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Review

Potential-sensitive molecular probes in membranes of bioenergetic relevance

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Contents

Abbreviations: ADP, adenosine 5′-diphosphate; ANS, 1-anilinonaphthalene 8-sulfonate; ARA-C, 1-β-D-arabinofuranosylcytosine; ATP, adenosine 5′-triphosphate; BHT, butylated hydroxytoluene; $BTPP^+$, butyltriphenylphosphonium cation; CAT_{12}, 4-(dodecyldimethylammonium)-1-oxyl-2,2,6,6-tetramethylpiperidine bromide; CC_6, 3,3′-dihexyl-2,2′-oxacarbocyanine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ΔG_p, phosphorylation potential; ΔpH, transmembrane pH gradient; $\Delta\psi$, transmembrane potential; $\Delta\mu_H$, transmembrane electrochemical gradient; di-4-ASP, *N*,*N*-dibutyl-4-*p*-aminostyrylpyridinium cation; di-6-ASPPS, *N*,*N*-dihexyl-4-*p*-aminostyrylpyridinium propylsulfonate; diBA-C_4-(3), bis[1,3-dibutylbarbituric acid-(5)]trimethineoxonol; diBA-C_4-(5), bis[1,3-dibutylbarbituric acid-(5)]pentamethineoxonol; diI-C_1-(5), 3,3′-dimethyl-2,2′-indodicarbocyanine; diI-C_3-(5), 3,3′-dipropyl-2,2′-indodicarbocyanine; diI-C_5-(3), 3,3′-dipentyl-2,2′-indodicarbocyanine; diI-C_6-(5), 3,3′-dihexyl-2,2′-indodicarbocyanine; diO-C_1-(3), 3,3′-dimethyl-2,2′-oxacarbocyanine; diO-C_2-(5), 3,3′-diethyl-2,2′-oxacarbocyanine; diO-C_3-(5), 3,3′-dipropyl-2,2′-oxacarbocyanine; diO-C_5-(3), 3,3′-dipentyl-2,2′-oxacarbocyanine; diS-C_2-(5), 3,3′-diethylthiodicarbocyanine; diS-C_3-(5), 3,3′-dipropylthiodicarbocyanine; diS-C_4-(5), 3,3′-dibutylthiodicarbocyanine; DMPC, L-α-dimysteroylphosphatidylcholine; DMPG, L-α-dimysteroylphosphatidylglycerol; DNP, 2,4-dinitrophenol; EDKC, *N*,*N*′-bis(2-ethyl-1,3-dioxolane)kryptocyanine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; ITP, inosine 5′-triphosphate; merocyanine 540 (M540, MC-I), 5-[(3-sulfopropyl-2(3*H*)-benzoxazolylidene)-2-butenylidene]-1,3-dibutyl-2-thiobarbituric acid; MNS, 2-(*N*-methyl-anilino)naphthalene-6-sulfonate; NAD(H), β-nicotinamideadenine dinucleotide; oxonol V, bis[3-phenyl-5-oxoisoxazol]-4-yl]pentamethineoxonol; oxonol VI, bis[3-propyl-5-oxoisoxazol-4-yl]pentamethineoxonol; Q, quinone; RH-160, 4-(4′-dibutylaminophenyl-1′:3′-diethyl)-1-δ-sulfobutylpyridinium hydroxide; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; TNS, toluidinonaphthalenesulfonate; TPP^+, tetraphenylphosphonium cation; WW781, 1,3-dibutylbarbituric acid (5)-1-(*p*-sulfophenyl)-3-methyl-5-pyrazolone pentamethineoxonol.

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I. Introduction

This review is concerned with the use of potential-sensitive molecular probes in energy-transducing preparations. The focus of the material and comments contained herein is on a number of current and long-standing issues and controversies associated with the probe approach to the detection, characterization, and quantitation of electrical potential gradients and the time course of their development in biological systems. The papers cited in this review have been chosen to illustrate both the advantages in the application of molecular probes as well as a number of problems associated with their use in such systems. In the latter context, the selection of cited investigations is inevitably somewhat subjective, and no attempt has been made to provide in this comparatively short review a comprehensive listing of the many applications of molecular probes in energy-transducing preparations. A number of sources for the latter type of information are currently available. A multiple-volume compilation dealing with the application of these probes in a variety of biological preparations varying from model membranes to tissue and organ level work has been edited by Loew [1]. Physiological applications have been covered in a monograph edited by DeWeer and Salzberg [2]. More specialized reviews by Waggoner [3], Cohen and Salzberg [4], and Bashford and Smith [5] have appeared. The early application of several probes of the oxonol class in energy-transducing membranes has been described by Chance et al. [6,7]; more recent summaries by Bashford et al. [8] and Smith et al. [9] have appeared, Tedeschi [10] has summarized the utility of a number of experimental techniques for determining membrane potentials in mitochondria, including molecular probes.

Molecular probes fall into two broad classes: those intrinsic to the preparation under investigation and extrinsic probes. Examples of the former class are the carotenoids and certain chlorophyll complexes; these examples are restricted virtually exclusively to photosynthetic systems. One of the few intrinsic probes in mitochondrial preparations is the energy-dependent shift in the cytochrome *c* oxidase Soret band [11]. Intrinsic indicators have the advantage of being nonperturbing to the functional and organizational stability of the biological system, but are present in a limited number and type of preparations and consist of only a few components as previously described. Large numbers of biological systems lack such intrinsic indicators and, if information regarding charge gradients is to be obtained in many of the latter systems, the addition of extrinsic indicators under carefully controlled conditions is necessary. Extrinsic probes are necessarily perturbing to the system but can often be employed at micromolar concentration levels or less so that the disruption of the structure/function operation of the energy transducing system is kept to a minimum – although, as will be described subsequently, the perturbation of certain probes can still be substantial, even under the latter conditions.

The contents of this review are organized around the following issue that are applicable to both intrinsic and extrinsic probes in a large cross-section of preparations including mitochondrial and related systems, photosynthetic preparations, sarcoplasmic reticulum, and a number of miscellaneous preparations: the ability of probes to respond to charge separation, the nature and amplitude of the associated signals and their calibration for quantitative work, the specificity of the probe to the membrane potential component of the electrochemical gradient. The relative sensitivity of the indicators to surface potentials, intra- and transmembrane charge gradients is an increasingly important consideration in view of the recent models for energy transduction proposed by Kell [12] and Malpress [13] involving membrane surface phenomena and the long-standing proposal of Williams [14] regarding local or intramembrane charge separation. The kinetic competence of probes to follow the formation of charge gradients is an important aspect in their application and is closely linked to the mechanism(s) responsible for the formation of energy-dependent signals exhibited by the probes. The preceding issues will be explored in the context of the behavior of specific probes in a variety of biological systems.

The characteristics of probe response to potential gradients, the mechanism(s) by which such signals occur, and certain liabilities associated with these indicators are discussed by probe class. The use of molecular probes in whole cells, tissue, and organ preparations especially when the origin of the probe signal appears to originate from specific organelles is considered in a separate section as is the issue of the nature of the potential gradient(s) to which molecular probes are sensitive, and the kinetic competence of these probes to follow elementary charge separation events.

II. Historical perspective

The vast majority of what may be considered molecular probes, especially those in the extrinsic probe class, are optical dyes, although a few spin labels have been employed in surface potential studies [15]. Hashimoto et al. [16] and Wojtczak and Szewczyk [17] have found that the cationic probe CAT_{12} used in the investigations described in Ref. 15 is reversibly accumulated in intact mitochondria and mitoplasts but not submitochondrial particles when ATP is present, a process that leads to signal loss due to the broadening of the EPR spectrum when the probe binds to the inner surface of the inner mitochondrial membrane. This probe is thus unsuitable for surface potential measurements in mitochondria when a transmembrane potential is present. Similar considerations probably also apply to the use of CAT_{12} in other preparations that generate inside-negative transmembrane potential gradients. Hartzog et al. [18] have recently described protocols that correct for the uptake and destruction of spin labels by the preceding membrane preparations when these probes are used in the measurement of surface pontentials. Hashimoto and Rottenberg [19] have recently reported that the phosphorescence signal from terbium may be a reliable indicator of membrane surface potentials in mitochondria and submitochondrial particles. Little or no change in this potential was observed upon energization of these preparations.

The anionic *N*-arylnaphthalenes have long been employed as potential-sensitive probes in energy-transducing systems including mitochondria and submitochondrial particles, chloroplasts, chromatophores and sarcoplasmic reticulum preparations. ANS and to a lesser extent MNS and TNS are the primary probes of this class that have been employed in charge separation investigations. Williams et al. [20] cite a number of investigations in which the probes have been used in both intact mitochondria and EDTA-type submitochondrial particles that lack the F_1 portion of the ATPase, and early descriptions of the behavior of ANS in membranes have been provided by Azzi [21] and Njus et al. [22]. This class of probes has the property of undergoing a drastic increase in fluorescence quantum yield when they are transferred from a polar to a nonpolar environment. They have thus been employed in investigations based on fluorimetry as opposed to absorbance or transmission spectroscopy. The basis of the energy-dependent fluorescence intensity changes exhibited in mitochondrial preparations has been explained on the basis of a change in binding constants such that, in intact mitochondria, this probe is ejected from the mitochondrial inner membrane to the bulk phase with a corresponding decrease in emission intensity, whereas in submitochondrial particles, ANS was transferred from the bulk phase to the membrane with a characteristic increase in fluorescence intensity, Azzi et al. [23] in an early investigation found that the introduction of substrate to an ANS-rat liver mitochondria system caused an increase in the Michaelis constant, K_m, characterizing the binding of the probe to the mitochondrial membrane, but that in EDTA-type submitochondrial particles, a decrease of some 3.6-fold in K_m was observed with no change in the total number of membrane binding sites available to the probe in either of the two preparations. It was possible to calibrate the ANS probe using K^+-valinomycin generated diffusion potentials. The fluorescence yield change associated with the diffusion potentials was linear over a 100 mM external K^+ concentration range and with the assumption that the internal K^+ concentration was 10 mM, succinate was found to support a $\Delta\psi$ of 180 mV in submitochondrial particles.

Further work by Williams et al. [20] demonstrated that ANS readily crosses the inner mitochondrial membrane since no additional probe–membrane binding was observed when the organelle membrane was disrupted by osmotic shock. Work by Barret-Bee and Radda [24] based on the quantum yield properties of ANS and MNS suggests that these probes may be sensitive to multiple membrane conformation states.

Based on the observation that the rate at which the MNS and ANS ATP-dependent fluorescence intensity increase develops in bovine heart submitochondrial particles is independent of the total ATPase activity that was systematically reduced by Nbf-Cl (NBD-Cl) addition and that the rate at which the fluorescence enhancement occurs does decrease when the more slowly hydrolyzable substrate ITP is substituted for ATP, Ferguson [25] has suggested that the probe fluorescence intensity enhancement observed in submitochondrial particles may not be sensitive to a delocalized potential gradient but to local phenomena at the level of the ATPase.

More recently, Robertson and Rottenberg [26] have reexamined the behavior of ANS in rat liver mitochondria. Binding studies based on the physical separation of bound and free probe suggest the presence of two classes of binding site: a high-affinity, low-capacity (K_D = 10–50 μM, n = 3-8 nmol ANS/mg protein) class in which bound ANS is strongly fluorescent and a low-affinity, high-capacity (K_D > 500 μM, n > 50 nmol ANS/mg protein) class with little probe fluorescence. The finding that the apparent ANS dissociation constant is dependent on the inner mitochondrial membrane surface potential has been employed to estimate the latter potential which appears either to remain constant during the oxidation of substrates or to undergo a change that is small in comparison to the effect of $\Delta\psi$ on the ANS signal, observations that are not compatible with the models of Kell [12] and Malpress [13] for energy transduction. The energy-dependent re-

duction in ANS fluorescence was found to be best explained as an expulsion of the probe from the mitochondrial membrane when $\Delta\psi$ is formed. The potential-dependent probe fluorescence changes were correlated with $\Delta\psi$ but tended toward saturation at high values of the potential gradient.

Haynes [27] has also found that, at low medium ionic strength, ANS binding is sensitive to surface potential changes in liposomes. The latter properties has been employed by Chiu et al. [28] to obtain values of the potential, relative to the external medium for both the inner and outer membrane surfaces of the Ca^{2+}-ATPase-rich and Ca^{2+}-binding protein fractions from the sarcoplasmic reticulum membrane. Binding to the inner and outer membrane vesicle surfaces was distinguished by the time-course of the ANS signal development in rapid mixing experiments. The association of probe with the outer membrane occurred during the time of mixing, whereas the binding of ANS with the inner membrane was dependent on the rate at which the probe could cross the bilayer, a process operating on a time-scale of seconds. No difference in the inner and outer membrane surface potentials was found in the ATPase vesicle fraction and a small difference in the potentials of the two surfaces in the Ca^{2+}-binding protein fraction was explained on the basis of asymmetry in the phospholipid composition of the two bilayer leaflets.

Lebedev et al. [29] have found that the interaction of ANS with *E. coli* cells treated with dinitrophenol as a function of ionic strength is governed by the membrane surface potential. The natural logarithm of the probe fluorescence intensity varies linearly with ionic strength up to 500 mM, and the surface potentials obtained from this dependence exhibit an inverse dependence on the square root of the medium NaCl concentration as predicted by the Gouy-Chapman theory. A model for the relationship between surface and transmembrane potential gradients in this system is proposed.

Because of the strong dependence of the quantum yield of *N*-aryl-naphthalene probes such as ANS on the local dielectric constant, these probes are sensitive to membrane and protein structural changes [30,31] that alter the probe environment but are not necessarily related to potential gradient formation. The preceding property of the *N*-arylnaphthalene probes and the uncertainty in their relative sensitivity to surface vs. transmembrane potential as illustrated for ANS and whether these probes are sensitive to local vs. delocalized potential gradients has lead to the development of a large number of probes that exhibit potential-sensitive properties. Much of the initial expansion has been carried out by L. Cohen and associates in work dealing primarily with the squid giant axon and intercell communication in ganglia. Unlike ANS and similar *N*-arylnaphthalenes, many of these probes exhibit potential-dependent changes in both the fluorescence emission and transmission/absorption spectra and can be employed in investigations utilizing both fluorescence and dual wavelength difference transmission/absorption spectroscopy that affords a correction for light-scattering problems associated with turbid samples [32].

Many of the more recently developed optical molecular probes are polyene type dyes in which a variable length conjugated carbon chain connects two ring systems of varying complexity that often contain substituents that affect the binding properties of the probes. The wavelength of maximum absorbance of these probes depends on the length of the conjugated chain [33] which can be varied to obtain probes the absorption spectrum of which does not strongly overlap the intense bands associated with intrinsic membrane pigments such as the soret band of cytochromes.

III. Cyanine probes

The thiodicarbocyanine class of dyes has been used extensively in a variety of preparations including mitochondria, sarcoplasmitic reticulum vesicles, and whole cells in which an inside negative potential gradient can be generated. Among the more heavily used probes of this class are diS-C_2-(5) and diS-C_3-(5). A listing of a number of studies using energy-transducing organelles in which these probes have been employed is provided by Smith [9].

