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Perturbations of Membrane Structure by Optical Probes: II. Differential Scanning Calorimetry of Dipalmitoyllecithin and Its Analogs Interacting with Merocyanine 540*

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Summary. Differential scanning calorimetry of multilamellar liposomes, interacting with the optical probe Merocyanine 540, yields quantitative information about perturbances of the bilayer structure induced by this dye. At low dye: lipid ratios, the dye perturbs primarily its own microenvironment, which is laterally separated from the unmodified lipid domain and exhibits modified thermotropic properties. A further increase in the dye concentration results in a perturbance of the whole lipid bilayer. The degree of perturbance is sensitive to structural modifications in the head-group region of the lipids. It is concluded that Merocyanine 540 reports in every case, even at infinite dilution, on localized events originating from a perturbed microenvironment.

Key words: Optical probes, lipid bilayer, differential scanning calorimetry, structural perturbance.

Numerous reporter molecules, "probes," have in recent years been used to evaluate by optical methods transmembrane potentials (Waggoner, 1976), as well as structural properties, of macromolecules (Gabel, Steinberg & Katchalski, 1971; Clarke & Nakai, 1972; Witz & van Duuren, 1973) of lipid model membranes (Vanderkooi & Martonosi, 1969; Sackmann & Träuble, 1972; Haynes, 1974; Tsong, 1975; Haynes & Simkowitz, 1977) and of biological membranes (Vanderkooi & Martonosi, 1969; Träuble & Overath, 1973). For a review of the role of fluorescent membrane probes, *see* Radda (1975). A glance through the literature of the past few years reveals that until very recently (Cadenhead et al., 1977; Lentz, Freire & Biltonen, 1978) only insufficient attention was paid to the possible structural perturbances induced by the presence of these probe molecules. The literature cited in this context (Vanderkooi & Martonosi, 1969; Gabel et al., 1971; Clarke & Nakai, 1972; Witz & van Duuren, 1973; Träuble & Overath, 1973; Sackmann & Träuble, 1972; Haynes, 1974; Tsong, 1975; Haynes & Simkowitz, 1977) refers without exception to conformational studies performed at molar ratios dye:lipid > 1:40. Frequently, the molar fraction of the dye exceeds 0.15 (Vanderkooi & Martonosi, 1969; Clarke & Nakai, 1972), while in some cases the experiments were performed with equimolar amounts of dye and lipids (Vanderkooi & Martonosi, 1969; Tsong, 1975; Haynes & Simkowitz, 1977). A special paper (Träuble & Overath, 1973) reports on conformational studies of biomembranes investigated at a 3–4-fold excess of dye molecules.

Any meaningful application of a probe to elucidate structural features has to be preceded by the clarification of the following points: (1) location of the probe; (2) mechanism by which it monitors the features under investigation and changes therein; and (3) degree of perturbances induced by the probe into its own microenvironment and/or the whole system.

We chose to investigate lipid:dye interactions exemplified by dipalmitoyllecithin (DPL) and its analogs and MC 540[1], a frequently used polar dye that has previously been shown to be sensitive to transmembrane, as well as to membrane surface potentials (Waggoner & Grinvald, 1977; Russell, Beeler & Martonosi, 1979; Aiuchi & Kobatake, 1979). Recently, we demonstrated that MC 540 is a sensitive probe of structural modifications in lipid membranes

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[1] *Abbreviations:* MC 540 – Merocyanine 540 (5-((3-sulfopropyl-2(3H)-benzooxazolylidene)-2-butenylidene(-1,3-dibutyl-2-thiobarbituric acid); DSC – differential scanning calorimetry; DPL – dipalmitoyllecithin (1,2-dipalmitoyl-*sn*-glycerol-3-phosphorylcholine).

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due to its location (Lelkes & Miller, 1980). However, we noticed significant differences between experimental data obtained on one and the same model system by MC 540 and other experimental approaches, including optical probes (Diembeck, 1976; Bach et al., 1978; Lelkes et al., 1979). It seemed, therefore, appropriate to look quantitatively into the degree of perturbance induced by MC 540 into lipid model membranes, using differential scanning calorimetry (DSC) as a sensitive, nonperturbing approach (Sturtevant, 1974; Bach & Chapman, 1979). In this paper we present evidence that MC 540, like various other probe molecules (Jain & Wu, 1977), reports on a modified phase laterally separated from the unperturbed bulk lipids, and that beyond a certain critical dye concentration, not only the microenvironment of the dye itself is modified but the lipid bilayer structure as a whole.

