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Dynamic fluorescence measurements on the main phase transition of dipalmytoylphosphatidylcholine vesicles

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Abstract. The kinetics of the main phase transition in dipalmytoylphosphatidylcholine (DPPC) vesicles have been investigated using our iodine laser-T-jump technique with fluorescence detection. A set of three fluorescent probes has been used to sense different parts of the bilayer hydrocarbon chain region. The well established membrane probes DPH and TMADPH as well as DPHPC, a labelled DPPC molecule. We report three relaxation signals in the μs and ms time range, which are detected with all three probes. This result supports our model of the main phase transition in DPPC vesicles.

Key words: Kinetics, laser temperature jump, fluorescence, phospholipid-bilayers, DPH analogue

Introduction

Fluorescence spectroscopic techniques are an important tool in the study of the thermotropic behaviour of lipid bilayers and biological membranes.

The label molecule most widely used for these studies is DPH, introduced to the literature by Shinitzky et al. (1971). A charged analogue, TMADPH, was introduced (Prendergast et al. 1981) as a probe which is more specificly located in the bilayer, because the positively charged trimethylamino group is anchored in the head-group region of the lipids (see Fig. 2). Recently a labelled lipid, DPHPC, was described (Morgan et al. 1982; Cranney et al. 1983), in which the DPH chromophore partly replaces one fatty acid chain (Fig. 1).

The probe's motion is described by the gaussian cone angle model (Jähnig 1979; Heyn 1979; Lipari and Szabo 1979; Blitterswijk et al. 1981). According to this theory the probe molecules "wobble" around their position normal to the bilayer surface, with a characteristic time, τ_c. The angle-distribution is described by a gauss profile with cone angle θ as the characteristic parameter. In the fluid state of the bilayer θ is bigger than in the crystalline state. Values for θ are given in Engel and Prendergast (1981).

The probe's motion causes a depolarisation of the fluorescent light. The anisotropy

$$r(t) = (I_{\parallel}(t) - I_{\perp}(t))/(I_{\parallel}(t) + 2I_{\perp}(t)) \quad (1)$$

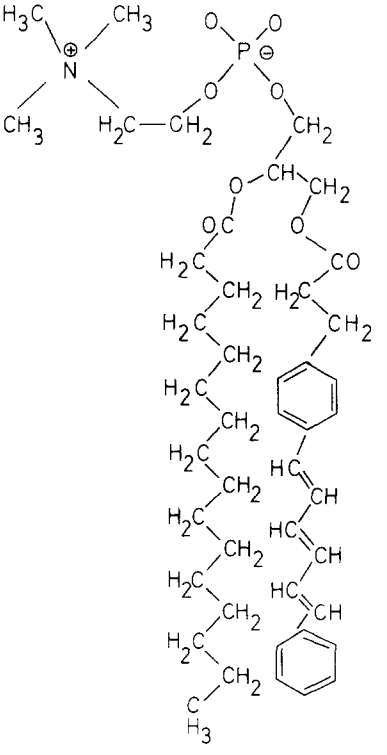

Fig. 1. Schematic bond structure of the DPHPC molecule

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Abbreviations: DMPC: Dimyristoylphosphatidylcholine; DPPC: Dipalmytoylphosphatidylcholine; DPH: 1,6-Diphenylhexa-1,3,5-triene; TMADPH: 1-[4-(Trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene; DPHPC: Diphenylhexatriene-phosphatidylcholine; T_m: Temperature of the main phase transition

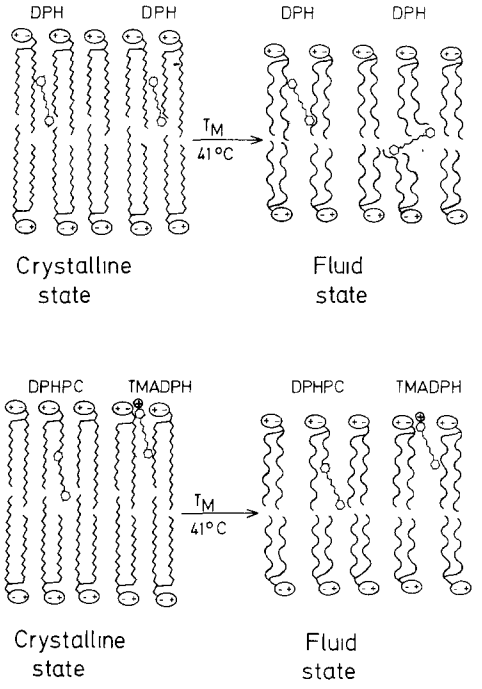


Fig. 2. Motion of the fluorescent labels inside the DPPC bilayer, according to the gaussian cone angle model. Recently Ameloot et al. (1984) were able to show that the probability is high that DPH lies between the two monolayers if the lipid is in the fluid phase

