Mechanism of Fluorescent Response of the Probe diS-C_3-(5) to Transmembrane Potential Changes in a Lecithin Vesicle Suspension

M. N. IVKOVA, V. A. PECHATNIKOV and V. G. IVKOV

Institute of Biological Physics, 142292 Pushchino, Moscow Region, USSR

Abstract. The dependence of both the magnitude and the sign of fluorescent responses of the probe diS-C_3-(5) in egg lecithin vesicle suspensions on the magnitude of the inside-negative transmembrane potential and on the total probe concentration in the sample volume has been studied. Results were compared with theoretical calculations made on the basis of the equilibrium thermodynamic model suggested earlier as well as on the observed concentration dependence of the probe fluorescence in aqueous media and membranes. It was shown that transmembrane potential results in a redistribution of the probe between the aqueous and the membrane phases and in the membrane interior. The model calculations showed that the probe concentrations in the external aqueous medium and in the outer lipid monolayer of a vesicle significantly decrease. At the same time, the dye concentration in the inner membrane monolayer increases significantly, which should lead to a marked quenching of the dye fluorescence. The changes in the fluorescence and absorption spectra are well explained in terms of the proposed mechanism. The highest responses of diS-C_3-(5) to changes of the transmembrane potential were observed in a shorter wavelength region of the fluorescence spectrum at the probe to lipid ratios in membrane of 15—20 moles of probe per 1000 moles of lipid. In the longwave region, the increase in fluorescence is not an obligatory indication of a decrease in the transmembrane potential and, under certain conditions, this process can take place when the transmembrane potential increases. The generation of a quasi-equilibrium diffusion transmembrane potential results in an increase in the average probe concentration in membranes if the signs of the probe charge and the potential inside the vesicles are opposite. Thus, the "on-off" mechanism, working under conditions of steady-state processes, is not valid under equilibrium conditions.

Key words: Transmembrane potential — Potential-sensitive probe diS-C_3-(5) — Lecithin vesicles — Liposomes — Fluorescent response — Mechanism of potential-dependent optical response — Probe redistribution

Introduction

The hypothetic mechanism of redistribution of positively charged amphiphylic probes in a membrane vesicle suspension under the action of transmembrane potential was discussed in our previous paper (Ivkov et al. 1983b). According to thermodynamic calculations, an inside negative transmembrane potential should result in a significant local increase in the probe concentration both in the inner monolayer of the vesicular membrane and in the internal aqueous volume of the vesicle. The equilibrium distribution of the probe diS-C_3-(5) in the absence of any potential and the concentration dependence of its fluorescence in both the lipid and aqueous phases were described earlier (Ivkova et al. 1982; Ivkov et al. 1983b). The model proposed allows to calculate the dependence of the values of the optical response to the transmembrane potential.

A comparison of the calculated values with experimental data shows how closely this mechanism approximates actual processes. This analysis was performed for a simple model system: a suspension of electroneutral lecithin vesicles sufficiently large to neglect the difference between the inner and outer monolayers. If the membrane is not symmetrical (due to its composition, or molecule packing) different partition coefficients have to be used for the inner and outer monolayers, respectively. The principle of the calculation apparently remains analogous.

Materials and Methods

Materials and methods used were essentially the same as described in the previous papers (Ivkova et al. 1982; Ivkova et al. 1983b). Here, a technique for generation of transmembrane potential using K^+-valinomycine will be presented.

Egg lecithin liposomes were formed by the detergent removal technique in a medium containing 0.2 mol/l KCl and 0.02 mol/l buffer Tris-HCl, pH 7.5. The initial lipid concentration in the suspension was 20 mg/ml. Ten μl of the suspension were added to the medium containing a mixture of KCl and NaCl(total salt concentration 0.2 mol/l in the same buffer) to obtain a necessary K^+ gradient between the internal and external aqueous volumes of the vesicles.

Following procedure was used: 1.99 ml of the medium were filled into a quartz cuvette and 10 μl of ethanol solution of the probe diS-C_3-(5) was added. The initial probe concentrations were 0.5, 0.2, or 0.1 mmol/l. The resulting solution was mixed using a magnetic stirrer. Fluorescence intensity was measured at $\lambda_f = 660$ nm and 700 nm, respectively; then 10 μl of the liposome suspension in KCl was added to the sample and fluorescence intensities F_{660} and F_{700} were measured after a delay of 3—4 minutes when they reached equilibrium values. Subsequently 5×10^{-8}—mol/l valinomycin (10 μl of ethanol solution, initial concentration of 10^{-5} mol/l) were added into the cuvette and values of F_{660}, F_{700} (or F_{665}) were determined again. In liposomes used in our experiments diffusion K^+-valinomycin transmembrane potentials, monitored by the probe fluorescence intensity, remained constant during about an hour.

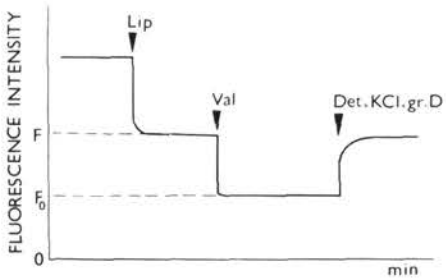

Fig. 1. Scheme of determination of the fluorescent response from experimental curves. Arrows point to the rise of the transmembrane potential and of its abolition by the addition of detergents, gramicidine D or KCl into the external aqueous medium (in the latter case, K^+ gradient between the internal and external media is abolished without breaking the membranes).

Values of transmembrane potential, $\Delta\psi$ (mv), were calculated by the Nernst relationship: $\Delta\psi = -58 \lg (c_{in}/c_{out})$, where c_{in} and c_{out} are the concentrations of K^+ inside and outside the vesicles, respectively. The fluorescent response of the probe was defined as a relative change in the probe fluorescence intensity under the action of transmembrane potential: $\Delta F/F_0 = (F_0 - F)/F_0$, where F_0 and F are the fluorescence intensities (at $\lambda_f = 660$ or 700 nm) for the vesicle suspensions without and with valinomycine, respectively (Fig. 1).

Detergents gramicidine D or KCl (initial concentration of the KCl solution 2 mol/l) were added to abolish the transmembrane potential. Membrane breaking by detergents (deoxycholate or Triton X-100) did not result in a full reversibility of the valinomycin effects on fluorescence apparently because of interactions between the probe and micelles of the detergent. The restoration of fluorescence induced by abolishing the potential with gramicidine-D was characterized by two phases: a fast and a slow one both of them being dependent on the probe-to-lipid ratio. No detailed investigations of this effect were made. When the potential was abolished by removing the K^+ gradient by the addition of KCl into the external medium, the fluorescence intensity returned to its initial level (± 5 %) as observed prior to the addition of valinomycin. The addition of valinomycin to the membrane suspension, with the concentrations of KCl both inside and outside the vesicles being the same, resulted in a fluorescence intensity change less than 3 % of the initial value. A similar effect was observed after the addition of KCl into the external medium in the absence of valinomycin.

