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# CHARGE-SHIFT PROBES OF MEMBRANE POTENTIAL

## Characterization of Aminostyrylpyridinium Dyes on the Squid Giant Axon

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**ABSTRACT** The characteristics of transmittance and fluorescence changes of 4-(*p*-aminostyryl)-1-pyridinium dyes in response to voltage-clamp pulses on the squid giant axon were examined. A zwitterionic styryl dye displays transmittance and excitation spectra on the voltage-clamped squid axon with shapes similar to those previously measured on a model membrane system and consistent with a postulated electrochromic mechanism. The speed of the transmittance response is faster than 1.2  $\mu$ s. The size of the fluorescence change is a factor of 40 lower than on the model membrane; this diminution can be rationalized in terms of the background fluorescence from Schwann cells and the nonoptimal geometric arrangement of the axon membrane. When the emission spectrum is dissected from the excitation response, a nonelectrochromic component is found. This component might result from molecular motion during the excited state lifetime. A positively charged dye permeates the axon membrane and displays complex response waveforms dependent on the method of application and the axon holding potential. This contrasts markedly with model membrane results where the behavior of the cationic and zwitterionic dyes were indistinguishable.

### INTRODUCTION

The *p*-aminostyrylpyridinium (ASP) chromophore can be incorporated into membrane probes whose absorption and excitation spectra may respond to membrane potential by an electrochromic mechanism (Loew et al., 1979a; Loew and Simpson, 1981; Loew, 1982). These probes were designed with the aid of a theoretical model (Loew et al., 1978) that predicted a large charge shift down the length of the chromophore upon photoexcitation; side chains of appropriate polarity can be appended to the chromophore at points that will ensure that any transmembrane electric field is parallel to the charge displacement vector (Loew et al., 1979b; Loew and Simpson, 1981), thus maximizing an electrochromic effect. Electrochromism, therefore, permits a theoretical approach to the development of potential sensitive probes. A complementary approach, which encompasses electrochromism as well as all other possible mechanisms, has depended upon screening a large number of dyes and optimizing relatively successful structures by successive synthetic modifications (Cohen et al., 1974; Ross et al., 1977; Gupta et al., 1981; Grinvald et al., 1982, 1983). In fact, a substantial fraction of the recently

synthesized dyes have ASP chromophores (Gupta et al., 1981; Grinvald et al., 1982, 1983; Hassner et al., 1984).

Several advantages might be anticipated for electrochromic probes. Since the coupling of an electric field to the electronic states of a chromophore is instantaneous on the timescale of ion currents or even intramolecular charge separations, electrochromism should faithfully follow the kinetics of a potential change associated with any physiological event; other mechanisms, which all involve potential dependent transitions between chemical states of the probes, are not guaranteed to be sufficiently fast for a given application. In addition, there is the possibility of a more uniform set of responses with electrochromic probes for different membrane preparations. Certain other mechanisms require a delicate energetic balance between two or more chemical states of the probe so that the equilibrium populations may be appreciably altered by the potential; this delicate balance may not be conserved over a range of membrane preparations (Ross and Reichardt, 1979; Warashina, 1980; Boyle et al., 1983). Under the assumption that the orientation of bound probes can be defined by the placement of hydrophilic and hydrophobic side chains rather than specific interactions with membrane proteins, an electrochromic response will not be especially sensitive to the particular membrane environment. Since the mechanism is a direct interaction with an electric field, the

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response size should be predictable after calibration with an appropriate model membrane system.

With a real probe, however, several factors may operate to limit these apparent advantages. Most seriously, there is no way of preventing additional nonelectrochromic mechanisms from complicating the probe response. These additional mechanisms may, in fact, be largely suppressed in the model membrane system used to calibrate the response but emerge only in a given experimental membrane system. If these nonelectrochromic responses are slow, kinetic experiments will be perturbed. A second, less important complication arises from the possible occurrence of conformation or orientation changes in the probe excited state that are beyond the ability of the current level of molecular orbital theory to predict. This will uncouple the internal charge displacement vector from the external electric field during the transition from the excited to the ground state. Thus, the electrochromic sensitivity of the absorption and excitation spectrum may not be mirrored by the emission spectrum of a membrane-bound probe. Also, the molecular relaxation that has time to occur in the excited states can render the emission spectrum more susceptible to nonelectrochromic mechanisms.

