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Interaction of *trans*-parinaric acid with phosphatidylcholine bilayers: comparison with the effect of other fluorophores

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The effect of the fluorophore *trans*-parinaric acid on the structure of lipid bilayer was studied and compared with the effect of other 'perturbants'. These include commonly used fluorophores (diphenylhexatriene, heptadecylhydroxycoumarin, *cis*-parinaric acid and two fatty acids, palmitic and oleic acids). Differential scanning calorimetry (DSC) and proton nuclear magnetic resonance techniques were used to evaluate structural changes in the lipid bilayers. The thermodynamic parameters of dipalmitoylphosphatidylcholine multilamellar vesicles obtained from the DSC thermograms suggest that *trans*-parinaric acid differs from the other 'perturbants'. *trans*-Parinaric acid has the most pronounced impact on the T_m, the width ($\Delta T_{1/2}$) and the index of asymmetry of the main gel to liquid crystalline phase transition without any effect on its transition, ΔH. The presence of *trans*-parinaric acid in the lipid bilayer of dimyristoylphosphatidylcholine small unilamellar vesicles influences the chemical shift difference between the choline protons of phosphatidylcholine molecules present in the two leaflets of the vesicle bilayer ($\Delta\delta H$). This suggests that *trans*-parinaric acid affects the head group packing in the bilayer. Its main effect is abolishing the major alterations in head group packing that occur through the phase transition. The above data indicate that *trans*-parinaric acid is concentrated in the gel phase domains, whereby it stabilizes the phase separation between the gel and liquid crystalline phases, probably by affecting lipid molecules present in the boundary regions between these two domain types.

Introduction

trans-Parinaric acid (all-*trans*-9,11,13,15-octadecatetraenoic acid) is a powerful probe used in physical characterization of lipid bilayers, biological membranes and lipoproteins [1–8]. The main advantage of this fluorophore is its preferential partition into gel-phase domains. This, together with the major increase in quantum yield of *trans*-parinaric acid present in the gel phase, gives *trans*-parinaric acid an advantage over most other

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Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance spectroscopy; T_m, temperature (°C) of maximum change in the specific heat capacity; ΔH, the enthalpic change during a phase transition (kcal/mol); $\Delta T_{1/2}$, the width (Cdeg) of the endotherm at half height at T_m.

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physical methods in monitoring phase separation [4,8]. Most other fluorophores have a gel/liquid crystalline partition coefficient equal to or smaller than 1.0 [9–11]. However, due to its high affinity for the more ordered domains of the system this probe becomes a real component of this region and may affect its physical properties. This problem is of special importance since the information monitored by the fluorophore may be misleading. This fluorophore has relatively low quantum yields, therefore higher fluorophore concentrations (1 : 200–1 : 50 fluorophore-to-liquid ratio) are required for most measurements. The purpose of this work is to characterize interactions and effects of *trans*-parinaric acid on lipid bilayers.

Materials and Methods

Materials

DPPC, DMPC and DSPC were purchased from Avanti, Birmingham, AL, U.S.A. and used without further purification. Based on analysis by thin layer chromatography (0.5–1 mg load per 2 cm) [12,13], these lipids are more than 99% pure. 1,6-Diphenyl-1,3,5-hexatriene, 4-heptadecyl-7-hydroxycoumarin, *cis*-parinaric acid and *trans*-parinaric acid were purchased from Molecular Probes. Palmitic acid and oleic acid (Sigma, St. Louis, MO, U.S.A.; grade 1) were more than 99% pure. All other reagents were of analytical or spectral grade.

Methods

Large multilamellar vesicles were prepared as follows: 10 μmol of the desired phospholipid (or phospholipid mixture) in chloroform was placed in a round-bottommed flask. The desired amount of any one of the above fluorophores or fatty acids in chloroform was added. The solvent was removed at room temperature by flash evaporator under reduced pressure. The sample was then 'frozen' in an ethanol bath and the mixture lyophilized for at least 4 h to ensure complete removal of the organic solvents. 50 mM KCl at 5–10Cdeg above T_m was then added and the lipid was dispersed at this temperature range using a vortex cyclomixer under N_2 atmosphere. Small unilamellar vesicles were prepared for NMR experiments as described by Barenholz et al. [14]. Absorbance and fluorescence measurements of liposomes containing *trans*-parinaric acid solubilized by isopropanol or ethanol show that no major changes in *trans*-parinaric acid spectral parameters, including spectra shape, molar absorption and fluorescence intensity, occur. Therefore the possibility of major degradation and/or peroxidation can be ruled out.

