Voltage Sensitivity of the Fluorescent Probe RH421 in a Model Membrane System

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ABSTRACT The voltage sensitivity of the fluorescent styrylpyridinium dye RH421 has been investigated in dimyristoylphosphatidylcholine vesicles by inducing an intramembrane electric field through the binding of the hydrophobic ion tetraphenylborate (TPB). To assess the probability of electrochromic and solvatochromic mechanisms for the dye response, the ground-state dipole moment of the dye in chloroform solution was determined from dielectric constant measurements to be 12 (±1) Debye, and the change in dipole moment upon excitation was calculated from measurements of the Stokes shift in solvents of varying polarity to be 25 (±11) Debye. As well as causing absorbance and fluorescence changes of membrane-bound dye, the TPB-induced electrical field was found to reduce significantly the pK_a of the dye. The pH at which experiments are carried out is, thus, an important factor in determining the amplitude of the voltage-induced absorbance and fluorescence changes. The observed absorbance changes induced by the field are inconsistent with a pure electrochromic mechanism. A reorientation/solvatochromic mechanism, whereby the electrical field reorients the dye molecules so that they experience a change in polarity of their lipid environment is likely to make a significant contribution to both the spectral changes and to the field effect on the acid-base properties of the dye.

INTRODUCTION

The voltage-sensitive styryl dye RH421 (see Fig. 1) and dyes of related structure are increasingly finding application in biochemical and biophysical research for the visualization of voltage transients in membrane preparations (Grinvald et al., 1988; Fromherz and Müller, 1993; Bühler et al., 1991). It is often stated that the dyes respond to changes in membrane potential by an electrochromic mechanism (see, e.g., Bammel et al., 1990; Pratap and Robinson, 1993; Haughland, 1992). The basic requirement for an electrochromic mechanism is that the dye undergoes a significant change in its electronic distribution upon absorption of a photon, so that the molecule has significantly different dipole moments in its ground and excited states. If the dye molecule has a fixed orientation within the membrane, a transmembrane electrical field will cause different degrees of stabilization or destabilization of the ground and excited states, resulting in a shift of the absorbance spectrum (Waggoner and Grinvald, 1977; Loew et al., 1978; Liptay, 1969). In the case of the styryl dyes RH421 and RH160, however, several authors have presented results that cannot be explained by electrochromism (Fluhler et al., 1985; Clarke et al., 1992; Nagel et al., 1991). Therefore, although for some of the styryl dyes electrochromism may be the major cause of the voltage-induced spectral changes, at least for the dyes RH421 and RH160 another mechanism must be playing an important role.

So far the only study of the response time of RH421 to membrane potential changes is that of Müller et al. (1986), who used the dye as a probe for the action potential in heart tissue. They found that the onset of the action potential could be detected by the dye within 0.5 ms. Chien and Pine (1991) likewise reported for a dye of very similar structure a response time in the sub-ms region. Loew et al. (1985) investigated the response time of a related aminostyrylpyridinium dye by means of voltage clamp pulses on the giant squid axon. Their measurements showed that the dye could react to the voltage pulses within 1.2 μs. It is possible that the true response time of the dyes is much faster, because the measurements cited above were limited by the time resolution of the apparatus. For an electrochromic mechanism one would expect a response time in the sub-ns region. Therefore, the measured response times are not inconsistent with electrochromism. Such response times could, however, also arise from molecular motion of the dye within the membrane, in which case a response time in the ns–ms range might also be expected.

In cell or organelle membranes it is possible that a direct interaction of the styryl dyes with membrane proteins may contribute to the spectral changes. In this case the dyes may not be acting as true indicators of membrane potential, but rather as indicators of protein conformational changes. Direct interactions of the dye RH160 with the Na^+,K^+-ATPase (Klodos and Forbush, 1988) and the H^+-ATPase of *Neurospora* (Nagel et al., 1991) have been suggested. The interaction of RH421 with proteins will be discussed by us in a future publication. Here we present an investigation of the voltage sensitivity of RH421 in pure lipid membranes. Intramembrane electrical fields have been induced by the binding of the hydrophobic ion tetraphenylborate (TPB), a method first applied by Bühler et al. (1991).

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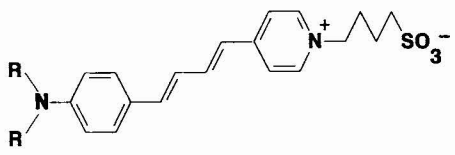

R = $CH_2CH_2CH_2CH_2CH_3$

FIGURE 1 Structure of RH421.

MATERIALS AND METHODS

N-(4-Sulfobutyl)-4-(4-(*p*-(dipentylamino)phenyl)butadienyl)-pyridinium inner salt (RH421) was obtained from Molecular Probes (Eugene, OR) and was used without further purification. An ethanolic solution was checked for the presence of fluorescent impurities by recording the fluorescence emission spectrum at varying excitation wavelengths. A single peak was observed with a maximum at 695 nm, which was independent of the excitation wavelength, indicating a single dye species (Zouni et al., 1994).

Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids (Alabaster, AL). DMPC unilamellar vesicles were prepared by the ethanol injection method described in detail elsewhere (Zouni et al., 1993, 1994). The final vesicle suspension contained no detectable trace of ethanol, i.e., [ethanol] $\leq$ 10 μM, according to a nicotinamide adenine dinucleotide/alcohol dehydrogenase enzymatic assay (Boehringer Mannheim, Indianapolis, IN). Dialysis tubing was purchased from Medicell International (London, UK). The phospholipid content of the vesicle suspensions was determined by the phospholipid B test from Wako (Neuss, Germany).

The majority of measurements with the vesicles were performed in a buffer containing 30 mM Tris, 1 mM EDTA, and 150 mM NaCl. The pH of the buffer was adjusted to 7.2 with HCl. All solutions were prepared using triply distilled water. The origins of the various reagents used were as follows: Tris-[(hydroxymethyl)amino]methane (99.9%, Sigma Chemical Co., St. Louis, MO), EDTA (99%, Sigma Chemical Co.), NaCl (analytical grade, Merck, Darmstadt, Germany), HCl (0.1 M Titrisol solution, Merck), ethanol (analytical grade, Merck), chloroform (analytical grade, Merck), sodium TPB (analytical grade, Merck), glycine (99%, Merck), sodium acetate trihydrate (99+%, Sigma Chemical Co.), acetic acid (2.0 *N* solution, Sigma Chemical Co.), dichloromethane (analytical grade, Merck), 1-octanol (>99.5%, Fluka, Buchs, Switzerland), 1-hexanol (>99%, Fluka), acetone (analytical grade, Merck), methanol (99.8%, Merck), acetonitrile (analytical grade, Merck) and 1-propanol (>99%, Fluka).