decays to the limiting value r_∞, according to the equation

$$r(t) = r_\infty + (r_0 - r_\infty) \exp(-t/\tau_c). \quad (2)$$

The decay time provides information about the probes local environment on a ns time-scale. Structural and dynamic contributions can be separated (Jähnig 1979). Experiments have been done for DPH in lipid bilayers and biological membranes (Lakowicz et al. 1979; Kinosita et al. 1981).

In the last decade measurements of the steady state anisotropy,

$$r_{ss} = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp) \quad (3)$$

have been carried out to investigate the main phase transition in phospholipd bilayers and biological membranes (Shinitzky and Barenholz 1978; Prendergast et al. 1981; Kinosita et al. 1981).

These measurements give no information about the transition kinetics. This is the reason why we have focused our attention mainly on relaxation experiments. With measurements from ns to ms using turbidity as the detection parameter we were able to demonstrate (Holzwarth et al. 1985) that three quarters of the total relaxation amplitude falls in the μs to ms time range. From an energetic point of view we therefore believe that these are important dynamic phenomena as far as lipid-lipid and lipid-protein interactions in biological membranes are concerned. For the relaxation experiments reported here we used an iodine laser-T-jump apparatus (Holzwarth 1979; Holzwarth et al. 1985). This technique and its advantages over the Joule heating arrangements are explained by Holzwarth et al. (1985).

It has been reported (Zimmermann et al. 1974; Shepard and Büldt 1978) that electric fields higher than $10\,kV \cdot cm^{-1}$, as they occur in Joule heating experiments, cause rupture of the phospholipid bilayer. This effect is completely avoided in our laser-T-jump measurements. The only Joule heating study using DPH as a probe molecule was done by Gruenewald (1982). For DMPC vesicles he reported two relaxation signals in the ms time range. In a preliminary study (Genz and Holzwarth (1985)) we used DPH and TMADPH to study the DMPC-bilayer with the iodine laser-T-jump. In the present work we investigated DPPC vesicles. The detection system has been improved, so that a better signal-to-noise ratio was achieved allowing for a lower probe/lipid ratio. Also the new DPHPC molecule was now available to us.

Materials and methods

Materials

DPPC and DPH were purchased from Fluka (Switzerland), TMADPH was from Molecular Probes (Texas) and DPHPC was from E. Thomas (University of Salford). The buffer solution (pH = 7.5) consisted of 0.01 *M Tris* (hydroxy-methyl) aminomethane (Fluka), 0.1 *M* NaCl (Merck, Germany) and 1 m*M* NaN_3 (Merck) in triply distilled water. All chemicals were of the highest purity available and were used without further purification.

Vesicle preparation

The vesicles were prepared by the "modified injection method" (Kremer et al. 1977). 30 μ*M* of DPPC were dissolved in 1 ml ethanol and slowly injected (20 min) with a Hamilton (Switzerland) syringe into 10 ml of pure buffer at 54 °C, and then dialyzed above T_m against pure buffer for at least 8 h. Vesicles were characterized by electron microscopy, laser light scattering and turbidity transition curves.

Fluorescence labelling

A few μl of DPH or TMADPH in methanol were added to the vesicle suspension giving a probe/lipid

ratio of 1/400. Incubation time was 45 min at a temperature of 65 °C to remove methanol. DPHPC was injected together with DPPC (ratio 1/400). The incorporation of the dyes was characterized by absorption and fluorescence spectra. Turbidity transition curves showed none of the effects of the probes reported for probe/lipid ratios greater than 1/250 (Genz and Holzwarth 1985). The suspensions were used immediately after preparation (stability was at least 2 days) and checked before and after the T-jump exeriments by absorption spectroscopy and turbidity transition curves on a Perkin Elmer 555 UV-Vis spectrometer; no changes could be detected.

