Biochimica et Biophysica Acta 940 (1988) 219–240
Elsevier

BBA 73984

Phosphatidylcholine-fatty acid membranes. I. Effects of protonation, salt concentration, temperature and chain-length on the colloidal and phase properties of mixed vesicles, bilayers and nonlamellar structures

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(Received 1 December 1987)

Key words: Phosphatidylcholine; Fatty acid; Phase behavior; Vesicle aggregation; Membrane fusion; Bilayer hydration

The phase and colloidal properties of phosphatidylcholine/fatty acid (PC/FA) mixed vesicles have been investigated by optical methods, acid-base titration, and theoretically as a function of temperature (5–80 °C), molar lipid ratio (0–1), lipid chain length (C_{14}–C_{18}), headgroup ionization ($1.5 \leqslant pH \leqslant 10$), vesicle concentration (0.05–32 μmol vesicle $\cdot$ dm^{-3}, and ionic strength ($0.005 \leqslant J \leqslant 0.25$). Increasing the fatty acid concentration in PC bilayers causes the phase transition temperatures (at $4 \leqslant pH \leqslant 5$) to rise until, for more than 2 FA molecules per PC molecule, the sample turbidity exhibits only two transitions corresponding to the chain-melting of the 1:2 stoichiometric complexes of PC/FA, and pure fatty acid. The former transition is into a nonlamellar phase and is accompanied by extremely rapid vesicle aggregation (with association rates on the order of $C_a \approx 10^7$ dm$^3 \cdot$ mol$^{-1} \cdot$ s^{-1}) and massive lipid precipitation. Fluid-phase vesicles with less than 2 FA per PC associate much more slowly ($C_a \approx 10^3$ dm$^3 \cdot$ mol$^{-1} \cdot$ s^{-1}), their aggregation being comparable to that of the ordered-phase liposomes. Under no conditions was the relation between the fatty acid concentration and the vesicle association rate for the fluid-phase vesicles linear. In contrast to the X-ray diffraction data, optical measurements reveal a 'pretransitional region' between the chain-melting temperature of the PC component and the temperature at which the gross transformation into a nonlamellar phase sets in. This is seen for all lipid mixtures investigated. On the relative temperature scale, lipids with different chain lengths behave qualitatively similarly; however, the effective association constants determined for samples of constant lipid concentration seem to decrease somewhat with the number of CH_2 groups per chain. Fatty acid protonation, which yields electrically neutral bilayers, invariably increases the rate of vesicle association; we have measured, for example, $C_a \approx 10^2$ at pH $\approx$ 7 and $C_a \approx 10^7$ dm$^3 \cdot$ mol$^{-1} \cdot$ s^{-1} at pH $\approx$ 4). Protonation of the phosphatidylcholine phosphate groups, which causes a net positive charge to accumulate on the lipid vesicles, initially increases ($C_a \approx 10^8$ dm$^3 \cdot$ mol$^{-1} \cdot$ s^{-1}) but ultimately decreases ($C_a \approx 10^7$ dm$^3 \cdot$ mol$^{-1} \cdot$ s^{-1}) the rate of association between PC/FA (1:2) mixed vesicles. The presence of ions also facilitates the association between PC/FA mixed vesicles, not only for

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; PE, phosphorylethanolamine; PC, phosphatidylcholine; FA, fatty acid; MA, myristic acid; PA, palmitic acid; SA, stearic acid; J, ionic strength; T_m, chain-melting phase-transition temperature; T_h, lamellar-to-nonlamellar transition temperature; NBD, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl); NMR, nuclear magnetic resonance; A, optical absorbance; TLC, thin-layer chromatography.

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charged but also for electrically neutral vesicles. This shows that the nonelectrostatic effects of protonation and ion screening, which probably arise from changes in the interfacial polarity (hydrophilicity), significantly affect the colloidal and phase properties of such mixed PC/FA membranes. Concomitantly, surface electrostatics and interfacial hydration together determine the titration properties of PC/FA (1:2) membranes, the latter effect in acidic suspensions being at least as strong as the former. From our C_a vs. pH data the pK values at ionic strength $J = 0.005$ (0.1) for FA and PC in the fluid phase are found to be 7.5 (≈ 7) and 1.5, respectively. This suggests the electrostatic and hydrational contributions to the pK shifts are 1.5 and 1 pH units, respectively, again indicative of the importance of interfacial hydration. Direct acid-base titrations yield similar results and provide evidence, moreover, for the occurrence of an isothermal, pH-induced phase transition at 65 °C around pH 6 for the C_{14} mixture.

Introduction

The concentration of total free (i.e., chemically unbound) fatty acids in certain biological membranes can be quite high: as much as 8% in rat liver plasma membranes [1] and 5% in the sarcolemmal membranes of cardiac muscle after myocardial ischaemia [2]. The local concentration of fatty acids may be substantially higher than this, however, since they are probably distributed nonuniformly within biomembranes.

Free fatty acids have been shown to alter a variety of membrane-mediated cellular functions such as lymphocyte mitogenesis [3], surface receptor capping [4], integral enzyme activity [5], calcium transport [6,7], membrane permeability [8,9] and fusion [10–12]. To date, however, little is known about the molecular origin of most of these effects.

The effects of fatty acids on the properties of lipid bilayer membranes have been examined in a number of studies. Verma et al. [13], for example, have investigated a series of phosphatidylcholine/fatty acid mixtures by Raman spectroscopy. They concluded that fatty acids with a sigle *cis*-double bond decrease, and those with a *trans*-double bond increase the chain-melting phase transition temperature of PC/FA mixed lipid bilayers, but also cause the chain melting to become a multiple process. Preferential partitioning of the palmitic acid component into ordered phase clusters at the transition was inferred on the basis of deuterium nuclear magnetic resonance data on DPPC/PA mixtures by Pauls and colleagues [14]. These authors have also confirmed, by using perdeuterated samples, that DPPC/PA (8:2) mixed vesicles exhibit complex chain-melting phase behaviour. Fatty acids, moreover, were found in this study to increase the average order parameter of the bilayer interior and to slow down the motions of the phospholipid side-chains. This conclusion is probably trustworthy, despite the fact that Klausner et al. [4] have claimed, on the basis of fluorescence polarization studies, that saturated fatty acids do not much influence the properties of the bilayer interior, but rather have an ordering effect on the interfacial region.

The phase behaviour of various phosphatidylcholine/fatty acid mixtures have been investigated in some detail by calorimetry [15,16] or spectroscopic methods [17,18]. Eliasz and collaborators [19] have reported the chain-melting phase-transition temperatures, as studied by the differential scanning calorimetry, to shift upwards upon the introduction of fatty acids into dipalmitoylphosphatidylcholine bilayers. Kantor and Prestegard [17,18] by using NMR have established phase diagrams of the peritectic type, indicating nonideal mixing, and provided circumstantial evidence for the formation of interlipid complexes. Mabrey and Sturtevant [15], in a more extended report, concluded that the palmitic acid molecules in such complexes pack with dipalmitoylphosphatidylcholine chains in a one-to-one ratio, resulting in a single, relatively sharp chain-melting phase transition at 61.5 °C. Finally, Marsh and Seddon [20] have shown, by X-ray diffraction and ^{31}P-NMR, that this transition is directly into a nonlamellar, inverted hexagonal phase.

The effect of fatty acids on phospholipid phase behaviour will be persued in extenso in a paper to follow, reporting the results of our detailed X-ray diffraction studies on phospholipid/fatty acid mixed systems (Seddon, J.M., Cevc, G. and Marsh,

D., unpublished data). Here we present the results of, to our knowledge, the first systematic study of the effect of fatty acids, in mixtures with homologous chain length phosphatidylcholines, on the physicochemical – particularly collodial – properties of such mixed vesicles as a function of fatty acid/phosphatidylcholine molar ratio, lipid chain length, temperature, pH and ionic strength. Based on a detailed theoretical analysis of the data we conclude that fatty acids do indeed form preferential 1 : 2 stoichiometric complexes with phosphatidylcholine molecules, and thereby modify both the electrostatic – and especially – the surface hydration properties of the resulting mixed systems. Because of this intimate intermolecular interaction, the dissociation constants of both of the lipid components are significantly altered compared to the values for the individual monomers; this effect may be important for the biological role of fatty acids. A further consequence of the formation of the fatty acid/phosphatidylcholine complexes is that vesicles consisting of these lipids can aggregate and associate very rapidly, nearly as fast, for example, as phosphatidylserine vesicles upon the addition of calcium. This provides insight into the mechanism of fatty acid induced membrane fusion, which will be the topic of further publications.