Absorbance measurements were performed with a Shimadzu (Kyoto, Japan) UV-2100 UV-visible recording spectrophotometer using a bandwidth of 5 nm. Steady-state fluorescence measurements were recorded with a Shimadzu RF-5000 recording spectrofluorophotometer. To minimize contributions from scattering of the exciting light and higher-order wavelengths a glass cutoff filter was used in front of the excitation monochromator. The temperature of the cuvette holders was thermostatically controlled. Integration of the fluorescence emission intensity was achieved by weighing the chart paper under the curve.

Measurements of the pH dependence of the dye absorbance were carried out using a series of 0.1 M acetic acid/sodium acetate buffers and a series of 0.05 M glycine buffers of varying pH (Dawson et al., 1986). NaCl (1.0 M) was added to the vesicle dialysis medium and to the buffers in order to prevent variations in the ionic strength on either side of the membrane. The determination of the pK_a values was carried out by fitting the data obtained to the Henderson-Hasselbalch equation (Stryer, 1988) using the commercially available nonlinear least squares program ENZFITTER. The program was purchased from Biosoft (Cambridge, UK) and was run on an IBM-AT/386 compatible personal computer (mey-soft, Berlin, Germany).

For all spectral measurements in the presence of vesicles 5 μl of an ethanolic dye solution was added to a quartz cuvette containing 1 ml of aqueous solvent. Small volumes ($\leq$10 μl) of sodium TPB were also added from stock solutions in ethanol. For absorbance measurements the effect of the small volume of ethanol added on the spectrum of membrane-bound dye was checked in separate control experiments and found to be negligible under the experimental conditions used. In the case of the fluorescence titrations with dye and TPB both were added from a series of ethanolic stock solutions. The final solutions measured thus contained a small and constant percentage of 1.0% ethanol. Above the gel-liquid crystalline phase transition temperature such a small concentration of ethanol is not expected to perturb the membrane structure significantly (Jain and Wu, 1977; Holzwarth et al., 1985).

The association of TPB to the lipid vesicles was analyzed according to a binding model. The apparent microscopic binding constant, K, is defined according to the mass action law by

$$K = \frac{c_{XL}^*}{(nc_L^* - c_{XL}^*)(c_X^* - c_{XL}^*)} \quad (1)$$

where c_L^*, c_X^*, and c_{XL}^* represent the total concentrations of lipid, hydrophobic ions (free and bound) and bound hydrophobic ions, respectively. n is the number of hydrophobic ion binding sites per lipid molecule. Rearranging Eq. 1, the concentration of bound hydrophobic ions, c_{XL}^*, can be calculated as follows.

$$c_{XL}^* = \frac{1 + K(c_X^* + nc_L^*) - \{[1 + K(c_X^* + nc_L^*)]^2 - 4K^2nc_L^*c_X^*\}^{1/2}}{2K} \quad (2)$$

The fluorescence change, ΔF, of the dye in the membrane caused by the addition of hydrophobic ions is assumed to be related to the concentration of bound hydrophobic ions by

$$\frac{\Delta F}{F_o} = f_{XL}c_{XL}^* \quad (3)$$

where F_o is the fluorescence before the addition of hydrophobic ions and f_{XL} is the value of $\Delta F/F_o$ per molar concentration of bound hydrophobic ions. Substituting Eq. 2 into Eq. 3 for c_{XL}^* and fitting the resulting equation to the fluorescence titration data of $\Delta F/F_o$ as a function of the total TPB concentration (c_X^*) allowed values of K and n to be determined. The nonlinear least-squares fit was carried out as for the pH titration data using the ENZFITTER program.

The dielectric constants of a series of RH421 solutions of varying concentrations in chloroform were measured at 20.2°C using a dipole meter, type DM01, from Wissenschaftlich-Technische Werkstätten (Weilheim, Oberbayern, Germany). The refractive indices of the solutions were determined using an Abbe refractometer from Zeiss (Oberkochen, Germany). Chloroform was chosen as the solvent on the basis of its relatively low polarity and because of the relatively high solubility of the dye in this solvent.

RESULTS

Dipole moment of ground-state RH421

The results of measurements of the dielectric constant, ϵ_{12}, of dye solutions in chloroform as a function of dye concentration are shown in Fig. 2. According to the method of Guggenheim (1949) and Smith (1950) for dilute solutions of polar solutes the dipole moment, μ, of the solute is given by Moll and Lippert (1954):

$$\mu^2 = \frac{27kT\epsilon_o}{N_A(\epsilon_1 + 2)^2} \lim_{c_2=0} \left\{ \frac{d\epsilon_{12}}{dc_2} - \frac{dn_{12}^2}{dc_2} \right\} \quad (4)$$

where k, N_A, and ϵ_o represent Boltzmann's constant, Avogadro's constant, and the permittivity of a vacuum, respectively. T is the absolute temperature and ϵ_1 is the dielectric constant of the solvent. n_{12} represents the refractive index of the solution and c_2 is the solute concentration in mol m^{-3}. Over the concentration

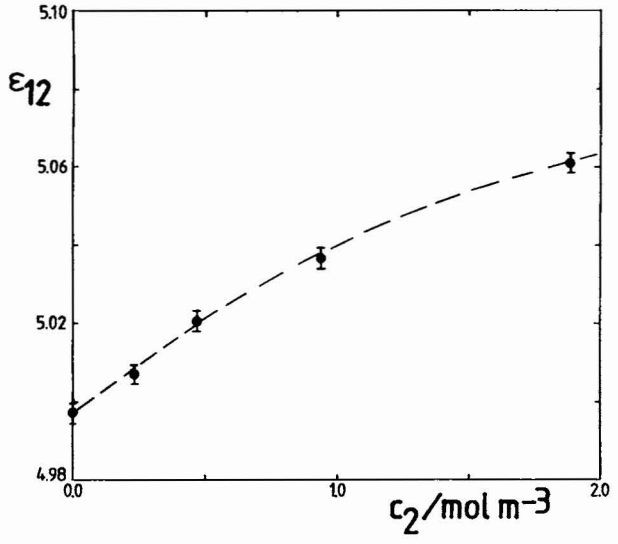

FIGURE 2 Dielectric constant, ϵ_{12}, of RH421 solutions in chloroform as a function of the dye concentration, c_2.

