developed due to the rapid depletion of the oxygen. (C): The computer fit to the data using a single exponential form of Eq. (4). (D): The decomposed log plot from which an apparent first-order rate constant of 5.66 sec^{-1} is obtained from this data. Medium: 0.25 M sucrose, 10 mM K-HEPES at pH 7.6, 25 mM K_2SO_4. Concentrations: 20 mM oxonol VI, 0.47 mg/ml submitochondrial particle protein, nominally 270 μM O_2 in medium (initial concentration). (E): A semilog plot illustrating the exponential decay of the probe red shift after the oxygen is consumed. The first-order rate constant is given in Table 1

of the rapid depletion of oxygen, the slower process did not develop significantly in these experiments. The data were accordingly fit by a single exponential version of Eg. (4). Typical results are presented in Fig. 8*a–e*. Note that the recorder trace returned to the initial baseline as the oxygen was depleted. The oxygen pulse experiments could thus be repeated in a regenerative fashion. The requirement of somewhat higher submitochondrial particle concentration in these experiments in order to reach anaerobiosis in a reasonable time period (15–30 min) and since the dye concentration could not be increased above approximately 20 μM because of excessive absorbance allowed data to be collected over a somewhat narrower range than in the preceding two substrate pulse cases. The apparent first-order rate constants, however, exhibited a linear dependence on dye concentration. The resulting second-order rate constant was not statistically different from those derived from

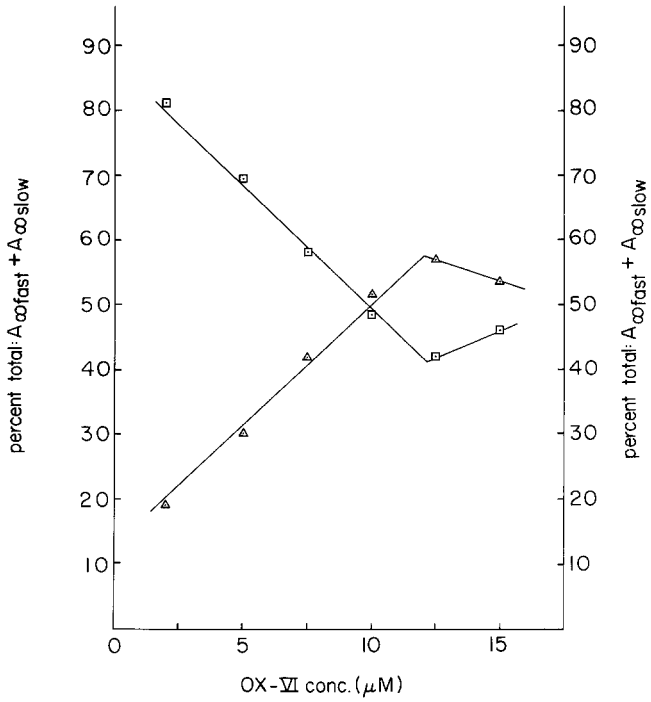


Fig. 9. The percentage contribution of the fast (□) and slower (△) processes to the total amplitude of the change induced by an NADH pulse to submitochondrial particles suspended at 0.15 mg/ml protein concentration in oxonol VI solutions of varying concentration plotted as a function of the dye-to-particle ratio. Medium: 25 mM K_2SO_4, 0.25 mM sucrose, 10 mM Na-HEPES at pH 7.6, 0.1 M NADH pulse (initial concentration). *See* text for interpretation

the previous two experiments. The oxygen pulse plot is included in Fig. 6, and the numerical value is given in Table 1. The decay of the oxonol VI red shift shown in Fig. 8*b* follows first-order kinetics with a halftime of about 3 sec; the first order plot is presented in Fig. 8*e*. The value of the rate constant is collected in Table 1.

In the ATP and NADH pulse experiments, the red shift decayed over a period of approximately 30 min and followed complex kinetics which could not be fitted by a first order plot. The onset of this decay can be seen in Fig. 7*b*, but it was not routinely monitored.

A number of control experiments were carried out using the uncoupler CCCP and valinomycin. At approximately 10 nmol CCCP per mg submitochondrial particle protein, the signal due to NADH pulses was virtually completely abolished. The addition of valinomycin greatly attenuated both the amplitude and rate of formation of the spectral shift resulting from NADH injection, but the signal was not completely abolished,

and the biphasic time course was still observed. The presence of valinomycin at approximately 1 μg per mg particle protein was necessary to reduce the signal amplitude by a factor of 5 to 10 and the rate constant by an order of magnitude in comparison to experiments carried out in the absence of the ionophore. The medium in the control experiments described above was as described in Table 1.