Materials and Methods

Chemicals. Diacylphosphatidylcholines DMPC, DPPC, DSPC, puriss., were purchased from Fluka (Neu-Ulm, F.R.G.); they were shown prior to be used in experiments to be pure by TLC on silicic acid plates using a solvent system of $CHCl_3$/CH_3OH/33%NH_3 (65 : 35 : 5) with molybdenum blue staining and subsequent sulphuric acid charring and were used without further purification; TLC after experiments revealed no marked deterioration products. MA, PA and SA (puriss.) were also from Fluka; NaOH, HCl and NaCl were products of Merck (Darmstadt, F.R.G.) and were p.a. The water was doubly distilled.

Vesicle preparations. To prepare mixtures of a controlled phosphatidylcholine/fatty acid ratio, appropriate amounts of the dry lipids were weighed into glass vials and then dissolved completely in $CHCl_3/CH_3OH$ (4 : 1). Subsequently, these specimens were dried at 45–50 °C under a stream of nitrogen, which was directed so as to stir the solution, and then desiccated in vacuo (10^2 Pa) for 24 h.

Lipid suspensions for the measurements of the association rates were prepared by adding a warm electrolyte solution to the dry lipid mixture, so that the final lipid chain concentration was 3 mM (unless stated otherwise) and the final pH value of the resulting lipid preparation at ambient temperature was approx. 10, with an ionic strength J = 0.0025. The lipid preparations were then suspended with an ultrasound transducer (Biosonik III (Bronwill, U.S.A.), setting 30, for 15 min at room temperature) until they became optically clear ($A_{400nm} \leqslant 0.03$ for 1 cm lightpath). After this treatment the suspensions contained essentially only small, unilamellar vesicles, as confirmed by gel chromatography on Sephacryl 200 columns *. A new stock of suspension was used for each series of measurements.

Vesicle suspensions were routinely aged for 2 days approx. 10 C° above the chain-melting temperature of the PC component prior to experiments. This was found to be necessary, since we noticed, by optical and microscopic observation, that the DMPC/MA (and to a lesser extent also DPPC/PA) samples which were kept at low temperatures reverted gradually into a different phase, characterized by the presence of threadlike (or occasionally needle-like) structures; these structures disappeared for the stoichiometric mixtures of DMPC/MA (1 : 2) (or DPPC/PA (1 : 2)) upon heating the suspensions above about 32 (about 50) °C.

For the investigations of the effects of salt a stock lipid suspension was split into two aliquots and the ionic strength of one of these was increased by the addition of dry NaCl followed by a brief, additional ultrasonication, to J = 0.25. Specimens of varying salt concentration prepared

* Vesicles consisting of PC/FA in 1 : 2 molar ratio, which are the main object of this work, exhibited only one, rather sharp phase transition. This proves that their membranes were not asymmetrical. We can not, however, exclude the possibility that for intermediate fatty acid concentrations the inner and outer lipid layers were of slightly unequal composition.

by mixing these two stock solutions were left to equilibrate in order to avoid the generation of transmembrane salt gradients.

Determination of phase-transition temperatures. Transition temperatures were determined by measuring the change in optical absorbance at 400 nm in a Beckmann 3400 UV/VIS spectrometer connected to an *xy*-recorder. Lipids were introduced as stirred suspensions in a 1 cm lightpath cuvette or used as concentrated samples inside flat mica containers positioned into cuvettes. The sample temperature was scanned by a programmable temperature controller, typically at a rate of 0.5 C° per min, and measured by a miniature Fe-Ni thermocouple immersed directly in the sample cuvette; this signal was fed into a linearized, digital thermometer (accuracy $\pm$ 0.2 C°) connected to the *xy*-recorder.

Measurement of the association constants. To measure the rate of vesicle association as a function of pH, salt and vesicle concentration, an aliquot of the stock lipid suspension with pH $\approx$ 10 was first heated in a cuvette in the spectrometer to the desired temperature. Subsequently, variable amounts (e.g., to achieve pH 3.5, approx. 400 μl) of 0.025 M HCl were injected into the cuvette under constant stirring. (This ensured that the pH value of the sample reached 90% of its final value within 0.5 s, and produced samples with ionic strengths $0.003 \leqslant J \leqslant 0.08$.) Optical absorbance A_{400} was plotted as a function of time on the strip-chart recorder and then analyzed as described in the Discussion section. The temperature variation within the sample, caused by the injection of the acid, was $\leqslant 1.5$ C° at the high, and essentially negligible at the low-temperature end. Ultimately, the pH value of the suspension was determined directly with an accuracy of 0.05 units by using a glass pH electrode and calibrated digital pH-meter.

Calculation of the association rates. The rates of vesicle association were determined from the measured A vs. t recordings by analyzing the data using the mass action kinetics model of vesicle aggregation of Bentz and Nir [22,23]. In brief, this is as follows.

Vesicles in the system, which initially consists of $v_1(t=0) \equiv v_0$, identical spherical particles may be assumed to aggregate according to the mass-action reaction

$$v_i + v_j \xrightarrow{C_a} v_{i+j} \tag{1}$$

where C_a is the rate of association; the kinetic equation describing this reaction is, assuming that all of the association steps are equivalent and also irreversible:

$$[\mathrm{d}v_n(t)/\mathrm{d}t] = -\sum_{i=1}^{m-n}(1+\delta_{in})[v_i(t)][v_n(t)]C_a + \sum_{i=1}^{n/2} C_a[v_i(t)][v_{n-i}(t)] \tag{2}$$

with $\delta_{in} = 1 \Leftrightarrow i = n$; m is the maximum allowed aggregate size [22].

Numerical integration of Eqn. 2 in terms of the concentration of n-mers, $[v_n(t)]$, yields the time-course of vesicle aggregation explicitly. However, it is also possible to describe accurately, albeit phenomenologically, the temporal variation of the equilibrium mass-average aggregate size, $\langle n \rangle \equiv \Sigma_{i=1}^{m} i^2[v_i(t)]/[v_0(t)]$, which results from the aggregation between vesicles, by the simple expression

$$\langle n \rangle = \sqrt{[1 + 4C_a v_0 t + (C_a v_0 t)^2]} \tag{3}$$

as has been shown by Bentz and Nir [22].

In the limit of validity of Rayleigh-Gauss-Debye scattering theory, which is applicable as long as the aggregate size is much smaller than the wavelength of the incident light [24], the quantity $\langle n \rangle$ is directly related to the sample turbidity:

$$\langle n \rangle = 1 + F \tag{4}$$

where F measures the increase in the optical absorbance of the sample, $F = A(t)/A(t=0)$. In terms of the time interval, t_F, during which the absorbance of the vesicle suspension increases by a factor $(1 + F)$, the association rate can, consequently, be written as:

$$C_a = \left\{[(1+F)^2 + 3]^{\frac{1}{2}} - 2\right\}/t_F v_0 \tag{5}$$

We have used Eqn. 5 throughout this work to obtain the vesicle association rates from our mea-

sured A vs. t curves; a further assumption was that the vesicle radius was initially $r_v = 80$ nm, i.e. that each vesicle consists of $5 \cdot 10^5$ acyl chains (assuming $A_{chain} = 0.3$ nm^2, which is tantamount to postulating that $[v_0] \equiv 0.2 \times$ (acyl concentration) $\times 10^{-5}$. By doing so we have neglected the variation of vesicle size with lipid chain length, an assumption which, at least for pure phosphatidylcholine vesicles, seems to be tolerable [25], and with time, as the latter aspect will be dealt with in a separate paper.

Alternatively, the rate of association can also be expressed in terms of the collision rate, C_c, and of the vesicle–vesicle interaction potential, U, approximately [26] as:

$$C_a \approx C_c \cdot \left\{ 2 \int_0^\infty dx (D^\infty/D) \exp(U(x)/RT) / [r_v (x/r_v + 1)^2] \right\}^{-1}$$

$$\approx C_c \exp(-U(d_{eqv})/RT)$$

$$= (4RT/3\eta) \exp(-U(d_{eqv})/RT) \quad (6)$$

where the dimensionless parameter $(D^\infty/D) \rightarrow (2d/r_v)$ [27] accounts for the viscous intervesicle interactions [26]. This result implies that the actual association rate is proportional to the diffusion-controlled association rate, $C_c \approx 8.5 \cdot 10^9$ dm$^3 \cdot$ mol$^{-1} \cdot$ s^{-1}, corrected for the effect of vesicle–vesicle interaction potential, U, here expressed per mol of vesicles. η is the bulk suspension viscosity, d_{eqv} an effective intervesicle separation (see further discussion), and R the gas constant.

