HEADGROUP-DEPENDENT MODULATION OF PHASE TRANSITION IN DIPALMITOYL LECITHIN ANALOGS

A fluorescence depolarization study

P. I. LELKES, A. KAPITKOVSKY, H. EIBL* and I. R. MILLER

Department of Membrane Research, The Weizmann Institute of Science, PO Box 26, Rehovot, Israel

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1. Introduction

Phase transition temperatures of synthetic DPL-analogs with an increased number of CH_2-groups in their polar headgroup regions vary significantly both in their values and in their dependence on the structural modification of the headgroup region, depending on the experimental approach [1,3,4]. In pure DPL excellent agreement between data obtained by DSC and by the fluorescence depolarization of DPH was reported [5,6]. We therefore chose the latter method to examine structural changes in those synthetic DPL analogs during temperature-induced phase transitions from the gel to the liquid–crystalline state.

Our findings suggest that every external probe molecule perturbs its membrane microenvironment and that the degree of perturbance is dependent on both the membrane structure and the chemical nature of the probe.

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; DPL, 1,2-dipalmitoyl-*sn*-glycerol-3-phosphorylcholine (dipalmitoyl-lecithin); DSC, differential scanning calorimetry; MC 540, merocyanine 540; NPN, *n*-phenylnaphtylamine; PN(*n*), synthetic DPL analog with *n* CH_2-groups between the phosphate and the quaternary ammonium in the polar headgroup; T_t, temperature of the main transition; ΔT, width of the transitional region; ΔT_t, difference between T_t values obtained in the heating and in the cooling modes (hysteresis)

* Permanent Address: Max-Planck-Institut für Biophysikalische Chemie, D-3400 Göttingen-Nikolausberg, FRG

2. Materials and methods

DPL was purchased from Fluka (puriss grade). The synthesis of the DPL-analogs PN(*n*), with $n = 2–11$, and some of their physicochemical properties are described in [1–3]. All these lipids were used without further purification. Multilamellar liposomes and single-walled vesicles were prepared in double-distilled water according to standard procedures, as in [4]. To 3 ml of the aqueous lipid suspensions ($\sim 5 \times 10^{-4}$ M) 1 μl 2 mM DPH in tetrahydrofuran (both reagents from Fluka) were added to give a final lipid:dye ratio of ~750:1. The samples were rapidly vortexed and maintained for ≥ 45 min at ~50°C, i.e., above their phase transition temperatures, in order to allow partitioning and equilibration of DPH within the lipid bilayers. The fluorescence depolarization of DPH was measured on a fluorescence polarimeter (Hubweiz, Flupol IV, Hubbrecht Labs., Utrecht). The method was essentially that one described in [5]. The temperature was set and controlled by a feedback temperature-regulation unit that is an integral part of this instrument. The actual temperature inside the cuvette was measured with an accuracy of ± 0.1°C.

Temperature scans were performed for each sample first in the cooling and subsequently in the heating mode, cycling, the temperature between 50°C and 20°C at a steady scan rate $\leq$ 20°C/h. The reproducibility of the data was $>$ 1% with the same sample. Different preparations yielded results that agreed within $\leq$ 3% at scan rates of ~20°C/h.

The mathematical formalism relating the fluores-

cence depolarization of DPH to membrane structure is detailed in [6]. The terms and abbreviations, as explained in [6], are used here. The phase transition temperatures T_t were determined either graphically [7] or from a plot of $d(\ln\eta)/d(1/T)$ against T [6]. With both methods the T_t values agreed within 0.3°C. Control experiments, as described in [8–10] were performed to rule out possible artifacts.