Cuvette and pipette tips were washed in a mixture of dichromate with sulfuric acid to diminish dye absorption on the walls. Other procedural hints as reported by Tsien and Hladky (1978); Krasne (1980) were also followed. Four identical preparations of liposome suspension were used. In every preparation 4—10 measurements were performed for every value of transmembrane potential. Average values of the fluorescent response and standard deviations were calculated.

Results

Dependence of the fluorescent response on the transmembrane potential

The fluorescent response of the probe diS-C_3-(5) to the action of transmembrane potential was investigated in a suspension of unilamellar lecithin vesicles of

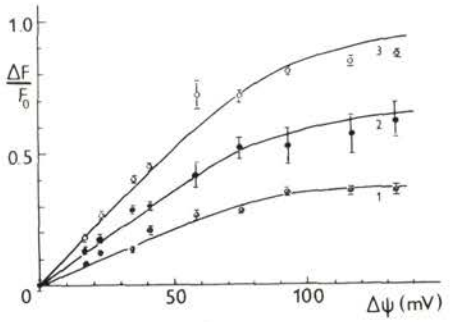

Fig. 2. Dependence of the fluorescent response of diS-C_3-(5) at $\lambda_f = 660$ nm on the transmembrane potential in egg lecithin vesicle suspension. Points are average values of 5—10 different preparations. Standard deviations are shown. The lipid concentration was 0.1 mg/ml. Total probe concentrations in the sample volume: 1: 0.5×10^{-6} mol/l, 2: 1×10^{-6} mol/l, 3: 2.5×10^{-6} mol/l.

large diameter at a constant lipid concentration and at three different total probe concentrations (Fig. 2). Inside-negative transmembrane potential was generated by K^+-valinomycin as described in the Methods. Fluorescence intensity was measured at $\lambda_f = 660$ nm. Under these experimental conditions the relationship between $\Delta F/F_0$ and $\Delta\Psi$ is approximately linear up to a potential of about -50 mV, and it reaches a plateau as the potential increases.

The higher the total probe concertration in the sample the greater the value of the optical response. In Fig. 2 theoretical dependences of ($\Delta F/F_0$) on $\Delta\Psi$ are represented by solid lines. They were calculated based on the model proposed in our previous work (Ivkov et al. 1984). The good coincidence of theoretical and experimental dependences allows the assumption that this model, in general terms, may correctly reflect the processes occurring in membranes under the action of transmembrane potential

Calculation of probe distribution in liposome suspension with no potential present

Concentrations of both of the lipid (c_l) and probe (c_p^o) in the sample are set. The partition coefficient characterizing the probe distribution between the membrane and the aqueous phases is $K_{p-o} = (5.70 \pm 0.95) \times 10^4$ for egg lecithin liposomes (Ivkova et al. 1983b). Partial specific density of lecithin is about 1 g . cm^{-3}. Thus, with a known c_l, the volume of the lipid phase (v_m) can be easily calculated. The diameter of large vesicles prepared by the detergent removal technique is about 100 nm with a bilayer thickness of about 5 nm. In this case, the membrane curvature can be neglected and the bilayer can be considered symmetrical. Thus, in the absence of electrical field, the probe will be evenly distributed between the outer and inner vesicle monolayers.

Table 1. Distribution of the probe diS-C_3-(5) in lecithin liposome suspension in the presence of a transmembrane potential (theoretical calculations). The amount of the probe is expressed in terms of volume concentrations (c_p^a and c_p^m) in aqueous and membrane phases respectively; $c_p^o = 0.5 \times 10^{-6}$ mol/l, the sample volume $v_{out} = 2 \times 10^{-3}$ l, the volume of the liposome $v_m = 2 \times 10^{-7}$ l, the inner vesicular volume $v_{in} = 6 \times 10^{-7}$ l, the partition coefficient in the absence of any potential $K_{p,o} = 5.7 \times 10^4$.

$\Delta\Psi$ (mV)	$c^a_{p,out} \times 10^8$ mol/l)	$c^a_{p,in} \times 10^8$ (mol/l)	$c^m_{p,out} \times 10^3$ (mol/l)	$c^m_{p,av} \times 10^3$ (mol/l)	$c^m_{p,av} \times 10^4$ (mol/l)
0	7.5	7.5	4.28	4.28	4.28
17.4	5.32	10.6	3.25	5.68	4.46
23.2	4.74	11.8	2.95	6.14	4.56
35.0	3.54	14.2	2.30	7.03	4.68
40.6	3.06	15.3	2.06	7.40	4.73
58.0	1.84	18.4	1.33	8.36	4.84
75	1.06	21.2	0.82	8.99	4.90
93	0.60	24.0	0.49	9.46	4.98
116	0.27	27.0	0.24	9.75	5.00
134	0.15	29.4	0.14	9.90	5.02
	(0.147)				

If such a vesicle is a sphere its internal aqueous volume $v_{in} \approx 3 v_m$. As a rule, it is significantly smaller than the external aqueous volume, v_{out}, (2 ml in our experiments). By definition, $K_{p,o} = c^m_{p,o}/c^a_{p,o}$, where $c^m_{p,o}$ and $c^a_{p,o}$ are the probe concentrations in the membrane and aqueous phases, respectively. The total amount of the probe in the sample is $n_p^o = c_p^o(v_{out} + v_{in} + v_m)$; on the other hand, it is $c^m_{p,o}\ v_m + c^a_{p,o}\ (v_{out} + v_{in})$ or $c^a_{p,o}(v_{out} + v_{in} + K_{p,o}\ v_m)$.

The quantity $v_{out} + v_{in} + K_{p,o}\ v_m$ can be regarded as an "effective volume" of the sample, where $K_{p,o}\ v_m$ is the effective volume of the membrane phase. The probe concentration in the aqueous phase is:

$$c^a_{p,o} = c_p^o \frac{v_{out} + v_{in} + v_m}{v_{out} + v_{in} + K_{p,o}\ v_m} \tag{1}$$

and that in the membrane phase:

$$c^m_{p,o} = K_{p,o}\ c^a_{p,o} \tag{2}$$

At a lipid concentration of 0.1 mg/ml used in our experiments, $v_m \approx 2 \times 10^{-4}$ ml and $v_{in} \approx 6 \times 10^{-4}$ ml, i.e. they are small as compared with v_{out}. In this case,

$$c^a_{p,o} \approx \frac{c_p^o}{1 + K_{p,o}\ v_m / v_{out}} \tag{3}$$

The probe concentration in these conditions reaches several mmole/l in membranes and it does not exceed 100 nmol/l in the aqueous medium (see Table 1).