Steady-state anisotropy measurements

These were done in the Max-Planck-Institute for Molecular Genetics (Berlin) on a SLM 8000 spectrometer. The intensities $I_{\parallel}$ and $I_{\perp}$ were calibrated, background noise (2%) was determined with pure DPPC vesicles. Scan-speed (Haake PG 20 control unit) was 0.25 K/min and the temperature was checked with a Ni-Cr-Ni thermoelement in control experiments.

Laser-T-jump experiments

Our iodine laser system (Holzwarth et al. 1977, 1985; Holzwarth 1979) was used in the oscillator mode with 2.4 µs relaxation time producing temperature jumps of 1 K in 150 µl heated volume. Details of the detection system are shown in Fig. 3.

Excitation light and the laser beam are in coaxial arrangement, this ensures optimal use of the T-jump energy because detected and heated volume are then nearly identical. The arrangement is very compact and yields a high light intensity and good anisotropy values nearly the same as those achieved in the steady-state measurements.

The excitation light of a watercooled Hg/Xe arc lamp (PTI, Princeton) is focused into the sample cuvette (Helma, Germany, 5×10 mm, QS) by an elliptical mirror without any lenses. The signals $I_{\parallel}$ and $I_{\perp}$ with respect to the vertical polarisation of the excitation light, (Glan-Thompson Prism from Halle, Berlin), were detected by two EMI 9558 QA multiplier tubes connected with Dialog (Düsseldorf) heads; overall rise time 1 µs. Both channels were calibrated for horizontally polarised excitation light; scattered light was below 5%, checked with vesicles containing no fluorescent dye. The difference signal, $I_{\parallel} - I_{\perp}$, was amplified in a Tektronix 7A22 and digitised as well as recorded by a Biomation 1010 waveform recorder in the dual time base mode. The relaxation signals were computer-fitted on an HP 9845 B. The program allows sampling of the signals to improve the signal-to-noise ratio and enables a wide choice of the fit range. The relaxation times are calculated with their error values.

The laser induce a white light flash more than one order of magnitude larger than the relaxation amplitude which is characterised in Fig. 4; this made signal recording in the first 20 µs impossible due to overshooting of the photomultiplier-amplifier chain. We monitored this light flash to check that the detection line had recovered after 10 µs.

Results and discussion

Equilibrium measurements

The phase transition of DPPC vesicles was characterised by steady-state anisotropy measurements using DPH, TMADPH and DPHPC as probe molecules (Fig. 5 a, b, c).

Such curves have been reported for DPH and TMADPH (Prendergast et al. 1981). A polarisation measurement with DPHPC as probe molecule has been published (Morgan et al. 1982).

We performed measurements with rising and falling temperature using a low scan speed (0.25

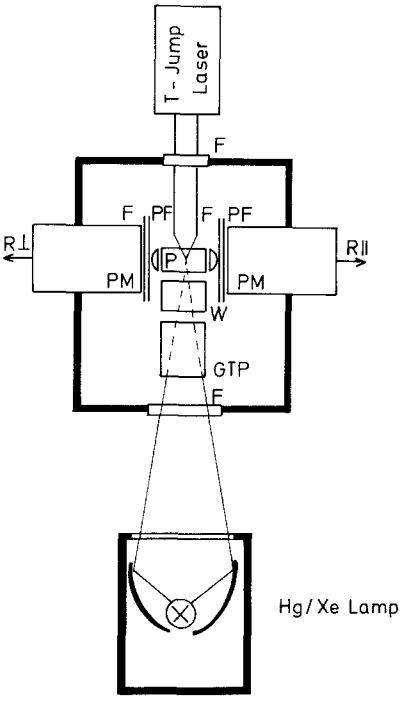


Fig. 3. The fluorescence detection unit. *F* = filters, *GTP* = Glan-Thompson prism, *W* = water filter, *PF* = polarization filters. *PM* = photomultiplier, *R* = registration unit

