# Kinetics of the Subtransition of Asymmetrically Substituted Phosphatidylcholines†

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ABSTRACT: The thermodynamics and kinetics of the subtransition $L_\epsilon \rightleftharpoons P_\beta$ of sonicated unilamellar vesicles of 1-myristoyl-2-stearoylphosphatidylcholine (1M-2S-PC) and of 1-stearoyl-2-myristoylphosphatidylcholine (1S-2M-PC) were studied by equilibrium cooling curves and temperature-jump relaxation spectrometry with an anthracenophane cryptand as a mobile fluorescent probe. The unilamellar vesicles exhibit the midpoint temperature $T_{sII}$ of the subtransition about 10 °C below the respective main transition. The kinetics of the subtransition in the time range between $10^{-4}$ and $10^3$ s is characterized by a cooperative relaxation process in the range of milliseconds and a further noncooperative process in the range of seconds. The slow process is assigned to the rearrangement of lattice defects. The fast process is evaluated in terms of a cyclic reaction scheme that consists of two pathways for the biomolecular association of probe and vesicle coupled with the conformational change of the lipid matrix during the subtransition. The analysis reveals that the fast process comprises the nucleation and growth of cluster. The cooperative lattice transformation of the subtransition follows a first-order rate law. The rate constants at $T_{sII}$ are 70 $s^{-1}$ for 1S-2M-PC and 170 $s^{-1}$ for 1M-2S-PC. Since the plots of the relaxation time vs. the degree of transition are in accordance with the predictions of the linear Ising model, it is concluded that clusters are propagated anisotropically in a linear fashion; e.g., fluidlike $P_\beta$ conformations grow along the ripple.

The thermotropic behavior of lecithin model membranes is characterized by several endothermic phase transitions; e.g., the symmetrically substituted lecithins like DPPC[1] and DSPC undergo a subtransition, pretransition, and main transition, separating the membrane phases $L_\epsilon$, $L_\beta$, $P_\beta$, and $L_\alpha$, respectively. Recently, Stümpel et al. (1983) studied the phase behavior of 13 different phosphatidylcholines by applying calorimetry and X-ray diffraction. The lecithins differed from each other with respect to length as well as to position of the acyl chains in the glycerol backbone. These authors observed that only those lecithins that were symmetrically substituted with the same fatty acid in positions 1 and 2 displayed three endothermic phase transitions. The phosphatidylcholines that were 1,3 substituted or that contained acyl chains of different length in the positions 1 and 2 exhibited two phase transitions, the main transition and one additional transition in the gel state, which was called subtransition II. It was identified as the symmetry change $L_\epsilon \rightleftharpoons P_\beta$ on the basis of X-ray diffraction data. The pretransition was found to be absent in these types of phospholipid. It was the aim of this study to characterize the thermodynamics and kinetics of this recently discovered subtransition II in order to provide a more refined understanding of the relationship between subtransition II and the previously known pretransition and subtransition I of the 1,2 symmetrically substituted lecithins. Since most work on the subtransition I was performed with DPPC, mixed-chain lecithins of the same molecular weight, namely, 1M-2S-PC and 1S-2M-PC, were chosen as the appropriate representatives for the investigation of subtransition II. Vesicle dispersions of the lecithins were used to perform equilibrium cooling curves and temperature-jump experiments. The transition was monitored by applying a fluorescent anthracenophane cryptand, which has been shown in the accompanying paper (Herrmann et al., 1984) to be a very sensitive probe for transitions below the main transition of lecithins.

## Materials and Methods

*Lipids.* The synthesis of 1M-2S-PC and of 1S-2M-PC will be published separately (H. Eibl, unpublished results). The purity of the compounds was established by thin-layer chromatography and elemental analysis to be better than 99%. The positional purity of the compounds was confirmed by determination of the phosphate to vicinal diol ratio and by gas chromatographic analysis of the fatty acids as methyl esters after hydrolysis with phospholipase $A_2$ (van Deenen & de Haas, 1963). Phosphate was determined according to Eibl & Lands (1969) and vicinal diol after fatty acid hydrolysis as described previously (Eibl & Lands, 1970). The ratio of phosphate to vicinal diol of 1:1 indicated the absence of 1,3-diacyl isomers. The positional purity of the mixed-chain lecithins was better than 98%. DMPC and DPPC were purchased from Fluka (Buchs, Switzerland).

*Fluorescent Dye.* The anthracenophane (Figure 1) was synthesized as described in the accompanying paper (Herrmann et al., 1984).

*Preparation of Model Membrane Systems.* Vesicle preparations were performed by a modification of Huang's procedure (Huang, 1969). At all times during preparation and storage, the vesicle dispersions were maintained 20 °C above $T_m$. Prior to the experiment, 2 μL/mL anthracenophane stock solution (1 mM) in tetrahydrofuran was added to the vesicle dispersion and incubated for 2 h at 20 °C above $T_m$. The contamination of the preparation by large multilamellar vesicles was checked in 24-h intervals by plotting its turbidity $A(\lambda)$ between 300 and 700 nm against the reciprocal fourth

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[1] Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; 1S-2M-PC, 1-stearoyl-2-myristoyl-*sn*-glycero-3-phosphocholine; 1M-2S-PC, 1-myristoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; $T_m$, $T_p$, $T_{sI}$, and $T_{sII}$, midpoint temperature of main transition, pretransition, subtransition I, and subtransition II.

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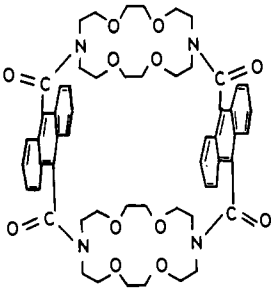


FIGURE 1: Anthracenophane probe.

power of the scattering wavelength (Barrow & Lentz, 1980).