Materials and Methods

DPL was purchased from Fluka (Buchs, Switzerland). The DPL-analogs with stepwise increase in the number n of $(CH_2)_n$ groups between the phosphate and trimethyl ammonium have been characterized elsewhere (Diembeck, 1976; Bach et al., 1978; Diembeck & Eibl, 1979; Lelkes et al., 1979. Lelkes & Miller, 1980). MC 540 was obtained from Eastman-Kodak as Na^+-salt and used without further purification. All experiments were performed in 10^{-2} M phosphate buffer (pH 7.0 ± 0.1); H_2O was bidistilled over $KMnO_4$ and the inorganic reagents of *p.a.* grade. Differential scanning calorimetry (DSC) of nonsonicated liposomes was performed on a DuPont 990 Differential Scanning Calorimeter as described previously (Bach et al., 1978). The lipid-dye interaction products were prepared by mixing appropriate amounts of the aqueous lipid suspension (75 mg/ml) with an aqueous solution of MC 540 (10^{-2} M) to give the desired molar ratio of dye/lipid. The interaction products were subsequently treated as described previously (Bach et al., 1978). The DSC experiments were performed 24–28 h after incubation, in order to ensure completion of the interaction process by allowing the dye molecules to diffuse through the lipid lamellae (P.L. Lelkes & I.R. Miller, *in preparation*). The enthalpy of melting, ΔH, was calculated by integration over the peak. The values for ΔH of the interacting region and for the unmodified lipid domain were obtained by graphical deconvolution, assuming that to a first approximation the shape of the lipid transition peak is not altered significantly.

Results

Endothermic thermograms for pure DPL and for DPL interacting with increasing amounts of MC 540 are shown in Fig. 1. The numbers denote the molar ratios lipid/dye for each sample. In order to facilitate quantitative comparison between the data, the curves were drawn after normalization for 1 mg lipid in each preparation. The thermal properties of aqueous suspension of pure DPL-liposomes are in agreement with

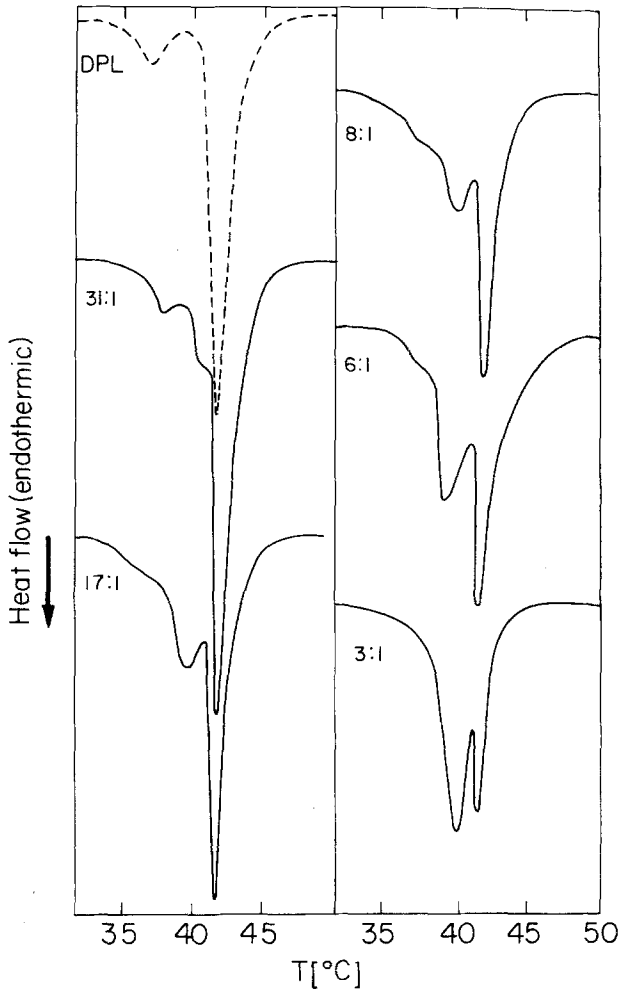

Fig. 1. Thermograms of dipalmitoyllecithin interacting with Merocyanine 540. Numbers refer to the molar ratio lipid/dye. The thermograms are reconstructed for 1 mg lipid in each preparation. Scan rate: 5 °C/min

values quoted in the literature (Jacobson & Papahadjopoulos, 1975; Suurkuusk et al., 1976; Lentz et al., 1978). In the presence of increasing concentrations of MC 540, an additional transition peak appears gradually at lower temperatures; this peak can be attributed to the dye:lipid interaction region. For lipid/dye ratios smaller than 4:1 this second peak eventually becomes broader and larger than the main transition peak. Within an experimental accuracy of ± 0.5 °C, the phase transition temperature T_m of the main, unmodified lipid peak is not affected by the probe. However, concomitant with the formation of the interacting peak, the region of the pretransition of DPL at ≈ 35 °C becomes less well-defined and becomes, at high dye concentrations, an undistinguishable part of the lower temperature slope of the interaction peak.