Here we explore these, as well as some related issues, for the ASP probes. We are able to contrast the behavior of several of the probes on the hemispherical bilayer system employed in our earlier work (Loew and Simpson, 1981; Loew et al., 1979a), with their responses to voltage-clamp steps across the membrane of the squid giant axon. These experiments allow us to detect problems of the type described above, to rationalize them in terms of the probe and membrane structures, and to begin to develop strategies to correct or circumvent them in future charge-shift probe design.

## EXPERIMENTAL PROCEDURES

The apparatus and methodology for obtaining voltage-dependent fluorescence or transmittance changes of dyes bound to a squid axon have been described (Cohen et al., 1974; Ross et al., 1977; Gupta et al., 1981). Pure excitation and transmittance response spectra were obtained by varying the wavelength of the incident light and monitoring voltage-dependent changes in emitted and transmitted light, respectively. Here we are also concerned with the sensitivity of the emission spectra of the probes. To eliminate the excitation component from the fluorescence response, an excitation wavelength is chosen at the point at which the transmittance response is null (i.e., the wavelength at which the voltage-induced absorbance change switches sign; this was at or near the maximum of the absorption or excitation spectrum). The emission response spectra are then obtained on a point-by-point basis with a set of interference filters that can be inserted into the emission path. In the squid axon experiments, the optical response to a voltage-clamp pulse is averaged directly; because the use of interference filters in the emission path reduces the normal intensity of the collected fluorescence, the signal-to-noise ratios were small and the results can be used only for qualitative comparison.

All of the internal perfusion experiments, and some of the other experiments, were carried out using a different arrangement of voltage-clamp electrodes and amplifiers from that described, and a horizontal chamber having floor, ceiling, and front made of glass, while the rear wall was of Delrin into which were fitted rectangular platinized platinum

electrodes for current passing and measurement (Salzberg and Bezanilla, 1983). The voltage electrode was located in the rear wall, immediately under the central plate, and the entire chamber was mounted on the mechanical stage of a compound microscope (Zeiss UEM; Carl Zeiss, Inc., Thornwood, NY) with focusable head. The internal electrode was of the "piggy-back" type described by Chandler and Meves (1965) consisting of platinized platinum-iridium wire 75–100  $\mu\text{m}$  in diameter for passing current and a 75–80- $\mu\text{m}$  pipette filled with 0.57 M KCl for measuring voltage. The pipette also contained an electrically floating 25- $\mu\text{m}$  platinum-iridium wire to lower the longitudinal impedance. The currents were guarded. Axons were perfused using a modification (Bezanilla and Armstrong, 1972) of the technique originally devised by Tasaki et al. (1962) but adapted to the optical chamber employed here. The usual internal perfusion solutions consisted of 350 mM sucrose, 20 mM HEPES, 300 mM K-glutamate, and 50 mM KF, adjusted to pH 7.3. For the experiments concerned with measurement of the speed of the response of di-6-ASPPS, the voltage-clamp circuitry was an improved version of that described by Bezanilla et al. (1982). The voltage amplifier settled in  $<1 \mu\text{s}$  and the clamp permitted compensation for at least the full value of the series resistance present. In these experiments, data could be acquired at 1.2  $\mu\text{s}$ /point, with a resolution of 12 bits. The dyes used in these experiments were synthesized according to a previously published procedure (Loew et al., 1979b). The dye structures are depicted in Fig. 1.

