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The phase diagram of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine/sucrose in the dry state. Sucrose substitution for water in lamellar mesophases

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The phase diagram of the binary system, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/sucrose, was determined by DSC. In contrast to dry DPPC, which exhibits chain melting at 342.5 K, the main feature of the DPPC/sucrose system is eutectic melting at 320 K. This was supported earlier by Crowe, J.H., Crowe, L.M. and Chapman, D. (Science 223 (1984) 701–703), who reported a drastic decrease in the chain-melting temperature of the dry lipid in the presence of some mono- and disaccharides. Electron microscopy suggests that the phase structures on either side of the phase transition are of the lamellar type. Definite sugar saturation concentrations can be derived from this phase diagram. Up to about 17 mol% sucrose, i.e., 1 mol of sucrose per 5 mol of lipid is adopted by DPPC in the low-temperature phase L_c. In the high-temperature phase L_α the saturation concentration is well above 90 mol% sucrose at 320 K (eutectic point) but decreases with increasing temperature. The lower limit of 50 mol% sucrose is reached at 455 K. At this temperature, peritectic melting of sucrose occurs. Because of some similarities in the phase diagrams of DPPC/sucrose and DPPC/water, it is possible to understand the sucrose substitution for water in dry lamellar mesophases.

Introduction

Sugar–lipid interactions are now of interest because it has been shown that some mono- and disaccharides are capable of stabilizing membranes during dehydration [1,2]. Some investigators have been able to demonstrate that even model systems like small and large unilamellar vesicles maintain their size and encapsulation after dehydration/rehydration in the presence of saccharides [2–5]. Furthermore, it has been shown recently that multilamellar liposomes can be formed in pure water-free glycerol and ethylene glycol [6].

As the substitution of water represents a problem of fundamental and practical interest, the aim of the present paper was to study the phase behaviour of the dry DPPC/sugar system. The dry state as the final state in water removal, especially, has not been well understood. Further, we will examine the hypothesis whether sugar is able to substitute for water in lamellar mesophases.

Materials and Methods

DPPC was purchased from Ferak (West Berlin). The lipid was pure as determined by TLC. The sucrose was pure and obtained from VEB Laborchemie Apolda.

The DPPC/sucrose mixtures were prepared in the following manner. Weighed amounts of dry DPPC and sucrose at the desired molar ratio were codissolved in a solvent mixture consisting of chloroform/ethanol/water (1 : 5 : 1, v/v/v). The DPPC/sucrose solution was dried by blowing with pure nitrogen gas. The sample was further dried carefully at high vacuum (under 10^{-3} Pa) for 24 h, transferred to the DSC capsules, then sealed and stored in a desiccator over P_2O_5 at high vacuum until use.

The DSC measurements were made using a DSC 2 (Perkin Elmer, U.S.A.). The heating rate was 5 K/min.

Abbreviations: DSC, differential scanning calorimetry; DPPC, 1-2-dipalmitoyl-*sn*-glycero-3-phosphocholine.

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The registered DSC scans are the first heating scans after storing the samples at 20 °C. The approach of constructing a binary phase diagram from the calorimetric data is well-known from the literature dealing with pseudobinary phospholipid systems [7–9]. According to this procedure, the onset and completion temperatures, i.e., the temperatures at which the calorimetric trace leaves and returns to the baseline, can be used after correction as solidus and liquidus temperatures, respectively. The error of this method was estimated to be about ±0.5 K. Further conclusions regarding the miscibility state were drawn on the basis of the DSC scan-line shape.

The samples for electron microscopy were prepared in different ways. For fracturing without freezing, the dry material was placed between freshly cleaved mica plates, rubbed with a glass rod and fractured by separation of the mica plates. Surface replication was done with the BALZERS apparatus BAF 400 D without cooling of the specimen desk (preparation at about 295 K) and using the highest possible heating of the specimen desk (323 K).

For freeze fracturing, samples of the material were either directly placed, or were suspended a short time before in paraffin oil then placed between copper specimen holders for the sandwich technique of Balzers, then equilibrated at the different temperatures for 30 min and quick-frozen by plunging into liquefied propane cooled to 88 K by liquid nitrogen. The specimens were fractured in a Balzers 400 D unit at 123 K using the double-replica device.

