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Perturbations of Membrane Structure by Optical Probes: I. Location and Structural Sensitivity of Merocyanine 540 Bound to Phospholipid Membranes

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Summary. The maximum monomer absorption wavelength of a frequently used external membrane probe, Merocyanine 540, can be related to the location of the binding site for the dye within lipid membranes. Solvent studies indicate the occurrence of very specific and mutual perturbances between the probe and its microenvironment, that are of relevance, when investigating structural and functional events in biomembranes with the aid of this dye. Merocyanine 540 (MC 540) is an excellent probe for structural alterations in the lipids including phase transitions. The extinction coefficient and λ_{max} place the location of the dye-chromophore slightly above the domain of the glycerol of backbone of neutral and charged phospholipids. This explains the sensitivity of MC 540 to structural variations in the head-group region of several synthetic dipalmitoyl-lecithin analogues. The major physical parameters involved in variations of the optical signals associated with changes in the membrane structure are the dye/lipid partition coefficient and the monomer-dimer dissociation constant of the dye bound to the lipids. A temperature dependent transition from the liquid-crystalline to the crystalline state leads mainly to an exclusion of the dye from the lipid phase with a concomitant dimerization of the dye molecules still in contact with the polar-head group region of the lipid. The relevance of this finding for the mechanism of transient optical signals in connection with the occurrence of action potentials in excitable membranes is discussed. Our findings underline the necessary caution when applying external optical probes and analyzing membrane features from the spectral data, because of inevitable perturbances in the microenvironment of every probe molecule.

Key words: Optical probes, lipid membranes, binding-sites, phase transitions, structural perturbances.

Our knowledge of structural and functional events on the molecular level in membranes has been greatly enhanced by the introduction of external probes into model lipid membranes as well as into biological membrane preparations. For a recent review, *cf.* Andersen (1978). To obtain maximal and reliable information from experiments with optical probes, three major questions have to be answered while analyzing the data. (i) Where in the membrane is the dye to be located? (ii) What is the mechanism by which the membrane associated signals are produced? (iii) How far does the presence of the probe perturb the original membrane system? A further point that has to be elucidated is whether the probe reveals overall membrane properties or whether it only senses its own microenvironment and changes therein.

Merocyanine 540 (MC 540) belongs to the class of cyanine dyes (Hamer, 1964) that have in recent years successfully been used as external optical probes to monitor both structural and functional changes in biological membranes. For an up-to-date review, *see* Cohen, Salzberg and Grinvald (1978) with further references to be obtained there. Merocyanine 540 is one of the nonpermanent polar dyes (Waggoner, 1976) that has proven to be a useful tool, not only in monitoring transient membrane action potentials (Davila *et al.*, 1973; Ross *et al.*, 1977), but also in elucidating subcellular features like transport and state of energization in chromatophore membranes of *Rhodospirillum rubrum* (Chance & Baltscheffsky, 1975) and in submitochondrial particles in *Rhodopseudomonas* (Chance, 1975). More recently MC 540 has found physiological applications as a probe of transmembrane electrical activity in the heart (Salama & Morad, 1976). Furthermore, it was shown that MC 540 selectively stains electrically excitable cells (Easton, Valinsky & Reich, 1978) and that this dye can be used as a specific indicator for leukemic and

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immature hemopoietic cells (Valinsky, Easton & Reich, 1978).

Whereas the mechanism by which the permeant cyanine dyes monitor membrane potentials has been fairly well understood (Sims *et al.*, 1974; Waggoner, 1976; Waggoner, Wang & Tolles, 1977), detailed information is still lacking as to both the location of MC 540 within the membrane as well as to the mechanism by which it reports transient changes in membrane potentials. Cyanine and oxonol dyes are understood to permeate through the membranes and to redistribute according to some potential-dependent accumulation processes (Waggoner & Grinvald, 1977). In the case of MC 540, its location in more or less polar membrane regions is not undisputed (Pohl, 1976; Waggoner & Grinvald, 1977; Easton *et al.*, 1978). As to the possible mechanisms, it is not clear whether the optically detectable changes are caused by a direct interaction of the dye and its state of aggregation with the electric field and/or the potential across the membranes (Tasaki & Warashina, 1976b; Waggoner & Grinvald, 1977; Dragsten & Webb, 1978), or whether they reflect additionally or even exclusively changes in the membrane structure (Pohl, 1976; Easton et al., 1978), that occur during changes in the membrane potential.

To clarify some of these questions, we investigated absorption spectra of MC 540 in various solvent systems and lipid model membranes. As the merocyanines are known to be potent solvent polarity indicators (Brooker *et al.*, 1964), we performed extensive solvent polarity studies covering the whole range of biologically occurring dielectic constants between $\varepsilon=2$ and $\varepsilon=80$. Certain informations from such experiments are applicable to characterize the polarity of the binding site of the dye within biological systems, such as lipid membranes (Pohl, 1976), proteins (Turner & Brand, 1968), or plasma membranes (Nakamaru, 1977). Gross conformational changes in membranes are observed, when the lipid regions undergo temperature dependent reversible phase transitions from the rigid to the fluid state. Some of the optical methods used to follow such structural changes involve spectroscopical investigation of the fluorescence (Träuble & Overath, 1973), the fluorescence polarization (Lentz, Barenholz & Thompson, 1976) or the absorbance (Träuble, 1972) of certain dyes that interact with the membrane preparations. Merocyanine 540 has recently been suggested as a useful candidate for such optical measurements of phase transition (Pohl, 1976). We measured the temperature dependence of MC-540 spectra adsorbed to lipid membranes that had some structural modification in the polar head-group region, i.e., exactly the moiety where we located the binding sites for MC 540. Close examination of the spectroscopic data will also enable us to discuss the mechanism by which MC 540 indicates structural changes in membranes.

A further crucial point to be discussed is the question how and to what extent the external dye molecule disturbs its own microenvironment and how far, therefore, membrane properties observed with the aid of such probes indeed reflect the true situation that one would encounter without the probe in the unperturbed matrix.

Materials and Methods

Merocyanine 540 was purchased as monosodium salt from Eastman Kodak Co., Rochester N.Y. Thin-layer chromatography on silica-gel stripes in a chloroform/methanol mixture (3:2 vol/vol) showed a single spot, and thus the dye was used without further purification.

All aequous solutions were prepared from bidistilled water at pH 6.5 ± 0.2.

For solvent polarity studies, the following reagents were used: ethanol, methanol, *n*-butanol, octonal, benzene and dioxan (all from MERCK, Darmstadt, W. Germany, all of them UVASOL grades); chloroform, ethyleneglycol and phenylaceticacid were obtained from FLUKA, Buchs, Switzerland, all in puriss grade. The dielectric constants of the different solvents are taken from the Handbook of Chemistry and Physics (57th edition, 1976–77). To cover the whole range of polarity between $\varepsilon=2$ and $\varepsilon=80$, homogenous solvent mixtures of ethanol-H_2O, dioxane-H_2O, chloroform-ethanol and methanol-dioxane were prepared. To obtain the dielectric constants of these mixtures, the data of Turner and Brand (1968) as well as the tables and figures in Landolt-Börnstein (1959) were consulted.

Merocyanine-540 stock solutions of 10^{-3} M/liter were freshly prepared daily either in H_2O or – for experiments with H_2O-insoluble solvents – in ethanol. Aliquots of the stock solutions were added to each solvent under investigation to yield the desired final concentration. Impurities induced by this method in no case exceeded 1%.

Phosphatidylcholine (PC) and phosphatidylserine (PS) (grade I) were purchased as monosodium salts in chloroform/methanol solutions from Lipid Products, Nutfield, England, and were used without further purification. Dipalmitoyl-lecithin (DPL) was obtained (puriss grade) from FLUKA. The synthesis and some physico-chemical properties of several DPL analogues with increased numbers of $(CH_2)_n$ ($n=2$–9) groups between the phosphate – and the trimetylammonium – molecules are described elsewhere (Diembeck, 1976).

In preparing multilammellar lipsomes by vortexing or single walled vesicles by sonicating the aequous lipid dispersions (5 mg/ml) (in a temperature controlled sonicating bath for 1 hr), we followed the standard procedures of Bangham, Hill and Miller (1974) for liposomes and of Huang and Thompson (1974) for vesicles, respectively. All preparations were carried out above phase transition temperatures, i.e., at approximately 25 °C in the case of PC and PS and at 50° C in the case of DPL and its analogues. Vesicle preparations could be used up to 2 days after sonicating, provided that they were stored above phase transitions to prevent fusion of the unilammellar vesicles into multilammellar liposomes (Suurkuusk, *et al.*, 1976).

