Interaction of the Voltage-Sensing Fluorescent Probe diS-C_3-(5) with Dipalmitoylphosphatidylcholine Liposomes

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Abstract. The interaction of the probe diS-C_3-(5) with dipalmitoylphosphatidylcholine (DPPC) liposomes has been studied using fluorescence and differential scanning calorimetry (DSC). The partition coefficients (K) of the probe for the lipid and the aqueous phase (in terms of molar part units) were $(1.20 \pm 0.04) \times 10^6$ at 45 °C and $(0.50 \pm 0.07) \times 10^6$ at 23 and 36 °C. In terms of volume concentration units, these values correspond to $K_p = (2.88 \pm 0.10) \times \times 10^4$ and $K_p = (1.20 \pm 0.17) \times 10^4$, respectively. DSC thermograms were practically identical both for large unilamellar and multilamellar liposomes. The main transition peak remained practically unchanged over the entire range of the probe concentrations used. The pretransition could be observed up to maximal probe concentrations applied and it widened and shifted from 35.4 °C in pure DPPC to ~ 32 °C at a probe/lipid ratio of 0.027. These results suggest that in both quasicrystalline and liquid crystalline lipid bilayers the probe molecules are included in "defects" between structurally ordered microregions (microdomains or clusters). The dependence of the fluorescence response on the transmembrane potential in a suspension of unilamellar DPPC vesicles suggest that the equilibrium thermodynamic model is valid for liquid crystalline bilayers.

Key words: diS-C_3-(5) — Fluorescent probe — Membrane potential — DPPC liposomes — Partition coefficients — Differential scanning calorimetry

Introduction

Voltage-sensing probes of various nature have widely been used in transmembrane potential studies (Waggoner 1979; Cafiso and Hubbell 1981; Loew and Simpson 1981). Modern techniques not only permit measuring of integral effects

in cellular and organelle suspensions, but studying space-time distribution of electric activity along "two-dimensional" cell complexes as well (Grinvald 1984). Most authors stress two principal difficulties of using probes: calibration of the fluorescent response in electric potential units, and estimation of effects of the probe used on cell membranes. The mechanism of the optical response of the probe, diS-C_3-(5), to transmembrane potential changes in a membrane vesicle suspension was described in a previous paper (Ivkova et al. 1984). Calculations were made based on the equilibrium thermodynamic model (Ivkov et al. 1984) and on the concentration dependence of the probe fluorescence in aqueous media and membranes (Ivkova et al. 1984).

Recently, Raines and Cafiso (1984) reported that potential-dependent fluorescent responses of dancylphosphonium probes could be described by the thermodynamic model used to estimate the transmembrane potential in electron spin resonance experiments (Cafiso and Hubbell 1978). The above two models, proposed independently, show general similarities, and they seem to reflect the actual physical processes occurring in membranes.

The aim of the present work was to study the influence of bilayer phase states on the probe behaviour in lipid suspensions and effects of the probe dissolved in a membrane on the bilayer structure. Due to this aim dipalmitoylphosphatidylcholine (DPPC) uni- and multilamellar liposomes were chosen as studied objects.

Materials and Methods

Identical materials and methods were used as those described in previous papers (Ivkova et al 1982, 1983, 1984) Dipalmitoylphosphatidylcholine (DPPC, Serva) and cholate (Serva) were used to prepare liposomes Carbocyanine dye diS-C_3-(5) was a gift by Prof A Waggoner Unilamellar liposomes were prepared by the detergent removal technique in 0 2 mol/l KCl and 0 02 mol/l Tris-HCl buffer, pH 7 5 The lipid concentration in suspensions was 20 mg/ml

Multilamellar liposome suspensions were prepared by shaking for 10 min in buffer at temperature exceeding that of DPPC phase transition (T_c) The lipid samples were equilibrated at temperatures above T_c for 30—60 min before loading them in a calorimetric cell Differential scanning calorimetry (DSC) was performed in a Privalov DASM-4 calorimeter The DSC thermograms are shown in Fig 1 Traces of detergent in membranes after dialysis did practically not affect in main phase transition, whereas the pretransition peak was shifted by $\sim 1\,5$ °C to lower temperatures and become wider than that in pure DPPC liposomes

Valinomycin ($0\,5 \times 10^{-8}$ mol/l) also shifted and widened the pretransition peak, it, however, had practically no effect on the main transition It is known that low concentrations of admixtures (cholesterol, paraffines, etc) abolish the pretransition peak in phosphatidylcholine phase transitions (Ivkov and Berestovsky 1981) The detergent concentration in membrane after more than 48 hours of dialysis is sufficiently low to be without any effect on the bilayer structure

