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Voltage-sensitive fluorescence of amphiphilic hemicyanine dyes in a black lipid membrane of glycerol monooleate

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Abstract

Amphiphilic fluorescent hemicyanine dyes were adsorbed to a hemispherical bimolecular membrane of glycerol monooleate. Their excitation spectra of fluorescence were as in water, their emission spectra were as in hydrocarbon. An AC-voltage was applied across the membrane and the relative changes of the spectra of excitation and of emission were recorded. For all dyes we observed a blue-shift of excitation with positive voltage on the opposite side of staining. The effect is compared with the blue-shift expected for electrochromism. For most dyes we observed a red-shift of emission and a drop of the fluorescence intensity. These effects are compared with the red-shift and the drop of quantum yield expected for a voltage-induced solvatochromism caused by a minute displacement of the dyes in the anisotropic environment at the membrane/water interface.

Key words: Fluorescence; Solvatochromism; Electrochromism; Membrane voltage; Hemicyanine dye; Lipid bilayer; Glycerol monooleate

1. Introduction

Amphiphilic fluorescent hemicyanine dyes are used as indicators of fast voltage transients in neuron membranes [1,2]. The mechanism of the voltage-sensitivity of fluorescence is unknown [3]. An understanding of the mechanism is the prerequisite to optimize systematically these probes. A study of voltage-sensitivity in the defined environment of an artificial membrane is a decisive step to solve the problem in conjunction with investigations in neuron membranes on one side [4,5] and in bulk solvents on the other side [6,7].

In the present paper we consider the voltage-sensitivity of the complete excitation and emission spectra of fluorescence for six hemicyanine dyes in a hemispherical bilayer (black lipid membrane, BLM) of glycerol monooleate. The study is based on previous investigations in planar BLMs with a restricted set of dyes and of spectral data [8,9] and on a similar study in neurons [4,5].

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2. Materials and methods

Dyes. We studied six amphiphilic hemicyanines (Fig. 1). They are homologs of BABP (dibutylaminophenylbutylsulfonatopyridinium). In BNBP and BNBIQ the aminophenyl and pyridinium are replaced by naphthylamine and isoquinolinium, respectively. In RH364 and RH160 one and two double bonds are inserted, respectively. Naphthylamine and one double bond are introduced in di4ANEPPS. The biaryl dyes BABP, BNBP and BNBIQ were described in Refs. [6,7]. The styryl-dyes RH364, RH160 and di4ANEPPS were introduced in Refs. [10–13]. They were obtained from Molecular Probes, Junction City.

Black lipid membrane. Membranes were formed on a bent glass pipette (tip diameter about 0.6 mm) (Fig. 2). The pipette was made hydrophobic with octadecyltrichlorosilane [14]. It was inserted into a patch-clamp holder [15]. (List, Darmstadt) on a micromanipulator (Leitz). Using a Hamilton syringe the pipette was filled with 500 mM NaCl (NaCl suprapure (Merck) in Milli-Q-water (Millipore)). About 2 μl of a 10 mM solution of glycerol monooleate (Sigma) in tetradecane (Sigma)

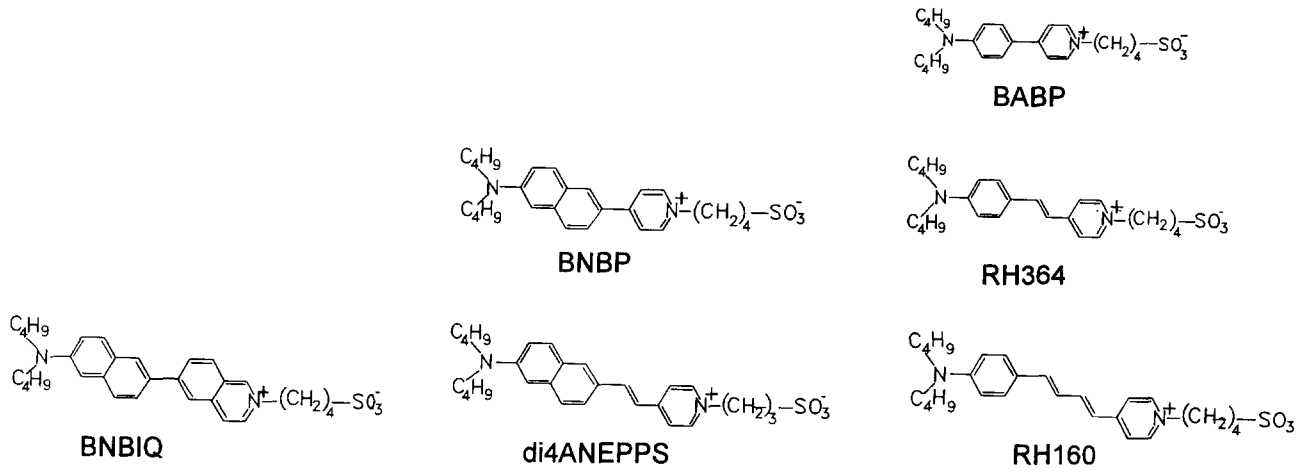

Fig. 1. Amphiphilic hemicyanine dyes. They are derived from dibutylaminophenylbutylsulfonatopyridinium (BABP) [6] by substitution of aminophenyl by naphthylamine and of pyridinium by isoquinolinium (biaryl dyes) as in BNBP [7] and BNBIQ [7] and by the intsertion of double bonds (styryl dyes) as in RH364 [10], RH160 [10] and di4ANEPPS [12].

were applied to the tip and sucked into the pipette. Then the pipette was dipped into the electrolyte in a silica cup. Pipette and bath were contacted by Ag/AgCl electrodes. An approximately hemispherical bubble (diameter about 500 μm) was blown up using the syringe [16–18]. Formation of the bubble was observed in an inverted microscope. Thinning of the membrane was checked electrically by applying voltage pulses and observing the current on an oscilloscope. After thinning, the membrane was stained by adding about 1 μl of a 1 mM methanolic solution of a dye to the electrolyte below the bubble. We studied between 5 and 10 membranes per dye.