Scanning calorimeter

The custom built differential scanning calorimeter used in these experiments was originally designed only for heating scans [13,15]. Recently a PDP 11/10 computer was interfaced to the calorimeter. The addition of computer control has made it possible to use this 'heating' calorimeter for precisely controlled cooling scans as well. In the cooling mode the heater of the adiabatic shield is turned off and cooling is initiated. The rate of cooling is then controlled by adjusting the sink heater to 'buck' the cooling sufficiently to maintain a constant rate of cooling. It is to be noted that the maximal cooling rate (−9 Cdeg/h) is limited by the capacity of the cooling coils. All experiments in the heating mode were performed at a scanning rate of 15 Cdeg/h.

The apparent heat capacity curves reported in this paper have not been corrected for the time response of the instrument. Therefore, all values of cooperative unit and transition width are apparent. This correction only provides for a true shape of the heat capacity curve and does not influence the measured enthalpy change (ΔH) and melting temperature (T_m) of the transition. All measurements were done under identical experimental conditions and the thermograms were found to be very reproducible (S.D. of less than $\pm 4\%$). Each sample of multilamellar vesicles was scanned at least four times, twice 6–12 h after their preparation and then twice 7 days after preparation; during the 1-week interim period, the multilamellar vesicles were kept under argon in the dark at 4°C.

Proton NMR measurements

Proton NMR measurements were performed by using a Bruker WH 300 pulse Fourier transform spectrometer. The desired temperature (± 0.5°C) was obtained by using the Bruker temperature

control unit. The instrument was locked on the ^2H_2O reference. Typical conditions were a spectral width of 3.2 kHz with 4 K data points in the frequency domain. Excellent signal-to-noise ratios were obtained for 10 mM phospholipids after 80–150 transients were averaged by using a recycle time of 2 s (identical spectra were obtained by using a recycle time of 5 s but not after 1 s). A pulse width of 1.5 μs was used. The relative number of protons was determined from the area of the separated resonances by integral height and by planimetry using the Apple II graphic tablet system.

Results and Discussion

Proton NMR spectroscopy

Proton NMR was used as a sensitive means to detect the effect of *trans*-parinaric acid on the packing of the phospholipid head groups in the lipid bilayer of small unilamellar vesicles. For convenience we used DMPC ($T_m = 20\,°C$ [12]). As discussed later, *trans*-parinaric acid was found to have very similar effects on the thermotropic behavior of DPPC and DMPC multilamellar vesicles. The DMPC small unilamellar vesicles were prepared as described in the Methods section. The effect on the head group packing was assessed by comparing the effect of *trans*-parinaric acid, diphenylhexatriene and 4-heptadecyl-7-hydroxycoumarin on the difference in chemical shift ($\Delta\delta H$) between inside and outside choline signals as described previously [10,16]. Due to the high resolution power of the Bruker 300-MHz NMR spectrometer no shift reagent was required to obtain the shift difference. In all cases $\Delta\delta H$ was found to be dependent on the temperature. Fig. 1 shows representative NMR spectra for the choline ($N^+(CH_3)_3$) protons of DMPC small unilamellar vesicles (Fig. 1A) and small unilamellar vesicles of DMPC containing 1 mol% fluorophore (diphenylhexatriene, Fig. 1B; heptadecylhydroxycoumarin, Fig. 1C; *trans*-parinaric acid, Fig. 1D).

Eigenberg and Chan [16] have proposed that the chemical shift difference between the inner and outer leaflets ($\Delta\delta H$) is due to differences in head group packing density in the two leaflets. From the geometrical consideration of the organization of these two leaflets [17,18] it was clear that

