Spectral Properties of Fluorescent Dyes in Lecithin Vesicles

Probes for the Structure of Lipid Bilayer Membranes and for Membrane Potentials

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Lecithin Vesicles, Fluorescent Probes, Phase Transitions, Membrane Potentials

Three fluorescent dyes (a merocyanin, a cyanin and a modified umbelliferon) have been incorporated into vesicles from three different lecithins. Their absorption spectrum, emission spectrum and fluorescence polarization in the lipid vesicles has been studied and compared with the spectral properties in homogeneous organic solutions. The reactivity of lipid bound dyes with reagents in the aqueous phase was investigated. The location of various dyes in the lipid membrane and their use as indicators of phase transitions or membrane potentials are discussed.

Introduction

The importance of membrane potentials for living organisms has been accepted since a long time. The relevance of phase transitions for the function of biomembranes is still controversial. In both cases direct measurements of the property in question (*e.g.* by microelectrodes in the case of the membrane potentials or by X-ray analysis in the case of phase transitions) are difficult or not possible in living organisms. Therefore, indirect methods have been widely applied. One of these methods is the application of optical probes. Changes in the absorption and emission spectra of various dyes which are not covalently bound to membranes have been used to monitor changes of membrane potentials in nerves [1, 2], erythrocytes [3, 4] and cell organelles [5]. The use of optical probes is not limited by the size of the studied objects, which is the case with microelectrodes, and therefore would be more convenient in many cases from a technical point of view. Fluorescent dyes are also widely used to get information on membrane structure and dynamics [6, 7]. Unfortunately the physical-chemical interpretation of dye-spectra in membranes is only possible in a very qualitative way. It seems posible to improve upon this situation by studying promising dyes in various solvents and in lipid model membranes. This was the purpose of the experiments reported here. Previously a merocynin dye and a cyanine dye have been used as potential indicators [2, 4] and a derivative of 7-hydroxycumarin (umbelliferon) has been used as a lipoid pH-indicator [8]. Here, these dyes have been studied in homogeneous solutions and in vesicles made from three different lecithins. Several other fluorescent probes have been studied less extensively for comparison. Absorption spectra, emission spectra and fluorescence polarization were measured. The reaction of two dyes with OH^- added to the aqueous phase was studied in the absence and presence of lecithin vesicles. The results show that the dyes may be partly protected against reagents added to the aqueous phase after incorporation into lipid membranes. The effect on the dye spectra of a potential created across the vesicle membrane by an ion gradient and valinomycin or gramicidin was investigated.

The obtained data are used to discuss the location of the probes within the lipid membrane and the reasons why certain dyes are good indicators for phase transitions in the lipid membrane while others are less suitable. The question is discussed whether the studied dyes indicate directly the membrane potential or if they probe certain structural changes in the membrane which may be induced by the electric field but also by other parameters (as temperature).

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Materials and Methods

1. Chemicals

Dyes (for chemical structure see Fig. 2): Merocyanin 540 (MC-540) was obtained from Eastman organic chemicals and used without further purification. 3,3′-Dipropylthiacarbocyanin [diS-C_3(5)] was a gift from Dr. A. S. Waggoner, Amherst College, Massachusetts. 4-Pentadecylumbelliferon (C_{15}-UBF) was synthesized and chromatographically purified by Dr. H. Alpes in our laboratory. The spectral pro-

perties of this substance were identical to that of 4-Heptadecylumbelliferon (C_{17}-UBF) which was a gift of Dr. P. Fromherz, Max-Planck-Institut für Biophysikalische Chemie, Göttingen. Umbelliferon (7-hydroxycumarin) (UBF) was a product from Merck, Darmstadt, and purified by column chromatography. 1,4-Diphenyl-benzene (DPB) from Fluka Buchs and 1,6-diphenyl-1,3,5-hexatriene (DPH) from Schuchardt, München, were used without purification.

Lecithins: Egg-phosphatidylcholine (egg-PC) which was prepared in our laboratory by K. Janko, 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine [di-(16:0)-PC] from C. Roth, Karlsruhe, and 1,2-di-hexadecyl-sn-glycerol-3-phosphorylcholine [di(16:0)-EPC] from Fluka, Buchs, gave all three a single spot in thin-layer chromatography.

Salts and solvents: 0.1 M KCl in double distilled water pH ~6 was used for most vesicle preparations. All inorganic compounds and organic solvents used were of the highest purity grade from Merck, Darmstadt, and used without purification.

2. *Vesicle preparation and electron microscopy*

Vesicles were prepared by two different methods based on the work of Huang [9] or Batzri and Korn [10].

Sonication-vesicles: 10 mg egg-PC per ml salt solution (usually 2 ml) were sonicated under argon at 4 °C for 30 min, with a Branson B-12 sonifier and microtip at the power setting 3. For di(16:0)-PC and di(16:0)-EPC vesicles 5 to 10 mg lipid per ml was used and sonicated under argon at 60 °C for 5 to 10 min. All vesicle suspensions were centrifuged at 25000 × *g* for 10 min, to remove titanium or undispersed lipids.

Injection-vesicles: An ethanol solution containing about 30 μmol egg-PC per ml was rapidly injected through a Nr. 732 needle of a Nr. 1725 Hamilton syringe into a well stirred 0.1 KCl solution at 20 °C. The final ethanol concentration in this solution was maximally 5%. For di(16:0)-PC and di(16:0)-EPC the same procedure was used, but at 60 °C and about 20 μmol lecithin per ml ethanol solution. All soltuions were centrifuged at 25000 × *g* for 10 min. Usually no pellet formed.

For separation of vesicles and unbound dye 1 ml of the vesicle solution was chromatographed on Sephadex-G-75 in a column of 30 cm length and 0.9 cm inner diameter. For a survey of the size distribution and determination of trapped volumes with $K_3[Fe(CN)_6]$ [11] the vesicle solutions were chromatographed on a thermostated K26 column of Pharmacia filled with Sepharose 4B (bed length 25 cm, inner diameter 2.6 cm). In the effluent the extinction at 240 nm or 420 nm was measured continually with a Zeiss DMR 10 spectrometer. Lipid concentrations were determined from the total phosphorus content by the method of Bartlett [12], $K_3[Fe(CN)_6]$ concentrations were measured by the method of Newman and Huang [11].

Electron microscopy: 1 to 3 drops of a vesicle suspension were spread in a Petri dish on 2% phosphotungstic acid pH ~6.1. The spread surface layer was marked by talcum powder. Copper grids with carbon coated formvar films were tipped on the surface to remove a drop of subphase with the surface layer. After drying the specimen were viewed in a Siemens Elmiskop I at 100,000 x magnification. Prints were made with 150,000 x magnification. A survey of different areas of at least three grids showed reproducible appearence of each preparation.

3. *Absorption spectra*

The measurements were all done with a Zeiss DMR 10 recording spectrophotometer in quartz cuvettes of 1 cm depth. Bandwidth was between 0.4 and 1 nm. To correct for scattering of vesicle suspensions the reference beam passed a solution identical to the probe, but without dyes.

The smallest detectable changes in extinction were $\Delta E \sim 5 \times 10^{-3}$ in vesicle suspensions ($\sim 1.5 \times 10^{-3}$ in solution) at a full scale deflection of $E = 0.1$. For the measurement of small ΔE values the scattering from sample and reference must be identical, a condition sometimes difficult to meet.

