Tanford C. (1962), *Adv. Protein Chem. 17,* 69-165.
Taylor, R. P., Vatz, J. B., and Lumry, R. (1973), *Biochemistry 12,* 2933-2940.
Timasheff, S. N. (1966), *Biopolymers 4,* 107-120.
Timasheff, S. N. (1969), *Arch. Biochem. Biophys. 132,* 165-169.
Timasheff, S. N. (1970), in Biological Polyelectrolytes, Veis, A., Ed., New York, N.Y., Marcel Dekker, p 1-64.
Tulinski, A., Vandlen, R. L., Morimoto, C. N., Mani, N. V., and Wright, L. H. (1973), *Biochemistry 12,* 4185-4192.
Vandlen, R. L., and Tulinski, A. (1973), *Biochemistry 12,* 4193-4200.
Verwey, E. J., and Overbeek, J. Th. G. (1948), Theory of the Stability of Lyophobic Colloids, Amsterdam, Elsevier.
Wetlaufer, D. B. (1962), *Adv. Protein Chem. 17,* 303-390.
Wilcox, P. E., Cohen, E., and Tan, W. (1957), *J. Biol. Chem. 228,* 999-1019.
Wilcox, P. E. (1970), *Methods Enzymol. 19,* 64-108.
Winstead, J., and Wold, F. (1965), *J. Biol. Chem. 240,* PC3694-PC3696.
Wright, H. T., and Weber, B. H. (1972), *Eur. J. Biochem. 24,* 583-592.
Wright, H. T., Kraut, J., and Wilcox, P. E. (1968), *J. Mol. Biol. 37,* 363-366.
Wright, C. S., Hess, G. P., and Blow, D. M. (1972), *J. Mol. Biol. 63,* 295-303.
Wyman, J. (1964), *Adv. Protein Chem. 19,* 224-285.
Yphantis, D. A. (1964), *Biochemistry 3,* 297-317.

Charge Shift Optical Probes of Membrane Potential. Theory†

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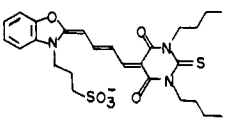
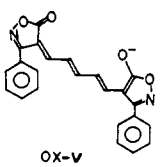
ABSTRACT: The chromophores of a series of known and unknown probes of membrane potential are subjected to molecular orbital calculations. These calculations are used to characterize the charge distribution and excitation-induced shift of electron density in the chromophores. This is used to predict or rationalize the magnitude of an electrochromic response to membrane potential. The predictions are consistent with more rigorous calculations on several selected systems as well as with the available experimental data. Emerging from the survey is a variation on previously considered forms of electrochromism involving a simple migration of the charge in an ionic chromophore. The intrinsic amphipathic structures of some of these systems may make them especially well suited for the construction of well oriented, highly responsive probes. A particularly promising charge-shift chromophore is the 4-(*p*-aminostyryl)pyridinium cation.

It has been well demonstrated (Tasaki et al., 1969a,b; Tasaki, 1974; Cohen et al., 1974; Ross et al., 1974, 1977; Conti, 1975) that electric potential changes, monitored by microelectrodes, are paralleled by changes in the absorption or fluorescence spectra of a wide variety of dyes applied to the squid giant axon. This pioneering work has encouraged many investigations into the utility of optical probes for the measurement of potential across the membranes of a variety of cells, organelles, and vesicle preparations that are inaccessible to microelectrodes (for recent reviews, see Chance et al., 1974; Chance, 1975; and Waggoner, 1976).

Unfortunately, dyes that appear particularly sensitive in one membrane system do not necessarily respond well in another. For example, M-540 (originally dye "I" in Cohen et al., 1974)



M-540



OX-V

is unresponsive in chromatophores (Chance and Baltscheffsky, 1975) but is quite sensitive in submitochondrial particles (Chance, 1975; Chance and Baltscheffsky, 1975). On the other hand, OX-V (Smith et al., 1976) is quite successful on chromatophores but inferior to M-540 on submitochondrial particles or squid axon (Chance, 1975; Chance and Baltscheffsky, 1975; OX-V is designated MC-V in these references). The reason for this problem is probably the diversity of mechanisms which are available to couple a spectral response to a change in membrane potential. Some of the mechanisms which have been postulated include:

(1) A potential-dependent partition of the dye between the cell interior and the external medium. Concentration of dye in the interior results in the formation of aggregates which are not fluorescent. Evidence for this mechanism has been found for the cationic highly permeant cyanine dyes in cell suspensions with a response time on the order of seconds (Sims et al., 1974).

(2) A potential-dependent partition between the external medium and the membrane. This requires an initial electrical potential to assure an asymmetric distribution of dye. This mechanism may have response times as fast as tens of microseconds, and the spectral change is thought to arise from the formation of dimers in the aqueous phase (Waggoner, 1976; Waggoner et al., 1977) [a solvatochromic effect may also be important (Pick and Avron, 1976)]. This mechanism has been associated with the cyanines in the squid axon (Cohen et al., 1974; Ross et al., 1974) by Waggoner (1976) and in chromatophores by Pick and Avron (1976).