The relative contributions of the fast and slower processes to the magnitude of the spectral changes induced by NADH pulses as the dye to submitochondrial particle ratio was varied was obtained from the ordinate intercepts resulting from the hand analysis of these experiments as previously described. The contribution of the slower process increased as the dye to particle ratio increased in experiments in which the dye concentration was held constant and the membrane concentration varied and in those in which the latter concentration was kept fixed and that of the dye varied. The approximate linear increase in the contribution of the slow process to the signal amplitude under the latter conditions is illustrated in Fig. 9. The plot exhibits saturation behavior at high dye concentrations. The significance of these findings with regard to possible permeation of the submitochondrial particle membrane by the dye will be considered in *Discussion.*

Equilibrium Binding Investigations

The binding of oxonol VI to energized and resting submitochondrial particles was also investigated under equilibrium conditions in order to provide additional insight into the time resolved studies. The interaction of the dye with a membrane under conditions in which the concentration of the dye is held constant and that of the membrane varied can be described by Eq.(5) (Bashford & Smith, 1978*a*)

$$\varepsilon - 1 = (\varepsilon_b - 1) - (K_d/n)\ (\varepsilon - 1)/m \tag{5}$$

where m is the membrane concentration, K_d the dissociation constant, and n the maximum number of binding sites available to the dye. The quantity ε is given by the ratio P/P_{initial} where P is some experimental parameter that is monitored as the membrane concentration is varied; P_{initial} is the value of the parameter in the absence of membrane. ε_b is the limiting value of ε obtained as m approaches infinity so that all dye is bound. Equation (5) indicates that a plot of $\varepsilon - 1$ *vs.* $(\varepsilon - 1)/m$ will be a straight line the slope of which is $-K_d/n$ and the intercept

on the ordinate $\varepsilon_b - 1$. In titrations of oxonol VI with submitochondrial particles, the monitored parameter P was chosen as the dye absorbance at 630 nm, which is sensitive to dye binding because of the spectral red shift illustrated in Fig. 2. K_d/n is obtained as a membrane concentration, the only independent variable in these titrations. Results of such titrations of oxonol VI with resting and ATP-$MgCl_2$ energized submitochondrial particles are illustrated in Fig. 10. Note that K_d/n is about an order of magnitude greater for resting particles than for the energized preparation. The latter observation indicates that more dye is associated with the energized submitochondrial particle membrane than with the resting preparation, although whether the enhanced binding is due to changes in K_d, n or both quantities cannot be determined from such plots.

More detailed information on the interaction of oxonol VI with the submitochondrial particle membrane can be obtained from titrations of fixed quantities of the membrane preparation with dye. Equations

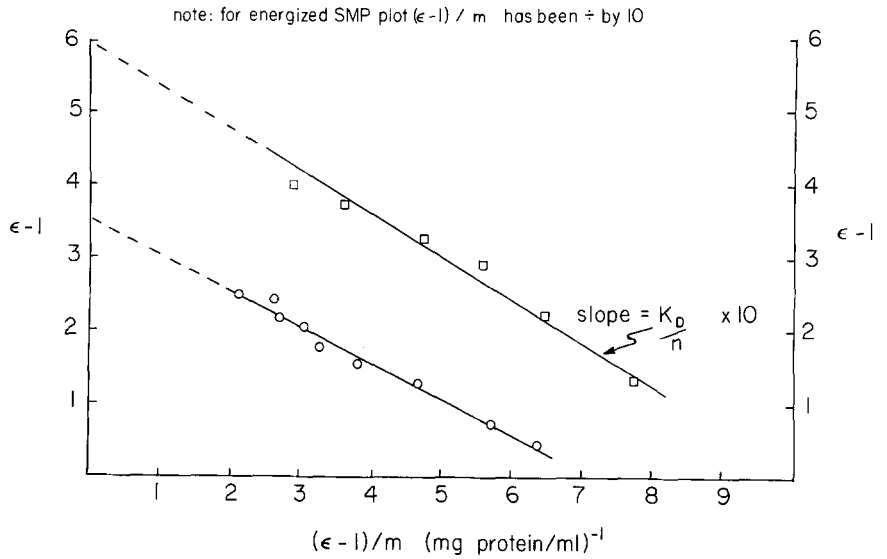


Fig. 10. Semireciprocal plots of data obtained from titrations of 2 μM oxonol VI with resting (○) and ATP-energized (□) submitochondrial particles. From Eq. (5), the slope of these plots gives $-K_d/n$ and the intercept on the ordinate $\varepsilon_b - 1$. K_d/n is about an order of magnitude larger for the resting preparation than for the energized one indicating that more dye is associated with the energized particles than with the resting ones. For the plot obtained using energized particles. $(\varepsilon - 1)/m$ has been divided by 10 for convenience in graphing the data. Values of K_d/n obtained from such plots are given in Table 2. Medium: 0.25 M sucrose, 10 mM Na-HEPES at pH 7.5, 25 mM K_2SO_4. For the energized particle case, the medium contained 28 mM ATP-$MgCl_2$

such as the Langmuir isotherm (McLaughlin & Harary, 1976) and the Scatchard relationship (Scatchard, 1949) which describe the hyperbolic nature of such titrations can be written in the following form