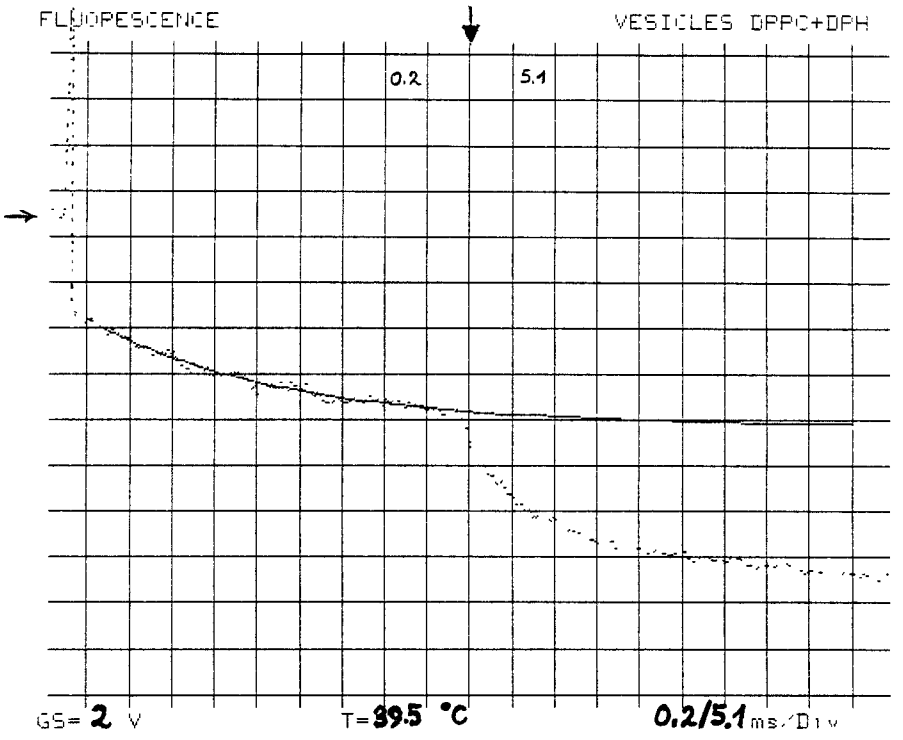


Fig. 4. Relaxation trace induced by the laser beam monitored by the change in fluorescence $(I_{\parallel} - I_{\perp})$ and recorded with a Biomation 1010 waveform recorder

K/min) to see if the vesicles show any hysteresis effect in their phase transition behaviour. None or only very weak hysteresis was detected within the accuracy of the observation. The phase transition curves are asymmetric, this has also been shown for turbidity curves (Holzwarth et al. 1985) and differential scanning calorimetry experiments (Genz, Holzwarth and Tsong to be published) using our preparations. The vesicles prepared by the injection method are approximately a factor of two larger in size, than sonified preparations which show no pre-transition. The T_m of our vesicles (39.7 ± 0.5) °C is lower than the values for large multilamellar aggregates (41.5 °C) which are commonly used for DSC and NMR experiments. This reduction of T_m is due to the curvature of the bilayer and not to impurities; this was checked by thin layer chromatography.

The anisotropy values can be understood by our diagram showing the space available for motion of the fluorescence labels inside the phospholipid bilayer (Fig. 2). TMADPH and DPHPC give higher anisotropy values in the fluid phase than DPH because one end of the probe is either fixed in the headgroup region by electrostatic interaction (TMADPH) or it is chemically bound to the fatty acid chain (DPHPC), while DPH is only limited in its "wobble" motion by the surrounding chains. In the crystalline phase all three probes are strictly limited in their motion by the all-trans conformation of the fatty acid chains; therefore the anisotropy values are nearly equal; only DPH shows slightly lower values (Fig. 5 a, b, c).

Kinetic measurements

The fact that the space available for the probe's "wobble" motion changes during the phase transition can be exploited for dynamic measurements of processes occurring during the phase transition, if these processes are slow compared with the fluorescence lifetime of the probes (5–10 ns, see Lakowicz et al. 1979; Prendergast et al. 1981; Cranney et al. 1983). The fluorescence of the probes changes its polarisation due to the "wobble" motion, which is 10 times faster than the fluorescence lifetime (Kawato et al. 1977). This means that the labels are probing their local environment on a ns time-scale and therefore they can register the time-dependent changes of this environment induced by the laser-T-jump. The rise of temperature, $\Delta T = 0.8 - 1.0$ K, causes a small increase in the cone angle, θ (Fig. 2), which is detected by time-dependent measurements of the difference signal, $I_{\parallel} - I_{\perp}$. $I_{\parallel}$ decreases strongly with increasing temperature in the phase transition region, while $I_{\perp}$ is nearly constant. The increase of $I_{\perp}$ which is due to more space becoming available for motion is compensated by the decrease of the signal intensity caused by a reduction of the fluorescence quantum yield for higher temperatures.