Optics and electronics. The assembly with the BLM was mounted on the stage of an inverted microscope (Axiovert, Zeiss/Oberkochen) (Fig. 2). The optical set-up was similar to that used in a study of neuron membranes [4]. The central part of the arc of a xenon high pressure lamp (150 W, Hamamatsu) was imaged onto the vertical segment of the membrane bubble through a continuous interference filter (Veril BL 200, Schott/Mainz), a shutter and a dichroitic beam splitter (FT 580 or FT 510, Zeiss) using the objective LD-Achroplane 32 × /0.40 (Zeiss). The area of illumination was restricted to a diameter of 120 μm by a diaphragm to avoid stray light from the anulus of solvent at the mouth of the pipette. Through the same objective the fluorescence was focussed onto the cathode (S-20) of a photomultiplier (type C31034, RCA) through the beam splitter, a cut-off filter (RG590 or RG520, Schott) and a second continuous interference filter. The width of the cone (in water) that illuminated the BLM and that detected the fluorescence from the BLM was about 33°. Spectra of excitation and emission were measured by scanning the two continuous interference filters. Before staining, reference spectra were scanned which were subtracted. Other details of the set-up and of calibration are described in Refs. [4,5].

To study the voltage-sensitivity we applied an AC-voltage with an amplitude of 100 mV and a frequency of 625 Hz across the membrane (Fig. 2). The modulated signal was fed into a lock-in amplifier (PAR 186, Princton Applied) which was triggered by the AC-voltage. The output of the lock-in amplifier – proportional to the voltage-induced change of fluorescence intensity ΔF – was read into a computer. The modulated output voltage of the photomultiplier was read directly into the computer. Its average was a measure of the total fluorescence intensity F.

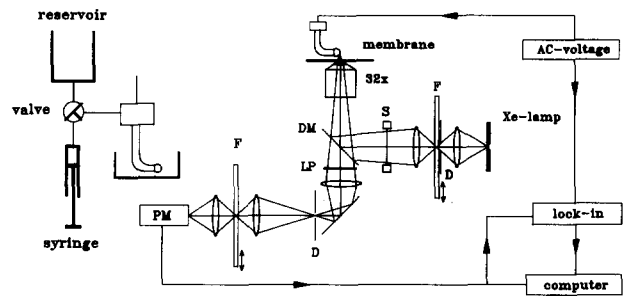

Fig. 2. Experimental set-up. A hemispherical bilayer of glycerol monooleate is blown up on the tip of a bent pipette using a syringe (inset). It is placed in the object plane of an inverted microscope and illuminated tangentially by a high-pressure Xenon lamp through a diaphragm (D), a continuous interference filter (F), a shutter (S) and a dichroitic mirror (DM). An AC-voltage (AC) is applied across the membrane. The fluorescence is detected by a photomultiplier (PM) through the dichroitic mirror, a low-pass filter (LP), a diaphragm (D) and a continuous interference filter (F). The electrical signal is fed into a lock-in amplifier. The measurement (shutter, motion of the continuous interference filters, data acquisition) is controlled by a computer.

Table 1
Maxima of the emission and excitation spectra of fluorescence M_{EM} and M_{EX} and spectral widths W_{EM} and W_{EX} for six hemicyanine dyes in a black lipid membrane of glycerol monooleate

Dye	M_{EM} (cm^{-1})	M_{EX} (cm^{-1})	W_{EM} (cm^{-1})	W_{EX} (cm^{-1})
BABP	18550	23250	3350	3150
BNBP	16700	22120	3320	3770
RH364	16720	21050	2640	3840
BNBIQ	15850	21850	3810	4500
di4ANEPPS	15270	20950	3210	4160
RH160	14900	19850	2920	4320

Protocol of measurement. Optical measurements were started when a membrane had a constant high capacitance for 3 min. After staining, the focus was adjusted, voltage was applied and the phase of the lock-in amplifier was optimized at a suitable pair of wavenumbers for excitation and emission. First a scan of the wavenumber of excitation $\bar{\nu}_{EX}$ was started (40 different wavenumbers) at a fixed wavenumber of emission $\bar{\nu}^*_{EM}$. At each wavenumber $\bar{\nu}_{EX}$ – after a delay of 0.2 s – thousand data points of the fluorescence F and of the voltage-induced change ΔF were recorded within 3 s. Then a similar scan of the wavenumber of emission $\bar{\nu}_{EM}$ was started at a fixed wavenumber of excitation $\bar{\nu}^*_{EX}$. Up to four pairs of sensitivity spectra were measured for each membrane. The fluorescence intensity F and the change ΔF were averaged at each wavenumber. ΔF and F were divided by each other and normalized by the applied voltage. The voltage sensitivity $\Delta F/F\Delta V$ was expressed with respect to positive voltage in the bubble.

The scans of the fluorescence intensity were fitted by exponential polynomes. From these spectra of excitation (proportional to absorption) and emission in arbitrary units $a(\bar{\nu}_{EX})$ and $e(\bar{\nu}_{EM})$ we determined the wavenumbers M_{EX} and M_{EM} of the maxima (reproducibility about 300 cm^{-1}) and the widths W_{EX} and W_{EM} at their semimaximal value. (As most excitation spectra had long tails in the blue we determined $W_{EX}/2$ from the maximum to the red.) By numerical differentiation we computed the derivatives $a'(\bar{\nu}_{EX})$, $e'(\bar{\nu}_{EM})$, $a''(\bar{\nu}_{EX})$ and $e''(\bar{\nu}_{EM})$ which were required to fit the voltage-sensitive spectra.

