

The Behavior of the Fluorescent Probe diS-C₃-(5) in Membrane and Aqueous Media

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Abstract. Based on an analysis of back fluorometric titration data a partition coefficient, $K_p = (5.70 \pm 0.95) \times 10^4$, and a partition constant, $K = (2.37 \pm 0.43) \times 10^6$, were found for the probe diS-C₃-(5) in egg lecithin vesicle suspension. The relative probe quantity in an aqueous medium and in liposomes was calculated using these parameters. The number of chromophore states in this system was computer-analysed and it was shown that the probe fluorescence could be described by two fluorescing dye forms, aqueous and membrane monomers. The dependence of fluorescence intensity on the probe concentration was studied in various salt media, and a dimerization (association) constant $K_a = 5 \times 10^4 \text{ mol}^{-1} \cdot \text{l}$ in the buffer, and $K_a = (8.1 \pm 1.5) \times 10^4 \text{ mol}^{-1} \cdot \text{l}$ in 0.1 or 0.2 mol/l salt medium (KCl or NaCl) was found. From the fluorescence and absorption data critical concentrations of the onset of large probe aggregate formation were calculated for various aqueous media. The concentration dependence of the probe fluorescence in the membrane phase was calculated. The critical concentration of interaction characterizing the efficiency of the fluorescence concentration quenching processes (CCI) was found to be approx. 5—6 mol probe per 1000 mol lipid. The top probe concentration in a membrane (the "saturation" concentration) was estimated from the slope of the initial linear parts of the back fluorometric titration curves, and was found to be equal to (59 ± 13) mol probe per 1000 mol lipid.

Key words: Lecithin liposomes — Potential-sensitive probe diS-C₃-(5) — Partition coefficient — Dimerization constant — Concentration fluorescence quenching — Probe aggregation

Introduction

The method of estimation of a potential-sensitive carbocyanine probe* distribution between an aqueous medium and membranes has been described in our previous article (Ivkova et al. 1982). It is based on the analysis of the initial parts of the back

* diS-C₃-(5): 3,3'-dipropyl-2,2'-thiadicarbocyanin

fluorometric titration curves. With a known partition coefficient it is possible to calculate the probe quantity both in the aqueous and lipid phase, and to estimate the contributions of the aqueous and membrane components to fluorescence.

Qualitative analysis of the fluorescence and absorption data for this probe type have shown that the concentration quenching of the dye fluorescence underlies a potential-dependent optical response (Sims et al. 1974; Waggoner et al. 1977; Waggoner 1979; Krasne 1980; Beeler et al. 1981). The local increase in a probe concentration in the internal vesicular aqueous volume or in the membrane interior is a result of probe redistribution due to the transmembrane potential. To describe the processes quantitatively it is necessary to know the dependence of fluorescence on the dye concentration in both the aqueous and membrane phase; this dependence is the main subject of the present article.

Material and Methods

Ten percent alcohol solution of lecithin-standard (Kharkov, USSR), and deoxycholate or cholate ("Fluka") were used to prepare liposomes. Unilamellar vesicles were obtained by the detergent removal technique. Tris-HCl buffer ("Serva") was used for media preparation.

Egg lecithin was tested spectrophotometrically for excessive oxidation. The oxidation index for the ethanol solution was checked from the optic density ratio at $\lambda = 215$ nm and 233 nm (Klein 1970).

The probe diS-C₃-(5) was a generous gift of Prof. A. Waggoner (USA). The stock ethanol solution of the probe was kept at -20°C in dark.

Absorption spectra were recorded with a Specord UV VIS. Fluorescence spectra were measured with a unique spectrofluorometer. Fluorescence of a thin layer (~ 1 mm) of the solution was recorded from the front surface of the sample. For a more detailed description of the instrument see our previous paper (Ivkova et al. 1982). It should be noted that the interference filter was replaced by a new one (Karl Zeiss) with better parameters (HBW 7 nm and $\tau_{\text{max}} = 23\%$).

Solutions were stirred with a magnetic mixer. All measurements were made at room temperature.