A more quantitative description of the structural

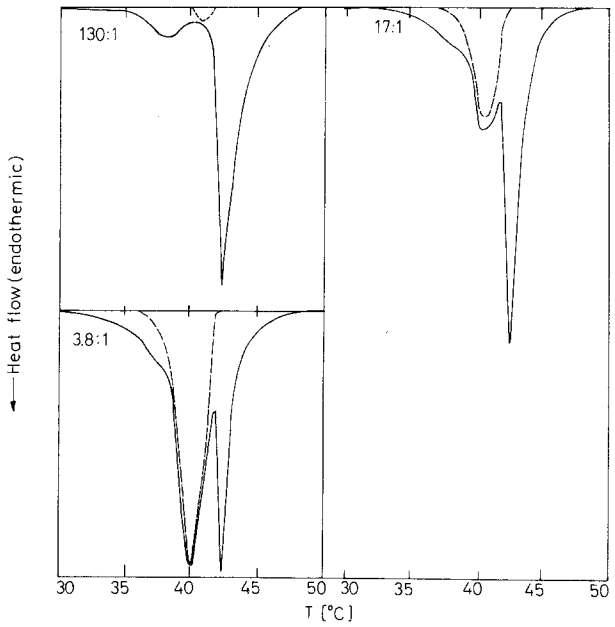

Fig. 2. Graphical deconvolution of the transition peak of the modified phase for three different lipid/dye ratios

perturbances induced by the dye is obtained by the graphical deconvolution of the two overlapping heat flow peaks (*cf.* Materials and Methods). For three different lipid/dye ratios it is demonstrated in Fig. 2 that, with increasing dye concentration, the contribution of the heat flow peak originating from the interaction region peak gains weight. From these reconstituted peaks we can obtain the thermotropic behavior of the two separate domains. Table 1 lists the experimentally determined total enthalpy of melting of the system (ΔH^{tot}) and the enthalpies of melting in the unmodified and the modified regions, ΔH^{lip} and ΔH^{int}, respectively. Table 1 furthermore contains the phase transition temperatures, T_m, of both the unmodified lipid phase T_m^{lip} and the perturbed interact region, T_m^{int}.

In Fig. 3 the enthalpy of melting, ΔH, is plotted against the molar fraction of dye. The total enthalpy of melting, ΔH^{tot}, as determined from the experimental curves, exhibits a biphasic behavior. It increases with increasing dye concentration to a maximal value at a molar fraction $mf \approx 0.12$, where ΔH^{tot} equals 13.5 kcal/mole lipid, approximately 40% more than the enthalpy of melting for pure DPL-bilayers. However, at higher dye concentrations, ΔH^{tot} is sharply reduced. For a lipid/dye ratio of 2:1, ΔH^{tot} is about 30% lower than the ΔH of pure DPL. This biphasic behavior for the enthalpies of melting as seen in Fig. 3 is due to the additive contributions of the pure lipid peak and of that of the modified interaction region, as obtained by the graphical deconvolution (Fig. 2, Table 1). While the enthalpy of melting of the unmodified lipid domain decreases monotonously with increasing dye concentration, ΔH^{int} exhibits a biphasic dependence on *mf*. Following a linear increase in ΔH^{int} with the dye concentration, the ΔH^{lip} and ΔH^{int} curves intersect at a lipid/dye molar ratio of $\approx 4:1$ ($\Delta H = 6.5$ kcal/mol), and decrease in parallel for any subsequent increase in the MC 540 concentration.

This biphasic nature of the interaction between MC 540 and DPL lipid bilayers is also reflected in

Table 1. Summary of the thermotropic properties of dipalmitoyllecithin interacting with Merocyanine 540