3. Results and discussion

Steady state measurements of the fluorescence depolarization of DPH can, under certain assumptions, be related to the 'apparent microviscosity η' of the microenvironment of the dye [5,6,8,9]. Plotting $\ln\eta$ against $1/T$ [6] for both liposomes and vesicles formed by DPL and its synthetic analogs, we find that a change in the headgroup conformation of these lipids influences the phase transition temperatures, the width of the transition region and most strikingly also the width of the hysteresis between the heating and cooling scans. In fig.1 this is exemplified for unsonicated, multilayered liposomes prepared from 3 different lipids, DPL, PN6 and PN8; similar graphs were obtained with sonicated vesicles. Our results for DPL agree in all their respects with the data obtained using DPH [8–10]. For the synthetic lipids with $n \geq 3$, the shape of the $\ln\eta$ versus $1/T$ curves is reminiscent of an apparent supercooling and superheating. However, as we know from the DSC measurements that such effects do not occur in the absence of external probes, we conclude that the alterations in the shape of the curves reflect modifications in the dye–lipid interactions due to the changes in the headgroup structure. Thus a close relation has to exist between the augmented polar headgroup and the site of location of DPH within these artificial DPL-analogs with $n \geq 3$. This argument is stressed furthermore by the fact that the agreement between T_t-values, as measured in pure DPL by DSC, DPH-fluorescence-depolarization and the fluorescence of NPN [8,10], was not found for the synthetic DPL-analogs with $n \geq 3$. Major discrepancies between the 'true' transition temperatures, as measured by DSC, and the data obtained by 3 different optical methods using external membrane probes are observed, except for the DPH-values for $T_t\downarrow$ (cooling mode) that are in the region of the $T_t\downarrow$ values obtained with NPN [1,3,4]. In fig.2 the phase transition temperatures of liposomes and vesicles, both in the heating and in the cooling mode, are plotted against the number n of CH_2-groups in the

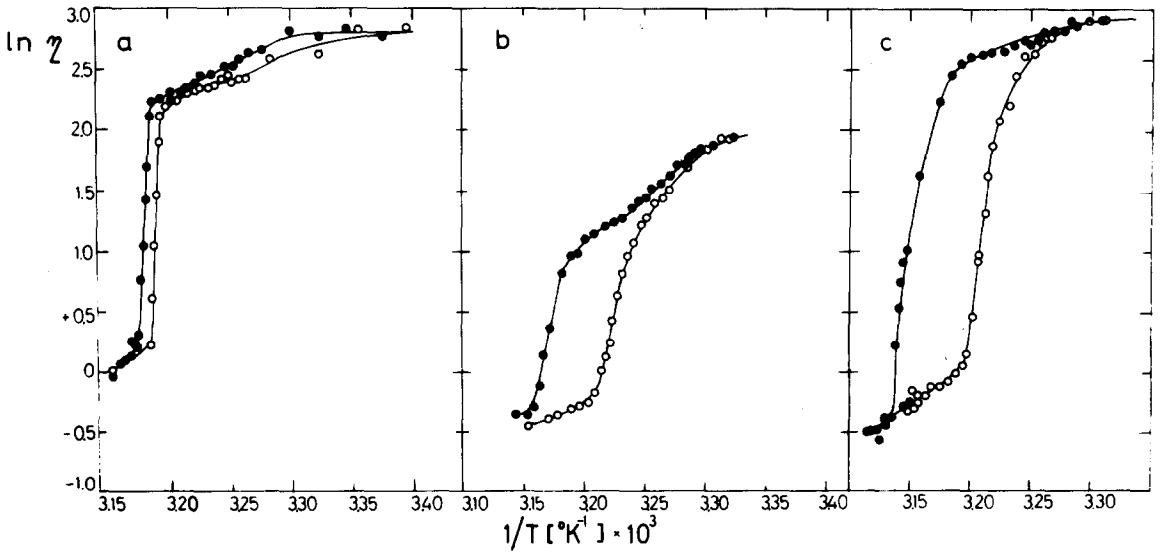

Fig.1. Temperature profile of the apparent microviscosity η obtained with multilamellar liposomes of DPL and two of its synthetic analogs PN6 and PN8: (o–o–o) cooling (↓); (•–•–•) heating (↑).