Evaluation. A change of the membrane voltage may alter the spectra of excitation and of emission. The voltage may change the amplitude, shift the maxima of the spectra and change the spectral shape. (Note that changes of spectral width are quite common for hemicyanine dyes in different environments [7].) The relative change of fluorescence $\Delta F/F\Delta V$ per voltage change ΔV is described by the shifts of the maxima per unit voltage $\Delta m_{EX} = \Delta M_{EX}/\Delta V$ and $\Delta m_{EM} = \Delta M_{EM}/\Delta V$ and by the specific changes of spectral width $\Delta w_{EX} = \Delta W_{EX}/\Delta V$ and $\Delta w_{EM} = \Delta W_{EM}/\Delta V$ according to Eqs. (1) and (2) as discussed in Refs. [4,5].

$$\frac{\Delta F(\bar{\nu}_{EX})}{F(\bar{\nu}_{EX})\Delta V} = \Delta t_{EX} - \frac{a'(\bar{\nu}_{EX})}{a(\bar{\nu}_{EX})}\Delta m_{EX} + \frac{a''(\bar{\nu}_{EX})W_{EX}}{a(\bar{\nu}_{EX})8\ln 2}\Delta w_{EX} \quad (1)$$

$$\frac{\Delta F(\bar{\nu}_{EM})}{F(\bar{\nu}_{EM})\Delta V} = \Delta t_{EM} - \frac{e'(\bar{\nu}_{EM})}{e(\bar{\nu}_{EM})}\Delta m_{EM} + \frac{e''(\bar{\nu}_{EM})W_{EM}}{e(\bar{\nu}_{EM})8\ln 2}\Delta w_{EM} \quad (2)$$

Δt_{EX} and Δt_{EM} are the relative changes of total amplitude per unit voltage. They include the relative changes of amplitude Δa_{EX} and Δa_{EM} of excitation and emission per unit voltage (Eqs. (3) and (4)). They account also for those effects which are induced in the excitation spectrum by the voltage-sensitivity of emis-

Table 2
Fit-parameters of voltage-sensitive fluorescence of six hemicyanine dyes in a membrane of glycerol monooleate

Dye	Δa (%/0.1 V)	Δm_{EM} (cm^{-1}/0.1 V)	Δm_{EX} (cm^{-1}/0.1 V)	Δw_{EM} (cm^{-1}/0.1 V)	Δw_{EX} (cm^{-1}/0.1 V)
BABP	−2.5	−11.3	3.2	1.9	0.9
BNBP	−3.0	−13.1	12.2	−1.0	4.9
RH364	−2.3	−8.9	12.7	−0.3	9.3
BNBIQ	−4.4	3.7	26.3	35.5	9.9
di4ANEPPS	−3.3	−7.2	34.1	11.9	7.2
RH160	−1.8	−16.9	34.0	−8.2	23.0

Relative change of amplitude per unit voltage $\Delta a = \Delta a_{EX} + \Delta a_{EM}$, shift of the maximum of emission per unit voltage Δm_{EM}, shift of the maximum of excitation per unit voltage Δm_{EX}, changes of spectral width per unit voltage for emission Δw_{EM} and for excitation Δw_{EX}. The data refer to positive voltage applied to the interior of the BLM-bubble stained from the outside. The reproducibility was better than 0.5% for the amplitudes and better than 5 cm^{-1} for the shifts and changes of widths.

sion at the wavenumber of emission $\bar{\nu}^*_{EM}$ and in the emission spectrum by the voltage-sensitivity of excitation at the wavenumber of excitation $\bar{\nu}^*_{EX}$ according to Eqs. (3) and (4).

$$\Delta t_{EX} = \Delta a_{EX} + \Delta a_{EM} - \frac{e'(\bar{\nu}^*_{EM})}{e(\bar{\nu}^*_{EM})}\Delta m_{EM} + \frac{e''(\bar{\nu}^*_{EM})W_{EM}}{e(\bar{\nu}^*_{EM})8\ln 2}\Delta w_{EM} \quad (3)$$

$$\Delta t_{EM} = \Delta a_{EM} + \Delta a_{EX} - \frac{a'(\bar{\nu}^*_{EX})}{a(\bar{\nu}^*_{EX})}\Delta m_{EX} + \frac{a''(\bar{\nu}^*_{EX})W_{EX}}{a(\bar{\nu}^*_{EX})8\ln 2}\Delta w_{EX} \quad (4)$$

We fitted a complete set of data of voltage sensitivity – for the excitation and for the emission spectrum together – by the five parameters $\Delta a = \Delta a_{EX} + \Delta a_{EM}$, Δm_{EM}, Δm_{EX}, Δw_{EM} and Δw_{EX} on the basis of Eqs. (1)–(4). The reproducibility was better than 0.5% for the amplitude, better than 5 cm^{-1} for the spectral shifts and the changes of width.

To present pure sensitivity spectra of excitation and of emission we have to displace the original data of voltage-sensitive excitation by $\Delta t_{EX} - \Delta a_{EX}$ – the perturbation from the emission – and of voltage-sensitive emission by $\Delta t_{EM} - \Delta a_{EM}$ – the perturbation from the excitation. However, the data themselves do not provide a partition of the fit parameter Δa into its components Δa_{EX} and Δa_{EM}. We assigned the observed change of amplitude exclusively to a change of emission: i.e., we assumed $\Delta a = \Delta a_{EM}$ and $\Delta a_{EX} = 0$. The issue is considered in the Discussion.

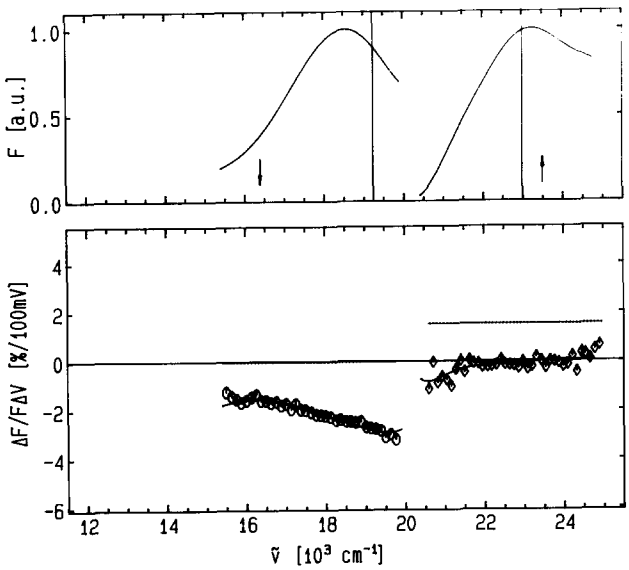

Fig. 3. Fluorescence and voltage-sensitive fluorescence of BABP in a membrane of glycerol monooleate. Top: Normalized spectra of emission and excitation. The maxima in propanol are indicated as vertical lines. The wavenumbers of excitation $\bar{\nu}^*_{EX} = 23500\ cm^{-1}$ and emission $\bar{\nu}^*_{EM} = 16400\ cm^{-1}$ are marked by arrows. Bottom: Relative change the fluorescence $\Delta F/F\Delta V$ per unit voltage for the emission and for the excitation spectrum. The data are fitted according to Eqs. (1)–(4) (fit parameters in Table 2). The dashed lines are the original abscissae of the data as discussed in the text.