*NMR Experiments.* $^1$H NMR experiments (Bruker WH 270) on vesicle dispersions (5 mM lipid) prepared in 10 mM NaCl/$D_2O$ were performed at 20 °C above $T_m$ in the phase $L_\alpha$ and 5 °C below $T_m$ in the phase $P_\beta$. The time-dependent change of the signal of the choline methyl protons was followed after the addition of 2 mM $Mn^{2+}$ ions and of 2 mM $Mn^{2+}$ ions together with 4 $\mu$M anthracenophane probe, respectively. The choline peak was observed for up to 3 h. The HDO signal was taken as a reference peak.

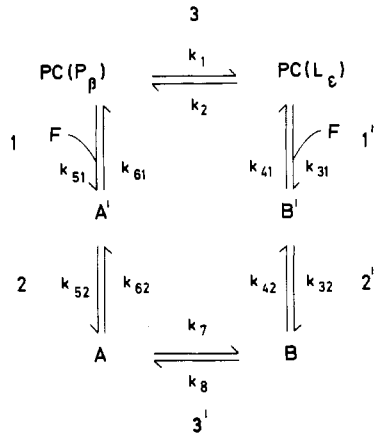
*Equilibrium and Kinetic Measurements.* Fluorescence cuvettes equipped with a water jacket (Fa. Hellma, Müllheim, FRG) were used for the equilibrium and the slow temperature-jump experiments. The temperature was controlled to within 0.03 °C by circulating water through the cuvette and the thermostated cell holder with two Haake thermostats, type FT. The fluorescence was measured in a Schoeffel RRS 1000 spectrofluorometer ($\lambda_{Ex}$ = 365 nm; $\lambda_{Em}$ = 422 nm). The equilibrium cooling curves were recorded with a rate of 6 °C/h. Slow temperature jumps were accomplished by switching the water circulation between sample chamber and water bath from one thermostat to the other. The height of the temperature jumps was adjusted to 0.1–1.0 °C depending on the slope of the equilibrium cooling curve. Both the fluorescence and the temperature were recorded in all temperature-jump experiments. Since the relaxation time $\tau_i$ of the observed process and the temperature rise time $\tau_s$ of 25–30 s were of the same order of magnitude, an exponential step function had to be used for the evaluation of the relaxation curves (Eigen & de Maeyer, 1963):

$$y(t) = y_{ko}\left[(1-\alpha)\frac{\tau_i}{\tau_i-\tau_s}e^{-t/\tau_i} - \frac{\tau_i-\alpha\tau_i}{\tau_i-\tau_s}e^{-t/\tau_s}\right] \quad (1)$$

$$\alpha = y_{fo}/y_{ko}$$

$\alpha$ is defined as the ratio of the amplitude of the fast relaxation process $y_{fo}$ to the overall amplitude $y_{ko}$. The lower limit for the determination of the chemical relaxation time was found to be about 20 s. Fast temperature-jump experiments were performed according to Coutts et al. (1975). If one monitored the fluorescence of the anthracenophane, temperature jumps of only 0.08 °C were sufficient to obtain resolvable relaxation curves. The collapse of the electrical field of 1 kV/cm during the temperature jump was much too small to damage the membrane irreversibly (Teissie & Tsong, 1981; Stulen, 1981; Grell, 1981). The relaxation curves were processed on-line and analyzed as described by Peters & Teschner (1979). The fast temperature jumps were performed in parallel with the slow temperature jumps at the same time after sample preparation.

*Model for the Relaxation Kinetics of Phase Transitions in Spherical Membrane Vesicles Monitored by a Mobile Dye.* If a phase transition in spherical membrane vesicles, e.g., the subtransition $P_\beta \rightleftharpoons L_\epsilon$, is followed by a mobile indicator F that is distributed between the aqueous and the lipid phases, the reaction scheme consists of at least two pathways for bimolecular association coupled with monomolecular interconversion:




(2)

The reactions 1 and 1′ describe the diffusion-controlled adsorption of the probe F from the external aqueous phase to the vesicle surface in phase $P_\beta$ (process 1) or $L_\epsilon$ (process 1′) followed by the translocation of the probe to a more apolar binding site (2 and 2′). Steps 3 and 3′ describe the rearrangement of the free and occupied binding sites during the phase transition. If further distinct binding sites of the probe in the lipid bilayer or intermediate structures during the phase transition can be identified, accordingly the reaction steps 2 and 2′ and 3 and 3′ are split into sequences of consecutive monomolecular interconversions.

Following Ruf & Grell's (1981) theoretical analysis, the relaxation times for steps 1 and 1′ are estimated to be in the time range of 10 and 100 $\mu$s if the initial phospholipid concentration is chosen to be $10^{-3}$ M. The relaxation time for the incorporation of the dye into a less polar surrounding typically amounts to some tenths of a millisecond as was measured, for example, for the azo dye o-methyl red (Ruf & Grell, 1981). If one assumes that the dye equilibrates between aqueous phase, boundary layer, and bilayer interior in less than 10% of the time required by the monomolecular interconversions 3 and 3′, the steps 1, 1′, 2, and 2′ enter into the expression of the slowest relaxation process by their respective equilibrium constants [cf. Kustin et al., 1965]:

$$\tau^{-1} = \alpha k_1 + \beta k_2 + \alpha' k_7 + \beta' k_8 \quad (3)$$

where

$$\alpha = \frac{Z_1}{N_1} \qquad \beta = \frac{Z_2}{N_2} \qquad \alpha' = \frac{Z_3}{N_1} \qquad \beta' = \frac{Z_4}{N_2}$$

$$Z_1 = K_1/(K_1 + \bar{c}_3 + \bar{c}_5) + K_2/\bar{c}_2$$

$$Z_2 = \frac{K_1}{\bar{c}_3} + \frac{K_2}{K_2 + \bar{c}_2 + \bar{c}_5}$$

$$Z_3 = \frac{\bar{c}_5}{K_1 + \bar{c}_3 + \bar{c}_5} + \frac{\bar{c}_5}{\bar{c}_2}$$

$$Z_4 = \frac{\bar{c}_5}{\bar{c}_3} + \frac{\bar{c}_5}{K_2 + \bar{c}_2 + \bar{c}_5}$$

$$N_1 = \frac{K_2 + \bar{c}_2 + \bar{c}_5}{\bar{c}_2} - \frac{\bar{c}_3}{K_1 + \bar{c}_3 + \bar{c}_5}$$