Molar ratio lipid/dye	Molar fraction, mf $\frac{(\text{dye})}{(\text{dye})+(\text{lipid})}$	Total enthalpy of melting, ΔH^{tot} (kcal/mole)	Enthalpy of melting of the pure lipid phase, ΔH^{lip} (kcal/mole)	Enthalpy of melting of the interaction region, ΔH^{int} (kcal/mole)	Phase transition temperature of the pure lipid, phase, T_m^{lip} (°C)	Phase transition temperature of the modified domain, T_m^{int} (°C)
Pure DPL	–	9.8	9.8	–	41.8	–
130 :1	0.008	8.5	8.3	0.2	42.3	40.9
85 :1	0.012	8.5	7.7	0.8	43.2	41.1
50 :1	0.020	9.6	8.8	0.8	42.3	40.9
31 :1	0.031	11.8	10.1	1.7	41.8	40.0
22 :1	0.043	11.8	9.4	2.4	42.1	40.4
17 :1	0.056	11.9	9.2	1.7	42.6	40.7
14.1 :1	0.067	12.2	10.0	2.2	42.2	40.6
10 :1	0.088	15.4	13.2	2.2	42.6	40.7
8.1 :1	0.111	10.5	7.6	2.9	41.9	40.0
6.2 :1	0.139	11.6	8.0	3.6	42.7	40.3
5.6 :1	0.152	12.6	8.6	4.0	41.7	39.5
5 :1	0.167	16.5	9.8	6.7	42.1	39.8
3.8 :1	0.208	14.0	6.6	7.4	42.4	40.1
3.8 :1	0.208	11.5	5.9	5.6	42.3	40.6
3 :1	0.250	8.0	3.1	4.9	41.8	40.0
2 :1	0.333	7.4	4.9	2.5	42.8	40.8

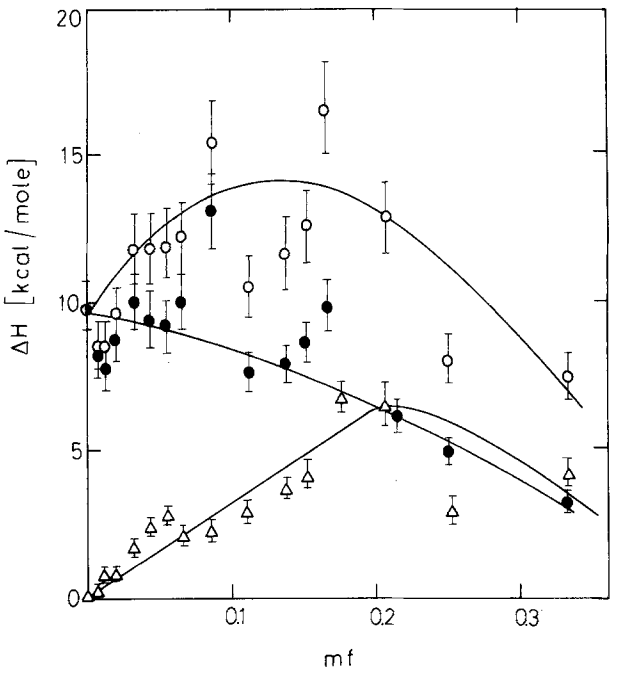

Fig. 3. Enthalpies of melting ΔH (kcal/mole) as a function of the molar fraction, *mf*, of the dye. —o— total enthalpy of melting, ΔH^{tot}. —●— ΔH^{lip} for the free lipid phase. —Δ— ΔH^{int} for the modified phase. Error bars represent the experimental accuracy

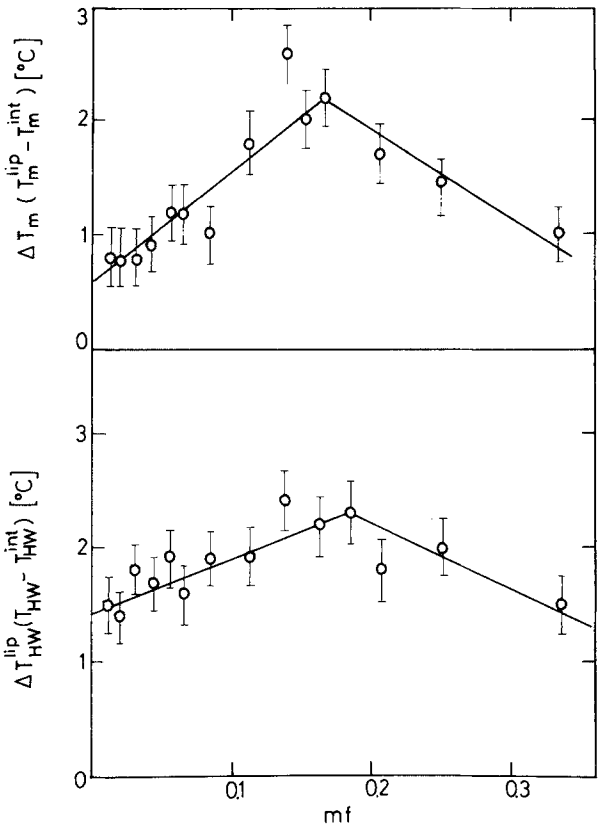

Fig. 4. Decrease in the melting temperature of the interact peak with respect to the melting temperature of the unmodified bilayer, ΔT, as a function of the molar fraction *mf* of the dye T_m = temperature at the maximum of the peak, T_{HW} = temperature at the midpoint of the half-width

the difference of the transition temperatures of the unmodified and the modified regions, T_m^{lip} and T_m^{int}, respectively (Fig. 4). A qualitatively similar picture is obtain when plotting the difference in the temperature at the midpoint of the half-width, T_{HW}, of the two heat flow peaks as a function of *mf*.