All spectroscopic measurements were performed on a Cary model 118 dual-beam spectrophotometer using the AUTO-SLIT mode with the gain set at 3. The 1-cm light-path precision cuvettes

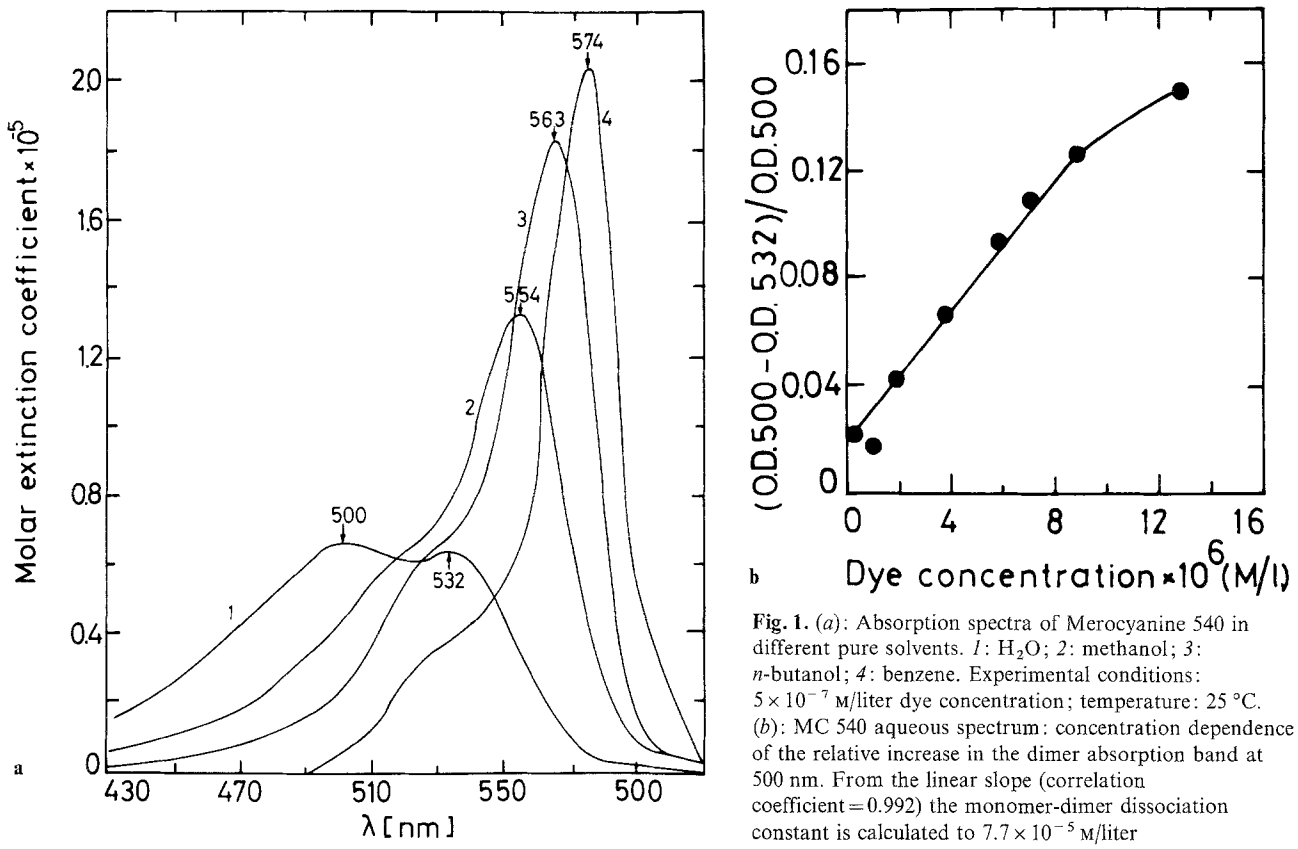


Fig. 1. (*a*): Absorption spectra of Merocyanine 540 in different pure solvents. *1*: H_2O; *2*: methanol; *3*: *n*-butanol; *4*: benzene. Experimental conditions: 5×10^{-7} M/liter dye concentration; temperature: 25 °C. (*b*): MC 540 aqueous spectrum: concentration dependence of the relative increase in the dimer absorption band at 500 nm. From the linear slope (correlation coefficient = 0.992) the monomer-dimer dissociation constant is calculated to 7.7×10^{-5} M/liter

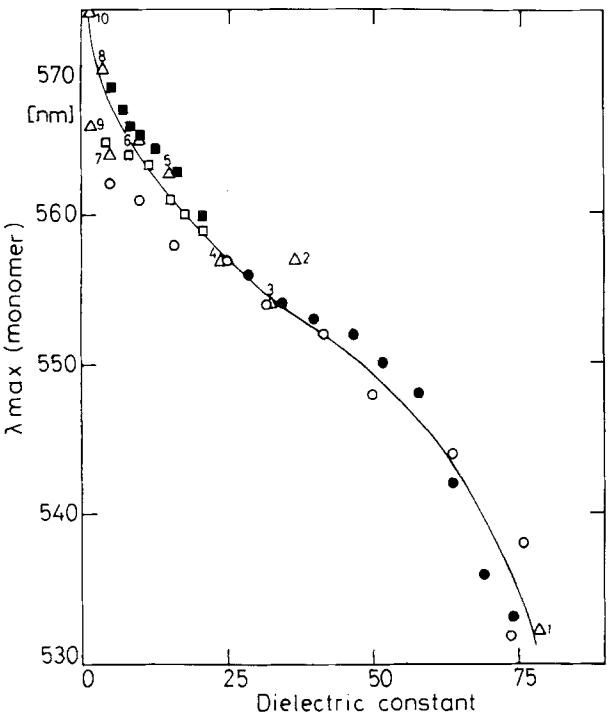
(Beckmann) were mounted in temperature-controllable cuvette-holders both for the reference and the sample cuvettes. Temperature regulation was achieved via a MGW-LAUDA (PTR-Regler R20/2 Electronic) temperature bath, whereby the temperature could be controlled electronically. The actual temperature inside the sample cuvette was continuously monitored via a copper-constantan thermocouple and could be read with a precision of 0.2 °C on a Philips GM 6020 voltmeter.

All solvent polarity studies were performed at 25 °C. The interaction between MC 540 and PC and PS, respectively, was measured equally at 25 °C. The temperature scans for measuring phase transitions were performed by stepwise changing the temperature. The scan rates both in the heating and the cooling modes amounted to less than 10 °C/hr. After reaching the preset temperature in each case, 5 min were allowed for a complete equilibration of the system. Between the experiments, additionally to the internal stirring by heat convection, several times air was bubbled through the fluid in the cuvettes via a 5 µl Peddersen-Pipette to reduced unstirred layer effects.

Results

Solvent Polarity Studies

Absorption spectra of MC 540 were measured in H_2O and in different pure organic solvents as well as in some homogeneous solvent mixture (Fig. 1). The aqueous MC 540 spectrum (Fig. 1a) exhibits at very low dye concentrations (1×10^{-7} M/liter) a double absorbance peak at 500 and 532 nm with approximately the same value of 0.6×10^5 mol^{-1} cm^{-1} for the molar extinction coefficient ε for both absorption bands. Increasing the dye concentration up to 1×10^{-6} M/liter does not alter the appearance of the MC 540 spectrum, which obeys Beer's law. Increasing the temperature from 20 to 40 °C results merely in a small bathochromic shift in both absorption-peak positions by about 2 nm. This can be accounted for by the slight decrease in the dielectric constant D of the solvent. A further increase of the dye concentration up to 2×10^{-5} M/liter enhances the molar extinction at the shorter wavelength band concomitant with a hypsochromic shift by 3 nm against a slight decrease in the molar extinction at 532 nm. At a given concentration (in this range), we additionally observe a decrease in the 500-nm peak against an increase in the optical density at $\cong$ 534 nm when increasing the temperature from 20 to 40 °C. Such behavior has been reported previously for other dyes from the same class of merocyanines (Brooker, 1966) and indicates that in this concentration range the longer wavelength band can be attributed to the dye monomer, whereas the absorption at the shorter wavelength should be due to a dimer. Plotting the differences in optical density at the two absorption bands (OD 500–OD 532) divided by the absorption at 500 nm