$$N_2 = \frac{K_1 + \bar{c}_3 + \bar{c}_5}{\bar{c}_3} - \frac{\bar{c}_2}{K_2 + \bar{c}_2 + \bar{c}_5}$$

$$K_1 = \bar{c}_3\bar{c}_5/(\bar{c}_4 + \bar{c}_7)$$

$$K_2 = \bar{c}_2\bar{c}_5/(\bar{c}_1 + \bar{c}_6)$$

$$K = \bar{c}_3/\bar{c}_2$$

$\bar{c}_i$ = equilibrium concentration of component $i$

$\bar{c}_1 = [A]$ $\bar{c}_2 = [PC(P_\beta)]$ $\bar{c}_3 = [PC(L_\epsilon)]$

$\bar{c}_4 = [B]$ $\bar{c}_5 = [F]$ $\bar{c}_6 = [A']$ $\bar{c}_7 = [B']$

$\bar{c}_2$ and $\bar{c}_3$ are the equilibrium concentrations of free binding sites for the dye F in the phase $P_\beta$ and $L_\epsilon$.

Equation 3 can be considerably simplified if the following conditions are satisfied. (1) The ratio of free to occupied binding sites is at least $10^2$:1. Hence

$$\bar{c}_2 + \bar{c}_3 \simeq c_2^\circ + c_3^\circ \gg c_5^\circ$$

and

$$\bar{c}_2 \gg \bar{c}_1 \quad \bar{c}_3 \gg \bar{c}_4 \quad \bar{c}_2 \gg \bar{c}_5 \quad \bar{c}_3 \gg \bar{c}_5$$

(2) The aqueous phase contains more than 75% of the total indicator concentration:

$$\bar{c}_5 > 3(\bar{c}_1 + \bar{c}_4)$$

Then the expression for the relaxation time reads

$$\tau^{-1} = k_1 + k_2 + \alpha''(k_7 + k_8) \qquad (4)$$

where

$$\alpha'' = \bar{c}(\text{occupied binding sites})/\bar{c}(\text{free binding sites})$$

$$\alpha = \beta = 1$$

For our kinetic experiments, both requirements could be easily fulfilled. The molar fraction of the anthracenophane in the aqueous phase varies as a function of temperature between 0.8 and 0.95, and the number of binding sites on the vesicle for the dye is high. Titrations of vesicle dispersions with anthracenophane revealed that there exists at least one binding site for the dye per 25 phospholipid molecules.

## Results

*NMR Experiments.* In the phases $L_\alpha$ and $P_\beta$ the signal of the choline methyl protons of the asymmetrically substituted lecithins in the vesicle dispersions was split into two components about 11 Hz apart (Figure 2). This splitting is due to the magnetic inequivalence of the head groups of the inner and outer shell of the vesicles (Kostelnik & Castellano, 1973; Bergelson, 1978). Upon the addition of $Mn^{2+}$ ions the low-field component of the signal was broadened beyond detection, whereas no significant change of the line width and the height of the signal of the inner choline head groups was observed. Neither $Mn^{2+}$ alone nor $Mn^{2+}$ plus the anthracenophane had any influence on the shape and height of the inner choline methyl peak during the time of observation (Figure 2). These results indicated that the vesicles were not leaky for $Mn^{2+}$ ions and did not fuse to larger coacervates in the presence of the anthracenophane, irrespectively of whether the membranes were in the gel or in the liquid crystalline state. This is in contrast to the behavior of DMPC vesicles, which show a disappearance of the inner choline signal, partly caused by $Mn^{2+}$ leakiness and fusion of the vesicles (Herrmann et al., 1984). This sensitive NMR test for vesicle fusion could not be extended to phase $L_\epsilon$ because already in the absence of $Mn^{2+}$ and anthracenophane the signal of the choline methyl resonances was very broad and was hidden in the asymmetric base line caused by the large HDO peak.

*Equilibrium Measurements.* The equilibrium cooling curves of the vesicle dispersions revealed a cooperative phase transition

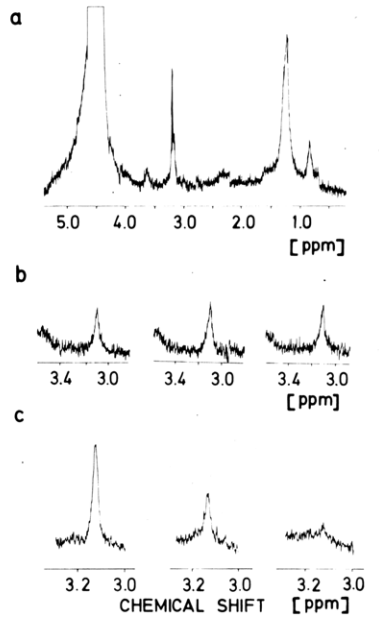



FIGURE 2: $^1H$ NMR spectra of small unilamellar vesicle dispersions of 4 mM 1M-2S-PC in 10 mM $NaCl/D_2O$ solution at 50 °C: (a) spectrum of the vesicles as prepared originally; (b) time dependence of the signal of the choline methyl protons of the inner head groups 1.5, 12, and 190 min after the addition of 2 mM $MnSO_4/D_2O$ and 4 $\mu$M anthracenophane/$CD_3CN$; (c) time dependence of the same signal observed with 7 mM DMPC vesicle dispersions in 10 mM $NaCl/D_2O$ 4, 12, and 30 min after the addition of 2 mM $MnSO_4/D_2O$ and 1.5 $\mu$M anthracenophane/$CD_3CN$.