4. *Fluorescence spectra and fluorescence polarisation*

All measurements were done with a Hitachi-Perkin-Elmer MPF 4 spectrofluorometer equipped with a polarization attachment. No correction for the photomultiplier sensitivity and the lamp spectrum was applied. This is justified when only emission maxima of the same dye obtained with identical settings of the fluorometer are compared (Table I). For polarization measurements the monochromator bandwidth was increased up to 6 nm. 1 cm square quartz cuvettes were used. The extinction of the solutions at the excitation wavelength usually did not exceed $E = 0.03$ so that inner filter effects could be neglected.

Fluorescence intensities were measured with 4 combinations of the polarizer and analyzer positions (incident beam vertically polarized and analyser vertically or horizontally oriented yielded J_{VV} and J_{VH}; in an analogous way, with horizontally polarized beam, J_{HV} and J_{HH} were obtained). The polarization of fluorescence was calculated from the measured intensities J_{VV}, J_{VH}, J_{HV}, J_{HH} by the method

of Chen and Bowman [13]

$$p = \frac{J_{VV} - \frac{J_{HV}}{J_{HH}} J_{VH}}{J_{VV} + \frac{J_{HV}}{J_{HH}} J_{VH}} .$$

Scattering from vesicle suspensions without dyes did not contribute more than $1-2\%$ to the measured fluorescence intensity. It was tested that this did not significantly affect the polarization values. Settings of excitation and emission monochromator will be given together with the results. The temperature was controlled by circulating thermostated water through the cell holder. Warming of the samples occured at about 1 °C/min, cooling at a slightly lower rate.

5. *Experiments with ion gradient and ionophores*

Ion gradients were obtained by diluting vesicle suspensions prepared in 10^{-1} M KCl (or 10^{-1} M LiCl) into an aqueous phase containing 10^{-1} M LiCl (or NaCl, KCl, RbCl, CsCl, NH_4Cl). The final lipid concentrations were usually 3×10^{-5} M. Thereafter diS-C_3(5) or MC-540 was added from ethanolic solution to give a dye concentration of approx. 5×10^{-7} M. Alternatively, the dye was added to the lecithin before sonication. The fluorescence intensity or the extinction of the solutions was followed using the settings specified under results. After 3 – 5 min valinomycin was added from ethanolic solution (3 or 30 µl 10^{-4} M solution made the aqueous phase 10^{-7} M or 10^{-6} M) and rapidly mixed. The change in fluorescence intensity or extinction was followed for up to 10 min. Alternatively, vesicles were diluted in 0.2 M sucrose and gramicidin added in the same way as valinomycin.

Results

1. *Characterization of di(16:0)-EPC vesicles*

While vesicles from egg-PC and di(16:0)-PC have frequently been studied and are well characterized, no studies on di(16:0)-EPC vesicles have been published. Therefore vesicles from the three lecithins have been compared by Sepharose-chromatography and electronmicroscopy. Both methods show that the vesicles have similar structure, but different size distribution. In Fig. 1 the efflux of the vesicles from a Sepharose 4B column is followed by measuring the extinction at 240 nm. The first large peak (representing large liposomes) which coincides with the void volume of the column as measured by dextran-blue 2000 is cut off in all records. In egg-lecithin

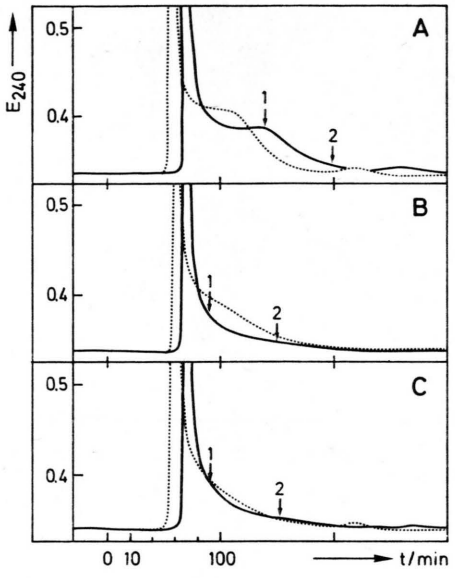

Fig. 1. Chromatography of lecithin vesicles on Sepharose 4B. The extinction at 240 nm *versus* elution time is plotted. At time zero 1 ml of vesicle suspensions (10 mg-PC/1 ml 10^{-1} M KCl) was applied to the column (i. d. 2.6 cm, length 25 cm). Elution was done either at 20 °C (——) or 35 °C (– – –). Flow rate was 1.2 ml/min at 20 °C and 1.5 ml/min at 35 °C. The first peak coincided with the void volume as determined by blue dextran 2000. Samples for determination of V_i/N were taken between points 1 and 2. A: egg-PC; B: di(16:0)-PC; C: di(16:0)-EPC.

vesicles a second smaller peak represents small unilamellar vesicles [9]. A third very small peak of unknown origin is consistently recorded. The second peak is not separated in di(16:0)-PC and di(16:0)-EPC vesicles, but is only indicated by a long trailing. Chromatography at 35 °C gives a better separation with di(16:0)-PC vesicles although the separation in egg-PC is poorer (due to increased diffusion and faster flux rates). With di(16:0)-EPC the effect of temperature increase is small. Similar records were obtained in the presence of $K_3[Fe(CN)_6]$ when measuring the extinction at 420 cm (only at 20 °C). Samples contained between points 1 and 2 were collected and analyzed for lipid and $K_3[Fe(CN)_6]$. Controls showed that $K_3[Fe(CN)_6]$ was not bound measurably to the lipid membrane. From these data the aqueous volume trapped inside the vesicles was calculated. It was 200 cm^3 per mol egg-PC, 500 cm^3 per mol di(16:0)-PC or di(16:0)-EPC (±20% in all cases). The size distribution of unfractionated di(16:0)-EPC vesicles studied by negative staining revealed the most frequent diameters. From 959 vesicles measured on 12 different plates 13% had a

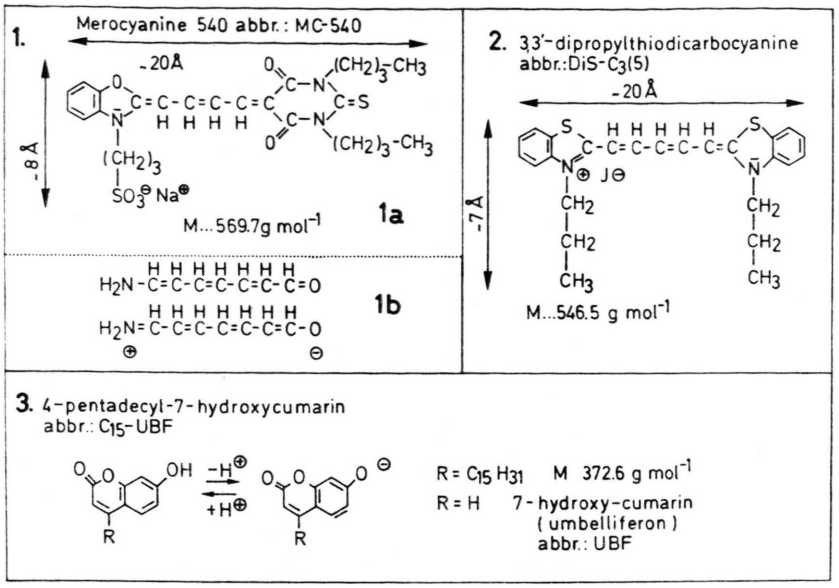

Fig. 2. Chemical structure of fluorescent dyes.

diameter of (140 ± 30) Å, 33% of (200 ± 30) Å, 19% of (270 ± 30) Å, 13% of (330 ± 30) Å, 8% of (400 ± 30) Å, 4% of (470 ± 30) Å and the remaining 10% were distributed between 800 and 1400 Å. Egg-lecithin vesicles formed stacked aggregates and only few single vesicles could be counted. From 100 counted particles about 20% were in the range (140 ± 30) Å, 20% in the range (200 ± 30) Å and the rest distributed between 250 and 1200 Å.

