CHARGE-SHIFT PROBES OF MEMBRANE POTENTIAL

A PROBABLE ELECTROCHROMIC MECHANISM FOR *p*-AMINOSTYRYLPYRIDINIUM PROBES ON A HEMISPHERICAL LIPID BILAYER

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ABSTRACT The characteristics of the spectroscopic responses to membrane potential are examined for a series of dyes based on the 4-(*p*-aminostyryl)-1-pyridinium chromophore. An apparatus using an oxidized cholesterol hemispherical bilayer and phase-sensitive detection provides response spectra in either transmission or fluorescence excitation modes. All the probes with good binding properties display biphasic response spectra that are similar in both shape and magnitude. Detailed analysis of the response spectra allows all the previously discovered mechanisms for extrinsic potential sensitive molecular probes, which require a change in the probe's chemical environment, to be ruled out. The data are consistent with an electrochromic mechanism. Polarized fluorescence intensities from the membrane-bound probes indicate that the chromophore is optimally oriented for an electrochromic response.

INTRODUCTION

Organic dye molecules have been found that display spectroscopic responses to membrane potential. Of the ~1,000 dyes that have been screened on the squid axon (Cohen et al., 1974; Ross et al., 1977), 20–30 have proven useful in various tissue or cell suspension preparations. These applications and mechanistic studies of the spectroscopic responses have been comprehensively reviewed (Cohen and Salzberg, 1978; Waggoner, 1979). It appears that all of the dyes respond by a mechanism that involves a motion of the molecule in response to the electric field; this field-induced change in the orientation or environment of the probe effects a perturbation of its spectral properties.

We have been engaged in a systematic effort to design probes that utilize only motion of electrons, rather than molecular motion, in the mechanism of their response to membrane potential (Loew et al., 1978). The rationale is to design molecules that will undergo large electronic redistributions upon excitation and will be oriented in the membrane so that this shifting charge interacts maximally with the field, i.e., linear electrochromism (Platt, 1956, 1961). Waggoner and Grinvald (1977) have shown that linear electrochromism is, in principle, capable of providing responses comparable in size to those of other "fast" mechanisms. Chance and co-workers (1974) have also discussed the possibility of utilizing electrochromism for the design of extrinsic potentiometric spectroscopic probes.

An advantage of an electrochromic mechanism, in some applications, is that the response is fast enough to follow the change in membrane potential associated with any physiological process, whatever the speed. Another advantage is that electrochromic probes should be

capable of a relatively uniform sensitivity in any tissue or cell system; one need only require that they have an amphipathic structure capable of strong binding to all membrane systems. Probes responding by molecular motion, on the other hand, require a delicate balance between at least two chemical states in equilibrium, so that a potential-induced perturbation in the free energy difference can result in a large change in the relative populations of the states (Waggoner and Grinvald, 1977). This delicate balance may easily be disrupted by attempts to apply successful probes to new systems, as has been demonstrated by Ross and Reichardt (1979). In a systematic study of dye responses from a series of neuronal preparations, they found that the magnitude and spectral distributions of the signals varied tremendously from species to species.

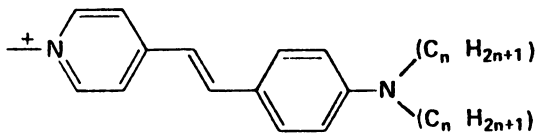
Perhaps the most important advantage is that a relatively simple theoretical procedure can be used to qualitatively screen a chromophore for large responses by an electrochromic mechanism before one goes to the trouble of synthesizing a probe (Loew et al., 1978). The *p*-aminostyrylpyridinium (ASP) chromophore was the first chromophore to be incorporated into a series of probes as a result of this theoretical screening. Fig. 1 gives the structures and our nomenclature for these compounds. The first "charge-shift probe" to be prepared was di-5-ASP and we have, accordingly, spent most of our initial effort characterizing this molecule (Loew et al., 1979*a*,*b*).

In this work, we examine the responses of both the absorption and excitation spectra of a series of eight ASP probes to an electric field across a hemispherical bilayer membrane. Besides providing additional evidence for an electrochromic mechanism, this allows an analysis of the way in which molecular structural variations affect the sensitivity of charge-shift probes.

METHODS

The probes were prepared according to the scheme outlined for di-5-ASP by Loew et al. (1979*a*); complete details on the synthesis, along with analyses of absorption, excitation, emission, and polarization spectra, will be submitted for publication elsewhere.