## RESULTS

### Response Spectra from Stained Squid Axon

The relative transmittance and excitation responses from a single axon stained with di-6-ASPPS are plotted for several wavelengths in Fig. 2. The relative transmittance change induced by a 50-mV hyperpolarization is determined by dividing the change in light intensity by the resting transmitted intensity at each wavelength after staining. The relative fluorescence change as a function of excitation wavelength was determined with emission at the maximum, 570 nm; the emission component at this wavelength was determined by employing a 470-nm excitation filter ( $\Delta\lambda = 0$ ) and this component was subtracted from the relative fluorescence response to obtain the values plotted in Fig. 2. As mentioned in the Methods section, the

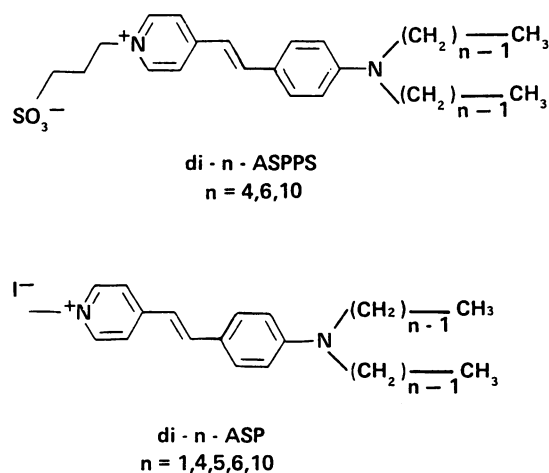


FIGURE 1 Structures of *p*-aminostyrylpyridinium probes are shown.

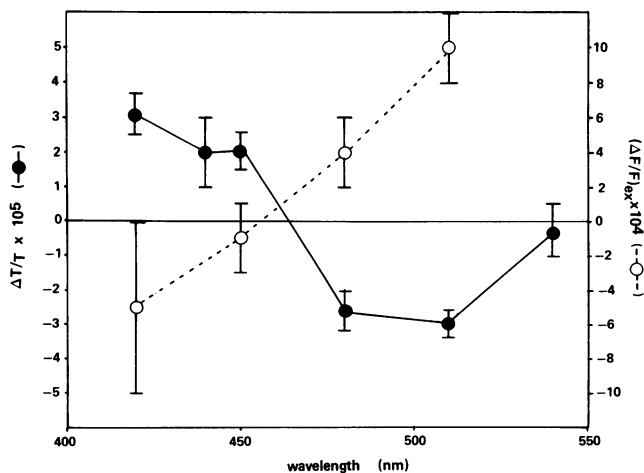


FIGURE 2 Relative transmittance and fluorescence excitation responses for di-6-ASPSS bound to squid axon. Voltage-clamp steps of  $-50$  mV were employed. Generally, 256 sweeps were accumulated to obtain the optical changes. The axon was stained with  $0.03$  mg/ml dye, for transmittance, or  $0.3$  mg/ml dye, for fluorescence, in deaerated seawater for  $10$  min. This dye solution was washed away before the data were acquired.

use of interference filters in the emission path severely limits the total fluorescence collected, thus reducing the signal-to-noise drastically (a  $550$ -nm cutoff filter passes  $40$  times more light than the  $570$ -nm interference filter). Only a limited number of points were obtained for  $(\Delta F/F)_{ex}$  and their precision is relatively poor. These data, however, are consistent with results from other axons so that several general conclusions about the excitation response may be reached. First, both the excitation and absorbance response spectra have the characteristic biphasic shapes and cross

zero near the maxima of the resting spectra as in the bilayer results (Loew and Simpson, 1981). However, while one might have expected the size of the relative excitation response  $(\Delta F/F)_{ex}$  to be similar in squid experiments and in hemispherical bilayer experiments, they are  $20$ – $40$  times lower in squid. A large part of this difference may be attributed to the contributions from the resting intensity of dye not specifically bound to the axon membrane. Gupta et al. (1981) have estimated that the background fluorescence from dye bound to the Schwann cells and connective tissue still present in the cleaned axon preparation is at least  $12$  times that of dye actually bound to the excitable membrane. Geometrical differences between the axon and the bilayer experiments must also be considered. Since the photoexcitation-induced charge displacement must be perpendicular to the membrane surface for a maximum electrochromic effect, and since the transition moments of these dyes are essentially parallel to these charge displacement vectors (Loew and Simpson, 1981), illumination of the effectively planar bottom of the hemispherical bilayer provides an ideal geometry for an optimal electrochromic response from both absorption and right-angle fluorescence. In the axon experiment, however, a cylindrical membrane is being sampled with its axis perpendicular to both the incident beam and the path of detected emission; this geometry severely diminishes contributions from dye chromophores that are oriented for optimal electrochromism. This geometry can also be modeled with the hemispherical bilayer by illuminating the side and middle. Indeed,  $\Delta F/F$  is lowered by a factor of  $\sim 4$  relative to that obtained from illumination of the bottom (Simpson, L., and L. M. Loew, unpublished results). The discrepancy in the sizes of the fluorescence changes in bilayer vs. axon can