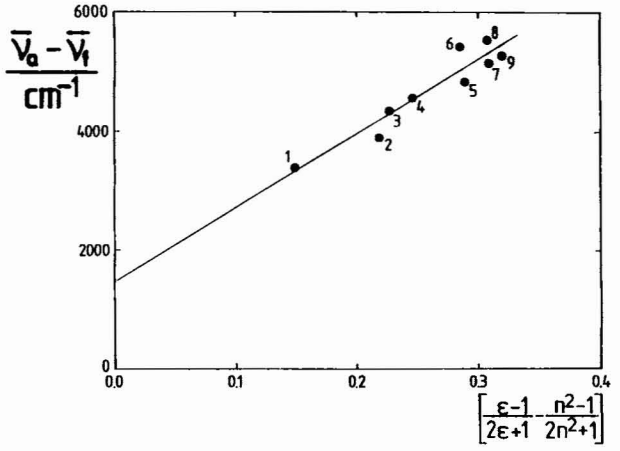

FIGURE 3 Lippert plot of the Stokes shift, $\bar{\nu}_a - \bar{\nu}_f$, of RH421 as a function of the solvent orientation polarizability, $[(\epsilon - 1)/(2\epsilon + 1) - (n^2 - 1)/(2n^2 + 1)]$. The points refer to the following solvents: (1)chloroform, (2) dichloromethane, (3) octanol, (4) hexanol, (5) ethanol, (6) acetone, (7) methanol, (8) acetonitrile, and (9) water.

range of dye used for the measurements of ϵ_{12} no significant variation in n_{12} was observed. Therefore, the final term in brackets in Eq. 4 can be neglected.

From the initial slope and intercept, respectively, of Fig. 2 $\lim_{c2=0}(d\epsilon_{12}/dc_2)$ and ϵ_1 are found to be 0.050 ($\pm$0.010) mol^{3}mol^{-1} and 4.997 ($\pm$0.002). Substitution of these values into Eq. 4 yields a dipole moment of RH421 of 4.0 ($\pm$0.4) $\times$ 10^{-29} C m, which is equivalent to 12 ($\pm$1) Debye.

The downward curvature of the plot of ϵ_{12} against c_2 at high dye concentrations is almost certainly due to dye dimerization. Measurements of the dye absorbance spectrum have shown that a blue shift occurs at mM concentrations and above. From the deviation from Beer's law the dimerization constant of the dye in chloroform has been calculated at 460 and 610 nm to be ~400 M^{-1} (data not shown). This is five orders of magnitude less than the value observed in water (Clarke et al., 1992). Based upon the above value of the dimerization constant it can be calculated that at dye concentrations <0.5 mM more than ~80% is present in the monomer form.

It is possible that the value of the dipole moment measured may depend on the solvent used. The dye possesses a flexible alkyl chain between the positively charged pyridinium moeity and the negatively charged sulfonate group. In a more polar solvent the attraction between these two parts of the molecule would be weakened because of screening from the solvent. This may lead to a more extended conformation of the molecule and hence a higher value of the ground-state dipole moment.

Dipole moment change upon excitation

A common requirement for both electrochromism and solvatochromism is a large charge shift upon electronic excitation. To determine the dipole moment change, $\Delta\mu$, caused by the excitation, the Stokes shift, $(\bar{\nu}_a - \bar{\nu}_f)$, i.e, the frequency difference between the maxima of the absorbance spectrum and fluorescence emission spectrum, has been measured in a range of organic solvents (see Fig. 3 and Table 1). In solvents such as water, in which the dye aggregates strongly (Clarke et al., 1992), the values of the Stokes shifts refer to dilute solutions so that frequency changes due to intermolecular interactions can be neglected.

In the 1950s Lippert (1955, 1957) developed the following relationship between the dipole moment change and the orientation polarizability, $[(\epsilon - 1)/(2\epsilon + 1)] - [(n^2 - 1)/(2n^2 + 1)]$, of the solvent:

$$10^2(\bar{\nu}_a^{sol} - \bar{\nu}_f^{sol}) = 10^2(\bar{\nu}_a^{gas} - \bar{\nu}_f^{gas}) + \frac{2}{4\pi\epsilon_o hca_w^3}\left[\frac{\epsilon - 1}{2\epsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}\right](\Delta\mu)^2 \quad (5)$$

where $(\bar{\nu}_a^{sol} - \bar{\nu}_f^{sol})$ and $(\bar{\nu}_a^{gas} - \bar{\nu}_f^{gas})$ represent the Stokes shifts in cm^{-1} in the solution and in the gas phase, respectively. The factor 10^2 has been introduced to convert the wavenumber from cm^{-1} to m^{-1}. h and c are the Planck's constant and the speed of

TABLE 1 Wavelengths of the absorbance and fluorescence emission maxima and the Stokes shift of RH421 in various solvents at 20°C and in a DMPC membrane at 30°C

Solvent	Orientation polarizability	λ_{Abs}^{max} (nm)	λ_{Em}^{max} (nm)	$\bar{\nu}_A$-$\bar{\nu}_F$ (cm^{-1})
Chloroform	0.149	549	674	3378
Dichloromethane	0.218	545	692	3898
Octanol	0.226	522	676	4364
Hexanol	0.245	522	686	4580
Ethanol	0.289	521	698	4867
Acetone	0.285	502	691	5448
Methanol	0.309	515	702	5172
Acetonitrile	0.308	508	708	5561
Water	0.320	485	652	5281
DMPC membrane		480	646–665	5350–5800
DMPC membrane (+50 μM TPB)		520	658–665	4030–4200

light in a vacuum, respectively. a_w represents the radius of the solvent cavity in which the fluorophore resides. For RH421 a_w was estimated from measurements on Stuart-Briegleb space-filling models to have a value of 8 ($\pm$2) $\times$ 10^{-10} m.