Eqn. 6 provides the means (by combining Eqns. 5 and 6) for calculating the intervesicle interaction potential from the measured optical absorbance data

$$U_{ex} = RT \ln\left\{ 4RT t_F v_0 / 3\eta \left\{ [(1+F)^2 + 3]^{\frac{1}{2}} - 2 \right\} \right\} \quad (7)$$

but it is, of course, also possible to evaluate this potential indirectly from the association rates by using

$$U_{ex} = RT \ln[4RT/3\eta C_a] \quad (8)$$

Direct and fluorescence microscopy. Samples for direct (phase contrast) and fluorescence microscopy contained 1% (weight) of 1,2-dipalmitoyl-*sn*-glycero-3-L-phosphorylethanolamine-*N*-(pyrenesulfonyl) (Calbiochem, Frankfurt, F.R.G.) as a marker. They were viewed at a magnification of 400 on a Zeiss Axiomat microscope equipped with a water-heated stage. For fluorescence measurements the excitation wavelength was 340 nm and the emission was filtered by a 380 nm cutoff filter. Alternatively, samples with 1% of NBD-PE were irradiated with 490 nm light and measured at 530 nm. Direct observations were made in the phase-contrast mode.

Acid-base titrations. These were performed by adding 10 μl amounts of 0.1 M HCl or NaOH to 25 ml of stirred, unbuffered, thermostated suspensions of DMPC/MA (with a molar concentration of 0.75/1.5 (1 : 2 mol/mol) and initial ionic strength of 0.0025 or 0.1); the bulk pH value was monitored directly with a glass electrode attached to a digital pH meter. After each titration step the reading was permitted to settle, but not longer than 15 min. Experiments were performed in duplicate or triplicate.

Results

Effect of bilayer composition

Depending on their detailed composition, vesicles consisting of homologous phosphatidylcholine/fatty acid (PC/FA) mixtures at pH $\leqslant$ 5.5 undergo several structural transitions in the temperature ranges of 8–57, 30–63, or 48–73°C, for the myristic, palmitic or stearic chain lipids, respectively.

By measuring the temperature dependence of the optical absorbance of lipid suspensions at 400 nm (data not shown) we have confirmed that the behaviour at or near both termini of the (pseudo-binary) phase diagram of these lipids is essentially identical to that of the pure phosphatidylcholine or fatty acid compounds as one would expect, the experimental sensitivity for the samples with high proportion of fatty acid being relatively low.

In PC/FA mixtures with 5 to about 35% fatty acid concentration the thermal behaviour of the system is as expected from the corresponding phase rules: up to five subsequent phase transitions are observed, indicative of the complex phase be-

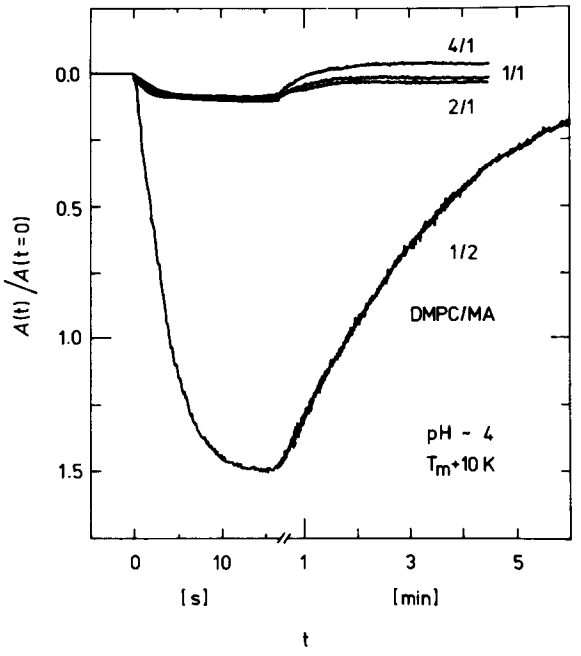

Fig. 1. Protonation-induced change in the relative optical absorbance of vesicle suspensions containing mixed vesicles of dimyristoylphosphatidylcholine and myristic acid (with a variable DMPC/MA molar ratio but at constant total chain concentration of 1.5 mM) 10 C° above the chain-melting transition temperature, T_m. The experiments were conducted by rapidly injecting 0.025 M HCl into the prewarmed, unbuffered lipid suspension with pH ≈ 10, while recording the absorbance at 400 nm on a strip-chart recorder.

haviour of such partly phase separated mixtures. Analysis of this behaviour will be one of the topics of a subsequent paper (our unpublished data) and Schullery and colleagues [16] have also published relevant data; here it will suffice to say that the (1 : 2) phosphatidylcholine/fatty mixtures or lipid systems containing fatty acids in excess of 65% (mol) typically exhibit only one, or at most two phase transitions between 5 and 80 °C: the 'strong', with fresh samples well-resolved transitions at 48, 62 or 73 ° C for the myristoyl, palmitoyl and stearoyl lipids; secondary transitions, which are never well resolved, show up in the absorption measurements occasionally, at 54, 63 and 70 °C for C_{14}, C_{16} or C_{18} chains, respectively. The former set of transition temperatures corresponds to the chain-melting transitions of the stoichiometric (1 : 2) PC/FA complexes and the latter to those of residual, phase-separated fatty acid.

Fig. 1 shows recordings of the optical absorbance of lipid suspensions 5 C° above the corresponding chain-melting phase transition as a function of time after a fast (within 0.5 s) pH-jump from 10 to ⩽ 4. Results obtained with various mixtures of the homologous phosphatidylcholine and fatty acids measured at 400 nm are shown, but using different wavelengths yields essentially identical results (data not shown).

The turbidity of pure phosphatidylcholine samples is hardly affected by such a pH-jump on the time-scale of our experiments; in contrast to this, the optical absorbance of the samples with a fatty acid proportion higher than 65% increases rapidly after the acidification of the suspension, indicative of an extensive, fast and (at least partly) irreversible vesicle association. The latter is accompanied by macroscopic, massive lipid precipitation and/or formation of sticky lipid clumps at high temperatures. Our X-ray studies (unpublished data) but also visual inspection in phase-contrast mode and fluorescence microscopy have shown that these clumps arise from lipids in the inverted hexagonal phase, H_{II}.

The data presented in Fig. 2 show that concentrations of fatty acid in excess of about 65 mol% as well as elevated temperature are prerequisites for fast lipid precipitation following acidification of the lipid suspension. Samples mea-

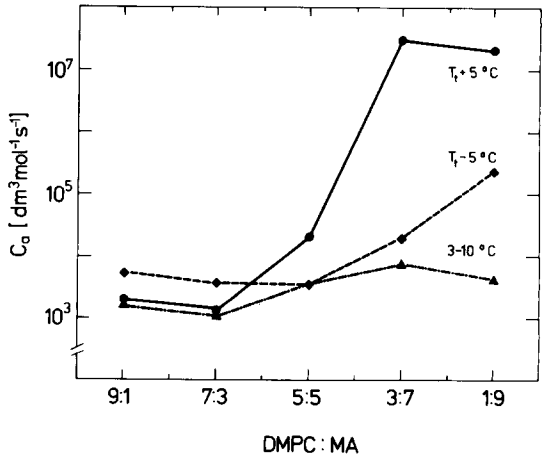

Fig. 2. Rate of protonation-induced association between small vesicles consisting of dimyristoylphosphatidylcholine/myristic acid (1 : 2) as a function of lipid molar ratio and relative temperature after a pH-jump from 10 to 3. The association constant has been evaluated from the measured temperature scans of the absorbance at 400 nm by means of Eqn. 5.

sured below the lipid chain-melting transition temperature and especially specimens kept at $T = 3–10\,^{\circ}C$ associate relatively slowly and poorly, the corresponding association rates being in the order of $2 \cdot 10^3 \leqslant C_a \leqslant 10^4$ $dm^3 \cdot mol^{-1} \cdot s^{-1}$, nearly independent of fatty acid concentration except, perhaps, at the highest FA/PC ratios. These observations suggest that a certain minimum number of fusogenic lipids, as well as some critical degree of lipid fluidization, must both be present before a gross vesicle precipitation can occur.

Temperature effects

To clarify the molecular mechanism of the effect of temperature on the colloidal behaviour of systems containing fatty acids we have investigated the temperature variation of the rate of association of (1 : 2) PC/FA mixed vesicles in some detail.