Probe redistribution under the action of transmembrane potential

The theoretical basis of the model was given in the previous paper (Ivkov et al. 1984). Therefore only the calculation procedure will be described. The effective volume of a sample increases by a factor of $(1/\beta)$, where

$$\beta = \frac{v_{out} + v_{in} + K_{p,o}\, v_m}{v_{out} + m v_{in} + \frac{1}{2} K_{p,o}\, v_m \left(p + \frac{m}{p}\right)} \tag{4}$$

The quantities m and p are related to transmembrane potential by:

$$m = e^{|zF\Delta\Psi/RT|}, \qquad p = e^{|zF\Delta\varphi/RT|} \tag{4a}$$

where z is the probe charge, F is the Faraday constant, R is the universal gas constant and T is the temperature. It is obvious that in this case m is the ratio of the respective KCl concentrations inside and outside the vesicles: $m = [K^+]_{in}/[K^+]_{out}$. As analyzed earlier (Ivkov et al. 1984), the difference in potential between the zone of the probe location in the membrane and the adjacent aqueous medium is $\Delta\varphi \approx 0.1\ \Delta\Psi$.

Thus $p \approx \sqrt[10]{m}$. The respective probe concentrations in the aqueous medium outside and inside the vesicles are:

$$c^a_{p,out} = c^a_{p,o} \cdot \beta, \qquad c^a_{p,in} = c^a_{p,o} \cdot \beta m, \tag{5}$$

and those in the outer and inner monolayers of the vesicular membrane:

$$c^m_{p,out} = c^m_{p,o} \cdot p\beta, \qquad c^m_{p,in} = c^m_{p,o} \cdot \frac{m}{p} \cdot \beta \tag{6}$$

where $c^m_{p,o}$ and $c^a_{p,o}$ are related by expression (2). Average probe concentration in a membrane is:

$$c^m_{p,av} = \frac{c^m_{p,out} + c^m_{p,in}}{2} = \frac{1}{2} c^m_{p,o} \cdot \beta \left(p + \frac{m}{p}\right). \tag{7}$$

An example of calculations of probe distribution in a liposome suspension at various transmembrane potential values in given in Table 1. Since the partition coefficient is considered to be identical for both monomers and aggregates, all the values in Table 1 increase in proportion to the increase of the initial probe concentration in the sample volume.

The data presented in the Table clearly indicate how the probe becomes redistributed in a membrane suspension under the action of a transmembrane potential:

1. the probe concentration in the external aqueous medium decreases significantly, by a factor of 50 at $\Delta\Psi \approx 130$ mV;

2. the probe concentration in the internal aqueous medium increases although not so markedly (by a factor of 4 at $\Delta\Psi \approx 130$ mV)

3. the probe concentration in the outer membrane monolayer decreases significantly (by a factor of 30 at $\Delta\Psi \approx 130$ mV);

4. the probe concentration in the inner monolayer increases by a factor of 2 at $\Delta\Psi \approx 60$ mV and by a factor of about 2.3 at $\Delta\Psi \approx 130$ mV;

5. the average probe concentration in the membrane increases by 17 % at $\Delta\Psi \approx 100$ mV.

Calculation of fluorescent response

The value of fluorescent response at chosen probe and lipid concentrations can be calculated from data as given in Table 1, and from the concentration dependences of probe fluorescence in the membrane and the aqueous phases (Ivkova at al. 1983b). Optical densities of the samples investigated did not exceed 0.02, and the fluorescence intensities of both the membrane and aqueous components of the dye were additively summed. In the absence of any potential:

$$F_o = F_m + F_a = q_m c^m_{p,\,o,\,mon} \cdot \frac{v_m}{v_{out}} + q_a\, c^a_{p,\,o,\,mon}, \tag{8}$$

where F_m and F_a are the fluorescence intensities of the membrane and aqueous components, respectively: q_m and q_a are the fluorescence intensities of 1 mole of membrane and aqueous dye monomers at the chosen wavelength, respectively; $c^m_{p,\,o,\,mon}$; and $c^a_{p,\,o,\,mon}$ are the effective monomer concentrations in membranes and aqueous medium, respectively. In calculating fluorescence intensity the factor v_m/v_{out} was used to account the average effective concentration of the probe membrane monomers in a sample volume rather than the probe concentration in the membrane.

In the presence of a transmembrane potential:

$$F = q_a\left(c^a_{p,\,out,\,mon} + c^a_{p,\,in,\,mon} \cdot \frac{v_{in}}{v_{out}}\right) + q_m(c^m_{p,\,out,\,mon} + c^m_{p,\,in,\,mon})\,\frac{v_m}{2v_{out}} \tag{9}$$

the factor v_{in}/v_{out} is also used to take into account the average concentration of "inner" aqueous monomers of the dye in the sample volume (in this case, it concerns the true rather than the effective concentration of the monomers).

The fluorescent response of the probe is:

$$\frac{\Delta F}{F_0} = \frac{F_0 - F}{F_0} =$$

$$= \frac{\left(c^{a}_{p,\,0,\,mon} - c^{a}_{p,\,out,\,mon} - c^{a}_{p,\,in,\,mon} \cdot \frac{v_{in}}{v_{out}}\right) + \frac{q_m}{q_a}\left(2c^{m}_{p,\,0,\,mon} - c^{m}_{p,\,out,\,mon} - c^{m}_{p,\,in,\,mon}\right) \cdot \frac{v_m}{2v_{out}}}{c^{a}_{p,\,0,\,mon} + \frac{q_m}{q_a} c^{m}_{p,\,0,\,mon} \cdot \frac{v_m}{v_{out}}} \qquad (10)$$

where $c^{m}_{p,\,0,\,mon}$, $c^{m}_{p,\,out,\,mon}$ and $c^{m}_{p,\,in,\,mon}$ are the effective concentrations of the fluorescing monomers in the respective membrane monolayers as determined from a plot of the fluorescence concentration dependence (Ivkova et al. 1984b).

Expression (10) incorporates a ratio of fluorescence intensities of equal amounts of membrane and aqueous dye monomers under identical experimental conditions, q_m/q_a.

As discussed earlier (Ivkova et al. 1982; Ivkova et al. 1983a, b), at probe concentrations used in our experiments the corrections for screening and reabsorption may be neglected. Therefore, q_m/q_a can be easily estimated for a chosen wavelength from the back titration experiments (Ivkova et al. 1983b). At $\lambda_f =$ 660 nm the ratio q_m/q_a is 0.46 ± 0.07. As a rule, in the range of probe and lipid concentrations used the dye occurs as a monomer in the aqueous medium.

Thus the fluorescent response at $\lambda_f = 660$ nm is:

$$\frac{\Delta F_{660}}{F_{0,660}} =$$

$$= \frac{\left(c^{a}_{p,\,0} - c^{a}_{p,\,out} - c^{a}_{p,\,in} \cdot \frac{v_{in}}{v_{out}}\right) + 0.46\left(2c^{m}_{p,\,0,\,mon} - c^{m}_{p,\,out,\,mon} - c^{m}_{p,\,in,\,mon}\right) \cdot \frac{v_m}{2v_{out}}}{c^{a}_{p,\,0} + 0.46 c^{m}_{p,\,0,\,mon} \cdot \frac{v_m}{v_{out}}} \qquad (11)$$

In many cases, it is more convenient to express probe concentration in membranes in terms of the probe/lipid ratio. For example, in the work by Ivkova et al. (1983), this concentration is given as numbers of probe molecules per 1000 molecules of the lipid. With the probe/lipid ratios commonly used in experiments, this value is approximately proportional to the mole fraction of the probe in the lipid phase, $\hat{X}^{m}_{p} \approx n^{m}_{p}/n_1$.