Oxidized cholesterol was prepared according to the procedure described by Tien (1974). The filtered octane solution, which is the final product of Tien's procedure, was evaporated and redissolved in half



di–n–ASP

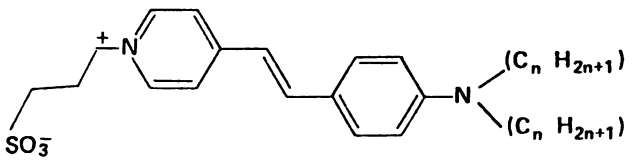


FIGURE 1 Molecule structures of the di-*n*-ASP and di-*n*-ASPPS probes.

the volume of decane (Aldrich Chemical Co. Inc., Milwaukee, Wis., Gold Label grade). This solution was used to form hemispherical bilayer membranes suspended from a silanized pasteur pipette tip in 100 mM KCl (Dragsten and Webb, 1978). The probes were added to the KCl bathing solution from 3-mM stock solutions in ethanol; in all experiments the ethanol concentrations were $\leq 0.5\%$.

Polarized fluorescence intensities were obtained by focusing the 442-nm beam (4 mW) of a He-Cd laser (model 4110H, Liconix, Mt. View, Calif.) through a 10 × achromat microscope objective onto the appropriate area of the hemispherical bilayer, which was suspended from the pipette in an ordinary 1 × 1-cm fluorescence cuvette. Emitted light was detected at 90° to the incident beam with an EMI 9558 QA photomultiplier tube (EMI Gencom, Inc., Plainview, N.Y.) in a thermoelectrically cooled housing after passing through a 570-nm interference filter and a polarizing filter; the photocurrent was read on an electrometer (Victoreen Inc., Sheller-Globe Corp., Cleveland, Ohio).

Absorption and fluorescence-excitation spectral responses to an applied voltage across the bilayer were obtained with the apparatus illustrated in Fig. 2. A 100-W tungsten-halogen lamp, powered by a stabilized DC source (DCR40-25B, Sorensen Co., Manchester, N. H.), was focused onto the 2-mm entrance slit of a scanning monochromotor (H20V, Instruments SA, Metuchen, N.J.) The exit slit of the monochromotor has been modified to provide a 2 × 2-mm emerging light beam which was focused onto the bottom of the hemispherical bilayer with a 10 × achromat objective, as for the laser fluorescence experiments; the beam width at the focus was ~250 μm. Transmitted light was collected by a matching objective and detected with a photodiode (model 500-UV, United Detector Technology, Inc., Santa Monica, Calif.). Fluorescence at 570 nm was detected at 90° with the cooled photomultiplier, as in the laser fluorescence experiments. The entire optical system was mounted by a 1-m optical rail and an aluminum bread board on a heavy table top, isolated from floor vibrations by four air-filled shock absorbers. The current output from either detector is converted to a voltage and sent into one channel of a Bascom-Turner 8110 recorder (Bascom-Turner Instruments, Newton, Mass.). In a branching path, the AC component of this total signal is amplified by a factor of 100 and sent into a lock-in amplifier (model 5203, Princeton Applied Research Corp., Princeton, N. J.). The 1-V reference signal for the lock-in amplifier is supplied by a square wave generator (B & K Precision, Chicago, Ill.), which also provided the ±50 mV, 200-Hz transmembrane potential through Ag/AgCl electrodes. The output of the lock-in amplifier is, thus, only the part of the optical signal that is in concert with the AC potential. This response spectrum was recorded simultaneously with the total optical signal on a second channel of the recorder and can be processed in several ways. The transmittance change was divided by the total transmittance to provide a display of the relative transmittance response, $\Delta T/T$, as a function of wavelength; since the solution absorbance is generally small and because $\Delta T/T \ll 10^{-2}$, this display is equivalent to $-2.3\,\Delta A$, the absorbance response to the potential as a function of wavelength (Waggoner

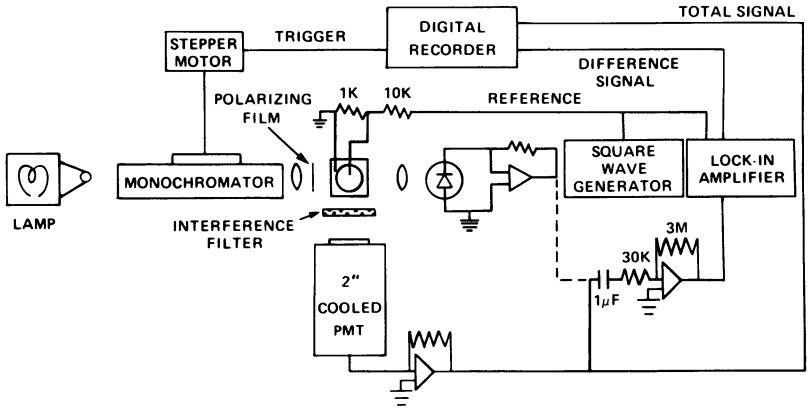


FIGURE 2 Block diagram of the apparatus for transmittance and fluorescence excitation-response spectra of dyes bound to a hemispherical lipid bilayer.