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(3) A potential-dependent dimerization within the membrane. Evidence for this process has been presented for a merocyanine, but a detailed mechanism was not proposed (Tasaki et al., 1976). Some recent data from polarization experiments on hemispherical bilayers indicate that dimerization follows reorientation of the probe (A. Waggoner, personal communication; Dragsten and Webb, 1977).

(4) A *direct* response to a *local* potential which develops subsequent to energization. This rationale has been developed mainly in response to the complex kinetic features of the spectral shifts of merocyanines and oxonols after flash photolysis of chromatophores (Chance et al., 1974; Chance and Baltscheffsky, 1975).

The original impetus for these investigations was, perhaps, the prediction (Platt, 1956, 1961) and experimental demonstration (see Liptay, 1969, for an early review) of a direct mechanism for electric field induced shifts of the energies of electronic transitions in dye molecules. The phenomenon is known as electrochromism (also as Stark effect) and has been described by eq 1 (Labhart, 1963; Reich and Schmidt, 1972; Chance and Baltscheffsky, 1975):

$$h\Delta\nu = (\vec{\mu}_g - \vec{\mu}_e)\cdot\vec{F} + \frac{1}{2}(\alpha_g - \alpha_e)F^2 \qquad (1)$$

where $\Delta\nu$ is the shift in frequency upon introduction of an electric field, $\vec{F}$, μ_g and μ_e are the dipole moments of the ground and photoexcited states, respectively, and α_g and α_e are the corresponding polarizabilities.

Equation 1 is directly applicable only when the chromophore dipoles are rigidly and anisotropically oriented with respect to the electric field, as pointed out by Reich and Schmidt (1972). In the case of unrestricted motion of the chromophores, the field will tend to align the dipoles so as to maximize $\vec{\mu}_g\cdot\vec{F}$ within the limitations of Maxwell-Boltzman statistics; this alignment effect leads to a strictly quadratic field dependence for electrochromism (Liptay, 1969) even for large $(\vec{\mu}_g - \vec{\mu}_e)$. This pseudoquadratic electrochromism is, of course, superimposed on the quadratic dependence derived from the second term on the right of eq 1; the latter, however, should be relatively insignificant at fields much below 10^7 V/cm, as pointed out by Fischer et al. (1976). These authors below 10^7 V/cm, as pointed out by Fischer et al. (1976). These authors further show that electrochromism in a rigid medium is maximized if the dipoles are uniformly oriented parallel to the field and that the change in optical density can become quite significant if all the conditions are optimized.

Using a merocyanine dye, Bucher et al. (1969), employed a multilayer film capacitor technique for the first demonstration of linear electrochromism; this technique gave the necessary rigid anisotropic dye distribution. Reich, Witt, and their co-workers have subsequently applied these techniques to the illumination-induced absorption changes of photosynthetic membranes. They obtained linear and quadratic electrochromism spectra of the individual photosynthetic pigments in the capacitor system (Schmidt et al., 1969, 1972; Schmidt and Reich, 1972; Reich and Scheerer, 1976). The weighted average of the linear spectra matched the flash photolysis induced absorption changes in chloroplasts (Junge and Witt, 1968; Emrich et al., 1969; Reich et al., 1976), indicating an in vivo electrochromic response. In essence, the carotenoids and chlorophylls are intrinsic electrochromic probes.

The success of the photosynthetic pigments is most likely attributable to a high local field and a rigid orientation in their membrane matrix. To date, it has not been possible to demonstrate a true electrochromic response for any of the hundreds of extrinsic probes that have been screened. The design of a probe to meet both the criteria of rigid orientation in a membrane and a large change in electronic structure upon photoexcitation should allow the electrochromic mechanism to be operative. Electrochromism has the advantage of being an instantaneous response on the time scale of any conceivable dynamic membrane event. It also offers the possibility of providing a set of universal probes, each of which need only be calibrated once for potential measurements in any membrane system.

Presented herein is a theoretical methodology for the rational design of electrochromic probes. This treatment predicts that several chromophores containing an ionic charge which experiences an excitation-induced shift across the long axis of the probe should meet the criteria for electrochromism. A simplified form of eq 1 may be introduced to describe the behavior of these probes:

$$h\Delta\nu = -q\vec{r}\cdot\vec{F} \qquad (2)$$

where q is the charge and $\vec{r}$ is the distance it migrates upon excitation. These charge-shift chromophores have the added advantage that the ground-state charge center can be used as the hydrophilic end in the construction of a well oriented amphiphilic probe.

Calculations

The extended Hückel molecular orbital method (Hoffmann, 1963) is the only practical all valence electron method for systems as large as the chromophores treated in this work. The program ICON-8, kindly provided by Professor Roald Hoffmann, was slightly modified for use in these calculations. CPU time on the SUNY at Binghamton IBM 370-158 computer ran between 1 and 2 min per chromophore.