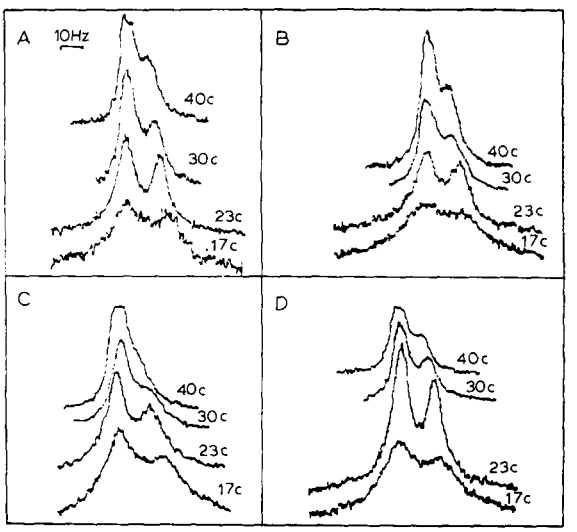

Fig. 1. The effect of the fluorophores on 300 MHz NMR spectra of $-N^+(CH_3)_3$ (choline) protons of DMPC small unilamellar vesicles. The small unilamellar vesicles were prepared as described in Materials and Methods. The NMR spectra were recorded at the specified temperatures. (A) No fluorophore; (B) 1 mol% diphenylhexatriene; (C) 1 mol% heptadecylhydroxycoumarin; (D) 1 mol% *trans*-parinaric acid. For more details see Materials and Methods.

monitoring the differential between the properties of the two leaflets is a very sensitive tool to determine change in head group packing density. $\Delta\delta H$ for small unilamellar vesicles is temperature-dependent, especially at the phase transition range (Fig. 2). An even more pronounced effect of temperature was found for DMPC-heptadecylhydroxycoumarin small unilamellar vesicles [10]. However, in the presence of *trans*-parinaric acid, $\Delta\delta H$ monotonically increases with decreasing temperature without any expression of DMPC phase transition, which is well expressed in the line width of the methylene protons (data not shown). Diphenylhexatriene has a similar though smaller influence than *trans*-parinaric acid in suppressing the effect of the phase transition on the measured $\Delta\delta H$. The effect of the fluorophore on the vesicle size was ruled out on the basis of the inside/outside choline proton signal intensity ratio [10,16]. These DMPC small unilamellar vesicles were small and seemed to reach their minimal size with larger curvature in the inner than the outer leaflet, which explains the $\Delta\delta H$ between the inner and outer choline protons [16]. This suggests that the probes which have

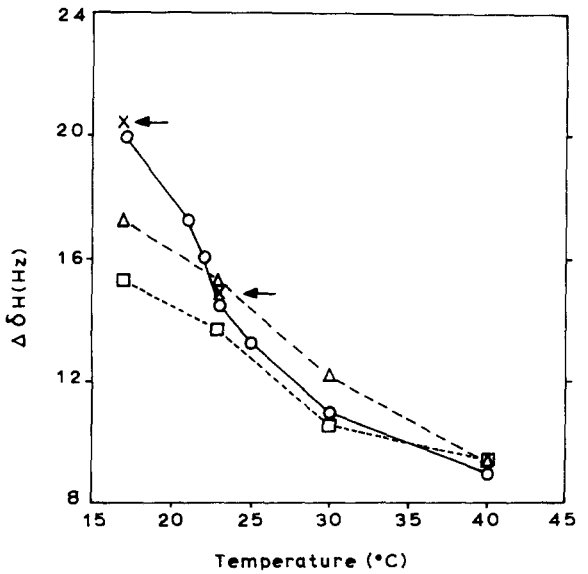

Fig. 2. The effect of fluorophores on the temperature-dependent inside/outside separation of DMPC small unilamellar vesicles choline proton signals ($\Delta\delta H$). O———O, DMPC small unilamellar vesicles without fluorophore (control); □-----□, 1 mol% diphenylhexatriene; x̄, 1% heptadecylhydroxycoumarin (at temperature above the phase transition, $\Delta\delta H = 0$); △– – –△, 1 mol% *trans*-parinaric acid. For more details see Fig. 1.

their fluorophore resides in the hydrophobic region of the lipid bilayer (diphenylhexatriene and *trans*-parinaric acid [5,4]) also perturb the head group region. The main effect of *trans*-parinaric acid is on the phase transition range, making the packing density of the choline protons of inner and outer layer more alike.