Conversion to volume concentrations is easy based on the volume of 1 mole of egg lecithin (about 0.75 l). In this case, the optical response is:

Table 2. Calculation of fluorescent response to transmembrane potential, $\Delta F_{660}/F_{0,660}$; for conditions, see Table 1.

$\Delta\Psi$ (mV)	$c_p^o = 0.5 \times 10^{-6}$ mol/l					$c_p^o = 1 \times 10^{-6}$ mol/l				
	$\dot{X}_p^m \times 10^3$					$\dot{X}_p^m \times 10^3$				
	$\frac{n_{p,out}^m}{n_l}$	$\frac{n_{p,in}^m}{n_l}$	$\frac{n_{p,out,mon}^m}{n_l}$	$\frac{n_{p,in,mon}^m}{n_l}$	$\frac{\Delta F}{F_o}$	$\frac{n_{p,out}^m}{n_l}$	$\frac{n_{p,in}^m}{n_1}$	$\frac{n_{p,out,mon}^m}{n}$	$\frac{n_{p,out,mon}^m}{n_1}$	$\frac{\Delta F}{F_o}$
0	3.21	3.21	2.90	2.90	0	6.38	6.38	4.60	4.60	0
17.4	2.44	4.26	2.44	3.50	0.07	4.84	8.46	3.90	5.10	0.12
23.2	2.23	4.60	2.23	3.75	0.09	4.43	9.2	3.60	5.10	0.16
35.0	1.74	5.27	1.74	4.10	0.15	3.46	10.5	3.10	5.10	0.25
40.6	1.54	5.55	1.54	4.20	0.17	3.07	11.0	2.80	5.10	0.30
58.0	1.00	6.27	1.00	4.60	0.25	1.97	12.4	1.97	5.00	0.42
75.0	0.62	6.74	0.62	4.70	0.31	1.22	13.4	1.22	4.85	0.52
93.0	0.37	7.10	0.37	4.85	0.34	0.73	14.1	0.73	4.75	0.58
116	0.18	7.31	0.18	4.90	0.37	0.36	14.6	0.36	4.70	0.63
134	0.10	7.42	0.10	5.00	0.38	0.21	14.8	0.21	4.65	0.65

$$\frac{\Delta F_{660}}{F_{0,660}} \approx$$

$$\approx \frac{\left(c^{a}_{p,o} - c^{a}_{p,out} - c^{a}_{p,in} \cdot \frac{v_{in}}{v_{out}}\right) + 0.61(2\hat{X}^{m}_{p,o,mon} - \hat{X}^{m}_{p,in,mon} - X^{m}_{p,out,mon}) \frac{v_m}{2v_{out}}}{c^{a}_{p,o} + 0.61\hat{X}^{m}_{p,o,mon} \cdot \frac{v_m}{v_{out}}} \quad (12)$$

Table 2 shows an example of calculations of fluorescent response for two total probe concentrations. These calculations were made in considering data reported in Table 1 as well as the concentration dependence of probe fluorescence in a membrane given in Fig. 6 in the paper by Ivkova et al. (1983).

It should be noted that fluorescence intensity expressed in terms of effective concentrations of fluorescing monomers conveniently describes the concentration dependences. However, it not always reflects true monomer concentrations, e. g. when the inductive-resonance transfer contributes to the quenching processes.

At a total probe concentration of 10^{-6} mol/l, the effective concentration of fluorescing dye monomers in the inner vesicular monolayer goes through the maximum and at $\Delta\Psi = 134$ mV, it yields the same value as in the absence of any potential. In this case, a decrease in the membrane dye fluorescence results from a sharp decrease in the dye concentration in the outer monolayer. Simultaneously, a minor increase or even a decrease in the effective concentration of fluorescing monomers in the inner monolayer occurs at a marked increase in total dye concentration in this monolayer.

Table 3. Relative contributions of the aqueous and membrane dye components, respectively to the potential-dependent fluorescent response ($\lambda_f = 660$ nm) of the probe diS-C_3-(5). For conditions, see Table 1. F_a/F_o and F_m/F_o are the contributions of the aqueous and membrane probes, respectively to the fluorescence in the absence of any transmembrane potential.

$\Delta\Psi$ (mV)	$c^{o}_{p} = 0.5 \times 10^{-6}$ mol/l		$c^{o}_{p} = 1 \times 10^{-6}$ mol/l		$c^{o}_{p} = 2.5 \times 10^{-6}$ mol/l	
	$\frac{\Delta F_a}{F_o}$	$\frac{\Delta F_m}{F_o}$	$\frac{\Delta F_a}{F_o}$	$\frac{\Delta F_m}{F_o}$	$\frac{\Delta F_a}{F_o}$	$\frac{\Delta F_m}{F_o}$
0	$\frac{F_{a,o}}{F_o} = 0.30$	$\frac{F_{m,o}}{F_o} = 0.70$	$\frac{F_{a,o}}{F_o} = 0.35$	$\frac{F_{m,o}}{F_o} = 0.65$	$\frac{F_{a,o}}{F_o} = 0.57$	$\frac{F_{m,o}}{F_o} = 0.43$
17.4	0.086	−0.017	0.10	0.01	0.16	0.04
23.0	0.11	−0.02	0.13	0.03	0.21	0.05
35.0	0.156	−0.004	0.18	0.07	0.30	0.075
40.6	0.175	0.007	0.20	0.09	0.34	0.085
58.0	0.22	0.024	0.26	0.16	0.43	0.17
75.0	0.25	0.058	0.30	0.22	0.49	0.24
93.0	0.27	0.07	0.32	0.26	0.53	0.29
116	0.28	0.08	0.33	0.29	0.55	0.35
134	0.29	0.09	0.34	0.31	0.56	0.37

The value of the response, $\Delta F_{660}/F_{0.660}$, is a sum of two quantities, $\Delta F_a/F_o$ and $\Delta F_m/F_o$, i.e. of the contributions of the aqueous and membrane components, respectively. Values of these components are given in Table 3. At all the probe concentrations used an increase in the potential above 100 mV resulted in a virtually complete "quenching" of the aqueous component, since a considerable portion of the dye passed from the aqueous to the membrane phase. In membranes, the quenching of the dye fluorescence is the more effective the higher the total probe concentration in the sample.

Negative values of the fluorescent response of "membrane" probes (an increase in the fluorescence of the membrane summand) at $c_p^o = 0.5 \times 10^{-6}$ mol/l result from the transition of aqueous monomers into the membrane; under similar conditions, no quenching in the membrane interior occurs.