Adsorption of the probe to the cuvette walls under our experimental conditions was quantitatively estimated. About 10% of the total probe quantity in the solution adsorbed to the quartz walls. Apparently the adsorption capacity of quartz is markedly less than that of glass. Probe adsorption generally depends on the ionic strength of the solution, on the presence of liposomes, and on the duration of the experiment. These factors were taken into account when performing our experiments; however, no corrections of the results were made since the influence of these factors lie within the limits of the experimental error.

Results

Probe distribution between the aqueous and lipid phase

The partition constant* (K) and the partition coefficient (K_p) of the probe

* We introduce the term "partition constant", as opposed to the partition coefficient, to describe the probe distribution over the molar part units in contrast to the concentration units.

diS-C₃-(5) in egg lecithin liposome suspension were calculated from data of 11 experiments. The values obtained [$K = (2.37 \pm 0.43) \times 10^6$; $K_p = (5.70 \pm 0.95) \times 10^4$] were by about one order of magnitude lower than the respective values for azolectin liposomes of those for SR vesicles (Ivkova et al. 1982). Back titration curves are not shown here since they are similar to those reported in our previous paper (Ivkova et al. 1982).

Using K_p , the relative probe quantity in an aqueous medium and in membranes can be easily estimated:

$$\frac{n_p^a}{n_p^o} = \frac{1}{1 + \frac{K_p v_m}{v_a}}; \quad \frac{n_p^m}{n_p^o} = \frac{1}{1 + \frac{v_a}{K_p v_m}}, \quad (1)$$

where n_p^a and n_p^m are the number of probe molecules in the aqueous and membrane phase, v_a and v_m are the volumes of these respective phases; n_p^o is the total probe quantity in the sample: $n_p^o = n_p^a + n_p^m$.

The v_m/v_a ratio can be expressed as the molar lipid concentration in the sample volume:

$$\frac{v_m}{v_a} = M \bar{V}_l c_l \cdot 10^{-3}, \quad (2)$$

where c_l is the lipid concentration (mol/l), \bar{V}_l is partial specific volume (cm³/g), and M is the mass of one mole of the lipid. The molecular mass of egg lecithin is about 775, its partial specific volume at 25 °C is 0.984 cm³·g⁻¹ (Reiss-Husson 1967).

Thus taking into account that $K \approx 5.7 \times 10^4$, the fraction of the "aqueous" probe is approximately:

$$\frac{n_p^a}{n_p^o} = \frac{1}{1 + 4.35 \times 10^4 \cdot c_l}, \quad \frac{n_p^m}{n_p^o} = 1 - \frac{n_p^a}{n_p^o} \quad (3)$$

Absorption spectra of diS-C₃-(5) in 0.2 mol/l KCl upon various lecithin additions are shown in Fig. 1. An increase in the lipid concentration results in a spectrum shift from the "aqueous" ($\lambda_{\max} = 647$ nm) to the "membrane" position ($\lambda_{\max} = 666$ nm). At the highest lipid concentration (spectrum 11) only 2.4 % of all the probe molecules are in aqueous medium, as it can be estimated from expression (4). At a lipid concentration of 26×10^{-6} mol/l (spectrum 4) the "aqueous" probe makes up about 50 per cent of all molecules.

Testing of the number of chromophore states

Fluorescence spectra of diS-C₃-(5) upon various liposome additions are shown in Fig. 2. Spectrum 1 (no lipids) and spectrum 13 (high lipid concentration) can be

of the spectrum shape (see spectra 1—4 in Fig. 2, and the segment "ab" in Fig. 3). An increase in the liposome concentration results in a red shift of the spectrum and in an increase in the spectrum halfwidth (curves 2,3 in Fig. 3A). Moreover, there is a decrease in the quenching efficiency (spectra 6, 7 in Fig. 2). At high lipid concentrations, the spectrum shift reaches a limit magnitude and the spectrum halfwidth decreases, again, to a constant value (segment "cd" in Fig. 3A).