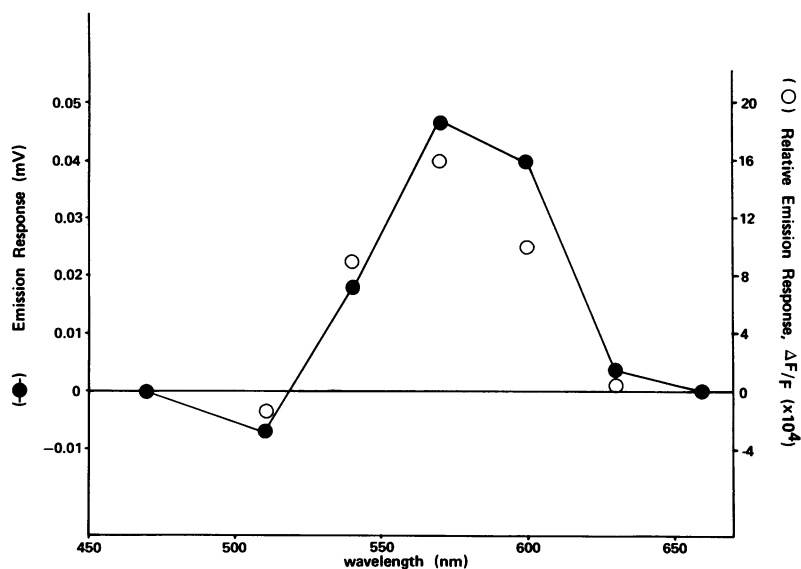


FIGURE 3 Relative and absolute (uncorrected) emission response spectra of squid axon stained with di-6-ASPSS. Voltage-clamp steps of  $-50$  mV and excitation at  $470$  nm. Generally, 256 sweeps were accumulated at each wavelength. The axon was stained with  $0.03$  mg/ml dye as in Fig. 2.

therefore be understood as the product of both of these factors that diminish the latter's relative response.

The wavelength dependence of the emission response from a di-6-ASPPS stained axon is shown in Fig. 3. Excitation was at 470 nm with a 10-nm bandpass interference filter; the emission filters were all 30-nm bandpass. A control experiment with a 470-nm emission filter indicated that light scattering does not significantly contribute to the response. The zero crossover point for the emission response is 520 nm—substantially blue shifted from the resting emission maximum and the crossover in the hemispherical bilayer experiment (560 and 540 nm, respectively; Loew, L. M., and L. Simpson, unpublished results). Also in contrast to the hemispherical bilayer results, the maximum fractional emission change is similar to the maximum relative excitation response. Although the data for the emission response on the axon are uncorrected, the photodiode has a relatively constant responsivity over this wavelength range and the emission interference filters have nearly uniform transmittance and bandpass specifications. Thus, the very asymmetrical biphasic shape of the response spectrum shown in Fig. 3 is probably real. It may result from a superposition of an electrochromic mechanism (which would lead to a simple biphasic spectrum) with a voltage-dependent change in quantum yield arising from an additional process.

#### Anomalous Behavior of Cationic Dyes on Squid Axon

On the hemispherical bilayer, the cationic ASP series of dyes (e.g., di-4-ASP [Fig. 1]) have the same response