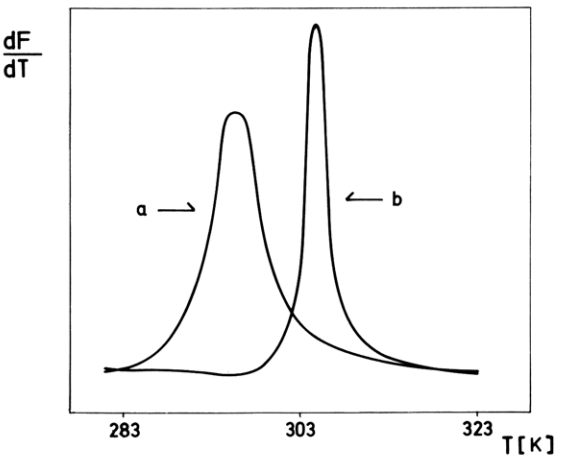



FIGURE 3: Differential cooling curves of vesicle dispersions of 2 mM 1S-2M-PC (a) and 1M-2S-PC (b) in 50 mM NaCl solution in the presence of 2 $\mu$M anthracenophane. The cooling rate was 0.1 °C/min.

between two gel phases for both mixed-chain lecithins, 1S-2M-PC and 1M-2S-PC, with transition temperatures $T_{sII}$ of 22.7 and 31.6 °C, respectively (Figure 3). This subtransition II occurred for both lipids approximately 10 °C below the respective main transition (Figure 4). It was detected by the fluorescence change of the anthracenophane probe in heating as well as in cooling curves. In the liquid crystalline phase 1 $\mu$M dye fluoresced in a 1 mM lecithin dispersion only 10 times stronger than in an aqueous saline solution. Below $T_m$, however, the fluorescence gradually increased by a factor of 3.5 over a temperature range of about 20 K. The maximum fluorescence increase occurred at the temperature $T_{sII}$ of subtransition II (Figure 3). No preincubation of the sample at temperatures below the subtransition was necessary. Vesicle dispersions that had been maintained 20 °C above $T_m$ after preparation displayed the subtransition II immediately during the first cooling scan. In contrast to the behavior of the

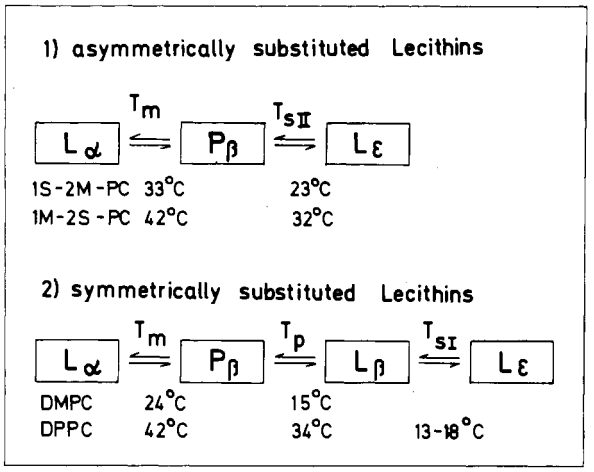



FIGURE 4: Phases and phase transition temperatures of symmetrically and asymmetrically substituted phosphatidylcholines. The transition temperature of subtransition I is taken from the literature (Chen et al., 1980; Füldner, 1981; Nagle & Wilkinson, 1982.

asymmetrically substituted lecithins, the 1,2 symmetrically substituted lecithin of the same molecular weight, DPPC, induced no significant increase of the fluorescence during the temperature range of subtransition I $L_\epsilon \rightleftharpoons L_\beta$ irrespectively of whether the sample was preincubated for 72 h below $T_{sI}$ or not.

Figure 3 shows the differential plots $dF/dT$ vs. $T$ of the experimental cooling curves of 1S-2M-PC and 1M-2S-PC. The nearly symmetrical graphs of Gaussian shape are characterized by a half-width $\Delta T_{0.5}$ of 3 °C for 1M-2S-PC and of 6.3 °C for 1S-2M-PC (Table I).

*Kinetic Experiments.* Since for the fast temperature-jump experiments on vesicle dispersions the applied electric field had to be as low as possible, the optimum concentrations of lecithin and dye of 1 mM and of 2 $\mu$M were chosen in order to obtain the maximum signal to noise ratio and the maximum change of signal with temperature (cf. Figure 3 of the accompanying paper). In addition, taking these concentrations, the complex

Table I: Equilibrium and Kinetic Parameters of the Subtransition II of Asymmetrically Substituted Lecithins

| | 1S-2M-PC | 1M-2S-PC |
|---|---|---|
| $T_{sII}$ (°C) | 22.7 ± 0.5 | 31.6 ± 0.3 |
| $\Delta T_{1/2}$ | 6.3 ± 0.5 | 3.0 ± 0.2 |
| $\Delta H_{cal}$ (kJ/mol)[a] | 33.1 | 27.6 |
| $\nu = \Delta H_{vh}/\Delta H_{cal}$ | 13 ± 2 | 27 ± 3 |
| $k_{T_{sII}}$ ($s^{-1}$) | 70 ± 10 | 170 ± 20 |
| $\Delta H^* k_1$ (kJ/mol) | −190 ± 40 | −400 ± 80 |
| $\Delta H^* k_1/\nu$ (kJ/mol) | −15 ± 2 | −15 ± 2 |
| $\Delta H^* k_2$ (kJ/mol) | 230 ± 20 | 310 ± 40 |
| $\Delta H^* k_2/\nu$ (kJ/mol) | 18 ± 2 | 11 ± 2 |

[a] Calorimetric data taken from Stümpel et al. (1983).

eq 3 for the slow relaxation time reduces to eq 4 (vide supra). As a consequence of this simplification, a reliable evaluation of the rate constants for the subtransition becomes possible.