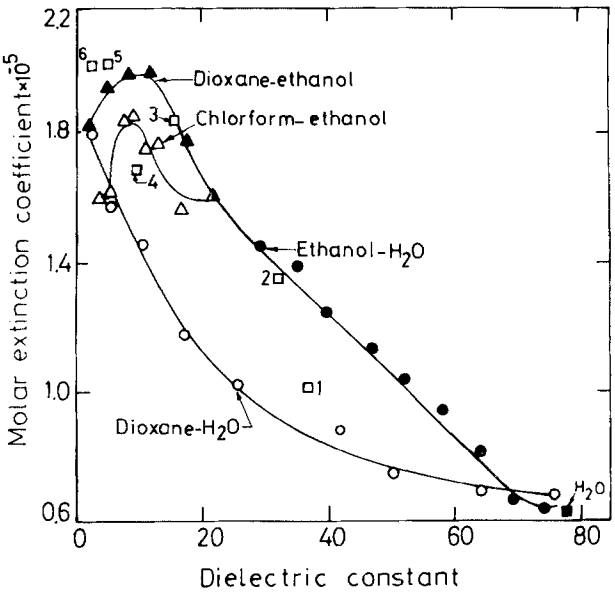


Fig. 2. Correlation between the maximum monomer absorption of MC 540 (λ_{max}) and the macroscopic dielectric constant of pure solvents and homogeneous solvent-mixtures (*cf.* Table 1): Pure solvents (△): *1*, H_2O; *2*, ethyleneglycol; *3*, methanol; *4*, ethanol; *5*, *n*-butanol; *6*, octanol; *7*, phenylacetate; *8*, chloroform; *9*, dioxane; *10*, benzene. Solvent mixtures: Dioxane-H_2O (○); ethanol-H_2O (●); ethanol-dioxane (□); and ethanol-chloroform (■). All experiments were carried out at 25 °C

Fig. 3. Molar extinction coefficient of MC 540 as a function of the macroscopic dielectric constant. Open squares (□) denote some of the pure solvents: *1*, ethyleneglycol; *2*, methanol; *3*, *n*-butanol; *4*, octanol; *5*, phenylacetate; *6*, benzene. Symbols used for solvent mixtures: Dioxane–H_2O (○); ethanol–H_2O (●); chloroform–ethanol (△); and dioxane–ethanol (▲). Temperature: 25 °C

(OD 500) against the total dye concentration, c, in the solution (Fig. 1b), we obtain a straight line in the concentration range between 1×10^{-7} and 1×10^{-5} M/liter. From the slope we calculate a monomer-dimer dissociation constant of 7.7×10^{-5} M/liter, which is in fair agreement with the data obtained for some other cyanines (West & Pearce, 1965; West & Carroll, 1966). We therefore conclude that at low dye concentrations ($<10^{-6}$ M/liter) the aequous spectrum of MC 540 originates from the dye-monomer, exhibiting a double absorption peak. For higher concentration, the spectrum represents a mixed state with an increased contribution from dimers and possibly also from higher aggregates (Waggoner & Grinvald, 1977).

In organic solvents we observe in the concentration range between 1×10^{-7}–5×10^{-6} M/liter monomeric dye spectra (West & Geddes, 1964), which follow fairly well Beer's law. The bands of maximum absorbance depend, both in their heights and their positions, strongly upon the solvents (Fig. 1*a*). The subsidiary maxima (or shoulders) at shorter wavelengths have been attributed to some vibrational bands originating from simultaneous excitation of atoms in the conjugated chains (Brooker, 1966). With decreasing polarity of the solvent, the monomer absorption band shifts towards longer wavelength concomitant with an increase of the molar extinction coefficient.

Thus we find the maximum monomer absorption (λ_{max}) of MC 540 at 554 nm in methanol (dielectric constant $D=32.6$), at 563 nm in butanol ($D=17$), and 574 nm in benzene ($D=2.2$). The corresponding values for the molar extinction coefficient ε (in units of 10^5 mol^{-1} cm^{-1}) are in H_2O: $\varepsilon=0.63$, in methanol $\varepsilon=1.35$, in butanol $\varepsilon=1.85$, in benzene $\varepsilon=2.1$.

The same type of spectral appearance of MC 540 can also be demonstrated for homogeneous solvent mixtures (Fig. 2). Plotting the maximum absorption wavelength λ_{max} against the dielectric constants D of the solvent mixtures, we find reasonable agreement of the λ_{max} values as obtained in the different solvents. A plot of the molar extinction coefficients ε of MC 540 against D, on the other hand, shows significant differences for the various solvent mixtures (Fig. 3). Such a behavior has been shown previously for several merocyanines, and it indicates the differential and specific influence of the solvents on both

the absorption-band position λ_{max} and upon the band-strength $\sim\int\varepsilon dv$ (Sheppard, 1942; McRae, 1958; West & Geddes, 1954).

Similar solvent specific discrepancies were obtained when correlating the spectral data to the solvent polarity parameters mentioned by West & Geddes (1964). The only reasonable correlation that could be established was the one between λ_{max} and D.

The general pattern of a nonlinear bathochromic shift in λ_{max} with increasing dielectric constant, seen in Fig. 2, is valid in numerous solvents and solvent mixtures, as listed in Table 1. The data for some of the pure solvents are in good agreement with those published by Pohl (1976). Plotting the wavelength of the maximum monomer absorption peak λ_{max} against the macroscopic dielectric constant D of the pure solutions and solvent mixtures (Fig. 2) shows that the correlation between λ_{max} and D is not a linear one. Deviations from linearity concomitant with a significant stray of the data exceeding the experimental error occur at values of very high and very low dielectric constants, respectively. This may indicate a preferential moiety for the MC-540 molecule in solvents of medium polarity with a dielectric constant somewhere between 15 and 45.

Based on these observations, we suggest to use the position of the monomer absorption band of MC 540, λ_{max}, as an indicator for the effective dielectric constant in the closest surrounding of the dye chromophore. It has to be stressed, however, that there is ample experimental evidence for the occurrence of some specific perturbances of the solvent-dye system which effect both the dye and its microenvironment and which may lead to structural perturbances in the vicinity of its binding site when applying MC 540 as an external membrane probe.

Interaction of MC 540 with Lipid Vesicles

Assuming a continuous gradient in the dielectric constant of lipid-model membranes between $D=2$–3 in the hydrophobic chore of the bilayer and $D=80$ in the free aqueous phase, we can utilize the results from the preceding solvent studies and use MC 540 as a monitor for the average dielectric constant of its microenvironment. The average dielectric constant thus measured will depend not only on the location of the dye but also on its orientation with respect to the dielectric gradient. Vice versa, the dielectric constant of the dye microenvironment as inferred from comparison of λ_{max} and/or of the extinction coefficient with those measured in different solvents is to be taken with caution, since the chromophore orientation

Table 1. Absorption spectroscopy of MC 540 in pure solvents and in homogeneous solvent mixtures (cumulative data)

No.	Solvent	Dielectric constant[a] D [250 °C]	Maximum absorption[b] λ_{max} [nm]	Molar extinction coefficient[c] ε [$\times 10^5$ mol^{-1} cm^{-1}]
1	Water	78.5	500/532	0.63
2	Ethyleneglycol	37.0	557	1.03
3	Methanol	32.6	554	1.35
4	Ethanol	24.3	558	1.54
5	*n*-Butanol	16.6	563	1.84
6	Octanol	10.0	565	1.68
7	Phenylacetate	5.1	564	2.03
8	Chloroform	4.0	570	1.60
9	Dioxane	2.2	566	1.83
10	Benzene	2.0	574	2.05
	Dioxane:H_2O[d]			
11	10:90	76	538	0.67
12	20:80	64	544	0.69
13	30:70	50	548	0.75
14	40:60	42	552	0.88
15	50:50	32	554	11.02
16	60:40	25	557	11.08
17	70:30	16	559	1.19
18	80:20	10	561	1.47
19	90:10	5	562	1.59
	Ethanol:H_2O[d]			
20	10:90	74	533	0.65
21	20:80	69	536	0.67
22	30:70	64	542	0.81
23	40:60	58	548	0.95
24	50:50	52	550	1.04
25	60:40	47	552	1.14
26	70:30	40	553	1.25
27	80:20	35	554	1.39
28	90:10	29	556	1.46
	Chloroform: ethanol[d]			
29	10:90	21	560	1.60
30	30:70	17	563	1.57
31	50:50	13	564.5	1.77
32	60:40	11	565.5	1.76
33	70:30	9	566	1.85
34	80:20	7.5	567	1.84
35	90:10	5.5	568.5	1.61
	Dioxane: ethanol[d]			
36	10:90	20.5	559	1.59
37	20:80	18.0	560	1.79
38	30:70	15.5	561.5	1.82
39	50:50	12.0	563.5	2.09
40	70:30	8.0	564	1.99
41	90:10	4.0	565	1.95

[a] Dielectric constants taken from the Handbook of Chemistry and Physics (1967/77) and from Landolt-Börnstein (1959).
[b] λ_{max} values within an experimental reproducibility of ± 0.5 nm.
[c] ε-values determined at a dye concentration of 5×10^{-7} M/liter. Limits of experimental accuracy: $=\pm 5\%$.
[d] Homogeneous solvent mixtures in (vol/vol)-%.