Results

Behaviour of the probe in membranes

Changes in probe fluorescence intensity were studied in unilamellar liposome suspensions at temperatures above (45 °C) and below (36 and 23 °C) that of DPPC phase transition (T_c = 41.5 °C). Spectral changes upon increasing lipid

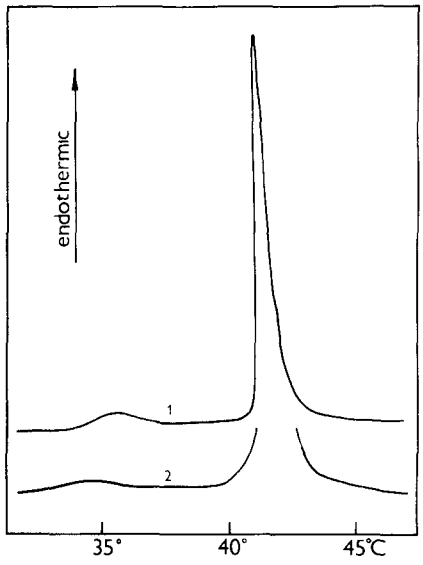

Fig. 1. DSC heating curves of multilamellar (1) and unilamellar (2) DPPC liposomes Heating rate = 0 5 °C/min The medium contained 0 2 mol/l KCl, 0 02 mol/l Tris HCl (pH 7 5) The lipid concentration was 0 4 mg/ml

concentration were similar to those observed in egg phosphatidylcholine (EPC) (Ivkova et al. 1983). The partition coefficients, K, for the distribution of the probe between lipid bilayers (quasicrystalline or liquid-crystalline) and the aqueous medium were calculated from the back fluorometric titration curves by the method described earlier (Ivkova et al. 1982). In terms of molar part units, the following values were obtained: $K = (1.20 \pm 0.40) \times 10^6$ at 45 °C, and $K = (0.50 \pm 0.07) \times 10^6$ at 23 and 36 °C; the respective values expressed in terms of volume concentration units were: $K_p = (2.88 \pm 0.10) \times 10^4$ at 45 °C, $K_p = (1.20 \pm 0.17) \times 10^4$ at 23 and 36 °C. In EPC suspension $K = (2.37 \pm \pm 0.43) \times 10^6$ at 20 °C (Ivkova et al. 1983), i.e. twice that for DPPC at 45 °C, although both lipids are in the liquid crystalline state.

The dependence of the fluorescence intensity on the probe concentration in the lipid phase can be calculated from the curves of back fluorometric titration as described earlier (Ivkova et al. 1983). Fig. 2 illustrates these dependences for two wavelengths (660 and 700 nm). Similarly to our previous works, the probe concentrations were expressed as probe to lipid ratios, and the fluorescence

intensities as the corresponding concentrations of effective fluorescent monomers, $n_{p\ mom}^{m}/n_l$. It can be seen that the concentration quenching at $\lambda_f = 660$ nm is more effective than that at $\lambda_f = 700$ nm.

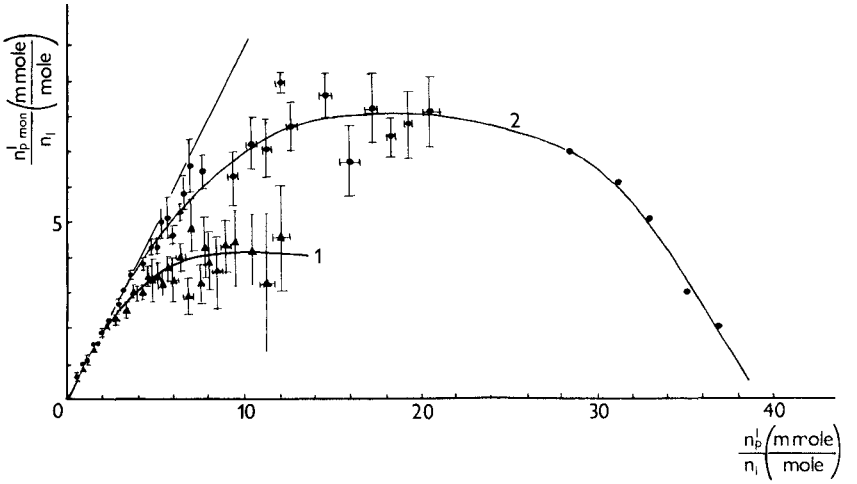

Fig. 2. Dependence of diS-C_3-(5) fluorescence intensity expressed in terms of fluorescing monomer concentration on the probe concentration in the lipid phase (1 — calculations from back fluorometric titration curves at $\lambda = 660$ nm, 2 — at $\lambda = 700$ nm), 45 °C