Implicit in the derivation of the Lippert equation is the assumption that solvent relaxation processes have reached equilibrium before emission. In the case of the solvents presented in Fig. 3 at room temperature this assumption is justified. Time-resolved fluorescence measurements of RH421 in a range of solvents have shown that solvent relaxation occurs in less than 0.1 ns, whereas the fluorescent lifetime of the dye is of the order of 1 ns (Visser et al., 1994).

A Lippert plot of the Stokes shift in solution against the orientation polarizability of the solvent (see Fig. 3) allows $\Delta\mu$ to be calculated from the slope and the Stokes shift in a vacuum from the intercept with the ordinate axis. For RH421 one obtains values of 25 ($\pm$11) Debye for $\Delta\mu$ and 1460 ($\pm$420) cm^{-1} for the Stokes shift in a vacuum. The large error in the value of $\Delta\mu$ is due to the estimate of a_w, which is present in the third power in the Lippert equation. In comparison with other dyes (Lakowicz, 1983) the value of $\Delta\mu$ is very high, which is advantageous for both electrochromism and solvatochromism.

For the aminostyrylpyridinium chromophore with a single double bond between the anilino and pyridinium moeities Loew and Simpson (1981) have estimated a value for $\Delta\mu$ of 16 Debye, which is comparable to the value calculated here for RH421. It should be noted that RH421 is a somewhat longer molecule, having two double bonds separating the anilino and pyridinium groups. The estimate of Loew and Simpson (1981) was made from the magnitude of the voltage-induced fluorescence change of dye bound to hemispherical lipid bilayers assuming the entire fluorescence change to be due to an electrochromic mechanism.

When bound to DMPC vesicles at a temperature of 30°C, RH421 exhibits a Stokes shift in the range 5350–5800 cm^{-1}. The exact value is dependent on the excitation wavelength used for the fluorescence measurement. According to the Lippert plot (see Fig. 3) such a value of the Stokes shift corresponds to an orientation polarizability comparable to water. The λ_{max} in the absorbance spectrum is also very close to that observed in water (see Table 1). As pointed out by Loew et al. (1979b) and by Fromherz and Schenk (1994), the aminostyrylpyridinium chromophore may undergo an anisotropic solvation within the membrane if the long axis of the dye lies perpendicular to the membrane surface. The similarity of the Stokes shift and λ_{max} values to those in water, therefore, suggests that the ground state of RH421 is stabilized by interaction of the positively charged pyridinium group with the polar headgroup region of the membrane. This result is in agreement with previously published measurements of the local dielectric constant based on the pK_a shift of the dye on binding to the membrane (Zouni et al., 1994).

Electrical field-induced absorbance and fluorescence changes

To obtain information about the voltage-sensitive mechanism of RH421 a simple model system is required, so that strong electrical fields can be produced within the membrane. A suitable system is a lipid vesicle with bound hydrophobic ions (Bühler et al., 1991), e.g., TPB. Hydrophobic ions bind a short distance from the water-membrane interface inside the membrane phase. Hence they produce strong electrical fields within the membrane, to which the dye can react. In Fig. 4 the absorbance change of RH421 produced by the binding of TPB is shown. Similar effects also occur on binding of another negatively charged hydrophobic ion, namely pentabromophenolate (data not shown). At a suitable excitation wavelength the fluorescence changes caused by the binding of TPB are even more distinct than the absorbance changes (see Fig. 5).

Maximal fluorescence changes after the addition of TPB are observed when the membrane is saturated with TPB (see Fig. 5 *A*). From the binding curve the apparent binding constant, *K*, and the number of binding sites/lipid molecule, *n*, have been calculated according to the method described under Materials and Methods to be 7 ($\pm$1) $\times$ 10^4 M^{-1} and 0.09 ($\pm$0.03) sites/lipid molecule, respectively. The value of *K* is an apparent value ignoring the effects of electrostatic repulsion due to buildup of a boundary potential as more TPB binds. Accordingly, the true binding constant would be expected to be somewhat higher. Taking into account electrostatic repulsion Benz (1988) has reported a value of 34 $\times$ 10^4 M^{-1} for the interaction of TPB with black lipid membranes made of dioleoyl phosphatidylcholine at 25°C. Smejtek and Wang (1990) have reported a true binding constant of 59 ($\pm$14) $\times$ 10^4 M^{-1} for the binding of TPB to dipalmitoyl phosphatidylcholine vesicles at 42°C.

The value of *n* of 0.09 ($\pm$0.03) sites/lipid molecule corresponds to a surface concentration at saturation of 1.2

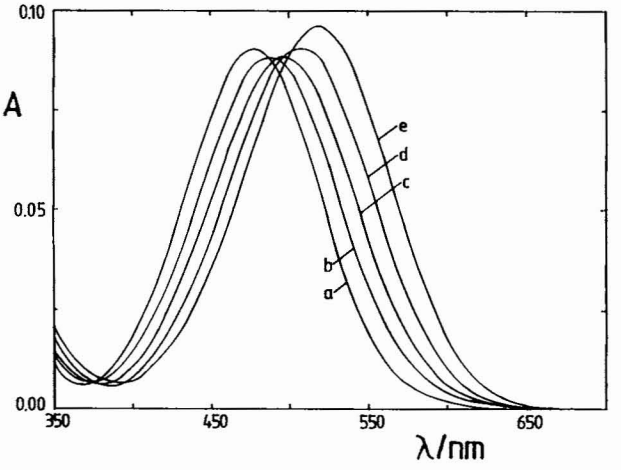

FIGURE 4 Absorbance spectra of 3.7 μM RH421 in the presence of 200 μM of DMPC in the form of unilamellar vesicles as a function of the TPB concentration: (*a*) 0, (*b*) 5 μM, (*c*) 10 μM, (*d*) 20 μM, and (*e*) 50 μM. T = 30°C, pH 7.2.