Differential scanning calorimeter

Table I shows that diphenylhexatriene, heptadecylhydroxycoumarin and *cis*-parinaric acid have minimal effects on the parameters which characterize the thermotropic behavior of DPPC multilamellar vesicles. T_m, ΔH and the shape of the main endotherm were almost identical to those of pure DPPC multilamellar vesicles and were unchanged even after 1 week at 4°C or at room temperature. Minor, though clear, effects were observed on the pretransition, which was slightly broadened by the presence of these probes in the DPPC bilayer. At 5 mol% of *cis*-parinaric acid in the T_m of the main transition was reduced by about 0.5 Cdeg with only a relatively minimal effect on the ΔH of the main endotherm. The slight lowering of the T_m by *cis*-parinaric acid and the fact that the endotherm shows slight broadening at its lower temperature side support the *cis*-parinaric acid preference for liquid crystalline domains. This result would not be expected if one assumes ideal mixing of the compounds, since *cis*-parinaric acid has a much higher melting point (85–86°C) than DPPC [1]. Palmitic (melting point 64°C) and oleic (melting point 14°C) acids at a concentration of 2 mol% were used as a control to assess the effect of long chain fatty acids on the thermotropic behavior of DPPC multilamellar vesicles. As expected, the two fatty acids differ in their effect. Oleic acid has almost no effect on the main transition or the pretransition. Palmitic acid, on the other hand, affects all the parameters of the DPPC thermotropic transition. The T_m of the pretransition and the main transition were raised by 1.8 and 0.5 Cdeg, respectively, and the endotherm was broadened as expressed in the change in $\Delta T_{1/2}$ (Table I). These data agree well with those previously described by Schullery et al. [19].

The effect of *trans*-parinaric acid (melting point 95–96°C [1]) on the thermotropic behavior of DPPC multilamellar vesicles differs from those observed for all other 'perturbants' tested by us (Table I). Representative thermograms for multilamellar vesicles composed of mixtures of DPPC and *trans*-parinaric acid (0–5 mol%) shown in Fig. 3 demonstrate the features of the main gel to liquid crystalline phase transition and the pretransition which are altered by *trans*-parinaric acid.

Fig. 4 shows that the T_m of the main transition is elevated with increasing mol% of *trans*-parinaric acid in a linear fashion, while the enthalpic change (ΔH) of the transition is unaffected. Fig. 5 shows similar effects of *trans*-parinaric acid on the pretransition. The T_m of the pretransition is elevated with increasing levels of *trans*-parinaric acid, but the effect is smaller than that observed for the main transition. ΔH of the pretransition is unaffected by the presence of *trans*-parinaric acid in the DPPC bilayer.

It is difficult to interpret changes in the characteristics of the pretransition due to the fact that this is a very slow process [20]. Thus, only dramatic changes in the thermodynamic parameters of the pretransition may influence the shape of the ap-

TABLE I

THE EFFECT OF FLUOROPHORES ON THERMOTROPIC BEHAVIOR OF DIPALMITOYLPHOSPHATIDYLCHOLINE MULTILAMELLAR VESICLES

The values obtained for 1-week-old multilamellar vesicles (see text) are given in parentheses. The maximal S.D. are ±0.05 °C; ±0.15 °C and ±0.2 kcal/mol for the pretransition, main transition and ΔH, respectively. a = asymmetric endotherms (see text).

System of multilamellar vesicles	Pretransition	Main transition		
	T_m (°C)	T_m (°C)	ΔH (kcal/mol)	$\Delta T_{1/2}$ (Cdeg)
DPPC – plain	35.50	41.69	8.46	0.6
	(35.70)	(41.74)	(8.51)	(0.6)
DPPC + 1.5 mol% diphenylhexatriene	35.50	41.74	8.28	0.75
	(35.50)	(41.80)	(8.21)	(0.71)
DPPC + 1.5 mol% heptadecylhydroxycoumarin	35.39	41.76	8.43	0.65
	(35.44)	(41.77)	(8.31)	(0.60)
DPPC + 2 mol% palmitic acid	37.26	42.23	8.48	0.845
	(37.37)	(42.27)	(8.48)	(0.845)
DPPC + 2 mol% oleic acid	35.93	41.57	8.8	–
	(36.00)	(41.50)	–	–
DPPC 1.5 mol% *cis*-parinaric acid	35.55	41.78	(8.56)	(0.6)
DPPC + 5 mol% *cis*-parinaric acid		41.12		0.75
		(41.39)		(0.75)
DPPC + 0.25 mol% *trans*-parinaric acid	35.53	41.74	8.6	a
DPPC + 0.5 mol% *trans*-parinaric acid	35.55	41.81	8.8	a
DPPC + 1.0 mol% *trans*-parinaric acid	35.67	41.81	8.4	a
DPPC + 2.0 mol% *trans*-parinaric acid	35.72	41.98	8.8	a
DPPC + 5.0 mol% *trans*-parinaric acid	35.96	42.36	8.2	a
	(36.03)	(42.39)	(8.4)	a
DMPC – plain	–	23.92	5.49	
DMPC + 2 mol% *trans*-parinaric acid	–	24.31	5.60	a

parent heat capacity curve. Also, it is likely that *trans*-parinaric acid molecules are evenly distributed in the bilayer plane, since all DPPC molecules are evenly distributed in the gel phase even during the pretransition. Thus it is expected that *trans*-parinaric acid will have a relatively minor effect on the pretransition. The fact that *trans*-parinaric acid affects the shape of this endotherm speaks against its complete phase separation, although a partial phase separation cannot be ruled out.