2. *Absorption and emission spectra*

The spectra of MC-540, diS-$C_3(5)$, C_{15}-UBF and UBF (for chemical structures see Fig. 2) have been measured in water, different *n*-alcohols, chloroform and dioxan-1,4. The longest wavelength absorption maxima (and extinction coefficients) are given in Table I. Spectra were also measured in solutions containing vesicles of three different lecithins. It is seen from Table I that a shift of >30 nm in the longest wavelength absorption band occures when the aqueous solution of MC-540 is mixed with vesicle suspensions and a shift of nearly 20 nm occurs in the case of diS-$C_3(5)$. These changes are due to binding of the dyes to the lipid membrane. It was confirmed by Sephadex-chromatography that at molar ratios of lecithin : dye about 1000 : 1 the dyes are completely bound to the vesicles. Extinction coefficients of the membrane-bound dyes were measured at this lecithin : dy ratio. C_{15}-UBF is completely bound to the vesicles at all lecithin : dye ratios studied (the smallest ratio was 50 : 1). Usually measurements were done at the ratio 100 : 1. UBF was used for comparison in homogenous solutions only.

The absorption spectrum of solutions containing vesicles and MC-540 (molar ratios lecithin : dye about 140 : 1) was measured at 20 °C and 45 °C. In Fig. 3 A the difference spectrum is shown. In di(16:0)-PC vesicles the maximum at 565 nm increased, the shoulder at 520 decreased. A very

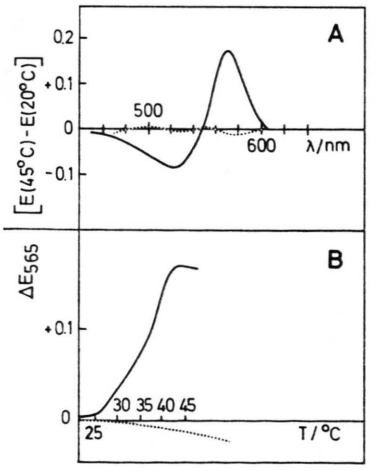

Fig. 3. Temperature dependance of MC-540 absorption spectrum in lecithin-visicles (lecithin: MC-540=150:1). A: extinction at 45 °C minus extinction at 20 °C plotted *versus* wavelength. ———, di(16:0)-PC vesicles; — — —, egg-PC vesicles. B: extinction at 565 nm *versus* temperature; ——— di(16:0)-PC vesicles; — — —, egg-PC vesicles.

Table I. Absorption- and fluorescence maxima of dyes in organic solvents and lecithin vesicles. (Values given in square brackets [] are uncertain because of limited solubility or spectral changes after dissoltuion. The dye concentrations used for the calculation of ε in vesicles refer to the total volume of the vesicle suspensions).

Dye →		MC-540			diS−C_3 (5)			C_{15}-UBF (C_{15}-UBF$^-$)			UBF (UBF$^-$)		
Solvent	Dielectric constant	Abs. max. [λ/nm] ($\pm$1 nm)	$\varepsilon/10^5$ [M^{-1} cm^{-1}] ($\pm$10%)	Fluor. max. [λ/nm] ($\pm$2 nm)	Abs. max. [λ/nm] ($\pm$1 nm)	$\varepsilon/10^5$ [M^{-1} cm^{-1}] ($\pm$10%)	Fluor. max. [λ/nm] ($\pm$2 nm)	Abs. max. [λ/nm] ($\pm$2 nm)	$\varepsilon/10^4$ [M^{-1} cm^{-1}] ($\pm$5%)	Fluor. max. [λ/nm] ($\pm$2 nm)	Abs. max. [λ/nm] ($\pm$1 nm)	$\varepsilon/10^4$ [M^{-1} cm^{-1}] ($\pm$5%)	Fluor. max. [λ/nm] ($\pm$2 nm)
water (pH=6)	80	532±2 [a]	0.6	570	648±2 [b]	1.5 [c]	666	[325 (365)]	—	[384] —	325 (369)	1.5 (1.9)	450 (452)
methanol	33	555	1.5	575	654	1.9	669	324 (368)	1.5 (2.0)	380 (450)	325 (369)	1.5 (2.0)	385 (452)
ethanol	25	559	1.6	579	657	2.0	675	326 (376)	1.5 (2.1)	380 (450)	326 (377)	1.5 (2.1)	386 (452)
propanol-1	20	561	1.7	580	660	2.0	676	326 (376)	1.5 (2.1)	379 (450)	327 (380)	1.5 (2.1)	384 (452)
butanol-1	18	563	1.8	581	661	2.1	678	326 (377)	1.5 (1.9)	379 (450)	329 (381)	1.5 (2.1)	383 (452)
hexanol-1	14	566	1.8	582	662	2.0	680	327 (377)	1.5 (1.9)	378 (450)	328 (380)	1.4 (1.9)	383 (452)
octanol-1	10	566	1.9	583	663	2.0	682	327 (377)	1.5 —	378 (450)	329 (380)	1.5 (2.1)	381 (452)
decanol-1	8	567	1.9	582	665	1.7	680	327 —	1.5 —	377 —	328 (380)	1.3 —	378 (452)
chloroform	4,8	571	0.9 [d]	587	666	1.9	680	323 —	1.5 —	375 —	324 —	1.5 —	379 —
dioxan-1,4	2.4	565 [e]	1.6	583	[670] [f]	—	—	[321] —	— —	[370] —	[322] —	— —	[370] —
egg-PC-vesicles		567±2	1.5	583	664±2	1.2	683	327 (380)	1.5 (1.7)	386 (445)			
di (16:0)-PC-vesicles [g]		565±2	1.4 [h]	582	662±2	1.2	680	326 (377)	1.5 (1.7)	386 (445)			
di (16:0)-EPC-vesicles		563±2	1.4 [h]	582	664±2	1.4	681	328 (380)	1.5 —	387 (445)			

[a] The spectrum is nearly independent of pH between 3 and 8. Above pH 9 the maximum at 532 nm disappears and a new maximum at 390 nm appears.
[b] The spectrum is nearly independant of pH between 3 and 12. Below pH 2 the maximum at 648 nm decreases.
[c] This value is a lower limit of ε. A continuous decrease of the extinction is observed in aqueous solutions, which becomes faster with increasing ionic strength.
[d] In the presence of approximately 5% ethanol a value of 1.6×10^5 M^{-1} cm^{-1} was found.
[e] A small shoulder at 600 nm appeared, with was absent in all other solvents.
[f] In the presence of approximately 5% ethanol the initial value was 2×10^5 M^{-1} cm^{-1}. This maximum at 670 nm continually decreased (to half its original size) and a new maximum at 480 nm appeared.
[g] Molar ratio dye: lecithin between 1:500 and 1:1500.
[h] Above the phase transition (50 °C) an $\varepsilon=1.7\times10^5$ M^{-1} cm^{-1} was measured.

similar spectral change has been found during the action potential with an analog of MC-540 bound to the nerve membrane by Ross *et al.*[2]. In Fig. 3 B the extinction at 565 nm is plotted at different temperatures, and shows a sharp increase in the phase transition region close to 40 °C. With egg-lecithin vesicles only very small spectral changes were observed (dashed curve).