and Grinvald, 1977). The fluorescence change, ΔF, can also be displayed relative to the total fluorescence, F, to provide a measure of the size of the response as a function of wavelength; before the $\Delta F/F$ operation was performed, the total fluorescence was corrected for a small amount of background by subtracting the excitation spectrum obtained in the absence of a membrane. A more theoretically meaningful fluorescence-excitation response spectrum is obtained by correcting the ΔF for variations in intensity of the incident light over the wavelength range of interest. This was accomplished by recording the emission, over this excitation range, of a concentrated rhodamine B solution in ethylene glycol (contained in a triangular cuvette to avoid inner filter effects) with a 620-nm cut-off filter in place of the 570-nm interference filter. The stored ΔF spectrum is then divided by this correction curve to provide ΔF_{cor}. The total fluorescence can also be corrected (after subtracting background), and a first derivative can be obtained for comparison with ΔF_{cor} or $\Delta I/I$ as described below.

The Pariser-Parr-Pople (PPP) self-consistent field π-molecular orbital method (Pariser and Parr, 1953; Pople, 1953) with configuration interaction was applied to the analysis of the ASP spectrum. The SCFMO computer program (Molnar, 1976) was used with the heteroatom parameters suggested by Greenwood (1972). The full calculation required 2 s of CPU time on the Itel-AS6 computer in State University of New York (SUNY) at Binghamton Computer Center.

RESULTS

Probe Orientation

The transition moment of the ASP chromophore to its lowest excited singlet is expected to be along the long axis of the π-system (Salem, 1966). This expectation was supported by PPP molecular orbital calculations (Greenwood, 1972; Molnar, 1976) that also provided a reasonable prediction of 521 nm for λ_{max}^{vacuum} ($\lambda_{max}^{ethanol}$ = 495 nm). These theoretical considerations thus allow estimation of probe orientation in the membrane from polarized fluorescence data.

A qualitative assessment of the preferred orientations of the membrane-bound probes is obtained according to the analysis of Yguerabide and Stryer (1971) and has already been applied to di-5-ASP (Loew et al., 1979*a*). Briefly, the vertically polarized laser is focused on either the bottom (*B*) or middle (*M*) of the hemispherical bilayer and the fluorescence is collected through a vertically oriented polarization filter. These intensities, *BV* and *MV*, are normalized for the variations in illumination area by dividing each by the horizontally polarized emission intensities, *BH* and *MH*, respectively (*BH* and *MH* are theoretically equivalent). Assuming, then, that there is little movement of the chromophore during its excited state lifetime and that the emission and excitation transition moments are parallel, the ratio (*BV*/*BH*):(*MV*/*MH*) provides a measure of the relative population of probes oriented perpendicular vs. parallel to the membrane surface. These assumptions can be justified on the basis of the high value of the polarization, *P*, determined for lipid vesicle-bound probes under standard isotropic conditions (*P* ranges from 0.32 to 0.26) and the constancy of *P* over the excitation spectrum (unpublished results). Also, the PPP calculations indicate a completely isolated lowest energy transition with the next absorption occurring 175 nm to the blue.

A completely random orientational distribution with respect to the membrane surface normal would dictate a 1:1 ratio. The results displayed in Table I indicate a strong preference for orientation of the probes perpendicular to the membrane surface. This is consistent with the blue-shifted absorption spectra of vesicle-bound probes relative to probes dissolved in water or organic solvents as discussed by Loew et al. (1979*a*). From a theoretical viewpoint, this orientational preference was expected on the basis of the amphiphilic rod-shaped

TABLE I
SUMMARY OF ASP PROBE CHARACTERISTICS

Probe*	(*BV/BH*):(*MV/MH*)	$\Delta F/F \times 10^2$‡	$\Delta T/T \times 10^5$‡
di-1-ASP	4.0 ± 0.8	−1.5	+2.8
di-4-ASP	3.6 ± 0.6	−2.8	+5.3
di-4-ASPPS	2.8 ± 0.4	−1.5	+3.8
di-5-ASP	3.4 ± 0.3	−3.0	+5.8
di-6-ASP	3.4 ± 0.4	−2.8	+5.3
di-6-ASPPS	2.7 ± 0.2	−2.0	+4.8
di-10-ASP	2.9 ± 0.2	−1.8	+3.8
di-10-ASPPS	2.6 ± 0.4	−1.3	+3.0

*All experiments were carried out with a 2-μM probe in the 100 mM aq KCl bathing solution.
‡Responses were determined at 490 nm with a 100 mV 200 Hz symmetrical square wave applied to the inside of the hemispherical bilayer. Errors were ±35% for di-1-ASP, di-10-ASP, and di-10-ASPPS and ±15% for the rest.

structures of the probes and is optimal for the realization of an electrochromic response to a transmembrane electric field (Loew et al., 1978).