Several examples of these results are presented in Fig. 3). After a pH-jump from 10 to the final value of about 4 at temperatures close to or below the chain-melting temperature of the phosphatidylcholine component, T_m, the sample turbidity is relatively insensitive to temperature: acidification of the suspension in this temperature range induces, at most a minute change in optical absorbance in the order of 0.4 A units, stemming from the macroscopic vesicle flocculation (Fig. 3, upper curve). In contrast to this, at temperatures close to or higher than T_h the sample turbidity increases by one or two orders of magnitude within seconds (Fig. 3, lower two curves); after having reached a maximum (see subsequent paragraphs) it then decreases again on the time-scale of 10 s because the suspension becomes depleted of lipid vesicles owing to the adhesion of the lipid material to the cuvette walls.

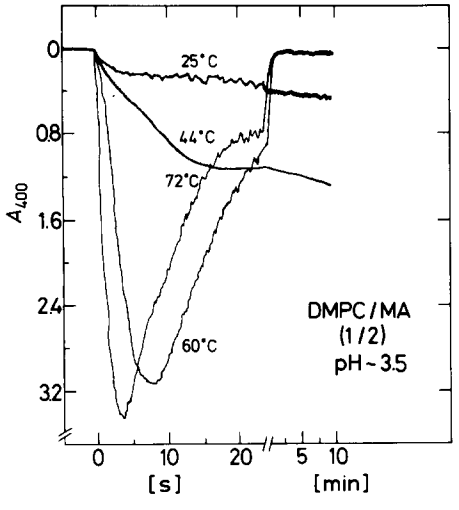

Fig. 3. Optical absorbance of dimyristoylphosphatidylcholine/myristic acid (1 : 2) mixed vesicles as a function of temperature and time following a fast ($\leqslant$ 0.5 s) jump in the pH value of the suspension: pH 10 → 3. The total acyl-chain concentration was 3 mM.

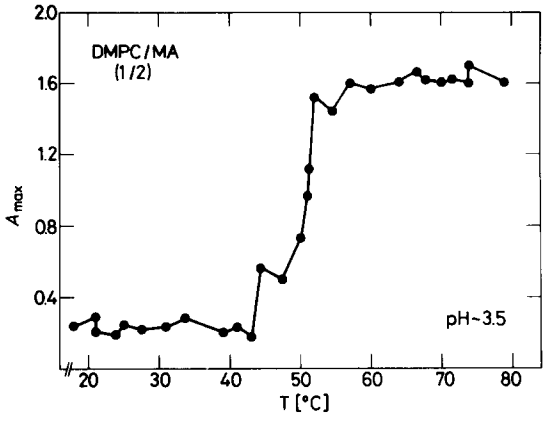

Fig. 4. Maximal absorbance of dimyristoylphosphatidylcholine/myristic acid (1 : 2) mixed vesicles measured after the pH of the prewarmed lipid suspension with a total chain concentration of 3.0 mM was changed from 10 to 3. (Obtained from recordings similar to those reproduced in Fig. 3.)

The appearance of a temporal turbidity maximum is affected by the composition, temperature, salt content, and pH of the system. In our experiments with phosphatidylcholine/fatty acid mixtures the appearance of such a maximum always coincided with the formation of structures in the nonlamellar, inverted hexagonal phase, the critical temperature for this being identical to the lamellar-to-nonlamellar transition temperature, for the myristic lipid mixtures at $T_h = 48\,^{\circ}C$ (Fig. 4).

To substantiate this conclusion we have performed a series of experiments with (1 : 2) phosphatidylcholine/fatty acid mixtures of various chain lengths. On the one hand, these have been characterized by X-ray diffraction (unpublished data); on the other hand, the maximal turbidity and the association rate of these vesicles were measured as a function of temperature and the transition temperature, T_h, which was identified

with the point of maximal increase in the C_a vs. T curve.

Chain length effects

Fig. 5 shows, for various chain lengths, the highest value of the optical absorbance as a function of temperature for phosphatidylcholine/fatty acid (1 : 2) mixed samples containing 3 mM total concentration of acyl chains. Results for the palmitoyl and stearoyl lipids are very similar to those found for DMPC/MA (1 : 2) mixtures (cf. Fig. 4): the absorbance abruptly increases exactly at the temperature at which the lipid mixture undergoes a transition into a nonlamellar phase, T_h [DMPC/MA (1 : 2)] $\approx 48\,^\circ$C, T_h [DPPC/PA (1 : 2)] $\approx 62\,^\circ$C and T_h [DSPC/SA(1 : 2)] $\approx 72\,^\circ$C. With the palmitoyl and especially with the stearoyl lipids, moreover, a small but significant increase in the maximal absorbance is detected at temperatures above the corresponding chain-melting phase transition of the phosphatidylcholine component, i.e., at $T \geqslant 42$ or $\geqslant 55\,^\circ$C, for C_{16} and C_{18} lipids, respectively.

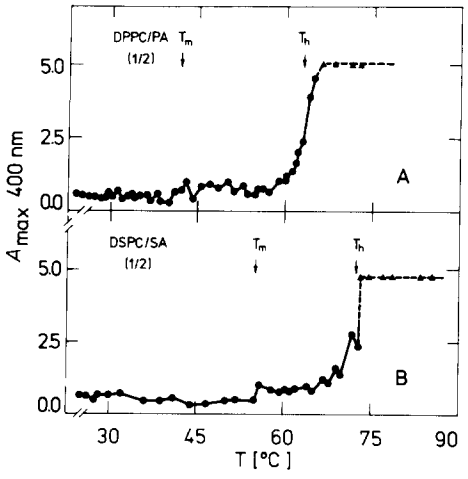

Fig. 5. Effects of acyl chain length and temperature on the protonation-induced maximum in the absorbance of suspensions of phosphatidylcholine/fatty acid (1 : 2) mixed vesicles. T_h and T_m denote the lamellar-to-non-lamellar and chain-melting phase transition temperature, of the 1 : 2 mixture and of the pure PC component, respectively. Note that the data points at $T \geqslant T_h$ in the case of palmitoyl and stearoyl lipids give only the lower limit to the real values, as with our instrument the maximum was unresolved in the case of 3 mM total chain concentration, which was kept constant throughout experiments. PA, palmitic acid; SA, stearic acid.

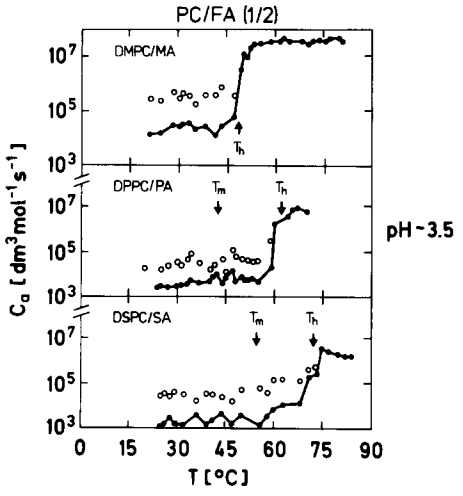

Fig. 6. Chain length and temperature effects on the rate of association between phosphatidylcholine/fatty acid (1 : 2) mixed vesicles at pH $\approx$ 3.5. T_h and T_m denote the lamellar-to-nonlamellar and chain-melting phase transition temperature, respectively. MA, myristic acid; PA, palmitic acid; SA, stearic acid.

Similar features are observed in studies of the temperature dependence of the rate of vesicle association (Fig. 6). Depending on whether the initial or the terminal parts of A vs. t curves are analysed (dots and solid curves in Fig. 6, respectively) the association constants for fluid-phase vesicles are found to increase between $2 \cdot 10^2$- and $\geqslant 10^4$-fold with respect to the corresponding low-temperature, ordered-phase values.

Owing to the relatively large scatter of our C_a data, which is substantially greater than that of the corresponding A_{max} points, it is not perfectly clear at present – but nevertheless probable – that the rate of vesicle association increases at the chain-melting temperature of the phosphatidylcholine component, as does the maximum absorbance value. It is definite, however, that the rate of association of phosphatidylcholine/fatty acid (1 : 2) mixed vesicles gradually decreases with increasing chain length.

Vesicle concentration effect

Eqn. 5 is strictly valid only in the Debye-Rayleigh limit, i.e., as long as the scattering particles are much smaller than the wavelength of the incident light. For our vesicles, which are rela-

tively large, and when massive vesicle–vesicle fusion is occurring, as with PC/FA vesicles at low pH and high temperatures, the quantitative reliability of Eqn. 5, as a basis for the determination of the rate of vesicle association must be doubted, however. Consequently, the validity of the interaction potential results derived therefrom could be questioned.