Since MC-540 reacts irreversible with OH^- under colourfading in water (pH $\sim$11), it was tested wether the dye is protected in lipid membranes. This was found in fact. When NaOH reacted with the dye in the absence and presence of excess lipid vesicles (lipid : dye ratio 1400 : 1) very different reaction rates were found by following the extinction decrease at 565 or 535 nm. In the absence of lipid vesicles the reaction was complete within 5 min (at 20 °C) and 1 min (at 50 °C). In the presence of excess egg-PC or di(16:0)-PC vesicles the reaction was biphasic. A fast exponential extinction decay was followed by a very slow reaction. The extinction decay of the slow reaction was approximately linear with time. Extrapolating this back to time zero gave a slowly reacting portion of MC 540 (about one third of the total amount). The faster exponential decay was completed within 70 min (in egg-PC vesicles at 20 °C) and within 45 min (in di(16:0)-PC vesicles at 20 °C) that is 10 – 15 times slower than without vesicles. No slowly reacting part of MC-540 was found at 50 °C and the exponential decay was complete within 25 min (in egg-PC vesicles) and 50 min (in di(16:0)-PC vesicles) about 30 – 50 times slower than without vesicles.

The absorption spectra of vesicles plus diS-C_3(5) showed a small temperature-dependance. The maximum at 660 nm decreased, the shoulder at 620 nm increased slightly.

The absorption spectra of vesicles plus C_{15}-UBF showed no temperature-dependance.

As seen from Table I, the absorption maxima of C_{15}-UBF and UBF depend only slightly on the polarity of the solvent. Therefore absorption spectra cannot be used to get information on the position of the chromophore in membrane-bound C_{15}-UBF. Addition of KOH to the aqueous phase showed that the chromophore is easily accessible to OH^- in contrast to membrane-bound MC-540. All C_{15}-UBF present in lecithin vesicles reacted according to the reversible scheme $ROH + OH^- \rightleftharpoons RO^- + H_2O$ during the mixingtime (<30 sec) when the pH was raised to 11. The pK of C_{15}-UBF in egg-PC vesicles and 100 mM KCl is 9.6 ± 0.2 (J. Wulf, personal communication). This is similar to the value of 10.1 given by Fromherz[8] for C_{17}-UBF in di(16:0)-PC-octadecane monolayers on 10 mM NaCl. The pK of UBF is 7.8 in the presence of egg lecithin liquid crystals[18].

Whereas the emission spectra of MC-540 and diS-C_3(5) have a similar shape in PC-vesicles and homogeneous organic solutions, this is not the case with C_{15}-UBF as seen in Fig. 4. Three emission peaks are evident, the main peak around 380 – 390 nm and two smaller peaks around 440 – 450 nm and 490 nm. Similar spectra are obtained by addition of small amounts of water to ethanolic solutions of C_{15}-UBF (Fig. 4, 5 A) and UBF. The fatty acid

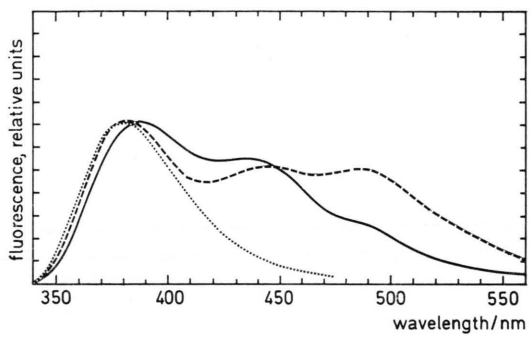

Fig. 4. Fluorescence emission spectrum of C_{15}-UBF, normalized to equal intensity at the main emission peak. ····, in dry ethanol (10^{-6} M C_{15}-UBF); – – –, in ethanol +10% water (10^{-6} M C_{15}-UBF); ———, in egg-PC vesicles (PC : C_{15}-UBF = 100 : 1).

chain apparently does not affect the position and relative intensities of the peaks appreciably. Since the absorption spectra of C_{15}-UBF are nearly identical in PC-vesicles and organic solvents, the fluorescence spectra are tentatively explained with the formation of different populations of fluorescent species. The 380 nm peak is attributed to the excited protonated species ROH*, the peak around 440 nm to the deprotonated species RO^{-*}, which is formed from ROH* in the presence of proton-acceptors. This mechanism has been shown to exist in aromatic oxycompounds by Förster[19] and Weller[20] and will be discussed later for this system.

A characteristic difference between solutions of C_{15}-UBF in ethanol plus water and in vesicles was found in the temperature dependance of the emission spectra (Fig. 5 A, B). In the first case all three peaks decrease with increasing temperature by

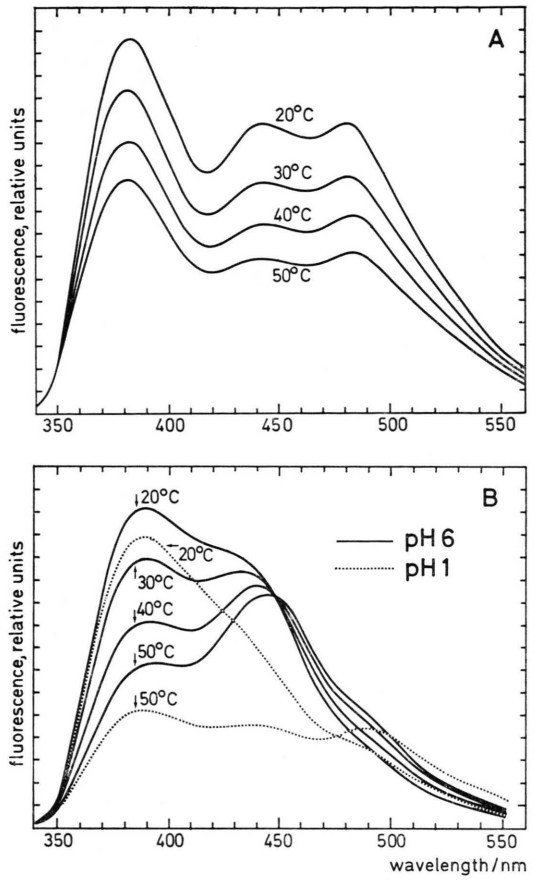

Fig. 5. Temperature dependance of the C_{15}-UBF emission spectra. A: 10^{-6} M C_{15}-UBF in ethanol + 10% water. B: C_{15}-UBF in di(16:0)-EPC vesicles (PC : dye = 100 : 1) in 10^{-1} M KCl, pH=6 (———); in 10^{-1} M H_2SO_4, pH = 1 (– – –).

roughly the same factor. In all vesicle suspensions the 390 nm peak decreases much more than the 445 nm peak. The 490 nm peak decreases very slightly in egg-lecithin suspensions, and increases slightly in di(16:0)-PC vesicle or di(16:0)-EPC vesicle suspensions. di(16:0)-EPC-vesicles were also studied at pH ~1. Under those conditions, the second peak was reduced and still decreased, whereas the third peak increased. At 50 °C all three peaks had about the same height (see Fig. 5 B). All experimental facts are consistent with the hypothesis of three fluorescent species ROH*, RO^{-}* and $(ROH \cdot RO^{-})$*. It would be interesting to study this system in more detail (*e. g.* in deuterated solvents) but this was not done up to now.