characteristics as the zwitterionic ASPPS dyes (Loew and Simpson, 1981). In our initial experiments with the squid axon, however, the ASP dyes gave very small fluorescence changes that were opposite in direction compared with the responses from the ASPPS series. A possible explanation for this result is that the negative resting potential or differences in surface potentials can drive these dyes, with their partially delocalized positive charge, into the inside of the axon, resulting in a higher internal concentration than external concentration after the dye solution is washed away. Indeed, at the end of experiments, the voltage-clamp electrode from the axon interior was found to be stained with dye after an application of a member of the ASP series but not after any of the ASPPS dyes. Further evidence for this behavior was obtained by altering the usual protocol for dye application to the axon. It was found that if the response is measured while the axon is still bathed in a solution of di-4-ASP, the fluorescence change had the same polarity as that obtained with di-6-ASPPS. The biphasic wavelength dependence of transmittance signals appeared to be preserved, as well, although the signals were quite small at the low wavelengths. As the axon incubates in the dye solution over a period of 25 min, the fluorescence signal slowly decreases, consistent with the idea that internal dye results in a signal of opposite sign. Finally, when the extracellular dye is purged by rinsing the axon with fresh seawater, the fluorescence signal reappears with reversed polarity.

The transmitted light response of di-4-ASP was further examined using the horizontal axon apparatus that permitted internal perfusion with the dye. No significant transmittance response was observed upon application of dye to

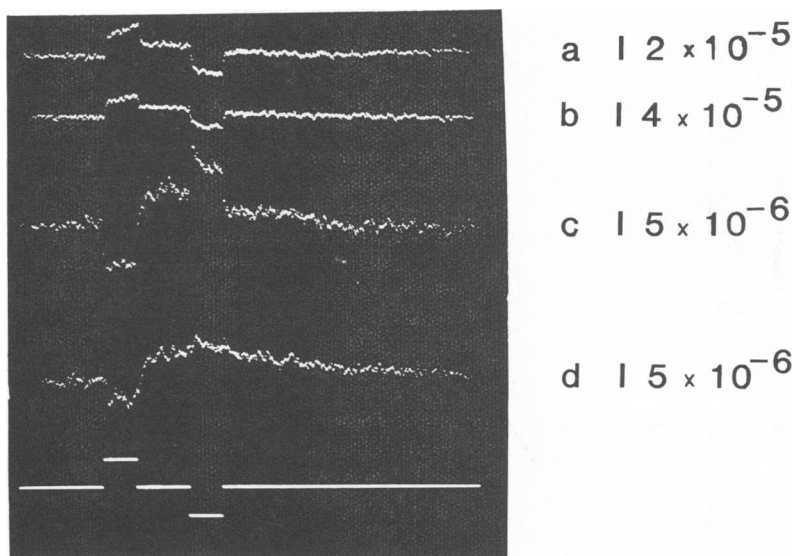


FIGURE 4 Kinetic traces of transmittance changes in response to voltage clamp pulses of 5-ms duration. A 50-mV depolarizing pulse is followed by a 50-mV hyperpolarizing pulse (*bottom* trace). The axon is incubated in 0.05 mg/ml di-4-ASP in seawater for 10 min after which the dye solution is replaced with fresh seawater. A holding potential of  $-50$  mV is maintained throughout. The bars indicate the magnitudes of the fractional transmittance changes at each wavelength. The displayed waveforms are accumulations of 512 sweeps: (a) 430 nm; (b) 440 nm; (c) 490 nm; (d) 510 nm.

the outside until the axon bathing solution was replaced with fresh seawater. Under these conditions (Fig. 4), a response is observed having polarity opposite to that obtained from zwitterionic dyes applied in the same way. These signals also have the appropriate wavelength dependence for dye bound to the interior surface of the membrane (i.e., opposite of ASPPS dyes applied outside). At longer wavelengths (Fig. 4 *d*), however, there is a slow component to the transmittance change that is superimposed on the normal fast response. Also, a more negative holding potential could increase the signal slightly and reduce the slow component (Fig. 5 *a*). This may reflect an increase in the amount of the positive dye driven to its binding sites on the inner membrane surface.