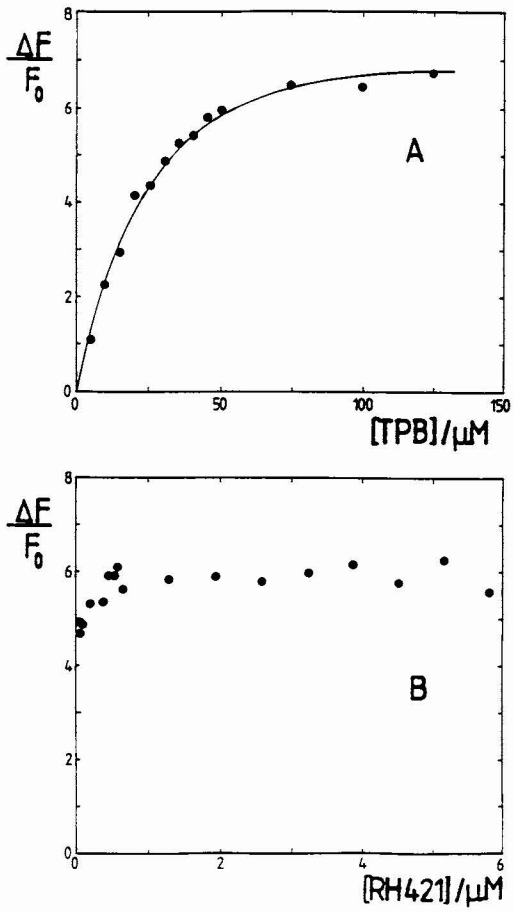

FIGURE 5 (*A*) Fluorescence change of 3.7 μM RH421 in the presence of 154 μM DMPC in the form of unilamellar vesicles after the addition of TPB as a function of the TPB concentration; λ_{ex} = 550 nm (+ OG530 cutoff filter), λ_{em} = 660 nm (+ RG645 cutoff filter), bandwidth = 3 nm, T = 30°C, pH 7.2. (*B*) Fluorescence changes after the addition of 50 μM TPB in the presence of 154 μM DMPC vesicles as a function of the RH421 concentration. All other experimental conditions are as for (*A*). $\Delta F/F_0$ represents the normalized fluorescence change, whereby F_0 is the fluorescence before the addition of TPB.

(±0.4) × 10^{17} ions per m^2 (see Appendix A). Smejtek and Wang (1990) have reported an adsorption site area of 4.1 nm^2 for the binding of TPB to dipalmitoyl phosphatidylcholine vesicles at 42°C. This corresponds to a surface concentration at saturation of 2.4 × 10^{17} ions/m^2. Flewelling and Hubbell (1986a) have reported a saturating surface concentration of 5 × 10^{16} ions/m^2 for the binding of TPB to planar phospholipid bilayers. The value of the saturating surface concentration determined here, thus, lies approximately midway between the value of Smejtek and Wang (1990) and that of Flewelling and Hubbell (1986a).

The magnitude of the fluorescence change induced by TPB was found to be almost completely independent of the dye concentration (see Fig. 5 *B*). Accordingly, a dye aggregation or disaggregation can only have a minor contribution to the voltage-sensitive response mechanism, if at all.

A comparison of the absorbance and fluorescence change induced by TPB allows the determination of whether the fluorescence response of the dye is due to an effect on the dye in the ground state or in the excited state. The integrated fluorescence emission spectra of dye-vesicle suspensions with and without TPB are related to the corresponding absorbance values according to Eq. 6 (Parker, 1968).

$$\frac{\int F_{TPB}\, d\lambda_{em}}{\int F_0\, d\lambda_{em}} = \frac{(1 - 10^{-A_{TPB}})}{(1 - 10^{-A_0})} \frac{q_{TPB}}{q_0}. \quad (6)$$

F_{TPB} and F_0 are the fluorescence intensities in arbitrary units of dye with and without TPB, respectively. A_{TPB} and A_0 are the corresponding absorbance values, and the quantum yields are represented by q_{TPB} and q_0. From experimental measurements of the integrated fluorescence intensities and the absorbance values with and without TPB, Eq. 6 allows the ratio q_{TPB}/q_0 to be determined. The experiments could not be extended to excitation wavelengths >520 nm because of the overlap of the exciting light with the emission spectrum. At excitation wavelengths >450 nm it was found that TPB induces an increase in the dye quantum yield, whereas at excitation wavelengths <450 nm a significant decrease is observed. The largest TPB-induced fluorescence changes are observed on excitation on the long-wavelength side of the absorbance spectrum. At 520 nm, e.g., a 176% increase in the integrated fluorescence intensity was observed. The change in absorbance, however, only predicts a 119% increase in fluorescence. Therefore, at 520 nm excitation ~67% of the observed change in fluorescence intensity can be explained by the absorbance change, i.e., an effect on the ground state of the dye, whereas 33% is due to an effect of the field on the quantum yield, i.e., an effect on an excited state reaction of the dye.

To elucidate the mechanism of the effect of electrical field on the excited state of RH421 time-resolved fluorescence measurements are necessary. To explain the electrical field effect on the absorbance spectrum one can imagine two possible mechanisms. The first is an electrochromic mechanism whereby the electrical field causes different degrees of stabilization or destabilization of the ground and excited states. The second is a reorientation/solvatochromic mechanism whereby the field causes an alignment of the dye molecules according to their dipole moments and an absorption change due to the change in polarity of the lipid environment. On the basis of the observed absorbance changes, however, (see Fig. 4) a pure electrochromic mechanism for the voltage dependence can be ruled out. For an electrochromic mechanism of a dye with a large permanent transition moment one would merely expect a shift of the absorbance spectrum with increasing TPB concentration (Liptay, 1969; Waggoner and Grinvald, 1977). In fact one finds both spectral shifts as well as intensity changes. As the TPB concentration is increased first a decrease in absorbance is observed. Afterward, at higher TPB concentrations, an increase in absorbance is observed. Such spectral changes are more consistent with the perturbation of an equilibrium of dye between different sites or orientations within the membrane, in accordance with a reorientation/solvatochromic mechanism.

Absorbance increases (hyperchromism) are also observed on transfer of the dye from methanolic solution to alcohols of longer chain length, whereby a maximum absorbance was found in propanol (see Fig. 6). The TPB-induced hyperchromism in the lipid membrane can, therefore, be explained by a reorientation of the dye in the direction of the membrane interior where it experiences a reduced polarity. The very low absorbance of the dye in aqueous solution is due to the fact that the dye aggregates strongly in water (Clarke et al., 1992; Zouni et al., 1994).

To rule out the possibility of a direct chemical interaction of TPB with the dye, the effect of TPB on the absorbance spectrum of the dye has been measured in aqueous and in ethanolic solution in the absence of vesicles. In aqueous solution TPB causes a significant decrease in absorbance, which is most likely due to the association of TPB with dye aggregates. In ethanol no effect of TPB on the absorbance spectrum of RH421 could be detected.