To check for the importance of this limitation, we have determined the rate of vesicle association as a function of vesicle (i.e., lipid) concentration in the range of 0.05 to 32 μmol vesicle $\cdot$ dm^{-3} at two different temperatures. The data are presented in Fig. 7 in the form of $[v_0]$ vs. t_F^{-1} plots; the range of validity of Eqn. 5 is that in which there is a linear relationship between these two variables. Temperatures 10 C° above and below the lipid lamellar-to-nonlamellar phase transition temperature, $T_h = 48$° C, were chosen.

We find that our experimental association rate data can be fitted with straight lines with correlation coefficients 0.99 and 0.91 for the high and low temperature, respectively (Fig. 7). The data of Fig. 7, which yield $C_a = 5.5 \cdot 10^7$ and $6.5 \cdot 10^3$ dm^3 $\cdot$ mol$^{-1} \cdot$ s^{-1}, and the corresponding values obtained from direct analysis of A vs. t curves, $C_a \approx 2 \cdot 10^7$ (cf. Fig. 4), moreover, are so similar

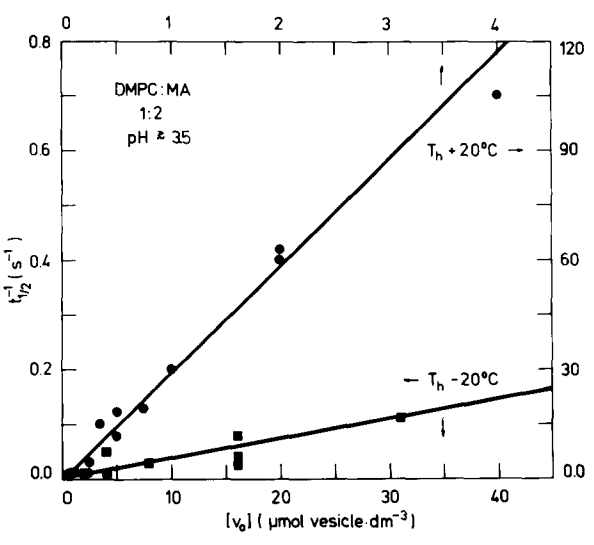

Fig. 7. Rate of association, C_a, between protonated PC/FA (1 : 2) mixed vesicles as a function of total vesicle concentration $[v_0]$ and relative temperature. Note that the scale for the high temperature data (the upper and right axes) is different from that pertaining to the lower temperature (lower and left axes).

that this vindicates the validity of Eqn. 5 for the whole range of concentrations and for the type of vesicles used in this study.

Lipid ionization and vesicle–vesicle association: pH titration effects

Our procedure for studying vesicle–vesicle interactions and bilayer phase behaviour is based largely on studying the effects of lipid ionization on the chain-melting or colloidal properties of the system (cf. Figs. 1 and 3). This is possible because for the PC/FA mixtures protons from the solution bind to lipid headgroups, change the intermolecular, and consequently the intervesicle, interactions, and ultimately cause vesicle–vesicle association, aggregation and – under certain conditions – fusion, depending on the solution pH value. pH titrations of the association rate can therefore provide valuable information about the mechanism of the interlipid and interbilayer interactions.

Fig. 8 documents this. Changing the final pH value of the suspension of DMPC/MA (1 : 2) mixed vesicles affects the sample turbidity as well as the rate of vesicle association dramatically. If lipids are suspended in a low-ionic-strength buffer, for example with $J = 0.005$, the rate of association climbs from a value of $C_a \approx 10^5$ dm$^3 \cdot$ mol$^{-1} \cdot$ s^{-1} at pH $\approx$ 8, where fatty acids in phosphatidylcholine bilayers are approx. 75% deprotonated, to nearly 10^7 dm$^3 \cdot$ mol$^{-1} \cdot$ s^{-1} at pH $\approx$ 5. In the region $4 \leqslant$ pH $\leqslant 5$ this value remains relatively constant, but it increases again upon further acidification of the suspension below pH $\leqslant$ 4, where values in the order of 10^8 dm$^3 \cdot$ mol$^{-1} \cdot$ s^{-1} are measured. Finally, the association rate drops by nearly one order of magnitude, to $C_a \approx 10^7$ dm$^3 \cdot$ mol$^{-1} \cdot$ s^{-1}, when the pH value approaches 1 (and the ionic strength starts to increase). The pH-dependence of the maximal optical absorbance of PC/FA (1 : 2) suspensions exhibits similar qualitative features (data not shown).

Ions partly mask the effects of surface electrostatics by accumulating in the counterion cloud near the charged bilayer surface. This explains, among others, the differences between Fig. 8A, which pertains to a relatively diluted electrolyte solution, and Fig. 8B. The distinctly biphasic character of the titration curve no longer appears in

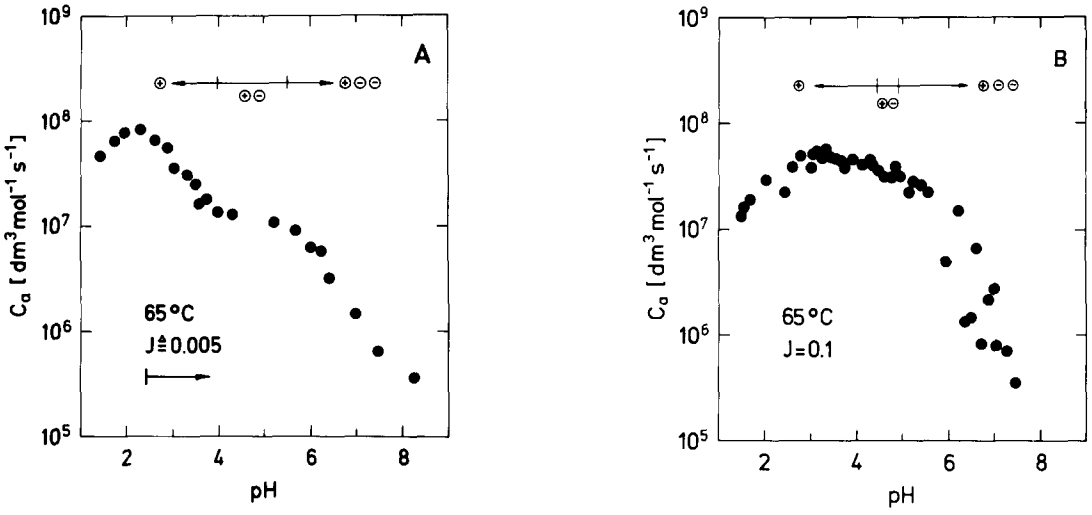

Fig. 8. pH-titration of the rate of association between PC/FA (1 : 2) mixed vesicles in the fluid phase (at 65 ° C) at low (A) and near physiological (B) ionic strengths J, of the electrolyte solution. Association rates were calculated by means of Eqn. 5 from the optical absorbance data, which were recorded as a function of time after a pH-jump from 10 to the indicated pH value.

the latter, because of the differences in the dissociation behaviour of lipids in diluted and 0.1 M salt solutions. From this it may be concluded that the titration region of fatty acid molecules is shifted towards lower pH values upon the addition of salt, the phosphate group of phosphatidylcholine begining to protonate at somewhat higher pH values in the presence of ions. Consequently, in Fig. 8B only one, broad maximum centred around pH 4 is seen, whereas in Fig. 8A both branches of the titration curve are resolved.

For $pH \leqslant 3$ the association rates are lower in the presence of salt, whereas for $3 \leqslant pH \leqslant 6$ the reverse is true.

Increasing the pH value of lipid suspensions decreases the chain-melting transition temperature of phosphatidylcholine/fatty acid (1 : 2) mixed vesicles (data not shown).

It is noteworthy that the highest association rates determined in this study, $10^7 \leqslant C_a \leqslant 10^8$ dm^3 · mol^{-1} · s^{-1} for $2 \leqslant pH \leqslant 5.5$, are as large as the values found with phosphatidylserine vesicles upon the addition of divalent ions, $10^7 \leqslant C_a \leqslant 10^8$ dm^3 · mol^{-1} · s^{-1} [28–31]; counterion effects are also similar in both systems.