The temperature-jump analysis of the subtransition II of 1M-2S-PC and 1S-2M-PC revealed one slow and two fast relaxation processes. The fastest noncooperative process had a small amplitude and a relaxation time $\tau_1$ of about 100 $\mu$s. The process was observed over the whole temperature range investigated; however, since the time of resolution of the apparatus interfered with the relaxation process, we were not able to measure the temperature dependence of the relaxation time unambiguously. A detailed study of the concentration dependence of this relaxation process could not be performed due to the limited availability of these lecithins. With DMPC, however, such a concentration dependence was measured in our studies on the kinetics of the pretransition (unpublished data). By use of the same concentrations of anthracenophane and lecithin, a relaxation process of similar amplitude and time constant was recorded. According to the dependence of the reciprocal relaxation time on the sum of free concentrations of dye and DMPC, this relaxation process was assigned to the binding and the translocation of the dye into the vesicle. The similarity of the time course and of the amplitude of the relaxation curves of DMPC, 1S-2M-PC, and 1M-2S-PC has led us to assign this fast noncooperative relaxation process of

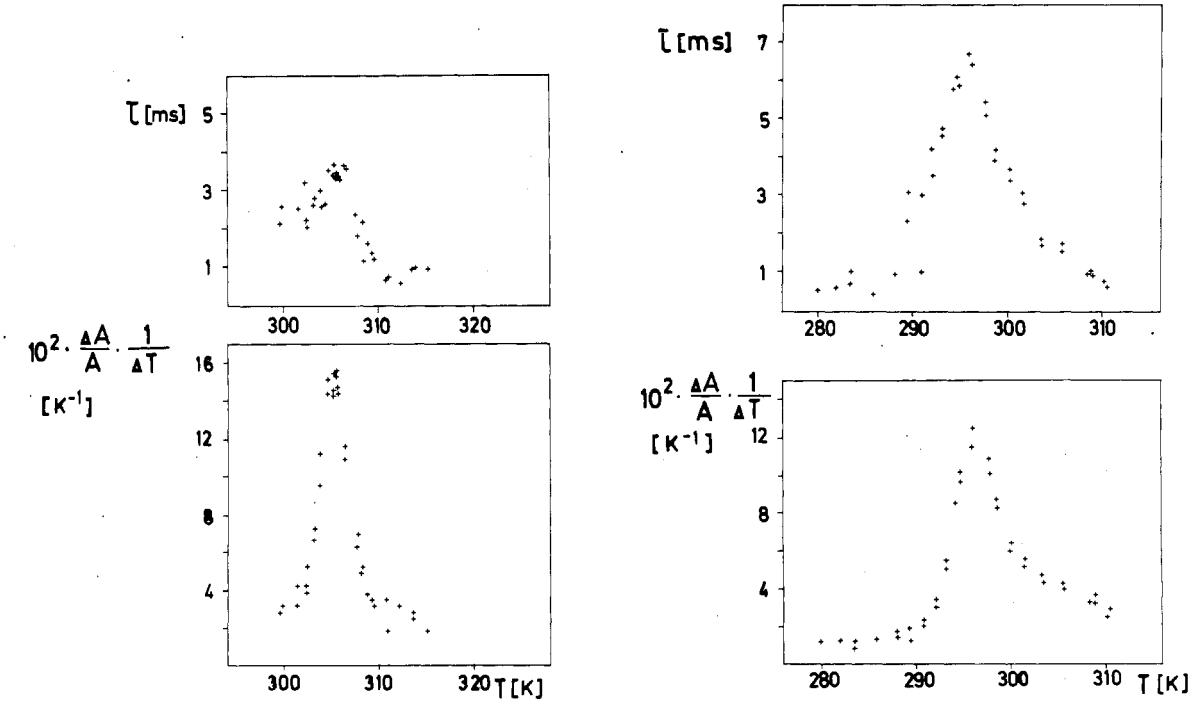



FIGURE 5: Relaxation times and normalized amplitudes of fast temperature-jump experiments with vesicle dispersions of 1 mM 1M-2S-PC (left) and 1 mM 1S-2M-PC (right) in 50 mM NaCl solution in the presence of 2 $\mu$M anthracenophane.

the mixed-chain lecithins to the steps 1, 1′, 2, and 2′ of the reaction scheme.

The second relaxation process of the fast temperature-jump experiments was of the order of a few milliseconds. This relaxation time $\tau_2$, as well as its amplitude, showed an approximated Gaussian behavior as a function of temperature (Figure 5) with a maximum at the subtransition temperature $T_{sII}$. The amplitude of the fast process ($A_2$) amounted to 70–80% of the total equilibrium amplitude. The remaining 10–20% of the overall signal change arose from a slower process in the time range between 20 and 90 s. In contrast to the faster relaxation process, this relaxation time was within experimental error independent of temperature in a range of about 6 °C below and above the subtransition II. With each lipid dispersion about 40 kinetic experiments were performed. Each jump was followed by an equilibration time of at least 20 min, in order to reestablish equilibrium conditions. The dispersions remained essentially clear throughout the experiments.

## Discussion

The anthracenophane proved to be an excellent reporter group for the investigation of the subtransition II of asymmetrically substituted lecithins. The localization of the probe and the study of probe–lecithin interactions have been the subject of the preceding paper. Upon the transition from the liquid crystalline to the gel phase, the anthracenophane becomes incorporated into the lipid bilayer. The increase of the fluorescence of the probe with decreasing temperature reflects the increase of its distribution coefficient between the lipid phase and the bulk aqueous phase. Since the relative quantum yield of the fluorophore is 100-fold higher in lecithin than in water, even minor changes of the partition ratio give rise to large changes of the fluorescence signal. The fluorophore is incorporated in the vicinity of the glycerol backbone.

By making use of the fluorescence intensity change of the anthracenophane, the subtransition II of the asymmetrically substituted lecithins was sensitively monitored. No significant signal increase, however, was observed at the temperature of the subtransition $T_{sI}$ of DPPC. It has been reported that the subtransition I of the 1,2 symmetrically substituted lecithins was only detectable after prolonged tempering at low temperatures and showed a pronounced hysteretic effect (Chen, 1980; Fülner, 1980, 1981). According to our results, such a refined pretreatment of the sample is not necessary for the detection of subtransition II. The transition was immediately recorded in the first cooling scan, and no hysteresis was observed in repetitive cooling and heating cycles.