Fig. 6 describes the broadening of the endotherms of the main transition and pretransition by *trans*-parinaric acid expressed as the apparent $\Delta T_{1/2}$. It is clear that *trans*-parinaric acid has a major influence on $\Delta T_{1/2}$ of the main transition with a much smaller effect on the pretransition half width. Most of the broadening effect is found in the extent of a high temperature asymmetry of the main transition endotherm (Fig. 3).

An index of asymmetry can be estimated by dividing the endotherm into two parts, one above and the one below the T_m, Since ΔH of the main transition remains unaltered (8.5 ± 0.2 kcal/mol), and by relating the calculated ratio of the above areas to that obtained for pure DPPC multilamellar vesicles, the ratio of DPPC molecules melting (in the heating mode) above and below T_m can be

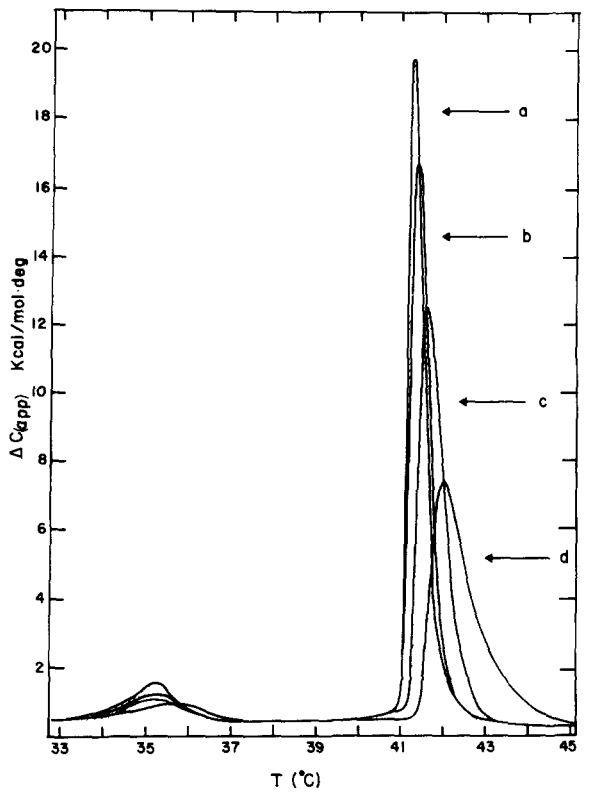

Fig. 3. Thermograms of DPPC multilamellar vesicles containing various mol% of *trans*-parinaric acid. All multilamellar vesicles were prepared in 50 mM KCl as described in Materials and Methods. (a) No *trans*-parinaric acid; (b) 0.5 mol% *trans*-parinaric acid; (c) 2 mol% *trans*-parinaric acid; (d) 5 mol% *trans*-parinaric acid. $C_{(app)}$ = apparent Cp. For more details see Methods and text.

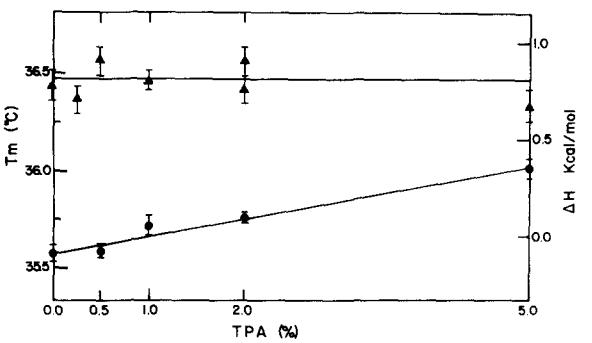

Fig. 5. The effect of *trans*-parinaric acid (TPA) on T_m (°C; ●) and ΔH (kcal/mol; ▲) of DPPC multilamellar vesicles pretransition. For more details see Fig. 3.