Fluorescence Response

Fig. 3 displays ΔF_{cor} obtained for di-6-ASPPS [4-(*p*-aminostyryl pyridinium propylsulfonate)] applied to the inside or outside of the bilayer. As would be expected, on the basis of the predicted electronic charge shift and as for di-5-ASP (Loew et al., 1979*b*), hyperpolarization causes a red shift of the excitation spectrum for probe applied to the outside of the bilayer. The most striking aspect of this experiment is the close similarity of the spectra obtained for

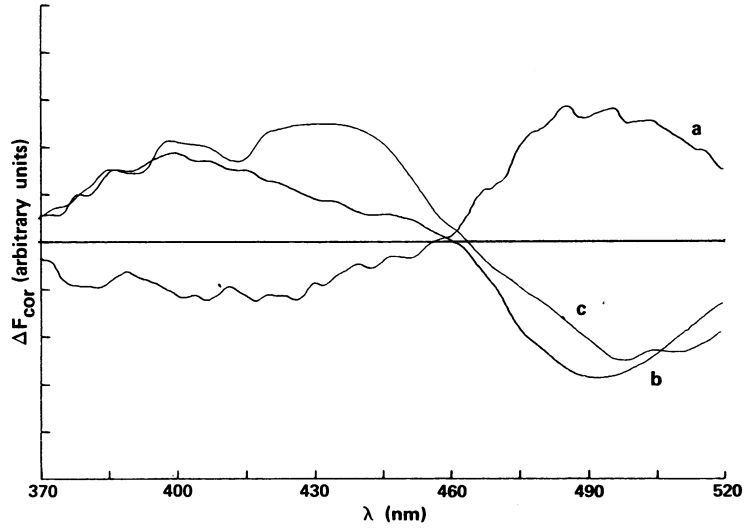


FIGURE 3 Corrected fluorescence excitation response spectra (ΔF_{cor}) of 2 μM di-6-ASPPS bound to (*a*) the inside and (*b*) the outside of the hemispherical bilayer. Voltage pulses at 200 Hz and 100 mV centered at ground are applied to the inside with the outside kept at ground. Trace *c* is the first derivative of the corrected fluorescence excitation spectrum of the membrane-bound probe.

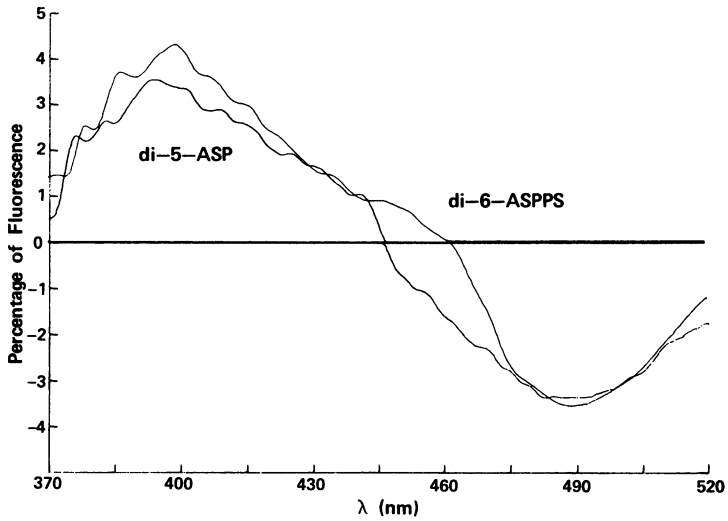


FIGURE 4 Ratio of the fluorescence change for a 100-mV, 200-Hz transmembrane potential (ΔF) to the total membrane fluorescence (F) as a function of exciting wavelength. 2μM di-5-ASP and 2 μM di-6-ASPPS applied to the outside of the hemispherical bilayer.

these two probes (Fig. 4, cf., Fig. 2 in Loew et al., 1979*b*). Any molecular motion response to the membrane potential for di-5-ASP, a positively charged ion, would be expected to be drastically altered for di-6-ASPPS, a zwitterionic molecule. The fact that the response spectrum is not changed in shape or in magnitude (Fig. 4) is, however, consistent with an electrochromic mechanism. Fig. 3 also illustrates the close match of ΔF_{cor} and the first derivative of the corrected excitation spectrum; this is another criterion for linear electrochromism (Liptay, 1969; Reich and Schmidt, 1972). The deviations from exact matchups in all of these comparisons can be ascribed partially to the error inherent in these measurements of small changes in a small fluorescent signal. There are also small differences in the resting spectra for the various ASP probes. In addition, it was found that a response that is reduced by an order of magnitude and does not have a wavelength dependence (i.e., does not change sign) replaces the normal response spectrum when the pipette tip requires reconditioning or the membrane-forming solution has aged. Although the origin of this effect is not understood, it, too, could provide small distortions of the recorded response spectra.