The isomers 1M-2S-PC and 1S-2M-PC differ with respect to their interchain interaction in a bilayer membrane. Regarding the bending of the acyl chain at the position 2 of the glycerol backbone (Seelig & Seelig, 1975), the effective chain length of the fatty acid in position 2 should be approximately six methylene groups shorter in the case of 1S-2M-PC and two methylene equivalents longer in the case of 1M-2S-PC than the acyl chain in position 1. Therefore, 1M-2S-PC, which has an additional van der Waals interaction of two more methylene groups, exhibits the higher phase-transition temperatures of the main transition and the subtransition II. The cooperativity of the subtransition was estimated from the cooperative unit $\langle \nu \rangle$ defined as the ratio of the van't Hoff enthalpy

$$\Delta H_{vh} = 4RT_{sII}/\Delta T_{0.5}$$

to the calorimetrically determined enthalpy $\Delta H_{cal}$ (Mabrey & Sturtevant, 1976, 1979; Chen et al., 1980). The values obtained were $\nu = 13 \pm 2$ for 1S-2M-PC and $\nu = 27 \pm 3$ for 1M-2S-PC at 50 mM NaCl (cf. Table I).

In the kinetic experiments three processes with time constants in the range of about 100 μs, some milliseconds, and about 0.5 min could be resolved. The sum of their individual amplitudes amounts to the total fluorescence amplitude of the equilibrium cooling experiment. For both phospholipids the cooperativity was mainly reflected in the millisecond process. Plots of relaxation times and amplitudes as a function of temperature resemble the differential cooling curves and fit very well to Gaussian curves with maxima at the respective midpoint transition temperatures (cf. Figure 5). On the other hand, within experimental error no temperature dependence was observed for the relaxation time of the slow process. Obviously, neither the type of lattice, $P_\beta$ or $L_\epsilon$, nor the cooperative lattice transformation during the subtransition had any influence on the rate of the slow process.

Since the slow process is noncooperative and contributes only 10–20% to the total amplitude, we attribute it to the rearrangement of lattice imperfections. This interpretation is in agreement with kinetic X-ray diffraction experiments of Akiyama et al. (1982), who reported that the reorganization of lattice defects in lecithins occurs considerably slower than the lattice transformation itself. Moreover, if the rate-limiting step of the slow process critically depends only on the number of molecules at the boundary between lattice defect and the bulk membrane phase, the rearrangement of lattice defects should take place at a similar rate for a $P_\beta$ cluster in phase $L_\epsilon$ and a $L_\epsilon$ cluster in phase $P_\beta$ provided that the clusters are of comparable size and shape in the two phases. This consideration would reasonably explain the observed independence of the rate of the relaxation process on temperature. Since the noncooperative process was found to be several orders of magnitude slower than the two fast relaxation processes in the microsecond and millisecond range, it was not considered for the evaluation of the rate constants of the fast processes.

These fast relaxation processes are discussed in light of the cyclic reaction scheme (eq 2) introduced above. Under our experimental conditions the diffusion-controlled binding of the fluorophore to the vesicle surface takes place in the time range of 10–100 μs, and the subsequent incorporation of the anthracenophane should occur in some tenths of a millisecond (vide supra). Accordingly, the fast noncooperative relaxation process of some hundred microseconds is assigned to the steps 1, 1′, 2, and 2′ of the reaction scheme. In addition to these reactions, the cooperative relaxation process in the millisecond time range contains the steps 3 and 3′ of the phase transition of the binding sites in the free and bound state, respectively. We chose the initial concentrations of fluorophore and of phospholipid in such a way that the expression for the relaxation time reduces to eq 4. With an uncertainty of at most 2%, $\alpha$ and $\beta$ are equal to 1, whereas $\alpha''$ varies between 0.0015 and 0.01 depending on temperature. If we assume that during the subtransition both the free and occupied sites change their respective conformational states with comparable rates, the term $\alpha''(k_7 + k_8)$ is small compared to $k_1 + k_2$. If in opposite the conformational change of the occupied binding sites contributed to a significant extent to the millisecond relaxation process, the rate constants $k_7 + k_8$ for the rearrangement of occupied binding sites had to be at least 1 order of magnitude larger than the corresponding term $k_1 + k_2$ for the free binding sites. This hypothesis was tested by the evaluation of rate constants from the experimental data according to eq 4. Strongly curved hyperbolic and parabolic plots of $\ln k_i$ vs. $T^{-1}$ were obtained when the ratio $(k_7 + k_8)/(k_1 + k_2)$ was chosen

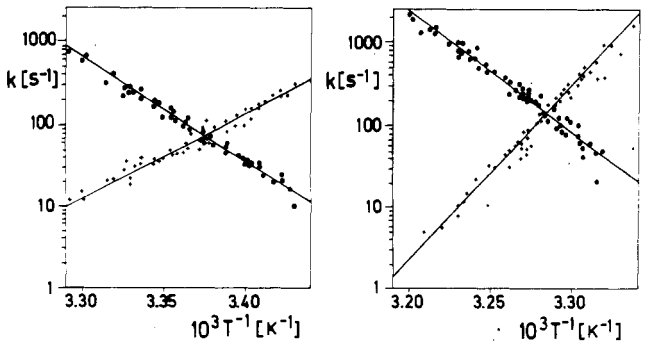



FIGURE 6: Arrhenius plots of the temperature dependence of the rate constants $k_1$ (crosses) and $k_2$ (circles) of the subtransition for 1S-2M-PC (left) and for 1M-2S-PC (right).

to be larger than 10. The nonlinearity arises from the coefficients $A_1$ and $A_2$ of the rate constants $k_7$ and $k_8$. The parameters $A_1$ and $A_2$ defined as

$$A_1 = \frac{[1 - x_5^2(T)](1 + K)}{1 + x_5(T)(1 + K + K^{-1})} \tag{5a}$$

$$A_2 = A_1 \frac{1 + K^{-1}}{1 + K} \tag{5b}$$

vary monotonously as a function of temperature between the limiting values 0 and $[1 - x_5^2(T)]/x_5$, with $x_5 = \bar{c}_5/c_5°$. Thus, a contribution of the term $A_1k_7 + A_2k_8$ in eq 4 that is larger than the error limit is not compatible with the experimentally observed symmetric Gaussian behavior of the temperature dependence of the relaxation times. It follows that the term $\alpha''(k_7 + k_8)$ can be neglected, and eq 4 reduces to

$$\tau^{-1} = k_1 + k_2 = k_2(1 + K) \tag{6}$$

Under the selected experimental conditions the reciprocal relaxation time is solely determined by conformational changes of the clusters of free phospholipid molecules:

$$\mathrm{PL}(\mathrm{P}_\beta) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{PL}(\mathrm{L}_\epsilon)$$

The rate constants were evaluated from eq 6 with the same $K(T)$ values obtained from differential cooling curves. As shown in Figure 6, the Arrhenius plots ln $k_i$ vs. $T^{-1}$ fit very well to straight lines for both phosphatidylcholines 1S-2M-PC and 1M-2S-PC.