The replicas were cleaned successively with benzene, acetone and distilled water and examined in a JEM 100 B (JEOLCO, Tokyo) or BS 500 (Tesla, Brno) electron microscopes.

Results

The first heating scans of different dry DPPC/sucrose mixtures are shown in Fig. 1. It is well-known that pure DPPC-monohydrate exhibits at least three transition peaks [10–12], which we also observed. Only the first 342.5 K indicates the chain-melting process, i.e., the transition of DPPC from the crystalline phase L_c to the rhombohedral liquid crystalline phase R_a [13,14] (see Fig. 1). The subsequent peaks were not considered for pure DPPC and were not detected for sucrose-containing samples. The DSC thermograms of the first two samples with sugar mol fractions of $x = 0.07$ and 0.17 show simple but broadened peaks compared to pure DPPC. The transition peak temperature for $x = 0.07$ sucrose (Fig. 1) is higher than for pure DPPC. This is an indication of nonideal miscibility. For the same reason, the transition temperatures of sucrose for $x = 0.49$ are also higher than for pure sucrose (see below). As is typical of eutectic melting, a decrease of the liquidus

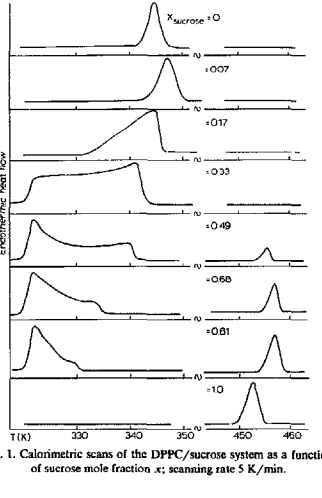

Fig. 1. Calorimetric scans of the DPPC/sucrose system as a function of sucrose mole fraction x; scanning rate 5 K/min.

temperatures with increasing sugar concentration was observed.

A further transition at about 455 K was observed at sucrose concentrations above a mole fraction of 0.49. The intensity of the transition increases with sugar concentration. We propose that this transition is due to the melting of excess sucrose, which is not miscible with DPPC in the 'molten' state L_m. Normally, the DSC scans were taken to only about 350 K to avoid chemical decomposition of the sample. The transition at about 455 K was obtained from the final heating scan. At this temperature, the sample decomposes and was not used for further experiments. However, the transitions at 455 K were obtained repeatedly. The melting temperature of pure sucrose is 452 K.

Restricted miscibility was also observed for the molten sugar and the 'molten' state DPPC. This is supported by the nearly horizontal coexistence line at 455 K indicating peritectic melting for sugar concentrations of x larger than 0.49. The results of the DSC measurements are summarized in the phase diagram in Fig. 2. The broken lines in the figure were drawn in accordance with the Gibbs phase rule. Nonhomogeneities in the baseline were observed for the sugar concentration of $x = 0.68$ at about 430 K (not shown in Fig. 1). We consider this to be an indication of the

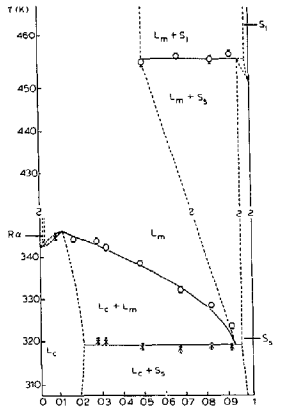

Fig. 2. Phase diagram of the DPPC/sucrose system. L_c, lamellar crystalline phase; L_m, lamellar phase of DPPC/sucrose mixture in the molten state; S_s, solid sucrose; S_l, liquid sucrose; R_α, rhombohedral liquid crystalline phase [13,14].

broken drawn coexistence line $L_m/L_m + S_m$ in Fig. 2. In order to separate the R_α region of DPPC-monohydrate known from the literature [13,14] and the L_m region (see below), a small miscibility gap was supposed in the low-sucrose region x less than 0.07.