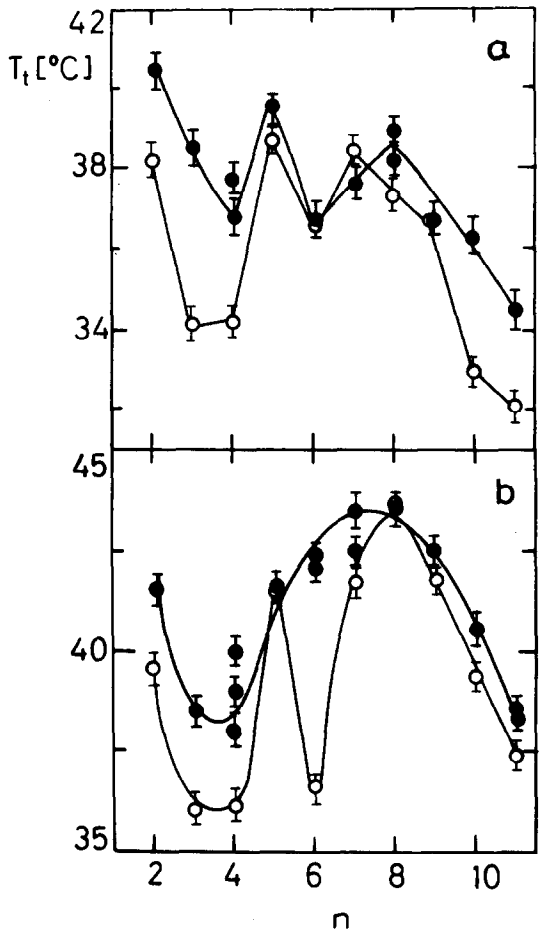

Fig.2. Dependence of the phase transition temperature T_t on the number of n of $(CH_2)_n$-groups in the polar headgroup region of synthetic DPL analogs: (a) cooling, (b) heating; (•) liposomes, (○) vesicles.

polar head of the synthetic DPL analogs. The differences between vesicles and liposomes, both numerically and in their quasioscillatory n-dependence, reflect the increased constraints in the molecular packing of the vesicles due to their smaller radius of curvature. This in turn will lead to modifications of the structural features of these lipids, as postulated from a similar periodical n-dependence of both the transition temperatures and the melting enthalpies, found by DSC-measurements [3]. $T_t\downarrow$ values obtained by the temperature-dependent change in absorption of MC 540 also showed a certain oscillatory pattern with no discrimination between liposomes and vesicles [4], while the NPN-data did not show any kind of oscillatory n-dependence of the transition temperatures [1].

A dependence on the number of (CH_2)-groups in the polar headgroup region is also detected for the width of the transition region, ΔT (fig.3). Besides certain numerical differences between the heating and the cooling modes, major differences are observed between liposomes and vesicles in their n-dependence. Vesicles exhibit a broad transition region ($\Delta T = 9°C$) for small numbers of methyl-groups in the polar head ($n = 2$–4); the value of ΔT decreases in an oscillatory manner to ~3°C, for PN 8–11; this value is in the region of the ΔT-values for the corresponding liposomes. The multilayered lipid membranes, on the other hand, exhibit a somewhat different n-dependence of their transition widths. ΔT-values for liposomes increase from 1.2°C for DPL to $\Delta T = 5.5°C$ for PN 4 and subsequently decrease again with increasing n.

These variations in ΔT should indicate parallel variations in the number of lipid molecules per cooperative unit [11–13]. However no such effect was reported either with DSC [3] or with the other external probes used up to now [2,4]. Therefore the relation between ΔT and the size of the cooperative unit might be questioned, especially when external probes were used to determine structural features like phase transitions in lipid membranes.

The hysteresis between the transition temperatures measured in the heating and in the cooling mode, respectively, ΔT_t, is shown in fig.4 as a function of n. Above a certain critical number of CH_2-groups in the polar head, ΔT_t varies quite abruptly. It increases by

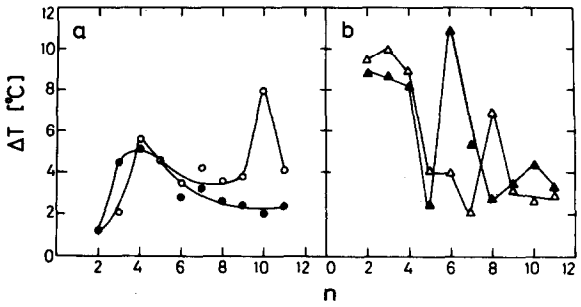

Fig.3. The width of the transitional region ΔT for the main phase transition of synthetic DPL-analogs as a function of n. ΔT was determined graphically from plots of $\ln\eta$ versus $1/T$ [6]. (a) liposomes, (b) vesicles; (•, ▲) cooling, (○, △) heating.