The linear Arrhenius plots verify our conclusion that the conformational change of the occupied binding sites does not contribute to significant extent to the cooperative relaxation process. Hence, the complexity of the reaction scheme introduced above is considerably reduced. The pseudomonomolecular reorganization of phospholipid molecules in the membrane matrix represents the rate-limiting step, which is coupled to the rapidly equilibrating indicator reactions of the binding and translocation of the probe.

The linearity of the Arrhenius plots (Figure 6) demonstrates that the cooperative lattice transformation during the subtransition II follows a first-order rate law. The rate constants in forward and backward direction at the subtransition temperature $T_{sII}$ were determined to be 70 $s^{-1}$ for 1S-2M-PC and 170 $s^{-1}$ for 1M-2S-PC. Despite its higher cooperativity, the subtransition of 1M-2S-PC takes place at a higher rate at the transition temperature than that of 1S-2M-PC. This result can be reasonably explained by the lower activation enthalpy of 1M-2S-PC per monomer for the transition from phase $L_\epsilon$ to the intermediate phase $P_\beta$ (Table I). In contrast, the apparent activation enthalpy per monomer for the crystallization to phase $L_\epsilon$ was calculated to be essentially the same for both

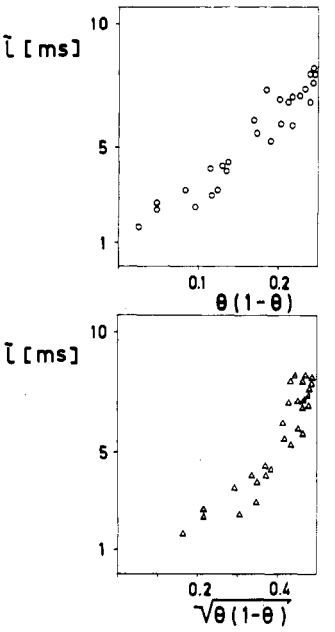



FIGURE 7: Relaxation times for a 1S-2M-PC vesicle dispersion as a function of $[\theta(1 - \theta)]^n$ ($n$ = 0.5 and 1). The equilibrium and kinetic data were taken from a set of experiments performed with the same vesicle preparation.

lipids. Since the value is negative, one has to demand a more complex reaction scheme for the transition to phase $L_\epsilon$, which includes fast exothermic preequilibria determining the negative sign of the activation enthalpy. Such fast processes in the nanosecond to microsecond time range are involved in the reorganization of water structure, head group rotations, and chain-isomerization reactions of individual lipid hydrocarbons, as it has been shown by ultrasonic relaxation measurements (Eggers & Funck, 1976; Gamble & Schimmel, 1978; Harkness & White, 1979) and the laser temperature-jump technique (Gruenewald et al., 1981).

The cooperativity of the millisecond subtransition process is indicated by the Gaussian behavior of relaxation time and amplitude with maxima at the transition midpoint of subtransition II. Such cooperative relaxation processes have already been found by other authors in kinetic studies on the main transition of lecithins (Träuble, 1971; Tsong & Kanehisa, 1977; Gruenewald et al., 1980; Inoue et al., 1981; Gruenewald, 1982). The gel to liquid crystalline phase transition is characterized by two cooperative processes in the 0.1–1- and 10–100-ms time range. Inoue et al. (1981) and Takemoto et al. (1981) showed that the cooperative unit and the relaxation time critically depend on the size of the liposome. The maximum relaxation time in DPPC liposomes increased about 2 orders of magnitude, from 1.4 to 80 ms, with increasing number of molecules per liposome from $3.0 \times 10^3$ to $33 \times 10^3$. Our experiments on the kinetics of the subtransition were performed on sonicated single-lamellar vesicles containing approximately (3–4) $\times 10^3$ molecules [cf. Watts et al. (1978) and the preceding paper]. The measurements yielded maximum relaxation times of about 10 ms, which demonstrates that the cooperative processes of the main phase transition and of subtransition II take place in a similar time range. The comparison of the kinetic data reveals a further similarity: in both cases the experimental relaxation times $\tau$ are symmetrical functions of the degree of transition $\theta$ with a maximum at $\theta$ = 0.5. Gruenewald et al. (1980) found for their data on the main phase transition in lipid vesicles that $\theta$ is a linear function of $[\theta(1 - \theta)]^{1/2}$. In the one-dimensional Ising model $\theta$ is linear with $\theta(1 - \theta)$, and the authors concluded that the behavior of

relaxation times of transitions in two-dimensional systems cannot be described by the linear Ising model.