Effect of TPB on the acid-base properties of RH421

A further interesting influence of TPB on the dye RH421 is that it reduces the pK_a value of membrane-bound dye from 3.1 ($\pm$0.1) in the absence of TPB to a value of 2.1 ($\pm$0.1) in the presence of 50 μM TPB (see Fig. 7). Apparently the protonation of the dye is more difficult in the presence of TPB. From electrostatic considerations alone one would have expected the opposite effect. The negatively charged TPB ions should attract H^+ ions from the aqueous solution so that protonation of the dye would occur at lower values of the bulk H^+ concentration. At the ionic strength of 1.0 M used for the experiment, however, the electrostatic forces are completely screened by the electrolyte (Holzwarth and Jürgensen, 1974; Clarke, 1993).

Two possible sources of the reduction in pK_a could be acting. The first possible explanation is a change in the electronic distribution along the dye molecule due to the TPB-induced field. According to quantum mechanical calculations of Loew et al. (1978, 1979a) the excitation of the dye is accompanied by a shift of positive charge in the direction of the amino nitrogen. From the wavelengths of the absorbance maxima in water for the protonated (370 nm) and deprotonated (490 nm) forms of the dye (Clarke et al., 1992) the theoretical pK_a in the excited state can be calculated according to the theory of the Förster cycle (Förster, 1950) to be approximately -9. Considering the assumptions of the basic Förster theory it is possible that the true excited-state pK_a may have a different value. Nevertheless, the calculation indicates that shift of positive charge toward the amino nitrogen should significantly increase the acidity of the dye. It is entirely possible that TPB also causes an electronic redistribution, so that a positive charge shift in the direction of the amino nitrogen occurs. The consequence would, therefore, be a reduction in pK_a, as experimentally observed.

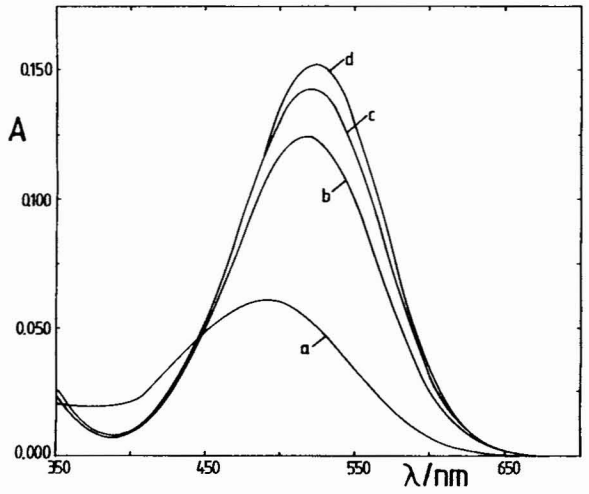

FIGURE 6 Absorbance spectra of 3.7 μM RH421 in (*a*) water, (*b*) methanol, (*c*) ethanol, and (*d*) propanol. T = 30°C, bandwidth = 5 nm.

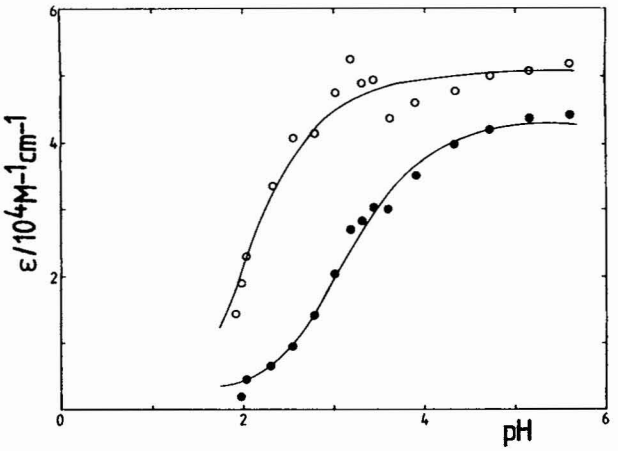

FIGURE 7 Variation of the molar absorptivity, ϵ, of RH421 at 500 nm in the presence of 500 μM of DMPC in the form of unilamellar vesicles and 1.0 M NaCl as a function of pH (●) without TPB and (○) after the addition of 50 μM of TPB. The solid curves represent nonlinear fits of the data to the Henderson-Hasselbalch equation. [RH421] = 3.7 μM, bandwidth = 5 nm, T = 30°C.

The second possible explanation for the reduction in pK_a in the presence of TPB is a field-induced reorientation within the membrane. If the dye is drawn further into the membrane by the induced field, as suggested on the basis of the absorbance changes, the polarity of its environment would be reduced, so that the protonation would become more difficult and the pK_a would decrease. It is informative to estimate how large the change in dielectric constant would have to be to cause the observed decrease in pK_a. The pK_a shift caused by the binding of TPB can be related to the change in local dielectric constant by the following equation:

$$pK_a^{TPB} - pK_a^{o} = -\frac{1}{2.303RT}\left[FU_b + \frac{e_o^2 N_A}{8\pi\epsilon_o r}\left(\frac{1}{\epsilon_m^{TPB}} - \frac{1}{\epsilon_m^{o}}\right)\right] \quad (7)$$

where pK_a^{TPB} and pK_a^{o} are the values of the pK_a in the membrane in the presence and absence of TPB, respectively. U_b

is the electrical boundary potential in the membrane produced by the binding of TPB. ϵ_m^{TPB} and ϵ_m^o represent the local dielectric constant experienced by the dye in the presence and absence of TPB, and r is the radius of an H_3O^+ ion. The value of r has been estimated to be 0.17×10^{-9} m (Cevc and Marsh, 1987). The derivation of Eq. 7 is described in detail elsewhere (Zouni et al., 1994). It is based upon the difference in electrostatic and Born energy contributions of H_3O^+ ions within the membrane with and without TPB ions.