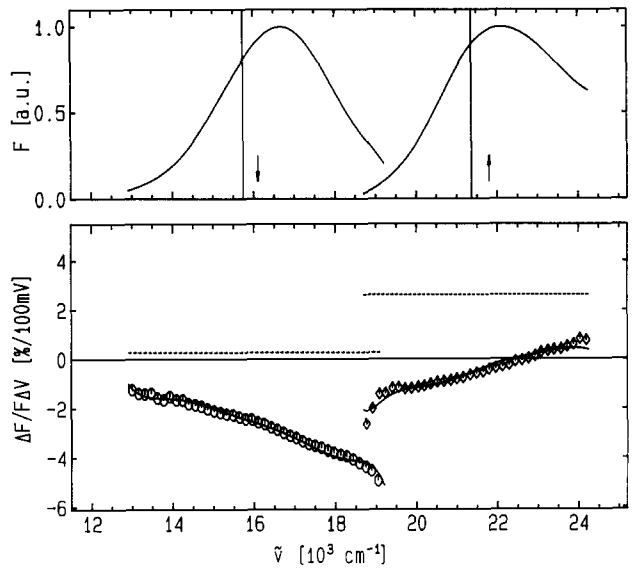

Fig. 4. Fluorescence and voltage-sensitive fluorescence of BNBP in a membrane of glycerol monooleate. Excitation at $\bar{\nu}^*_{EX} = 22800\ cm^{-1}$, emission at $\bar{\nu}^*_{EM} = 16100\ cm^{-1}$.

3. Results

In this section we present the excitation and emission spectra of fluorescence for the six hemicyanine dyes in glycerol monooleate. The spectral maxima of the dyes in propanol are marked as references. We present typical spectra of voltage-sensitive fluorescence and evaluate them according to Eqs. (1)–(4). We begin with the smallest dye BABP, proceed with the two dyes of intermediate size BNBP and RH364 and end up with the three largest dyes BNBIQ, di4ANEPPS and RH160. The spectral data and the fit-parameters of voltage sensitivity are summarized in the Tables 1 and 2.

BABP

The fluorescence spectra and the spectra of the voltage-induced change $\Delta F/F\Delta V$ of fluorescence are shown in Fig. 3. The maximum of excitation is slightly in the blue of the reference in propanol, whereas the emission is distinctly in the red of the reference. The prominent feature of voltage-sensitivity is a drop of the amplitude $\Delta a = -2.5\%/100$ mV. There is also a distinct red-shift of the emission $\Delta m_{EM} = -11.3\ cm^{-1}/100$ mV, but only small blue-shift of the excitation $\Delta m_{EX} = +3.2\ cm^{-1}/100$ mV.

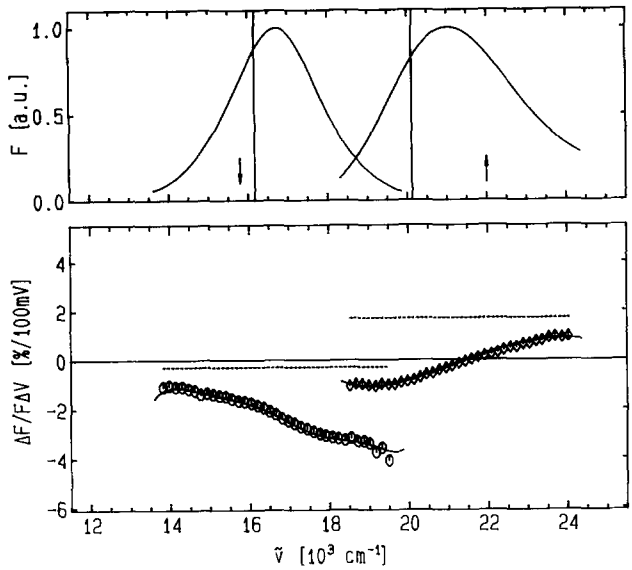

Fig. 5. Fluorescence and voltage-sensitive fluorescence of RH364 in a membrane of glycerol monooleate. Excitation at $\bar{\nu}^*_{EX} = 22000$ cm^{-1}, emission at $\bar{\nu}^*_{EM} = 15800$ cm^{-1}.

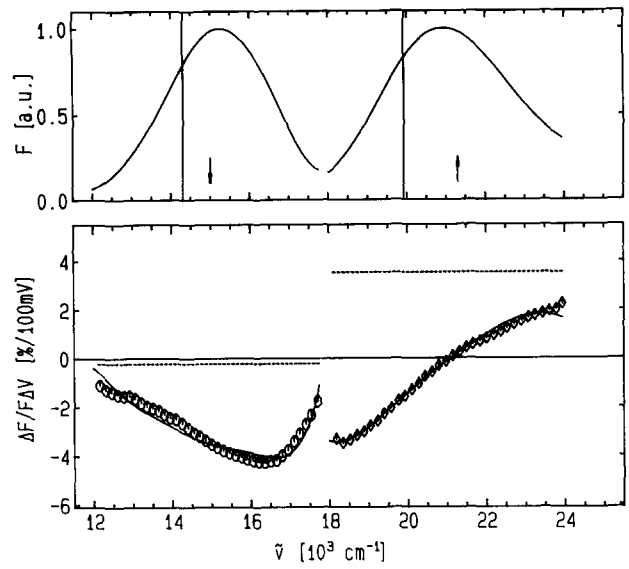

Fig. 7. Fluorescence and voltage-sensitive fluorescence of di4ANEPPS in a membrane of glycerol monooleate. Excitation at $\bar{\nu}^*_{EX} = 21300$ cm^{-1}, emission at $\bar{\nu}^*_{EM} = 15000$ cm^{-1}.