Electron microscopy of replicas from fractured DPPC/sucrose below and above the phase transition temperature (345 K) for the system with a sucrose mole fraction of 0.49 is shown in Fig. 3. The replicas always show the basic structure of lamellar multibilayers. Below the phase transition temperature, the layers are flat with sharp fracture steps from layer to layer. This was observed using the preparation without freezing at 295 K (Fig. 3a) and at 323 K (Fig. 3b), as well as using the paraffin oil suspension technique after freezing from 295 K (Fig. 3c), 310 K (not shown) and 330 K (Fig. 3d). In the unfrozen fractured samples the formation of curved fracture steps is striking, often resulting in circular pieces of lamellae (Fig. 3a, b), whereas, with the paraffin oil suspension technique, the lamellae are characterized more by an irregular tilting, especially after freezing from 310 K and 330 K (Fig. 3d).

In the preparation frozen without paraffin oil from above the phase transition temperature at 353 K, the steps between the fractured lamellae are mostly more or less 'molten' (Fig. 3e and the right-hand side of Fig. 3f), but there are also areas with distinct edges of the steps (Fig. 3f). In the preparations with the paraffin oil and those frozen from 350 K, no 'molten' fracture steps of the lamellae were found. The lamellae are only partially flat and smooth, and a subdivision of the lamellae in smooth areas with rough features was mainly observed (Fig. 3g, h).

Discussion

The phase diagram of DPPC/sucrose constructed on the basis of the first DSC heating scans reveals a complex miscibility behaviour. In the low-temperature phase L_c, 5 mol of DPPC are able to adopt about 1 mol of sucrose. At higher sucrose concentrations a drastic decrease of the main phase transition temperature was observed. The addition of solid sucrose at x higher than 0.17 is accompanied by the formation of a miscibility gap, i.e., DPPC containing about 0.2 mol of sucrose per mol coexists with a nearly pure sucrose phase.

DPPC in the molten phase, L_m, adopts much more solid sucrose than in the low-temperature state, L_c. Our phase diagram (Fig. 2) suggests that 'molten' DPPC adopts sucrose up to about $x = 0.95$ at the eutectic temperature. This amount decreases with increasing temperature (indicated by the broken inclined line in Fig. 2) up to a sucrose mole fraction of 0.5, i.e. 1 mol per mol DPPC. This is also the limiting concentration of molten sucrose which is adopted by the 'molten' DPPC at about 455 K.

It should be mentioned that the second and subsequent heating traces differ from those in Fig. 1 (data not shown). They are most likely to represent thermodynamically unstable states of the DPPC/sucrose system. A lack of stability was also found in the DPPC/water system at very low water content [15]. Reversibility in the DPPC/sucrose system can be achieved by annealing the samples at 20 °C for about 1 week. These results are in accordance with DSC on dry DPPC/trehalose mixtures [16]. Lee and co-workers termed the transition 'conditionally' reversible. Nevertheless, the reported DSC scans of the DPPC/trehalose system (see also Ref. 1) exhibit single broadened peaks but no demixing phenomena were described. This, however, may be a problem of DSC sensitivity. Furthermore, the influence of the initial kind of solution or dispersion from which the lipid/sugar mixture was dried should also be taken into account [17].

DSC measurements do not reveal the phase structure. This is why we undertook some additional investigations. At the temperatures indicated, the electron microscopy replicas of the dry DPPC/sucrose system show features typical of lamellar multibilayers of hydrated lecithin/water dispersions. The bilayer arrangement in the dry state is also known from analyses of minimally hydrated phospholipid crystals [18,19].