Taking our data on the subtransition $L_\epsilon \rightleftharpoons P_\beta$, we plotted the relaxation times $\tau_i$ of the millisecond process vs. $[\theta(1-\theta)]^n$ varying $n$ between 0.1 and 2. The best fit of the data to a straight line was obtained when the exponent was chosen to be $n = 1$ (Figure 7). This empirical treatment leads to the surprising result that our kinetic data of the subtransition of asymmetrically substituted phosphatidylcholines can be interpreted in terms of the linear Ising model of "infinite" chains. According to (Zimm & Bragg, 1959; Schwarz, 1965)

$$\tau = \frac{1}{\theta k_F}\theta(1-\theta) \qquad (7)$$

the slope of the graph gives the reciprocal product of the nucleation parameter $\sigma$ times the rate constant $k_F$ for the forward growth reaction. At a first glance it seems paradoxical that the processes of nucleation and propagation of clusters in a two-dimensional lattice should proceed anisotropically in a linear fashion. However, our empirical finding can be reasonably explained in the light of recently published NMR data (Wittebort et al., 1982) and theoretical calculations (Falkovitz et al., 1982) on the structure of the undulated $P_\beta$ phase. According to their results, the $P_\beta$ phase has a locally varying structure and has simultaneously both solid- and fluidlike membrane properties. Moreover, the model proposed by Falkovitz predicts that the clusters of lecithin molecules in solid- and fluidlike conformation vary periodically in accordance with the periodicity of the ripples. Hence, if in a $L_\epsilon$ domain fluidlike conformations of $P_\beta$ appear, the nucleus can grow without any restriction along the ripple; however, in transversal direction across the ripples only the nearest neighbors can transform into fluidlike conformations. This anisotropic growth of clusters during the subtransition II supports the idea that the $P_\beta$ phase is structurally heterogeneous.

In conclusion, our kinetic study indicates that in the time range of $10^{-4}$–$10^3$ s the subtransition of asymmetrically substituted lecithins in single-lamellar vesicles is characterized by one process in the time range of milliseconds and a further process in the time range of seconds. The fast cooperative process comprises the nucleation and growth of clusters and represents the lattice transformation, whereas the slow noncooperative process is assigned to the rearrangement of lattice defects.

**Registry No.** 1M-2S-PC, 76343-22-1; 1S-2M-PC, 20664-02-2; anthracenophane, 90763-91-0.

## References


Akiyama, M., Terayama, Y., & Matsushima, N. (1982) *Biochim. Biophys. Acta 687*, 337–339.

Barrow, D. A., & Lentz, B. R. (1980) *Biochim. Biophys. Acta 597*, 92–99.

Bergelson, L. D. (1978) *Methods Membr. Biol. 9*, 275–335.

Chen, S. C., Sturtevant, J. M., & Gaffney, B. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5060–5063.

Coutts, S. M., Riesner, D., Rabl, C. R., & Maass, G. (1975) *Biophys. Chem. 3*, 275–289.

Eggers, F., & Funck, T. (1976) *Naturwissenschaften 63*, 280–285.

Eibl, H., & Lands, W. E. M. (1969) *Anal. Biochem. 30*, 51–57.

Eibl, H., & Lands, W. E. M. (1970) *Biochemistry 9*, 250–258.

Eigen, M., & de Maeyer, L. (1963) *Tech. Org. Chem. 8*, 916–921.

Falkowitz, M. S., Seul, M., Frisch, H. L., & Mc Connell, H. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3918–3921.

Füldner, H. H. (1980) Ph.D. Thesis, University Ulm, Ulm, FRG.

Füldner, H. H. (1981) *Biochemistry 20*, 5707–5710.

Gamble, R. C., & Schimmel, P. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3011–3014.

Gruenewald, B. (1982) *Biochim. Biophys. Acta 687*, 71–78.

Gruenewald, B., Blume, A., & Watanabe, F. (1980) *Biochim. Biophys. Acta 597*, 41–52.

Gruenwald, B., Frisch, W., & Holzwarth, J. F. (1981) *Biochim. Biophys. Acta 641*, 311–319.

Harkness, J. E., & White, R. D. (1979) *Biochim. Biophys. Acta 552*, 450–456.

Herrmann, U., Tümmler, B., Maass, G., Mew, P. K. T., & Vögtle, F. (1984) *Biochemistry* (preceding paper in this issue).

Huang, C. (1969) *Biochemistry 8*, 344–352.

Inoue, S., Nishimura, M., Yasunaga, T., Takemoto, H., & Toyoshima, Y. (1981) *J. Phys. Chem. 85*, 1401–1405.

Kostelnik, R. J., & Castellano, S. M. (1973) *J. Magn. Reson. 9*, 291–295.

Kustin, K., Shear, D., & Kleitman, D. (1965) *J. Theor. Biol. 9*, 186–212.

Mabrey, S., & Sturtevant, J. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3862–3866.

Mabrey, S., & Sturtevant, J. M. (1979) *Methods Membr. Biol. 9*, 237–274.

Peters, F., & Teschner, W. (1979) *Comput. Programs Biomed. 10*, 125–132.

Ruf, H., & Grell, E. (1981) *Mol. Biol., Biochem. Biophys. 31*, 333–376.

Schwarz, G. (1965) *J. Mol. Biol. 11*, 64–77.

Seelig, A., & Seelig, J. (1975) *Biochim. Biophys. Acta 406*, 1–5.

Stulen, G. (1981) *Biochim. Biophys. Acta 640*, 621–627.

Stümpel, J., Eibl, H., & Niksch, A. (1983) *Biochim. Biophys. Acta 727*, 246–254.

Takemoto, H., Inoue, S., Yasunaga, T., Sukigara, M., & Toyoshima, Y. (1981) *J. Phys. Chem. 85*, 1032–1037.

Teissie, J., & Tsong, T. Y. (1981) *Biochemistry 20*, 1548–1554.

Tien, H. T., & Diana, A. L. (1968) *Chem. Phys. Lipids 2*, 55–101.

Träuble, H. (1971) *Naturwissenschaften 58*, 277–284.

Tsong, T. Y., & Kanehisa, M. I. (1977) *Biochemistry 16*, 2674–2680.

Tsong, T. Y., Tsong, T. T., Kingsley, E., & Siliciano, R. (1976) *Biophys. J. 16*, 1091–1104.

van Deenen, L. L. M., & de Haas, G. H. (1963) *Biochim. Biophys. Acta 70*, 538–553.

Watts, A., Marsh, D., & Knowles, P. F. (1978) *Biochemistry 17*, 1792–1801.

Wittebort, R. J., Blume, A., Huang, T. H., Das Gupta, S. K., & Griffin, R. G. (1982) *Biochemistry 21*, 3487–3502.

Woolley, P., & Diebler, H. (1979) *Biophys. Chem. 10*, 305–318.

Zimm, B. H., & Bragg, J. K. (1959) *J. Chem. Phys. 31*, 526–535.