Effect of salt concentration on vesicle association

Increasing salt concentration can facilitate intervesicle aggregation and association, as can be seen from Fig. 9. If the concentration of sodium chloride in the suspension is increased from 0.0025 to 0.1 M, the protonation-induced maximal increase in the optical absorbance of PC/FA (1 : 2) mixed vesicles is increased by approximately a

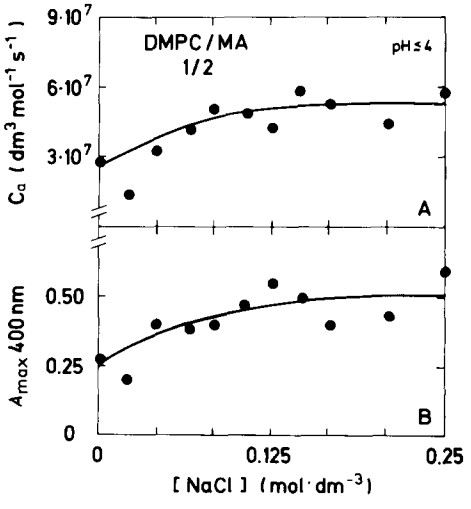

Fig. 9. Effect of salt concentration on (A) the rate of vesicle association and (B) the corresponding maximal optical absorbance of a suspension of the fluid-phase dimyristoylphosphatidylcholine/myristic acid (1 : 2) mixed vesicles after a pH-jump from 10 to 4.

factor of 2. The rate of vesicle association also increases correspondingly.

Owing to the prohibitively high experimental scatter in the concentration range [NaCl] $\geqslant 0.125$ M we have not extended our optical studies beyond $J = 0.25$. The reason for this is that between $J = 0.1$ and 0.2 strong vesicle flocculation sets in, which has also been observed by Egret-Charlier by NMR; this suggests that the bulk ionic strength ceases markedly to affect the pK of fatty acid in PC bilayers in the range 0.1 or more [32].

Acid-base titration of DMPC/MA (1 : 2) mixtures

We have also performed acid-base titrations with suspensions of dimyristoylphosphatidylcholine and myristic acid in the stoichiometric (1 : 2) ratio at 25 and 65 °C for low ionic strength $0.0025 \leqslant J \leqslant 0.008$ and for $J \approx 0.1$ (Fig. 10).

Starting at high pH, the titration curve pertaining to ambient temperature (Fig. 10, lower part) first changes its slope near the endpoint of the titration, the position of the latter being also confirmed by independent optical absorbance measurements. Such inflection of the curve is for lipid suspensions in 0.1 M NaCl solution occasionally accompanied also by a cup-like invagination. Upon further acidification a nearly linear titration curve is measured, up to the point at which approximately 2 equivalents of the acid (note that each complex contains two fatty acid molecules) has been added and the pH value is approaching 5. Here, another inflection is observed, causing the pH to change rapidly with subsequent addition of acid. The final change in slope at pH $\approx$ 3.5 signals the onset of the phosphate protonation. Addition of salt causes the titration curve of fatty acid to shift upwards by approx. 1 pH unit, the corresponding pK values being approx. 7.1 and 8.1, for $J \geqslant 0.0025$ and 0.1, respectively.

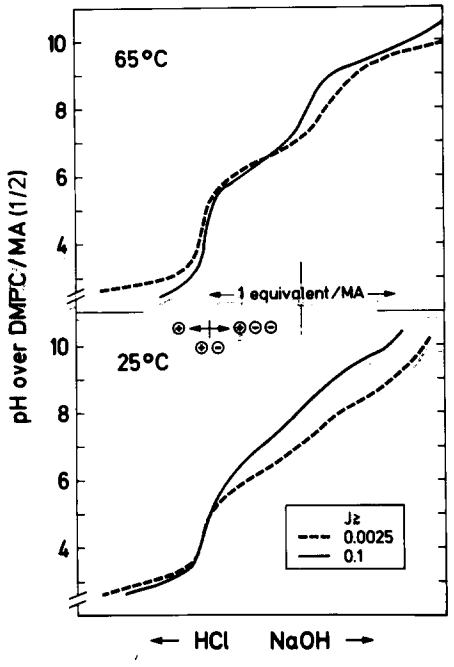

Fig. 10. Acid-base titration curves of dimyristoylphosphatidylcholine/fatty acid mixed systems at 25 (lower) and 65 °C (upper). The ionic strength was at least 0.0025 (dashed line) or 0.1 (full line). Additional features in the upper curves are indicative of an isothermal phase transition between a nonlamellar and a lamellar fluid bilayer phase. (Magnitude of the equivalent is shown in the body of the figure.)

Acid-base titration curves pertaining to (1 : 2) phosphatidylcholine/fatty acid mixtures at 65 °C (Fig. 10, upper part) differ from those measured at 25 °C and typical of ordered bilayer phase in that they exhibit another point of inflection near pH 7–8, i.e., near the point of 50% ionization; we believe this to be diagnostic of the isothermal phase transition at this pH, as will be argued in the Discussion.

Discussion

Temperature effects

The effects of fatty acids on the bilayer phase behaviour and, in some instances, on the fusion of their mixtures with phosphatidylcholines have been studied previously by others [15–18,35,36]. However, most studies of phosphatidylcholine/fatty acid mixtures to date were restricted to low PC/FA molar ratios, and all have been performed at constant absolute temperature. Moreover, very little attention has as yet been paid to the characterization of the physicochemical properties of interacting membranes containing fatty acids and neither a detailed theoretical analysis nor an appropriate data analysis of the intermolecular and intervesicle interactions has been attempted for such systems.

To avoid some of these shortcomings we measure our samples, whenever appropriate and possible, at constant relative temperature. This allows a

TABLE I

EFFECTIVE INTERACTION POTENTIAL

Effective interaction potential, $U_{ex}(d)$ (kJ) (calculated from Eqn. 7 using the experimental data of Fig. 2), between PC/FA mixed, small vesicles and apparent electrostatic surface potential, $\phi_0(d)$ (italics, in mV), as a function of the phosphatidylcholine/fatty acid molar ratio and temperature relative to the chain-melting phase transition, T_m. $\phi_0(d)$ was evaluated by using Eqn. 6 (last row) and Eqn. 13 by assuming that vesicles interact purely electrostatically over a separation, d (in nm). The two values for d were chosen so as to agree with previous estimates (e.g., Ref. 53) or fall in the upper range of Table III.

DMPC/MA: 1:0		9:1	7:3	5:5	3:7	1:9	
3–10° C	44.1	43.5	44.2	40.6	39.0	40.5	$U_{ex}(d)$
	632.2	*627.9*	*632.9*	*606.6*	*594.5*	*605.9*	$\phi_0(d = 30)$
	22.3	*22.1*	*22.3*	*21.4*	*21.0*	*21.4*	$\phi_0(d = 2.5)$
T_m–5 C°	43.8	39.8	40.7	40.6	36.6	29.3	$U_{ex}(d)$
	526.1	*501.5*	*507.1*	*506.5*	*480.9*	*430.3*	$\phi_0(d = 30)$
	21.9	*20.9*	*21.2*	*21.1*	*20.1*	*18.0*	$\phi_0(d = 2.5)$
T_m + 5 C°	43.5	43.0	43.7	36.0	15.8	16.4	$U_{ex}(d)$
	448.7	*446.1*	*449.7*	*408.2*	*270.4*	*275.5*	$\phi_0(d = 30)$
	21.6	*21.5*	*21.7*	*19.7*	*13.0*	*13.3*	$\phi_0(d = 2.5)$

correlation between the structural and colloidal properties to be made and makes a scrutiny of the physicochemical properties of the investigated system possible.

We find no simple, general relation to exist between the rate of vesicle association and fatty acid concentration (cf. Fig. 2 and Table I), in contrast to the statements by other authors. Rather than this, our data suggest the main effect of fatty acids on the colloidal properties of PC/FA mixed vesicles is an indirect one, acting via effects such as the creation of activated sites with a high local fatty acid concentration (and correspondingly low defect edge and intermembrane interaction energies). This assumption is supported by the observation that the rate of vesicle association in independent of fatty acid concentration in the low-temperature region, at 3–10 °C, where no phase transition occurs on the time-scale of our measurements.

Close to the chain-melting phase-transition temperature, which is the best studied region, the situation is more complicated. At temperatures slightly below that of the chain-melting of the phosphatidylcholine component, T_m, the rate of vesicle association as a function of the relative fatty acid concentration increases gradually but nonlinearly. This could be due to isothermal phase transitions between various lamellar phases, as concluded on the basis of the (pseudo) binary phase diagram; the time evolution of the turbidity of PC/FA mixed samples, such as are presented in Figs. 2 and 5, is consistent with this interpretation.