On the basis of the measurements of the absorbance spectra and the Stokes shift of the dye in different solvents (see Table 1) it appears that in the absence of TPB the chromophore experiences a ground-state stabilization in the membrane very similar to that in water, consistent with a location of the pyridinium group at the membrane-aqueous phase interface. Therefore, let us assume a local dielectric constant, ϵ_m^o, of 70 for the situation before the addition of TPB. The value of U_b for saturating concentrations of TPB has been calculated (see Appendix A) to be -0.05 V. Inserting these values of ϵ_m^o and U_b into Eq. 7, upon rearrangement it can be shown that the pK_a shift of -1.0 pH units is consistent with a dielectric constant in the presence of 50 μM TPB of 25. This would correspond to a change in polarity of the dye environment equivalent to transferring the dye from water to ethanol. Within the lipid headgroup region of the membrane there is a steep change in the dielectric constant from 78 in the adjacent aqueous solution to a value of 2–3 in the membrane interior (Flewelling and Hubbell, 1986b). A small change in the angle of the dye within the membrane would, therefore, suffice to produce the large reduction in pK_a observed.

The first explanation for the pK_a reduction would suggest an electrochromic mechanism for the voltage dependence, the second a reorientation/solvatochromic mechanism. It is, however, also possible that both mechanisms are contributing.

The fact that an intramembrane electrical field shifts the acid/base equilibrium of membrane-bound RH421 raises the possibility of a further voltage-sensitive mechanism. Protonation of RH421 leads to drastic spectral changes (Clarke et al., 1992). Therefore, coupling of the voltage-sensitive response of the dye observed at neutral pH to its acid-base equilibrium by working at a pH midway between the pK_a values of dye in the presence and absence of an electrical field, i.e., at a pH of ~2.5, significantly increases the magnitude of the electrical field-induced absorbance and fluorescence changes (data not shown). Such a pH value is not of physiological relevance, so that for RH421 this coupled mechanism is normally not functional. In the case of a voltage-sensitive dye with a pK_a in the physiological pH range, however, such a mechanism could have a major influence.

DISCUSSION

Two possible mechanisms have been proposed to explain the voltage dependence of the absorption spectrum of RH421 when bound to lipid membranes: 1) an electrochromic mechanism and 2) a reorientation/solvatochromic mechanism. To decide which of the mechanisms is likely to be making the major contribution, let us consider the absorbance changes that would be predicted by the two mechanisms separately on the addition of TPB.

First we shall discuss the electrochromic mechanism. In the case of a large charge shift on excitation of the dye, effects due to a change in dye polarizability caused by the electrical field can be neglected, and the frequency shift, $\Delta\nu$, expected on application of the field, is given by Waggoner and Grinvald (1977):

$$\Delta\nu = -\frac{\Delta\mu}{h} E \cos\theta \tag{8}$$

where $\Delta\mu$ is the change in dipole moment of the dye on excitation and θ is the angle between the vector of the dipole moment change and the vector of the electrical field. The maximum frequency shift would be observed when the direction of the charge shift on excitation of the dye is parallel or antiparallel to the field lines, i.e., $\theta = 0°$ or 180°. $\Delta\mu$ has been calculated from the Lippert plot to have a value of 25 ($\pm$11) Debye. The electrical field strength within the membrane at saturating concentrations of TPB has been calculated in Appendix A to be 2.4×10^8 V m^{-1}. Thus, the maximum frequency shift ($\theta = 0°$) that could be expected for such a field strength can be calculated from Eq. 8 to be 3.0×10^{13} Hz ($\equiv$ 1010 cm^{-1}). For RH421, which has an absorbance maximum when bound to the membrane in the absence of TPB of 480 nm, this corresponds to a wavelength shift of 24 nm. In fact, the experimentally determined absorbance maximum at saturating concentrations of TPB (see Fig. 4) occurs at 520 nm, i.e., a wavelength shift of 40 nm. The experimental wavelength shift is thus almost twice that of the maximum shift that could be produced by an electrochromic mechanism. Furthermore, the absorbance increase observed at high TPB concentrations (see Fig. 4) and the electrical field-dependent changes in quantum yield cannot be explained in terms of an electrochromic mechanism, which predicts only a frequency shift for a dye with a large permanent transition moment (Liptay, 1969). An electrochromic mechanism alone can, therefore, not explain the observed absorbance and fluorescence changes.

Now let us consider the reorientation/solvatochromic mechanism. In the Results section it was shown that the pK_a shift of bound RH421 due to TPB was consistent with a change in polarity of the dye environment approximately equivalent to transferral of the dye from water to ethanol. For such a change in polarity, how large would be the expected solvatochromic wavelength shift? From Table 1 it can be seen that transferring the dye from water to ethanol causes a red shift of 36 nm. The TPB-induced wavelength shift of 40 nm could, therefore, be accounted for by a change in dye environment from a water-like environment in the absence of TPB to an alcohol-like environment in the presence of TPB.

The very low value of the Stokes shift of the dye in the presence of TPB (see Table 1) would seem to imply an even

less polar dye environment. Comparison with the values in Fig. 3 suggests a polarity approximately equivalent to octanol. It is, however, possible that dye reorientation could also cause a decrease in microscopic mobility, which may also reduce the Stokes shift. An increase in viscosity can result in a lower Stokes shift because of the reduced rate of solvent relaxation in the excited state. Another possible cause for the low value of the Stokes shift may be the anisotropic solvation of the dye in the membrane (Fromherz and Schenk, 1994). The solvation energies in the ground and excited states could be different because of the charge shift from the pyridinium to the aniline group, which may be located at different depths within the membrane.

The hyperchromic effect induced by TPB is also consistent with a decrease in polarity of the environment, because similar effects are observed in isotropic solvents (cf. Figs. 4 and 6). The experimentally observed changes in quantum yield could likewise be explained in terms of a reorientation/solvatochromic mechanism, since it is known that the quantum yield and the fluorescence lifetime of such dyes depends on the polarity of the environment (Ephardt and Fromherz, 1989; Visser et al., 1994).