Changes in absorption and fluorescence spectra under the action of transmembrane potential

Absorption spectra of the probe diS-C_3-(5) both without and with liposomes in the absence or presence of a transmembrane potential, respectively, are shown in Fig. 3. At all the probe concentrations introduction of a potential resulted in a shift of the monomer peak ($\lambda_{max} = 670$ nm) to red. This process was accompanied by an increase in absorption in the dimer band region (610 nm). As illustrated in Table 1, generation of a transmembrane potential results in a decrease in the probe quantity in the aqueous medium and thus in a minor longwave shift of the spectrum. Obviously a significant increase in the probe concentration in the inner membrane

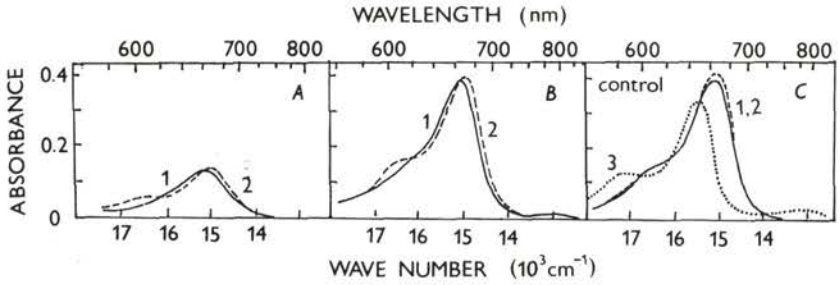

Fig. 3. Effect of transmembrane potential on absorption spectra of diS-C_3-(5) in egg lecithin vesicle suspension. 10^{-5} l of the suspension (20 mg/ml of lipid in 0.2 mol/l KCl; 0.02 mol/l Tris-HCl; pH 7.5) was added to the medium containing: *A*, *B* — 0.2 mol/l NaCl, *C* — 0.2 mol/l KCl in the same. The potential in the presence of 5×10^{-8} mol/l valinomycin was 134 mV. Total probe concentrations: *A*: 1×10^{-6} mol/l, *B*, *C* — 3.3×10^{-6} mol/l. Spectra: 1: dye in liposome suspension with no potential, 2: the same as in 1 but with a potential, 3: dye in the aqueous medium in the absence of lipid.

monolayer must increase the possibility of dimerization. Changes in the absorption spectra due to these processes are particularly prominent at total probe concentrations exceeding 2×10^{-6} mol/l (Fig. 3*C*).

A longwave shift is also characteristic for fluorescence spectra, the spectrum position being typical for the membrane dye (Fig. 4). The shape of the difference spectrum (curves 3 in Fig. 4) shows that under the action of potential the relative decrease in fluorescence of the aqueous probe is greater than that of the membrane probe, this is in agreement with the theoretical predictions.

At low total probe concentrations only a spectrum shift occurs, without any quenching of the membrane dye fluorescence (Fig. 4*A*).

Wavelength dependence of the fluorescent response nature (some practical recommendations)

The dependence of fluorescent response on probe concentration in the lipid phase (at a transmembrane potential of 134 mV) is shown in Fig. 5 for three different wavelengths. Obviously, both the magnitude and the sign of the response depend on the dye concentration as well as on the fluorescence wavelength. At low probe concentrations, fluorescence intensity increases at $\lambda_f = 685$ and 700 nm. At these wavelengths the amplitude of the response is much lower. The highest responses are observed at probe concentrations between 15 and 20 moles of the probe per 1000 moles of lipid. When the probe concentration in membranes exceeds 30 moles per 1000 moles of lipid, the permeability of the lipid bilayer for ions increases. As a result, a faster dissipation of the transmembrane diffusional potential occurs.

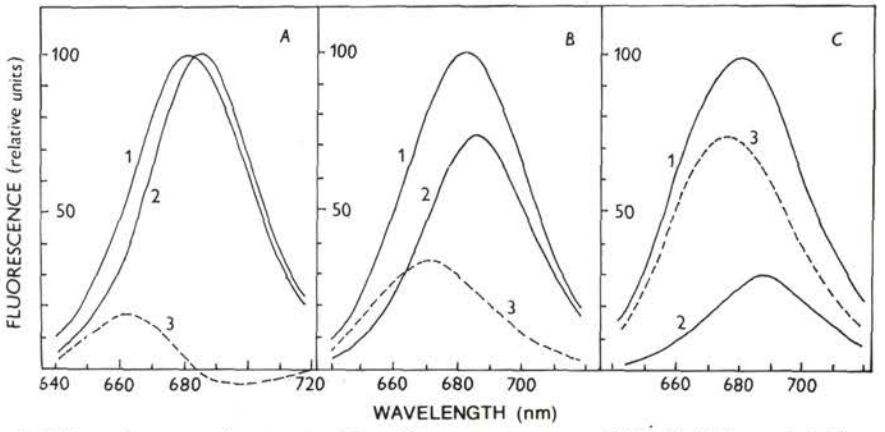

Fig. 4. Effect of transmembrane potential on fluorescence spectra of diS-C_3-(5) in egg lecithin vesicle suspension under conditions as specified in Fig. 3*A*. Probe concentrations: *A* — 0.5×10^{-6} mol/l, *B*: 1×10^{-6} mol/l, *C*: 2×10^{-6} mol/l. Spectra: 1: with no potential, 2: with a potential, 3: differential spectra.

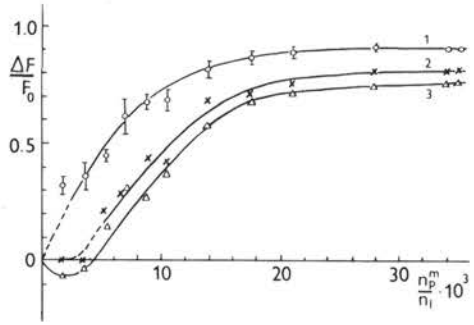

Fig. 5. Experimental dependence of the optical response of diS-C_3-(5) on the probe/lipid ratio in membranes of lecithin vesicles for three different fluorescence wavelength: 1 — 660 nm, 2 — 685 nm, 3 — 700 nm. The lipid concentration was 0.1 mg/ml, the transmembrane potential was 134 mV. Measurements were performed at total probe concentrations ranging from zero to 5×10^{-6} mol/l.

Experimentally obtained curves of fluorescent responses ($\lambda_f = 700$ nm) to various potentials are shown in Fig. 6. In this case, the membrane dye component makes up the major contribution to the fluorescence. For a total probe concentration of 0.5×10^{-6} mol/l no response was registered at this wavelength. A minor increase in fluorescence (less than 10 %) that remained unchanged with increasing $\Delta\Psi$ was a result of the valinomycin effect. This effect has not been further analyzed.

At a total probe concentration in the sample of about 1×10^{-6} mol/l fluorescent response was occurred at $\Delta\Psi > 50$ mV. A detectable response appeared at $c_p^w \approx 2.5 \times 10^{-6}$ mol/l at any value of transmembrane potential.