Thus three different levels of the probe/lipid ratio can be distinguished: 1) a segment "ab", where only the "aqueous" monomer fluorescence occurs, 2) a segment "cd", where the "membrane" monomer fluorescence is mainly observed, 3) an intermediate segment "bc", where the existence of the two fluorescing forms results in an increase in spectrum width and in a gradual longwave shift of the spectrum.

The computer analysis has shown that all the intermediate spectra can be described as the superposition of only two fluorescing chromophore forms with an error below 3 %. The relative fluorescence contributions of these forms are shown in Fig. 3B.

The absence of the isoemission points in the fluorescence spectra (Fig. 2) is a result of nonfluorescing aggregate formation. At a total probe concentration in the sample of $(1-2) \times 10^{-6}$ mol/l contribution of the "aqueous" monomers may be neglected if the probe/lipid ratio is below 0.01. At ratios exceeding 0.1, the contribution of "membrane" monomers can be neglected.

Dependence of fluorescence on the probe concentration in the aqueous medium

Since our considerations concern sufficiently diluted probe solutions, the dimerization (association) constant can be expressed as concentrations:

$$K_a = \frac{|d|}{|m|^2} \quad (4)$$

where $|m|$ and $|d|$ are the monomer and dimer concentration, respectively. In this case, the total probe concentration is:

$$c_p^a = n_p^a / v_a = |m| + 2K_a |m|^2 \quad (5)$$

The measured fluorescence intensity is proportional to the monomer concentration as shown earlier:

$$F = q \cdot |m|, \quad (6)$$

where q is the proportionality coefficient equal to the fluorescence value of one mole of the probe monomers measured under the given experimental conditions. This coefficient may be calculated from the slope of the initial segment of the concentration dependence curve of the probe fluorescence.

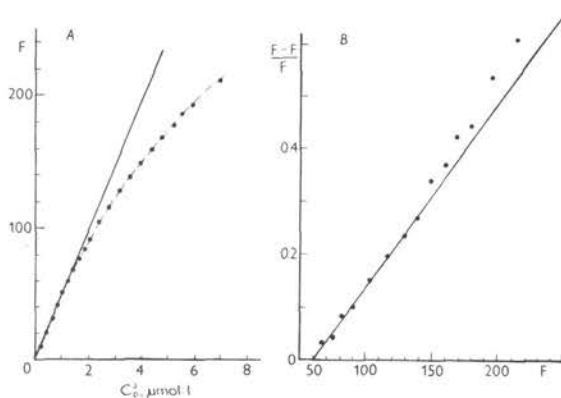


Fig. 4. Effect of dimerization on the fluorescence of the probe diS-C₃-(5) in an aqueous medium containing 0.1 mol/l NaCl, 0.2 mol/l tris HCl (pH 7.5). A: dependence of the fluorescence intensity (in relative units) at $\lambda_r = 690$ nm on the probe concentration. Points represent experimental data, the full line represents the theoretical value of F_0 at the absence of dimerization. B: plot for calculation of the dimerization constant.

The equation for estimation of K_a may be received from (5) and (6):

$$\frac{F_0 - F}{F} = \frac{2K_a}{q} \cdot F, \quad (7)$$

where F_0 is the fluorescence intensity which may occur in the absence of dimerization, and F is the actual fluorescence intensity.

Dependence of fluorescence on the probe concentration in the medium containing 0.1 mol/l NaCl is shown in Fig. 4A. The full line represents the F_0 plot. The slope of this line gives the value of q .

The linear relationship of $(F_0 - F)/F$ and F (Fig. 4B) at low dye concentrations shows that dimerization occurs in the concentration range of $\sim 1 \times 10^{-6}$ to $\sim 4 \times 10^{-6}$ mol/l. The dimerization (association) constant K_a can be obtained from the line slope according to the formula (7). K_a depends on the ionic strength: it is $\sim 5 \times 10^4 \text{ mol}^{-1} \cdot \text{l}$ in the buffer ($2 \times 10^{-2} \text{ mol/l}$ Tris. HCl), and $(8.1 \pm 1.5) \times 10^4 \text{ mol}^{-1} \cdot \text{l}$ in the salt medium containing 0.1 or 0.2 mol/l KCl (NaCl).