The anionic form C_{15}-UBF^{-} (after addition of twice the stöchiometric amount KOH to C_{15}-UBF) shows nearly identical emission spectra in methanol, ethanol (with or without water) and lecithin vesicles (emission maximum 450 ± 2 nm), with a small temperature dependance.

3. *Fluorescence polarization*

In Table II polarization values p are given for several dyes in organic solutions and incorporated into different PC-vesicles. Some values taken from the literature are included for comparison. MC-540 and diS-C_3(5) show the highest p-values with small differences between egg-lecithin and the saturated lecithins. C_{15}-UBF (at pH 6) and C_{15}-UBF^{-} (at pH 11) show significantly lower p values in egg-lecithin vesicles, similar to all other dyes given in Table II. For MC-540, diS-C_3(5), C_{15}-UBF and C_{15}-UBF^{-} the temperature dependance of p was measured between 20° and 50 °C. Fig. 6 shows the results. MC-540 shows a small and diS-C_3(5) (not shown) a negligable drop of p between 35 and 40 °C in the saturated lipids at the lipid : dye ratio 1500 : 1. (This ratio was chosen to be sure that the dyes were completely bound to the vesicle.) The fluorescence intensity of MC-540 in di(16:0)-PC and di(16:0)-EPC-vesicles slightly increases between 20 and 35 °C, which can be explained by the extinction change at 560 nm as illustrated in Fig.

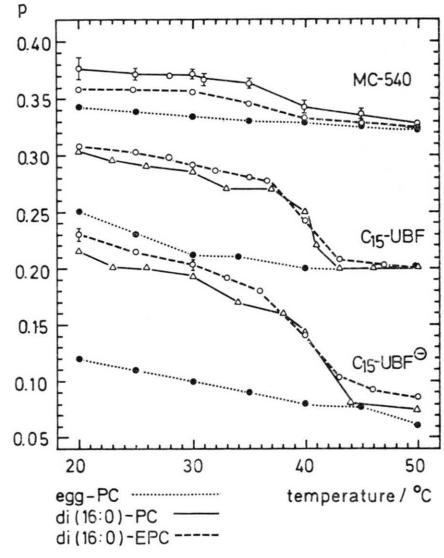

Fig. 6. Temperature dependance of fluorescence polarization of three dyes in lecithin-vesicles. The p-values of MC-540, C_{15}-UBF, and C_{15}-UBF^{-} in vesicles made of three different PCs are plotted *versus* temperature. MC-540: excitation 565 nm emission 585 nm. C_{15}-UBF: excitation 330 nm emission 390 nm. C_{15}-UBF^{-}: excitation 380 nm emission 450 nm.

Table II. Polarization p of several dyes in organic solvents and lecithinvesicles ($T=21$ °C).

Dye	Organic solutions: Solvent	Excitation → emission wavelength [nm] (long pass filter)	p (±0.005)	Vesicle suspensions in 0.1 M KCl, pH∼6: Lecithin	Molar ratio PC : dye	Excitation → emission wavelength [nm] (long pass filter)	p [a] (±0.01)
MC-540	methanol	565 → 585	0.176	egg-PC	1500:1	565 → 585	0.35
					600:1		0.33
	chloroform		0.088	di(16:0)-PC	1500:1		0.37
				di(16:0)-EPC	1500:1		0.36
diS-C_3(5)	methanol	660 → 680	0.019	egg-PC	1500:1	660 → 680	0.34
					600:1		0.32
	chloroform		∼0	di(16:0)-PC	1500:1		0.32
				di(16:0)-EPC	1500:1		0.32
C_{15}-UBF	methanol	325 → 380 (350)	0.014	egg-PC	100:1	330 → 390 (350)	0.25
				di(16:0)-PC	100:1		0.30
				di(16:0)-EPC	100:1		0.30
C_{15}-UBF$^-$	methanol	370 → 450 (430)	∼0	egg-PC	100:1	380 → 450 (430)	0.12
				di(16:0)-PC	100:1		0.22
				di(16:0)-EPC	100:1		0.21
3-octadecyl-lumiflavin (flavin 1)	ethanol	465 → 515 (430)	0.020	egg-PC	150:1	465 → 510 (430)	0.26
				di(16:0)-PC	150:1		0.33
1,4-diphenyl-benzene (abbr.DPB)	cyclo-hexane	290 → 340 (310)	0.012	egg-PC	150:1	285 → 340 (310)	0.20
				di(16:0)-PC	150:1		0.31
1,6-diphenyl-1,3,5-hexa-triene (abbr.DPH)	cyclo-hexane	375 → 450 (390)	0.022	egg-PC	150:1	375 → 450 (390)	0.14 [b]
				di(16:0)-PC	150:1		0.34
dansyl-PE	—	—	—	egg-PC	100:1	366 → >450	0.13 [c]
				di(16:0)-PC	100:1		0.21
perylene	limiting value of p in viscous solutions [5]		0.464	egg-PC	900:1	410 → 468	0.06 [d,e]
				di(16:0)-PC	1250:1		0.21

[a] The standard error for samples out of the same vesicle preparation is ±0.005. For different vesicle preparations (prepared under identical conditions) it is ±0.012.
[b] The same value as found in this work is given by Shinitzky and Barenholz [14].
[c] From the work of Faucon and Lusson [15].
[d] From the work of Papahadjopoulos *et al.* [16].
[e] From the work of Jacobson and Wobschall [17].

3 A. It decreases in the normal way above 40 °C. In egg-PC vesicles it continually decreases between 20 and 50 °C. diS-C_3(5)-fluorescence shows a similar behaviour in all lipid vesicles. C_{15}-UBF both in the neutral and negative form shows a characteristic drop in p close to the well-known phase transition temperature of di(16:0)-PC-bilayers around 40 °C. C_{15}-UBF$^-$ clearly yields lower p-values than the neutral form. The temperature dependance of p was not followed for all the other molecules listed in Table II. But they all show a higher p-value in saturated lecithin vesicles at 20 °C which presumably is due to immobilization below the phase transition.

When p-values are used to determine the orientation of dyes in the lipid membrane, it is necessary to know the angle between the transition moments for absorption and emission of each dye. In MC-540, diS-C_3(5) and C_{15}-UBF these transition moments are nearly parallel because the extrapolation of $1/p$ *versus* $T/\eta \to 0$ (Perrin-plot) in solvents of different viscosity η yielded a limiting polarization p_0 between 0.45 and 0.50 for all three dyes.