Analysis of the probe fluorescence response

The diffusion transmembrane potential is determined by the gradient of K^+ established between the aqueous media inside and outside the liposomes. It can be generated by valinomycin in liquid crystalline, but not in quasicrystalline (solid) bilayer vesicles (Krasne et al. 1971). The potential formed accross a liquid crystalline membrane will be retained upon solidation of the lipid if the ion gradient does not dissipate at the phase transition temperature. It is known that the ion permeability of a lipid bilayer increases greatly at T_c (Papahadjopoulos et al. 1973). The question arises whether similar effects would evoke the dissipation of transmembrane ion gradients in large unilamellar DPPC liposomes prepared by the detergent removal technique.

To elucidate this question, a number of experiments was carried out. Fig. 3 shows fluorescence intensity (F_{660}) changes induced by alternately cooling and heating the vesicle suspension (continuous recording over ~ 1 hour). The initial decrease in fluorescence upon liposome addition results from the dye distribution between the aqueous and the membrane phase ($\lambda_{max}^{f} \simeq 670$ nm in water and 685 nm in membranes). Valinomycin generated a diffusion transmembrane potential (~ 130 mV at a concentration ratio of $[K^+]_{in}/[K^+]_{out} = 200$) if it was added at temperatures higher than that of DPPC phase transition (Fig. 3 *a*, *c*).

This potential induced a decrease in fluorescence intensity due to the redistribution of positively charged dye molecules between the aqueous and the membrane phase and in the membrane interior (Ivkova et al. 1984). Cooling of the sample from 45 °C to 34 °C resulted in an increase in fluorescence intensity at $\lambda_f = 660$ nm due to the ejection of the probe from bilayers to water during DPPC phase transition. Repeated heating of the lipid suspension to 45 °C practically restored F_{660} to the original level.