Among the earliest applications of this class of probes was the use of diS-C_3-(5) by Laris et al. [34] in hamster liver mitochondria preparations. In the intact mitochondria, this cyanine probe undergoes an uncoupler-, ionophore- or electron transport inhibitor-sensitive reduction in fluorescence intensity of up to 80% when succinate or ATP is supplied. In submitochondrial particles, an increase in diS-C_3-(5) is observed when substrate is added. The energy-dependent fluorescence reduction signal observed in intact mitochondria was calibrated using K^+-valinomycin diffusion potentials. The plot of percent diS-C_3-(5) fluorescence quenching vs. K^+ concentration was linear over an external concentration range of 1 to 40 mM. The probe signal, however, became nonlinear at K^+ concentrations below 1 mM and tended toward a plateau at concentrations below the latter value. Using 100 mM as the matrix concentration of K^+, -64 mV and -45 mV were respectively obtained for the resting state $\Delta\psi$ of mitochondria suspended in a K^+- and an Na^+-containing medium. From the K^+ diffusion potential calibration plot for diS-C_3-(5), succinate was estimated to generate a $\Delta\psi$ between -150 and -180 mV. Similar values for $\Delta\psi$ in mitochondria have been reported by Zinchenko et al. [35] using diS-C_2-(5) and diS-C_3-(5) calibrated with K^+ diffusion potentials. These investigators observed a decrease of only 3–8 mV in $\Delta\psi$ during ATP

synthesis but a reduction of 10 to 150 mV during Ca^{2+} accumulation.

Using the probe CC_6, Tedeschi et al. [36] have reported a small positive value for $\Delta\psi$ (10–20 mV) in mitochondria from *Drosophilia*; similar values have also been obtained by these investigators from microelectrode measurements on large mitochondria from copper-deficient rat liver. Laris et al. [34] have obtained by use of CC_6 values for $\Delta\psi$ similar to those obtained using diS-C_3-(5) as a probe and have also been able to detect hyperpolarization of the mitochondrial matrix using ANS whereas Tedeschi et al. [36] did not detect an energy-dependent response with the latter probe. These discrepancies in part appear to be due to a strong dependence of the magnitude of the energy-dependent probe signal on the probe-to-membrane concentration ratio as well as in general on certain aspects of probe calibration procedures. Tedeschi [10] has questioned the use of K^+-valinomycin diffusion potentials in calibrating probe signals in mitochondria, citing the lack of changes in the K^+ gradient during metabolism which gradient is described as a Donnan potential. Correction for osmotic effects on the matrix volume that produce substantially altered calibration plots when diffusion potentials are employed as calibration tools in this system have also been employed.

More recently, Schummer and Schiefer [37] have shown that, within experimental error, the same calibration curves are obtained for the potential-dependent diS-C_3-(5) fluorescence signal in *Mycoplasma mycoides* subsp. *capri* and enterococci when the external K^+ concentration to be used in the Nernst equation is calculated directly from added quantities of K^+ and when the external K^+ concentration as well as the corresponding probe fluorescence signal level are obtained from an independent null point titration procedure that provides this concentration at Donnan equilibrium where no net K^+ flux is present. Ca^{2+} was used to vary the cell membrane potential in the null point procedure, and the internal K^+ concentration was independently measured using atomic absorption. The diS-C_3-(5) calibration curves were similar to those previously described in that they were approximately linear in the range of -100 to -40 mV. The mycoplasma membrane potential was estimated to be -68 mV under cell growth conditions.

Cabrini and Verkman [38] have developed a semiquantitative model for the potential-dependent behaviour of diS-C_3-(5). For preparations that generate inside-negative potential gradients and have small intraventricular volumes such as the mitochondrion, the primary process responsible for the energy-dependent diS-C_3-(5) response is predicted to be translocation of membrane bound dye species to binding sites on the matrix side of the inner mitochondrial membrane. The accumulation of bound probe at these sites would apparently raise the local dye concentration such that the formation of bound dye aggregates is favored. Since such aggregates are either weakly or non-fluorescent [39] the energy-dependent decrease in diS-C_3-(5) emission is explained. Additional contributions to the decrease in the probe fluorescence intensity from association of this cyanine with matrix proteins and other components, however, cannot be excluded in this case.

Ivkova et al. [40] have developed a quantitative model for the diS-C_3-(5) fluorescence quenching generated in azolectin and sarcoplasmic reticulum vesicles by inside negative K^+ diffusion potentials based on the premise that the majority of the control level probe fluorescence originates from membrane-bound monomers that permeate the preparation and accumulate on the inner surface of the bilayer. The resulting increment in the probe local concentration accounts for the observed fluorescence quenching caused by the diffusion potential. The model does not consider potential-dependent differential probe-membrane binding per se but treats diS-C_3-(5) essentially as a permeant membrane-bound indicator. The formation of weakly or non-emitting probe aggregates from probe monomers in the aqueous medium was predicted to account for only 10% or less of the observed quenching. Calculations based on a 100 mV potential indicated that the on/off mechanism proposed by Waggoner [3] operating in these preparations such that the probe is ejected from the inner surface of the membrane bilayer when an inside-negative diffusion potential was established would not account for the observed percentage quenching of the probe control level fluorescence, the contribution of this mechanism accounting for only a few percent of the total potential-dependent probe emission intensity loss.

The reorientation of membrane bound diS-C_3-(5) monomers by the torque exerted by the electric field acting on the probe dipole moment followed by the formation of probe dimers was discounted based on probable electrostatic repulsion between probe monomers aligned by the electric field. Model-building studies indicated that the probe trimers could not be accommodated in membrane bilayers without serious disruption to the packing of the surrounding lipids, thus causing an increase in the membrane ion conductivity as observed in sarcoplasmic reticulum preparations when the diS-C_3-(5) to membrane mole ratio is high [41,42]. Fig. 1 contains a reconstruction of the model for the location of diS-C_3-(5) in phospholipid bilayers suggested by Ivkova et al. [40]. The program MACROMODEL was employed to generate the illustrated complex using the AMBER force field parameters. The energies of the DMPC and diS-C_3-(5) probe were separately minimized prior to the assembly of the complex, the energy of which was then subjected to minimization. Based on the minimal perturbation of the gel to liquid-crystalline phase transition in multilamellar DMPC pre-

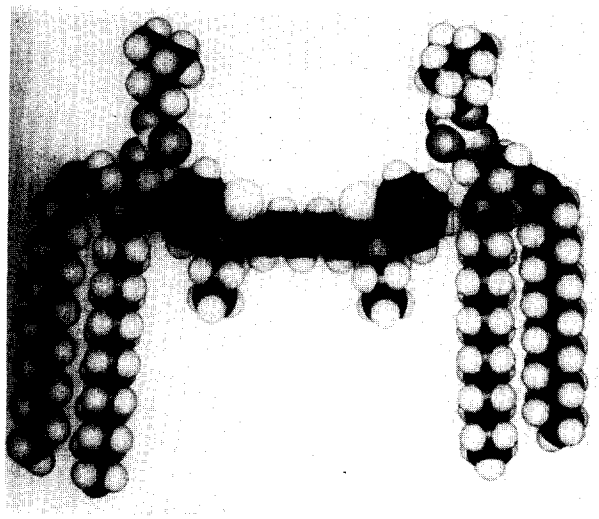

Fig. 1. A reconstruction of a model for the location of diS-C_3-(5) in phospholipid bilayers suggested by Ivkova et al. [40]. The molecular mechanics package MACROMODEL was employed to generate the illustrated structure using the AMBER force-field parameters. The DMPC lipid and the diS-C_3-(5) probe were separately subjected to energy minimization prior to the assembly of the complex, the energy of which was then minimized. Based on a minimal diS-C_3-(5) perturbation of the excess heat capacity profile describing the gel to liquid-crystalline phase transition in multilamellar DMPC preparations and a substantial probe effect on the pretransition in the latter system, a location in which the dye is at or near the bilayer surface, however, is implied, at least in the DMPC preparation in the absence of a membrane potential. See Section XII and Ref. 140.

parations and a substantial perturbation of the pretransition in this preparation, however, a location in which the dye is located at or near the surface of the bilayer is implied. See Section XII and Ref. 140.

Bammel et al. [43] have demonstrated that the rate at which the ATP-dependent cytochrome-*c* oxidase soret band shift signal develops in pigeon heart mitochondria and the signal amplitude associated with this shift are monotonically decreased as the diS-C_3-(5) to membrane protein ratio is increased. Since the Soret band shift is an energy-requiring process [11], the dye-induced retardation of this process suggests that the probe is being translocated by an electrophoretic process that competes with that responsible for the Soret band shift for energy from the electrochemical gradient generated by ATP hydrolysis. The latter observations are compatible with the mechanism suggested by Cabrini and Verkman [38] and Ivkova et al. [40] for the potential-dependent behavior of the diS-C_3-(5) fluorescence in systems that generate an inside-negative potential.

The cyanine class of probes, however, has been found to exert a number of undesirable side-effects in mitochondrial preparation. The rate at which the glutamate-dependent diS-C_3-(5) absorbance change developed in pigeon heart mitochondria was found to be inversely dependent on the probe concentration at fixed membrane concentration [43]. This observation was attributed to an inhibition of NADH-linked respiration by the probe and confirmed by measurement of respiration rates using a Clark electrode. Conover and Schneider [44] have found that diS-C_2-(5) and several other cyanine-type probes are inhibitory to NADH-linked coupled respiration driven by ATP or succinate. A number of other probes such as safranine O, merocyanine 540 and rhodamine B, however, were without effect on the respiration rate. Pechatnikov et al. [45] have reported a rotenone-like inhibition of respiration by both diS-C_2-(5) and diS-C_3-(5) in rat liver mitochondria; such inhibition has been reported to be prevented in lymphocytes by TMPD when used with ascorbate as an electron donor [46]. Montecucco et al. [47] have found that a number of probes, including diS-C_2-(5), diS-C_3-(5), diO-C_2-(5), and diI-C_6-(5), were inhibitors of site I respiration in rat liver mitochondria and that the inhibition of diS-C_3-(5), which was studied in detail, was probably due to the accumulation of the probe on the matrix side of the inner membrane or in the matrix volume itself to a level sufficient to cause the observed inhibition; a similar conclusion was reached by Conover and Schneider. None of the probes investigated by Montecucco et al., however, inhibited site II respiration. Auichi et al. [48] have reported inhibition of respiration by diS-C_3-(5) in synaptosomes.

Kovac and Pliachova [49] have found that at 2–5 μM concentrations, diS-C_3-(5) uncouples and inhibits respiration in intact *Saccharomyces cerevisiae* cells and at 6 μM induces the formation of respiratory-deficient *rho*$^-$ mutants, indicating that this probe does reach the cell mitochondria in situa and affects the replication of the mitochondrial DNA. The concentration threshold at which the preceding deleterious effects were observed, however, were above the dye concentrations normally used in short-duration experiments designed for the measurement of plasma and/or mitochondrial membrane potentials.

Singh et al. [50] have found that in reconstituted cytochrome-*c* oxidase vesicles or liposomes in low ionic strength medium, diS-C_3-(5) is subject to destruction by cytochrome-*c*-mediated lipid peroxidation, since a marked FCCP-insensitive decrease in the probe fluorescence in these systems was observed upon cytochrome *c* addition. The destruction of the probe could be prevented or reduced by high ionic strength (50 mM sodium phosphate buffer), prior addition of ascorbate, or the preparation of phospholipid vesicles in butylated hydroxytoluene (BHT), an antioxidant. Ca^{2+} and La^{3+} ions also prevented the destruction of the probe, since they prevent the binding of cytochrome *c* to the membranes. The use of high ionic strength is recommended when diS-C_3-(5) is used to monitor $\Delta\psi$ in these systems, or if low ionic strength medium is necessary, ascorbate should be added prior to cytochrome *c*. The cyanine

diO-C_5-(3) was not susceptible to destruction by the former processes in phospholipid vesicles.

A number of cyanines have been used in monitoring ion translation in sarcoplasmic reticulum membranes that generates diffusion potentials detectable by these probes. Examples of this application are the work of Young et al. [51] and Meisner and Young [52] based on diO-C_5-(3). The Pechatnikov group [41,42] have employed diS-C_3-(5) in sarcoplasmic reticulum membrane permeability studies and have noted that at probe concentrations somewhat higher than normally employed in these investigations, diS-C_3-(5) increased the permeability of the membrane in a manner similar to that caused by temperature increases or detergents.

Beeler et al. [53] have investigated the behavior of diI-C_1-(5) and diS-C_2-(5) in sarcoplasmic reticulum vesicles from rabbit skeletal muscle. The energy-dependent signals from these probes, monitored by dual wavelength absorption spectroscopy, were calibrated by K^+ or Cl^- diffusion potentials. The diI-C_1-(5) signals generated by diffusion potentials were linear over a ± 30 mV range whereas the diS-C_2-(3) response was linear from zero to -100 mV. The latter probe exhibited significantly reduced sensitivity to inside-positive charge gradients in this preparation. The presence of passively loaded Ca^{2+} reduced the absolute magnitude of the optical probe signal generated by inside-negative diffusion potentials. This effect was essentially the same as that observed during ATP-driven Ca^{2+} uptake in the sarcoplasmitic reticulum preparation. The magnitude of the signals from the two probes was approximately proportional to the amount of Ca^{2+} accumulated, and the reduction of the signals persisted as long as Ca^{2+} was retained by the vesicles.

Probe optical signals that were qualitatively indistinguishable from those generated by diffusion potentials were observed when Ca^{2+} was added under conditions where no such potentials were present. These observations suggest that the optical signals from the indicated probes are, at least in part, sensitive to the direct effect of ion flux on membrane properties such as the surface potential. In order to correct for the direct contribution of ion flux to the probe signals, the difference in the ATP-generated probe signal in this preparation with and without valinomycin was taken as a measure of any probe signal due to a transmembrane potential gradient on the assumption that the surface potential component in the probe signal was unaltered by the ionophore. Results obtained with diI-C_1-(5) using the preceding procedure indicating that a small inside-negative potential of approx. 10 mV is present during Ca^{2+} accumulation. In related work based on measurements with diO-C_3-(5), Meissner [54] has concluded that the formation of an inside-positive potential during ATP-dependent Ca^{2+} accumulation is prevented by compensating ion flux during this process and that the accumulation of Ca^{2+} can be enhanced by a pH gradient or inside negative diffusion potential. Dupont [55] has postulated from work with diS-C_2-(5), calibrated with diffusion potentials over a ± 100 mV range, that in the absence of oxalate to complex the Ca^{2+} ions, the first few cycles of the Ca^{2+} pump generate an inside-positive potential of approx. 60 mV that is inhibitory to ATPase activity. The electrogenic nature of the Ca^{2+} accumulation pump was found to be maintained as long as an inside-negative potential was maintained but was gradually reduced with increasing vesicle depolarization, a conclusion in agreement with the work of Zimniak and Racker [56] on a reconstituted Ca^{2+}-ATPase system based in part on the use of ANS as an potential-sensitive indicator.