4. Experiments with ion gradients and ionophores

The results of Sims *et al.* [4] could be reproduced with sonication- and injection-vesicles and several additional observations were made. Instead of following the fluorescence of diS-C_3(5) one can also use the extinction of the dye at 650 nm to obtain equivalent results. The new absorption peak to the

red of 700 nm which should appear according to Sims *et al.*[4] after addition of valinomycin was not clearly evident in our experiments. The addition of cholesterol to egg-PC was found unnecessary to get a stationary fluorescence response after addition of valinomycin. Nearly the same effect could be obtained by reducing the valinomycin concentration from 10^{-6} M to 10^{-7} M (see Fig. 7 A, B). Even at

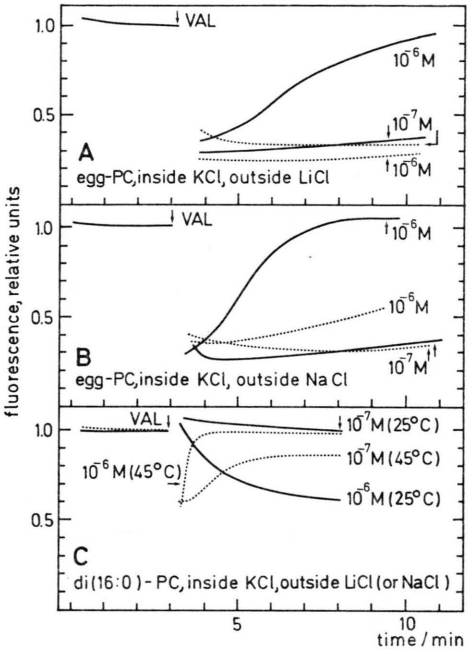

Fig. 7. A, B, Indication of membrane potentials by diS-C_3 (5); excitation 622 nm, emission 670 nm. T=21 °C, 10 μl of vesicles prepared in 10^{-1} M KCl, pH=6, were diluted with 3 ml of 10^{-1} M LiCl or NaCl. 15 μl of 10^{-4} M diS-C_3 (5) in ethanol were added. At the points marked by an arrow (↓) either 30 μs or 3 μl 10^{-4} M valinomycin in ethanol were added. ——, vesicles prepared from egg-PC; — — —, vesicles prepared from egg-PC+cholesterol (molar ratio 3:1). The signals are normalized to equal size before addition of valinomycin. C: Conditions as in A, B. Vesicles prepared from di(16:0)-PC. ——, 25 °C; — — —, 45 °C.

the lower valinomycin concentration there would be about 10 carrier molecules per vesicle, sufficient to establish the membrane potential, which is negative inside the vesicles.

When the potential was made positive inside by filling the vesicles with 0.1 M LiCl diluting with 0.1 M KCl, NaCl, RbCl etc. and adding valinomycin only a small fluorescence increase (ΔF max. 20%) was observed.

Vesicles from di(16:0)-PC were studied below and above the phase transition (Fig. 7 C). At 25 °C 10^{-7} M valinomycin gave no effect, whereas 10^{-6} M valinomycin showed a slow fluorescence decrease. At 45 °C a fast fluorescence decrease was followed by a reincrease, which was faster at the higher valinomycin concentration. Nearly identical results (not shown) were obtained when the vesicles were diluted with 0.2 M sucrose (to keep the osmolarity constant) instead of 0.1 M LiCl. The following experiments (Fig. 8) were done with 2×10^{-7} M ionophore concentrations. In the presence of a K^+-gra-

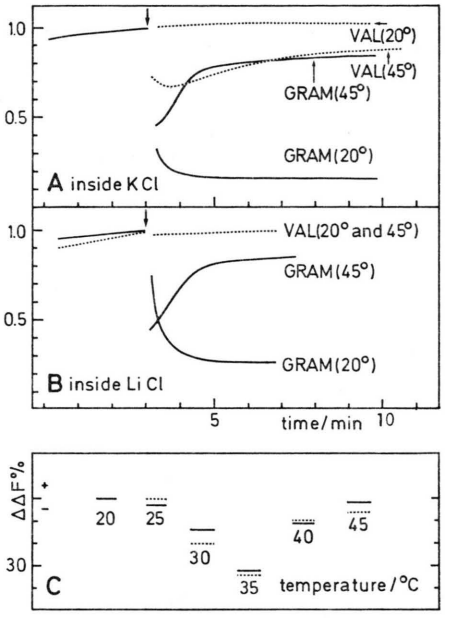

Fig. 8, A, B. Indication of membrane potentials by diS-C_3 (5); excitation 622 nm, emission 670 nm. 10 μl vesicles prepared from di(16:0)-PC in 0.1 M KCl (LiCl) pH=6, were diluted with 3 ml 0.2 M sucrose in water pH=6. 15 μl of 10^{-4} M diS-C_3(5) in ethanol was added. At the points marked by an arrow (↓) 6 μl of 10^{-4} M ionophore (either valinomycin or gramicidin) in ethanol was added. The original records were normalized to equal signal size before addition of ionophores. C. Temperature dependence of the fluorescence decrease of diS-C_3(5) induced by the membrane potential in di(16:0)-PC vesicles. Measuring conditions as in A, B but 0.1 M LiCl for dilution (only valinomycin 2×10^{-7} M) $\Delta\Delta F$ was obtained from two series of measurements. In the reference solution a: dilution was done with 0.1 M KCl (no K^+ gradient), b: ethanol without valinomycin was added. In the sample solution c: valinomycin was added to the solution after dilution of KCl vesicles with LiCl. d: valinomycin was added to the undiluted KCl vesicles. The solution was warmed to 45 °C for 5 min, cooled to 20 °C again and diluted with LiCl. The fluorescence changes ΔF were recorded in all 4 solution at the indicated temperatures (3—5 min were necessary to obtain a constant signal at each temperature). Signals were normalized to equal size at 20 °C. $\Delta\Delta F$ was obtained by subtracting ΔF in the sample, from ΔF in the reference (given in % of the 20 °C signal). — — —, $[\Delta F(a)-\Delta F(c)]$; ——, $[\Delta F(b)-\Delta F(d)]$.

dient valinomycin did not give effects at 20 °C but did at 45 °C (Fig. 8 A). As expected from its specificity, valinomycin did not work in the presence of a Li^+-gradient at either temperature (Fig. 8 B). Gramicidin gave effects under all conditions (Fig. 8 A, B) but at 45 °C the time course of the fluorescence change was different. Fig. 8 C (see legend for description) shows that valinomycin effects become measurable at 30 °C. Above 35 °C the effects decrease again which is expected from the results in Figs 7 C and 8 A. The effects with MC-540 were always smaller than with diS-C_3(5). When the molar ratio lecithin :dye was 60 : 1 or higher insignificant effects were observed when analog experiments were done as with diS-C_3(5). At molar ratios of about 20 : 1 the effects were qualitatively the same as with diS-C_3(5), but the fluorescence decrease was only between 10 – 20% compared to about 70% with diS-C_3(5) (Fig. 7). The size of the effect always varied with different valinomycin concentrations. Therefore, MC-540 seems less suitable than diS-C_3(5) as a potential indicator in lipid vesicles.

Vesicles containing C_{15}-UBF did not show significant spectral changes when ion-gradients were formed across the vesicle membrane and valinomycin was added.