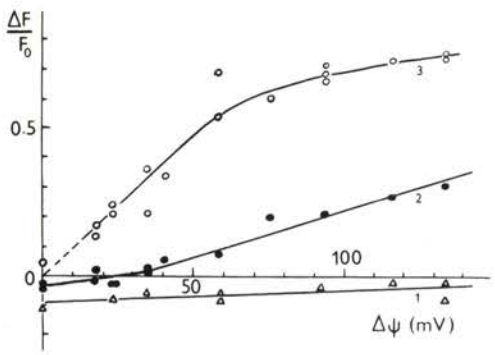

Fig. 6. Dependence of the fluorescence response of diS-C_3-(5) ($\lambda_f = 700$ nm) on transmembrane potential in egg lecithin vesicle suspension. The lipid concentration was 0.1 mg/ml. Total probe concentrations in the sample volume: 1: 0.5×10^{-6} mol/l; 2: 1×10^{-6} mol/l; 3: 2.5×10^{-6} mol/l.

The shortwave region of the spectrum thus seems more sensitive to changes in transmembrane potential (i.e. measurements can be performed at lower probe concentrations). It should be emphasized that the increase in fluorescence does not necessarily indicate a decrease in transmembrane potential. Such a process may occur when the magnitude of the potential increases, as demonstrated earlier.

Discussion

The present work provides an experimental support for the hypothetic model of the potential-dependent behaviour of the fluorescent probe diS-C_3-(5) in egg lecithin liposome suspension. This model (Ivkov et al. 1984) is applicable to quasi-equilibrium transmembrane potential on the condition that an almost equilibrium dye distribution between the aqueous medium and membranes and between both the inner and the outer aqueous volumes of vesicles is achieved during the measurement.

It has been suggested that lipid membranes have no discrete sites for probe binding. Thus the membrane may be regarded as a continuous phase to which general equations for interphase distribution of probe molecules can be applied. Low dye concentrations in the aqueous medium and membranes allow to consider the dye solutions in the corresponding phases to be close to ideal ones (the solubility of monomers, dimers and aggregates are supposed to be identical). This model is based on following:

1. probe concentrations in the outer and inner vesicular aqueous spaces, respectively, are related by the Nernst equation for ion distribution between compartments separated by a semipermeable membrane;

2. equilibrium probe distribution between a membrane monolayer and the aqueous medium adjacent to it is determined by the Nernst relationship for the interphase distribution of a dissolved substance;

3. the partition coefficient is determined by the difference in the electrochemical potentials of the dye between the respecting monolayers and aqueous medium adjacent to it. For this purpose effective volume quantities of the sample both in the absence and presence of diffusion transmembrane potential may conventiently be used.

This model allows to calculate probe redistribution under the action of a transmembrane potential by analogy with Waggoner's "concentration polarization" (Waggoner et al. 1977); they used it to describe the optical response of the dye to potential changes under steady-state conditions.

The probe is apparently located in the membrane interior near the carbonyl groups of fatty acid residues, where the polarity of the dye environment is similar to that of alcohols. The chain of conjugated bonds in the dye molecule is obviously

oriented almost parallel to the membrane surface. From an analysis of the probe structure and of the physico-chemical properties of egg lecithin vesicles, approximately 0.1 of the magnitude of the transmembrane potential may be suggested to fall on the region between the chromophore position in the membrane and the aqueous medium adjacent to the membrane surface. This is in agreement with the suggestion of Waggoner and coworkers (Waggoner et al. 1977).

The theoretical dependence of the fluorescent response on the magnitude of transmembrane potential is nearly identical to the dependence observed experimentally (Fig. 1). Essentially the present model reflects correctly actual physical processes occurring in the membrane suspension under the action of transmembrane potential. The mechanism of the optical response may be formulated as follows: a) transmembrane potential changes the electrochemical potential of the probe in both the external and internal membrane monolayers relative to the adjacent aqueous medium. At an inside-negative potential, the partition coefficient for the outer monolayer increases while decreases for the inner one; b) the probe concentration in the aqueous phase inside the vesicles increases and it decreases in the outside phase; c) the probe is redistributed between the outer and the inner lipid monolayers separated by a rather highly hydrophobic barrier (Waggoner et al. 1977).

As a result, a significant fraction of the "aqueous" probe is injected into the membrane's inner monolayer. At low initial probe concentrations ($\sim 0.5 \times 10^{-6}$ mol/l), the fluorescent response is practically determined by a spectrum shift to red that leads to a fluorescence decrease at $\lambda = 660$ nm and to its increase at $\lambda = 700$ nm. At higher concentrations, this process is accompanied by a concentration quenching of the dye fluorescence in the inner membrane monolayer.

Problems of the mechanism of quenching are beyond the scope of the present paper. It is obvious that nonfluorescing dimers or larger aggregates are partly responsible for these events (West and Pearce 1965). Contributions of the inductive-resonance transfer or of dynamic quenching (Waggoner et al. 1977; Krasne 1980) cannot be ruled out either. It should be emphasized that the expression of concentration dependence of fluorescence quenching in terms of effective concentration of fluorescing monomers is merely a mathematical approach enabling an easy description of the quenching processes in a general form.

Undoubtedly, the ratio of the contributions of both the aqueous and membrane components of the dye to the fluorescent response depends primarily on the concentration of the lipid phase. At membrane concentrations of about 1 mg/ml the main events apparently occur in the membrane interion (Ivkova et al. 1983a) whereas at lipid concentrations of about 0.01 mg/ml the contribution of the aqueous component to the optical response predominates (the "aqueous" dye is injected into membranes where it becomes quenched).

In the absence of any transmembrane potential, an increase in the dye concentration leads to a spectrum shift to blue (Fig. 4): $\lambda_f = 686$ nm and $\Delta\lambda_f = 43$ nm at $c_p^o = 0.5 \times 10^{-6}$ mol/l, the respective values are: 683 and 49 at 2×10^{-6} mol/l (Fig. 4*C*), and 670 and 48 at 4×10^{-6} mol/l (Fig. 4*D*). This is explained by fluorescence quenching of the membrane rather than of aqueous probes at higher concentrations.

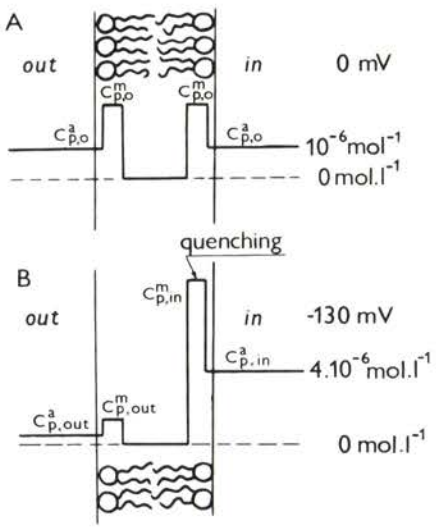

Fig. 7. Illustration of concentration changes of the permeant dye diS-C_3-(5) under the action of a transmembrane potential. Ordinate: quasiequilibrium dye concentrations as functions of the distance from the membrane bilayer surface (no relationship could be observed): *A* — in the absence of a transmembrane potential, *B* — in the presence of a potential.

Generation of a transmembrane potential leads to a "unification" of all the spectra (Fig. 4*A*, *B*, *C*, *D*, curves 2). As a result, $\lambda_f^{max} = 689 \pm 1$ nm and $\Delta\lambda_f = 42 \pm 1$ nm, i.e. the spectrum becomes that of a "membrane". This confirms that the above calculations are correct (Table 3).