For qualitative judgements, the standard EH[1] calculation was performed. This method is quite successful at predicting trends in the properties of related compounds. In particular, it quite accurately reproduces the shapes (i.e., electron probability distributions) of molecular orbitals produced by ab initio calculations (Jorgensen and Salem, 1973; Loew and MacArthur, 1977). Given that the most useful and prominent transitions in the dyes are at the longest wavelength, we need only examine the redistribution of electron density upon removal of an electron from the highest occupied molecular orbital (HOMO) and its placement in the lowest unoccupied molecular orbital (LUMO); this exactly determines (within the framework of the extended Hückel formalism) the shift in charge distribution in the excited state relative to the ground state, and, thus, the probability (eq 1 and 2) that the chromophore will display an electrochromic response.

Once a chromophore has been identified, an iterative self-consistent charge method can be used to characterize its total electron distribution. In this method, an EH calculation is used to generate a charge distribution; the coulomb integral of each atomic orbital basis function is then adjusted as a linear function of the electron occupancy, each orbital type having been assigned a unique function based on atomic spectra. The EH calculation is then repeated with the new coulomb integrals, and the cycle is repeated until charge self-consistency is attained. This procedure is detailed by McGlynn et al. (1972). The probes require between 8 and 30 min of CPU time on the IBM 370-158 with this method.

[1] Abbreviations used: EH, extended Huckel; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital.

TABLE I: Calculated Electrochromism Ratings of Some Dyes with Large Response in Excitable Membranes.

structure	reference	*S/N*	rating
(1) M-540	Cohen, 1974	5	G
(2)	Cohen, 1974	1	G
(3)	Cohen, 1974	8	G
(4)	Cohen, 1974	2	F
(5)	Cohen, 1974	2	E
(6)	Cohen, 1974	1.0	E
(7)	Cohen, 1974	0.03	E
(8)	Cohen, 1974	0.07	E
(9) diO-C_6-(3)	Cohen, 1974	1.0	P
(10) WW-375	Salzberg, 1976	50 (abs)	F

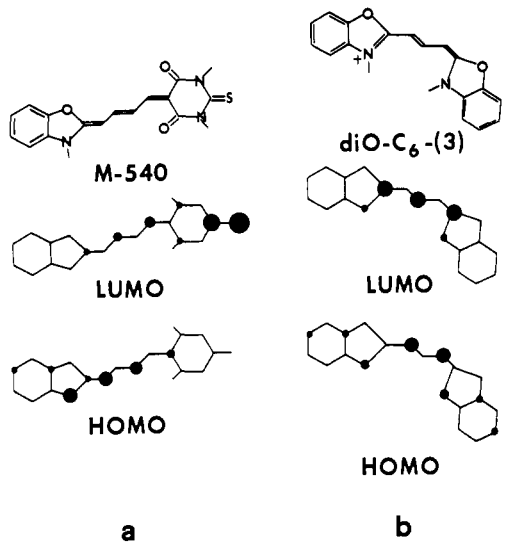


FIGURE 1: (a) MO's of M-540 (Table I, **1**); (b) MO's of the diO-C_6-(3) chromophore (Table I, **9**).

TABLE II: Calculated Ratings of Some Available Dyes.

structure	source	rating
(1)	Brooker, 1941	P
(2)	Platt, 1961	F
(3)	Platt, 1961	G
(4)	Eastman	E
(5)	Smith, 1976	P
(6)	this work	E

Results

Survey of Some Known Probe Chromophores. Two of the most popular membrane potential probes are M-540 and di-O-C_6-(3), neither of which are believed to respond via electrochromism (Waggoner, 1976; Waggoner et al., 1977). By way of illustration, the LUMO's and HOMO's of the chromophores in these probes are depicted in Figure 1. The HOMO of M-540 has its largest electron concentration around the center of the chromophore; a substantial shift to the C=S end of the chromophore is observed for the LUMO. The cyanine dye shows a symmetrical distribution of charge in both the HOMO and LUMO; an electrochromic response is precluded since there is no shift from one end of the chromophore to the other upon excitation. Based on these considerations, M-540 might be rated a good and di-O-C_6-(3) a poor prospect for an electrochromic response. We have not, as yet, considered orientations.

Tables I and II give the ratings of some familiar probe chromophores based on similar examinations of the shapes of the MO's involved in the electronic transitions. The qualitative scale of E (excellent), G (good), F (fair), and P (poor) was established to summarize the relative magnitudes of the shift in electron density upon excitation; a more quantitative comparison would be inappropriate at this level. To fully understand why an electrochromic mechanism is rarely dominant, we must consider, as well, the orientational effects of the probe side chains.