In a previous paper we demonstrated the sensitivity of MC 540 to structural variation in the head group of DPL analogs (Lelkes & Miller, 1980). Three of these DPL analogs, with 4,7 and 8 CH_2 groups between P and N (PN4, PN7, PN8), were chosen to study their interaction with MC 540 by DSC. Figure 5 shows the thermograms of these lipids interacting with MC 540 at a molar ratio lipid/dye ≈9:1 in each case. The transition peak of PN4 appears slightly broadened (1.2 °C for the half-width as compared to 0.6 °C in the unperturbed PN4). PN8 exhibits a similar behavior as DPL in Fig. 1. The presence of the probe leads to the abolition of the pretransitions and the appearance of an interaction peak at lower temperatures than the transition of the unperturbed lipid. In the case of PN7 both the endothermic (heating) and the exothermic (cooling) scans were performed. While the endothermic peak yields a single T_m value of 40.5 °C, we find in the cooling scan a multiphasic transitional behavior. The main transition is shifted to about 34 °C, 7 °C below T_m in the heating scan and by 11–12 °C below the T_m values of PN7 in the cooling scan in the absence of the dye (Bach et al., 1978). The influence of MC 540 upon the enthalpy of melting of these synthetic lipids is different in its concentration dependence for each of the DPL analogs investigated (Fig. 6). PN4 behaves essentially similarly to DPL (40% increase in ΔH^{tot} for a molar ratio lipid:dye of ≈9:1). For PN8, on the other hand, the thermogram of which is similar to that of DPL (Fig. 5), the ΔH seems to be largely independent upon increasing concentrations of MC 540 (at a molar ratio of 9:1, ΔH increases by ≈15%). The enthalpy of melting of PN7, however, is strongly reduced by the presence of the probe (at a molar ratio of 9:1 by ≈50%). Augmented incorporation of MC 540 into the lipid bilayer leads to a further decrease in ΔH, up to an *mf* value of 0.18, when no lipid phase transition is discernible any more.

Table 2 summarizes the DSC data on the analogs interacting with MC 540. The presence of the dye results, for all three lipids, in a concentration dependent decrease in T_m concomitant with an increase in the half-width of the transitional region. For PN7 a substantial hysteresis of the T_m values obtained in

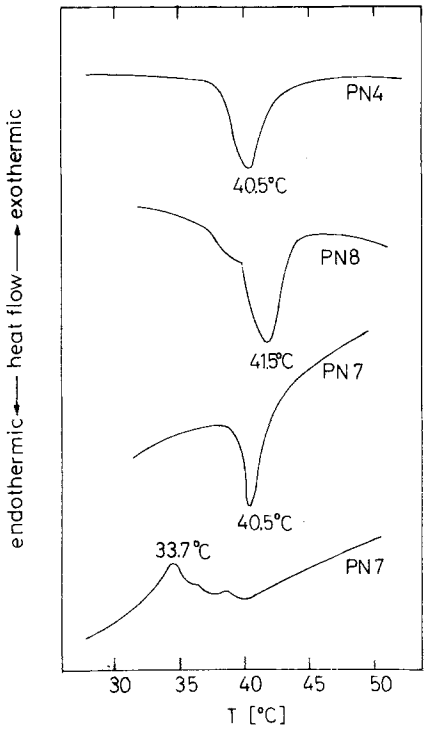

Fig. 5. Thermograms of several dipalmitoyllecithin analogs with a modified head group structure (PN(n)) interacting with Merocyanine 540 n(=4,7,8): number of CH_2 groups between P and N. Scan rate: 5 °C/min. Sensitivity: PN4, PN8:0.1 mcal/sec inch. PN7:0.02 mcal/sec inch

Table 2. Thermotropic properties of dipalmitoyllecithin analogs interacting with Merocyanine 540, cumulative data