$$n = s + (s/A)K_d \tag{6}$$

where s is the amount of dye bound per unit membrane and A is the free dye concentration; the other quantities have been previously defined. In developing the direct linear procedure for analyzing data described by a hyperbolic relationship, Cornish-Bowden and Eisenthal (Eisenthal & Cornish-Bowden, 1974; Cornish-Bowden & Eisenthal, 1974; Porter & Trager, 1977) have shown that for any experimentally observed s_i and A_i in exact agreement with Eq. (6), n and K_d will lie on a straight line through the points $(-A_i, 0)$ and $(0, s_i)$ in $n-K_d$ space. Thus a pair of lines corresponding to two experimental observations s_i, A_i and s_j, A_j will intercept at a point in $n-K_d$ space and provide values for n and K_d. For k pairs of points there will be $\frac{1}{2}k(k+1)$ such intersections. Since in practice, the experimental observations will contain errors, the intersections will occur over a finite area in $n-K_d$ space. In the direct linear method, the values of n and K_d which best fit the experimental data are the median values in the series of intersections arising from the several sets of data. The latter conclusion assumes only that the errors are as likely to be negative as positive and avoids the necessity of a detailed knowledge of error distribution which must be taken into account in fitting the various forms of hyperbolic binding equations by a least squares procedure.

Since the number of data points in the dye titrations was approximately 20, a computer routine was used to evaluate the values of n and K_d corresponding to the large number of intersections. A sorting routine was then employed to obtain the median values of n and K_d. Experimentally, since the total dye concentration is known as well as the membrane concentration, the desired quantities A and s can be calculated if the fraction of bound dye can be determined. The latter quantity can be obtained from $(\varepsilon-1)/(\varepsilon_b-1)$ (Bashford & Smith, 1978*a*) where ε_b-1 is determined from the plots described previously; ε was obtained from the ratio of the dye absorbance at 630 nm in the presence of submitochondrial particles to that of the free dye at the same concentration. Typical results of the binding investigations using both types of titrations are summarized in Table 2. The maximum number of binding sites is smaller and the dissociation constant is larger for the resting membrane preparation than for the energized one. Note the agreement of the ratios

of K_d/n for the energized preparation relative to that for the resting one obtained from the two titration procedures.

Table 2. Summary of results from equilibrium binding titrations using the oxonol VI submitochondrial particle system

State of submitochondrial particle preparation	Submitochondrial particle titration of oxonol VI K_d/n (mg SMP protein/ml)	Oxonol VI titration of submitochondrial particles	
		K_d (μM dye)	n (nmole dye) / mg SMP protein
Resting	0.49	13.6	16.5
ATP-$MgCl_2$ energized	0.058	4.4	42.5
	(K_d/n) resting/ (K_d/n) energized = 8.4	(K_d/n) resting/ (K_d/n) energized = 8.0	

Medium: 0.25 M sucrose, 10 mM Na-HEPES, pH 7.5, 25 mM K_2SO_4; submitochondrial particle concentration (dye titration): 0.087 mg protein/ml; oxonol VI concentration (submitochondrial particle titration): 2 μM. The same stock submitochondrial particle preparation was used in both titrations.

Discussion

The time course of the oxonol VI spectral red shift induced by NADH, ATP, and oxygen pulses in suspensions of submitochondrial particles in dye solutions of varying concentration is distinctly biphasic for the NADH and ATP cases, whereas that for the oxygen pulse is well fitted by a single exponential analysis since the oxygen is consumed sufficiently fast that the slower process is not developed significantly. In each of the three cases described above, the faster process is characterized by a second-order rate constant of approximately 3×10^5 M^{-1} sec^{-1}; for the ATP pulse experiments, the slower process is described by a first-order rate constant of approximately 0.3 sec^{-1}. The decay of the spectral change induced by oxygen pulses is also characterized by a first-order process with $k \sim 0.2$ sec^{-1}.

Analysis of the binding of oxonol VI to resting and energized submitochondrial particles by a number of procedures has led to the conclusion that more dye is associated with the energized membrane preparation than with the resting one. The preceding observation plus the second-order rate law describing the faster process suggest that the rate-limiting step in this process is the transfer of dye from the aqueous phase to the submitochondrial particle membrane.