The short time limit of 20 µs for our present dynamic measurements is caused by the overshooting of the detection arrangement (see method) induced by the light flash (Fig. 4). The long time limit lies in the range of several seconds and is caused by cooling of the sample following the

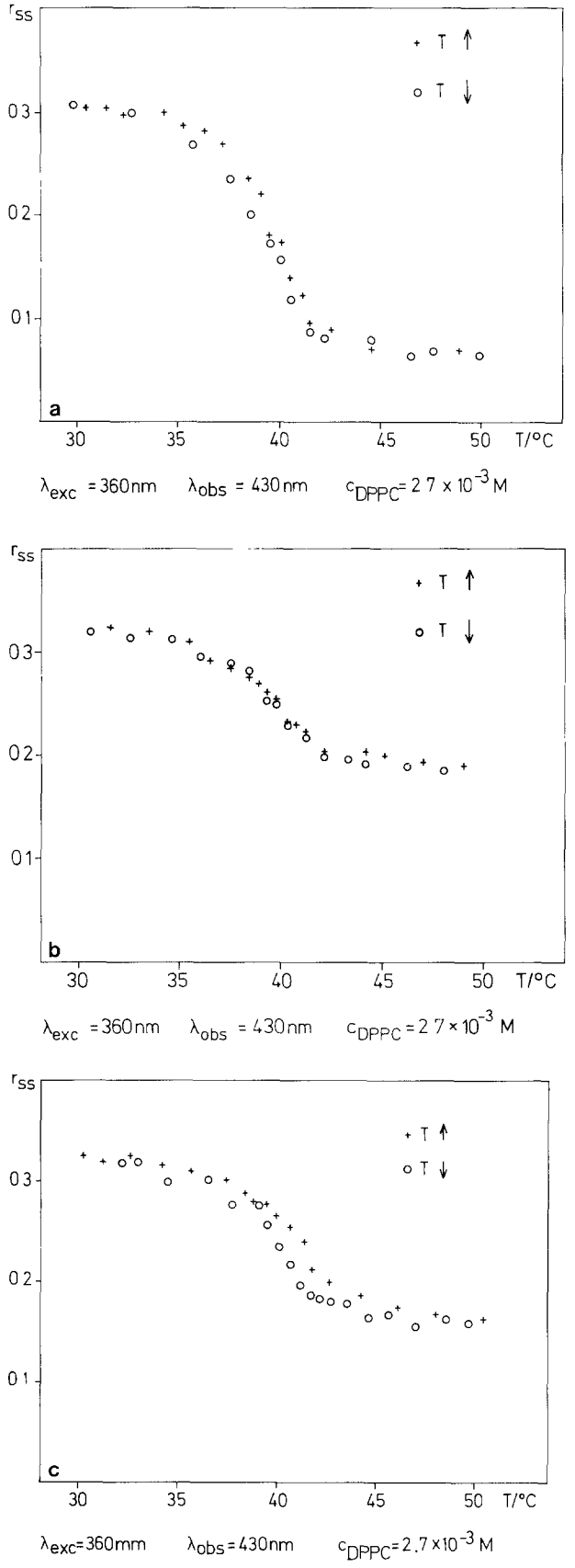
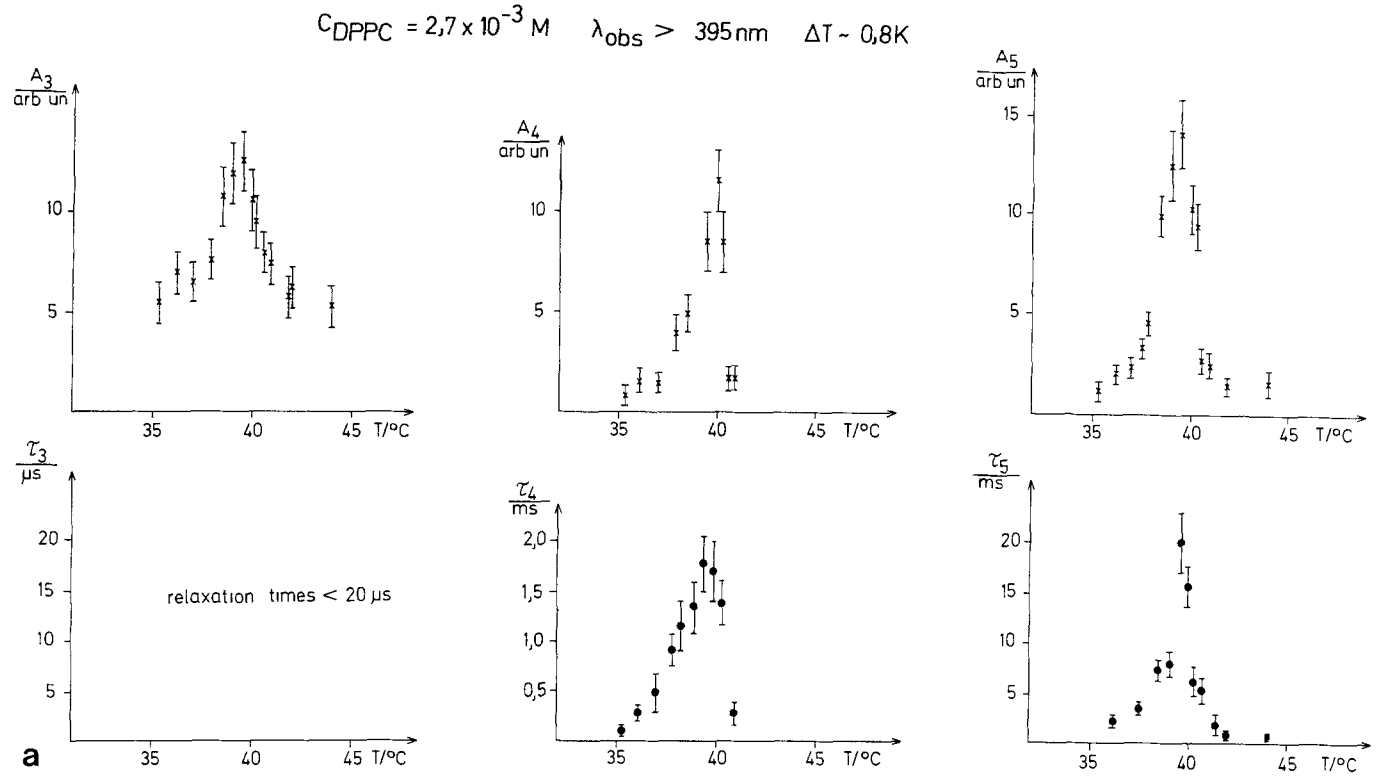