In earlier work, Russell et al. [57] investigated the behaviour of a series of oxycarbocyanine dyes diO-C_n-(3), where $n = 3,4,5$ and 6, and diO-C_3-(5) in sarcoplasmic reticulum vesicles under conditions similar to those described by Beeler et al. [53]. Conclusions similar to those of the latter investigators regarding the relative sensitivity of the probes to direct ion flux effects, presumably the membrane surface potential, and transmembrane potentials were reached: although the ATP-dependent signals contain a component that is sensitive to transmembrane potential, a significant portion of the total probe signal is derived from the association of Ca^{2+} and ATP to the sarcoplasmic reticulum vesicles and that the optical signals associated with the two process are qualitatively very similar.

diS-C_3-(5) has been used extensively in work with the red cell and red cell ghosts. A characteristic decrease in the dye fluorescence intensity is observed upon addition of the preceding preparations to the free probe solution; the fluorescence decrease has been ascribed by Sims et al. [58] to a high concentration of the probe in the interior of these cells that favors the formation of nonfluorescent probe aggregates. Hladky and Rink [59] and Tsien and Hladky [60] have found that the uptake and binding of the probe in these preparations is very sensitive to the presence of oxyhemoglobin. In the intact cell, the binding of the probe in the intracellular volume is dominated by the latter protein, a process that leads to the formation of nonfluorescent probe dimers. The residual fluorescence observed after probe uptake in this system was ascribed to membrane-bound monomers based on the characteristic absorption spectrum of this species. The behaviour of diS-C_3-(5) in erythrocytes differs from that predicted by the model of Cabrini and Verkman [38], which does not take into account large cell volume or probe binding by intracellular components such as proteins. Using measurements based on a null point K^+ titration technique (discussed in the probe calibration section), Hoffman and Laris [61] have obtained from the signal point calibration of the CC_6 probe fluorescence signal a value

of −15 mV for the *Amphiuma* red cell resting potential; the K^+ distribution was found to be within a few percent of the Cl^- distribution ratio; the potentials calculated from either K^+ or the Cl^- distribution were in agreement with the −15 mV value obtained by microelectrode measurements of the resting potential of the *Amphiuma* red cell. In the human red cell, the resting potential was estimated to be −9 mV from a calibration generated from valinomycin-K^+ diffusion potentials but with the Cl^- membrane permeability explicitly included in the calibration procedure by use of the constant field equation with $P_{K^+}/P_{Cl^-} = 20$ when valinomycin was present. The resting potential value so obtained is in agreement with the Cl^- distribution obtained in the absence of the ionophore. Freedman and Hoffman [62] have more recently used the Jacobs-Stewart theory for Donnan equilibria in the red cell modified for nonideal solutions by the use of osmotic coefficients for hemoglobin and for salts to accurately predict Donnan potentials as a function of medium composition. The Donnan potentials calculated from the theory have been used to calibrate the fluorescence signal from diS-C_3-(5) and diI-C_3-(5) in the human red cell. It was further shown that the calibration plot for diS-C_3-(5) fluorescence signal generated by varying the Cl^- distribution was coincident with that obtained from diffusion potentials calculated from the constant field equation when a value of 20 for the P_{K^+}/P_{Cl^-} ratio was employed. These and other studies are discussed in detail by Laris and Hoffman [63] and by Freedman and Laris [64].

The cationic probe safranine exhibits energy-dependent absorption spectrum shifts in mitochondria and has been shown to be specifically sensitive to $\Delta\psi$ in that the absolute magnitude of the absorbance change induced by succinate is enhanced by nigericin + K^+, and the total energy-dependent signal is completely abolished by valinomycin [65]. Akerman and Wikström [65] have found that the absorption spectrum shift of safranine is linear with diffusion potential values calculated from the Nernst equation over a −40 to −170 mV range. Within experimental error, the probe response was identical to potentials generated with either valinomycin + K^+ or by pH jumps in the presence of

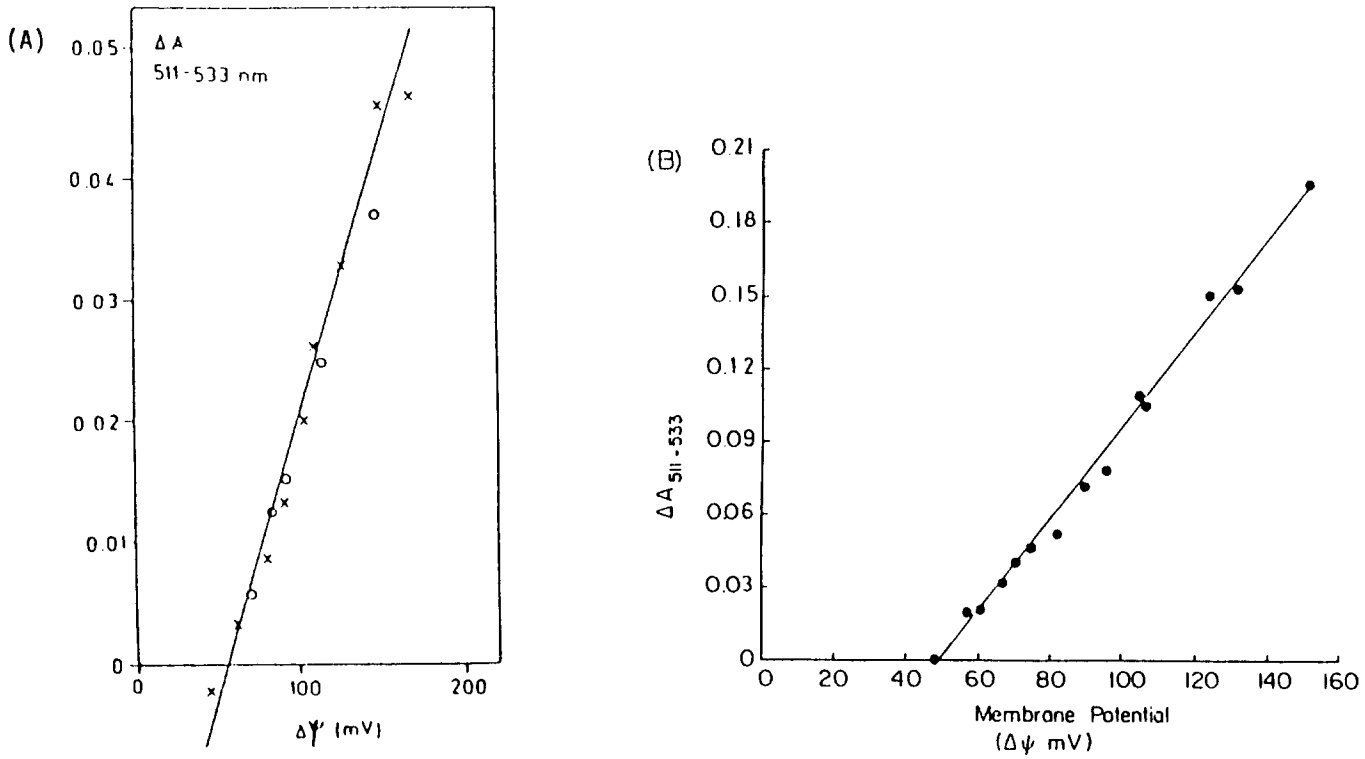

Fig. 2. Calibration plots derived from the differential absorbance change at the indicated wavelength pairs exhibited by the cationic molecular probe safranine to diffusion potentials generated in rat liver mitochondria, panel (A), and in mung bean mitochondria, panel (B), illustrating that the probe absorbance signal is a linear function of potential from nominally 40 to 170 mV. In panel (A), the circles and crosses, respectively, correspond to H^+ and K^+ diffusion potential values, whereas K^+ potentials only were generated in panel (B). The ionophores FCCP and valinomycin were used in the generation of the H^+ and K^+ diffusion potentials, respectively. Deviations from linearity occur when the potential exceeds nominally 170 mV. Note that in each plot the intercept on the abscissa corresponding to no probe absorbance change corresponds to a $\Delta\psi$ of nominally 50 mV; since an uncoupler was present in both experiments, the $\Delta\psi$ value is presumably cancelled by the pH term in the electrochemical gradient. The $\Delta\psi$ value of 50 mV in mung bean mitochondria has been ascribed to a Donnan potential. The data shown in panels (A) and (B) are from Refs. 65 and 148, respectively.

FCCP. Above the 170 mV value, the probe response tended to underestimate the value of the potential calculated from the Nernst relationship, possibly because the K^+ leaked from the mitochondrial matrix became comparable to the smaller amount of added K^+ in the medium when the formation of high K^+ gradients is attempted in this case. Representative calibration plots based on diffusion potentials are shown in Fig. 2. Since Zanotti and Azzone [66] have suggested that the mechanism responsible for the energy-dependent safranine absorption spectrum shift is probe permeation of the mitochondrial membrane followed by stacking on the inner side of the membrane, the probe response to high potentials may also begin to saturate, since the binding sites on the membrane may be essentially completely occupied.

The plot of the safranine absorption spectrum shift vs. potential calculated from the Nernst equation intersects the abscissa near -50 mV, indicating the presence of a membrane potential of the latter magnitude in uncoupled rat liver mitochondria, determined by the high K^+ levels used in these measurements, that is cancelled by an inside acid pH gradient, Fig. 2. Nicholls [67] has obtained a similar value of $\Delta\psi$ under similar conditions using an ion distribution technique. Laris et al. [34] (see above) have also found that, in media containing high levels of K^+, resting mitochondria, presumably in state 4, support similar values of $\Delta\psi$ from measurements with diS-C_3-(5) but noted an increase in $\Delta\psi$ that fell in the nonlinear portion of the probe calibration plot when succinate was present.

Akerman and Saris [68] have been reported that Ca^{2+} and, to a 10-fold greater extent, La^{3+} inhibit the K^+ diffusion potential-induced safranine absorption spectrum shift due to probe stacking in phospholipid vesicles. The origin of this inhibition appears to be a competition of the Ca^{2+} and La^{3+} ions with safranine for binding sites on the outer and inner negatively charged surfaces of the bilayer.

In mitochondria, Akerman [69] has found that Ca^{2+} uptake depolarizes this preparation as monitored by safranine. The initial rate of Ca^{2+} translocation was a linear function of $\Delta\psi$ and was half-maximal at 10 μM Ca^{2+}. It was suggested that calcium is transported with one net positive charge.

More recently, Valee et al. [70], however, have found that in rat liver mitochondria, the uptake and retention of Ca^{2+} induced by phosphate or acetoacetate is reduced by safranine, especially when Mg^{2+} and ATP are not present in the medium. Safranine-hindered Ca^{2+} transport was accompanied by a decrease in $\Delta\psi$ and an increase in the respiration rate. These deleterious effects of the probe were much reduced when Ca^{2+} transport was initiated in mitochondria incubated with Mg-ATP, Valle et al. [70] have suggested that the use of safranine to monitor $\Delta\psi$ during Ca^{2+} transport be avoided or that at least special care should be exercised in potential measurements with this probe under ion-translocating conditions.

Storey [71] has found that the diS-C_3-(5) and safranine probes exhibit spectral changes in leaky submitochondrial particles from skeletal muscle similar to those responses observed in intact mitochondria [24,65] when the leaky particles are energized by oxygen pulses. The latter particles have membrane permeability properties similar to those of open fragments of the inner mitochondrial membrane, so a delocalized $\Delta\mu_H$ cannot be maintained, yet they retain a capacity for energy coupling [72]; the energy-dependent probe signals observed in this preparation suggest the existence of limited energy-coupling domains such as those proposed by Ernster [73] and imply a probe sensitivity to local charge separation processes.

IV. Oxonol probes

A number of negatively charged probes of the oxonol class have proved to be useful indicators of charge separation in systems that generate inside-positive potentials such as submitochondrial particles, chloroplasts, sarcoplasmic reticulum vesicles under certain conditions, and several reconstituted preparations.

Oxonols V and VI undergo an uncoupler-sensitive, potential-dependent fluorescence quenching and a red shift in the probe absorption spectrum in submitochondrial particles [74,75]. Oxonol V exhibits the larger energy-dependent fluorescence quenching, up to 80 percent of the initial signal, whereas oxonol VI undergoes the larger spectral shift, 15–20 nm, in the latter preparation. These oxonols are specifically sensitive to $\Delta\psi$ in that NH_3 and nigericin + K^+ enhance the energy-dependent signal amplitude that can subsequently be returned to the control level by valinomycin and permeant ions such as SCN^-.

The oxonol V and VI energy-dependent signals, however, are not readily calibrated in submitochondrial particles by diffusion potentials. The plots of the absorbance shift signal vs. the potential gradient calculated from the Nernst equation are nonlinear. The difficulties appear to be due in part to the leakiness of the particle membranes. Bashford and Thayer [75] in a double probe experiment in which oxonol VI and 9-aminoacridine were used to monitor $\Delta\psi$ and ΔpH, respectively, in submitochondrial particles were able to obtain a value of the electrochemical gradient, $\Delta\mu_H$, from a null point titration procedure in which the phosphate potential was varied in the presence of NADH. Under certain assumptions, values of 57–78 mV and 93–102 mV were obtained for $\Delta\psi$ for submitochondrial particles in a low- and a high-salt medium, respectively. In investigations of *Rhodopseudomonas spheroides* chromatophores in which oxonol VI was

employed as the membrane potential indicator, Bashford et al. [76] have calibrated the extrinsic probe signal by comparing it with that from the carotenoids in order to obtain values of the electrochemical gradient.

The origin of the oxonol energy-dependent optical changes in submitochondrial particles are linked to an enhancement in the quantity of membrane-associated probe when a potential is formed. In the case of oxonol VI, a decrease in the dye-membrane dissociation constant and an increase in the maximum number of membrane-binding sites, n, is observed in ATP-energized submitochondrial particles. The oxonols are also able to permeate the particle membrane and apparently bind to the inner surface of the membrane as well as accumulate in the internal volume of these particles. The membrane permeation process can be detected by noting the dye-mediated retardation of the extent and rate of the ATP-dependent cytochrome-*c* oxidase soret band shift in this preparation [77]. The initial transfer of oxonol VI to the membrane and the permeation process can be distinguished kinetically, as will be discussed in Section X.

In a comparison of the behavior of oxonol VI in complex V, Kiehl and Hanstein [78] have found an uncoupler-sensitive and valinomycin-reversible ATP-induced absorption spectrum red shift in the complex V preparation that contains quantities of cholate and desoxycholate that are sufficient to disrupt the submitochondrial particle membrane. The formation of a delocalized potential gradient in this system would appear to require the existence of vesicular structures highly resistant to detergents, perhaps because of the absence of phospholipids in the unreconstituted complex or a volume with solvent-like properties formed by the proteins of this enzyme system similar to that suggested by Wagner et al. [79] for the CF_1. In the absence of conclusive evidence for such unusual structures, other possibilities for the origin of the ATP-dependent oxonol VI signal must be considered. The association of protons or cations with the complex would tend to reduce any negative surface charge density and promote the association of the negatively charged oxonol VI with this preparation thereby generating an absorption spectrum red shift. Since the ATP-dependent probe spectral shifts were observed in the presence of 10 mM $MgSO_4$, however, the medium ionic strength was probably sufficient to screen any surface charges such that these changes have a negligible contribution to the probe signal. The association of the probe with the F_1 portion of the ATPase may be affected by the binding of ATP to the complex. Thus, the probe signal in complex V may be in part originating from processes operating at the level of the ATPase.

Oxonol VI also responds to the energization of leaky skeletal muscle submitochondrial particles [71] by O_2 pulses by a spectral red shift that develops with a halftime of less than 0.4 s. The probe thus appears to be sensitive to local charge separation that originates from energy coupling occurring in limited domains in this preparation as also sensed by cyanine probes described in the previous section.