The probes have a strongly enhanced fluorescence upon binding and an emission maximum at 570 nm that is 40 nm to the blue of the aqueous probe fluorescence. The use of the 570-nm interference filter thus cuts down background fluorescence (which is routinely subtracted anyway as noted in Methods). However, in some preliminary experiments with other emission filters, it does appear that the zero cross-over point in the excitation-response spectrum may shift if fluorescence is monitored from the maximum emission wavelength; this suggests that the emission spectrum is sensitive to membrane potential as well and also provides an additional reason for deviations in the comparisons described above.

The fluorescence response of the other ASP probes was qualitatively similar to those displayed in Figs. 3 and 4 and the data for all of them are summarized in Table I. Di-1-ASP did not bind well to the membrane and displayed a correspondingly weak response, as might

have been predicted from its structure. The two probes with 10-carbon chains, on the other hand, appear to have large binding constants but require >30-min incubations to reach a significant level of binding. During this long period, transmembrane "flip-flop" of these lipophilic probes could become a factor in reducing the observed $\Delta F/F$.

Aggregate formation is often implicated in the response mechanism of potential-sensitive probes (Waggoner, 1979). We were prompted, therefore, to examine the dependence of membrane fluorescence on the concentration of probe in the bathing solution. Fig. 5 displays the results for di-6-ASPPS. Intensities were corrected for background and for inner filter effects. At high concentrations, the fall in fluorescence is indeed indicative of aggregate formation; aggregates of organic dyes are characterized by a blue-shifted absorption spectrum and a quenching of fluorescence. The experiment illustrated in Fig. 3 was performed at a probe concentration well below that of the onset of aggregation. Further evidence against the involvement of aggregates in the response mechanism is presented below.

Transmission Response

Polarized transmission response spectra are displayed in Figs. 6 and 7 for di-6-ASP and di-6-ASPPS, respectively; a similar experiment for di-5-ASP has been reported by Loew et al. (1979*b*). Once again, the response is essentially independent of the charge on the probe; the small 10- to- 12-nm difference in these spectra may be ascribed to slight differences in the resting absorption spectra of the probes bound to the hemispherical bilayer (although lipid vesicle spectra for the two probes are admittedly identical). All the probes show strongly dichroic responses for illumination of approximately the bottom eighth of the hemispherical

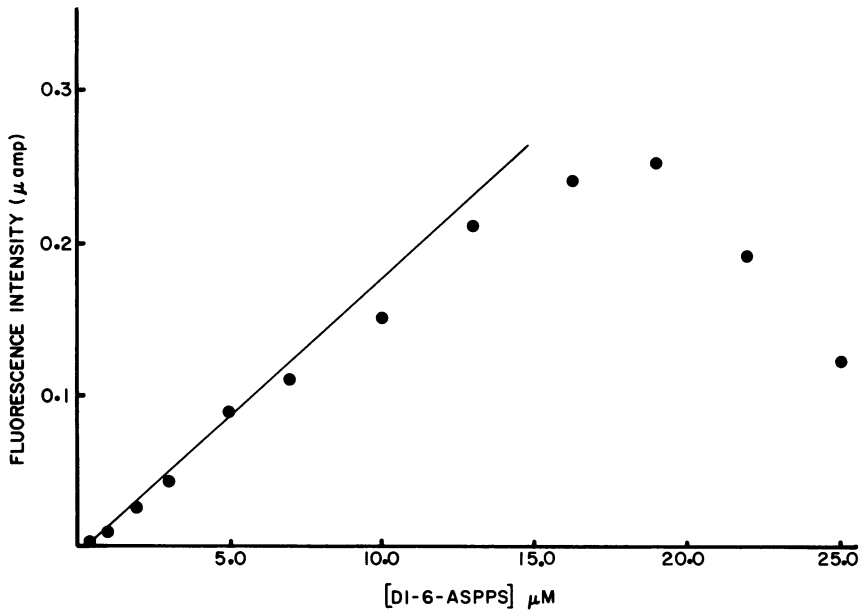


FIGURE 5 Fluorescence intensity of membrane-bound di-6-ASPPS as a function of concentration in the outer bathing solution. Excitation at 465 nm; emission at 570 nm. Intensities were corrected for absorption of the exciting light by the bathing solution and for background levels of fluorescence.