In Table I, the chromophore of M-540 (entry 1) rates a G, as has been discussed. Chromophores **2–4** (XV, IX, and XVI, in Cohen et al., 1974) are structurally similar to M-540 and display similar excitation-induced shifts; the lower rating for **4** is probably due to the absence on oxygen of low-lying d orbitals, which act as electron acceptors in the excited states of the sulfur-containing chromophores. The *n*-butyl side chains are the same for all of these probes and would tend to draw this end of the molecules into the hydrophobic interior; this, how-

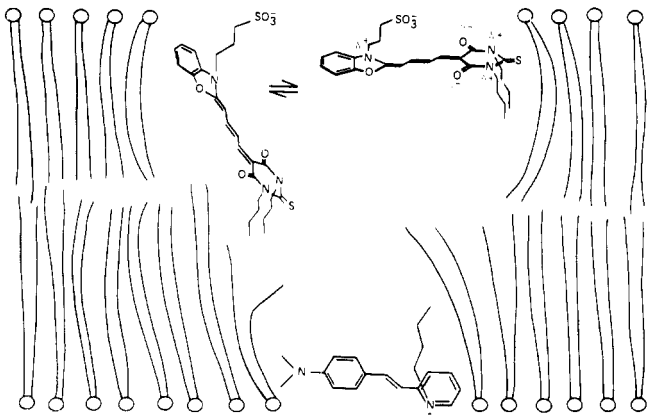


FIGURE 2: Probe orientations in a membrane which should maximize hydrophilic and lipophilic interactions.

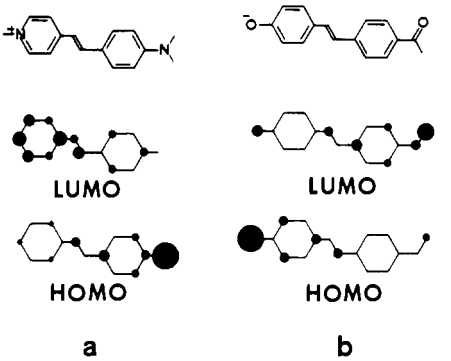
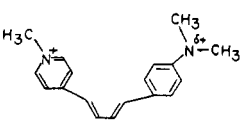


FIGURE 3: (a) MO's involved in the lowest energy transition of the 4-(*p*-aminostyryl)pyridinium chromophore (Table II, **6**); (b) MO's of Table III, **3**.

ever, is countered by the polarity of the amide linkages in the chromophore. The net effect may be an equilibrium distribution of parallel and perpendicular alignments with the sulfonate acting as a pivot. This is illustrated at the top of Figure 2. These illustrated orientations have recently been proposed (Dragsten and Webb, 1977) to account for fluorescence polarization measurements on M-540 stained hemispherical lipid bilayer membranes. This mobility of M-540 and its analogues thus minimizes a direct electrochromic response to a transmembrane potential. Dragsten and Webb invoke a reorientation mechanism at short time scales, which is also consistent with some unpublished experiments on polarization effects in the absorption action spectra (Alan Waggoner, personal communication).

Chromophores **5–8**, *p*-aminostyrylpyridinium or *p*-aminostyrylquinolinium chromophores, all show E ratings. These are incorporated into probes VI, 207, 213, and 217, respectively, in Cohen et al. (1974). The quinolinium systems are not rod shaped and have their most polar site, the quinoline nitrogen, shielded by the fused benzene ring and an ethyl group. The probe containing chromophore **7** has an *n*-butyl group bonded to the pyridinium nitrogen. This counteracts the hydrophilicity of this portion of the molecule and suggests a most stable, albeit mobile, orientation as shown at the bottom of Figure 2. The probe containing chromophore **8** has methyl groups at the pyridinium and amino nitrogens. According to simple resonance theory as well as our EH calculations, the pyridinium nitrogen bears relatively more of the charge than the amino nitrogen; this might suggest a favorable orientation perpendicular to the plane of the membrane with the amino group stretched out into the hydrophobic region. On the other hand, this probe can adopt a conformation (by rotation around the central single bond) which allows both of the partially charged nitrogens to be stabilized at the aqueous interface:



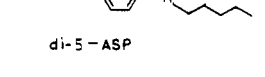
Thus, despite the high ratings of their chromophores, none of these probes have the optimal structural features for a proper rigid orientation in the membrane.

As has been discussed (Figure 1b), the symmetrical charge distribution of chromophore **9** precludes an electrochromic mechanism. The two hexyl groups of di-O-C_6-(3) should orient the chromophore parallel to the membrane plane.

The last entry of Table I shows an F rating for the chromophore of the probe WW-375, which has been highly successful in absorbance experiments on squid axon (Salzberg et al., 1976). The "F" rating can be attributed to the same reluctance of an amide carbonyl to accept excited-state electron density as mentioned for chromophore **4**. In summary, this representative sampling of probes used on excitable membranes does not contain a compound which is ideally constructed to display a direct electrochromism.

In Table II, some of the available "Brooker dyes" were examined in order to obtain more of a feel for the important features which may lead to a successful probe. The merocyanines (**2–4**) are good in general but have two polar ends which force random orientation. A small difference in the two ends as in the cyanine of chromophore **1** is not sufficient to give a good dipole moment in either ground or excited states. Chromophore **5** is the chromophore of OX-5 which has been used extensively by Chance and his co-workers (Chance, 1975; Chance and Baltscheffsky, 1975; Smith et al., 1976). The negative charge is distributed symmetrically between the two carbonyl oxygens; the probable orientation of OX-5, governed by the hydrophobic phenyl groups, has the long axis parallel to the membrane surface; this is consistent with the acute angle found experimentally (Smith et al., 1978).