When the dye is applied directly to the inner surface of the membrane by perfusion (Figs. 5 *c-f*), much larger signals are obtained and the slow component is accentuated. Fig. 5 is a collection of experiments showing variations in the optical waveforms at 510 nm under various conditions. This wavelength is at the edge of the

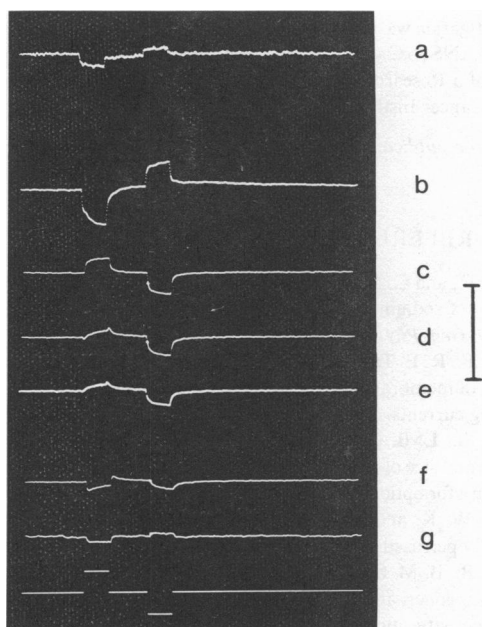


FIGURE 5 Kinetic traces at 510 nm under various conditions of dye application and holding potential. (a) Di-4-ASP applied outside as in Fig. 4; holding potential is  $-120$  mV. (b) Di-4-ASP applied outside as in Fig. 4; holding potential raised to  $-40$  mV. (c) A  $0.02$  mg/ml solution of di-4-ASP in perfusion buffer is allowed to perfuse  $10$  min. This trace was recorded during perfusion with the dye at a holding potential of  $-60$  mV. (d) Same axon and conditions as in *c* but after flushing with dye-free perfusion buffer. (e) Same as *d* but holding potential lowered to  $-120$  mV. (f) Same as *d* but holding potential raised to  $-20$  mV. (g) Axon perfused with  $0.02$  mg/ml di-4-ASPPS followed by flushing with dye-free buffer; holding potential is  $-60$  mV. A fractional transmittance change of  $2 \times 10^{-4}$  is indicated by the bar at the right. This applies to all the traces except *a* and *b* where the bar corresponds to  $5 \times 10^{-5}$ . The bottom trace shows the pair of  $50$  mV depolarizing and hyperpolarizing pulses as in Fig. 4. All of the displayed waveforms are accumulations of 512 sweeps.

absorption band ( $\lambda_{\max} = 465$  nm) and showed considerable variability. The method of application, dye concentration, and holding potential all affect the character of the di-4-ASP response; this sensitivity, as well as the superposition of fast and slow kinetic components in some of the traces, can only be explained in terms of several concurrent molecular mechanisms. A fast response with a biphasic wavelength dependence similar to that seen in Fig. 4 can be discerned under all of these conditions and is, in fact, dominant at the blue end of the spectrum. In a separate experiment shown in Fig. 5 *g*, di-4-ASPPS was applied to the axon interior by perfusion. In contrast to its cationic relative, this zwitterionic dye signal displays no slow component under any of the conditions of holding potential or method of application. In addition, the wavelength dependence for perfused di-4-ASPPS was simply biphasic, with a transmittance response spectrum opposite to that (Fig. 2) obtained with di-6-ASPPS applied externally.

### Time Course

While the hemispherical bilayer allowed more accurate determination of the spectral characteristics of the dye response, the axon apparatus permitted better time resolution in monitoring the optical signals. An experiment designed to characterize the response kinetics of the zwitterionic dye is shown in Fig. 6 and demonstrates that the absorption changes are essentially as fast as the potential step within the  $1.2$ - $\mu$ s resolution imposed by the electronics. This result is consistent with an electrochromic mechanism that must be essentially instantaneous. The first two points of both the on and off response appear to go in the