BNBP

The fluorescence spectra and the voltage-induced change $\Delta F/F\Delta V$ of fluorescence are shown in Fig. 4. The excitation and the emission are displaced to the blue with respect to propanol. The voltage-sensitivity, is dominated again by a large drop of the amplitude $\Delta a = -3\%/100$ mV and also by a distinct red-shift of the emission $\Delta m_{EM} = -13.1$ cm^{-1}/100 mV. Now the excitation is shifted to the blue by $\Delta m_{EX} = +12.2$ cm^{-1}/100 mV.

RH364

The fluorescence spectra and the voltage-induced change $\Delta F/F\Delta V$ of fluorescence are shown in Fig. 5. The maxima of excitation and of emission are displaced again to the blue with respect to propanol. Also the voltage-sensitivity is similar to BNBP: We observe a – somewhat smaller – drop of the amplitude $\Delta a = -2.3\%/100$ mV and opposite shifts of emission and excitation $\Delta m_{EM} = -8.9$ cm^{-1}/100 mV and $\Delta m_{EX} = +12.7$ cm^{-1}/100 mV.

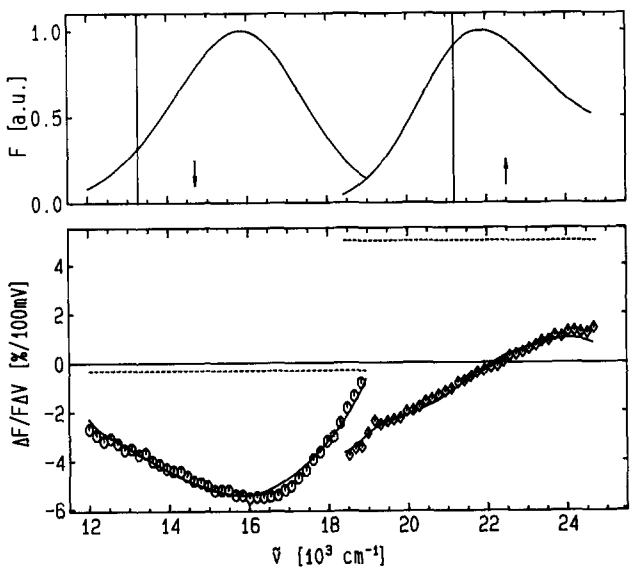

Fig. 6. Fluorescence and voltage-sensitive fluorescence of BNBIQ in a membrane of glycerol monooleate. Excitation at $\bar{\nu}^*_{EX} = 22500$ cm^{-1}, emission at $\bar{\nu}^*_{EM} = 14700$ cm^{-1}.

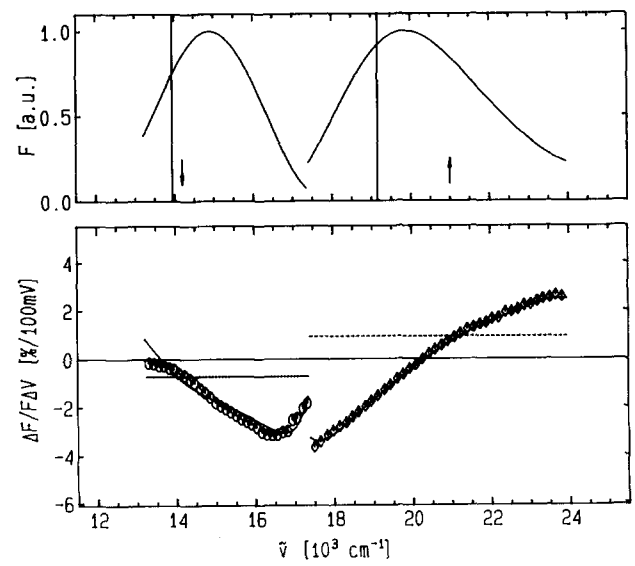

Fig. 8. Fluorescence and voltage-sensitive fluorescence of RH160 in a membrane of glycerol monooleate. Excitation at $\bar{\nu}^*_{EX} = 21300$ cm^{-1}, emission at $\bar{\nu}^*_{EM} = 15000$ cm^{-1}.

BNBIQ

The fluorescence spectra and the spectra of voltage-induced change $\Delta F/F\Delta V$ are shown in Fig. 6. The maxima of excitation and of emission are displaced to the blue with respect to propanol. The displacement of emission is enormous. The voltage induces an enormous drop of amplitude $\Delta a = -4.4\%/100$ mV. Also the blue-shift of excitation $\Delta m_{EX} = +26.3\ cm^{-1}/100$ mV is larger than in BNBP and RH364. The fit provides a minute blue-shift of emission $\Delta m_{EM} = +3.7\ cm^{-1}/100$ mV.

di4ANEPPS

The fluorescence spectra and the spectra of the voltage-induced change $\Delta F/F\Delta V$ of fluorescence are shown in Fig. 7. Considerable displacements of excitation and emission to the blue are observed with respect to propanol. The voltage-induced blue-shift of excitation is now even larger than for BNBIQ $\Delta m_{EX} = +34.1\ cm^{-1}/100$ mV. Again only a minute shift of emission – now to the red – by $\Delta m_{EM} = -7.2\ cm^{-1}/100$ mV is observed. The drop of amplitude $\Delta a = -3.3\%/100$ mV is similar as for the other dyes.

RH160

The fluorescence spectra and the voltage-induced change $\Delta F/F\Delta V$ of fluorescence are shown in Fig. 8. The excitation and the emission are displaced again to the blue as compared to propanol. The voltage-induced blue-shift $\Delta m_{EX} = +34\ cm^{-1}/100$ mV is similar to di4ANEPPS. A distinct red-shift of the emission $\Delta m_{EM} = -16.9\ cm^{-1}/100$ mV is observed as in RH364 and BNBP. The drop of amplitude $\Delta a = -1.8\%/100$ mV is relatively weak.

4. Discussion

We consider first the fluorescence spectra of excitation and emission. From the analysis we obtain information about anisotropic solvation of the dyes at the membrane/water interface. Then we discuss the spectra of voltage sensitivity, i.e., the spectral shifts of excitation and emission and the changes of amplitude. We consider electrochromism and field-induced solvatochromism as possible mechanisms of voltage-sensitive fluorescence.