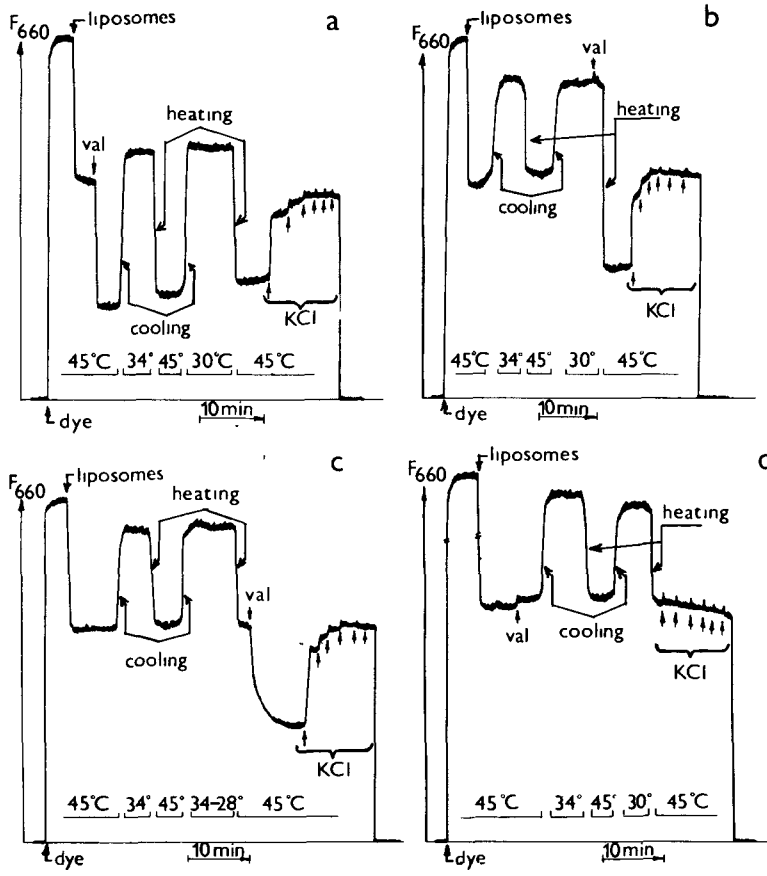

Fig. 3. Changes of the fluorescence intensity at $\lambda = 660$ nm (continuous registration) induced by various agents. The probe concentration in the sample was 0.7 μmol/l. 10 μl of DPPC liposomes (20 mg/ml in 0.2 mol/l KCl, 0.02 mol/l Tris · HCl, pH 7.5) were added to the medium (2 ml) containing 0.02 mol/l Tris · HCl and 0.2 mol/l NaCl (*a*, *b*, *c*) or 0.2 mol/l KCl (*d*). The valinomycin (val) concentration was 5×10^{-8} mol/l. The gradient $[K^+]_{in}/[K^+]_{out} = 200$, $\Delta\psi \simeq 130$ mV in (*a*, *b*, *c*) and $\Delta\psi = 0$ in (*d*). The arrows indicate a series of KCl additions (20 μl, 3 mol/l KCl).

The final effect was almost entirely independent of the moment of the valinomycin addition (Fig. 3 *a*, *c*). The addition of valinomycin to "solid" liposomes (Fig. 3 *b*) left fluorescence intensity (F_{660}) unchanged; however, subsequent heating had a similar effect as that shown in Fig. 3 *a*, *c*. A comparison of these results with those shown in Fig. 3 *d* (with no potassium ion gradient between the inner and outer aqueous media in the latter) shows that F_{660} decreased as a result of generation of a transmembrane potential and not of the addition of valinomycin.

At the end of each experiment, the transmembrane potential was decreased by repeated additions of KCl (20 μl, 3 mol/l KCl) for control purposes. KCl had no effect when no transmembrane potential was present, due to $[K^+]_{in} = [K^+]_{out}$ (Fig. 3 *d*); similar result was also obtained with a gradient of K^+ between the inner and outer aqueous media with no valinomycin in the suspension (not shown).

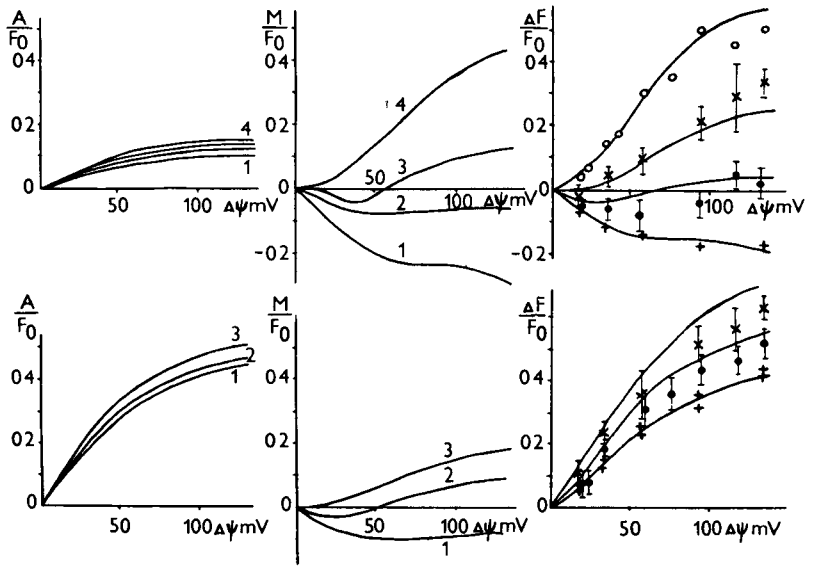

Fig. 4. Dependence of the fluorescence response of diS-C_3-(5) at $\lambda_f = 700$ nm (*a*, *b*, *c*) and $\lambda_f = 660$ nm (*d*, *e*, *f*) on the transmembrane potential in unilamellar DPPC liposome suspension The theoretical calculations are by the solid lines *a*, *d* — the contributions of the aqueous probe component, *b*, *f* — the contributions of the membrane probe component, *c*, *e* — the total fluorescence response $\Delta F/F_0 = (A + M)/F_0$. The numbered curves were calculated for the following dye concentrations 1 — 0 25 μmol/l, 2 — 0 5 μmol/l, 3 — 1 μmol/l, 4 — 2μmol/l Points are average values of 2—5 identical preparations. Standard deviations are given Total probe concentrations in the sample volume; + — 0 25 μmol/l, × — 1 μmol/l, ● — 0 5 μmol/l, ○ — 2 μmol/l

The value of the fluorescence response to K^+-valinomycin induced transmembrane potential was calculated using a simple thermodynamic model (Ivkov et al. 1984). Fluorescence intensities for different concentrations of the probe in membrane phase were devided from concentration dependences shown in Fig. 2. The expected and actually obtained results for two wavelengths at 45 °C are shown in Fig. 4.