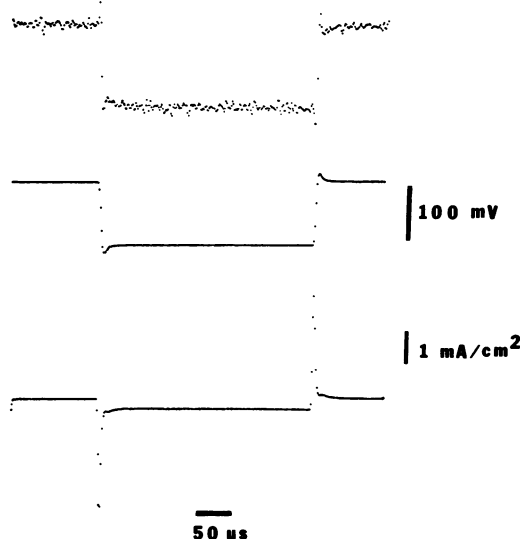


FIGURE 6 Response of di-6-ASPPS to a fast voltage-clamp pulse. The resolution of the experiment is  $1.2$   $\mu$ s. The hyperpolarizing pulse is of  $250$ - $\mu$ s duration and  $120$ -mV amplitude from a holding potential of  $-20$  mV. The dye was applied at  $0.02$  mg/ml as in Fig. 2. The seawater also contained  $200$  nM tetrodotoxin and  $1$  mM 3,4-diaminopyridine. The optical signal is an accumulation of  $6,000$  sweeps at  $520$  nm.

opposite direction. This very early component may represent a transient phase of the optical response or it may include a fast coupling artifact. The time resolution shown in Fig. 6 is substantially better than the resolution of 100  $\mu$ s previously possible with the hemispherical bilayer experiment (Loew et al., 1979a). However, several nonelectrochromic mechanisms could provide responses that are faster than this experimental limit. Indeed, using the same apparatus several other dyes displayed absorption responses not resolvable from the time course of the voltage change (Salzberg, B.M., F. Bezanilla, and A. L. Obaid, manuscript in preparation); these include dyes whose spectral shifts suggest nonelectrochromic mechanisms. Had the response of di-6-ASPPS been slower, electrochromism would have been eliminated from consideration as a primary contributing mechanism.

## DISCUSSION

The responses of di-6-ASPPS and di-4-ASP on the squid axon are different in many respects from the results obtained in hemispherical bilayer experiments. Several conclusions may be drawn. (a) The negative fixed charge of an appended sulfonate group is necessary to prevent slow penetration of the axon by the positive ASP chromophore under the influence of the axon resting potential. In the hemispherical bilayer, neither ASPPS nor ASP probes flip across the membrane even in the presence of chemical or electrical potentials to drive them. In general, one would expect the neutral zwitterionic probes to be less prone to complicating chemical or pharmacological effects; in fact, one feature of electrochromism is that it does not discriminate against neutral probes. (b) In rationalizing the magnitude of the probe response, account must be taken of the background fluorescence from dye associated with nonexcitable membrane. Also, the inherent anisotropy of fluorescence requires that the membrane geometry must be considered. (c) The emission response is most likely to display nonelectrochromic features. This is simply because the inherently weak electrochromism in emission may be overwhelmed by competing mechanisms. By choosing a null point in the emission response or by correcting for it, one can still use the fractional fluorescence change contributed by the shift of the excitation spectrum as a more reliable measure of an electrochromic response.

The fluorescence response is composed of three components: shifts of the excitation and emission spectra and a change in emission quantum yield. The latter is of unknown origin but the spectral shifts have met all the experimental criteria for electrochromism on either the squid axon or the hemispherical bilayer.

In practice, voltage-sensitive dyes are used by choosing a cutoff filter to collect maximal fluorescence and an excitation wavelength to maximize the potentiometric response. For di-6-ASPPS this leads to a  $\Delta F/F$  of  $10^{-3}$  (cf. Gupta et al., 1981). Alternatively, a complete analysis of the wave-

length dependence of the voltage-dependent response, as illustrated in Figs. 2 and 3, affords a guide to the best choice of wavelengths for an optimal fractional fluorescence change; a factor of 2–3 improvement in  $\Delta F/F$  might be expected for  $\lambda_{ex} \sim 500$  nm and  $\lambda_{em} \sim 570$  nm, at the expense perhaps of the signal-to-noise ratio of the measurement. For either of these two experimental designs, electrochromism appears to underlie the major component of the voltage-sensitive fluorescence signal.

Our results on the squid axon can be rationalized on the basis of the model membrane experiments. Despite this, however, it appears that some of our earlier hopes that electrochromism would lead to responses that could be calibrated on a model membrane with simple generalization to natural systems, seem now to have been unrealistic. While one can always apply the same thorough spectroscopic analysis presented above for the squid axon, we suspect it would be simpler to devise a direct calibration protocol for each new application.

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