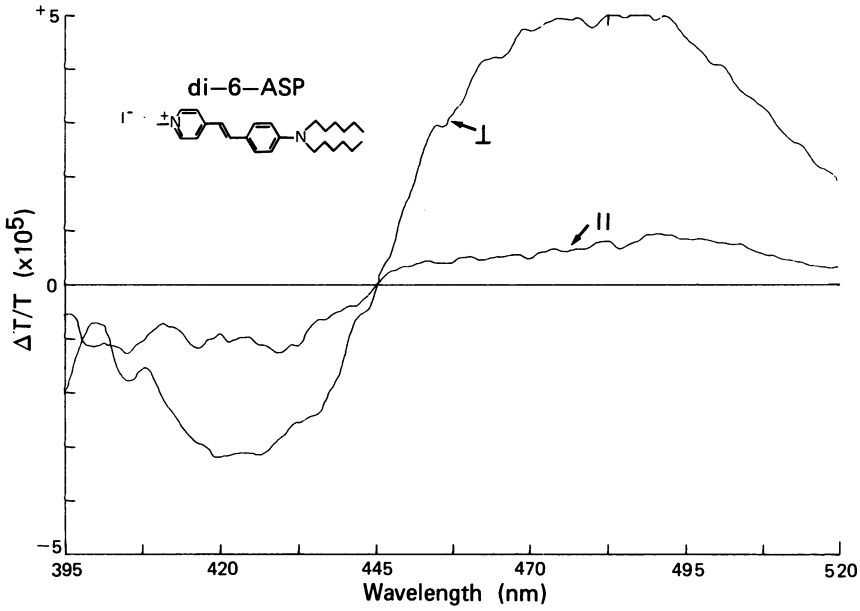


FIGURE 6 Transmittance response spectrum of di-6-ASP bound to the outer surface of a hemispherical bilayer. The bathing solution was 2 μM di-6-ASPPS. The two traces correspond to incident light polarized parallel ($\parallel$) and perpendicular ($\perp$) to the bottom surface of the membrane. Approximately the bottom eighth of the hemisphere is illuminated. The responses are presented as the ratio of the change in transmittance (ΔT) to the total transmittance (T). 200 Hz, 100 mV.

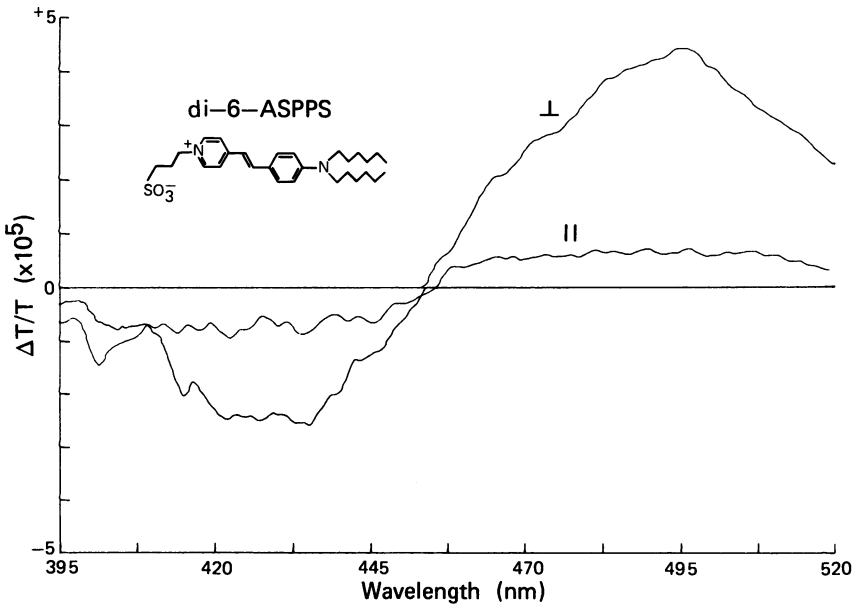


FIGURE 7 Transmittance response spectrum of di-6-ASPPS. The conditions are the same as for Fig. 6.

bilayer. This dichroism results from the preferred orientation of the probes, as discussed above, and is reinforced by the dependence of electrochromism on the dot product of the electric field with the charge displacement vector (Loew et al., 1978). (Note that if it were possible to illuminate along the plane of a truly planar membrane, the dichroism exhibited in the $\Delta T/T$ spectra would be even greater.) These spectra rule out a rotational reorientation mechanism (Dragsten and Webb, 1978), because that would require responses of opposite sign for the two polarizations.

These spectra, taken together with the fluorescence excitation-response spectra, also argue against the involvement of dye aggregation in the response mechanism. As in the case of most polyene dyes (West and Pearce, 1965; Emerson et al., 1967), the ASP probes display a blue shift of the absorption spectrum (Loew et al., 1979*a*) and quenched fluorescence (Fig. 5) upon aggregation. The $\Delta T/T$ response spectra could be consistent, therefore, with an aggregation of probe on the outer membrane surface in response to depolarizing pulses. If this were the case, the ΔF_{cor} spectra (Fig. 3) should reflect not only this blue shift but also a superposed drop in the total fluorescence in response to a depolarizing pulse. Thus, if aggregation were involved in the mechanism, the fluorescence-excitation response spectra and the transmittance-response spectra would have to have very different shapes (this is well illustrated in Fig. 7 of Ross et al., 1977). Comparison of our Figs. 3 and 7 shows that the excitation spectrum and the absorption of di-6-ASPPS respond equivalently to the membrane potential. This is true of all the ASP probes, providing more evidence against the involvement of aggregation in the response mechanism.