Finally we should consider whether or not a reorientation/solvatochromic mechanism is feasible based on energetic considerations. A reorientation and overall realignment of the dye molecules within the membrane can only occur if the reorientational energy supplied by the electrical field is significant in comparison to the thermal energy, which tends to randomize the orientation. For an isotropic medium it can be shown that the orientational distribution of an ensemble of dipoles in an electrical field is given by Tanford (1961) and Atkins (1983):

$$\frac{\Delta N}{N_{tot}} = \frac{e^{x\cos\theta} - e^{x\cos(\theta+\Delta\theta)}}{e^{x} - e^{-x}} \tag{9}$$

where

$$x = \frac{\mu E}{kT} \tag{10}$$

ΔN represents the number of dipoles within a cone bordered by the angles θ and $\theta + \Delta\theta$, where θ is the angle between the vector of the dipole moment μ and the vector of the electrical field strength E. In an isotropic medium an alignment of the dipoles due to the field can be expected if $x \geq 1$, i.e., the reorientational energy is greater than or of the same order of magnitude as the thermal energy. Based on the calculated field strength of 2.4×10^8 V m^{-1} (see Appendix A) for a TPB-saturated vesicle and the dipole moment of RH421 determined in the ground state in chloroform solution of 12 Debye, the value of x can be calculated for 30°C to be 2.3. The reorientational energy is, thus, more than twice that of the thermal energy. Under these conditions in an isotropic medium a realignment of the dipoles must take place. In a lipid membrane realignment is still not certain to occur, because interactions between the dye and its surrounding lipid molecules could feasibly prevent a reorientation. Nevertheless, the necessary condition for reorientation in the membrane that $x \geq 1$ is satisfied.

Whether the value of x in a cell membrane can be expected to be >1 depends on the magnitude of the local electrical field strength experienced by the dye. Recently it has been shown by Gross et al. (1994) that the aminostyrylpyridinium dye di-8-ANEPPS is sensitive to changes in the intramembrane dipole potential, Ψ_D. For phosphatidylcholine membranes Ψ_D has a value of ~300 mV and the entire voltage gradient occurs over a distance of ~5 Å. This corresponds to an electrical field strength of 6×10^8 V m^{-1}. Under these conditions the value of x for RH421 can be calculated for 30°C to be 5.8. Therefore, even in the absence of an applied field a dye alignment could occur as a result of the dipole potential alone.

Summarizing, it has been found that a reorientation/solvatochromic mechanism, whereby the electrical field reorients the dye molecules so that they experience a change in polarity of their lipid environment, is likely to make a significant contribution to both the spectral changes and to the field effect on the acid-base properties of the dye. The results cannot be explained by an electrochromic mechanism alone. Using aminostyrylpyridinium dyes with shorter conjugated systems Loew and Simpson (1981) found evidence suggesting electrochromism to be the sole contributing voltage-sensitive mechanism. In the case of RH421 its extra double bond would, therefore, seem to allow it more orientational flexibility. A contribution from electrochromism arising from dye reorientation, which could enhance the interaction of the field with the charge shift caused by the excitation is a further possibility. In this case the time resolution of the voltage-sensitive response of the dye would, however, be limited by the time necessary for the reorientation, which would, depending on the temperature and the viscosity of the membrane, lie in the ns–ms range. Finally, the fact that the acid-base properties of RH421 are altered by an intramembrane field shows that pH can play an important role in determining the amplitude of electrical field-induced spectral changes of voltage-sensitive dyes.

APPENDIX A

The electrical boundary potential U_b and the electrical field strength E within a vesicle membrane induced by the binding of TPB can be estimated by using the three-capacitor model of the lipid membrane (Clarke, 1993). According to the model the boundary potential produced between the position in the membrane where the hydrophobic ions bind and the external aqueous solution is given by the following (cf. Eq. 21 in Clarke, 1993):

$$U_b = \alpha U_m + \frac{\alpha^2 e_o}{C_m}\left(\frac{r_o}{A_o} - \frac{r_i}{A_i}\right) - \frac{\alpha e_o}{C_m}\frac{r_o}{A_o} \tag{A1}$$

where U_m is the total membrane potential and α is the ratio of the total membrane capacitance, C_m, to the capacitance of the boundary region, C_o. r_o and r_i represent the number of hydrophobic ions bound to the external and internal monolayers of the vesicle, respectively. A_o and A_i are the external and internal surface areas of a vesicle and e_o is the elementary charge. In the absence of a total membrane potential the concentration of TPB ions on both sides of the membrane are equal and

Eq. A1 reduces to

$$U_b = -\frac{e_o}{C_o}\frac{r_o}{A_o} \quad \text{(A2)}$$

The field strength E in the external boundary layer where the dye binds is therefore given by

$$E = -\frac{e_o}{C_o}\frac{r_o}{A_o}\frac{1}{d} \quad \text{(A3)}$$

where d is the thickness of the boundary layer.

According to conductance studies with planar bilayers in the presence of TPB (Andersen et al., 1978) the capacitance of the boundary region, C_o, is $\sim 40\ \mu F\ cm^{-2}$. The thickness of the boundary layer, d, must at least be less than half the thickness of the membrane as a whole, i.e., $d < 2$ nm. Theoretical calculations of the potential energy profile of TPB across the membrane (Flewelling and Hubbell, 1986b) suggest that the ions bind within the membrane at a distance of 0.2–0.3 nm from the membrane-aqueous solution interface. This is also in accordance with an estimate of d of 0.2 nm based on fluorescence titrations of Bühler et al. (1991).

The value of r_o/A_o is the surface concentration of adsorbed TPB ions in the external monolayer of a vesicle. For a vesicle saturated with TPB, fluorescence titrations (see Results section) have yielded a value of 0.09 TPB ions/lipid molecule. The surface area occupied by a phosphatidylcholine headgroup in the external monolayer of a vesicle has been determined by Huang and Mason (1978) to be $74 \times 10^{-20}\ m^2$. Therefore, multiplying the number of TPB ions/lipid molecule by the area/lipid molecule yields a saturating surface concentration of TPB of $1.2 \times 10^{17}/m^2$.

Insertion of the values of C_o, r_o/A_o, and d given above into Eqs. A2 and A3 yields a boundary potential U_b of -0.05 V and an electrical field strength, $|E|$, in the external boundary region of the membrane of $2.4 \times 10^8\ V\ m^{-1}$. This corresponds to a transmembrane potential difference (assuming a membrane thickness of 4 nm) of ~ 1 V. Dielectric breakdown of cell membranes occurs at a membrane potential of ~ 1.6 V, corresponding to an electrical field strength in the membrane of $4 \times 10^8\ V\ m^{-1}$ (Zimmermann et al., 1974). The electrical field strength generated by the binding of saturating concentrations of TPB is, therefore, approaching the maximum achievable field strength. Significantly greater values would lead to membrane rupture.

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