In sarcoplasmic reticulum vesicle preparations, Beeler et al. [53] have found that the oxonol VI absorption spectrum shift is linear with K^+ diffusion potentials from zero to 100 mV (inside positive) and that the probe signal obtained with these potentials was enhanced by passively loaded Ca^{2+} in the 0 to 50 mM range. A similar Ca^{2+} enhancement of the ATP-induced probe signal was also observed in this preparation. As in the case of the cyanines previously discussed, a portion of the oxonol VI signal was due to the direct effect of Ca^{2+} translocation, presumably via changes in the vesicles surface potential, and the latter signal was qualitatively identical to that due to transmembrane potentials. Several other oxonols, including WW781 and the barbituric acids [53] diBA-C_n – $(2m + 1)$ where $n = 4$, $m = 2$ and $n = 4$, $m = 1$ were not sensitive to diffusion potentials in this system. The ATP-induced signals observed using these indicators thus apparently originated from surface potential changes associated with ion translocation.

In chloroplasts, actinic illumination generates the characteristic red shift in the oxonol VI absorption spectrum [80]. Using K^+ diffusion potentials as calibrations Schuurmans et al. [80] estimated that a $\Delta\psi$ of 90 mV was initially developed upon illumination of a chloroplast suspension and that the potential decayed to 50 mV when steady-state illumination conditions were achieved. The oxonol VI probe response developed within 20 ms after illumination of the suspension and corresponded to the faster phase decay of the carotenoid band shift signal, i.e., the development of the oxonol VI spectral shift lagged behind that of the carotenoid band shift signal.

Admon et al. [81], in additional work with oxonol VI in chloroplasts, demonstrated that the probe was specifically sensitive to $\Delta\psi$ in that nigericin enhanced the probe absorption spectrum shift that could then be abolished by valinomycin. The latter investigators, however, found that the oxonol VI absorbance signal was sensitive to medium ionic strength and concluded that it could not be calibrated with K^+-valinomycin diffusion potentials. An attempt was made to calibrate the probe signal with proton diffusion potentials, and a value of approx. 50 mV was obtained for $\Delta\psi$ under steady-state actinic illumination. The oxonol VI response to these apparent proton diffusion potentials, however, was not sensitive to valinomycin in the presence of K^+ but could be abolished by detergents. The value of $\Delta\psi$ obtained in these experiments must therefore be treated with caution. The oxonol VI absorbance change caused the diffusion potential generated by proton influx into

the interior of azolectin vesicles, however, was reversible by K^+-valinomycin.

In Photosystem-I-enriched subchloroplast vesicles, under conditions in which the redox potentials were adjusted for optimum electron transport, Peters et al. [82,83] found that the time development of the oxonol VI absorbance change signal and the slower phase carotenoid signal was similar but not identical. Under the latter conditions, nigericin was without effect on the probe signal, suggesting that the probe may be responding to the total $\Delta\mu_H$ or, more likely, that $\Delta\psi$ is maximal in this case. Under suboptimal conditions, nigericin did enhance the probe signal, indicating that proton displacement was contributing to $\Delta\mu_H$. The carotenoid slower phase decay and the oxonol VI signals were considerably more sensitive to the presence of valinomycin, uncouplers and to digitonin than was the faster decay phase of the carotenoid band shift. These observations suggest locations for both the carotenoid and the oxonol VI probes near the membrane surface. Since the activation energies for the response of the two indicators to charge gradient formation were somewhat different, the locations of the two probes are probably not identical. Peters et al. [82] found no change in oxonol VI fluorescence lifetime or in probe-membrane binding during continuous illumination of the suspension.

Since in spinach chloroplasts, oxonol VI inhibits ATP synthesis under flash illumination conditions, Galmiche and Gerault [84], however, have suggested that the potential-dependent response of oxonol VI to $\Delta\psi$ be self-limited under the latter conditions by the electrophoretic movement of the probe across the thylakoid membrane with a resulting attenuation of $\Delta\psi$. The dye-to-membrane concentration ratio was some 250- to 500-times higher in the latter work than in that by Peters et al. [82], so the likelihood of sufficient free probe being available to distribute onto the thylakoid membrane with subsequent permeation of the membrane is significantly higher in the latter investigation.

Bashford et al. [76] have also employed oxonol VI in chromatophores from *Chromatium vinosum* and *Rhodospirillum rubrum* S-1. An actinic-light-induced oxonol VI absorbance decrease was detected at 587 nm, an isosbestic point in the carotenoid band shift signal. The probe response could also be enhanced by nigericin + K^+, indicating probe specificity to $\Delta\psi$ in these systems. The presence of oxonol VI was observed to accelerate the decay of the carotenoid band shift, indicating that, in the chromatophore preparations at least, the probe does redistribute across the membrane, presumably in accordance with $\Delta\psi$. These observations differ from those of Peters et al. [82] (see above), but the oxonol VI concentrations employed by Bashford et al. was 5-times greater than that employed by the former investigators. The light-induced oxonol VI signal observed in the chromatophores was linear with potentials calculated from the carotenoid band shifts. The energy-dependent oxonol VI signal time-course was monophasic and followed a second-order rate law in these membrane preparations with a rate constant $k_2 = 10^6$ M(dye)$^{-1} \cdot$ s^{-1}.

Krab et al. [85] have developed a model that accounts for the thresholds observed in the oxonol VI response to K^+ diffusion potentials in a number of systems. The model predicts such a threshold if a pre-existing inside-negative potential is present; the magnitude of the threshold is predicted to increase as vesicle size and the internal K^+ concentration decrease. On a semiquantitative basis, the model accounted for the behavior of oxonol VI in small (20–30 nm diameter) sonicated vesicles and in larger (200–300 nm diameter) vesicles prepared by a dialysis technique using both azolectin or lipids from *Synechococcus* 6716 for both types of preparation. A significantly larger threshold in the probe response to inside-positive K^+ diffusion potentials was observed in the smaller vesicles than in the larger ones.

The observed behavior of oxonol VI in this system does not appear to be dependent upon the mechanism of the probe response, and thresholds in ATP synthesis have been observed in small proteoliposomes that are significantly larger than those observed in larger reconstituted preparations; oxonol VI was used to monitor potential gradients used to drive the ATP synthesis. The extension of this model to preparations such as submitochondrial particles, mitochondria and chloroplasts is complicated by uncertainties in the ion activities in the internal volumes of these preparations and by the effect of probe association with matrix proteins. A threshold in the safrinine response to calibrating diffusion potentials in rat liver mitochondria has also been observed by Akerman and Wikström [65] and interpreted in terms of a pre-existing potential gradient, as has Nichols et al. [67] using an independent technique as discussed in the section on cyanine probes.

V. Merocyanines

Merocyanine 540 is a member of the latter class of probes included in an extensive survey conducted by Cohen et al. [4,86] of dyes as possible indicators of the action potential in the giant axon from the squid *Loligo peali*. The probe fluorescence response to the development of the action potential in the latter preparation was coincident with the signal from electrodes and, as subsequently demonstrated by Ross et al. [87], an increase in the signal-to-noise ratio over that obtained from fluorescence measurements was possible using absorption spectroscopy to follow the probe response to this potential. Salama and Morad [88] have also demonstrated that merocyanine 540 emission accurately follows the time-course of the action potential in frog heart muscle strips.

Waggoner [3] has suggested that the rapid optical changes displayed by fast responding probes are due to a rapid association/dissociation dye–membrane interaction that requires a differential probe concentration at the two bulk phase interface regions of the bilayer. Conti [89] and Dragston and Webb [90] have suggested, however, that the loss in merocyanine 540 fluorescence intensity observed in a number of preparations may be due to the rotation of membrane-bound merocyanine 540 monomers into the plane of the membrane due to the torque resulting from the interaction of the electric field with the large dipole moment of this probe.

Verkman [91] has developed a mechamism, compatible with the preceding merocyanine 540–membrane interaction model, for the probe fluorescence intensity loss that involves the association of the dye with vesicle bilayers within 1 ms, then a reorientation of the bound monomers in less than 1 ms followed within 10 ns by the formation of dimers with the long axis of the probe perpendicular to the lipid acyl chains and located deep in the bilayer.

In submitochondrial particles as measured by dual wavelength absorbance spectroscopy [92], the energy-dependent merocyanine 540 optical signal follows a second order rate law with $k_2 = 10^4$ M(dye)$^{-1} \cdot$ s^{-1}; at micromolar concentrations, the probe signal thus develops much less rapidly ($t_{1/2} = 70$ s) in the submitochondrial particle system than in the previously cited investigations. No unresolved faster phase signal could be observed in rapid mixing measurements in the latter preparation at 5 ms time resolution.

Binding studies indicate that the origin of the merocyanine 540 energy-dependent signals in submitochondrial particles is enhanced association of the probe with the particle membrane [92]. The probe, however, does not electrophoretically cross the latter membrane as judged by the lack of an effect of the presence of the probe on the ATP-dependent cytochrome-*c* oxidase Soret band shift signal or on the rate of formation of NADH or cytochrome *c* oxidation both via ATP-driven reversed electron transport; the latter three processes cover a time span of 20 ms to 20 min.

The binding constants characterizing the interaction of merocyanine 540 with submitochondrial particles are highly sensitive to medium ionic strength, suggesting that the probe signal may arise in part from membrane surface potential alterations. In related work, Russell et al. [57] have also found that the latter probe signal as well as that from a number of other merocyanines and oxonols observed during Ca^{2+} translocation in sarcoplasmic reticulum preparations appears to contain a significant surface potential component.

Merocyanine 540 also responds to energization of leaky skeletal muscle submitochondrial particles [71] by O_2 pulses; a slowly developing fluorescence intensity decrease was observed and implies a probe sensitivity to local charge gradients.

Using absorbance, fluorescence and circular dichroism spectroscopy, Lavie and Sonenberg [93] have found evidence for the direct interaction of merocyanine 540 with valinomycin at high (153 mM) KCl when no membrane was present. The dye–ionophore interaction was much reduced when the KCl concentration was reduced to 5 mM and no evidence for such interaction between the probe and valinomycin could be detected when the medium contained 153 mM NaCl. Thus, caution must be exercised in calibrating merocyanine 540 optical signals with K^+-valinomycin diffusion potentials.

Masamoto et al. [94] have compared the light-induced optical response of the supposedly impermeable merocyanines NK2272, NK2273, and NK2274 with the carotenoid band shift in chromatophores from *Rhodopseudomonas sphaeroides*. The extrinsic dye signal was linearly proportional to the carotenoid signal; salt-induced merocyanine signals were small in comparison to those generated by illumination, suggesting that a small change in the membrane surface potential occurs upon energization of this preparation, an observation not in accord with the theory of Malpress [13]. The behavior of the merocyanine and carotenoid signals in the presence of uncouplers was also found to be inconsistent with the existence of a potential gradient across the unstirred layer adjacent to the chromatophore membrane as required in the energy transduction model of Kell [12].

Novak and Freedman [95] have employed the merocyanine-rhodamine probe WW781 in experiments in which the red-cell resting potential was determined from the proton distribution in the presence of an uncoupler and the anion-exchange inhibitor DIDS.

VI. Miscellaneous extrinsic probes

A number of investigations based on potential-sensitive probes that do not fall into the preceding categories devoted primarily to work with polyene-type dyes are contained in this section.

Tomov [96] has recently shown that the fluorescence of the cationic probe pyronin G undergoes an ATP- and succinate-dependent quenching in rat liver mitochondria that can be reversed by oligomycin and 2,4-dinitrophenol, respectively. A model to explain the behavior of this probe has been developed based on the assumption that the probe distributes across the inner mitochondrial membrane in accordance with the Nernst relationship and that the decrease in fluorescence intensity is due to the formation of nonfluorescing dimers due to the increment of the probe concentration in the matrix; higher-order aggregates were not considered,

nor was probe–membrane association, unlike the model developed by Ivkova et al. [40] for the behavior of diS-C_3-(5) in sarcoplasmic reticulum preparations. A method for quantitatively determining the membrane potential has been developed that is dependent on the ratio of the fluorescence intensity observed in the presence and absence of a potential gradient, on the total probe concentration, and on the volume fraction due to the membrane preparation under study. This procedure does not require a calibration plot relating probe fluorescence and membrane potential to be developed and is applicable to potentials in the range −120 to −220 mV. Membrane potentials values of −175 and −200 mV, respectively, supported by ATP and succinate in the rat liver mitochondria preparation were obtained by the preceding method.

Mikes and Dadak [97] have suggested that a number of cationic probes of the berberine class may be sensitive to charge gradients and related phenomena at the outer surface of the mitochondrial inner membrane, but no attempt at calibrating the probes has apparently been carried out. An FCCP-sensitive increase in the probe fluorescence intensity is generated by succinate oxidation; this increase has been attributed to an increase in the probe quantum yield apparently due to a potential-driven relocation of the probe to more hydrophobic regions of the membrane, although a small increase in the quantity of membrane-bound probe was observed when the mitochondrial preparation was energized. The largest fluorescence signal increase was observed with the 13-methyl derivative. The more hydrophobic berberine probes, however, inhibited NAD-linked respiration but had no effect on succinate oxidation up to a probe concentration of 10 μM. The latter inhibition has been investigated in detail by Mikes and Yaguzhinskij [98].

VII. Carotenoids

At least a fraction of the carotenoid pigments [99] and certain chlorophyll complexes found primarily in photosynthetic membranes undergo an absorption spectrum band shift according to the relationship [100]:

$$h\Delta\nu = -\Delta\mu \cdot F - (1/2)\Delta\alpha F^2 \qquad (1)$$

in which $\Delta\mu$ and $\Delta\alpha$ are, respectively, the difference in ground and excited state dipole moments and polarizabilities. F is the electric field strength associated with a potential gradient, $\Delta\nu$ is the absorption or emission spectrum shift in frequency units and h is Planck's constant. Since the electrochromic, charge shift, or Stark effect operates on the time-scale of an electronic transition, 10^{-15} s, these intrinsic probes are able to follow the time-course of rapidly developing charge separation events in the aforementioned membranes. From measurements based on chlorophyll absorption signals, Dutton et al. [101] have shown that charge separation occurs in reaction centers within 10 ps following an actinic light flash.

Packham et al. [102] have investigated the behavior of the light-induced carotenoid band shift in relationship to electron transport in chromatophores from *Rhodopseudomonas sphaeroides*. When cytochrome c_2 was available for oxidation by the reaction center, the decay of the carotenoid signal caused by short-duration light flashes was slow, having a halftime of nominally 10 s. The decay rate was significantly faster when cytochrome c_2 was unavailable for oxidation – halftime of approx. 1 s – and the decay in this case could be accelerated 20-fold by *ortho*-phenanthroline via a probable back-reaction involving the reduction of the chlorophyll dimer. In each of the preceding instances, the carotenoids signal decay but not the electron transport could be accelerated by submicromolar concentrations of valinomycin or FCCP, indicating that the induced charge separation in the chloroplast reaction center is rapidly delocalized and can then be dissipated by the back-reactions involving the dimer or by electrophoretic ion flux. When cytochrome c_2 was unavailable for oxidation, the FCCP-sensitive proton efflux process could be decreased by valinomycin plus K^+, indicating that the dimer → Q electron transfer generates a delocalized membrane potential as sensed by the carotenoids even though electron transport may proceed across only a portion of the membrane dielectric.

Symons et al. [103] have estimated the contribution of surface potential changes to the carotenoid signal induced by the formation of a K^+ diffusion potential across the membrane of *Rhodopseudomonas capsulata* chromatophores to be 20%. The surface potential contribution could be corrected for by employing Na^+ to maintain a constant ionic strength when calibrating K^+ diffusion potentials were developed. Additional corrections were proposed as necessary for pH effects possibly due to proton diffusion potentials as well as for suboptimal valinomycin to membrane concentration ratios; if the latter corrections were not applied, an error approaching a factor of 2 in the determination of the membrane potential was postulated.