The presence of a potential results in both a redistribution of the probe between the membrane monolayers (and quenching of its fluorescence in the inner monolayer) and an increase in the average probe concentration in membranes that leads to an "igniting" of membrane component fluorescence. The resulting fluorescence is determined by both processes and, depending on the conditions, a decrease or an increase in fluorescence intensity may be observed at $\lambda_f > 680$ nm. Thus it is more preferable to study transmembrane potential at $\lambda_f = 660$ nm than that at $\lambda_f = 700$ nm.

Let us estimate roughly the time required for equilibrium probe redistribution in a membrane suspension after the potential step. The rate of probe diffusion through membrane is determined by the central hydrophobic barrier (Waggoner et al. 1977) and can be estimated from the steady-state current of the probe. For diS-C_3-(5) at the average probe concentration in the aqueous medium of 1.5×10^{-6} mol/l and an average transmembrane potential of 37 mV, the steady-state current across a glycerylmonooleate planar membrane is $i=6\times10^{-8}$ A/cm^2 (Waggoner et al. 1977) or $i\simeq6\times10^{-13}$ mol/s . cm^2 for positively charged ions of diS$^+$.

The change in the dye concentration in the inner vesicular volume: $\Delta c=\Delta n/v_{in}$, where $\Delta n=i\,.\,t\,.\,S$ (t is the time; S is the area of the membrane). For a sphere, $S/v_{in}=6/D$ where D is the diameter of the vesicle; for a vesicle of 100 nm in diameter, this value is 6×10^5 cm^{-1}. Thus, $\Delta c\simeq6\times10^{-13}\times6\times10^5t\simeq3.6\times10^{-7}t$ (mol . s/cm^3) or $\sim3.6\times10^{-4}\,t$ (mol . s/l), i.e. under these conditions probe concentration may change by about 360×10^{-6} mol/l during 1 s. It is a very rough estimation since at the same time the outer concentration decreases as well.

In a cell of 10^{-3} cm in diameter, the ratio $S/v_{in}=6\times10^3$ cm^{-1}. In this case, $\Delta c\simeq4\times10^{-6}t$ (mol . s/l). i.e. at an outer dye concentration of about 1.5×10^{-6} mol/l an identical change in the intracellular dye concentration will occur during 100 sec.

The mechanism of the potential-dependent response is shown schematically in Fig. 7. Layout of the scheme is identical with that in Fig. 6 in the paper by Waggoner et al. (1977) to enable an easy comparison of the nature of probe distribution at equilibrium with that at steady-state.

This pattern is much like that given by Waggoner for the behaviour of a probe in a bilayer lipid membrane on the hole (BLM) except for the probe distribution in the internal aqueous vesicular volume. It is just this effect that leads to results contrary to those found by Waggoner under steady-state conditions, when a small volume of the lipid phase (BLM) separates two large aqueous volumes so that the dye concentration in water remains practically constant in the course of the experiment. In this system, the fluorescent response is determined by the aggregation of the dye in the "unstirred" aqueous layers near the membrane; the dye is ejected here from the membrane by the electric field (the "on-off" mechanism). In a system of closed vesicles, the probe is drawn into the membranes and not thrown out into water by the "on-off" mechanism.

Another difference concerns the absence of a nearmembrane probe concentration increase which is evident from simple estimations. At a lecithin concentration of 0.1 mg/ml, the "effective volume" of the lipid phase in the sample ($K_{p,o}v_m$) is about $5.7\times10^4\times2\times10^{-7}=11.4\times10^{-3}$ l, at a volume of the aqueous medium of 2×10^{-3} l. (The total effective volume is always larger than the sample volume). In

this case, $v_{cell} = 6.7\ v_{out}$ (v_m and v_{in} are $10^{-4}\ v_{out}$ and $3 \times 10^{-4}\ v_{out}$ respectively, and it can be neglected).

The probe concentration in the aqueous phase ($c^a_{p,o}$) is about 0.9×10^{-6} mol/l at a total probe concentration of 6×10^{-6} mol/l. As a result of the potential-induced redistribution, concentrations of the "aqueous" probe inside and outside the vesicles are about $4c^a_{p,o}$ and $0.02\ c^a_{p,o}$, i.e. 3.6×10^{-6} mol/l and 0.018×10^{-6} mol/l, respectively (see Table 1 in the paper by Ivkov et al. (1984)).

With respect to this, the probe concentration inside the vesicles is rather high; however, how many molecules of the probe are inside one vesicle? The inner volume of a vesicle of 100 nm in diameter is $\pi D^3/6 \simeq 0.5 \times 10^{-18}$ l. The amount of the probe molecules is $n_m \simeq 3.6 \times 10^{-6} \times 6 \times 10^{23} \times 0.5 \times 10^{-18} \simeq 1.1$ molecule per one vesicle.

Apparently, there is no increase in the probe concentration near the outer membrane surface. Diffusion transmembrane potential is generated due to the ejection of a fraction of cations from the inner to the outer aqueous medium. The net charge of these cations, $\Delta Q = C\Delta\psi$, where C is the membrane capacity and $\Delta\Psi$ is the magnitude of the transmembrane potential. At $C = 1 \times 10^{-6}$ F/cm^2 and $\Delta\Psi = 130$ mV, $\Delta\Psi = 1.3 \times 10^{-7}$ c/cm^2 or $\sim 0.8 \times 10^{12}$ ions/cm^2. For a 100 nm vesicle, with an area of about 3×10^{-10} cm^2, $\Delta Q = 4 \times 10^{-17} c$ or ~ 240 ions. There are two permeant cations in the system, K^+ and diS-C_3-(5); their concentrations differ sharply: $[K^+] = 0.2$ mol/l, $c^a_{p,out} \simeq 2 \times 10^{-8}$ mol/l. The cation fraction in the "near-membrane" layer depends on the ratio of their bulk concentrations and, undoubtedly, all the 240 "near-membrane" ions are K^+ ions.

Some possible mechanisms for the fluorescent response of carbocyanine dyes to changes in transmembrane potential were suggested by Sims and coworkers (Sims et al. 1974). They showed that hyperpolarization of erythrocytes resulted in an additional absorption by these cells of the probe from the aqueous medium. On the other hand, depolarization of these membranes resulted in the probe ejection from cells into water. Sims et al. also suggested similar events to occur in phospholipid vesicles, where the fluorescent response results from aggregation--disaggregation processes.

Ross and coworkers (Ross et al. 1974) showed that changes in the absorption spectra of the dye might be determined, in principle, by aggregation of the "membrane" dye. Based on the analysis of absorption and fluorescence spectra, Krasne assumed that "...these dyes monitor transmembrane potential changes by a redistribution mechanism". Moreover, depending on the ratio of the dye to membrane concentrations, "...the major redistribution may occur within the membrane, between the aqueous phases, or between the membrane and aqueous phases" (Krasne 1980).