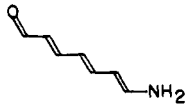
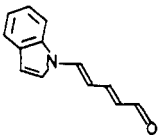
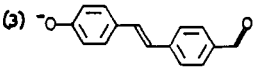
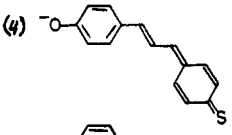
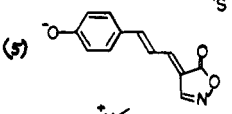
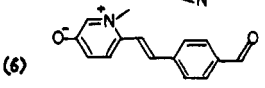
Screening of New Chromophores. The LUMO and HOMO of entry 6 of Table II are shown in Figure 3a. As with the *p*-aminostyryl probes in Table I, this chromophore shows a large difference in the shapes of these molecular orbitals and is assigned an E rating. The advantage of this chromophore is its highly localized, well-exposed ground-state charge at the pyridinium nitrogen and its rigid-rod-shaped structure. We have taken advantage of these features in the design and synthesis of a new probe which has been successful in some model-membrane studies (Loew et al., 1978). The structure of this probe, 4-(*p*-dipentylaminostyryl)-1-methylpyridinium iodide (di-5-ASP), enhances the binding at the amino end with two pentyl chains, while retaining the rod-shaped structure of the chromophore.



di-5-ASP

A theoretical screening of some as yet unknown probes is shown in Table III. The merocyanine-like chromophore **2** shows promise if it could be built into a molecule which could impart good orientation properties. Chromophores **3** and **6** have E ratings (Figure 3b) and would seem to have the intrinsically amphipathic structures necessary for proper orientation; a straight hydrocarbon chain can easily be built onto

TABLE III: Screening of Some Chromophores for Electrochromic Responsiveness.

chromophore	rating
(1) 	G
(2) 	E
(3) 	E
(4) 	F
(5) 	F
(6) 	E

the carbonyl group to enhance orientation. Chromophore **6** has the advantage of assuring that the phenolate oxygen will be unprotonated at physiological pH. It also avoids transport of cations by the probe across the membrane; this phenomenon has been detected for negatively charged oxonol probes (A. Waggoner, personal communication).

Self-Consistent Charge Calculations. This method allows the fixed parameters of the EH calculation to readjust themselves in response to charge asymmetries within a molecule. The highly exaggerated charge separations, which make the EH method unrealistic for polar systems, are smoothed out and make possible quantitative predictions. Thus, at the expense of increased CPU time, we may assess the reliability of the qualitative arguments presented in the previous sections.

The charge distributions in several representative chromophores were calculated to check for consistency with our predictions on orientation. Figure 4 shows the total charge distributions (Mulliken, 1955, 1962) of the chromophore of M-540 (Table I, chromophore **1**), the 4-(*p*-aminostyryl)pyridinium chromophore (Table II, chromophore **6**), and the 4-(*p*-formylstyryl) phenolate chromophore (Table III, chromophore **3**), respectively; to simplify these figures, the generally small charge on the hydrogens was added to the charge associated with their adjacent carbon or nitrogen atoms. The large negative charge concentration on the well-exposed carbonyl oxygens in Figure 4a strengthens the arguments presented above for the partial orientation of M-540 parallel to the membrane surface. Similarly, the positive charge is highly concentrated in the pyridinium ring of the aminostyrylpyridinium system (Figure 4b), as can be inferred from qualitative resonance and electronegativity reasoning (vide supra) (it is instructive, however, to note that the formal positive charge on the pyridinium nitrogen leaks substantially to the less electronegative neighboring carbons); the perpendicular orientation of di-5-ASP is further assured by the addition of the two pentyl chains to the already nonpolar amino end of this amphipathic chromophore. The charge distribution in Figure 4c is again consistent with the resonance theory conclusion that