The respective dissociation constant K_d is $\sim 2 \times 10^{-5}$ and $(1.27 \pm 0.23) \times 10^{-5} \text{ mol/l}$. These values are close to the dissociation constant $K_d \approx 1.5 \times 10^{-5} \text{ mol/l}$ estimated by West and Pearce from the analysis of the absorption data for the probe diS-C₂-(5) in water (West and Pearce 1965).

The deviation of the $(F_0 - F)/F$ plot from the straight line at probe concentrations exceeding $4 \times 10^{-6} \text{ mol/l}$ is a result of aggregation processes. Within this range the estimation of the magnitude of concentration quenching can be made from the concentration dependence of F (Fig. 4A).

With a known value of K_a the monomer concentration, and consequently the

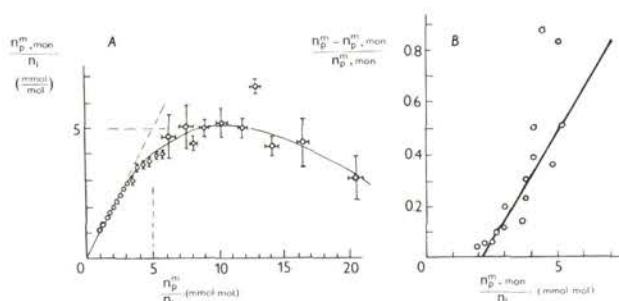


Fig. 5. Effect of aggregation on the fluorescence of the probe diS-C₁₂(5) in egg lecithin membranes. **A:** fluorescing monomer concentration dependence calculated from back fluorometric titration data, the crossing of the dotted lines gives the CCI value. **B:** plot to check the existence of dimerization.

fluorescence intensity, can be calculated from (5) and (6):

$$F = q \cdot \frac{\sqrt{8K_a c_p^a + 1} - 1}{4K_a} \quad (8)$$

at probe concentrations (c_p^a) between 1 to $\sim 4 \times 10^{-6}$ mol/l. In liposome suspension the probe concentration in the aqueous medium depends on the lipid concentration. It can be calculated using Eqs. (1)–(3).

Aggregate formation may be also recorded in the absorption spectra from the presence of the wide longwave maximum (J-peak) and of the spread shortwave peak (H-peak) (West and Pearce 1965; Sims et al. 1974).

Dependence of fluorescence on the probe concentration in the lipid phase

At a high liposome concentration the fluorescence and absorption spectra remain in fact unchanged upon the subsequent addition of the lipid (see spectra 11–13 in Fig. 2, and 8–11 in Fig. 1), i. e. the bulk of the probe molecules is in the membranes in the monomer form. At the maximum lipid concentration (spectrum 13 in Fig. 2) e. g. the dye quantity in the aqueous medium does not exceed 3 % of its total quantity. This permits calculations of the effective fluorescence intensity, q_m , of 1 mole of dye monomers in the lipid phase.

The relative probe quantity in membranes and the aqueous medium can be estimated according to Eqs. (1)–(3), and the fluorescence contribution of the aqueous component (F_a) according to expression (6) or (8). Thus the fluorescence intensity of the membrane component $F_m = F - F_a$, and the fluorescing dye monomer concentration is F_m/q_m .

The dependence of the probe fluorescence on its concentration in the membrane is shown in Fig. 5. It was calculated from the back fluorometric titration

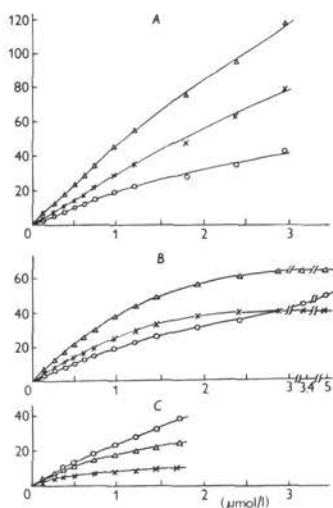


Fig. 6. Direct fluorometric titration curves of the probe diS-C₃-(5) in a medium containing 0.2 mol/l KCl and 0.02 mol/l tris. HCl, pH 7.5. Fixed lecithin concentrations in the sample: A, 1.3×10^{-3} mol/l; B, 0.13×10^{-3} mol/l; C, 0.013×10^{-3} mol/l. Fluorescence intensity was measured at: 660 nm (circles); 685 nm (triangles); 700 nm (crosses). Abscissa: Probe concentration, ordinate: fluorescence (in arbitrary units).