Effects of the lipid-phase dissolved probe on the membrane structure

Disturbances in the lipid bilayer structure induced by "admixtures" of the probe can be analysing DSC thermograms. As mentioned above (see Materials and Methods), the main phase transition peak remains practically unchanged at low admixture concentrations; the pretransition peak is however very sensitive to admixtures. Fig. 5 and Table 1 show effects of various probe concentrations on phase transition in multilamellar DPPC liposomes. The probe concentrations in the lipid phase were calculated according to the partition coefficient for solid bilayers:

$$\alpha_l = \alpha_0 \frac{K_p v_m}{v_{out} + K_p v_m},$$

where α_0 and α_l are the probe/lipid ratios in the sample and in lipid phase, respectively; v_m and v_{out} are the volumes of the lipid and the aqueous phase (usually $v_{out} \gg v_m$); K_p is the partition coefficient.

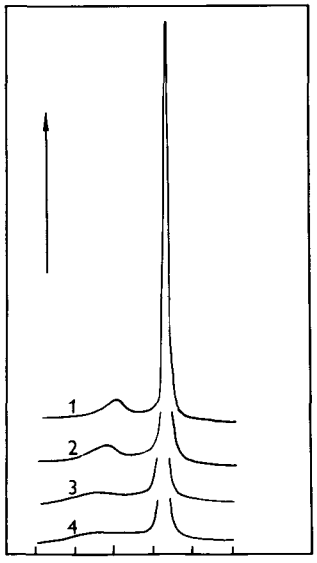

Fig. 5. DSC heating curves of multilamellar DPPC liposome suspension at various probe/lipid ratios in membranes The medium contained 0 1 mol/l KCl, 0 1 mol/l sucrose, 10 mmol/l imidazole HCl, pH 7 0 The lipid concentration was 0 4 mg/ml Heating rate = 1 °C/min Probe concentrations in lipid phase 1 — without probe, 2 — 3 mmols of probe per 1 mol of DPPC, 3 — 8 mmols of probe per 1 mol of DPPC, 4 — 27 mmols of probe per 1 mol of DPPC

Table 1. Phase transition parameters of multilamellar dispersions of dipalmitoylphosphatidylcholine (DPPC) at various ratios of diS-C_3-(5) and DPPC in the membrane T_p and T_c are transition temperatures for pretransition and main transition, respectively, $T_{1/2}$ is the width of the main transition peak at half its height, n_p/n_l is the probe/lipid ratio in the lipid phase For experimental conditions, see Fig 5

	Medium	$\frac{n_p(\text{mmol/l})}{n_l(\text{mol/l})}$	T_p (°C)	T_c (°C)	$T_{1/2}$ (°C)
1	H_2O	0	35 4	41 3	0 6
1a	0 1 mol/l sucrose,	0	35 4	41 4	0 7
	100 mmol/l KCl,	3	34 9	41 4	0 7
	10 mmol/l imidazole HCl,	8	32 9	41 2	0 8
	pH 7 0	27	32 2	41 2	0 7
1b	0 1 mol/l surcose, 100 mmol/l KCl,	0	35 4	41 7	0 7
	10 mmol/l imidazole HCl,	3	33 4	41 2	0 8
	pH 7 0, 1 mmol/l $MgCl_2$,	8	33 4	41 2	0 7
	0 1 mmol/l $CaCl_2$	27	—	40 8	0 8
2a	0 3 mol/l sucrose,	0	35 8	41 8	0 7
	10 mmol/l imidazole HCl, pH 7 0	3	33 5	41 1	0 7
		8	32 8	41 1	0 7
		27	31 1	41 1	0 8
2b	0 3 mol/l sucrose,	0	35 0	41 2	0 7
	10 mmol/l imidazole HCl, pH 7 0,	3	32 2	41 2	0 8
	1 mmol/l $MgCl_2$, 0 1 mmol/l $CaCl_2$	8	32 2	41 2	0 8
		27	31 9	42 1	0 8

Discussion

In the absence of transmembrane potential distribution of the probe in a liposome suspension is characterized by the partition coefficient, which depends on both the membrane charge (Ivkova et al. 1982) and hydrocarbon chain packing in a liquid crystalline lipid bilayer. The latter is mainly determined by the ratio of saturated to unsaturated fatty acid residues. In this respect, differences in partition coefficients between DPPC and EPC suspensions are highly significant. Such a large difference cannot be explained by a greater average area per molecule in EPC bilayers (0.7 nm^2) as compared with that in DPPC liquid crystalline bilayers ($\sim 0.68\,nm^2$). An ever more pronounced difference in the area per molecule between DPPC bilayers above ($\sim 0.68\,nm^2$) and below ($\sim 0.53\,nm^2$) phase transition (Ivkov and Berestovsky 1981) is accompanied by a decrease in the value of K as high a factor as 2.4.