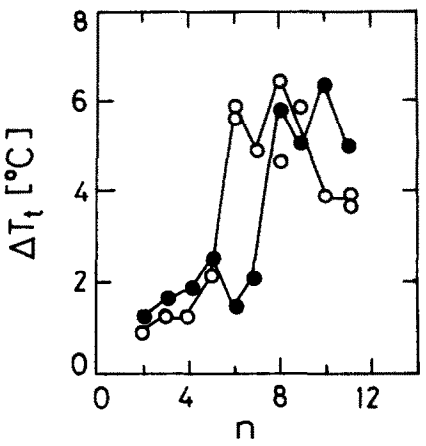

Fig.4. Hysteresis of the main transition temperatures obtained from scans in the heating and cooling mode (ΔT_t) plotted against n. (○) liposomes; (●) vesicles.

a factor of ~4, in the case of liposomes between $n = 5$ and $n = 6$ and for vesicles between $n = 7$ and $n = 8$, as compared to $2 \leq n \leq 5$.

Several conclusions can be drawn from the experiments:

1. The presence of DPH at a molar ratio dye:lipid of 1:750 perturbs the lipid structure, as judged from the significant deviations from the main transition temperatures obtained by DSC in the absence of DPH [3]. Similar results have been reported [9] for DPL:DPH ratios of 500:1, while for certain sphingomyelins even at lipid:dye ratios of 5000:1 structural perturbances were detected crystallographically (Y. Barenholz, private communication). The degree of perturbation depends for the synthetic DPL analogs investigated on the headgroup conformation and is more pronounced when measuring in the cooling mode. The extent of perturbance of the same bilayer organization induced by different optical probes, as inferred from their effect on T_t is: DPH < NPN < MC 540.
2. The degree of cooperativity was shown to be related to the width of the transition region [11,12]. Our results seem to indicate that the introduction of additional methyl-groups into the polar headgroup of DPL might cause conformational changes in the gross lipid body. The variations, both in ΔT and ΔT_t, hint to changes both in the packing and in the size of the cooperative units [11–13]. However, the occurrence of these effects only in the presence of the different probes strongly suggests that additional effects have to be considered when determining 'cooperative units' with the aid of external probes. The actual size of the cooperative units and their packing depend very much on the degree of perturbation introduced by different experimental approaches [9].
3. The pronounced hysteresis effects observed with DPH were not found in [1,3,4]. Re-examination of these data shows that the hysteresis (ΔT_t) in the DSC measurements is equal for all DPL-analogs ($\Delta T_t = 1.3 \pm 0.3$°C). The data from the absorption spectroscopy of MC 540 indicate nearly a doubling of the hysteresis to $\Delta T_t = 2.5 \pm 1.2$°C. Introducing a hydrophobic probe, however, seems to increase the hysteresis drastically. For NPN there appears to be a slight dependence on the number n of $(CH_2)_n$ groups in the polar head. The mean value for PN 3–11 is found to be $\Delta T_t = 4.0 \pm 0.9$°C. When using DPH as external probe, both for liposomes and vesicles, the average hysteresis for small values of n ($n = 3$–5) of $\Delta T_t = 1.4 \pm 0.5$°C is not significantly different from the one of pure DPL. Above a certain critical headgroup length/conformation, the hysteresis is increased by a factor of ~4. The average value for liposomes yields $\Delta T_t = 5.1 \pm 1.0$°C (for PN 6–11) and in the case of vesicles it is $\Delta T_t = 5.6 \pm 1.2$°C (PN 8–11). The relevance of hysteresis in biological macromolecules has been discussed [14] in terms of stability of the system. It was shown that such pronounced hysteresis phenomena, like those induced by the presence of hydrophobic membrane probes, are related to the occurrence of structural metastability [15]. Our data confirm that external membrane probes, even at infinite dilution [4], visualize macroscopically detectable changes in cooperativity, packing and stability which occur within the microenvironment of these probes only and that therefore the experimental outcome will vary considerably, depending on the type of probe used and its specific interaction with the system under investigation.

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