Discussion

Vesicles prepared from di(16:0)-EPC are an interesting system for several reasons. Firstly, the ether lecithin can be used at extreme pH values where normal esterlecithins are hydrolyzed. Secondly, the lack of carbonylgroups should influence the phospholipid-sterol interaction, because Huang [21] has recently stressed the importance of the carbonylgroups for interaction of lecithin and cholesterol (*versus* epicholesterol). Thirdly, it may be studied if a relatively small structural change in the lecithin-molecule has detectable consequences for the structure of vesicles and the phase transition in the lipid bilayer. To characterize the di(16:0)-EPC vesicles they were compared with egg-PC vesicles and di(16:0)-PC vesicles by chromatographic methods and electronmicroscopy. The 25 cm Sepharose 4B column used did not separate large and small vesicles as good as Huangs 50 cm column [9], but a thermostated column of sufficient length was not available. Also the separation of the peaks is reduced with increasing temperature as seen from Fig. 1 A. Chromatography on Sepharose 4B showed that the aqueous volume trapped inside the vesicles is very similar in di(16:0)-EPC vesicles and about 2 – 3 times larger than in egg-PC vesicles. When assuming a uniform size of the vesicles in the fraction collected between points 1 and 2 of Fig. 1 (A, B, C), an area per molecule lecithin of about 60 Å (as known from X-ray and monolayer work) and a thickness of the lipid bilayer of about 40 Å, it is possible to calculate the vesicle-diameter from the measured trapped volume V_i. By straight forward calculations the following relation is obtained:

$$\frac{V_i}{N} = \frac{L \cdot a_s \cdot r^3}{3(r^2 + R^2)} . \qquad (1)$$

V_i, Volume trapped inside of uniform vesicles formed from N moles lecithin;
r, $R = (r + 40\,\text{Å})$ internal and external radius of the vesicles;
a_s, area per lecithin molecule;
L, Avogadro-Loschmidt number.

From the experimentally determined values of V_i/N an external vesicle radius $R = 156 \pm 24$ Å is calculated for di(16:0)-EPC and di(16:0)-PC vesicles. This is in reasonable agreement with the electron microscopic finding that more than 50% of the unfractionated vesicles have a diameter between 170 and 300 Å. For egg lecithin visicles a $R = 102 \pm 12$ Å was calculated. In the electronmicrographs most free vesicles had a diameter between 140 and 200 Å.

An interesting observation was made when Sepharose chromatography was performed at 35 °C. A higher proportion of small vesicles was eluted than at 20 °C in the case of di(16:0)-PC vesicles (Fig. 1 B). This directly confirms the interpretation of Yi and MacDonald [22] that a decrease in the turbidity of di(16:0)-PC dispersions around 32 °C is due to a disaggregation phenomenon. A similar effect was smaller with di(16:0)-EPC (Fig. 1 C) and absent with egg-PC vesicles (Fig. 1 A).

The optical properties of dyes in lecithin vesicles will be discussed next. The dyes were usually incorporated into unfractionated vesicles. In all tested cases the results were the same with chromatographed vesicle fractions.

MC-540 belongs to a group of dyes which exhibit large responses to changes of the solvents dielectric constant. These dyes possess a strong electron-donating and an electron-accepting group linked by conjugated double bonds, such that charge

separation is possible as schematically shown in Figs. 2, 1 b. If this zwitterionic resonance structure significantly contributes to the electronic structure of the molecule, a large dipole moment results. For complete charge separation and dimensions of the dye as obtained from model building one calculates a dipole moment $\mu = 40$ debye for MC-540. Lippert [23] has measured a ground state dipole moment of $\mu_g = 33$ debye with a similar dye and a drecrease of μ in the excited state ($\mu_e < \mu_g$). For molecules with $\mu_e < \mu_g$ a blue shift of the absorption maximum should result with increasing polarity of the solvent. This was found experimentally for both MC-540 and diS-$C_3(5)$. In analogy to the simple approach of Waggoner and Stryer [24] one can use such molecules as probes for the polarity of their microenvironment in lipid membranes. The advantage of absorption spectra is that they are not influenced by reorientation of dipoles in the probe environment, which is the case for fluorescence spectra as discussed by Radda and Vanderkooi [6] or Azzi [7]. Absorption occurs within about 10^{-15} sec, while relaxation times for dipole-reorientation are always longer than 10^{-12} sec. The position of the absorption maxima given in Table I indicates that MC-540 and diS-$C_3(5)$ in lecithin-bilayers are located in an environment of medium polarity (like octanol-1). Chance *et al.* [25] have given a tentative location of MC-540 in mitochondrial membranes, with the sulfonate-group anchored in the region of the polar head group and the rest of the molecule extending about 15 Å deep through the ester bond region into the apolar part of the lipid bilayer.

The Stokes-shifts for both dyes in vesicles are also nearly identical to those found in octanol-1, which means that solvent reorientation (which does not influence the absorption process) is either absent or influences the emission equally in the membrane and in the alcohol. In contrast to MC-540 and diS-$C_3(5)$ the C_{15}-UBF molecule shows an additional red shift of the fluorescence maximum when incorporated into lecithin membranes which may be caused by dipol reorientation in the environment of the excited molecules. The complex emission spectrum of C_{15}-UBF shows that the probe environment in the membrane is more polar than dry ethanol or methanol and the polarity still increases with increasing temperature. The chromophore should be located on the outside of the membrane in the region between the glycerol-portion and the polar head groups but not directly in the aqueous phase. This is consistent with its good accessibility to OH^- ions, whereas MC-540 is clearly protected against OH^- in the lipid membrane. (It should be kept in mind that the reaction rate of C_{15}-UBF with OH^- may be slowed down in vesicle suspensions but still proceed so fast that the reaction is complete during the mixing period.) As already mentioned under "Results", Förster [19] and Weller [22] have first described a pK-shift in excited molecules. Applying their approach to C_{15}-UBF one may calculate the pK* of the excited species from the pK of the ground state and the energy differences between ground state and excited state of the protonated form ROH (ΔE) and the anion RO^- ($\Delta E'$). ΔE and $\Delta E'$ may be estimated from the longest wavelength absorption maxima and shortest wavelength fluorescence maxima

$\Delta E = 1/2\,(h\,\nu_A + h\,\nu_F)$; $\Delta E' = 1/2\,(h\,\nu_A' + h\,\nu_F')$ (with ν_A, ν_F, ν_A', ν_F' the frequencies of the absorption or emission maxima of ROH and RO^-).

From the equation

$$pK - pK^* = \frac{\Delta E - \Delta E'}{2.3\,k\,T} \qquad (2)$$

one calculates for C_{15}-UBF in egg lecithin vesicles a $pK^* = 1.4 \pm 0.4$ ($pK = 9.6 \pm 0.2$). C_{15}-UBF is a strong acid in the excited state. Due to its incorporation into the membrane the emission of the excited protonated form ROH* is still observed whereas UBF in water only shows the emission of the deprotonated form RO^{-*}. The origin of the 490 nm peak is not clear. If one thinks of excimer formation it is surprising that it should occur at rather low dye concentrations (10^{-6} M in ethanol-water 9/1, v/v). On the other hand in the presence of water some kind of cluster formation could occur and increase the local dye concentration. In the vesicle membrane the local dye concentration is high anyway (close to 10^{-2} M at a lipid:dye ratio 100:1).

The method of incorporating molecules into lipid membranes and studying their reactivity in a highly anisotropic surrounding seems interesting. This is probably a good model for reactions occurring in biological membranes. The reaction of membrane bound MC-540 with OH^- reveals that about one third of the dye reacts very slowly compared to the rest. This is interpreted as representing two pools of dye, one in the outer monolayer of the vesicle membrane and one in the inner monolayer. The outer surface of a vesicle with 200 Å diameter is about twice as large as the inner surface in agree-

ment with the ratio of the fast reacting *versus* the slow reacting dye pool. The two pools apparently exchange very fast at 50 °C.