In contrast to the value of $\sim 3 \times 10^5$ M^{-1} sec^{-1} obtained in energization experiments, the rate constant for the passive binding process is some 30 times larger, $\sim 9 \times 10^6$ M^{-1} sec^{-1}. There are at least three possible explanations for the difference in rate constants: (i) the concentration of the free dye in the energization experiments has been significantly reduced by passive association with the submitochondrial particle membrane prior to the introduction of substrate or oxygen; (ii) the concentration of the dye in the volume immediately adjacent to the membrane surface is lower than that of the dye in the bulk phase because of electrostatic repulsion due to an increased surface potential caused by binding of the dye prior to the injection of substrate or oxygen; and (iii) the intrinsic affinity of the membrane binding sites for the dye has been changed as a consequence of energization. The amount of dye bound to resting submitochondrial particles can be obtained from the dye titration data for fixed particle membrane concentration approximately the same as that used in the rapid mixing experiments. At 10 μM total dye concentration, the lower limit for which pseudo first-order conditions were usually present, the amount of bound dye is less than 30% of the total. If this percentage were valid for all the dye concentrations used in obtaining the second-order rate constant for the faster energization process, the revised figure would become $\sim 4 \times 10^5$ M^{-1} sec^{-1}, which is clearly well below that for passive binding. The percent of bound dye will decrease, however, as the total dye concentration is increased due to the saturation tendency of the dye binding curve. Thus, the passive binding of oxonol VI prior to energization is unlikely to sufficiently lower the effective dye concentration to account for the 30-fold difference in the second-order rate constants for the faster energization process and for passive binding.

The second possibility for the difference in rate constants is that passive binding of the dye anions increases the magnitude of the particle surface charge density σ. The electrostatic potential Ψ_0 in the aqueous phase immediately adjacent to the membrane is related to the surface charge density by (McLaughlin & Harary, 1976)

$$\sinh (F\Psi_0/RT) = A\sigma/\sqrt{C} \tag{7}$$

where F, R, and T have the usual definitions; C is the electrolyte concentration in molar units, and A is a constant dependent on temperature and dielectric constant. Since at equilibrium, the concentration of dye at the surface of the particle $[D]_0$ is related to the bulk dye concentration $[D]$ by the Boltzmann relationship

$$[D]_0 = [D] \exp [F\Psi_0/RT] \quad (8)$$

an increase in σ at constant electrolyte concentration will increase the magnitude of Ψ_0 which is negative of course and reduce the effective dye concentration $[D]_0$. The location of the dye suggested by orientation studies using a model membrane system (Smith & Powers, 1978) indicates that the dye occupies a region near the the polar headgroup-bulk phase interface with the charged chromophore in likely contact with the aqueous medium. Such a location of the dye in the submitochondrial particle membrane would be expected to increase the surface charge density and supports the argument presented above.

Equation (7) suggests that the electrostatic potential Ψ_0 may be altered if the electrolyte concentration C is varied, provided that the surface charge σ does not change in a compensating fashion. The second-order rate constant observed for the faster membrane energization process would be altered because of the effect of such a change on the effective dye concentration $[D]_0$. The value of the second-order constant obtained from ATP pulse experiments in the absence of K_2SO_4 was only barely statistically different from that obtained in the presence of the salt and is clearly well below that for passive binding (*see* Table 1). However, since the surface charge may also decrease when the salt is removed from the medium because of reduced shielding of the electrostatic repulsion between neighboring bound dye molecules, the two effects could essentially cancel leaving Ψ_0 virtually unchanged. The binding constants of oxonol VI and derivatives of it are known to be quite sensitive to electrolyte concentration from investigations of the binding of these oxonols to phospholipid vesicles (Smith & Powers, 1978). In a carefully controlled experiment, the effect of the K_2SO_4 on the binding of oxonol VI to submitochondrial particles was investigated using the procedures described in *Results*. The ratio of the maximum number of binding sites, n, obtained in the presence and absence of 25 mM K_2SO_4 in a 0.25 M sucrose, 10 mM Na-HEPES medium at pH 7.5, 2.28, was nearly equal to the ratio of the square root of the electrolyte concentrations, 2.45. Since n is proportional to σ, the preceding result suggests that Ψ_0 is maintained essentially constant as the electrolyte concentration is varied, thereby explaining the independence of the second-order rate constant observed in the ATP pulse experiments on the latter concentration. Since the sulfate ion is divalent, the electrolyte concentration in Eq. (7) should more properly be replaced by the ionic strength. For the experiment described above, the ratio of the square roots of the

ionic strengths is 4, and the agreement with the ratio of the values of n becomes less satisfactory. Equation (7), however, is strictly valid only if the initital surface charge on the membrane is zero and if the distribution of the charge due to dye binding is uniform. These conditions are unlikely to be rigorously met in the case of the submitochondrial particle membrane, especially the latter one because of the highly invaginated nature of the membrane due to the protruding ATPase complex.

In view of the preceding results, the increase in the membrane surface charge density due to passive dye binding remains a viable explanation for the difference in rate constants for the faster energization process and passive dye binding. It should be noted, however, that charge effects such as those above would be difficult to distinguish from a change in the intrinsic affinity of the dye binding sites as a consequence of membrane energization.