data. The plot is similar to aggregation curves for micelle formation (Tanford 1973). Values of the critical interaction concentration (CCI), analogous to critical micelle concentration (CMC) can be determined.

Similar calculations have been made based on data of the direct fluorometric titration (titration with a dye at a constant lipid concentration). Examples of these curves are shown in Fig. 6, and the calculated dependence of fluorescing monomer concentration on the total probe concentration in the membrane phase is given in Fig. 7. This dependence is generally analogous to that of the curves calculated from back fluorometric titration data and it is mainly determined by probe aggregation in membranes.

Although the mechanism of the probe fluorescence quenching in membranes is not completely understood it may be assumed that aggregation plays a key role in this process. A sharp increase in the nonspecific membrane permeability at the probe/lipid ratios at which maximum quenching is observed, and the rapid fall of the membrane potential produced may be regarded as an indirect evidence of the formation of probe aggregates in membranes. This assumption may also be confirmed by the analysis of the absorption spectra where the marked decrease in the "aqueous" monomer absorption (spectra 2, 3 in Fig. 1) is not compensated for by a corresponding growth of the absorption of the "membrane" monomers and dimers.

The CCI value can probably characterize the onset of aggregate formation.

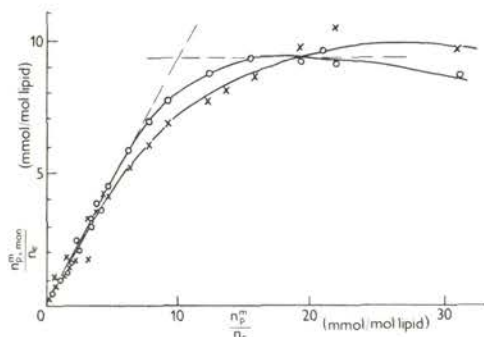


Fig. 7. Calculated dependence of the fluorescing monomer concentration in membranes on the total dye concentration in the lipid phase. The curves were obtained from direct fluorometric titration data shown in Fig. 6 at two wavelengths: 660 nm (circles); 700 nm (crosses).

For lecithin membranes, the CCI is about 5 mol probe per 1000 mol lipid (Fig. 5A) and it is of the same order as the values for some other membranes (Ivkova et al. 1983). Probe aggregation in membranes may be studied in analogy with the analysis of probe dimerization in an aqueous medium.

For probe concentrations in lipid phase the expression (5) can be written as molar portions:

$$\frac{n_p^m}{n_l} = \frac{n_p^m \text{ mon}}{n_l} + 2K_a^m \cdot \left(\frac{n_p^m \text{ mon}}{n_l} \right)^2, \quad (9)$$

where n_p^m is the total probe quantity in the lipid, n_l is the lipid quantity, and $n_p^m \text{ mon}$ is the number of probe monomers. This equation is transformed into:

$$\frac{n_p^m - n_p^m \text{ mon}}{n_p^m \text{ mon}} = 2K_a^m \frac{n_p^m \text{ mon}}{n_l} \quad (10)$$

The linear relationship between $\frac{n_p^m - n_p^m \text{ mon}}{n_p^m \text{ mon}}$ and $\frac{n_p^m \text{ mon}}{n_l}$ indicates the existence of a bimolecular process, and the slope of the line gives the value of the dimerization constant (Fig. 5B). A deviation from this line points to the onset of aggregation at probe concentrations about 4–5 mol probe per 1000 mol lipid. A somewhat larger CCI value could be obtained from direct titration data (Fig. 7). In this case however the average and not individual value of the partition coefficient was used to calculate the concentration dependence.