It should be emphasized that similar changes in K (by a factor of 2—2.5) are typical of dyes such as ANS and BTB (Sackmann and Träuble 1972). Smaller

changes in K were observed in dimyristoylphosphatidylcholine (DMPC) liposome suspension for n-butyramide, ethyl acetate and acetone (Katz and Diamond 1974).

Molecules of amphiphilic probes are usually arranged along the boundary between the polar and the hydrocarbon region of the bilayer (Vladimirov and Dobretsov 1980; Ivkova et al. 1984). They may be localized in the "defects" between more closely packed phospholipid clusters in "liquid", and in microdomains in solid bilayers. No reliable data are available concerning how many probe molecules can be dissolved in the "liquid" lipid phase without appreciable disturbances in the membrane structure. However, based on DSC thermograms, some conclusions can be drawn for quasicrystalline DPPC bilayers.

Typical thermograms are shown in Fig. 5. It is striking that the main transition peak remains practically unchanged at any probe concentration used. The maximum concentration, 27 probe molecules per 1000 lipid molecules, is close to the limiting probe concentration, at which a complete concentration quenching of fluorescence occurs (Fig. 2). The absence of detectable changes in the main phase transition points to the fact that both the microdomain size (cooperative unit) and the hydrocarbon chain packing in these microdomains were retained at the probe concentrations studied. The probes were probably not included into clusters in the liquid crystalline bilayer either. Unlike the main transition, pretransition is extremely sensitive to low concentrations of admixtures. Its presence up to a probe concentration of 30 moles per 1000 moles of lipid (Table 1) suggests that the chain tilt and the orientation order are preserved in sufficiently large microdomains.

It is very difficult to determine the complete relationship between fluorescence and the probe concentration in the lipid phase as illustrated in Fig. 2. At low lipid concentrations, when much less probe molecules are in membranes than in water, the data show a significant discrepancy. The problem can probably be solved by increasing the number of experiments; however, no sufficiently reliable results were obtained at high probe concentrations in membranes at $\lambda_f = 660$ nm. We believe that all this are the reasons for the discrepancy between the theoretical and experimentally observed $(\Delta F/F_0)$ dependences on $\Delta\psi$ at high probe concentrations (Fig. 4*f*).

At $\lambda_f = 700$nm the fluorescence intensity is almost exclusively determined by membrane probes, and the curve of the concentration quenching can be plotted more exactly. As a result, the theoretical $(\Delta F/F_0)$ dependence on $\Delta\psi$ is closer to the experimental one. At this wavelength, a negative response (fluorescence increase) is observed at small initial probe concentrations ($\sim 0.25 \times 10^{-6}$ mol/l). At a probe concentration of $\sim 0.5 \times 10^{-6}$ mol/l a negative fluorescent response is observed at $\Delta\psi \approx 50$ mV. The increase in fluore-

scence is due to the redistribution of the probe from the aqueous into lipid phase without quenching at the inner membrane monolayer.

It is obvious that the curves shown Fig. 4 (*c*,*f*) represent a sum of both probe components, the aqueous and the lipid phase. To analyse the behaviour of each component, we calculated theoretically their respective fluorescences. Fig. 4 (*a*—*c*) illustrates the results of the calculations for $\lambda_f = 700$ nm and Fig. 4 (*d*—*f*) those for $\lambda_f = 660$ nm. The fluorescence of the aqueous component always decreases at an inside-negative transmembrane potential. The fluorescence of the membrane component depends on two opposite processes: the ejection of the probe from the aqueous to the lipid phase under the action of a transmembrane potential, and redistribution of the probe between the outer and the inner membrane monolayer. Their ratio determines the nature of the potential dependent fluorescent response.

Thus, the fluorescent probe diS-C_3-(5) even at maximal concentrations does not produce any appreciable distortions in a lipid bilayer. The probe partition coefficients are similar for temperatures both above and below the phase transition temperature; this allows to suggest that the probes are included in "defects" between structurally ordered microregions (microdomains or clusters) without any detectable effects on the inner structure of these regions.

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