Slower Phase

The contribution of the slower process to the total amplitude of the optical signal caused by the spectral red shift on energization of the submitochondrial particle suspensions suggests a possible mechanism by which the process occurs. In both the NADH and ATP pulse experiments, the percentage contribution of the slow phase increases as the dye-to-particle ratio increases. The appearance of a slower phase has also been observed by Ross *et al.* (1977) in experiments with the giant axon from the squid, *L. peali,* when the concentration of the bathing solution was increased. In the NADH case in which the oxonol VI concentration was varied at constant membrane concentration (Fig. 9), the contribution of the slower process was observed to saturate in the 10–15 μM dye concentration range. A plausible but not unique interpretation of the slower process suggested by the saturation behavior is that the dye is moving through the membrane and binding to the inner surface of the bilayer, the binding sites of which are eventually saturated as the concentration is increased at constant membrane concentration. Evidence for permeation of membranes by a number of dyes of the oxonol and cyanine classes has been obtained. Bashford, Chance and Prince (1978*a*) have recently reported that the decay of the carotenoid band shift in *Rhodopseudomonas sphaeroides* chromatophores is accelerated in the presence of oxonol VI. Since the bandshift is an electrochromic indicator of membrane potential, the acceleration of the decay of this shift implies that

the potential is being reduced by movement of the negatively charged dye across the membrane bilayer and into the inside-positive inner volume of the chromatophore vesicle. Oxonol VI and derivatives of it are also known to permeate black membranes in proportion to the groups attached to the isoxazolone rings (S. Krasne, *personal communication*). Waggoner *et al.* (1977) have also observed that a number of cyanines and oxonols (of somewhat different structure than oxonol VI but negatively charged) are able to cross a black lipid membrane under the influence of a potential gradient applied by electrodes.

A mechanism in which the rate-limiting step is the occupation of sites the concentration of which exceeds that of the dye available to occupy them would be expected to be independent of dye concentration as observed for the slower process. If the occupation of such sites were followed by a rapid movement of the charged dye through the hydrophobic portion of the bilayer, a large fraction of these sites would be free at any given time; such a process would also avoid the accumulation of an appreciable concentration of the charged probe in the hydrophobic region which is energetically unfavorable. The increase in the contribution of the slower phase to the total signal amplitude as the dye to particle ratio increases would in this scheme result from the enhanced probability of occupation of the sites from which permeation of the hydrophobic core occurs. Whether the occupying dye species would come directly from the aqueous phase or from another previously occupied membrane binding site could not be distinguished in this model.

Haynes and Simkowitz (1977) have discussed a mechanism for ANS permeation of phospholipid vesicle bilayers involving the intercalation of the probe between headgroups followed by rapid movement across the hydrocarbon phase of the bilayer. Such a mechanism would not be inconsistent with the information available on the slower portion of the oxonol VI energy-linked response in submitochondrial particles. The rate constant for this process in ATP pulse experiments is comparable to those reported by Haynes and Simkowitz for valinomycin-facilitated transport of ANS across the vesicle bilayer membrane. Although the similarity of rate constants may be coincidental, it is clearly possible that the shielding action of valinomycin and K^+ may compensate for the localized charge that ANS bears whereas the negative charge of oxonol VI is delocalized over the conjugated system of the molecule. Waggoner *et al.* (1977) have found, for example, that in black lipid membranes across which a potential gradient is applied, the current density due to M-540 which bears a localized negative charge is much

lower than that of a number of cyanines and oxonols whose charges are delocalized.

Recently, direct evidence for the permeation of the submitochondrial particle membrane by oxonol VI has been obtained. Using a time-shared double beam spectrometer, the time course of the oxonol VI red shift and the energy-requiring shift of the cytochrome *c* oxidase Soret band were monitored in hand mixing experiments in which ATP was added to a submitochondrial particle suspension in an oxonol VI solution. The halftime of the energy-linked oxidase shift was increased from approximately 7 sec in the absence of oxonol VI to about 12 sec in the presence of 5 μM dye (J.C. Smith, C.L Bashford, and M.K.F. Wikström, *unpublished*). This observation indicates that a portion of the membrane potential developed from ATP consumption is used to pump the dye across the membrane. The approximately 5-sec increase in the halftime for the Soret band shift is about the same as the halftime for the slower dye process as one would expect for an energy-requiring dye permeation mechanism. It was also noted that the Soret band shift did not appear to develop until the faster process was largely complete. Whether this observation will require a faster permeation process than so far considered will require additional work.

Decay Kinetics

The decay of the oxonol VI spectral red shift induced by an oxygen pulse to an anaerobic submitochondrial suspension follows a first-order rate law with a halftime of approximately 3 sec. The value of the rate constant is included in Table 1. The exponential decay of the probe red shift in the submitochondrial particle system is in contrast to the second-order nature of the time course of the decay of the dye spectral shift caused by light activation in chromatophores from photosynthetic bacteria (Bashford *et al.*, 1978*a*). The second-order rate law in the latter case suggests that the dye is rate limiting and probably reflects the association of the free dye in the inner volume of the vesicle with the inner membrane surface followed by rapid movement of the probe across the bilayer. If in the oxygen pulse case oxonol VI does permeate the submitochondrial particle membrane following the initital binding step in the redistribution process, then the decay of the dye spectral shift appears to be limited by the rate at which the dye can move through the particle membrane, although contributions from other processes such

as the simple dissociation of bound dye from the membrane surface cannot be excluded from the first order decay. The difference in the rate laws characterizing the decay of the dye spectral shift in chromatophores and submitochondrial particles suggests that the latter preparation may be less easily permeated by the dye than the former one.