At low lipid concentrations (spectra 2, 3 in Fig. 2) the fluorescence of “aqueous” monomers is only observed. Fluorescence of the membrane probe is in fact completely quenched at these probe concentrations which are somewhat below the top probe concentration in the lipid phase, i. e. the concentration of

"saturation". Completion of the aggregation can be determined immediately from back titration curves, or from the concentration dependence of the intensity ratio at the spectrum slopes (F_{700}/F_{660}) (Ivkova et al. 1982).

At a total probe concentration exceeding $\sim 1 \times 10^{-6}$ mol/l the back fluorometric titration curve has a linear initial part similar to that obtained earlier for SR vesicles (Ivkova et al. 1982). The slope of this line gives the value of the "saturation" concentration. The latter is apparently governed by the effect of the electrostatic repulsion of the probe molecules in the membrane. For egg lecithin liposomes this value is about (59 ± 13) mol probe per 1000 mol lipid.

It should be noted that the marked divergence of the experimental data (Fig. 5) can result from a slow and uncontrolled aggregate breakdown in membranes and from probe redistribution between liposomes.

Discussion

The transmembrane potential obviously effects probe redistribution between the external and internal aqueous vesicular volume. The potential-dependent dye redistribution can probably occur in the membrane interior, and also between the aqueous and lipid phase is the first and necessary step in the description of the potential-dependent response mechanism.

The quantitative parameter characterizing the probe partition in the membrane suspension is calculated from the initial segments of back fluorometric titration curves, where of both the lipid and probe concentrations in the lipid phase are of the same order. The partition coefficient expressed as molar parts, i. e. according to our terminology "the partition constant" is more accurate for this analysis. However, for the concentration range over which potential-dependent optical response is measured, the probe/lipid ratio in membranes is markedly below 0.1 and the ordinary partition coefficient (expressed in volume concentration units) may be used; this simplifies the calculations.

Let us estimate relative probe quantities in the aqueous and membrane phase for some lipid concentrations usually used in experiments.

The partial specific volume of lecithin is $\sim 1 \text{ cm}^3 \cdot \text{g}^{-1}$, and the values of v_m/v_a in Eq. (1) can easily be estimated. Calculations show that the aqueous probe fraction is about 1.7 % for a lipid concentration of 1 mg/ml, about 15 % for 0.1 mg/ml, and 64 % for 0.01 mg/ml.

These estimations would be valid if the correct probe to lipid ratio was picked out. Data on the back fluorometric titration indicate that membranes can accommodate a limited quantity of the probe only (Ivkova et al. 1982). Saturation effects are observed at probe/lipid ratios exceeding 1/18, and, as a result, at a lipid concentration of 0.01 mg/ml ($\sim 13 \times 10^{-6}$ mol/l) calculation according to Eqs. (1)–(3) can give a marked mistake if the total probe concentration exceeds approx. 2×10^{-6} mol/l.

Quenching of the probe fluorescence in the aqueous medium is determined by dimerization and aggregation processes. As it has been shown earlier at a dye concentration of $(1-4) \times 10^{-6}$ mol/l only dimers are formed. The linear relationship between $(F_0 - F)/F$ and F (Fig. 4B) shows within this concentration range a bimolecular process occurs. The deviation from the line shows that the aggregates consist of more than two molecules, and the dimerization constant can be calculated for a limited concentration range.

The rate of aggregate production and breakdown depends on the probe concentration, ionic strength of the medium, temperature, and other experimental conditions. Also it may depend on aggregate dimensions. Dimers and small aggregates, for instance, are formed and broken up rapidly enough as related to the time-scale of an experiment. Production of large aggregates is however a very slow process which continues for tens of minutes, or even hours.

This process becomes enhanced when the concentration reaches some critical value. For example, in 0.1 mol/l NaCl or KCl, the aggregation time decreases to minutes at a probe concentrations of $(6-7) \times 10^{-6}$ mol/l. At this concentration the probe monomer concentration and the fluorescence intensity reaches maximum, and it sharply decreases with a further increase in the probe concentration.