Michels et al. [104] have found that $\Delta\psi$ determined by SCN^- distribution in chromatophores from *Rhodopseudomonas sphaeroides* is approx. 20% less than that obtained from carotenoid band shift measurements; apparently, the difference is due to a surface potential component in the carotenoid signal in this preparation. Similar observations have been reported by Ferguson et al. [105].

Clark and Jackson [106] have performed a comparative study of the light- and respiration-generated membrane potentials in *Rhodopseudomonas sphaeroides* whole cells. In order to calibrate the carotenoid signals

with K^+-valinomycin diffusion potentials, it was necessary to use chromatophores obtained from the whole cells, which are resistant to the effects of ionophores. The corrections employed by Symons et al. [103] described above, however, were found to be unnecessary in fresh chromatophores suspended in a medium of higher ionic strength than that used by Symons et al. No dependence of the diffusion potentials on the valinomycin to membrane concentration ratio was observed over a 10-fold range. The use of Rb^+ in lieu of K^+ when generating diffusion potentials reported by Symons et al. as necessary to avoid surface potential changes was found to be unnecessary by Clark and Jackson when a high salt medium was employed. These differences were ascribed to chromatophore membrane permeability changes probably associated with aging or storage conditions employed by Symons et al. The membrane potentials generated by light and respiration were larger than those obtained from $BTPP^+$ (butyltriphenylphosphonium ion) distribution. This discrepancy could not be explained on the basis of a surface potential change associated with a decrease in the internal thylakoid pH, since the medium contained NH_4SO_4, and the ΔpH contribution to $\Delta\mu_H$ was expected to be no more than 50 mV. The carotenoids may in this case sense the potential difference between membrane interfaces that in the model of Kell [12] exceeds the bulk phase potential gradient that is reported by the $BTPP^+$ ion distribution. A qualitative discrepancy in the light- or respiration-induced cytoplasmic $\Delta\psi$ in *Rhodobacter sphaeroides* cells as measured by TPP^+ distribution and by the carotenoid band shift has been reported by Crieland et al. [107]. Simultaneous measurements of $\Delta\psi$ based on TPP^+ distribution and the carotenoid signal, respectively, indicate that the cytoplasmic membrane is depolarized and hyperpolarized. The ATPase inhibitor venturicidin reduces the $\Delta\psi$ as measured by TPP^+ distribution but causes an apparent increase in $\Delta\psi$ as indicated by the carotenoid signal. These results suggest that at least one of these methods is seriously in error as used in this system.

Matsuura et al. [108,109] have shown that carotenoid band shift signals in cells, spheroplasts and chromatophores from *Rps. sphaeroides* can be caused by the addition of a number of monovalent and divalent salts and/or by changes in medium pH when an uncoupler is employed to control any transmembrane potential gradient. The salt- and pH-induced signals were ascribed to a change in the membrane surface potential that was estimated to be as high as 90 mV.

Raman spectroscopy has been employed in a number of studies of carotenoid behavior in photosynthetic systems. Koyoma et al. [110] have evaluated the signal from neurosporene, the dominant carotenoid in the G1C mutant of *Rps. sphaeroides*. When excitation was at 472.7 nm, the ratio of the two most prominent band intensities was very sensitive to small shifts in the neurosporene absorption spectrum induced by diffusion potentials or by FCCP, oligomycin, or desoxycholate. The Raman band intensity ratio was temperature dependent and contained a signal component due to the excitation beam acting as an actinic light source.

Johnson et al. [111] have incorporated the carotenoid β-carotene into phospholipid vesicles containing bacteriorhodopsin from *Halobacterium halobium*. A light-induced membrane potential that developed within 14–22 μs was monitored using the technique of kinetic Raman spectroscopy. The membrane potential sensitivity was found to be derived from an electrochromic response of β-carotene, since the latter sensitivity was dependent on the excited state properties of the carotenoid. This investigation is an example of the retention of the electrochromic character of carotenoids in reconstituted systems. Presumably a preferential orientation of β-carotene was assumed in the vesicle bilayer, since the first term of Eqn. 1 averages to zero if the orientation of the carotenoid is isotropic and the much smaller signal generated from the polarizability difference $\Delta\alpha$ would be the primary remaining source for electrochromism in this case.

Resonance Raman spectroscopy has been employed by Szalontai [112] to monitor membrane potential formation in the R_1M_1 mutant of *H. halobium* by measuring the effect of the carotenoid absorption band shift on the Raman spectrum associated with the 482.5 and 586.1 nm absorption region. The light employed in the Raman measurements also served to drive the photochemical cycle of bacteriorhodopsin. The ratio of the intensity of the Raman bands to that in spectral regions where no Raman signal was present was used to minimize light scattering artifacts from the whole cell suspensions. Analysis of the data yielded a time constant of 80 ($\pm$20) μs for membrane potential formation; this value was taken as the upper limit for the $BR_{570} \rightarrow M_{412}$ transition and is similar to the value of 50 μs reported for this transition in purple membrane fragments [113].

VIII. Charge shift extrinsic probes

The carotenoid pigments found primarily in photosynthetic systems respond to the formation of a potential gradient by direct interaction of the pigment electronic structure with the electric field associated with the potential difference present across or within the membrane; see Eqn. 1. The reconstitution of the carotenoids in membrane preparations that lack them, especially in non-photosynthetic systems such as the mitochondrial membrane, such that the electrochromic sensitivity of the pigments is preserved has often been unsuccessful apparently because the preferred orientation of the carotenoids in the membrane bilayer necessary for the first term in Eqn. 1 to be nonzero is not

assumed. Since the electrochromic mechanism operates on a time-scale sufficiently rapid to allow a probe responding by such a process to faithfully follow the time-course of virtually any charge separation process of physiological significance, the need for extrinsic probes that operate on the charge shift principle that could be employed in membranes lacking carotenoids is obvious.

Loew and associates have addressed the latter problem by using in several probe syntheses the *p*-aminostyrylpyridinium (ASP) chromophore that is predicted from molecular orbital calculations to have a large difference in the permanent dipole moment in its ground and excited electronic states. The probes di-6-ANPPS and di-4-ASP that contain the ASP chromophore have been synthesized by Loew et al. [114] and were shown to assume a preferred orientation in hemispherical bilayers and to undergo characteristic small shift in response to an electrical potential gradient applied via electrodes; these spectral shifts are compatible with an electrochromic mechanism. The compounds di-*n*-ASPPS ($n = 4,6,10$) and di-*n*-ASP ($n = 1,4,5,6,10$) have also been prepared by Loew et al. [115] and, when employed in the squid giant axon, respond within 1.2 μs to the action potential in this preparation; the observed shifts in the transmission and fluorescence emission spectrum of the probes are consistent with an electrochromic mechanism. The percentage change in the transmission or fluorescence reference signal was a factor of 40 smaller in the axon preparation than that observed in the aforementioned model membrane system, possibly due to staining of nonexcitable tissue in the axon by these dyes. These probes contain a sulfonate group that renders them zwitterionic near neutral pH and hence are not susceptible to electrophoretic forces that would tend to cause them to cross the axon membrane; the localized charge on the sulfonate moiety renders such a process particularly unfavorable.

Grinvald et al. [116] have synthesized some twenty styryl class probes and tested their sensitivity to the action potential in cultured neuroblastoma cells. The signal-to-noise ratio from some of these probes was as high as 90, larger than that obtained with any probe employed in previous work; fluorescence changes as large as 14 percent/100 mV were observed. Although the mechanism(s) responsible for the potential-dependent signals observed in this system may include electrochromism, the origin of these signals is not entirely clear, especially in view of the substantially longer time-scale of these experiments in comparison to those of Loew et al. [114,115] previously described.

The probe RH-160 has been employed by Ehrenberg,

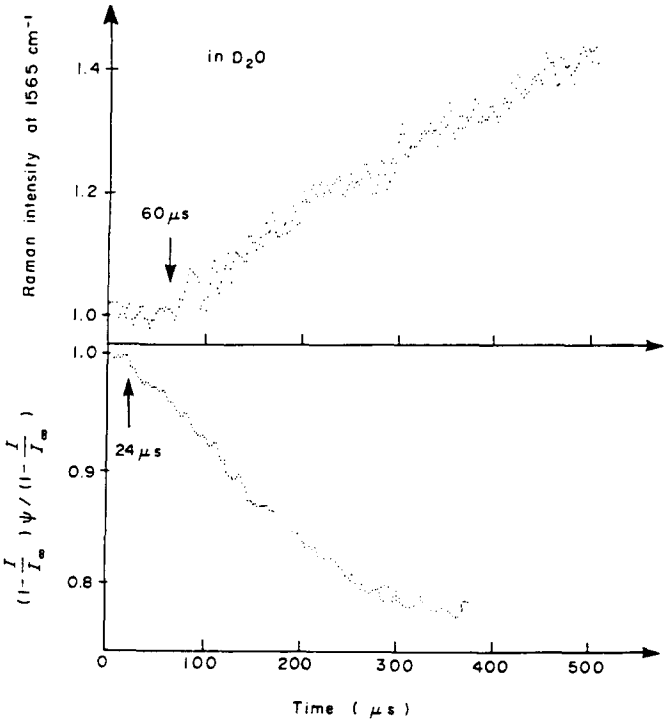

Fig. 3. The time-course of the M_{412} intermediate formation (upper panel) in the photocycle of *Halobacterium halobium* in a D_2O medium is compared to the response of the charge shift extrinsic molecular probe RH 160 to transmembrane potential development (lower panel). The M_{412} intermediate formation was followed by monitoring the Raman scattering signal at 1565 cm^{-1} generated from a 250 μs duration 0.15 W light flash at 457.9 nm. The RH 160 fluorescence signal has been corrected for possible surface potential contributions to the probe response. The numerical values given in the panels are the formation times for M_{412} and for an apparently new intermediate in the photocycle suggested by the extrinsic probe time-course. A substantial isotope effect is observed in these experiments, indicating that the photocycle involves proton kinetics. The M_{412} formation time in H_2O was found to be 22 μs. From Ref. 117.

Meiri and Loew [117] in work on the photochemical cycle of bacteriorhodopsin from *H. halobium* using Raman spectroscopy. The ligth-induced, apparently electrochromic signal develops on a microsecond time-scale and analysis of the results requires the existence of a photochemical intermediate X, the formation of which precedes the development of the M_{412} species or even the conversion of K_{590} to L_{590}. A comparison of the time-course of the M_{412} intermediate development and that of the response of the RH 160 probe to $\Delta\psi$ is illustrated in Fig. 3.

Thus far, however, electrochromic signals from charge shift probes have not been observed in mitochondrial or related reconstituted systems, although comparatively little work has been performed with such probes in these preparations.

IX. Applications of extrinsic probes to cultured cells, tissues and organs

A number of probes appear to be primarily sensitive to potential gradients associated with organelles in situ, primarily the mitochondrion.

Using the technique of fluorescence microscopy, Johnson et al. [118] have found that several cationic probes such as diO-C_2-(5) and a number of related cyanines, safranine O, and several rhodamines are preferentially accumulated in the mitochondrial matrix of culture-grown cells of various types. A number of neutral or anionic dyes, however, did not exhibit fluorescence associated with the mitochondria in these cells. The association of rhodamine 123 with in situ mitochondria was studied in detail; the enhanced probe fluorescence intensity could be reduced by both uncouplers and electron transport inhibitors and was increased upon the addition of nigericin to cells previously stained with this probe. Mitochondria associated with cells grown under anaerobic conditions were only weakly stained with rhodamine 123, but the probe fluorescence level observed from the mitochondria reached that detected from these organelles in aerobically grown cells when oxygen was introduced to the former anaerobically grown cultures. Oligomycin did not affect the rhodamine 123 fluorescence intensity from the mitochondria when it was introduced to prestained cells; relative to control levels, a slight increase in the probe fluorescence was observed, however, if the inhibitor was introduced prior to the staining of the cells with this dye. The preceding series of observations suggest that the accumulation of rhodamine 123 in mitochondria in situ is controlled by the mitochondrial membrane potential. Considerable heterogeneity in the degree of mitochondrial staining, however, was observed among the several types of cells employed in these investigations. The latter observation suggests that pools of mitochondria with differing membrane potentials may exist in situ.

In an extension of the previous investigations, Davis et al. [119] have contrasted the uptake of rhodamine 123 and the permeant cation TPP^+ in normal CV-1 cells from the African green monkey kidney epithelium with that in transformed MCF-7 cells from human breast adenocarcinoma. In media containing either 3.6 mM or 137 mM K^+, uptake and retention of the probe by the MCF-7 cell line, as monitored by fluorescence microscopy, was enhanced relative to that observed in the normal CV-1 cells, indicating that the plasma and/or the mitochondrial membrane potential was elevated in the transformed cell line. The difference in rhodamine and TPP^+ uptake in the two cell lines at the higher K^+ concentration, that dissipates the plasma membrane potential, relative to that at the lower medium K^+ concentration suggests the existence of an enhanced mitochondrial membrane potential in the MCF-7 cells. In the latter cell line, neither the TPP^+ nor the rhodamine uptake was enhanced by nigericin in the presence of ouabain, employed to prevent a plasma membrane potential increase, but the uptake of the two probes was markedly enhanced in the CV-1 line, indicating that $\Delta\psi$ is the primary contributor to the mitochondrial $\Delta\mu_H$ in the former cell line whereas ΔpH may be an appreciable component of $\Delta\mu_H$ in the CV-1 line. When nigericin alone was employed with the CV-1 cells, both the plasma and mitochondrial membrane potentials were maximal and the uptake of TPP^+ approached that observed in the transformed MCF-7 cells; these observations suggest that the total $\Delta\mu_H$ in MCF-7 cells is not different from that in the normal CV-1 cell line. From the ratio of TPP^+ uptake at the high and low K^+ medium concentrations, it was concluded that the plasma membrane potential was also elevated in the MCF-7 cells relative to that of the CV-1 line, and that mitochondrial staining in the MCF-7 line by the rhodamine was enhanced at the lower K^+ concentration due to the preconcentration of the dye in the cell cytoplasm. Since rhodamine 123 appears to be selectively toxic to transformed cells, the use of the probe as a therapeutic agent is discussed. Typical data are illustrated in Fig. 4.

Lampidis et al. [120] have, however, found that the cationic rhodamines 123 and 6G inhibit beating in cultured cardiac cells from newborn Sprague-Dawley rats and ultimately kill these cells, whereas the neutral rhodamines 116 and B had no such effect. The cationic rhodamines also lowered the respiratory control ratio of isolated rat liver mitochondria, whereas no effect was found with the two neutral probes. The toxic effect of rhodamines 123 and 6G on the cardiac cells may be related to enhanced intracellular accumulation of the cationic probes due to the elevated plasma membrane potential (-80 to -90 mV) in these cells relative to that of most other cell types.