Oxonol Responses on a Microsecond Time Scale

Oxonol VI and the phenyl derivative oxonol V respond to potential changes with a halftime of approximately 10 μsec when used in the squid giant axon (L.Cohen, *personal communication;* Chance, 1975). A number of other charged dyes of the oxonol, cyanine, and merocyanine classes are also capable of such rapid spectral responses to changes in applied potential in the giant axon system (Cohen *et al.*, 1974; Ross *et al.*, 1977; Cohen & Salzberg, 1978). Waggoner *et al.* (1977) have also been able to observe such rapid spectral responses to a train of voltage pulses applied across a black lipid membrane by electrodes in the bathing dye solution.

Although such rapidly developing signals cannot be time resolved in the rapid mixing experiments, they should appear as vertical displacements from the baseline during the flow time. In attempts to detect such signals in the submitochondrial particle-oxonol VI system, the sweep rate of the oscilloscope was increased so that the time from start to the cessation of flow occupied about half the scope display. The reactant concentrations were increased so that a substantial signal developed during the flow time. The observed signal, however, was clearly time resolved under these conditions. Although the possibility that the small microsecond signals are masked by the much larger but more slowly developing signals described in this communication cannot be excluded, the instrument as routinely used in this work is capable of detecting changes in transmission corresponding to $\Delta A \simeq 10^{-3}$ and is capable of detecting signals at least as small as $\Delta A = 10^{-4}$. Since the double beam spectrometer is capable of monitoring dye signals of comparable magnitude to those observed in experiments on the squid giant axon and black lipid membranes, the preceding results suggest that the microsecond signals do not occur in the isotropic membrane suspensions used in the present investigation.

Several mechanisms have been proposed to explain the microsecond spectral changes observed in bilayer and excitable membrane work. Wag-

goner *et al.* (1977) have suggested that the fast changes are due to association/dissociation of the dye with the bilayer from a volume of the bathing solution immediately adjacent to the bilayer surface. More recently, Dragston and Webb (1978) have argued that the potential-dependent microsecond changes in the M-540 fluorescence observed in experiments with bilayers are due to a rotation of the dye into the plane of the bilayer followed by the formation of a nonfluorescent dimer. Ross *et al.* (1974, 1977) and Tasaki, Warashina and Pant (1976) have alternatively proposed that the M-540 spectral changes observed in the squid axon and in the crab nerve, respectively, are due to a shift in the monomerdimer equilibrium of the membrane-bound fraction of the dye.

The following arguments are offered against the operation of these mechanisms in a submitochondrial particle suspension. Using the usual relationship

$$\ln 2 = k_1 t_{1/2} \tag{9}$$

the apparent first-order rate constant that corresponds to the observed 10 μsec halftime can be obtained. The effective concentration of the dye that would lead to the second-order rate constant given in Table 1 for passive binding of oxonol VI to submitochondrial particles can be calculated from Eq. (3). The value of the effective dye concentration so obtained is approximately 7 mM. If the second-order rate constant for the faster energization process is used, the required concentration is increased by a factor of 30. In either case, the required dye concentration is greatly in excess of the highest bulk dye concentrations employed in the experiments with submitochondrial particles. Thus, a region of high local concentration near the membrane surface is required by Waggoner's proposed mechanism. A dc bias potential was required for optical signal generation by the voltage pulses applied across the black membranes used by Waggoner *et al.*, and the excitable membrane systems used by Cohen *et al.* also maintain resting potentials. Such potentials may be sufficient to lead to the formation of the required local dye concentration by a diffusion mechanism, but the presence of such preexisting potentials in submitochondrial particles is unlikely since this preparation is essentially free of endogenous substrate. The latter proposal is supported by the observation that the oxonol VI absorption spectrum in the presence of submitochondrial particles without added substrate could be virtually completely recovered by the addition of an uncoupler to the energized preparation (Fig. 2) implying the absence of a pre-existing membrane potential. The data obtained for the passive

binding of oxonol VI to submitochondrial particles was the same in the absence and presence of the uncoupler CCCP (Fig. 4).