Lipid	Molar ratio lipid/dye	Phase transition temperature T_m (°C)		Enthalpy of melting
		Heating	Cooling	ΔH^{tot} (kcal/mole)
PN4	Pure PN4[a]	41.8± 0.7	41.0± 0.7	10.2
	79:1	42.0± 1.0	–	10.8
	9:1	40.0± 1.2	–	14.0
PN8	Pure PN8[a]	42.5+ 0.7	41.2+ 0.7	9.7
	73:1	43.5± 1.2	–	10.0
	9:1	40.5± 1.5	–	11.0
		(38.2± 1.2)[b]	–	
PN7	Pure PN7[a]	45.0± 0.7	43.2± 0.7	13.0
	112:1	45.0± 0.6	37.2± 1.0	12.0
	45:1	41.0± 1.2	37.2± 1.2	11.1
	22:1	40.5± 1.6	36.2± 1.7	8.7
	9:1	40.0± 1.0	33.7± 1.5	6.8
	5:1	35.0–44.0	29.0–43.0	0.7

[a] Data from Bach et al., 1978.
[b] T_m value of the heatflow peak originating from the perturbed region.

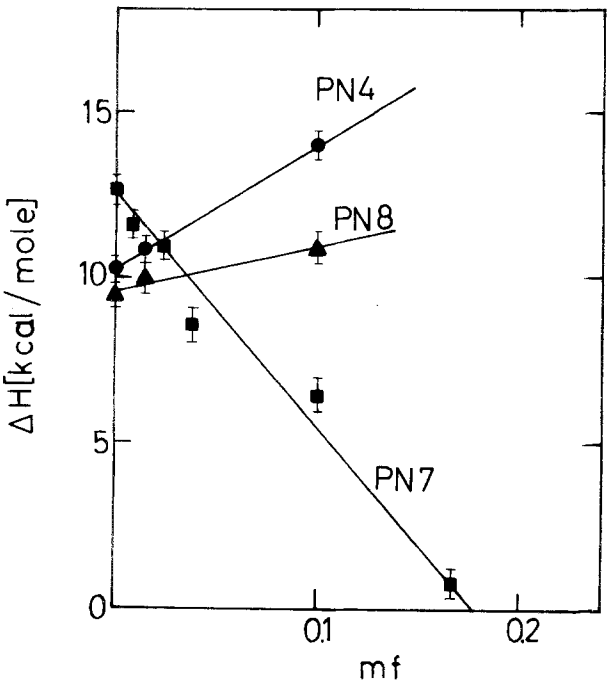

Fig. 6. Total enthalpy of melting ΔH (kcal/mole) of the dipalmitoyllecithin analogs as a function of the molar fraction of Merocyanine 540

the heating and cooling modes is induced by the probe. The variance of the enthalpy of melting with the dye concentration is dependent on the structure of the lipids (*cf.* Fig. 6).

Discussion

Our differential scanning calorimetry results show that the structural organization of lipid bilayers is perturbed by the presence of external probe molecules, like Merocyanine 540. The second peak at lower temperatures can be attributed to a lipid-dye interaction complex with modified thermotropic and, hence, structural properties as compared to the pure lipid bilayer (Jain & Wu, 1977).

The occurrence of two clearly separated heat flow peaks, which can be attributed to the unmodified lipid and the modified lipid-dye interaction phases, respectively, is due to a lateral phase separation between two different domains within the lipid bilayer (Jacobson & Papahadjopoulos, 1975; Teissié, Tocanne & Baudras, 1976; Hartmann, Galla & Sackmann, 1977). The two different regions coexist independently up to a certain dye concentration, which is determined by the structure of both the dye and the lipid (*cf.* below). This lateral phase separation indicates that the dye molecules are not distributed uniformly and randomly over the whole membrane surface, but they form, especially at higher dye concentrations, clustered "interaction complexes"

with their surrounding lipid molecules. A similar phase separation is introduced by the structurally related dye, ANS (l-anilino-8-naphthalene sulphonate), as revealed by DSC (Jain & Wu, 1977).

The mode and degree of modifications induced by small molecules into lipid membranes were classified by Jain and Wu (1977) according to the location of the perturbing molecules within the bilayer. We may, therefore, envisage MC 540 to be located near the glycerol backbone of the phospholipids, increasing the spacing of the lipids and reducing the transition temperature of the interaction domain (Jain & Wu, 1977). The conclusion confirms our previous tentative location of the binding site of MC 540, based on spectroscopic evidence (Lelkes & Miller, 1980). Thus we suggest that for zero membrane potential the main axis of the dye-chromophore is oriented parallel to the plane of the membrane, allowing stacking at higher dye concentrations, at a site with an average dielectric constant of 5–15, depending on the lipid head-group conformation. The apolar butyl chains of MC 540 protrude into the hydrocarbon region of the lipids (up to C_2–C_4) while its anionic SO_3–group is anchored between the quaternary ammonia near the aqueous interface (*cf.* Fig. 8 in Lelkes & Miller, 1980). In addition to the nature of this binding site, the dye-stacking at higher dye concentrations may be responsible for the modifications in the thermotropic properties of the interaction domain.