Bernal et al. [121] have demonstrated that the ex-

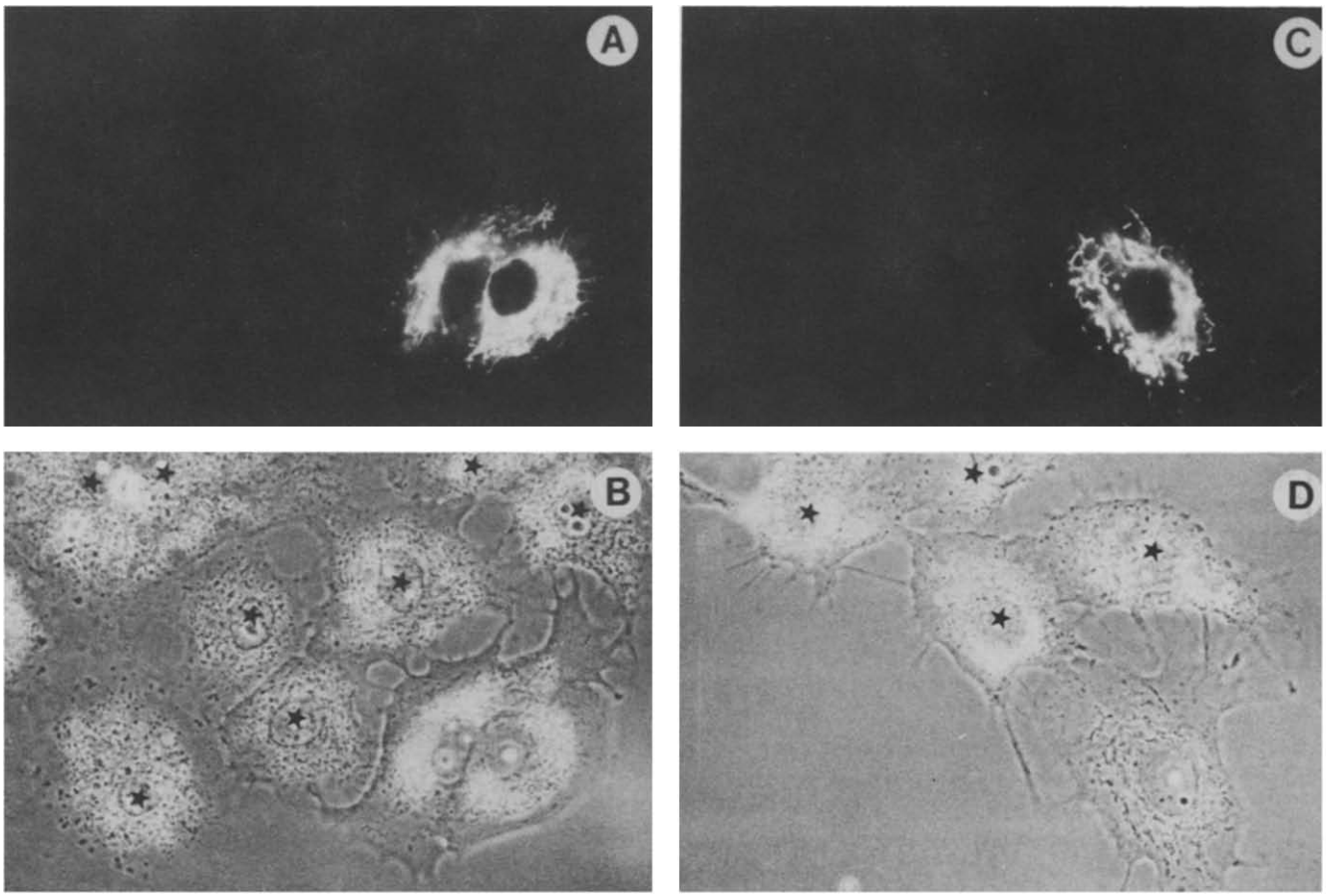

Fig. 4. Data illustrating the primarily mitochondrial uptake and retention of rhodamine 123 by MCF-7 and CV-1 cells in a medium containing 137 mM KCl used to dissipate the plasma membrane potential. Panels A and B illustrate the uptake of the rhodamine probe and C and D the retention of the cationic dye. Panels A and C are phase contrast micrographs, whereas panels B and D are fluorescence micrographs. In the phase contrast micrographs, the CV-1 cells are labeled with stars. The uptake and retention of rhodamine 123 is clearly elevated in the transformed MCF-7 cells. The magnification factor was 540. From Ref. 119.

posure of culture-grown L1210 leukemia cells to the cancer chemotherapeutic agents ARA-C, methotrexate and 5-fluorouracil results in the release of rhodamine 123 taken up by the mitochondria prior to the treatment of the cells with the preceding agents. The loss of mitochondrial rhodamine 123 fluorescence preceded changes in the plasma membrane permeability, and hence potential, as judged by the exclusion of trypan blue. Approx. 80% of the cell population exhibited a loss of rhodamine 123 fluorescence from the mitochondria, which loss could not be restored by removal of the chemotherapeutic agents by washing and prolonged incubation of the cells in growth medium. An increase in the fluorescence from the cell mitochondria could not be generated by the addition of nigericin to cells exposed to the preceding agents whose action thus appears to be the elimination of mitochondrial respiratory activity rather than a reduction in $\Delta\psi$ since nigericin is known to increase $\Delta\psi$ at the expense of the mitochondrial pH gradient.

Mokhova and Rozovskaya [46] have examined the behavior of diS-C_3-(5) and rhodamine 123 in intact lymphocyte suspensions. The quenching of the fluorescence observed upon addition of TMPD/ascorbate to these suspensions could be reversed by low concentrations of the uncouplers FCCP and 2,4-dinitrophenol in experiments of 3–5 min duration, a time span over which these uncouplers have no effect on the plasma membrane potential [67,122]. These results indicate that the two probes are responding to changes in the mitochondrial membrane potential in situ. Oligomycin caused an increase in the TMPD/ascorbate-dependent diS-C_3-(5) fluorescence quenching apparently due to an increase in the mitochondrial membrane potential when the ATP synthase activity was inhibited by this agent; a corresponding increase in the probe fluorescence level over that observed in control experiments in which oligomycin was not present was observed when 2,4-dinitrophenol was added. Oligomycin had no effect on the fluorescence of rhodamine 123, presumably because the

probe itself may be inhibiting the ATPase; such inhibition has been observed in isolated mitochondria [123]. The presence of TMPD minimized the rotenone-like inhibition of diS-C_3-(5) previously described in the section on cyanine probes [43,44].

From measurements based on fluorescence microscopy, Divo et al. [124] have demonstrated that rhodamine 123 is also taken up by the single mitochondrion of the malarial parasite, *Plasmodium falciparum*, over the entire intraerythrocyte cycle of this parasite, suggesting that the organism maintains a substantial mitochondrial potential over the latter cycle. Rhodamine 123 was released from the cells by the addition of several uncouplers, by valinomycin and nigericin, by ATPase inhibitors, and by inhibitors of electron transport. Exposure of the probe-stained parasites to uncouplers reduced the probe fluorescence signal from both the cytoplasm and the mitochondrion as expected for the dissipation of both the plasma and mitochondrial membrane potentials. The use of electron transport inhibitors, however, diminished only the mitochondrial-associated probe fluorescence intensity, as expected, whereas the inhibitor DCCD at low concentration caused the organelle-associated fluorescence intensity to increase, since the ATP synthase activity is blocked and the membrane potential consequently increases. The physiological role of the mitochondrial membrane potential is considered in the context of the finding from other investigations that glycolysis is the principal source of ATP in this parasite.

Using the fluorescence microscopy technique, Korchak et al. [125] have observed a loss in the probe diO-C_6-(5) fluorescence intensity when the peptide fMet-Leu-Phe or phorbol myristate is present in human neutrophils; this emission loss originates primarily from the mitochondria in these cells. The signal loss could be prevented by pretreatment of the cells with electron transport inhibitors or with uncouplers; oligomycin, however, had no effect on the fluorescence intensity decrease previously described. Some difference in the membrane potential values obtained using the lipophilic cation $TPMP^+$ and diO-C_6-(5) were noted. These discrepancies have been ascribed to nonidentical distribution properties of the two probes.

Wilson et al. [126] have, however, found that in lymphocytes, the cyanine probes diO-C_5-(3) and diI-C_5-(3) exhibited both plasma membrane and mitochondrial toxicity at concentrations above 5 nM and that specific plasma membrane toxicity was observed with type B cells. The use of a 15 min dye–cell incubation period, as employed by Johnson et al. [118] in measurements of in situ mitochondrial $\Delta\psi$ in transformed cells, was found to induce rapid dye toxicity.

Oseroff et al. [127] have evaluated several carbocyanine and rhodamine dyes for use in selective carcinoma cell photolysis therapy. The carbocyanine probe EDKC exhibited the greatest efficiency in the 680–700 nm light-induced killing of EJ (MGH-U1) bladder carcinoma cells grown in culture. The origin of the cell toxicity appeared to be related to the enhanced uptake of this probe by the transformed EJ and several other transformed cells relative to that observed in normal CV-1 kidney epithelial cells. The cell photolysis effect apparently originated from toxic probe degradation products including singlet oxygen and originated with the cell mitochondria; the latter observation may indicate that enhanced uptake of the dye results from elevated plasma and mitochondrial membrane potentials in transformed cells as suggested by Chen and associates [118].

The interpretation of the fluorescence intensity signals from probes that accumulate in the internal volumes of cells and organelles is compromised in many cases by uncertainties in the fraction of these signals that is due to membrane-bound probe. To circumvent these difficulties, Ehrenberg et al. [128] have synthesized a series of 'Nernstian' probes that bear delocalized (usually) positive charge, a property that facilities membrane permeation, and have short alkyl groups on the dye ring systems in order to minimize membrane binding. These probes also have high quantum yields, so they can be employed at low concentrations in experiments based on fluorescence microscopy. An application of these probes to the measurement of the plasma membrane potential in several types of cells is described in the probe calibration section.

Kauppinen and Hassinen [129] have employed the probe safranine in investigations of the Langendorff perfused rat heart. The probe fluorescence when corrected for reflectance and motional artifacts was found to be a sensitive and selective qualitative indicator of the mitochondrial membrane potential in this preparation. The latter conclusion was supported by the probe fluorescence response to changes in the extracellular K^+ concentration. When this concentration was increased to 18 mM, the plasma membrane potential was decreased, but the mitochondrial membrane potential is expected to increase due to a decrease in ATP consumption caused by the cessation of the mechanical activity of the heart. Under the latter conditions, a quenching of the probe fluorescence on the time-scale of electron transport in the respiratory chain was observed which is consistent with a hyperpolarization of the mitochondrial membrane. When the heart was maintained quiescent by the omission of Ca^{2+} from the perfusate, variation of the K^+ concentration did not affect the probe fluorescent intensity, as would be expected if the plasma membrane potential were contributing to the probe signal. Readmission of Ca^{2+} to the perfusing medium after a period of Ca^{2+}-free perfusion of the heart generated a characteristic and very rapid collapse of the mitochondrial membrane potential that could be moni-

tored by the pronounced increase in the probe fluorescence intensity. The addition of the uncoupler CCCP to the perfusate also generated an irreversible increase in the safranine fluorescence intensity. The direction of the safranine emission changes was qualitatively similar to membrane potential changes from the distribution of the permeable cation $TPMP^+$, indicating that a probable quantitative correlation between the safranine fluorescence intensity behavior and the membrane potential of in situ mitochondria exists.

The anionic probe oxonol V is responsive to the development of ischemia in the perfused rat heart preparation. In investigations based on flying spot surface fluorimetry [130], the control probe fluorescence intensity histogram becomes bimodal under conditions of partial ischemia, then becomes unimodal when complete ischemia is imposed on the heart [131]. The latter observations indicate that the oxonol V fluorescence intensity signal may be useful as an indicator of membrane potential in the perfused heart preparation, but a mitochondrial component in the ischemia-induced probe fluorescence signal loss is yet to be unequivocally established.

Oxonol V is also sensitive to electrical activity changes that accompany anoxia and seizure activity in the exposed cerebral cortex of the mongolian gerbil [132]. The probe signal monitored by surface fluorescence via fiber optics light guides is similar in its temperol development to that of the pyridine nucleotide fluorescence intensity changes, which originate primarily from the cortex mitochondria, during the onset of anoxia or to seizure episodes initiated by either the topical application of 1 M KCl or by the injection of the KCl or bicuculline into the lateral ventricle. The cyclic behavior of both the pyridine nucleotide and the oxonol V emission signals during seizure activity can be eliminated by the uncoupler CCCP, which would dissipate both plasma and mitochondrial membrane potentials, as well as by more specific electron transport inhibitors such as retenone. The oscillating bicuculline-induced fluorescence signals from both the pyridine nucleotides and oxonol V when eliminated by rotenone injection into the lateral ventricle could not be reinitiated by subsequent injections of bicuculline. The periodic, bicuculline-induced decrease observed in the oxonol V fluorescence signal, digitally smoothed and corrected for possible capillary network blood volume change artifacts, was found to precede a similar decrease in the corrected, smoothed pyridine nucleotide signal. These data suggest that enhanced neuronal firing and the resulting increased demand for ATP synthesis that occurs during seizures causes a decrease in the mitochondrial potential, detected by oxonol V, and that this reduction precedes the stimulation of respiration and hence the depletion of the reduced pyridine nucleotide level which depletion is observed as a dimunition in the intensity from these fluorophores. In unfiltered data, high-frequency signals are present in the oxonol V fluorescence record, apparently due to neuronal bursting activity, that correspond to temporal regions of diminished pyridine nucleotide intensity. Oscillations in the signal from both fluorophores characteristic of spreading depression have also been observed when the gerbil is allowed to recover from virtual total anoxia. A portion of the preceding observations is illustrated in Fig. 5.

X. Kinetic competence of molecular probes

Since one of the more attractive possible uses of potential-sensitive molecular probes is the follow the time-course of processes leading to potential gradient formation, that are normally too fast for classical ion distribution methods to detect, the question of the characteristic response time of these indicators to initial charge separation is an important issue.

A favorable case for evaluating the kinetic competence of molecular probes is one in which an intrinsic indicator or other means of independently monitoring potential gradient formation is afforded by the preparation under investigation. The ability to follow the time-course of the action potential in the squid giant axon or in heart muscle [88] afforded by electrode recordings is one example of the latter case. The fluorescence signal response to action potential for a number of probes has been shown to be coincident with the signal detected by an electrode-based recording circuit [4,86,133]. Such coincidence of the optical signal from the extrinsic probes and the electrode recordings in these cases indicates that the probes have a sufficiently low response time to follow the time development of the latter potential, at least on the millisecond time scale of the reference electrode-based signal.

In photosynthetic systems, the carotenoid response to actinic light, which probably offers the highest time resolution available, provides a reference to which the response time of extrinsic indicators may be compared. In those cases thus far investigated, the time-course of extrinsic dye signals usually lags behind that of the carotenoid, the fast developmental phase of which may be due to local charge separation not necessarily detected by extrinsic probes sensitive to delocalized potential gradients. The light-induced oxonol VI signal observed in chromatophores has, for example, been found to be approximately coincident with the initial portion of the decay phase of the carotenoid band shift signal [76,80].

The probe RH-160, however, has been shown to respond to potential gradients on a microsecond timescale in the bacteriorhodopsin photochemical cycle in *H. halobium* [117]. The probe response time in this case was sufficiently short to allow the detection of an apparent additional intermediate in the latter cycle.