Another possible source of the pH-induced changes in the optical absorbance of phosphatidylcholine/fatty acid mixed suspensions is the micelle–liquid-crystal transitions which may occur upon a pH-jump from 10 to 4, and which may involve fatty acid molecules previously not imbedded into lipid bilayers.

Such a mechanism of the turbidity change can be expected to be particularly prominent for samples containing excess amounts of fatty acid; it could explain, for example, why samples with 90% myristic acid (cf. Fig. 2) appear to associate nearly 50-times as fast as those containing less than 30% of the fatty acid; a considerable proportion of the fatty acid molecules in the former case is not incorporated in vesicles. The second phase transition, that is frequently observed at temperatures close to the corresponding chain-melting phase transition temperature of the pure fatty acid component (see, for example, Ref. 33), is presumably due to this partial phase separation.

Association rates on the order of 10^7 $dm^3 \cdot mol^{-1} \cdot s^{-1}$, typical of temperatures above the chain-melting phase-transition temperature, are measured only with phosphatidylcholine-fatty acid mixtures containing more than 65% FA. This sug-

gests the notion that phosphatidylcholine and fatty acids associate in the preferred stoichiometric ratio of 1 : 2 (mol/mol). It is noteworthy that a similar stoichiometry has also been reported by Hui and Barton to apply to mixtures of phosphatidylcholine and fatty alcohols [34].

According to Kantor and Prestegard [18], introduction of even a small number of fatty acid molecules into phosphatidylcholine bilayers should cause detectable changes in the rate of vesicle–vesicle association. Such effects are not apparent in Fig. 2. This discrepancy is either due to the experimental scatter of our data or, which we find more likely, reflects the fact that our particular experimental setup excludes the possibility of artifactual changes, originating in the 'isothermal' phase transitions. Concluding from the DMPC/MA phase diagram, the latter, for example, could well originate from an isothermal transition from a rippled $P_{\beta'}$ phase to an $L_{\beta'}$, phase.

We have discovered that the sample turbidity, which is proportional to the degree of vesicle–vesicle association, increases for all three chain lengths investigated by more than three orders of magnitude if the temperature is approached at which the PC/FA (1 : 2) mixtures adopt an inverted hexagonal structure, H_{II} (cf. Figs. 4 and 6). A new, rather simple and inexpensive method for the determination of the lamellar-to-nonlamellar transition temperature thus can be proposed. It should be possible to read off the transition temperature, T_h, directly from the A_{max} vs. t curves, or, alternatively, from the corresponding C_a vs. t data. Simple optical measurements, therefore, could be used for studying transitions into nonlamellar lipid structures, under the proviso that identification of phases would be confirmed for each system (at least once) by already established and more costly, but also more direct methods, such as X-ray diffraction or electron microscopy.

An unprecedented finding which emerges from our optical studies of PC/FA mixtures is that even relatively short-lived nonlamellar structures may greatly affect the rate of vesicle–vesicle association: the 'pretransitional' increase of the maximal optical absorbance of PC/FA (1 : 2) samples shown in Fig. 5 documents this.

This parallels the outcome of phosphorus nuclear magnetic resonance, ^{31}P-NMR, experiments (cf. Fig. 3 of Ref. 20). The major increase in the optical absorbance of the rate of vesicle association, which co-appears with the massive lipid clumping and the formation of nonlamellar structures, occurs simultaneously with the change of sign and halving in magnitude of the phosphorus chemical shift anisotropy, at $T = T_h$ [20]. Qualitatively similar observations for palmitoyl and stearoyl lipids (Fig. 5) prove that this is not an experimental artifact.

We believe that the fact that vesicles consisting of PC/FA (1 : 2) complexes associate prior to undergoing a major reorganization into the nonlamellar phase indicates that transient structures exist below T_h but above the chain-melting phase-transition temperature of the PC component, which exhibit properties similar to those of the samples that in toto are in a nonlamellar phase. Such structures appear to serve as the nucleation centres and promoters of fusion and thus could be termed 'fusogenic excitations'; they may be related to the 'inverted micellar intermediates', postulated to exist in the vicinity of, but below the transition temperature into the nonlamellar phase, T_h [37]. If the two are identical, their formation is strongly favoured by the presence of the fatty acid molecules, probably owing to the 'edge activity' of the latter.

The 'pretransitional' bilayer destabilization and the concomitant onset of vesicle association both depend crucially on the presence of fatty acid molecules within the lipid membranes. Our optical experiments have revealed no comparable increase in the vesicle–vesicle association rate of pure lipids (such as phosphatidylethanolamine (data not shown)) below the chain-melting phase transition, in agreement with the lack of 'pretransitional' effects in the ^{31}P-NMR chemical shift anisotropy for such lipids [20].

It remains to be seen whether or not the increase of the suspension turbidity and the anomalous increase in vesicle association rate observed for the phosphatidylcholine/fatty acid mixtures at $T \geqslant T_m$ (PC) are at least partly also due to a local demixing of the two lipid components; in light of the strong association of these two lipids in the 1 : 2 stoichiometric complexes this possibility is not very likely, however.

pH titration

The results of our potentiometric titrations at ambient temperature, shown in Fig. 10, indicate that: (1) suspensions of DMPC/MA (1 : 2) with pH ≈ 10 consist predominantly of lipid vesicles without major micelle contamination, and (2) during the titration of myristic acid the demixing between fatty acids and their soaps within bilayers is not a serious problem. The first of these two conclusions is substantiated by the fact that no clearly defined plateau is seen in the high-pH region of the titration curve: in the worst case, a minute change in the slope of this curve is found at pH ⩾ 10. The second statement is confirmed by the linearity of the titration curve for 5.5 ⩾ pH ⩾ 10; we believe that the marginal deviations from linearity that do appear in Fig. 10 originate from incomplete sample equilibration (according to Cistola et al. [38], equilibrium for fatty acids may take weeks to be reached).

In contrast to this, our acid-base titrations of dimyristoylphosphatidylcholine fatty acid (1 : 2) mixtures at 65 °C are markedly nonlinear at neutral pH, which suggests that an isothermal phase transition occurs near pH 7 at such elevated temperatures. Based on the Gibbs phase rule, it must then be postulated that three different phases exist in the PC/FA mixtures in the neutral pH region, the number of phases in the flanking pH regions being two.

We know that above 65 °C and at 3.5–4 ⩽ pH ⩽ 5.5 the DMPC/MA (1 : 2) complexes are in the 'inverted hexagonal' phase. At a pH close to 10, they may be taken to comprise almost exclusively of bilayer vesicles consisting of a mixture of phosphatidylcholine and fatty soap molecules. Consequently, somewhere in the intermediate pH region, i.e., at 6 ⩽ pH ⩽ 8, two different lipid phases should coexist with an excess water phase, with an isothermal phase transition between them being possible. We surmise that the two lipid phases are: an inverted hexagonal phase containing PC/FA (1 : 2) complexes, and a fluid bilayer phase that consists of phosphatidylcholine with different proportions of the fatty soap (and fatty acid?) molecules, their ratio varying with the precise pH of the suspension.

Such a conclusion could be relevant for our understanding of the distribution of fats, of fat–membrane or fat–albumin interactions, and of fat digestion in biological systems. A noteworthy example is that of oleic acid, which at subneutral but still physiological pH associates preferentially with phosphatidylcholine membranes even in the presence of high amounts of serum albumin [39].

Titration data thus yield valuable information about the structure of PC/FA mixed systems. If properly analysed, moreover, they can also highlight the molecular origin of the interfacial and interlipid interactions.

Lipid ionization

There are two groups on phosphatidylcholine/fatty acid mixed bilayers which can change their state of ionization if the pH of the suspension is varied: the carboxylic group of the fatty acid molecules and the phosphate group of phosphatidylcholine. Changing the pH therefore strongly affects the phase and colloidal behaviour of PC/FA vesicles, and also the rate of association between them.

On the one hand, it affects the electrostatic properties of the lipid/solution interface, thus modifying the intervesicle repulsion arising from the net lipid charge. On the other hand, it influences the effective interfacial polarity, which is strongly pH-dependent because lipid protonation changes the water affinity of the surface polar residues, thus modulating the lipid–lipid and the lipid–water interactions, which then in turn govern the thermodynamic and colloidal properties of the system.