Spectra

The polarity at the interface of lipid membranes and micelles, where amphiphilic dyes are bound, was compared to bulk solvents with a dielectric constant around $\epsilon = 20$ as, e.g., in propanol [19.20]. All spectra of excitation and emission of the six hemicyanine dyes in glycerol monooleate, however, are displaced to the blue with respect to propanol (Figs. 3–8) – with the exception of the emission of BABP. It is known that the excitation of hemicyanines is displaced to the blue by solvents of enhanced polarity and that their emission is displaced to the blue by solvents of reduced polarity [7]. We come to the conclusion that these dyes see two different environments in the membrane with respect to excitation and emission – the one more polar than propanol and the other less polar than propanol.

For a discussion of the two-valued polarity we summarize the basis of solvatochromism for the hemicyanines. Physicochemical details will be considered elsewhere (Fromherz, P., in preparation). The excitation starts from a ground state which is stabilized mainly by solvation of the positive pyridinium (isoquinolinium). The emission starts from an excited state, which is stabilized mainly by solvation of the aniline (aminonaphthalene) where the positive charge is located after excitation [7]. The energies of solvation are lost in the Franck-Condon transitions of excitation and emission: The positive charge is removed instantaneously from its solvation shell by intramolecular charge shift. An enhanced solvation in polar media displaces the excitation to the blue and the emission to the red. For the maxima the effect is described by Eqs. (5) and (6).

$$M_{EX} = \bar{\nu}_{EL} + \bar{\nu}_{IM} + \bar{\nu}_{SV} = \bar{\nu}_{EL} + \bar{\nu}_{IM} + \sigma_{SV} \log \epsilon \quad (5)$$

$$M_{EM} = \bar{\nu}_{EL} - \bar{\nu}_{IM} - \bar{\nu}^{*}_{SV} = \bar{\nu}_{EL} - \bar{\nu}_{IM} - \sigma_{SV} \log \epsilon \quad (6)$$

$\bar{\nu}_{EL}$ is the electronic excitation energy. $\bar{\nu}_{IM}$ is the intramolecular reorganization energy. $\bar{\nu}_{SV}$ and $\bar{\nu}^{*}_{SV}$ are the solvation energies in the ground state and in the excited state. In bulk solvents the displacement of excitation and emission is symmetrical for enhanced polarity [7] with identical solvation energy $\bar{\nu}^{*}_{SV} = \bar{\nu}_{SV}$. This solvation energy was found to be proportional to the logarithm of the dielectric constant ϵ with constant slope $\sigma_{SV} = d\,\bar{\nu}_{SV}/d \log \epsilon$ as expressed by Eqs. (5) and (6). The parameters $\bar{\nu}_{EL}$, $\bar{\nu}_{IM}$ and σ_{SV} for the six hemicyanine dyes are given in Table 3.

Table 3
Parameters of solvatochromism for six hemicyanine dyes derived from spectral data in bulk solvents ([7] and P. Fromherz, publication in preparation)

Dye	$\bar{\nu}_{EL}$ (cm^{-1})	$\bar{\nu}_{IM}$ (cm^{-1})	σ_{SV} (cm^{-1})
BABP	21000	1000	725
BNBP	18500	1000	1300
RH364	18000	500	1150
BNBIQ	17100	1325	2050
di4ANEPPS	16800	500	2100
RH160	16400	820	1500

$\bar{\nu}_{EL}$ is the electronic excitation energy. $\bar{\nu}_{IM}$ is the intramolecular reorganization energy. The solvatochromic sensitivity $\sigma_{SV} = d\,\bar{\nu}_{SV}/d \log \epsilon$ is the change of solvation energy per logarithmic unit of the dielectric constant ϵ.

We assign the unusual features of the hemicyanines in glycerol monooleate to a difference of the solvation energy in the ground state and in the excited state with $\bar{\nu}^*_{SV} \neq \bar{\nu}_{SV}$. We assume that environments of different polarity dominate the solvation in the ground state and in the excited state. We evaluate two effective dielectric constants from the maxima M_{EX} and M_{EM} in the membrane (Table 1) using Eqs. (5) and (6) as shown in Fig. 9.

The dielectric constants obtained from excitation scatter around 80. The dielectric constants evaluated from the emission are close to 2 – with the exception of BABP where both values are high: i.e., in the ground state the charged pyridine (isoquinoline) is solvated by an environment like water, whereas in the excited state the charged aniline (aminonaphthalene) is solvated by an environment like hydrocarbon.

Such a two-valued polarity is expected for a dye which is oriented in the membrane with the pyridinium (isoquinolinium) at the membrane surface and with the aniline (aminonaphthalene) in the core. The change of solvation energy $\Delta\bar{\nu}_{SV} = \bar{\nu}^*_{SV} - \bar{\nu}_{SV}$ depends on the displacement Δz_{SV} of the dominant site of solvation across the membrane, on the polarity gradient at the interface and on the solvatochromic sensitivity σ_{SV} according to Eq. (7). The z-direction is chosen normal to the membrane from the surface to the core.

$$\Delta\bar{\nu}_{SV} = 2\bar{\nu}_{EL} - M_{EX} - M_{EM} = \sigma_{SV}\frac{d\log\epsilon}{dz}\Delta z_{SV} \qquad (7)$$

As an estimate for the displacement of the solvation site we use the intramolecular shift of charge caused by excitation. For BNBP we obtain from an MNDO computation [7,21,22] a shift $\Delta s_{EX} = 0.33$ nm from pyridine to aminonaphthalene. With $\Delta z_{SV} = 0.33$ nm, $\sigma_{SV} = 1300\ cm^{-1}$ and $\Delta\bar{\nu}_{SV} = -1820\ cm^{-1}$ (from Eqs. (5) and (6) with Tables 1 and 3) we evaluate a polarity gradient $d\log\epsilon/dz = -4.2\ nm^{-1}$: i.e., the change from water to hydrocarbon occurs within about 0.4 nm. Note, that the conclusion about anisotropic solvation cannot be drawn on the basis of the absorption spectrum alone which indicates only an unexpected polar environment of the pyridinium (isoquinolinium) moiety [23].