Fig. 5 a – c. Steady-state anisotropy transition curves for DPPC vesicles containing **a** DPH, **b** TMADPH and **c** DPHPC with increasing (+) and decreasing (○) temperatures

temperature jump. We report three relaxation signals in the μs and ms time range. The shortest relaxation could not be time-resolved because it occurs in the 5–20 μs time range, but the relaxation amplitudes were determined. For the two slower signals, which lie in the ms range, relaxation times and amplitudes were measured. Figure 6 a–c show the temperature dependence of these parameters for all three fluorescent probes. All processes are strongly cooperative, they show strong maxima of relaxation times and amplitudes at the phase transition temperature. An attempt has been made to explain this phenomena in the framework of dynamic scaling, a modern phase transition theory (Holzwarth and Rys 1984).

The most interesting result of our kinetic measurements is the fact that all three label molecules, although localized in different parts of the bilayer, show very similar relaxation signals. This means that the dynamic processes underlying the relaxation signals must be due to cooperative changes of the bilayer structure and cannot be explained by single conformational changes of dynamically independent phospholipid molecules. This is underlined by measurements with an absorption probe in the headgroup of the phospholipid (paper in preparation) which shows very similar relaxation signals. These results support our model of the phase transition in DMPC and DPPC vesicles (Holzwarth et al. 1985). Two fast relaxation processes in the ns range, measured with turbidity detection (Holzwarth et al. 1985) are attributable to almost individual changes of single molecules. In a first very fast step (4 ns) simple rotational isomers (kinks) are formed in the hydrocarbon tails. This is followed by a weakly cooperative process around 300 ns which causes a first expansion of the local environment. The next three steps (τ_3, τ_4, τ_5) are highly cooperative. A further expansion (τ_3) of the bilayer is induced by the formation of more complex rotational isomers in the hydrocarbon tails, giving more space to the "wobbling" of the fluorescent probes and causing an expansion in the head group region. The two slowest signals (τ_4 and τ_5) are attributed to the coexistence of domains of different order in the phase transition region. This idea was first introduced by Adam (1968) and Tsong and Kanehisa (1977) and further developed by Mouritsen (1983) and Mouritsen and Zuckermann (1986) using a microscopic interaction model which includes a variable number of lipid-chain conformational states. According to this model, fluid areas coexist with more rigid clusters consisting of a mixture of all-trans isomers together with the intermediate lipids. We attribute our signal 4 to the growth of the fluid areas associated with a decrease associated with a decrease in size of the more



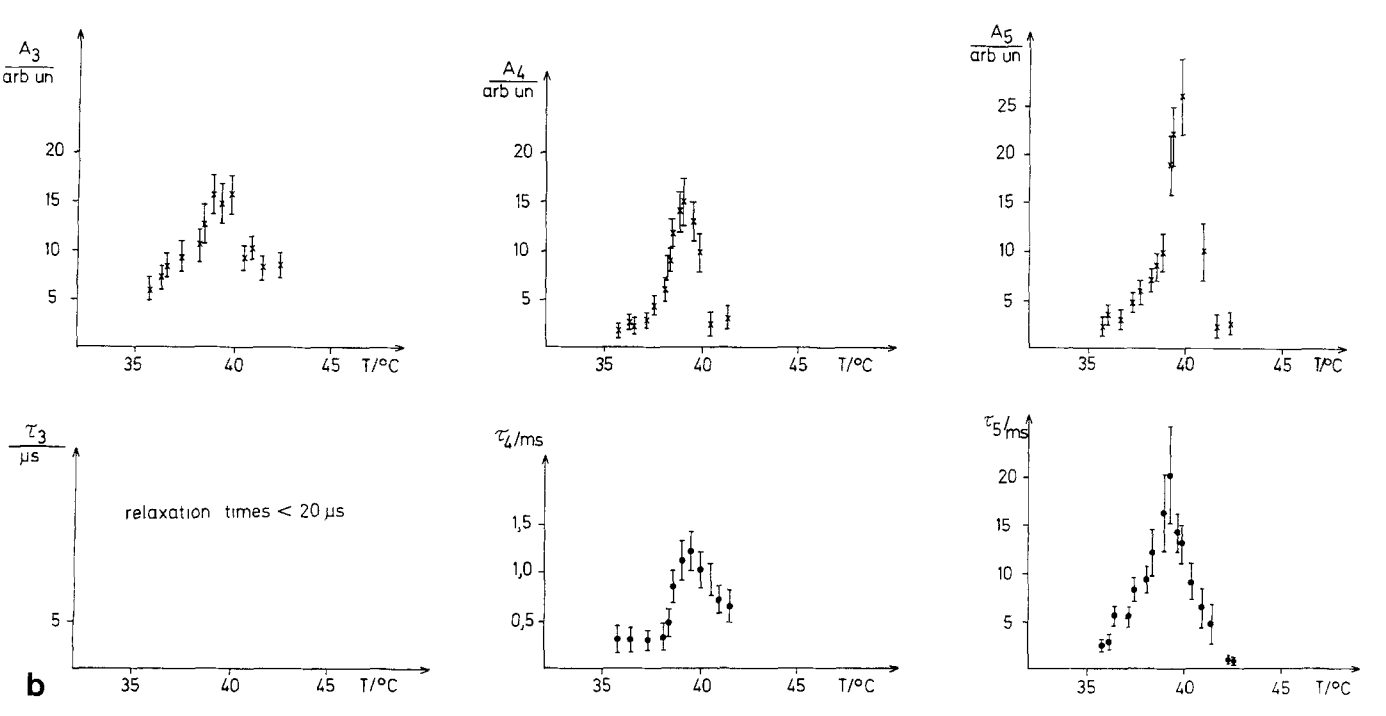


Fig. 6 a – c. Temperature dependence of relaxation times, τ, and their corresponding amplitudes A. Measured as a decrease of the difference signal $I_{\parallel} - I_{\perp}$ for **a** DPH, **b** TMADPH and **c** DPHPC containing vesicles ratio lipid/probe = 400. The presence of maxima in both amplitudes and relaxation times is indicative of cooperativity. Values are given for the temperatures where the T-jump started