Unfortunately, a qualitative analysis has only been presented in the above works and this makes a comparison and an analysis of the results obtained in terms

of the present model difficult. The probe distribution between the aqueous and membrane phase was not estimated quantitatively either. It is therefore interesting to compare our results with the data of Bashford and coworkers (Bashford et al. 1979), who have estimated the binding of oxonol dyes to membranes of sonicated phospholipid vesicles in terms of the Scatchard model.

In this case, the number of binding sites (n) per membrane unit (Bashford et al. 1979) corresponds to the concentration of "saturation" of the membrane by the dye in terms of our model (Ivkova et al. 1983b). The "affinity" of the dye for the lipid (K_d/n) is the reciprocal of the partition coefficient.

This is evident from the formula for the dye and the binding site dissociation constant K_d, Eq. (8) in the work (Bashford et al. 1979):

$$K_d = (d - b)n \cdot m/b,$$

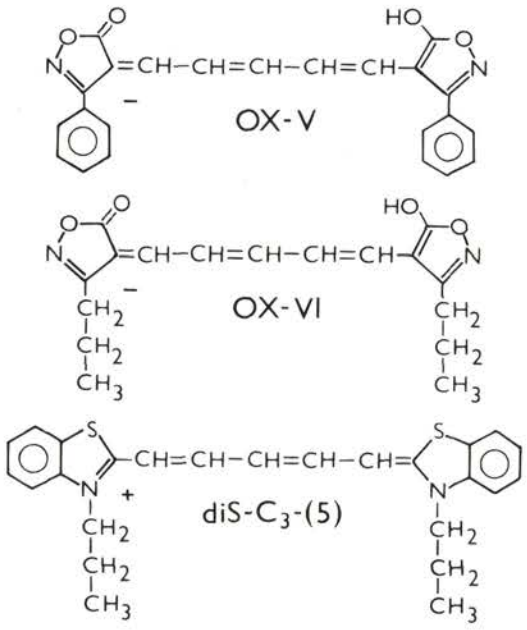

Fig. 8. Structural formulae of oxonol and carbocyanine probes, mentioned in the text.

where d is the initial dye concentration in the sample; m is the membrane concentration; n is the number of binding sites per membrane unit; b is the concentration of the occupied sites (in the sample volume). In this case, $K_d/n = (d - b)/(b/m)$, where $d - b = c_p^a$ and $b/m = c_p^m$, assuming that the "occupied sites" correspond to the dye dissolved in the membrane. Thus, $K_d/n = c_p^a/c_p^m$, or $n/K_d = K_p$.

The quantities n and K_d are expressed in concentration units, if the membrane amount is expressed in volume units. This is easily done since partial specific density of phospholipids is approximately 1 g/cm^3, i.e. 1 mg of the lipid has a volume of 10^{-3} ml. The affinity of the probe for egg lecithin depends on the structure of the oxonol dyes: $K_d/n = 0.0072$ mg/ml for OX-V and 0.11 mg/ml for OX-VI (Bashford et al. 1979). This corresponds to a K_p 1.4×10^5 and 0.9×10^4 for OX-V and OX-VI, respectively (structural formulae of the dyes are given in Fig. 8).

As discussed above, for diS-C_3-(5) in egg lecithin liposomes $K_p \approx (5.70 \pm 0.95) \times 10^4$. The value of K_d/n increases with the increasing concentration of cholesterol in lecithin/cholesterol vesicles (Bashford et al. 1979), i.e. the partition coefficient decreases.

Top probe concentration in membranes (the "saturation" concentration) may also be estimated from the number of binding sites, n, (Bashford et al. 1979) assuming an average molecular weight of the phospholipid of about 750. In this case, 1 mg of the lipid corresponds to 1.3×10^{-6} mole and a concentration $n = 147.9$ nmol/mg (Bashford et al. 1979) for OX-V in liposomes of total soybean phospholipids (azolectin) corresponds to a saturation concentration of 1 mole of the probe per 8.8 moles of lipid. We have obtained similar values ($\sim 1/5$-$1/8$) for top concentration of diS-C_3-(5) in sarcoplasmic reticulum membranes and azolecithin liposomes; for egg lecithin this value was somewhat less, about 1/18 (Ivkova et al. 1983b).

Apparently, this may be explained by a higher solubility of OX-V in the hydrophobic region of the membrane as compared with diS-C_3(5), and by a repulsing interaction between the negatively charged oxonol dyes and the azolectin bilayer.

The present model may actually be used to analyse the behaviour of any permeant lipid-soluble charged probe. Although the nature of redistribution of such probes is much similar, the nature of fluorescence changes may be rather different due to different contributions of static, dynamic, or inductive-transfer mechanisms of fluorescence quenching.

Acknowledgement. The authors wish to thank Prof. A. Waggoner for his kind providing the probe diS-C_3-(5).

References

Bashford C. L., Chance B., Smith J. C., Yoshida T. (1979): The behavior of oxonol dyes in phospholipid dispersions. Biophys. J. **25**, 63—85

Ivkova M. N., Ivkov V. G., Pechatnikov V. A., Pletnev V. V., Bukolova-Orlova T. G., Aphanasiev V. N. (1982): Distribution of potential-sensitive probe diS-C_3-(5) in membrane suspensions. Gen. Physiol. Biophys. **1**, 209—219

Ivkova M. N., Pechatnikov V. A., Ivkov V. G., Pletnev V. V. (1983a): About the mechanism of fluorescent response of the carbocyanine diS-C_3-(5) probe to changes in transmembrane potential. Biofizika **28**, 160—170 (in Russian)

Ivkova M. N., Pechatnikov V. A., Ivkov V. G. (1983b): The behavior of fluorescent probe diS-C_3-(5) in membrane and aqueous media. Gen. Physiol. Biophys. **2**, 473—486

Ivkov V. G., Pechatnikov V. A., Ivkova M. N. (1984): Redistribution of positively charged probes in membrane suspension under the action of transmembrane potential. Gen. Physiol. Biophys. **3**, 19—30

Krasne S. (1980): Interactions of voltage-sensing dyes with membranes. II Spectrophotometric and electrical correlates of cyanine dye adsorption to membranes. Biophys. J. **30**, 441—462

Ross W. N., Salzberg B. M., Cohen L. B., Davil H. V. (1974): A large change in dye absorption during the action potential. Biophys. J. **14**, 983—995

Sims P. J., Waggoner A. S., Wang C. H., Hoffman J. F. (1974): Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. Biochemistry **13**, 3315—3330

Tsien R. J., Hladky S. B. (1978): A quantitative resolution of the spectra of a membrane potential indicator, diS-C_3-(5) bound to cell components and to red blood cells. J. Membrane Biol. **38**, 73—97

Waggoner A. S., Wang C. H., Tolles R. L. (1977): Mechanism of potential-dependent light absorption changes of lipid bilayer membranes in the presence of cyanine and oxonol probes. J. Membrane Biol. **33**, 109—140

West W., Pearce S. (1965): The dimeric state of cyanine dyes. J. Phys. Chem. **69**, 1894—1903

Received June 15, 1983/Accepted November 3, 1983