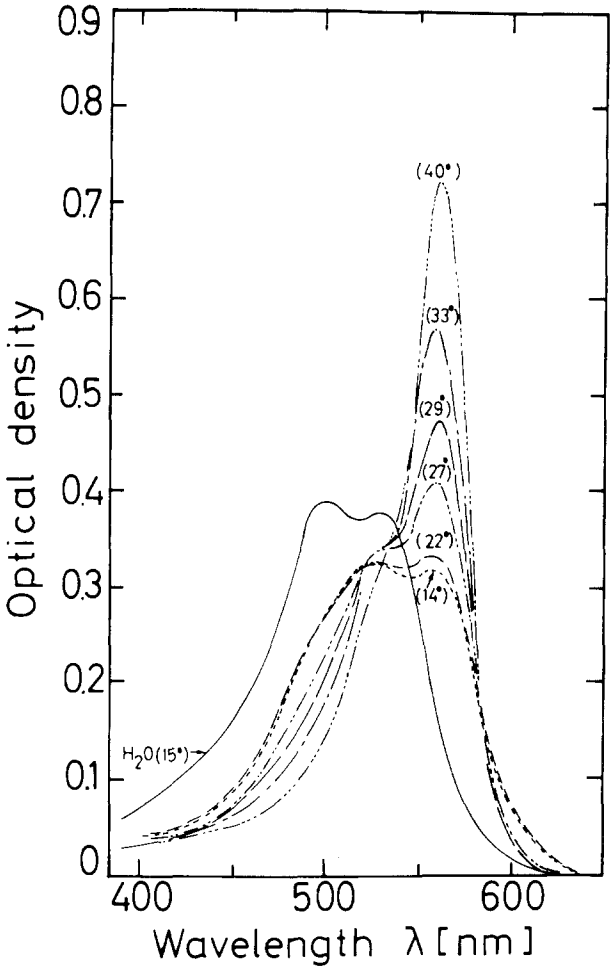


Fig. 4. Temperature-dependent absorption spectra of MC 540 bound to phospholipid vesicles. The dye was added at 14 °C to an aqueous suspension of PN 4 vesicles, the temperature was gradually increased to 40 °C. Solid line (——): aqueous spectrum at 15 °C. Details are in the text. Experimental conditions: 4.5×10^{-4} M/liter PN 4; 3×10^{-6} M/liter MC 540

in the anisotropic dielectric medium may affect the results. One has, however, to take into account that even at infinite probe dilutions the single dye molecules and their microenvironments may mutually perturb each other. This could effectively disturb the optical response of the probe and thus result in an incorrect estimation of the dye location. Therefore, only approximate ranges of the effective dielectric constant in the dye microenvironment can be inferred from Fig. 2.

In Table 2 the monomer emission wavelength λ_{max} and the molar extinction coefficient ε are listed for MC 540 interacting with PS and PC-vesicles both in H_2O and 200 mM KCl. The experimental results for PC agree with the values that have been reported by Waggoner and Grinvald (1977) as well as those by Pohl (1976). For a molar ratio lipid/dye 150:1

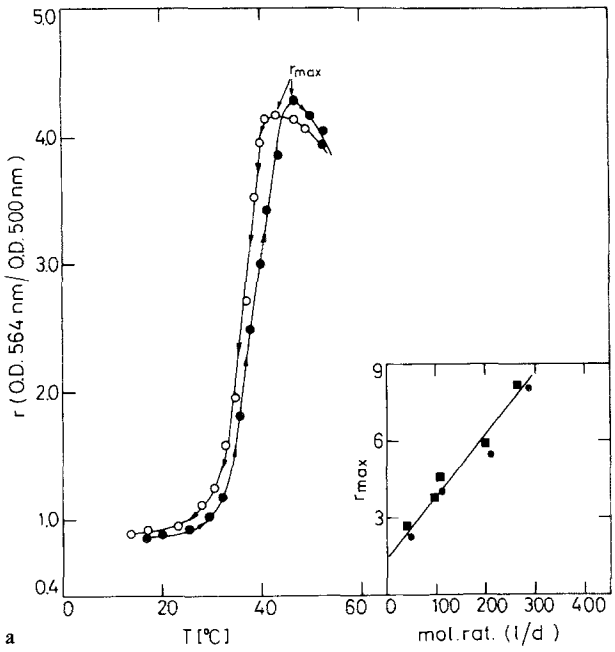
Table 2. Spectral characteristics of MC 540 bound to phospholipids

Lipid	H_2O		200 mM/liter KCl	
	λ_{max} (nm)	ε ($mole^{-1}cm^{-1}$)	λ_{max} (nm)	ε ($mole^{-1}cm^{-1}$)
PC	567	1.8×10^5	567	1.6×10^5
PS	561	0.81×10^5	564	1.5×10^5

we find both in H_2O and in 200 mM KCl the monomer absorption peak of MC 540 bound to PC-vesicles at 567 nm; this corresponds, according to Fig. 3, to a local dielectric constant of the dye binding site of approximately 6–8. In the presence of the salt, the molar extinction coefficient is somewhat reduced (by 12%) from 1.8×10^5 to 1.6×10^5 mol^{-1} cm^{-1} although the location of the dye in the membrane is not changed.

This reduction in ε can be caused by a decrease in the dye/lipid partition coefficient due to the increased ionic strength in the solution. Our data are in line with the results of the aforementioned investigators, who found ε-values of 1.7×10^5 mol^{-1} cm^{-1} (PC-vesicles in 100 mM KCl) and 145×10^5 mol^{-1} cm^{-1} (PC-liposomes in 500 mM NaCl), respectively.

As would be expected from the interaction of the negatively charged dye with a negatively charged lipid, the electrostatic repulsion between them prevents the penetration of MC 540 into the more hydrophobic core of PS. Thus in H_2O we find a maximum of the monomer absorption at 561 nm, which according to Fig. 2 indicates that the dye is located in a PS moiety with an effective dielectric constant of approximately 20. The molar extinction coefficient was found to be 0.81×10^5 $mol^{-1}cm^{-1}$, i.e., considerably lower (less than 50%) than in PC-vesicles under the same experimental conditions. Increasing the ionic strength in the solution screens the negative charges both in the dye and in the head-group region of PS and then should allow a stronger interaction between MC 540 and the lipid. Indeed in 200 mM KCl we find the maximum of the monomer absorption peak located at 564 nm, corresponding to a range of the effective dielectric constant D of ~8–12. Concomitantly, the molar extinction coefficient of the absorbed dye increases to 1.5×10^5 $mol^{-1}cm^{-1}$, this value being only somewhat lower than the molar extinction coefficient of MC 540 bound to PC in the presence of 200 mM KCl. These observations can be interpreted that at higher ionic strength more MC 540 has been incorporated in a more hydrophobic region of PS than in pure H_2O. However, neither in PC nor in PS does MC 540 penetrate right into the hydrocarbon core of the lipid bilayer.



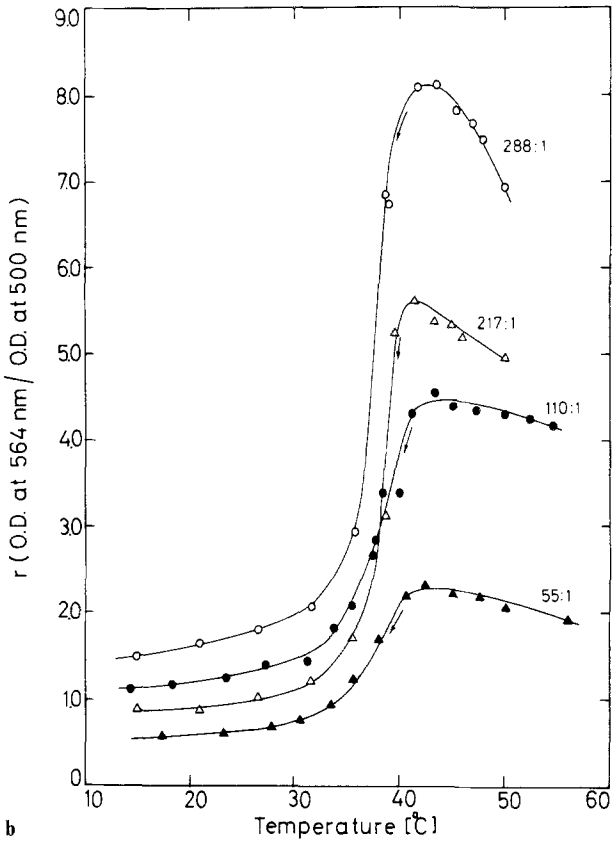


Fig. 5. The temperature dependence of the ratio $r = (\text{OD } 564/\text{OD } 500)$. (*a*): PN 2 (lipid/dye ratio 100:1): hysteresis in the temperature dependence of r, when cycling between heating (●) and cooling (○) of the sample. Phase transition temperatures were determined (for details, *cf.* text) to $T_t\uparrow = 38.5$ °C and $T_t\downarrow = 37.5$ °C. (*b*): Temperature dependence of r at several lipid/dye (l/d) ratios (PN 2). The scans were performed in the cooling mode. $T_t\downarrow$ values varied between 37. 2 °C ($l/d = 55:1$) and 38.4 °C ($l/d = 288:1$). With the other DPL analogs similar results were obtained, as can be seen from the inset, which shows the dependence of r_{max} on the l/d ratio, e.g., for PN 2 (●) and PN 4 (■)