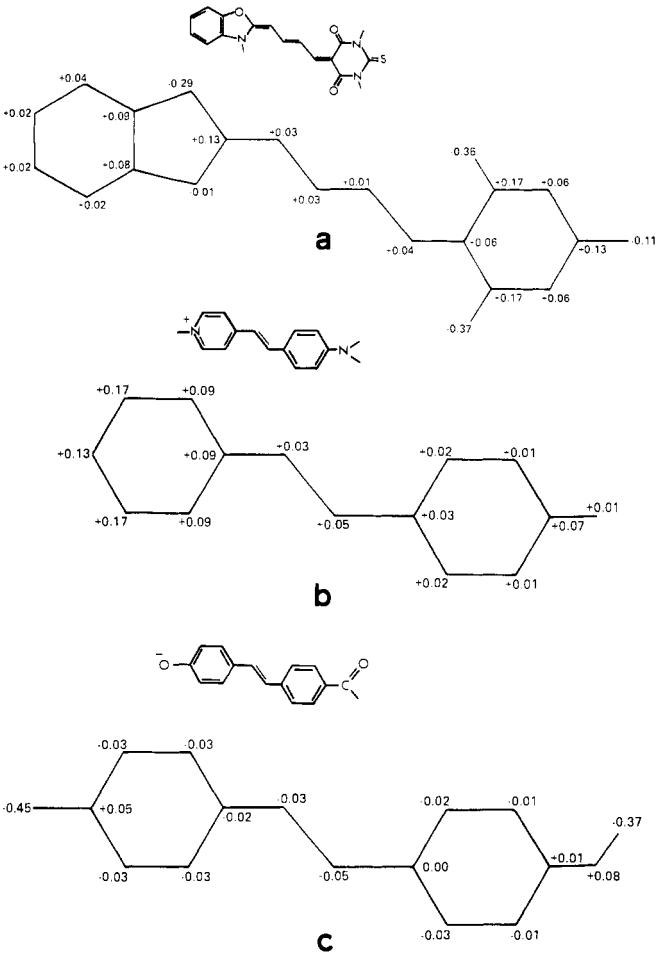


FIGURE 4: (a) Charge distribution in the chromophore of M-540; (b) charge distribution in Table II, **6**; (c) charge distribution in Table III, **3**.

the phenolate should bear most of the negative charge; quantitatively, however, Figure 4c reveals that in this case a significant portion of the charge is delocalized to the other end of the molecule. While, on this basis, it would be incorrect to characterize the 4-(*p*-formylstyryl)phenolate chromophore as amphipathic, a probe constructed by proper placement of alkyl chains at the carbonyl end should display a dominant orientation perpendicular to a membrane surface.

Calculations were also performed separately on both the ground and lowest energy $\pi^* \leftarrow \pi$ excited states of several of the chromophores. The excitation-induced shift of electron density can thus be assessed directly, rather than inferred from the HOMO and LUMO. The results were somewhat limited by the failure of some of the chromophores to give convergent iterations in the excited states. It was possible to achieve convergence for three of the new charge-shift candidates, and the differences between the ground- and excited-state total charge distributions are displayed in Figure 5a,b,c. Comparison with Table II, entry **6**, and Table III, entries **4** and **5**, respectively, clearly shows that the ratings based on the shapes of the HOMO and LUMO are, indeed, consistent with the total shifts in charge density derived from the more rigorous calculations.

It is instructive to translate the large charge shift for the aminostyrylpyridinium chromophore (Figure 5a) into an electrochromic effect for a typical membrane electric field ($\sim 10^5$ V/cm). The magnitude of $qr \cos\theta$, assuming perpendicular orientation, is 14×10^{-18} esu·cm, leading to (eq 2) a $\Delta\nu$ of 24 cm^{-1}. Monitoring the optical density of di-5-ASP at

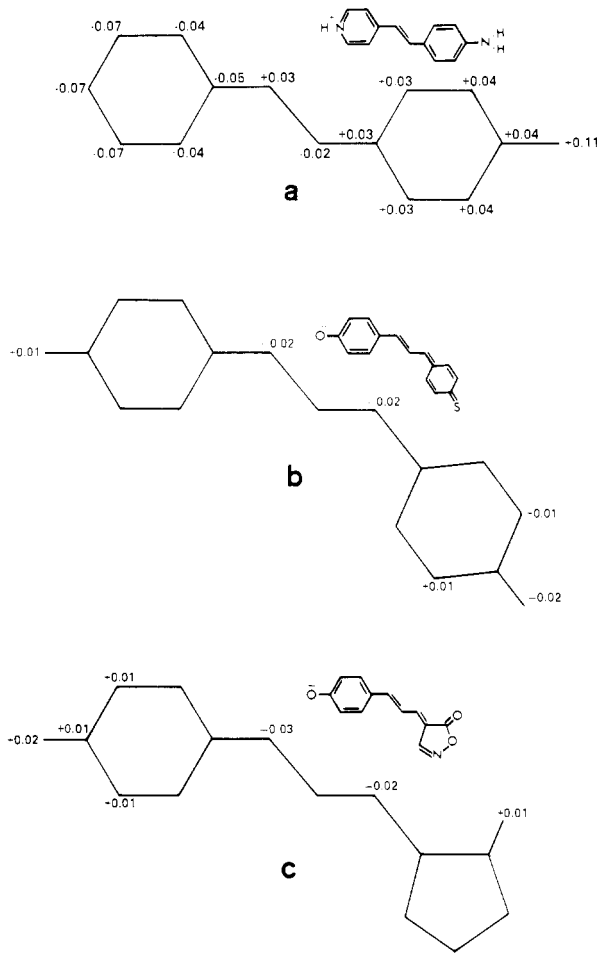


FIGURE 5: (a) Charge shift upon excitation of the 4-(*p*-aminostyryl)-pyridinium chromophore derived from self-consistent charge calculations; (b) charge shift for Table III, **4**; (c) charge shift for Table III, **5**.

500 nm, this corresponds to a shift of 0.6 nm; from the shape of the absorption band (L. M. Loew and S. Scully, unpublished results) of di-5-ASP, this, in turn, can be translated into a change in OD of ~1.3% at this wavelength. It should be strongly emphasized, of course, that the self-consistent charge method is not nearly of experimental accuracy; it is encouraging, nonetheless, that the calculated electrochromic effect is so much larger than the limits of detectability by either absorbance or fluorescence experiments.