Consistent with this location of the dye, we suggest that the sensitivity of MC 540 to modifications in the head-group structure of DPL analogs might be based on the different mode of interaction of this dye with the various lipids. The $\Delta H/mf$-curves for PN4 and PN8 qualitatively follow the one for DPL, whereas with PN7 a sharp decrease in ΔH is observed even for low dye concentration (Fig. 6). Extrapolating, for PN7, to $\Delta H=0$, we obtain from the intersect with the abscissa (0.17) that each dye molecule perturbs approximately 5 molecules of the surrounding lipid bilayer. A similar dependence on the head-group conformation has been detected in the interaction of basic polypeptides with the same DPL analogs (Bach et al., 1978). An $\approx 50\%$ decrease in ΔH was observed for the interaction product of PN7 with an equimolar amount of a random copolypeptide of lysine and phenylalanine, while for the interaction product of the copolymer with PN4, ΔH decreased only by $\approx 20\%$, as compared to the pure compounds.

Perturbances of bilayer properties induced by the presence of probe molecules have been noticed in our system previously (Bach et al., 1978; Lelkes et al., 1979; Lelkes & Miller, 1980). The phase transition temperatures, T_t, detected optically, deviated significantly from those measured by DSC (T_m). The concentration dependence of the shift in T_t, as well as the occurrence of substantial hysteresis, were shown to be macroscopic evidence for the specificity of the lipid:dye interaction, depending on the chemical nature of both interaction products (Lelkes et al., 1979). We concluded that at the lipid:dye ratios investigated (100:1–1000:1), the perturbing effect of the dye molecules is limited to the interaction region only. The optically detected membrane parameters originate from a structurally modified phase and do not necessarily reflect the actual situation in the unperturbed bulk lipid domain. This view is consistent with the picture emerging from the DSC data for DPL, that at all the measured dye:lipid ratios the two phases coexist and that the melting parameters of the unmodified domain do not change within experimental accuracy. Comparison of the DSC data to previous optical measurements of the transition temperatures of the same lipids using Merocyanine 540 at a lipid:dye ratio of 120:1 (Lelkes & Miller, 1980) underlines this conclusion and furthermore stresses the specificity of the dye/lipid interaction. Thus the T_m values of PN4, as determined by DSC, do not differ substantially in the absence (41.8 °C) and in the presence of 10% (wt/wt) MC 540 (40.0 °C), while the optically determined $T_t\uparrow$ is at 33.6 °C. For PN8, on the other hand, we find a good agreement between $T_t\uparrow$ and the T_m of the heat flow peak at 40.5 °C. In the case of PN7, however, where even low concentrations of MC 540 induce a strong degree of perturbance, we conclude from the large hysteresis between heating and cooling scans and the agreement of the $T_t\uparrow$ and T_m values that a few percent of dye already suffice to modify the entire bilayer.

The analysis of the concentration-dependent influence of MC 540 on the enthalpy of melting in the different domains yields a quantitative assessment of the perturbances induced by the presence of MC 540. From Figs. 3 and 4 the biphasic nature of the lipid:dye interaction is evident. In the case of DPL up to a dye/lipid ratio of 1:4 ($mf=0.2$) the enthalpy of melting in the intact lipid phase decreases by $\approx 30\%$, indicating that 1–2 lipid molecules are removed from the intact region by each dye molecule. Concomitantly the enthalpy of melting in the modified region, ΔH^{int}, increases linearly. From the intersect between the $\Delta H/mf$-curves of the two domains, we can estimate that the melting enthalpy in the modified region is ≈ 26 kcal/mole lipid, as compared to 9.8 kcal/mole for pure DPL (Suurkuusk, et al., 1976; Lentz et al., 1978). A similar increase in the enthalpy of melting of the interacting region was observed for the interaction of gramicidin S with dipalmitoyl- and dimyristoyllecithin multilammellar vesicles for molar ratios Gramicidin S/phospholipid up to 1:5 (Wu