Phase Transitions

The previous experiments, as to locating MC 540 within lipid membranes, were performed at 25 ± 1 °C, i.e., at a temperature where both phospholipids are known to be in a fluid phase. Monitoring the transitions between fluid-rigid phases of phospholipid model membranes by optical methods has been widely used, and just recently the use of MC 540 as an indicator for such phase transitions has been suggested (Pohl, 1976). A series of dipalmitoylecithin analogs with an increasing number of (CH_2)-groups between the phosphate and the trimethylammonium has recently been synthesized (Diembeck, 1976). Phase-transition temperatures of these DPL-analogs have been determined by differential scanning calorimetry (Bach *et al.*, 1978) and by the fluorescence of the hydrophobic probe N-phenyl-1-naphthylamine (NPN) (Diembeck, 1976). The results obtained with the two experimental methods showed distinct discrepancies, especially when the measurements were performed in the cooling mode, i.e., a temperature scan from the temperature above the phase transition to temperatures below it.

It might be useful to clarify whether these discrepancies between the different experimental approaches to measuring phase transitions reflect some sort of generality. This is done by investigating a pure system by differential scanning calorimetry on one hand and inserting an external probe for optical measurements on the other hand. The differences in the phase-transition temperatures obtained by the different methods are considered to be macroscopically measurable expressions of the perturbations in the dye microenvironment induced by the presence of the probe. Figure 4 demonstrates the temperature-dependent absorption spectra of MC540 bound to lipid vesicles formed by a DPL analogue with 4 (CH_2) molecules in the polar head-group region (PN 4). The molar ratio lipid/dye was approximately 150:1. The dye was added at 14 °C, i.e., the lipids being in the gel-phase. Compared to the aqueous spectrum of MC 540 at this temperature, there is an instantaneous change in the spectral appearance of the dye adsorbed to the lipid membranes. The absorption peak at 500 nm ("water peak") is diminished, whereas a new absorption band at 562 nm shows up, which can be attributed to the dye monomers interacting with the lipids (Waggoner & Grinvald, 1977). In the temperature region below the phase transition temperature, a second peak at 525 nm reveals the existence of mem-

Table 3. Phase transition temperatures T_t[°C] of DPL analogues as monitored with different experimental approaches

Lipid	DSC[a]		NPN[b]		MC 540[c]	
	$T_t\uparrow$	$T_t\downarrow$	$T_t\uparrow$	$T_t\downarrow$	$T_t\uparrow$	$T_t\downarrow$
DPL	42.0	40.7	–[d]	–[d]	38.5	37.5
PN2	42.1	40.5	41.8	41.0	38.3	37.8
PN3	41.0	39.2	41.8	38.5	–[d]	36.5
PN4	41.8	41.0	41.8	37.5	33.6	31.0
PN5	43.5	42.5	41.7	37.3	36.3	34.3
PN6	43.0	41.6	41.7	37.2	–[d]	31.0
PN7	45.0	43.7	41.7	37.0	40.5	36.5
PN8	42.5	41.2	41.7	37.0	40.5	39.5
PN9	43.2	42.0	41.7	36.9	–[d]	36.5

$T_t\uparrow$: heating mode; $T_t\downarrow$: cooling mode.
[a] From Bach *et al.* (1978).
[b] From Diembeck (1976).
[c] This work, molar ratio lipid/dye $\geq 120:1$.
[d] Not determined.

Table 4. Phase-transition temperatures of several DPL analogues obtained by measuring the absorption of MC 540 at different lipid/dye ratios

PN 3		PN 6		PN 8	
Molar ratio	$T_t\downarrow$	Molar ratio	$T_t\downarrow$	Molar ratio	$T_t\downarrow$
16:1	31.5	15:1	31.0	30:1	35.0
32:1	32.0	46:1	31.2	60:1	37.5
65:1	33.0	62:1	31.0	120:1	39.5
131:1	36.0	124:1	30.5		
195:1	36.5	190:1	29.0/32.0		
		248:1	29.0/32.0		

brane-bound dye aggregates that were identified as MC-540 dimers (Waggoner & Grinvald, 1977; Warashina & Tasaki, 1975). The monomer-dimer equilibrium of the adsorbed dye molecules depends on the membrane fluidity: increasing the temperature results in a sharp increase in the lipid monomer band concomitant with the disappearance of the dimer-band. Additionally, the position of the monomer absorption λ_{max} is shifted from 562 nm (at 20 °C) to 564 nm (at 40 °C). The temperature-dependent increase in the membrane-monomer absorption is accompanied by a further decrease in the absorbance at 500 nm, indicating an augmented partitioning of the dye molecules into the lipids in the fluid phase. Similar results were obtained in analogous experiments, when the dye was added at temperatures above the phase transition of the lipids. The spectra of MC 540 showed the same kind of temperature dependence: The lipid-monomer-peak, characteristic of MC 540 bound to vesicles in the liquid-crystalline phase decreased with decreasing temperature concomitant with a slight hypsochromic shift, whereas at the same time the 500-nm water-absorption peak increased. Below the phase transition, the dimer band was clearly discernable.

The phase transition and above all the existence of a phase transition temperature T_t can be obtained from a number of spectroscopical data. In the case of MC 540 a plot of the membrane monomer band at $\simeq 565$ nm or equivalently of the water-peak at 500 against temperature revealed two distinct phases with a transition point T_t between them. We propose to take the ratio of the two absorption bands $r \equiv$ (OD lipid-monomer/OD H_2O) as the most suitable indicator for phase transitions in lipid membranes, since the dye distribution between H_2O and the membrane changes abruptly at T_t.

Figure 5*a* shows the typical shape of the temperature dependence of r (ratio of the optical densities of the lipid monomer peak and the water peak) measured in PN 2 at a lipid-dye molar ratio of 120:1. The dye was added at low temperatures, the preparation gradually heated beyond the phase transition temperature, and then slowly cooled down again in order to follow up the hysteresis phenomenon. The temperature dependence of r shows at low and at high temperatures two regions with linear slopes interconnected by the transition region. The phase-transition temperatures T_t were determined according to the graphical method of Träuble and Overath (1973) and/or the derivative method (dr/dT) as mentioned by Jacobson and Papahadjopoulos (1975). T_t values obtained from both methods agreed within ± 0.3 °C. As with other experimental approaches, the phase transition during heating appears always at higher temperatures than during the cooling. From Fig. 5*a* we calculated for PN 2 transition temperatures $T_t\uparrow$ (heating) = 38.5 °C and $T_t\downarrow$ (cooling) = 37.5 °C.

For DPL and several of its analogues, temperature scans were performed both with unilammellar vesicles as well as with multilammellar liposomes. No differences between the two lipid preparations could be detected with respect to both $T_t\uparrow$ and $T_t\downarrow$ as measured by the temperature dependence of the MC-540 spectra. In both vesicles and liposomes, these values agreed within the experimental accuracy of 0.3 °C, although there are differences in the uptake characteristics for the dye. In vesicles there is an instantaneous adsorption of the dye with no further change in the spectrum up to 8 hr, in liposomes the diffusion-controlled uptake of MC 540 by the different layers can be followed up spectroscopically for a period of several days (details are to be published elsewhere).