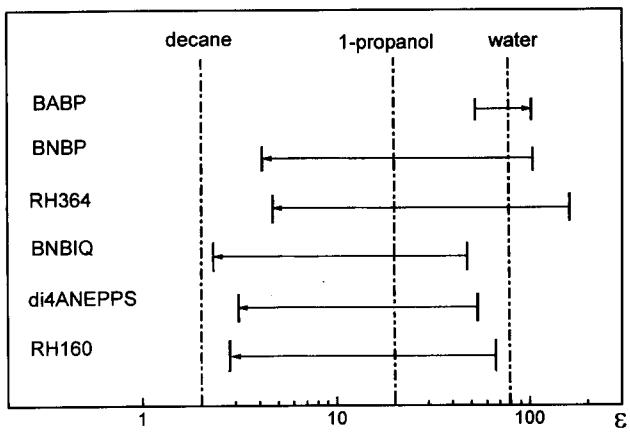

Fig. 9. Anisotropic solvation. Effective dielectric constants of six amphiphilic hemicyanine dyes in a membrane of glycerol monooleate derived from the maxima of the excitation and emission spectra of fluorescence. The high value reflects the solvation of the pyridinium (isoquinolinium) moiety in the ground state, the low value reflects the solvation of the aniline (aminonaphthalene) moiety in the excited state. The dielectric constants of decane, propanol and water are indicated.

Mechanism of voltage-sensitive fluorescence

We use two concepts to rationalize voltage-sensitive fluorescence. (i) Electrochromism: The electrical field in the membrane interacts with the charge shift along the chromophores which are oriented in the membrane [24–26]. (ii) Field-induced solvatochromism: The electrical field affects the environment of the dyes – by a displacement of the chromophores or by a rearrangement of the membrane.

We write the total shift of the spectral maxima as a superposition of electrochromic and solvatochromic shifts according to Eqs. (8) and (9).

$$\Delta M_{EX} = \Delta\bar{\nu}^{EC}_{EX} + \Delta\bar{\nu}^{SC}_{EX} \qquad (8)$$

$$\Delta M_{EM} = \Delta\bar{\nu}^{EC}_{EM} + \Delta\bar{\nu}^{SC}_{EM} \qquad (9)$$

(i) A positive voltage ΔV is applied to the inside of the BLM-bubble which is stained from the outside. Excitation shifts positive charge against the electrical field, emission shifts positive charge in field direction. Thus electrochromism gives rise to a blue-shift of excitation as well as of emission with $\Delta\bar{\nu}^{EC}_{EX} > 0$ and $\Delta\bar{\nu}^{EC}_{EM} > 0$.

(ii) The electrical field may displace the positive chromophore towards the membrane/water interface, i.e., to an environment of higher polarity. Field-induced solvatochromism leads to a blue-shift of excitation and to a red-shift of emission with $\Delta\bar{\nu}^{SC}_{EX} > 0$ and $\Delta\bar{\nu}^{SC}_{EM} < 0$.

Thus in general the change of excitation ΔM_{EX} is a superposition of two blue-shifts whereas the change of emission ΔM_{EM} is a superposition of an electrochromic blue-shift and a solvatochromic red-shift.

Spectral shift of excitation

For all dyes we observe a blue-shift of excitation (Table 2). We see no straightforward way to discriminate electrochromism and field-induced solvatochromism (Eq. (8)).

The expected electrochromic blue-shift per unit voltage is given by Eq. (10) with the charge shift $\Delta s_{EX} > 0$ by excitation, the change of electrical field strength $\Delta E < 0$ and the angle θ between the molecu-

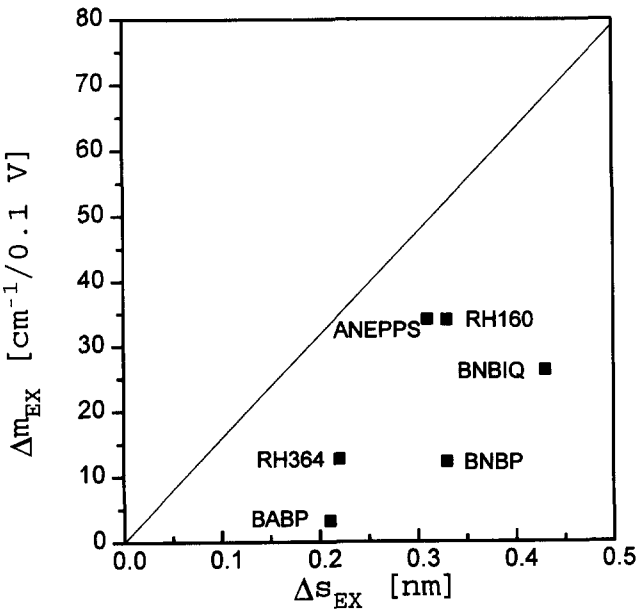

Fig. 10. Experimental shift Δm_{EX} of the maximum of excitation per unit voltage versus the intramolecular charge shift Δs_{EX} of excitation obtained from an MNDO calculation for six hemicyanine dyes. The line indicates the theoretical value of electrochromism for a perfect orientation of the dyes and a homogeneous voltage drop across a membrane of thickness 5 nm.

lar axis and the membrane normal. $\langle\cos\theta\rangle$ is the average projection.

$$\Delta m_{EM} = \frac{e_0}{hc}\frac{-\Delta s_{EX}\langle\cos\theta\rangle\Delta E}{\Delta V} \quad (10)$$

In Fig. 10 we have plotted the spectral shift Δm_{EX} from the experiments versus the charge shift Δs_{EX} from an MNDO computation. For comparison we have drawn the electrochromic shift expected for perfect order $\langle\cos\theta\rangle = 1$ and for a homogeneous voltage drop across the membrane of thickness d_M with $\Delta E = -\Delta V/d_M$ using $d_M = 5$ nm. All experimental data are below the theoretical line. There is no need to take into account field-induced solvatochromism, although we cannot exclude it.

We observe an almost linear relation of spectral shift and charge shift for the biaryl-dyes BABP, BNBP and BNBIQ. However, the difference of experiment and theory cannot be expressed by a constant factor. We cannot assign it exclusively to an incorrect estimate of the field strength ΔE. It must be related to an individual effect of orientation. Neglecting a correction of the field, we evaluate fairly low values of the order parameter $\langle\cos\theta\rangle = 0.09$, 0.23 and 0.39 for BABP, BNBP and BNBIQ. For RH364, di4ANEPPS and RH160 we obtain $\langle\cos\theta\rangle = 0.36$, 0.68 and 0.65, respectively.