The rotation-dimerization mechanism is also unlikely to be applicable to dyes like oxonol VI because the permanent dipole moment of these probes is small due to delocalization of the charge in the conjugated systems. Thus such dyes would not be susceptible to appreciable torque when a transmembrane electric field is present. The oxonols of the type used in this study also show little tendency to aggregate (Smith *et al.*, 1976) at least when free in solution, thereby suggesting that the mechanism involving a shift in the dye monomer-dimer equilibrium in the membrane bilayer is also unlikely in the case of oxonol VI. Thus, the conditions necessary for the mechanisms proposed for the microsecond process to operate do not appear to be present in the oxonol VI-submitochondrial particle system. An additional consideration in the discussion of microsecond dye kinetics in a submitochondrial particle suspension is that the enzyme turnover rates are unlikely to be large enough to allow the full membrane potential to be developed in a microsecond scale.

The Effect of Valinomycin

The magnitude and rate of development of the oxonol VI red shift on energization of submitochondrial particles in the presence of valinomycin are much slower than in comparable experiments in which it is absent. The biphasic nature of the probe response to NADH pulses, however, persists with both rate constants being reduced by as much as an order of magnitude when the ionophore is present. These results suggest that the rate of dye response may be proportional to the magnitude of the potential, which has been attenuated by the equilibration of the K^+ gradient by the ionophore before the probe response to $\Delta\Psi$ can develop significantly, and once the membrane potential is reduced, it cannot increase significantly with time because the pH gradient, to which the dye is insensitive, has been increased in order to maintain a constant electrochemical gradient.

The Effect of Probe Permeation on Calibration Procedures

Although the tendency of a number of charged probes of the oxonol and cyanine classes to cross the membrane of vesicular preparations

is unlikely to significantly affect the steady-state membrane potential in respiring systems since a source of energy is available to maintain the charge gradient, the permeation of the dyes does pose formidable problems when calibration of these probes is attempted using ionophore-induced diffusion potentials. The commonly used potassium or proton diffusion potentials induced by valinomycin or and uncoupler, respectively, involve transient charge separation since there is no source of energy present to maintain an electrical gradient. The movement of anionic probes in the same direction as the cations generating the initial charge separation in these calibration experiments tends to reduce the magnitude of the calibrating potential gradient in the process of responding to the generation of it. The magnitude of the probe response to diffusion potentials when used to quantitate steady-state potentials will likely lead to an underestimate of the latter potentials. The calibration problem becomes especially acute if an anionic probe forms a complex with the ionophore and is transported across the membrane. Gains and Dawson (1975) have reported that ANS forms such a complex with valinomycin, and Haynes and Simkowitz (1977) have concluded that ANS does not respond to membrane potential because of the latter transport problem. The latter interpretation, however, is at variance with that of Bakker and van Dam (1974). The problem of calibrating potential-sensitive probes has been considered in greater detail by Bashford and Smith (1978*b*).

The authors have benefited from a number of discussions with Drs. C.L. Bashford and W.S. Thayer and from the assistance of Dr. M. Pring in the data analysis. This work was supported by grants NIH GM 12202-15 and NINCDS NS-10939-06.

References

Bakker, E.P., Dam, K. van 1974. Influence of diffusion potentials across liposomal membranes on the fluorescence intensity of 1-anilino-8-naphthalene sulfonate *Biochim. Biophys. Acta* **339**:157

Bashford, C.L., Chance, B., Prince, R. 1978*a*. Oxonol dyes as monitors of membrane potential: Their behaviour in photosynthetic bacteria. *Biochim. Biophys. Acta* **545**:46

Bashford, C.L., Chance, B., Smith, J.C., Yoshida, T. 1978*b*. The behaviour of oxonol dyes in phospholipid dispersions. *Biophys. J.* **25**:63

Bashford, C.L., Smith, J.C. 1978*a*. The determination of oxonol-membrane binding parameters. *Biophys. J.* **25**:81

Bashford, C.L., Smith, J.C. 1978*b*. The use of optical probes to monitor membrane potential. *In:* Methods of Enzymology. S. Fleischer, editor. Vol. LV, pp. 569–586

Bashford, C.L., Thayer, W.S. 1977. Thermodynamics of the electrochemical proton gradient in bovine heart submitochondrial particles. *J. Biol. Chem.* **252**:8459

Chance, B. 1973. Rapid Flow Methods in Investigations of Rates and Mechanisms of Reactions, Part II: Investigations of Elementary Reaction Steps in Solution and Very Fast Reactions. G.G. Hammes, editor. p. 42. John Wiley & Sons, New York

Chance, B. 1975. Electron transport and energy-dependent responses of deep and shallow probes of biological membranes. *In*: Energy Transducing Mechanisms. (Biochemistry Series One). E. Racker, editor. Vol. 3, p. 1. University Park Press, Baltimore

Chance, B., Baltscheffsky, M. (with an Appendix by Cheng, W.W.) 1975. Carotenoid and merocyanine probes in chromatophore membranes. *In:* Biomembranes. H. Eisenberg, E. Katchalski-Katzir, and L.A. Mason, editor. Vol. 7, p. 33. Plenum, New York