The magnitude of the observed changes in the spectra during phase transitions, i.e., the absolute value of r is strongly dependent on the molar ratio

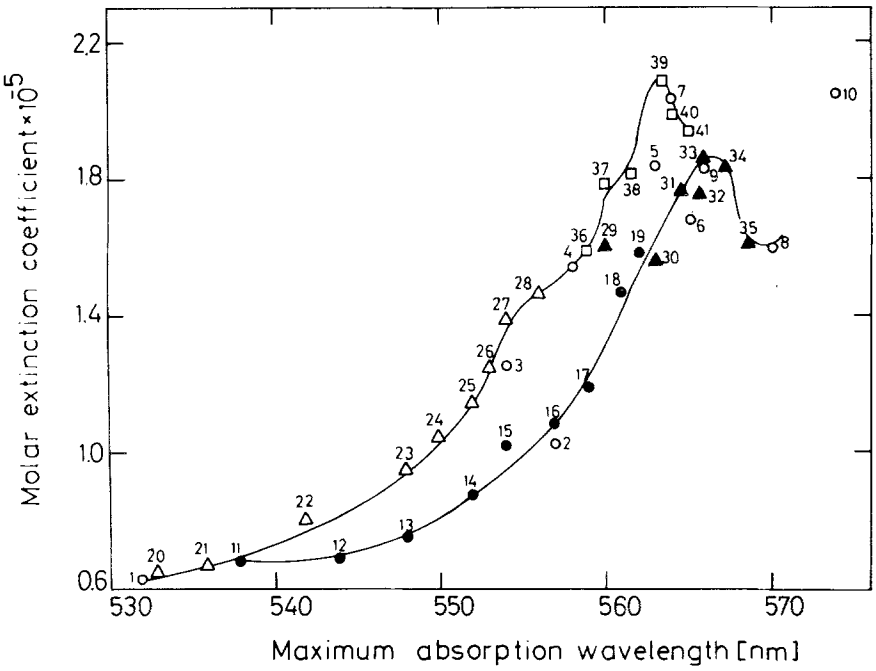


Fig. 6. The molar extinction coefficient of MC 540 determined in the solvent studies plotted against the corresponding maximum absorption wavelengths. The numbers refer to the solvents as listed in Table 1. The following symbols were used: pure solvents (○), dioxane-H_2O (●), ethanol-H_2O (△), ethanol-chloroform (□), and dioxane-ethanol (△). All experiments were carried out at 25 °C

lipid/dye. Figure 5*b* shows for PN 2 the temperature dependence of r at molar ratios between 55:1 and 228:1. The data were taken from experiments performed in the cooling mode. $T_t\downarrow$ varies between 37.2 °C (55:1) and 38.4 °C (288:1), the mean value being 37.8 ± 0.6 °C. The inset shows the dependence of the maximal r values, r_{max}, on the molar ratio lipid/dye for PN 4 and for PN 2. From such curves one could – in principle – estimate the amount of lipids in preparations with unknown lipid content. In Table 3 the phase-transition temperatures both in the cooling mode ($T_t\downarrow$) and in some cases also in the heating mode ($T_t\uparrow$) are listed for DPL and for nine synthetic DPL analogues; the number of (CH_2) groups between the phosphorous and tertiary ammonium increases steadily from 2 to 9. All values are taken from experiments where the molar ratio between lipid and dye amounted approximately to 120:1. Compared to the $T_t\downarrow$ values that were obtained by differential scanning calorimetry (Bach et al., 1978) the phase transitions, as monitored with MC 540, occur at significantly lower temperatures. In the case of the intermediate PN distances, e.g., like PN 4 or PN 6, the discrepancy between the $T_t\downarrow$ values can be up to 10 °C. For shorter PN distances (PN 2, 3) and for longer ones (PN 8, 9), the differences between the T_t values, $\Delta T_t = T_t(\text{DSC}) - T_t(\text{MC 540})$ as obtained by the two different experimental approaches is significantly smaller, e.g., (PN2: $\Delta T_t = 2.2$ °C, or, e.g., PN 8: $\Delta T_t = 1.7$ °C). Compared to $T_t\downarrow$ values as measured by light scattering of unstained liposomes at 400 nm and/or fluorescence of the hydrophobic probe NPN incorporated into the liposomes, respectively (Diembeck, 1976), the $T_t\downarrow$ values obtained with MC 540 are remarkably low for short and intermediate PN distances. The major discrepancies (up to 6.5 °C) are again to be found in PN 4 and PN 6. For long PN distances (PN 7–9) the $T_t\downarrow$ values show good agreement in both optical methods.

The transition temperatures, as measured with MC 540, depend on the amount of the monitoring probe present in the system. In Table 4 the influence of the molar ratio lipid/dye is demonstrated with three different DPL analogues. In the presence of a high MC-540 concentration both with PN 3 and with PN 8, the phase transition seems to occur at temperatures 4–5 °C, lower than those measured in the presence of only a small amount of MC 540. In PN 6 (and similarly in PN 7, not shown in the Table) at low molar ratios (up to 120:1) there seems to be no marked influence of the dye concentration upon the transition temperature $T_t\downarrow$. However, increasing the lipid excess to about 200-fold results in the appearance of two transition regions, as obtained from the temperature dependence of r. These two T_t values seem to be located approximately symmetrically towards lower and higher temperatures compared to the T_t values obtained in the presence of a higher dye concentration.

Discussion

Some relevant answers to the major questions posed in connection with the site of action and the response

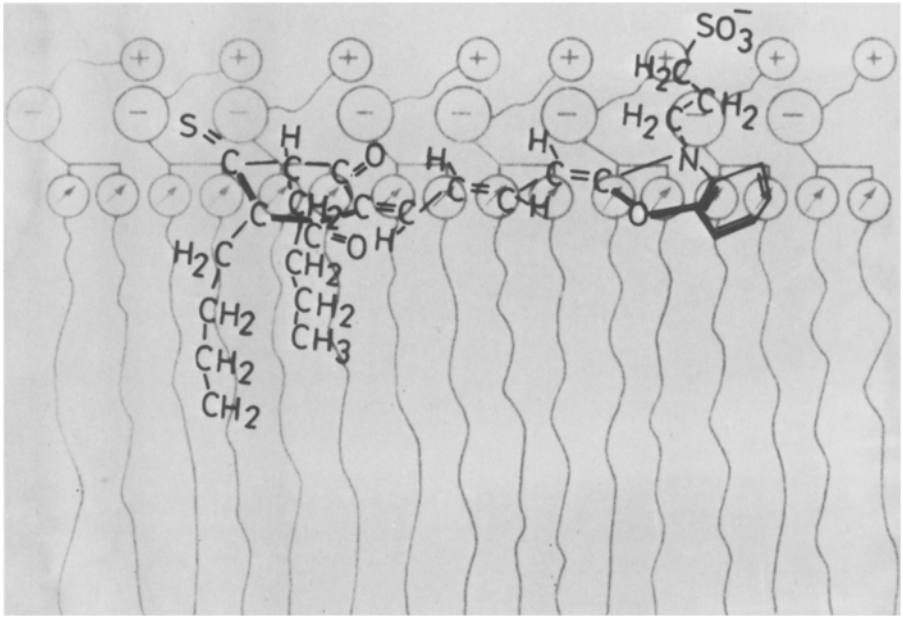


Fig. 7. Suggested binding site for MC 540 in phospholipid membranes. Details are in the text. (The size of MC 540 is exaggerated with respect to lipids)

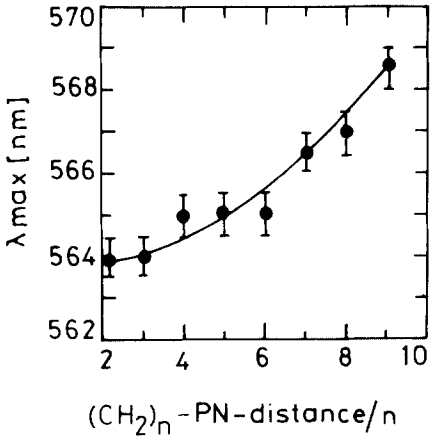


Fig. 8. Bathochromic shift of the maximum monomer absorbance λ_{max} of MC 540 bound to DPL analogs, depending on the number n of $(CH_2)_n$ molecules in the polar head group of the lipid. The bars represent the extent of experimental reproducibility (± 0.5 nm). All data were taken from experiments with a lipid/dye ratio $\geqq 120:1$ and from scans at 41 ± 1 °C, i.e., the lipids being in the liquid-crystalline phase

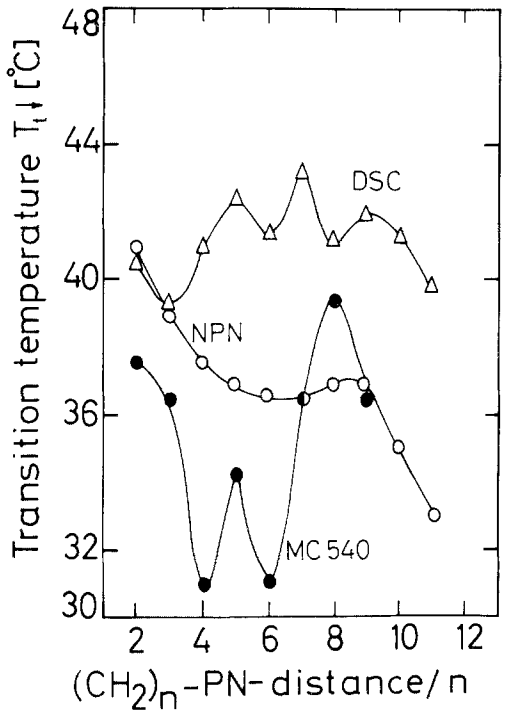


Fig. 9. Phase-transition temperatures of synthetic DPL analogs as a function of the number n of $(CH_2)_n$ molecules in the polar head-group region measured by different experimental approaches: differential scanning calorimetry (△), fluorescence of NPN (○), and absorption-spectroscopy of MC 540 (●) (*cf.* Table 3). Only transition temperatures obtained during cooling the samples ($T_t\downarrow$) are plotted here

of optical probes to membrane properties can be found, when studying the spectroscopic behavior of these dyes in solvents.