used as an index of asymmetry. This asymmetry index was found to be 1.14 ± 0.02, 1.23 ± 0.02 and 2.10 ± 0.02 for 0.5, 1.0 and 5.0 mol% *trans*-parinaric acid, respectively. The fact that T_m is elevated as a linear function of mol% *trans*-parinaric acid (Fig. 4) and the apparent asymmetry of the endotherm increases (Fig. 3) indicates that *trans*-parinaric acid is preferentially soluble in the gel phase. This result is consistent with previous results [4]. Heating scans were also compared to cooling scans using a scanning rate of 5 Cdeg/h for both, and the results using multilamellar vesicles of pure DPPC and of DPPC containing 2 mol% *trans*-parinaric acid are shown in Fig. 7, where the broadening effect as well as the induction of asymmetry by *trans*-parinaric acid on both cooling and heating scans without any apparent hysteresis can be observed.

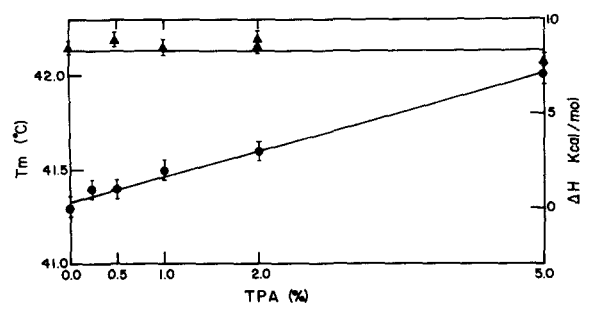

Fig. 4. The effect of *trans*-parinaric acid (TPA) on T_m (°C; ●) and ΔH (kcal/mol; ▲) of the main DPPC multilamellar vesicles gel to liquid crystalline phase transition. For more details see Fig. 3.

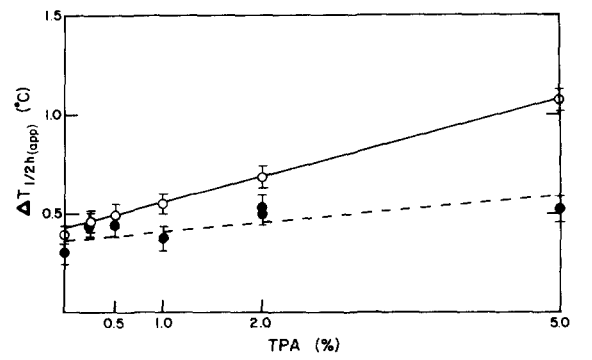

Fig. 6. The effect of *trans*-parinaric acid (TPA) on the width of the endotherm at half height at T_m for the main transition (○) and the pretransition (●). For more details see Fig. 3.

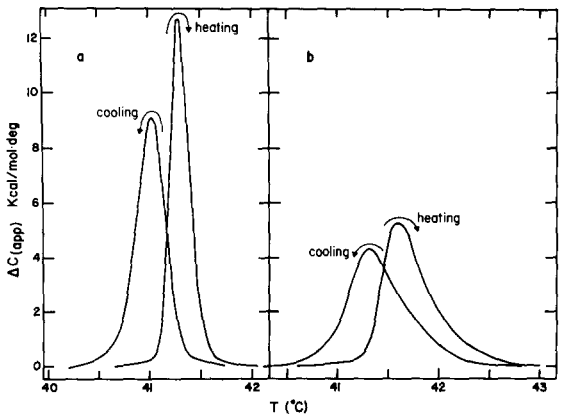

Fig. 7. Comparison between cooling and heating DSC scans for plain DPPC multilamellar vesicles (a), and for DPPC multilamellar vesicles containing 2 mol% *trans*-parinaric acid (b). For more details see Materials and Methods.

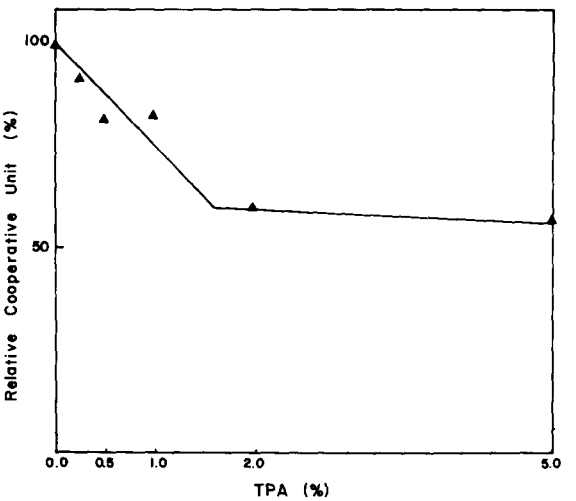

Fig. 8. Relative cooperativity (N) calculated as described by Mabrey and Sturtevant [21]. N for plain DPPC multilamellar vesicles is described as 100 (see Methods and text).