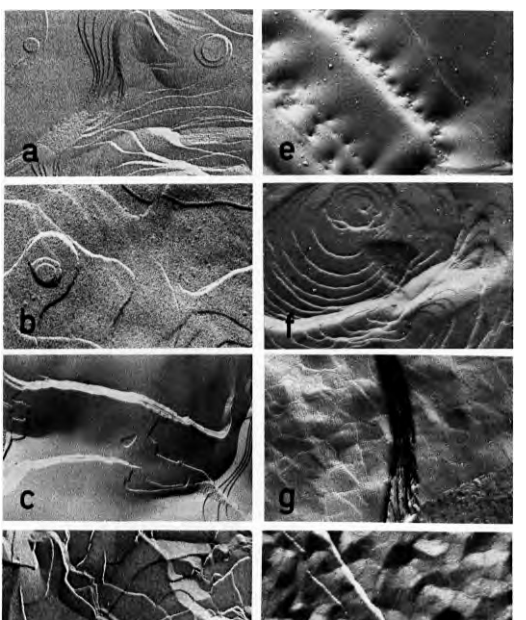

Fig. 3. Electron micrographs of fractured DPPC/sucrose, mole fraction of sucrose 0.49, below (a–d) and above (e–h) the phase transition temperature of this system. Replicas of unfrozen material at 295 K (a) and at 323 K (b), of material frozen after heating to 353 K (e, f) and of material suspended in paraffin oil and frozen from 295 K (c), 330 K (d) and 350 K (g, h). Orientation of the micrographs with the direction of shadowing from bottom to top. Magnifications: 50000× (a, b, g, h), 30000× (c–f).

For the molten state of dry DPPC/sucrose a real 'molten' appearance was found after freeze fracturing without suspending the sample in paraffin oil (Fig. 3e). It seems that the sample was fractured accidentally before freezing. The freezing thereby preserved the melting of the fracture steps. This explanation is supported by the contamination of the revealed faces with small particles. Where the specimen was really fractured in the frozen state, a transformation into a crystal-like state becomes visible. This is clearly demonstrated by the occurrence of screw dislocations (Fig. 3f). Unfortunately, at high temperatures, paraffin oil affects the host material because penetration into lipidic structures takes place. Freeze-fracture images corresponding to those in Figs. 3g and 3h were presented by McIntosh [20] for DPPC liposomes containing hexane.

Previously, we have done experiments on the stability and drug encapsulation of small unilamellar vesicles during dehydration [4]. At the end of the dehydration process the vesicles contain almost no water but sugar. Sucrose at concentrations higher than 1 mol of sugar per mol of egg phosphatidylcholine must be present on

both sides of the vesicle bilayers to maintain an existing concentration gradient, i.e., to maintain the bilayer structure in the dry state. In agreement with previously published results [2,5], these experiments show that vesicles may retain their bilayer arrangement in the dry state.

Based on the above arguments, we assume that the DPPC/sucrose phases are indeed lamellar phases. Furthermore, the first solid-state NMR experiments on a similar system, dry DPPC/trehalose, demonstrated that lamellar phases exist below and above the phase transition [16]. However, the motional properties of these lamellar phases differ from those in hydrated DPPC.

Tsvetkov et al. [17] have performed X-ray diffraction on DPPC-dihydrate/trehalose. They found that at room temperature the gel phase, L_β, prevails and, above the transition, the L_α phase prevails.

To summarize, at the transition temperature, T_c, the DPPC/sucrose system crosses from the crystalline phase, L_c, to the lamellar 'molten' phase, L_m.

On the basis of our experimental findings, we propose that the phase diagrams of DPPC/sucrose and DPPC/water exhibit some similarities. Fig. 4a is a section of the phase diagram of Fig. 2 omitting the low- and high-sucrose-containing regions. Fig. 4b shows a section of a schematic DPPC/water phase diagram (based on Refs. 15 and 21). The latter diagram is more complex because of the presence of an additional low-temperature phase, the ripple phase $P^{\prime}$, and of additional liquid-crystalline phases at lower water content influencing the course of the coexistence lines in this concentration range (not shown in Fig. 4b, see Refs. 10–12). Despite the differences between DPPC/water and DPPC/sugar phase diagrams, neglected in our schematic representation, some similarities are obvious.

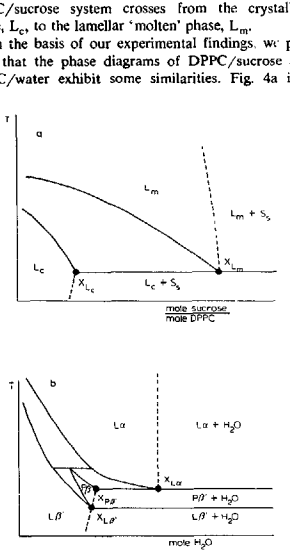

Fig. 4. Schematic phase diagram of (a) the DPPC/sucrose system and (b) the DPPC/water system. Concentrations are expressed in mol per mol. $x\mathrm{L}_{\beta'}$, $x\mathrm{P}_{\beta'}$ and $x\mathrm{L}_\alpha$ denote the amount of water adopted by DPPC in $\mathrm{L}_{\beta'}$, $\mathrm{P}_{\beta'}$ and L_α phases. $x\mathrm{L}_c$ and $x\mathrm{L}_m$ denote the amount of solid sucrose adopted by DPPC in crystalline and 'molten' states, respectively.