As a direct consequence of their amidic character, the merocyanines are remarkably solvent-sensitive dyes (Brooker, 1966); however, it has not been possible to generalize the correlation between a single solvent property and the optical data that were obtained from numerous dyes of this class. A number of different parameters have been proposed that should account for and be linearly correlated to the solvent-dependent spectral behavior of these dyes. Such parameters include the refractive index and some composite functions thereof, like the Bayliss function or the polarizability function (West & Geddes, 1964), transition energies, like E_T or Kosowers Z-number (Brooker, 1965), and finally the macroscopic dielectric constant D (McRae, 1957) of the solvent. Quantum chemical reasoning has shown (Liptay, 1965) that it is rather impossible to generalize

results obtained with a single dye and to deduct therefrom some solvent specific generalized functions, like the Z-number (Kosower, 1968) or the x_R and x_B values (Brooker, 1965). Therefore, for each dye the possible correlations of its spectral appearance and the solvent polarity parameters have to be checked individually. If deviations from the linearity are observed, they are to be interpreted as some specific solvent-solute interactions (Brooker, 1965). In the case of MC 540 the evaluation of the absorption-spectroscopic data showed that in no case could a totally linear relationship be established between the two principal parameters of the spectrum, i.e., the maximum emission band λ_{max} and the bandstrength $\int \varepsilon dv$ (ε = molar extinction coefficient), and any of the aforementioned parameters. Fitting the scattered data to any analytical curve shows the best outcome for a correlation between the maximum monomer absorption band λ_{max} and the macroscopic dielectric constant of the solvent, D, (Fig. 2). It has to be stressed, however, that the deviations of the experimental data from the fitted curve indicate a significant and specific mutual solvent-dye interaction. The scatter is pronounced in the region of extremely high and extremely low solvent polarities. The domain between $D=15$ and $D=45$ in the correlation between λ_{max} and D exhibits the least scatter in the experimental data, and therefore we can assume a preference of MC 540 for this range of dielectric media.

For a dye molecule interacting with a lipid membrane, this would indicate a preferential location in the interfacial region of the polar head groups. This preference is in keeping with the limited solubility of MC 540, similar to other merocyanines, both in strongly polar and in highly nonpolar solvents (Brooker *et al.*, 1951*a*; Tasaki *et al.*, 1976.)

The specificity of the dye-solvent interactions manifests itself also in the differential ways in which both λ_{max} and ε are influenced by the solvents. Plotting λ_{max} against ε (Fig. 6) yields for MC 540, like for other merocyanines, solvent and dye-specific curves. A coherent theory to explain the observed behavior has not been developed yet, but there have been attempts to interpret the shape of the curves qualitatively in terms of a solvent-dependent stabilization of the assymetry within the structural extremes of the resonance hybrids of the individual dyes (Brooker *et al.*, 1951*b*; McRae, 1957, 1958; Brooker, 1966).

An immediate conclusion from these observations has to be that, in whatever surrounding we observe the dye, the resulting spectra are determined to a great extent by very specific solvent-solute interactions, which may perturb mutually both the dye and its microenvironment. Therefore, the local dielectric constant inferred from such measurements should be taken with caution and may have just a comparative value. This note of caution is underlined by the fact that, even in apparently homogeneous bulk solutions, mixed solvents of the same dielectric constant but of different chemical structure may give somewhat different values for λ_{max} and for ε. This has obviously to be taken into account when using these dyes as external probes embedded in a highly structured "membrane solvent". For any given dye a specific energetic equilibrium is established, which determines its exact position within its ordered membrane moiety and which determines the degree of mutual perturbances. Any change in this delicate balance of forces between the probe and its microenvironment, either by inducing changes in the membrane structure, in the dye orientation, or the potential distribution, e.g., via a transient action potential in excitable membranes, leads to a new perturbed state accompanied by corresponding spectral changes. At infinite small dye concentrations, however, the number of dye molecules per membrane constituents may be so small that mutual dye-dye interactions as well as gross perturbances of the overall membrane structure can be excluded. In the case of a MC 540/lipid ratio of 1:150 inside the membranes, we can estimate that there are approximately 30 dye molecules in each vesicle and the average distance between two dye molecules on the membrane surface is approximately 60Å. In the absence of phase separation, the dye-dye interaction will be under these conditions minimal. Nevertheless, no matter how small the dye/membrane ratio might be, the optical signals of the dye probes report local events originating in the perturbed nonisotropic, heterogeneous microenvironment of the dye. It should be pointed out, therefore, that results obtained by spectroscopic means, using external probes, must be evaluated with great care, when attempting to translate them into structural and functional features of biological membranes.

No differences can be detected between the temperature-dependent spectra of MC 540 in single lamellar vesicles and multilayered liposomes provided we let the liposome-dye system equilibrate for $\geqq 24$ hr in order to allow the dye to penetrate through the lipid layers (P.I. Lelkes & I.R. Miller, *in preparation*). This indicates that the perturbed dye microenvironments are essentially identical in the two systems. The structural perturbances in the dye-binding moiety induced by the presence of the probes may be so substantial that they screen any existing differences in the conformational organization of the two aqueous lipid dispersion systems. With other optical indicators (8-anilino-1-naphthalenesulfonate (ANS) and Bromthymol Blue (BTB) that were located in the polar head-group region ($D \simeq 30$–40) of sonicated

DPL-vesicles, Sackmann and Träuble (1972), in contradiction to our results, found phase-transition temperatures which were in agreement with T_t values of unsonicated DPL-liposomes as detected by DSC measurements (Suurkuusk *et al.*, 1976).

The dependence of the absorption spectra of MC 540 interacting with PC- and PS-lipid membranes upon the ionic strength of the solution (Table 2) is consistent with previous experimental results. S.A. Simon and co-workers (1975) reported that for $T > T_t$ the presence of up to 1 M of univalent cations, like Na^+, K^+ or Li^+ did not change the transition parameters of egg lecithin and did not cause any alteration in the electrostatic charge distribution in the polar head-group region. In agreement with these findings, we do not observe any shift in the membrane monomer maximum band λ_{max}, whether we perform the experiments in H_2O or in the presence of 200 mM KCl. This result is also in agreement with recent data that were obtained in 100 mM KCl and 500 mM NaCl and that yielded for MC 540 the same wavelength of 567 nm for λ_{max}, in PC vesicles and in PC liposomes, respectively (Pohl, 1976; Waggoner & Grinvald, 1977). In consistence with these investigators, we find a slight reduction of the molar extinction coefficient ε in MC 540 bound to PC vesicles in the presence of the salt. Additionally, Easton *et al.* (1978) report a reduction of the fluorescence intensity of MC 540 bound to PC vesicles in the presence of 5 mM $CaCl_2$. A possible modification of the partition coefficient lipid dye in the presence of salt might explain these findings (Rubalcava, de Munos & Gitler, 1969). Likewise, the fluorescence intensity of this dye is reduced by 50–75% if 5 mM $CaCl_2$ and 5 mM $LaCl_3$, respectively, are added to an aqueous solution of 2×10^{-5} M MC 540 (Easton *et al.*, 1978).

Combining the solvent polarity studies with the spectral appearance of MC 540 bound to phospholipids (Table 2), we can, in accordance with several authors, attempt to locate the dye in the vicinity of the glycerol backbone region of the lipid molecules of a fluid bilayer membrane; the chromophore of the probe reports from a moiety with an effective dielectric constant of approximately $D = 6$–8 in the case of PC and of $D = 8$–10 in the case of PS, when the ionic strength in the Stern-layer adjacent to the aqueous solution is sufficient to shield the negative charges of the ionic groups of the phospholipids and to facilitate the penetration of the dye into the region of the ester-bonds. In view of its chemical structure, we view MC 540 to be located (Fig. 7) with its polar sulfonate group towards the more polar outer surface of the head-group region and the rest of the rodlike dye ranging through the ester bonds, anchored with the two butyl groups in the hydrocarbon chain region. The polyconjugated core of the chromophore is assumed to be in a uniform environment and therefore oriented parallel to the surface. This picture is in keeping with the views of Dragsten and Webb (1978) for MC 540 and of Ross *et al.* (1977) for some related cyanine dyes in the case of zero membrane potential. Upon imposing an electric field across the membrane, the dyes might reorient directly or in conjunction with the monomer-dimer equilibrium. A similar, even though somewhat deeper, location was used as a working hypothesis by Chance *et al.* (1974) and by Easton *et al.* (1978). In the case of PS we find a dependence of the depth of penetration of the dye on the electrostatic repulsion between the sulfonate group of the dye and the negatively charged head groups of this lipid. In any case, we observe spectra that are indicative for the effective dielectric constant of the chromophore microenvironment near the polar head group region of the phospholipids.