Spectral shift of emission

We observed red-shifts of the emission for all dyes except for BNBIQ with a minute blue-shift (Table 2). Similar effects were seen in planar BLMs [8,9]. Also in a neuron membrane we found red-shifts of emission for BABP, BNBP and RH364 [5]. Considering Eq. (9) we must conclude that an electrochromic blue-shift does not exist and/or that it is overcompensated by a solvatochromic red-shift.

The electrochromic blue-shift of emission per unit voltage is given by Eq. (11) with the charge shift $\Delta s_{EM} < 0$ of emission and with the order parameter $\langle\cos\theta\rangle^*$ in the excited state.

$$\Delta m_{EM} = \frac{e_0}{hc}\frac{\Delta s_{EM}\langle\cos\theta\rangle^*\Delta E}{\Delta V} \quad (11)$$

The electrochromic shift could disappear for a vanishing charge shift Δs_{EM} or for a vanishing order parameter $\langle\cos\theta\rangle^*$. The first effect is impossible because Δs_{EM} must be similar to $-\Delta s_{EX}$ considering the structure of the dyes [7]. Also a randomized orientation in the excited state is unlikely, considering the anisotropic solvation discussed above. We conclude that the electrochromic effect exists. It must be, however, overcompensated by a red-shift of field-induced solvatochromism according to Eq. (9).

The red-shift of emission by field-induced solvatochromism may be related to a displacement of the dye per unit voltage $\Delta z_{FI}/\Delta V$ according to Eq. (12) as obtained from Eq. (6).

$$\Delta m_{EM} = -\sigma_{SV}\frac{\mathrm{d}\log\epsilon}{\mathrm{d}z}\frac{\Delta z_{FI}}{\Delta V} \quad (12)$$

As an example we consider BNBP. The total red-shift is $-\Delta m_{EX} + \Delta m_{EM} = -25.3$ cm^{-1}/100 mV (Table 3) assuming that an electrochromic blue-shift is hidden in the emission in the order of Δm_{EX}. With $\sigma_{SV} = 1300$ cm^{-1} (Table 3) and a polarity gradient $\mathrm{d}\log\epsilon/\mathrm{d}z = -4$ nm^{-1}, as estimated above, we obtain $\Delta z_{FI}/\Delta V = -5$ pm/100 mV! Considering this minute displacement it is obvious that molecular details of the environment in the membrane are crucial for a quantitative understanding of field-induced solvatochromism. In the light of this result, the individuality of the dyes with respect to voltage-sensitivity is certainly not surprising.

Change of amplitude

We found a drop of the amplitude of fluorescence for all dyes in the range $\Delta a = -1.8$ to -4.4%/100 mV. This change may be due to a change of excitation or of emission. In both cases it may be caused by a change of intrinsic properties of the dyes – of the extinction coefficient or of the quantum yield of fluorescence, respectively – or to a reorientation of the transition moment of the dyes which affects their inter-

action with the exciting light beam or with the light detector [17].

We exclude a major field-induced reorientation of the dye in the ground state as well as in the excited state. It would bring the aniline (aminonaphthalene) moiety to a polar environment. Large red shifts of fluorescence would be expected which are not observed. The extinction coefficient of the hemicyanine dyes is most insensitive with respect to extreme changes of the environment [7]. Thus it is unlikely that a change of the extinction coefficient plays a role here.

We attribute the observed drop of amplitude to a drop of the quantum yield of fluorescence Φ_F alone. (For that reason we used $\Delta a_{EX} = 0$ and $\Delta a_{EM} = \Delta a$ in Eqs. (3) and (4) for the presentation of the data in Figs. 3–8.)

It is known that the quantum yield Φ_F of hemicyanine dyes is lowered dramatically in polar solvents [7]. That change is described by a negative constant slope d log Φ_F/d log ϵ for a wide range of polarities [7]. It is due to enhanced solvation of a twisted state in the excited molecule with enhanced charge accumulation in the aniline (aminonaphthalene) moiety. A field-induced change $\Delta\Phi_F/\Phi_F$ may be caused by a displacement Δz_{FI} of the aniline (aminonaphthalene) moiety to a more polar environment in the membrane. We may calibrate the experimental shift in terms of an equivalent change in bulk solvents. In analogy to Eq. (12) we obtain Eq. (13) using $\Delta\Phi_F/\Phi_F = 2.3\ \Delta$ log Φ_F.

$$\frac{\Delta\Phi_F}{\Phi_F \Delta V} = \frac{2.3\ \mathrm{d}\log\Phi_F}{\mathrm{d}\log\epsilon}\,\frac{\mathrm{d}\log\epsilon}{\mathrm{d}z}\,\frac{\Delta z_{FI}}{\Delta V} \qquad (13)$$

As an example we consider BNBP. The field-induced drop of amplitude is $\Delta a = \Delta\Phi_F/\Phi_F \Delta V = -0.03/100$ mV (Table 2). With a sensitivity of the quantum yield d log Φ_F/d log $\epsilon = -2.3$ [7] and a polarity gradient d log ϵ/d$z = -4$ nm^{-1} as estimated above we obtain $\Delta z_{FI}/\Delta V = -1.5$ pm/100 mV. Again a minute field-induced displacement could account for the effect.

Spectral broadening

We used the changes of spectral width as fit parameters in the evaluation of the data (Table 2). In principle the shape of spectra can be changed by solvatochromism [7] as well as by electrochromism [25]. At the present moment we are not able to present a coherent discussion of the individual changes for the different dyes.

5. Conclusions

The fluorescence spectra in bilayers of glycerol monooleate and their voltage-induced changes provide some insight into the mechanism of hemicyanine dyes as voltage-sensitive probes. Besides electrochromism, field induced solvatochromism is of crucial importance. It seems that the details of molecular solvation in the complex environment of the anisotropic membrane/water interface are important to attain a quantitative understanding of voltage sensitivity. Each dye is an individual problem with a superposition of several mechanisms.

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