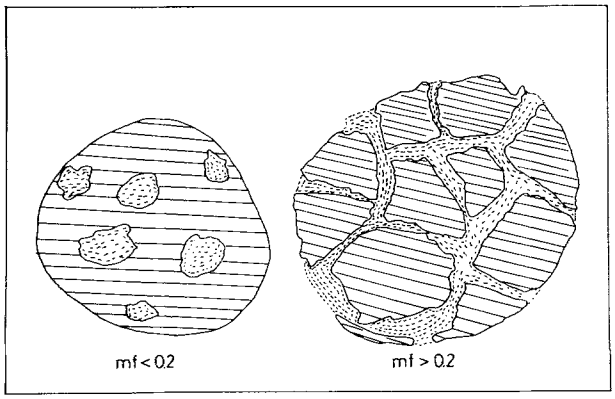

Fig. 7. Model for the perturbation of the lipid membrane structure at different concentrations of Merocyanine 540. The dotted area symbolizes the modified dye/lipid interaction phase

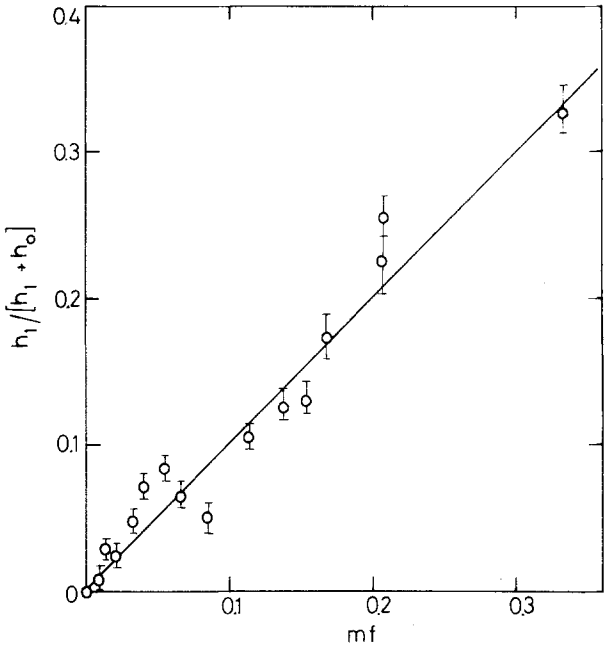

Fig. 8. Fractional contribution of the enthalpy of melting of the perturbed phase $h_i/(h_i+h_o)$ in dependence on the molar fraction of the dye, *mf*, whereby $h_i = \Delta H^{int}/\Delta H^{int}_{mole}$ and $h_o = \Delta H^{lip}/\Delta H^{lip}_{mole}$. ΔH^{int}_{mole} and ΔH^{lip}_{mole} are the molar enthalpies of melting in the modified phase (26 kcal/mole) and in the pure lipid phase (9.8 kcal/mole), respectively

et al., 1978). For the interaction of MC 540 with DPL, a further increase in the dye concentration beyond a molar fraction of 0.2 results in a sharp decrease in the enthalpy of melting in the interact region, concomitant with a reversal of the separation of the two phases, as seen in the decrease of ΔT_m and Δ_{HW}, respectively (Fig. 4). This behavior indicates a substantial change in the stoichiometry of the dye-lipid complex, a mutual modification of the two domains, and ultimately a drastic alteration in the bilayer structure as a whole, and is probably due to the strong tendency of MC 540 to form dimers and higher aggregates at high concentrations (West & Caroll, 1966). Beyond a critical dye concentration, the dye molecules do not remain in patches of perturbed interacting zones, separated from the unmodified domains, but due to stacking of the dyes, form continuous chains over the entire bilayer surface (Fig. 7). Such an arrangement forms long boundary lines perturbing the structural organization of the surrounding "free" lipids as well (Marsh, Watts & Knowles, 1976). This phenomenon of mutual interaction between the previously separated domains ultimately leads to a complete abolition of the original bilayer structure. A plot of the fractional contribution of the enthalpy of melting per lipid molecule in each region against *mf* (Fig. 8) yields essentially a straight line with a slope of ≈ 1.0, indicating that the area of the modified region is growing monotonously with increasing dye concentration. However, if the contribution of the "defects" in bilayer organization exceeds $\approx 20\%$ ($mf > 0.2$), the whole membrane is affected. From the data presented here and in a previous paper (Lelkes et al., 1979), it is evident that these quantitative assessments for the system DPL-MC 540 cannot be generalized for other lipids and dyes. The experiments with the DPL analogs already exemplify the quantitative differences. For PN7, for instance, we calculate that approximately five molecules of lipid are perturbed by one molecule of MC 540. In turn, a breakdown of the original bilayer structure occurs if the fractional contribution of the "defect-region" exceeds $\approx 10\%$.

It is evident that reliable usage of optical probes has to be preceded by quantitative studies of perturbances introduced by the probes into each system under investigation. It has be stressed that there is no "safe" concentration of dye, as the dye in every case, even at infinite dilution, will report from a perturbed microenvironment.

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