Firstly, the interaction of DPPC with water or sugar results in a drastic decrease of the main phase transition temperature (eutectic melting). Secondly, in both phase diagrams one finds different saturation concentrations for the low- and high-temperature phases. The respective saturation points are denoted by $x\mathrm{L}_{\beta'}$, $x\mathrm{L}_\alpha$ for the DPPC/water system and are well-known [22,23]. The saturation points for the DPPC/sucrose system are denoted by $x\mathrm{L}_c$ and $x\mathrm{L}_m$, respectively. Thirdly, in both diagrams, DPPC adopts more of the second component, H_2O or sucrose, in the high-temperature phase L_α or L_m.

We believe that these similarities in the phase diagrams of DPPC/water and DPPC/sucrose yield the thermodynamic explanation why sucrose or some mono- and disaccharides in general substitute for water in lamellar phases and biological systems in the dry state.

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References

1 Crowe, J.H., Crowe, L.M. and Chapman, D. (1984) Science 223, 701–703.
2 Crowe, L.M., Crowe, J.H., Rudolph, A., Womersley, C. and Appel, L. (1985) Arch. Biochem. Biophys. 242, 240–247.
3 Strauss, G., Schurtenberger, P. and Hauser, H. (1986) Biochim. Biophys. Acta 858, 169–180.
4 Henischel, M. and Volke, F. (1985) in Fortschritte der Onkologie Vol. 13; Liposomen-Eigenschaften-Anwendung (Arndt, D. and Fichtner, I., eds.), Akademie-Verlag, Berlin.
5 Madden, T.D., Bally, M.B., Hope, M.J., Cullis, P.R., Schieren, H.P.a nd Janoff, A.S. (1985) Biochim. Biophys. Acta 817, 67–74.
6 McDaniel, R.V., McIntosh, T.J. and Simon, S.A. (1983) Biochim. Biophys. Acta 731, 97–108.

7 Doerfler, H.D. and Brezesinski, G. (1983) Colloid Polymer Sci. 261, 427–433.
8 Doerfler, H.D. and Brezesinski, G. (1983) Colloid Polymer Sci. 261, 286–292 and 241–244.
9 Mabrey, S. and Sturtevant, J.M. (1976) Proc. Natl. Acad. Sci. USA 73, 3862–3866.
10 Doerfler, H.D. and Brezesinski, G. (1983) Colloid Polymer Sci. 261, 245–250.
11 Kodama, M., Kuwaba, M. and Seki, S. (1982) Biochim. Biophys. Acta 689, 567–570.
12 Chapman, D., Williams, R.M. and Ladebrooke, B.D. (1967) Chem. Phys. Lipids 1, 445–475.
13 Tardieu, A., Luzzati, V. and Reman, F.C. (1973) J. Mol. Biol. 75, 711–733.
14 Diehl, M. (1980) PhD thesis, MLU Halle/S.
15 Doerfler, H.D. and Brezesinski, G. (1983) Colloid Polymer Sci. 261, 329–334.
16 Lee, C.W.B., Waugh, J.S. and Griffin, R.G. (1986) Biochemistry 25, 3737–3742.
17 Tsvetkov, T.D., Tsonev, L.I., Tsvetkova, N.N., Koynova, R.D. and Tenchov, B.G. (1989) Cryobiology, in press.
18 Dorset, D.L. (1987) Biochim. Biophys. Acta 898, 121–128.
19 Pascher, I., Sundell, S., Harlos, K. and Eibl, H. (1987) Biochim. Biophys. Acta 896, 77–88.
20 McIntosh, T.J. and Costello, M.J. (1981) Biochim. Biophys. Acta 645, 318–326.
21 Ulmius, J.H., Wennerstrom, H., Lindblom, G. and Arvidson, P. (1977) Biochemistry 16, 5742.
22 Kjellander, R.J. (1978) Colloid Interface Sci. 66, 42.
23 Janiak, M.J., Small, M.D. and Shipley, G.G. (1979) J. Biol. Chem. 254, 6068–6078.