The transmittance responses for all the available ASP probes are summarized in Table I. As in the fluorescence experiments, the poor binding behavior of di-1-ASP and di-10-ASP can be blamed for their small and unreliable transmittance responses. It should be emphasized that unlike the fluorescence response, where only the small membrane-associated optical signal is detected, the transmittance experiment monitors the light that is not absorbed by the membrane-bound probe. The smaller values for $\Delta T/T$ relative to $\Delta F/F$ are simply related, therefore, to the relative sizes of their denominators.

Another consequence of this difference in the mode of detection is that $\Delta T/T$ should be sensitive to probe concentration whereas $\Delta F/F$ should not. This is because as probe concentration (and, therefore, the amount of bound probe) increases, ΔF and F should both increase in proportion, but ΔT will increase while T decreases. The experiments of Figs. 6 and 7 were designed to examine the shape of the spectral response; low probe concentrations were used to avoid distortions due to light absorption by unbound probe and possible aggregate formation. Fig. 8 reveals these distortions but also shows that $\Delta T/T$ values as high as 10^{-4} can be achieved.

DISCUSSION

The probe responses are affected primarily by structural factors that influence binding and orientation. The poor response for di-1-ASP results simply from the small amount bound to the membrane. The 10-carbon chain probes require long incubations to achieve a reasonable level of binding (determined by the fluorescence level); these lipophilic molecules may be able to permeate the membrane on this time scale, accounting for a lowered $\Delta F/F$, but no attempt was made to quantify such a process. All the probes were designed to preferentially orient

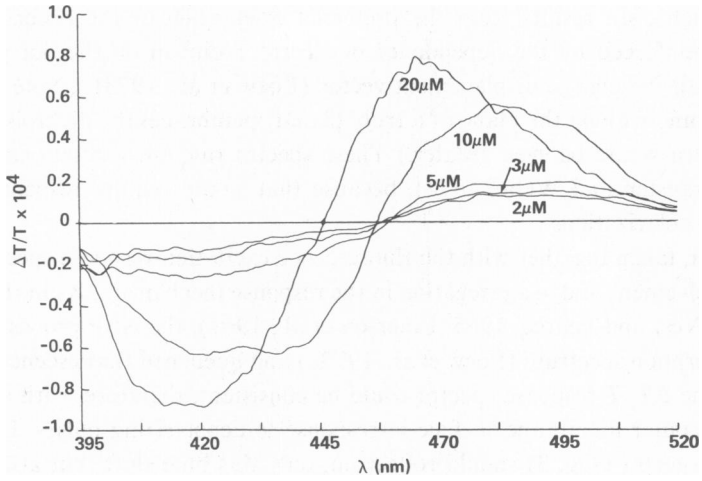


FIGURE 8 Transmittance response spectra for di-6-ASPPS as a function of probe concentration 100 mV, 200 Hz.

perpendicularly to the membrane surface. ASP dyes with the conjugated chain attached at the 2 position of the pyridinium ring and/or with long alkyl side chains on the pyridinium rather than the anilino nitrogen would not be expected to orient well for an electrochromic response (Loew et al., 1978); indeed, such dyes were among those originally screened on the squid axon and gave generally mediocre signals (Cohen et al., 1974).

The insensitivity of the ASP probe responses to the net charge on the probe is most significant. The positive charge of the chromophore is concentrated in the pyridinium ring in the ground state (Loew et al., 1978); thus, covalent linkage of the negative countercharge to this end of the chromophore in the ASPPS probes maintains their amphiphilic character and does not disrupt the orientation of the chromophore. An electrochromic mechanism, therefore, allows the ASP and the ASPPS probes to respond essentially identically to a membrane potential. The data presented here argue strongly for electrochromism, but other mechanisms are conceivable. For example, it might be possible that a motion of the probe from one site to another within the membrane and without any appreciable reorientation could account for the observed potential-dependent spectral shifts. The thermodynamics of this motion would have to be largely independent of the side chains on the ASP chromophore and the overall charge of the probe; in other words, the alkyl chains on the amino end would have to have essentially no depth-dependent interaction with the surrounding lipid and the negatively charged sulfonates could not be experiencing the changing electric field that moves the positively charged chromophore. Clearly, this set of circumstances is highly improbable. We are aware of only one previous neutral dye that provided a useful response;[1] this dye has an aminostyryl-quinolinium chromophore that may also be electrochromic. It is impossible to be certain that no other mechanism that might fit the data exists, or whether a small component of the response might be associated with a different mechanism (e.g., a small amount of reorienta-

[1]A. Grinvald. Personal communication.

tion), but we feel that linear electrochromism is the simplest, most obvious, and most reasonable choice to explain the primary responses of the ASP probes.