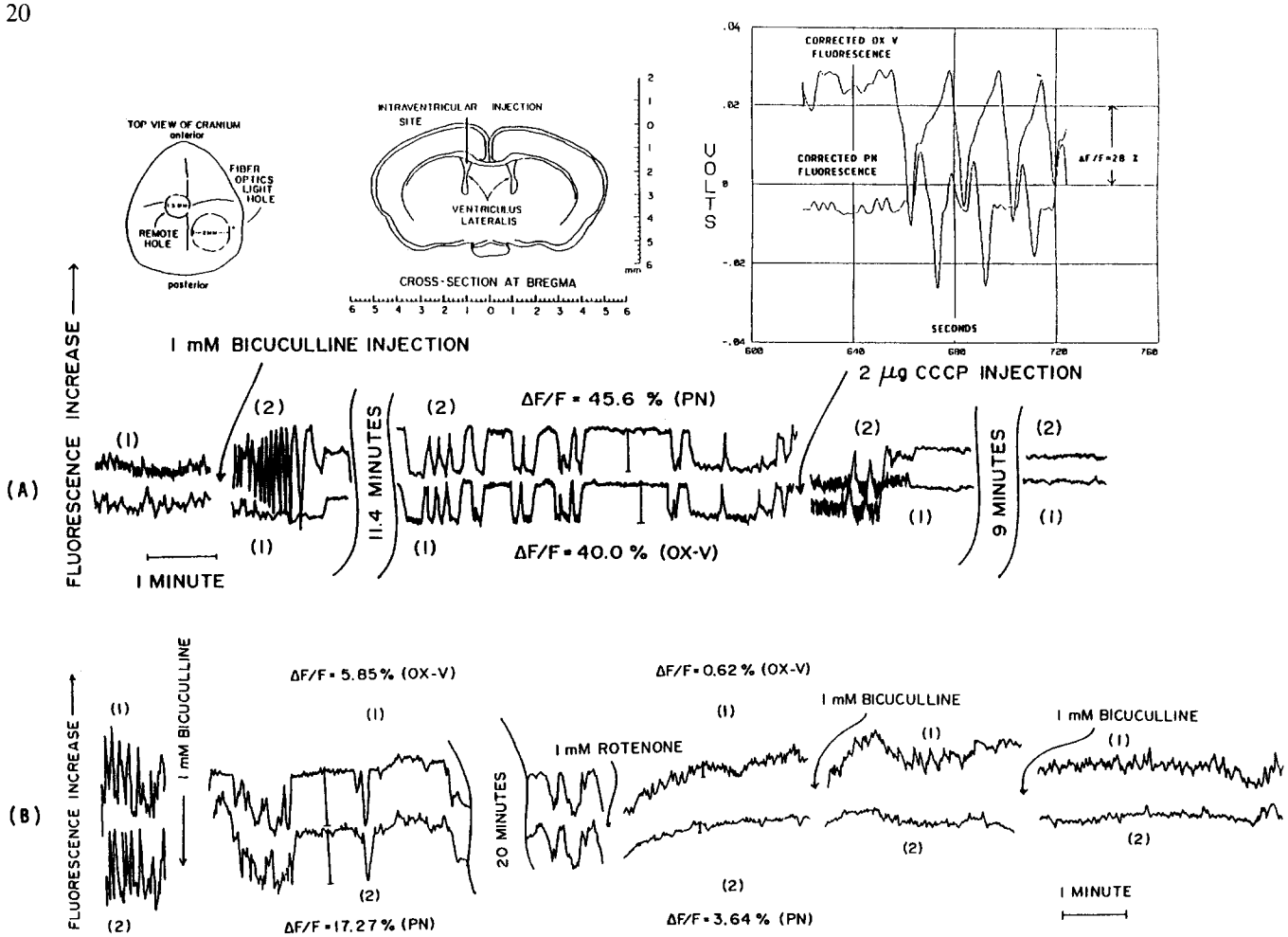

Fig. 5. In panel (A), the effect of intraventricular injection of bicuculline on the pyridine nucleotide and oxonol V fluorescence from the cerebral cortex of the mongolian gerbil is illustrated. Characteristic seizure-dependent oscillations in both signals are observed and can be eliminated by the injection of the oxidative phosphorylation uncoupler, CCCP. In panel (B), the bicuculline-induced pyridine nucleotide and oxonol V fluorescence signal changes are eliminated by the injection of rotenone and once so inhibited could not be reinitiated by subsequent multiple injections of bicuculline. The latter observation suggests a primarily mitochondrial origin for both fluorophore signals. The right inset above panel (A) contains an expanded section of a record from a different experiment in which the bicuculline-induced fluorescence signals have been corrected for possible capillary network blood volume change artifacts by subtracting the corresponding reflectance signal from the oxonol V and pyridine nucleotide raw fluorescence data obtained using a computer-based acquisition system interfaced to a Johnson Foundation MB 3 (eight filter) spinning disk spectrometer. Note that the decrease in the corrected oxonol V fluorescence signal precedes that in the corrected pyridine nucleotide signal; the latter phase difference is also apparent upon close inspection of panel (A). (The location of the two traces in panel (A) and that in the inset are reversed.) The significance of this phase difference is discussed in the text. The location of the skull openings used for optical measurements and for intraventricular injections are illustrated in the left inset above panel (A). The cortex tissue was stained with a 100 μM oxonol V stock solution in physiological saline for approx. 30 s; excess dye was then removed. The pyridine nucleotide fluorescence was excited at 365 nm and monitored using a Corning 3-73 cutoff filter. The oxonol V dye was excited at 580 nm and the fluorescence detected with a Corning 2-60 cutoff filter. In panels (A) and (B), traces (1) and (2) are the oxonol V and pyridine nucleotide signals, respectively; the quantity of bicuculline injected ranged from 0.5 to 10 nmol. 2 μg of CCCP and 10 nmol of rotenone were employed in the experiments described by panels (A) and (B). The diameter of the light guide employed in collecting the data shown in panels (A) and (B) was 1 and 2 mm, respectively. The RC time constant used in the latter collection was 600 ms. A data point was collected approx. every 100 ms in the record shown in the right inset. Adapted from Ref. 132.

A rapid probe response time in excitable tissue preparations, however, does not guarantee a similar response time capability in other preparations, especially mitochondrial and photosynthetic systems. Oxonols V and VI have been shown by Cohen (personal communication) to be capable of following the time-course of the action potential in the squid axon, but the time-course of the energy-dependent signal in submitochondrial particles is significantly slower. This difference in response time is likely due to differing mechanisms by which the probe potential-dependent signals develop in the latter two rather disparate preparations. In the submitochondrial particle case, a redistribution of the probe from the bulk phase to the membrane is responsible for the spectral red shift and decrease in fluorescence intensity observed when a membrane potential is generated in this preparation. Since the initial probe transfer process appears to be the rate-limiting step for the faster phase of the probe signal, the oxonol optical response can develop no faster than the time frame of a

diffusion controlled reaction. At micromolar probe concentrations and under conditions where the probe is the reactant in excess, half-times of a few tenths of a millisecond to several milliseconds are to be expected. Although these values are comparable to the dye response time in the excitable membranes, the observed rate constants governing the energy-dependent signals in energy-transducing membranes are two or three orders of magnitude lower than the rate constants expected for a diffusion controlled process [76,77] and hence give rise to a correspondingly more slowly developing signal. In the case of oxonol VI, the behavior of the energy-dependent signal derived from the probe absorption spectrum shift in submitochondrial particles is complicated by the presence of a slow phase signal component that obeys a first order rate law and develops on a time scale of tens of seconds; it is apparently associated with the permeation of the particle membrane by this probe.

It should be noted that the experimental protocol usually employed with excitable membrane preparations involves staining the tissue with the dye, then washing away the excess probe. This procedure does not favor a mechanism based on transfer of dye from the bulk to the membrane as is frequently observed in energy-transducing mitochondrial and photosynthetic systems discussed above, since there is little free probe left in the bulk phase to transfer to the membrane when a potential gradient is formed.

Shorter apparent response times can be achieved in the case where the energy-dependent signal is generated by a redistribution process in which the probe is transferred from the bulk phase to membrane binding sites by raising the dye concentration at constant membrane content. The use of elevated probe concentrations, however can seriously disrupt the membrane and in the case of diS-C_3-(5) and similar cyanines seriously inhibit NAD-linked respiration.

The difference in the time-course of the potential-dependent merocyanine 540 signal in excitable tissues and in submitochondrial particles is particularly notable, suggesting that quite different mechanisms govern the response of this indicator to charge separation. In the submitochondrial particle preparation, merocyanines 540 responds to membrane potential formation by an apparent rather slow redistribution of the probe onto the membrane that follows a second-order rate law with $k_2 = 10^4$ M(dye)$^{-1} \cdot$ s^{-1}, but does not appear to permeate the membrane and accumulate in the internal volume of the particles [92].

Waggoner et al. [3] have suggested a mechanism for the response of certain extrinsic indicators to the action potential based on a rapid probe–membrane association/dissociation process in excitable membranes that appears to require both high and differential local probe concentrations [134] on either side of the membrane

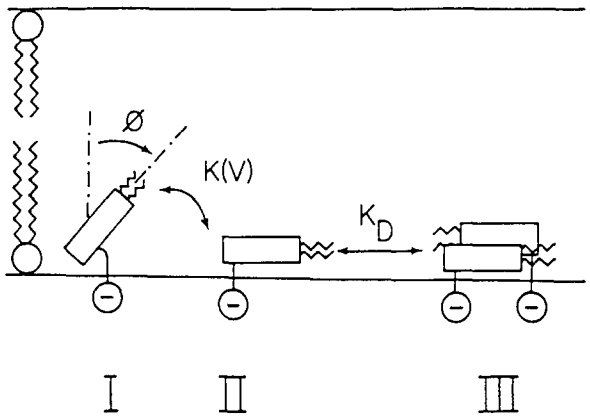

Fig. 6. A model for the location and orientation of merocyanine 540 (M540) in a lipid bilayer suggested by Wolf and Waggoner [135]. The deep penetration of the probe into the hydrocarbon region of the bilayer and the tilt of the probe long axis relative to the bilayer normal is expected to be highly perturbing to the lipid organization in the bilayer. The observation of large perturbations of the excess heat capacity profile describing the DMPC gel to liquid-crystalline phase transition at low dye-to-lipid mole percentages [140] is consistent with such a location. The validity of the illustrated potential-dependent dimerization of the membrane-bound probe (labeled II and III in the figure) in energy transducing organelles is at present unknown, but the observation of a potential-dependent M540 fluorescence quenching in submitochondrial particles with no change in the probe fluorescence lifetime (unpublished data collected by the author) is compatible with the latter mechanism.

bilayer, perhaps the unstirred layer. Current mechanisms for the merocyanine 540 signal in this preparation, however, are based on a change in the equilibrium between membrane-bound monomers and dimers that operates on a millisecond time scale. As previously mentioned, Verkman [91] has suggested a similar process following rapid association of merocyanine 540 with model membranes when diffusion potentials are generated across vesicle bilayers. Wolf and Waggoner [135] have also recently proposed such a model for the sensitivity of merocyanine 540 to action potentials in the squid axon preparation or in leach ganglia based on work with the probe in hemispherical bilayers; see Fig. 6.

Nernstian probes and similar probes designed to permeate and cross the membranes of cells and organelles are unlikely to function on a time-scale suitable for detecting and following elementary steps in charge separation. These probes, however, have not been designed for the latter application.

In summary, the response time of redistributing type probes such as a number of oxonols, cyanines, safranine, merocyanines and rhodamines that frequently give rise to large easily detectable signals in energy-transducing preparations is probably too long to allow the faithful monitoring of initial charge separation events in these systems at probe concentrations in the micromolar range necessary to avoid undue perturbation of the membrane integrity. The need for probes that function on the

charge-shift or electrochromic principle in these preparations, such as the apparent example of RH-160 in the *H. halobium* system, is quite clear.

XI. The relative sensitivity of probes to surface and transmembrane potentials

The issue of the sensitivity of extrinsic and intrinsic molecular probes to change in membrane surface potential in relation to the use of these indicators in the detection and quantitation of transmembrane potentials or to local gradients within the bilayer [14] has become increasingly important. A number of models for energy transduction also involve changes in the surface potential. Malpress [13] has suggested that the electrochemical gradient in energy-transducing membranes is related to a surface potential alteration caused by the formation of fixed charges on the membrane surface as electron transport occurs. The model of Kell [12] for energy transduction is also dependent on surface phenomena, and the transfer of energy from the respiratory chain to mobile ATPase complexes via a collisional process dependent on lateral diffusion as proposed by Slater et al. [136] would also likely be sensitivity to surface charge.

The use of extrinsic indicators to detect and measure transmembrane potentials under conditions of massive ion flux such as Ca^{2+} translocation in mitochondria or sarcoplasmic reticulum membrane vesicles is particularly problematical. As previously described in the sections on cyanine and oxonol probes, Beeler et al. [53] and Russell et al. [57] have found in sarcoplasmic reticulum preparations that the signal from indicators of several classes contain components from both surface potential alterations caused by the direct effect of ion translocation and from transmembrane potential gradients generated either by calibrating diffusion potentials or by ATP hydrolysis and furthermore that the signals produced by the latter two types of potential were qualitatively quite similar. In the sarcoplasmic reticulum preparations, the probe WW 781 was insensitive to diffusion potentials; the signal obtained from the later indicator appears to originate entirely from the direct effect of Ca^{2+} translocation on the surface potential.

The behavior of ANS in mitochondria has recently been re-evaluated by Robertson and Rottenberg [26], who concluded that if the probe is responding to substrate-driven surface potential changes, as predicted by the Malpress model, such a component is masked by the effect of the transmembrane gradient. At low ionic strengths, however, Haynes [27] has found that this probe is specifically sensitive to the surface potential in phospholipid vesicles; the latter property has been exploited [28] to obtain values for the surface potential on each bilayer leaflet of two sarcoplasmic reticulum preparations as described in a previous section.

Tedeschi and associates [26] have proposed that the energy-dependent signal from ANS and a number of carbocyanine probes observed in intact mitochondria originate at least in part from metabolically induced surface potential changes.

The value of the membrane potential obtained by ion distribution techniques and those derived from the carotenoid band shift signal in photosynthetic signals differ, with the larger value being explained on the basis that the carotenoid signal contains a contribution from the membrane surface potential [40,108].

The question of the relative sensitivity of extrinsic probes to the several forms of potential gradients depends in part on the location that these indicators assume in the membrane bilayer. Charge shift probes, for example, can respond only to the portion of a potential gradient and the accompanying electric field that falls across the dimensions of the probe molecule. Several experiments conducted in the author's laboratory [137] bear on the latter location issue. The effect of several anionic oxonols, merocyanine 540, and the cations diS-C_3-(5) and diS-C_4-(5) on the ^{31}P-NMR signal from unilamellar DPMC vesicles has been investigated. The anionic probes are without effect on the ^{31}P-NMR signal, indicating that they may be prevented, possibly by charge repulsion, from approaching sufficiently close to the phosphate group to affect the motion of this group. The cyanines broaden the ^{31}P spectrum and reduce the relaxation times. The latter effect is in part due to a dye-mediated vesicle fusion process, although an additional contribution from the perturbation of the phosphate local motion by these cationic indicators cannot be excluded [137]. The effects of the cyanines is enhanced when negative charge in the form of DMPG is introduced into the vesicles [138]. The cyanines thus appear to occupy sites at or very near the surface of the bilayer, where they apparently screen negative charge associated with the phosphate groups and thereby promote vesicle fusion, even though the medium electrolyte concentration is high, 160 mM KCl.