The apparent cooperativity of the transition was calculated according to Mabrey and Sturtevant [21]:

$$N = \frac{RT_m^2(Cp_{max})}{\Delta H}$$

where N is the average number of molecules per cooperative unit, R is the gas constant, T_m is the temperature of maximum change in the specific heat capacity (Cp). Cp_{max} is the Cp at the T_m. ΔH is the calorimetrically determined enthalpy change for the phase transition. N for the pure DPPC multilamellar vesicles is described as 100 (apparent value – see Methods). *trans*-Parinaric acid reduces the cooperative unit but the relationship between N and mol% *trans*-parinaric acid is not linear (Fig. 8). It is of interest that while a concentration of *trans*-parinaric acid greater than 2% causes a further increase in T_m, it has no measurable effect on N. The meaning of this observation is not clear.

The extent of overlapping between endotherms obtained in the absence and presence of *trans*-parinaric acid (Fig. 3) can be used as another way of determining how many DPPC molecules are affected by the presence of *trans*-parinaric acid and how this is related to the level of *trans*-parinaric acid. Using a computerized graphic tablet we found that most of the *trans*-parinaric acid effect occurs between 0 and 2 mol%. These data agree well with the effect of *trans*-parinaric acid on the apparent cooperative unit (N) (Fig. 8). These two lines of experimental approach suggest that most of the DPPC molecules in the bilayer are already affected by the presence of 2 mol% *trans*-parinaric acid in the bilayer.

A recently designed pressure perturbation calorimeter [22] can be used to determine the average rate of gel to liquid crystalline phase transition. For DPPC multilamellar vesicles this rate is in the order of 1 s^{-1}. The rate of transition can affect the shape of the endotherm as measured by the high sensitivity differential scanning calorimeter. For example, if *trans*-parinaric acid reduces the rate of transition, the heat capacity curve would broaden. However, it was found that 2 mol% *trans*-parinaric acid did not have any measurable effect on the transition rate of DMPC multilamellar vesicles, while its effect on the thermotropic behavior of DMPC as evaluated by a high sensitivity differential scanning calorimeter was almost identical to the DPPC-*trans*-parinaric acid system (see Table I). Thus the observed changes induced by *trans*-parinaric acid do not appear to be due to changes in kinetics of the transition. Rather, all the effects of *trans*-parinaric acid on the main transition appear to be thermodynamic in nature. The maximization of the *trans*-parinaric acid effect on N and the shape of

the endotherms at 2 mol% reflects the ability to stabilize the lipid molecules present in the boundary regions between the gel and liquid crystalline domains. Thus its influence is amplified compared to its effect being due to a one-to-one *trans*-parinaric acid-DPPC interaction. In this respect *trans*-parinaric acid resembles local anesthetics. It should be noted that *trans*-parinaric acid causes high temperature broadening whereas anesthetics, which reduce the T_m, cause low temperature broadening. The cause of this asymmetrical broadening in either case is not fully understood, but it appears to be correlated to whether the perturbant preferentially stabilized the gel (*trans*-parinaric acid) or liquid crystalline phase [23].

Preliminary data on the effect of *trans*-parinaric acid on the melting behavior of mixed systems, such as multilamellar vesicles made of DMPC/DSPC mixtures, indicate that, at temperatures between the T_m of the two components, *trans*-parinaric acid interacts mainly with the DSPC. This, together with previous data [8], suggests that differences in T_m rather than in chain length will determine *trans*-parinaric acid distribution in the bilayer plane.

In conclusion, *trans*-parinaric acid can be used as a specific stabilizer of phospholipid molecules in the gel phase. It can also be used as an excellent probe to monitor phase separation in lipid bilayers and biological membranes. However, one must be careful with the interpretation of the melting profile of the lipid molecules and the properties of the gel state domains using *trans*-parinaric acid as the probe.

Acknowledgement

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