Chance, B., Baltscheffsky, M., Vanderkooi, J., Cheng, W. 1974. Localized and delocalized potentials in biological membrane. *In*: Perspectives in Membrane Biology. C. Gitler, editor. p. 329. Academic Press, New York

Chance, B., Mayevsky, A., Smith, J.C. 1976. Localized and delocalized potentials in the rat brain cortex. *Neurosci. Abstr.* **2:**133

Cohen, L.B., Salzberg, B.M. 1978. Optical measurements of membrane potential. *Rev. Physiol. Biochem. Pharmacol. (in press)*

Cohen, L.B., Salzberg, B.M., Davila, H.V., Ross, W.N., Landowne, D., Waggoner, A.S., Wang, C.H. 1974. Changes in axon fluorescence during activity: Molecular probes of membrane potential. *J. Membrane Biol.* **19:**1

Cornish-Bowden, A., Eisenthal, R. 1974. Statistical considerations in the estimation of enzyme kinetic parameters by the direct linear plot and other methods. *Biochem. J.* **139:**721

Dragston, P.R., Webb, W.W. 1978. The mechanism of membrane potential sensitivity of merocyanine M-540. *Biochemistry (in press)*

Eisenthal, R., Cornish-Bowden, A. 1974. The direct linear plot, a new graphical procedure for estimating enzyme parameters. *Biochem. J.* **139:**715

Gains, N., Dawson, A.P. 1975. Transmembrane electrophoresis of 8-anilino-1-naphthalene sulfonate through egg lecithin lyposome membranes. *J. Membrane Biol.* **24:**237

Hansen, M., Smith, A.L. 1964. Studies on the mechanism of oxidative phosphorylation VII. Preparation of a submitochondrial particle (ETP_H) which is capable of fully coupled oxidative phosphorylation. *Biochim. Biophys. Acta* **81:**214

Haynes, D.H., Simkowitz, P. 1977. 1-Anilino-8-naphthalenesulfonate: A fluorescent probe of ion and ionophore transport kinetics and trans-membrane asymmetry. *J. Membrane Biol.* **33:**63

Hoffman, J.F., Laris, P.C. 1974. Determination of membrane potentials in human and *Amphiuma* red blood cells by means of a fluorescent probe. *J. Physiol. London* **239:**519

Laris, P.C., Bahr, D.P., Chaffee, R.R.J. 1975. Membrane potentials in mitochondrial preparations measured by means of a cyanine dye. *Biochim. Biophys. Acta* **376:**415

McLaughlin, S., Harary, H. 1976. The hydrophobic adsorption of charged molecules to bilayer membranes: A test of the applicability of the Stern equation. *Biochemistry* **15:**1941

Porter, W.A., Trager, W.F. 1977. Improved nonparametric statistical methods for the estimation of Michaelis-Menten kinetic parameters by the direct linear plot. *Biochem. J.* **161:**293

Renthal, R., Lanyi, J.H. 1976. Light-induced membrane potential and pH gradients *Halobacterium halobium* envelope vesicles. *Biochemistry* **15:**2136

Ross, W.N., Salzberg, B.M., Cohen, L.B., Davila, H.V. 1974. A large change in dye absorption during the action potential. *Biophys. J.* **14:**983

Ross, W.N., Salzberg, B.M., Cohen, L.B., Grinvald, A., Davila, H.V., Waggoner, A.S., Wang, C.H. 1977. Changes in absorption, fluorescence, dichroism, and birefringence

in stained giant axons. Optical measurement of membrane potential. *J. Membrane Biol.* **33**:141

Salama, G., Morad, M. 1976. Merocyanine 540 as an optical probe of transmembrane electrical activity in the heart. Science **191**:485

Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51**:660

Sims, P.J., Waggoner, A.S., Wang, C.H., Hoffman, J.F. 1974. Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidyl choline vesicles. *Biochemistry* **13**:3315

Smith, J.C., Chance, B. 1976. Energy-linked spectral responses of the probe MC-V in membranes. *Biophys. J.* **16**:20*a*

Smith, J.C., Powers, L. 1978. Orientation studies on a series of oxonol probes in dipalmitoyl phosphatidylcholine multilayers. *Biophys. J. (in press)*

Smith, J.C., Russ, P., Cooperman, B.S., Chance, B. 1976. Synthesis, structure determination, spectral properties, and energy-linked spectral properties of the extrinsic probe oxonol V in membranes. *Biochemistry* **15**:5094

Tasaki, I., Warashina, A., Pant, H. 1976. Studies of light emission, absorption and energy transfer in nerve membranes labelled with fluorescent probes. *Biophys. Chem.* **4**:1

Waggoner, A. 1976. Optical probes of membrane potential. *J. Membrane Biol.* **27**:317

Waggoner, A.S., Wang, C.H., Tolles, R.L. 1977. Mechanism of the potential dependent light absorption changes of lipid bilayer membranes in the presence of cyanine and oxonol dyes. *J. Membrane Biol.* **33**:109