Conclusion

The electrochromism of di-5-ASP along with chromophores **3** and **6** of Table III is governed by the expression on the right of eq 2. These charge-shift systems may have some special advantages for the design of electrochromic probes. The ground-state charge concentrated at one end of these rod-shaped systems anchors the probe to the membrane surface. A sulfonated side chain has been used as a flexible charged anchor for merocyanine probes. The placement of the anchor as part of the chromophore in the charge-shift probes has an important advantage, however; one needs simply to lengthen the opposite end of these rigid chromophores with straight-chain alkyl groups to assure a dominant orientation of the long molecular axis perpendicular to the membrane surface. The association of the charge with the membrane–water interface should sensitize the chromophore to potential changes associated with variations in local concentrations of ions; this will enhance a charge-shift probe's overall responsiveness, since changes in transmembrane potential are, after all, induced by variations in surface ion concentrations.

A methodology has been presented which allows the prediction of orientation and electrochromism for optical probes of membrane potential. These qualitative predictions, based on a simple molecular orbital theory approach, are consistent with the results of a more quantitative MO treatment as well as available experimental data.

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References

Brooker, L. G. S., and Sprague, R. H. (1941), *J. Am. Chem. Soc. 63,* 3203.

Bücher, H., Wiegand, J., Snavely, B. B., Beck, K. H., and Kuhn, H. (1969), *Chem. Phys. Lett. 3,* 508.

Chance, B. (1975), *MTP Int. Rev. Sci., Biochem., Ser. One,* 1.

Chance, B., and Baltscheffsky, M. (1975), *Biomembranes, 7,* 33.

Chance, B., Baltscheffsky, M., Vanderkooi, J., and Cheng, W. (1974), *Perspect. Membr. Biol., Mex. Soc. Biochem. Symp., 1st,* 329.

Cohen, L. B., Salzberg, B. M., Davilla, H. V., Ross, W. N., Landowne, D., Waggoner, A. S., and Wang, C. H. (1974), *J. Membr. Biol. 19,* 1.

Conti, F. (1975), *Annu. Rev. Biophys. Bioeng. 4,* 287.

Dragsten, P. R., and Webb, W. W. (1977), *Biophys. J. 17,* 215a.

Emrich, H. M., Junge, W., and Witt, H. T. (1969), *Z. Naturforsch. 246,* 1144.

Fischer, J. K., von Brüning, D. H., and Labhart, H. (1976), *Appl. Opt. 15,* 2812.

Hoffmann, R. (1963), *J. Chem. Phys. 39,* 1397.

Jorgensen, W. L., and Salem, L. (1973), "The Organic Chemists Book of Orbitals," Academic Press, New York, N.Y., p 53.

Junge, W., and Witt, H. T. (1968), *Z. Naturforsch. B 23,* 244.

Labhart, H. (1963), *Tetrahedron 19,* supplement 2, 223.

Liptay, W. (1969), *Angew Chem., Int. Ed. Engl. 8,* 177.

Loew, L. M., Bonneville, G. W., Scully, S., Surow, J., Hassner, A., and Alexanian, V. (1978), *Biophys. J. 21,* 206a.

Loew, L. M., Bonneville, G. W., Scully, S., Surow, J. Hassner, A., and Alexanian, V. (1978), *Biophys. J. 21,* 206a.

McGlynn, S. P., Vanquickenborne, L. G., Kinoshita, M., and Carroll, D. G. (1972), "Introduction to Applied Quantum Chemistry", Holt, Rinehart and Winston, Inc., New York, N.Y., pp 98–139.

Mulliken, R. S. (1955), *J. Chem. Phys. 23,* 1833.

Mulliken, R. S. (1962), *J. Chem. Phys. 36,* 3428.

Pick, V., and Avron, M. (1976), *Biochim. Biophys. Acta 440,* 189.

Platt, J. R. (1956), *J. Chem. Phys. 25,* 80.

Platt, J. R. (1961), *J. Chem. Phys. 34,* 862.

Reich, R., and Schmidt, S. (1972), *Ber. Bunsenges. Physik. Chem. 76,* 589.

Reich, R., and Scheerer, R. (1976), *Ber. Bunsenges. Phys. Chem. 80,* 542.

Reich, R., Scheerer, R., Sewe, K.-U., and Witt, H. T. (1976), *Biochim. Biophys. Acta 449,* 285.

Ross, W. N., Salzberg, B. M., Cohen, L. B., and Davilla, H. V. (1974), *Biophys. J. 14,* 983.