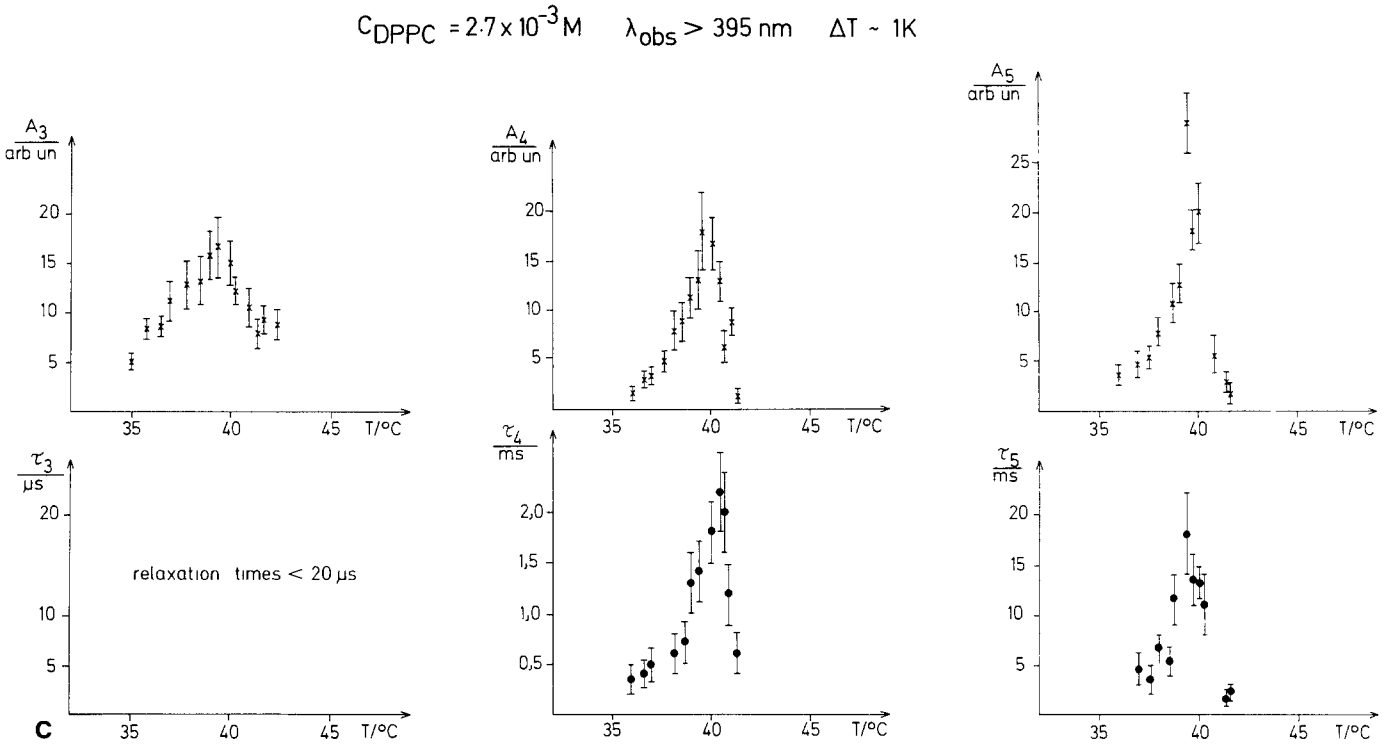


Fig. 6 c

rigid clusters and signal 5 to the disappearance of whole clusters. The last signal (τ_5) should be the most cooperative as verified by the dynamic measurements presented here. During the two slowest processes "frozen" fluorescent probes are released from the "cluster ice" and therefore lower the value $I_{\parallel} - I_{\perp}$. Further experiments with cholesterol and peptide molecules incorporated into the lipid bilayers are under way to refine our model.

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