The polarization measurements show that MC-540 and diS-$C_3(5)$ give high *p*-values in vesicles and very small differences above and below the gel-liquid crystalline phase transition. This indicates a restricted mobility of the dyes in the membrane. It is well known that molecular motions within the lecithin bilayer are minimal in the glycerol-region [26]. Therefore binding of the dyes in this region would explain the observed polarization and would be consistent with the observed absorption spectra. Small changes in the fluorescence polarization during the phase transition of the bilayer are also expected when the transition moments of the dye are oriented perpendicular to the membraneplane. This may be the case with MC-540 for which a nearly perpendicular orientation has been suggested by Chance *et al.* [25]. The orientation of the dyes cannot be calculated from one polarization value because several orientation models would yield the same polarization [27]. Measurements with planar, oriented membranes would give more information and allow to calculate the orientation of dyes. Once the orientation is known this information can be used in vesicle experiments. In fact the value of $p = 0.26$ found for flavin 1 in egg-PC-vesicles is consistent with a rectangular distribution of the dipoles around $\varepsilon_0 \pm \Delta\varepsilon$ during the excited state, with $\Delta\varepsilon = 50° - 60°$, as found by Frehland and Trißl [28] in planar lipid membranes. Once this has been established more complex experiments can be done with vesicles than with single black lipid membranes.

It is seen from the results presented here that several fluorescent molecules which are either embedded in the hydrocarbon-layer or have their hydrocarbon tail inserted into the membrane can be used as probes for phase transitions by measuring their fluorescence polarization. C_{15}-UBF and C_{15}-UBF^- indicate that the phase transition in small vesicles is shifted to lower temperatures and broadened (35 – 43 °C) compared to the calorimetrically determined phase transition in multilayered liposomes (42 ± 1 °C). Vesicles from di(16:0)-PC and di(16:0)-EPC behave very similar. In the di(16:0)-PC vesicles a slight indication of the calorimetrically determined pretransition (at 35 °C) [29] may be seen which is absent in the case of di(16:0)-EPC vesicles.

In the case of dye molecules bound in the glycerol region, the fluorescence polarization is a poor indicator of phase transitions. On the other hand the absorption spectrum of MC-540 shows a large change (Fig. 3) which is related to the phase transition. The temperature range over which the extinction change (at 565 nm) occurs (Fig. 3 B) is considerably broader than that of the phase transition as determined by turbidity changes [22] or calorimetry [29]. This was not due to the small size of the sonicated vesicles. Control-measurements have shown that the turbidity curves are not significantly influenced by the MC-540 concentrations used. Whereas the turbidity shows hysteresis below 34 °C during cooling [22], heating and cooling curves of MC-540 absorption are nearly identical under our experimental conditions. Therefore the influence of aggregation-disaggregation phenomena on the MC-540 absorption are probably small. It seems possible that the extinction increase between 25 and 40 °C is caused by structural changes in the choline- and/or glycerol-region of the lecithin-bilayer. Also the so-called pretransition [29] may contribute to these changes. The temperature where the extinction reached its maximal value was about (41 ± 1) °C. The corresponding temperatures were (25 ± 1) °C for di(14:0)-PC vesicles and (54 ± 1) °C for di-(18:0)-PC vesicles. This point of the extinction curve seems therefore to indicate the main phase-transition.

Measuring membrane potentials with dyes is a highly complex problem. Of all the dyes studied in this paper only diS-$C_3(5)$ showed large changes in absorption or fluorescence when potentials were created across vesicle membranes by an ion gradient and ionophores. But even there the question remains wether the potential alone or some concomitant structural change in the membrane make the effect. A direct effect of the electric field on the spectral properties of the dye (Stark effect) seems unlikely, because spectral changes were only observed with a lipid to dye molar ratio 60:1 and were absent at the ratio 600:1. This is true for addition of dye before or after preparation of the vesicles. In the case of di(16:0)-PC vesicles different effects were observed below and above the phase transition (Figs 7 C, 8). The different results with 10^{-6} and 10^{-7} M valinomycin (Fig. 7) might indicate that high valinomycin concentrations induce structural changes in the vesicle membrane. In the case of egg-PC this ef-

fect is prevented (Fig. 7 A, B) by addition of cholesterol which is known to reduce molecular motions in the hydrocarbon layer of the membrane. In order to minimize possible effects of the ionophore on the membrane structure all following experiments (Fig. 8) were done at about 10^{-7} M ionophore concentration. The result was that valinomycin is inefficient at 20 °C (Fig. 8 A, C). The reasons might be that valinomycin is not incorporated into the membrane under the experimental conditions used or its effectiveness as a carries is strongly reduced in the gel-phase of the membrane. Fig. 8 C shows that the effectiveness of valinomycin becomes measurable well below the main phase transition a finding in conflict with the interpretation of similar results with black lipid membranes [30]. Another study [31] with black lipid membranes had already shown that valinomycin is still effective below the phase transition (as found in lipid vesicles or lipid-water mixtures). This study shows that it does work apparently even below the phase transition in lipid vesicle. Additional structural changes in the membrane like those observed during the calorimetric pretransition [29] may be the reason for this phenomenon. In contrast to valinomycin, gramicidin works equally well at all temperatures and concentrations tested. One may speculate that pores can transport cations well quite independent of the membrane structure, whereas the effectiveness of carriers depends on structural changes (not necessaryly the main phase transition) in the membrane. Experiments like those reported here may become a possibility to discriminate between carriers and pore formers in vesicle systems. The reincrease of the fluorescence at high temperatures may be due to a collapse of the membrane potential (Nernst potential of about 140 mV) by increased leakiness of the vesicles. An alternative which seems more likely is based on the explanation of the fluorescence effects by Sims *et al.* [4]. These authors suggest an asymmetric distribution of the dye on the external and internal surface of the membrane due to the membrane potential. This distribution may become unstable at high temperature by a rapid dye exchange across the membrane as suggested earlier in this paper for MC-540.

The potential effects with MC-540 are smaller and more complicated than with diS-$C_3(5)$ as already mentioned under "Results". The partition coefficient of the dye between water and the membrane is apparently influenced both by valinomycin itself and by the membrane potential, with both factors creating spectral changes of opposite direction. High dye concentrations were necessary (molar ratio 20:1) to obtain significant fluorescence changes after addition of valinomycin. This high MC-540 concentration may strongly influence the structure of the lipid membrane. On the other hand, as discussed above, MC-540 in molar ratios between 100:1 and 1000:1 indicates phase transitions in di(16:0)-PC membranes by absorption changes (Fig. 3). These changes are qualitatively similar to changes observed during an action potential in nerve membranes [2], whereas application of a potential difference across the vesicle membrane by a K^+-gradient and valinomycin did not induce comparable absorption changes. From this observation it may be speculated that changes of probe properties during the action potential are not due to a direct influence of the electric field but are a consequence of structural changes in the membrane which are similar to changes during a phase transition in lipid bilayers.

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Note added in proof:

When this paper was in the press, two articles by Tasaki *et al.* [32, 33] on the use of MC-540 for studying nerve excitation appeared. The optical properties of MC-540 in organic solvents given in these papers agree with my results.

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