In comparison to the effect of several anionic probes, diS-C_3-(5) was found to have the smallest perturbation on the gel to liquid-crystal phase transition in DMPC multilamellar preparations. The excess heat capacity profile could quite remarkably be fitted to the ideal solution theory [139] when the probe was present at 12 mol% [140]. This probe thus appears to perturb the bilayer to a limited extent in the absence of a potential gradient. A surface location would tend to render the cyanine probe sensitive to changes in the membrane surface potential as observed in sarcoplasmic reticulum preparations. Other anionic probes such as oxonols V and VI, merocyanine 540, and the charge shift probe RH-160 were much more perturbing to the DMPC phase transition than was diS-C_3-(5). The cooperativity of the latter transition was effectively eliminated by

merocyanine 540 at only 0.3 mol% dye; this large perturbation is consistent with a location proposed by Wolf and Waggoner based on polarized light measurements in hemispherical bilayers in which the long axis, or optical chromophore, of the probe monomer is tilted relative to the bilayer normal, since the lipid cluster size undergoing the phase transition would likely be substantially reduced by the merocyanine probe in such an orientation. All of the probes investigated were excluded from the interior of the DMPC bilayer in the gel phase to a greater extent than they were when the bilayer was in the liquid crystal phase.

Investigations currently in progress in the author's laboratories have approached the probe location problem using primarily magnetic resonance techniques. The effect of lipophilic nitroxide spin labels on the relaxation times of fluorine labels covalently bound to a potential-sensitive probe of the styryl class has been used to determine the distance from the N–O group of the nitroxide label to the trifluoromethyl group of the probe. The structures of the fluorinated probe and two spin labels employed in these investigations are shown in Fig. 7. The correlation time necessary for the latter determination was estimated from analyses of probe fluorescence lifetime and polarization data. The spin label locus was determined from the effect of the unpaired electron on the linewidths of the lipid ^{13}C resonances the assignment of which was established for the DMPC unilamellar vesicle system employed in these measurements. From a knowledge of the distance of the probe fluorine moiety to the spin label site and the locus of the latter site in the bilayer, a model for the location of the probe in the model membrane has been generated as illustrated in Fig. 8 for the case of two DMPC lipids near the probe in a monomer configuration. This model places the upper end of the probe optical chromophore just below the glycerol backbone of the lipids with the remainder of the fluorophore extending well into the hydrocarbon portion of the bilayer. The model suggests that the optical properties of this probe should not be unduly sensitive to alterations in the bilayer surface charge density. The probe fluorine NMR and fluorescence lifetime properties suggest that the indicator occupies two distinct classes of sites, possibly located in the inner and outer leaflets of the DMPC vesicle bilayer.

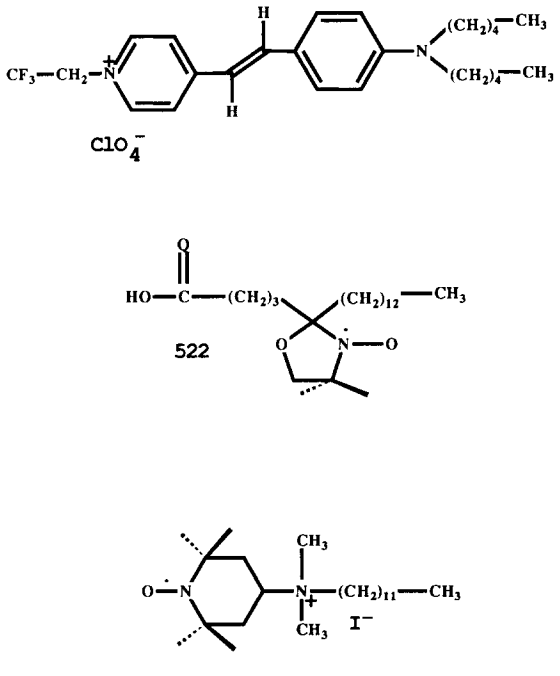

Fig. 7. The structure of a styryl probe labeled with a trifluoromethyl group and that of two lipophilic nitroxide spin labels used in developing the probe location model.

The relative sensitivity of molecular probes to surface vs. transmembrane potential gradients is a highly contentious issue dependent on experimental conditions, the nature of the membrane preparation under investigation, and numerous other factors which must be borne in mind when assessing possible applications of these indicators or interpreting the potential-dependent signals from them, especially when media having low ionic strength which offers minimal screening of surface charge is employed.

XII. The probe calibration issue

Quantitative investigations of potential gradients require that the optical signals generated by charge separation be calibrated, one of the more difficult aspects in the application of molecular probes to small volume preparations. A favorable case is one in which known potentials can be imposed via electrodes or alternatively where potentials generated by the preparation under investigation can be independently measured with electrodes and compared to the probe optical signal in order to develop a calibration plot. Examples of the former case are probe studies involving black lipid membranes or hemispherical bilayers [114], and work involving the action potential in excitable tissues such as the squid axon, heart muscle, or the large red cells from *Amphiuma*. In latter cases, one may wonder if the use of extrinsic optical indicators is not redundant. Such studies, however, have historically preceded the use of extrinsic probes in ensembles of cells, such as ganglia [2,4,141], designed to initiate work on intercellular communication and related matters.

Many whole cells as well as energy transducing organelles are considered to be too small for reliable measurements of potentials to be obtained with microelectrode-based techniques. A notable exception is the large mitochondria from copper-deficient rat liver investigated by Tedeschi and associates [10,36]. In these

small-volume preparations, an alternative calibration method must be employed. The diffusion potential technique has been virtually universally employed in the latter systems with varying degrees of success. This procedure usually involves the assumption that the membrane can be rendered selectively permeable to a single ion by the use of ionophores such as valinomycin in the case of K^+ or Rb^+ or in the case of H^+ by protonophores that are usually uncouplers of oxidative phosphorylation. The assumption that a single ion permeability is dominant, however, is questionable in those membranes containing ion transport systems. The case of Cl^- in the red cell is a well known example; Laris and Hoffman [61] found it necessary to specifically include the Cl^- permeability in their calculations of diffusion potential values by the use of the Goldman-Katz-Hodgkin constant field equation [142,143]. Concentrations must usually be used in the Nernst equation instead of activities, since the ion activity coefficients appropriate for the internal volumes of these preparations are usually not known.

A number of technical problems are associated with the use of diffusion potentials as calibration tools. The direct interaction of several probes with valinomycin has been reported [93,144], so a check for probe–ionophore or probe–protonophore interaction is necessary when they are to be used in the generation of diffusion potentials; these checks are usually possible only in the absence of a membrane and do not allow the exclusion of such interaction on or within the membrane bilayer.

The variation in the medium ionic strength that results from the necessary alteration of salt concentration in order to obtain a calibration plot for the probe signal based on diffusion potentials, however, can also alter the membrane surface charge density and produce a probe signal than is not readily distinguished from that due to the transmembrane potential; Admon et al [81] and Shahak et al. [145] have noted, for example, that the oxonol VI absorbance signal was sensitive to variation in the medium K^+ concentration and have attempted an alternative calibration based on proton gradients established by pH jumps; the probe signals observed in the latter instance, however, were not re-

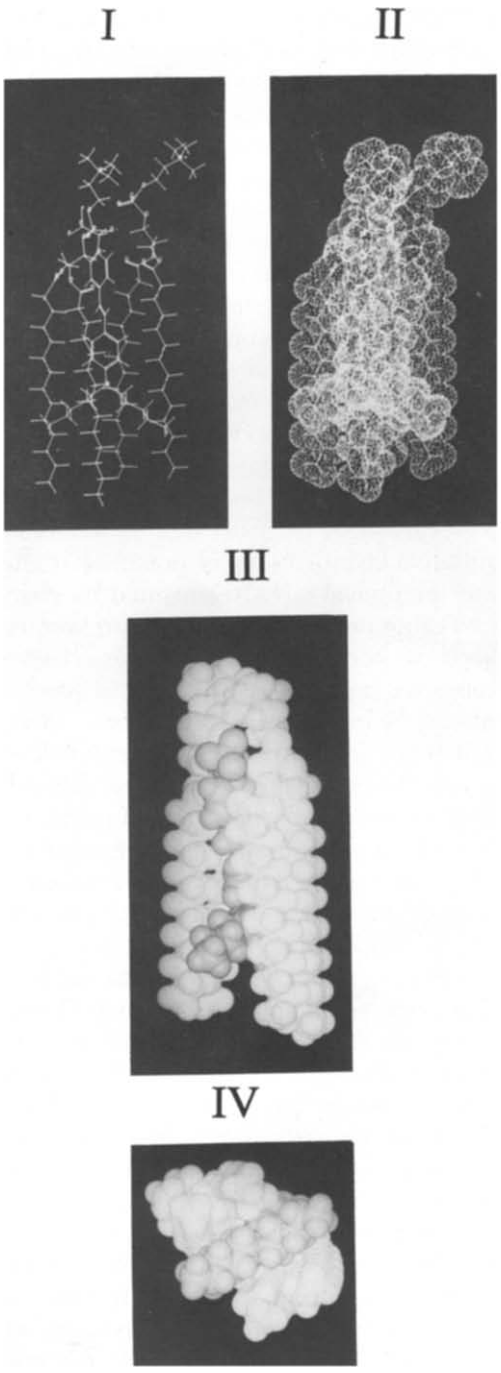

Fig. 8. A probe location model derived from a knowledge of the spin label locus in the bilayer and the distance from the latter site to the probe fluorine moiety. Since this distance was projected along the bilayer normal, the styryl probe location corresponds to the minimum probe penetration into the model membrane compatible with the experimental results. In panel I, the dye is shown intercalated between two DMPC lipids. Panel II illustrates the same complex but with Van der Waal's radii added; the dye is shown at higher density than the DMPC lipids. In Panel III, the dye is shown as the more densely shaded structure between the lipids in a CPK model format. Panel IV is a bottom view of the preceding complex; the dye is again shown as the more dense component. The structure of the preceding probe-DMPC complexes was optimized by energy minimization using the molecular mechanics package MACROMODEL. In these calculations, the energy of the dye and a DMPC molecule was separately minimized using the MM-2 and AMBER force-field parameters, respectively. The complex shown in panels I to IV was then assembled using the docking facility of the package and the energy of the complex minimized. The initial choice of locating the ring structures and *n*-pentyl groups of the styryl probe in the hydrocarbon region of the bilayer was based on energy considerations and the known sensitivity of the DMPC gel to liquid-crystalline phase transition to the presence of this probe (unpublished observations) and similar styryl probes such as RH 160 at low dye-to-lipid mole percentages [140].

versible by K^+-valinomycin and must be used with reservations in probe calibration plots. The use of diffusion potentials to calibrate probe response signals under conditions of massive ion flux that can significantly alter the membrane surface potential is also highly problematical as the work of Beeler et al. [53] and Russel et al. [57] has demonstrated.

Since many of the charged probes presently in use respond to potential gradient formation by mechanisms that involve enhanced association of the probe with the membrane, there is only a limited range of potential values over which the probe response is approximately linear. The probe response tends toward saturation at either end of the latter range, since at the low-potential end, a threshold usually exists below which the potential gradient is too small to drive the probe onto the membrane and at the other extreme, all membrane binding sites are occupied. When the probe response mechanisms involves expulsion of the indicator from the membrane, a threshold potential must be exceeded to drive the probe from the membrane and response of the probe cannot exceed that obtained when virtually all probe is forced off the bilayer, regardless of how large the potential becomes. The specific potential gradient range over which the probe signal is linear is a function of the binding affinity of the probe for the membrane under study, and probes from a structurally homologous series should ideally be used to obtain the desired range of linear indicator response.

An alternative probe calibration procedure is based on the null point titration technique. When the membrane permeability for a single ionic species is much larger than that for all other ions, the value of the external ion concentration can be varied until a concentration is found such that no change in an extrinsic probe signal is observed upon addition of an appropriate ionophore. This approach has been employed by Hoffman and Laris [61] in red cell work in which the external K^+ concentration was determined such that valinomycin addition produced no change in the fluorescence signal from the probe CC_6. Assuming that valinomycin renders the K^+ permeability much greater than that of other ions, the membrane potential is obtained directly from the initial external to internal K^+ concentration ratio. This procedure, however, affords only a single point calibration of the probe optical signal. A similar approach has been employed by Schummer and Schiefer [77] (see the Cyanine Probe section) to obtain the external K^+ concentration in whole cell preparations at various membrane potentials controlled by the medium Ca^{2+} content. Another null point procedure may be employed in systems that contain an ATP synthase. This procedure involves observing the effect of varying the phosphate potential on a probe signal generated by an alternative substrate such as NADH or actinic light [75]. The null point in the phosphate potential variation procedure, i.e., the value at which no change in the background probe signal is observed, allows the prevailing value of the phosphate potential to be calculated. According to the chemiosmotic model, however, in order to convert this value to one for the electrochemical gradient, the $2H^+/ATP$ ratio must be either known or assumed. It should be noted that in mitochondria various values of this stoichiometry have been reported [146]. If the electrochemical gradient is independently known in the latter preparation, knowledge of the phosphate potential allows the $2H^+/ATP$ stoichiometry to be established. Even if the ratio is known, the total value only of the electrochemical gradient is obtained by the null point titration procedure. In some cases, the use of dual probes each specific for ΔpH or $\Delta\psi$ may be employed [75] to obtain independently a value of one or both of the components of the electrochemical gradient and the remaining component calculated by subtraction.

An additional methodology that is currently under development is that of 'Nernstian' probes that are designed to distribute across membranes according to the prevailing membrane potential in a manner similar to permeant ions used in classical studies. These probes are particularly well suited for work based on fluorescence microscopy since their distribution across the plasma as well as various organelle membranes can be observed in situ using single cell measurements based on video technology [2]. The membrane potential would ideally be calculated from the ratio of the fluorescence intensity from the probe on either side of the membrane, since the latter intensity would be proportional to the indicator concentration assuming that the probe concentrations are sufficiently low that artifacts resulting from the inner filter effect and concentration quenching of the emission are negligible. The Nernstian probes, however, suffer from the same liabilities as the non-emitting permeant species used in classical investigations, namely nonspecific membrane binding. Emaus et al. [147] have reported, for example, that a substantial amount of accumulated rhodamine 123 in rat liver mitochondria is membrane-bound. This problem is particularly acute in the case of the optical indicators because the fluorescence quantum yield of membrane-bound and free probes is often quite different. It may be possible in some cases to correct for membrane binding by observing the residual probe emission after the deliberate dissipation of the potential gradient under study, but this correction assumes that the same quantity of probe is associated with the membrane regardless of whether a potential gradient is present; many of the probes presently in use respond to such gradients by a change in the fraction of membrane-associated probe. Ehrenberg et al. [128] have, however, recently used several rhodamine derivatives with high quantum yields to measure the plasma membrane

potential of culture-grown HeLa cells, mouse macrophage cells and neutrophils. It was necessary to correct for fluorescence contributions originating from outside the cell volume to the probe emission signal from within the cell and to correct for binding to internal cellular components. The latter correction was based on the assumption that the equilibrium constant describing the distribution of bound and free probe within the cell was independent of the plasma membrane potential and that the probe quantum yield was not affected by binding of the indicator to intracellular components, a condition not satisfied by one cyanine, diO-C_1-(3), tested in HeLa cells. The plasma membrane potential values obtained by this technique fell within the range of potentials measured by independent techniques. The previously described work of Chen and associates [118–121] with rhodamines in transformed cells that appear to have elevated mitochondrial membrane potentials is an example of the use of Nernstian-type indicators in intact cells.

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