A modification of the effective dielectric constant and of the structural stability of the head-group region of DPL can be achieved by enlarging the *P-N* distance. While increasing the number of CH_2 molecules between the phosphate and the trimethylammonium, we enhance the hydrophobicity of the polar region and its interface with the hydrocarbon layer which affects the spectral properties of MC 540 embedded into this region. Indeed, the bathochromic shift with increasing number n of CH_2 molecules, as evident from Fig. 8, indicates an increase in hydrophobicity of the microenvironment of the MC 540 chromophores. The effective dielectric constant of this microenvironment as inferred from the shift in λ_{max} varies from $D \simeq 20$ (for $n = 2$) to $D \simeq 4$–6 (for $n = 9$). There are remarkable differences between the T_t values of the DPL analogs as obtained by NPN (Diembeck, 1976) and by MC 540 for varying PN-distances (Fig. 9). While NPN shows a constant $T_t\uparrow$ of about 41.6 °C in the heating mode (gel to liquid crystal transition) between $(CH_2)_2$ and $(CH_2)_8$ and a $T_t\downarrow$ of about 37 °C in the cooling mode (liquid crystal to gel transition) between $(CH_2)_4$ and $(CH_2)_9$, strong oscillations in $T_t\downarrow$ with MC 540 are observed. This oscillation is reminiscent of that obtained by differential scanning calorimetry (Bach *et al.*, 1979). There it was shown that T_t and ΔH (the phase transition enthalpy) of these DPL analogs oscillate when the P-N distance is increased from $(CH_2)_2$ to $(CH_2)_{11}$. This almost periodic variation of these two quantities with the N-P distance (Fig. 9, Table 3) was interpreted in terms of a didimensional quasi-crystaline structure with the negatively charged phosphate groups in one plane and the positively charged ammonia between the negative charges but lifted towards the aqueous phase. The overall oscillation with the N-P distance

is retained by the perturbing MC 540 but the oscillation pattern is changed (Fig. 9). Thus MC 540 is more sensitive to the variations in structural stability in these lipids, due to a change in the P to N distance, than is NPN. The difference in the sensitivity to lipid structural alterations of the two probes may be related to their own sterical arrangement. In NPN two planar naphthyl and phenyl rings are connected via an aminic nitrogen and the whole dye structure may be located in the plane of the ester bonds of the phospholipids, and therefore a perturbance of the lipid structure by the probe is little affected by the N-P distance. MC 540, on the other hand, has a negatively charged sulfonate connected by a propyl chain to one end of the chromophore, while the other end has two strongly hydrophobic butyl chains. Thus the chromophore may be in the interface between the polar and nonpolar regions of the lipid layer with the butyl residues penetrating into the hydrocarbon region and the sulfate protruding into the layer of the quaternary ammonia (Fig. 7). This localization of MC 540 differs somewhat from the one tentatively proposed by Chance *et al.* (1974). These authors suggested the location of the dye in a more hydrophobic part of the acyl chains, the chromophore being located between the ester-bonds and the methylene groups 5–15 Å inside the hydrocarbon layers and the butyl-chains penetrating further towards the terminal methyl groups.

In contrast to the remarkably low T_t values reported both by MC 540 and NPN, the fluorescence depolarization of DPH indicates phase-transition temperatures of DPL in fair agreement with the results of DSC measurements (Jacobson & Papahadjopoulos, 1975; Suurkuusk *et al.*, 1976). However, while investigating by DSC the thermothropic behavior of phosphatidylcholine multilayers in the presence of DPH, Lentz, Freire and Biltonen (1978) observed even at lipid/probe ratios of 500:1 a slight lowering in T_t. Similar results were obtained by us with the phosphatidylcholine analogues with different P-N distances. Taking the experimental evidences together with the deliberation following the solvent studies, we conclude that even at infinite dilution every probe molecule causes *eo ipso* a certain perturbance of its own microenvironment. The spectroscopic data obtained report on the state of the probe microenvironment, which does not necessarily resemble the unperturbed membrane matrix. The degree of perturbation depends on the chemical nature of the probe and on its location in the lipid layer.

Finally to be discussed is the mechanism by which MC 540 monitors the structural changes in the lipid membrane, which are induced by the phase transition from the liquid-crystalline to the rigid state. A close examination of the temperature-dependent spectra (Fig. 4) reveals that for high lipid/dye ratios ($\simeq 100:1$) at temperatures above the transition temperature T_t virtually all dye molecules are bound to the membrane, as evident from the predominance of the membrane monomer band. At temperatures well below T_t a major portion of the dye seems to be in the aqueous phase, as inferred from the spectra characteristic of the aqueous dye solution. In our experiments we found – depending on the individual DPL analog – a 3- to 10-fold increase in the partition coefficient lipid/dye, when increasing the temperature above the phase transition. Similar behavior was reported for ANS and BTB by Sackmann and Träuble (1972). Furthermore, it appears that also the monomer-dimer equilibrium constant of the dye absorbed to the membrane is strongly dependent on the membrane fluidity, as can be seen, especially at very high lipid/dye ratios ($\geqq 300:1$), where we observe a temperature-dependent aggregation of the membrane-bound dye when we increase the membrane microviscosity. In this case at temperatures $T < T_t$ most of the dye molecules remain associated with the membranes, but they tend to be in their dimeric form with a characteristic maximum wavelength of the absorption band at 525 nm (Waggoner & Grinvald, 1977; Warashina & Tasaki, 1975). In any case – at high or low lipid/dye ratios – we observe a gradual hypsochromic shift of the membrane monomer maximum absorption band with decreasing temperature: this can be interpreted that, concomitant with the transition of the membrane from the fluid to the rigid phase, the dye is excluded from its nonpolar binding site ($D \simeq 10$) in the glycerol backbone region towards a more polar microenvironment.

These observations may be of importance when discussing the possible mechanisms of MC 540 while reporting rapid optical changes during transient action potentials. Currently the hypothesis is favored that at high MC-540 concentration the optical signal results from potential-dependent changes in the state of dye aggregation localized on the membrane (Waggoner & Grinvald, 1977; Tasaki & Warashina, 1976b; Ross *et al.*, 1977). However, other mechanisms, like potential-dependent reorientation of the membrane associated chromophore (Dragsten & Webb, 1978; Warashina & Tasaki, 1975), or a potential-dependent relocation of the dye molecule within the membrane (Waggoner & Grinvald, 1977) cannot be excluded. It is evident that "the mechanism for the squid axon optical change is more complicated than the two-state monomer-dimer mechanism" (Waggoner & Grinvald, 1977; Ross *et al.*, 1977).

In view of our results, we might suggest that the potential-dependent changes in the MC-540 signal

during a transient excitation of the membrane could be partially a consequence of structural alterations in the membrane evoked by the action potential. The occurrence of such potential-dependent modifications of the membrane structure has repeatedly been postulated (Sherebrin, McClement & Franco, 1972; Neumann & Nachmansohn, 1975; Adey, 1975; Tasaki & Warashina, 1976*b*).

With respect to the small size of the potential-dependent optical signals, e.g., in the squid giant axon experiments, one could conclude that these changes occur only at a small localized fraction of the membrane surface, e.g., the site of ion-channels or the gating particles (Keynes, 1976). Such a localized structural change could be envisaged as a change in the local membrane fluidity, which could lead to a concomitant change in the membrane-dye partition coefficient and/or the equilibrium constant for the state of dye aggregation.

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Note added in proof: The calculation of the monomer-dimer dissociation of MC540 in H_2O (Fig. 1*b*) was done for the low concentration range, where the dye is predominantly in its monomeric form and the fraction in the dimeric form $\alpha \ll 1$. Since at infinite dilution $OD500 \approx OD532$ and thus $OD500 \approx k(c_{mon} + c_{dim})$. Therefore, with $\alpha = \frac{OD500 - OD532}{OD500}$, we obtain:

$$K c_{dye} = \frac{\alpha}{(1-\alpha)^2} \approx \alpha = \frac{OD532 - OD500}{OD500}.$$