A rough estimate of the magnitude of the excitation-induced charge shift can be obtained for the ASP chromophore from $\Delta F/F$. Rearrangement of Eq. 3 in Waggoner and Grinvald (1977) allows expression of the charge shift in terms of the experimentally determined linear electrochromic change in the optical extinction of the bound probe ($\Delta\epsilon$) in response to an electric field, E(V/cm):

$$qr\cos\theta = \left(\frac{hc}{E\lambda^2}\right)\left(\frac{\Delta\epsilon}{d\epsilon/d\lambda}\right)(\text{electron}\cdot\text{cm}). \tag{1}$$

In Eq. (1), q is the electronic charge, r is its excitation-induced displacement (in centimeters), θ is the angle between this displacement and E, h is Planck's constant (4.1×10^{-15} eV · s), c is 3×10^{10} cm/s, λ is the wavelength expressed in centimeters, and $d\epsilon/d\lambda$ is the slope of the absorption band at the wavelength of the measurement. Because the extinction is proportional to the fluorescence, the following relationships are obeyed:

$$\frac{\Delta\epsilon}{\epsilon} = \frac{\Delta F}{F} = \frac{\Delta F_{cor}}{F_{cor}} \tag{2}$$

and

$$\frac{\Delta\epsilon}{d\epsilon/d\lambda} = \frac{\Delta F_{cor}}{dF_{cor}/d\lambda}. \tag{3}$$

Note that Eqs. 2 and 3 hold strictly only if the emission spectrum is insensitive to E (this is unlikely), or if the emission wavelength at which fluorescence is being monitored is at the zero-crossover point; we have not as yet confirmed this, but our choice of emission wavelength, 570 nm, is approximately at the maximum for the corrected emission spectra of the probes bound to phosphatidyl choline vesicles; this should be close to the crossover point in the emission response. With this reservation, the problems of precisely measuring the concentration and optical density of dye bound to the membrane can be avoided by substituting Eq. 3 into Eq. 1:

$$qr\cos\theta = \left(\frac{hc}{E\lambda^2}\right)\left(\frac{\Delta F_{cor}}{dF_{cor}/d\lambda}\right). \tag{4}$$

For convenience, F_{cor} is expressed as a fraction of the fluorescence intensity at λ_{max} of the excitation spectrum. At 490 nm, $F_{cor} = 0.75\,F_{max}$, $dF_{cor}/d\lambda \simeq 1.7 \times 10^5\,F_{max}$/cm and $\Delta F_{cor}/100$ mV $= 0.023\,F_{max}$. Assuming a bilayer thickness of ~50 Å (Tien, 1974), $E = 2 \times 10^5$ V/cm. Applying Eq. 4, $qr\cos\theta = 3.4 \times 10^{-8}$ (electron·cm) $= 16 \times 10^{-18}$ esu·cm (this is equivalent to 16 Debye units, but since a charge movement rather than a dipole moment change is involved, we have chosen not to express this quantity in Debye units). Since θ for all the probes is close to zero, this provides an experimental value for the charge shift in the ASP chromophore and compares favorably with the theoretically derived value of 14×10^{-18} esu·cm (Loew et al., 1978).

The response sizes are quite comparable to the best of the "fast dyes" screened on the squid axon (Cohen and Salzberg, 1978; Gupta et al., 1980). (The "slow dyes," used frequently for

cell suspensions rather than single-cell preparations, can, of course, provide signals an order of magnitude larger than those reported here.) An electrochromic response is limited in speed only by the time required to absorb a photon. In earlier experiments on di-5-ASP (Loew et al., 1979*b*) we reported a response coincident with the relatively slow 100-μs charging time of the hemispherical bilayer. We have not measured the response times for the rest of the ASP probes, although they are able to easily keep time with the 200-Hz train of voltage pulses used to generate the response spectra. The measurement of the response time with an apparatus capable of <1-μs resolution should provide an additional confirmation of the electrochromic mechanism.

It also remains to be seen whether the ASP probes can give consistent responses in other membrane preparations. In some preliminary experiments with the squid axon,[2] di-6-ASPPS provides both transmittance and fluorescence responses qualitatively and quantitatively consistent with those reported here for the hemispherical bilayer; $\Delta F/F$ is lowered by $\sim$10-fold because of background fluorescence from the Schwann cells, as found for other dyes, but the wavelength dependence is the same. Diffusion potentials across phospholipids vesicle membranes also elicit comparable responses.[3]

There are many membrane systems to which the ASP probes cannot be applied simply because the absorption bands of their intrinsic chromophores may overlap the 420- to 500-nm region and swamp out or distort any response. It is important, therefore, to expand our repertoire of probes to include chromophores with different absorption wavelengths. One might anticipate even larger effects for chromophores with extended conjugation since the charge would have a physically longer distance to travel and because the higher wavelength range exaggerates the energy shift of the transition. These advantages may be offset by the increased likelihood of photolability, which has not been significant with the ASP probes.[4] New charge-shift probes are being developed along these lines.

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