Ross, W. N., Salzberg, B. M., Cohen, L. B., Grinvald, A.,

Davilla, H. V., Waggoner, A. S., and Wang, C.-H. (1977), *J. Membr. Biol. 33,* 141.
Salzberg, B. M., Cohen, L. B., Ross, W. N., Waggoner, A. S., and Wang, C.-H. (1976), *Biophys. J. 16,* 23a.
Schmidt, S., and Reich, R. (1972), *Ber. Bunsenges. Phys. Chem. 76,* 1202.
Schmidt, S., Reich, R., and Witt, H. T. (1969), *Z. Naturforsch. B. 24,* 1428.
Schmidt, S., Reich, R., and Witt, H. T. (1972), in Proceedings of the 2nd International Conference on Photosynthesis Research, Forti, G., Avron, M., and Melanchi, A., Ed., Dr. W. Junk N. V. Publishers, The Hague, p 1089.
Sims, P. J., Waggoner, A. S., Wang, C. H., and Hoffmann, J. F. (1974), *Biochemistry 13,* 3315.
Smith, J. C., Russ, P., Cooperman, B. S., and Chance, B. (1976), *Biochemistry 15,* 5094.
Smith, J. C., Powers, L. S., and Claddis, P. (1978), *Biophys. J. 21,* 67a.
Tasaki, I., Carnay, L., Sandlin, B., and Watanabe, A. (1969a), *Science 163,* 683.
Tasaki, I., Carnay, L., and Watanabe, A. (1969b), *Proc. Natl. Acad. Sci. U.S.A. 64,* 1362.
Tasaki, I., (1974), *Ann. N.Y. Acad. Sci. 227,* 247.
Tasaki, I., Warashina, A., and Pant, H. (1976), *Biophys. Chem. 4,* 1.
Waggoner, A. (1976), *J. Membr. Biol. 27,* 317.
Waggoner, A. S., Wang, C. H., and Tolles, R. L. (1977), *J. Membr. Biol. 27,* 317.

Tissue Distribution and Subcellular Localization of Bovine Thioredoxin Determined by Radioimmunoassay†

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ABSTRACT: A double antibody radioimmunoassay for calf liver thioredoxin has been developed based on the use of rabbit antiserum and ^{125}I-labeled thioredoxin. The method permits determinations of as little as 2 ng (0.2 pmol) of thioredoxin in crude extracts, thus allowing measurements not possible with previous enzymatic determinations of thioredoxin. Since no competition is observed with thioredoxin from *Escherichia coli,* yeast, rat liver, Novikoff hepatoma, or man, the radioimmunoassay is specific for bovine thioredoxin; i.e., it is species specific. The immunoreactive thioredoxin in a week-old calf was determined in cell-free homogenates from different tissues, including platelets and erythrocytes. All tissues examined contain thioredoxin with the highest content in liver, kidney, thymus, erythrocytes, and brain. Immunoreactive thioredoxin in the different crude extracts represents from 0.03 to 0.07% by weight of the total protein; i.e., calf thymus contains 8×10^5 copies of thioredoxin per cell. Levels in bovine plasma were detectable but were below 0.05 mg per L. Calf liver and thymus were subjected to standard differential centrifugational techniques to separate subcellular fractions. Thioredoxin is present in homogenates of nuclei, mitochondria, microsomes, membranes, and in the 100 000*g* postmicrosomal supernatant. A homogenate of the plasma membrane fraction contains immunoreactive thioredoxin and the level increases several fold after treatment with 1% Triton X-100. This strongly suggests that a fraction of thioredoxin is associated with membrane structures or enclosed in vesicles. The ubiquitous presence of thioredoxin in tissues and subcellular fractions has important implications in view of the general thiol–disulfide oxidoreductase activity of the protein.

Thioredoxin is a well-characterized protein of 11 700 molecular weight (Laurent et al., 1964; Holmgren et al., 1975), containing a cystine residue, which is the electron acceptor in the transfer of electrons from NADPH catalyzed by the enzyme thioredoxin reductase (Thelander, 1968). The reduced form of thioredoxin, thioredoxin-$(SH)_2$, was until recently (Holmgren, 1976) the only known hydrogen transport system for the enzymatic formation of DNA precursors catalyzed by the essential enzyme (Fuchs et al., 1972), ribonucleotide reductase. The discovery of a second hydrogen transport pathway in *Escherichia coli* utilizing glutathione and a novel protein called glutaredoxin (Holmgren, 1976) raises questions about the function of thioredoxin in this reaction. Other functions for thioredoxin different from DNA precursor biosynthesis and as a general dithiol reductant have been described, e.g., the capacity to catalyze reduction of protein disulfide bonds in insulin (Moore et al., 1964; Engström et al., 1974; Holmgren, 1977), human choriogonadotropin (Holmgren & Morgan, 1976), or fibrinogen (Blombäck et al., 1974). Furthermore, a regulatory role has been suggested for thioredoxin in photosynthesis in spinach chloroplasts (Wolosiuk & Buchanan, 1977; Holmgren et al., 1977), and *E. coli* thioredoxin is known to be a subunit of T7 virus induced DNA polymerase (Mark & Richardson, 1976). Clearly more studies on the distribution and function of thioredoxins are required.

Thioredoxin was recently isolated in homogeneous form from calf liver (Engström et al., 1974), using an assay based on its capacity to promote disulfide reduction in insulin by NADPH and the homologous thioredoxin reductase (Holmgren, 1977). Since a thioredoxin has no obvious enzymatic activity by itself, measurements of thioredoxin have so far been performed by enzymatically coupled reactions using purified thioredoxin reductase or ribonucleotide reductase. These determinations have serious limitations since they cannot be applied to crude extracts without their prior purification. For

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