In 0.2 mol/l NaCl or KCl, the sharp decrease in the fluorescence resulting from the production of large aggregates starts at a probe concentration of $(2.8 \pm 0.4) \times 10^{-6}$ mol/l.

The study of breakdown processes of large aggregates using the dilution technique shows that disaggregation is very slow as related to the time-scale of an experiment. It apparently continues for tens of minutes, or even several hours. The rate of this process also depends of the probe concentration.

It has already been mentioned that probe aggregation in membranes can be characterized by the value of the critical interaction concentration (CCI). It is approx. 5 mol probe per 1000 mol lipid for egg lecithin liposomes, approx. 8 per 1000, and 9 per 1000 for azolectin liposomes and SR vesicles, respectively; all these values were obtained for salt media (Ivkova et al. 1983).

In the lipid phase probe aggregates are formed at significantly higher concentrations as compared with the aqueous medium. It is possible to estimate the chemical potential dependence for a probe monomer in any solvent, and for a probe molecule in an aggregate (micelle) suspended in the same solvent. Rough estimations can be made from the CCI value according to the expression (Tanford 1973):

$$\Delta\mu = \mu_{\text{agg}}^{\circ} - \mu_{\text{mon}}^{\circ} = 2.3 RT \lg \text{CCI}, \quad (10)$$

where μ_{agg}° and μ_{mon}° are the chemical potentials of a probe molecule in aggregates and in the monomer form respectively, R is the universal gas constant, T is the

temperature (K), and CCI is the critical interaction constant expressed as molar parts.

For the aqueous phase, CCI can be determined as the concentration at which large aggregates begin to form: it is approx. $7 \cdot 10^{-6}$ mol/l in 0.1 mol/l KCl, and approx. 3×10^{-6} mol/l in 0.2 mol/l KCl (the same values approx. for NaCl). Expressed in the units of molar parts, these values are approx. 1.3×10^{-7} and 5.3×10^{-8} mol probe per 1 mol water. According to (9), $\Delta\mu$ is approximately -12 and -10 kcal/mol for 0.1 and 0.2 mol/l salt solution.

In the lipid phase, CCI is approx. 10^{-2} mol probe per 1 mol lipid, and the respective value of $-\Delta\mu$ is below 3 kcal/mol. This approximate estimation gives only the order of the energy decrease due to aggregation.

The rate of formation and breakdown of aggregates apparently depends on many factors not checked during the experiment. It especially influences the experimental results of back fluorometric titration of the probe solution by lipid additions with in the small lipid concentration range. In those experiments small aliquots of liposomes without probe were added to the suspension of liposomes saturated by probe aggregates. In this case, redistribution through the aqueous solutions occurs which "smoothes" the concentration drop.

Aggregation may not be the only mechanism of fluorescence quenching. The inductive — resonance transfer effects may contribute on this process as well (Krasne 1980). Diffusion of the probe molecule along the membrane is much slower than in water; on the other hand, the average molecule separation in the membrane is 40–50 Å at a probe concentrations of 40–25 mol per 1000 mol lipid, i. e. it is of the order of the Forster radius (Parker 1972).

In conclusion, it can be noted that the observed concentration dependences of the probe fluorescence in both membranes and the aqueous medium allow an approximative estimation of the value of the potential-dependent optical response, provided that the probe redistribution under the transmembrane potential action is known.

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Correction

to the paper M. N. Ivkova et al. (1982): "Distribution of potential-sensitive probe diS-C₃-(5) in membrane suspension" *Gen. Physiol. Biophys.* **1**, 209-219.

In the manuscript of the above paper, Eq. (11) for an appropriate estimation of the partition constant, K, was mistakenly reprinted by the authors. Eq. (11) should read:

$$K = \frac{\gamma}{\gamma - 1} \cdot \frac{n_p^0 + n_a}{n_p^0},$$

where n_p^0 is the total amount of the probe in the sample, n_a is the amount of water molecules, γ is the derivative $\partial x / \partial n_i$ at $n_i = 0$, where x is the number of probe molecules in membranes and n_i is the number of lipid molecules in the sample. The authors wish to apologize for this error.