The bilayer electrostatic potential in dilute electrolyte solutions is nearly unscreened, so that all of the electrostatic effects are fully expressed. This is part of the reason why our titration of the rate of vesicle association measured in the electrolytes of low ionic strength (cf. Fig. 8A) reveal different states of lipid ionization much more distinctly than the data for vesicle suspensions in a 0.1 M solution of sodium chloride ($J = 0.1$, Fig. 8B).

Starting on the right-hand side of the titration curve, one can follow the protonation of the fatty acid molecules complexed in a 2 : 1 stoichiometric ratio with phosphatidylcholine, and how this affects the colloidal properties of the system. Beginning at high pH, increasing the concentration of

hydronium ions causes the rate of vesicle aggregation to increase owing to the gradual elimination of the net negative charge from the vesicle surfaces. It is not clear from Fig. 8A precisely where this process sets in, but independent, long-term observations of the turbidity of the suspensions of DMPC/MA (1 : 2) as a function of pH indicate that for $J \leqslant 0.1$ and $pH \geqslant 8$ this happens around $pH \leqslant 9.5$ (data not shown).

The plateau in the C_a vs. pH curves seen at $4 \leqslant pH \leqslant 5.5$ defines the range of electroneutrality of PC/FA (1 : 2) mixed membranes, which now consist of zwitterionic phosphatidylcholine and fully protonated fatty acid molecules. The further increase of the C_a values below pH 4 must be a consequence of proton binding to the phosphate group of the phosphatidylcholine molecules.

The C_a titration data presented in Fig. 8 thus imply that the apparent pK of the myristic acid in DMPC/MA (1 : 2) mixtures at 65 °C is 7.5 ± 0.2; the titration curve of the phosphate group in such mixtures is not resolved in integro in our type of experiments, but the pK value of this group can be deduced from the known onset of PO_4 ionization to be approx. 1.5 ± 0.5. This compares favourably with the result of our potentiometric acid-base titrations done under comparable experimental conditions, which yield $pK_{h,p}^{COO^-} \simeq 7.3$ (cf. Fig. 10).

With some uncertainty, our titration experiments suggest, that the apparent dissociation constant of the fatty acid molecules in 1 : 2 mixtures with phosphatidylcholine is shifted downwards by approx. 0.75 ± 0.25 pH units upon increasing the ionic strength from about 0.005 to 0.1, whereas the pK of the phosphate group, which is more difficult to estimate and correspondingly less accurate, is shifted upwards by 0.75 ± 0.5 pH units.

A survey of experimental pK values for fatty acids, for fatty acids in PC/FA mixtures, for phosphatidylcholine phosphate groups in such systems, and for some model compounds is given in Table II. It is seen that our estimates for the dissociation constant of the former, $pK_a^{COO^-}$, are similar to the values quoted by other authors, particularly if differences in the experimental temperature, which can lower the apparent pK, are taken into account [32].

The dissociation constant of the phosphate group of phosphatidylcholine molecules in mixture with fatty acids is reported here for the first time. Its value is surprisingly high, compared to that of pure PC, probably in consequence to the intermolecular fatty acid–phosphate interactions, as will be argued later.

Phosphate protonation initially causes the rate of vesicle association to increase, rather than to decrease, with the degree of surface ionization. At first sight, this appears counterintuitive: surface charge inevitably gives rise to an electrostatic repulsion which, one could expect, should decrease the rate of vesicle association. The most obvious reason for the observed increase would be the screening effects arising from the ionic strength variations at low pH. Indeed, in Fig. 8A and B, the measured C_a values are seen to be affected strongly by the ion concentration. However, the highest observed association rates in diluted acidic solutions, where lipid membranes bear quite high net positive charge, are significantly higher than those typical of more concentrated electrolytes. This implies that the observed increase in C_a at low pH and low ionic strength is due only partly to the variation of surface electrostatics with pH and salt content. A solution to this dilemma is provided by the inclusion of solvent effects in the analysis and the description of the dissociation process.

The lipid affinity for proton binding is commonly described in terms of the so-called dissociation constants, K_H, i.e., given by the characteristic pK_a ($= -\log K_H$) values of the component groups. Previously we have used the following phenomenological expression for the calculation of pK values

$$pK_a = pK_a^0 \pm \Delta pK_{h,Born} + \Delta pK_{el} \tag{9}$$

where pK_a^0 is the intrinsic pK of isolated groups and $\Delta pK_{h,Born}$ (in our preceding articles written as ΔpK_i^p) and ΔpK_{el} are the shifts in pK value due to surface hydration and electrostatic effects, respectively.

Here we will use the more general version of this result

$$pK_a = pK_a^0 \pm \Delta pK_{h,Born} + \Delta pK_{h,p} + \Delta pK_{el} \tag{10}$$

which will be derived and discussed in detail in a

TABLE II

APPARENT DISSOCIATION CONSTANTS OF VARIOUS PHOSPHATIDYLCHOLINE/FATTY ACID MIXTURES AND RELEVANT MODEL COMPOUNDS

AB, acid-base; CTAB, cetyltrimethylammonium bromide; egg-PC, egg-yolk phosphatidylcholine; ESR, electron spin resonance; OA, oleic acid.

Substance	pK	T (°C)	J	Method	Ref.
Butyric acid	4.85	25		AB titration	54
Octanoic acid	4.8	25		monomer titration	54
Potassium laurate	(8)	25	≈ 0.1	AB titration	33
Potassium oleate [a]	7.8	40	≈ 0.1	AB titration	33
66% MA in DMPC vesicles	≈ 7.1	25	$\geqslant 0.0025$	AB titration	this work
66% MA in DMPC vesicles	≈ 8	25	$\geqslant 0.1$	AB titration	this work
66% MA in DMPC vesicles	7.3 ± 0.2	65		AB titration	this work
66% MA in DMPC vesicles	7.5	65	0.005	C_a titration	this work
66% MA in DMPC vesicles	≈ 7	65	0.1	C_a titration	this work
20% MA in egg-PC vesicles	8.2	35	– [b]	^{13}C-NMR	18
10% MA in egg-PC vesicles	7.25	27	0.1	^{13}C-NMR	56
10% SA in egg-PC vesicles	7.25	27	0.1	^{13}C-NMR	56
5% OA in egg-PC vesicles	7.6	35	0.15	^{13}C-NMR	39
0.8% SA in egg-PC vesicles	7.1	29		^{13}C-NMR	32
Dipropyl phosphate	1.59			AB titration	57
Dimethyl phosphate	1.22			AB titration	57
PC vesicles	$\leqslant 1$			T_m titration	this work
PC in DMPC/MA vesicles	1.5	65	0.005	C_a titration	this work

[a] Small and co-workers [33] have found that potassium oleate begins to form lamellar structures near pH $\leqslant 9$, a finding which is also consistent with the data for other fatty acids by Hargreaves and Deamer [55].

[b] Kantor and Prestegard [18] report, surprisingly, that this value is independent of ionic strength.

forthcoming theoretical paper (Cevc, G., unpublished data). Consequently, in this work only its main aspects will be briefly talked over.

The first contribution to the hydration shift, $\Delta pK_{h,Born}$, corresponds to the so-called (electrostatic) polarity shift; it was first introduced, as a phenomenological quantity, by Fernandez and Fromherz [41]. Its origin lies in the work that must be done to bring a charge into the interfacial region, whose dielectric constant is invariably lower than that of the bulk solution because of the electrostatic dielectric saturation effect [42,43] resulting in a stabilization of the more highly charged state.

The second part of the hydration shift, $\Delta pK_{h,p}$, has not been discussed before. It arises because protonation of the surface polar residues changes the interfacial hydration. The magnitude of $pK_{h,p}$ thus depends strongly on the detailed molecular structure and effective hydrophilicity (polarity) of the titratable groups, the sign of the shift for phospholipids always being such that it disfavours headgroup protonation and the shift magnitude being no more than 1.5 [44].

The electrostatic shift, conversely, is always positive for negatively charged bilayers and negative for positive membranes, owing to the fact that protons in such cases behave as co-ions or as counterions, respectively. From standard diffuse double layer theory the electrostatic shift is found to be

$$\Delta pK_{el} = (F\phi_{el}/RT) \log e$$

$$\equiv \pm e(2/2.3) \sinh^{-1}\left\{e/\left[20A_c(20\epsilon\epsilon_0 cRT)^{\frac{1}{2}}\right]\right\} \quad (11)$$

or, with numerical values inserted,

$$\Delta pK_{el} = \pm 0.869 \ln\left[(2.09 \cdot 10^{-2} A_c)(cT)^{\frac{1}{2}}\right] \quad (12)$$

where salt concentration, c, is in (mol · dm^{-3}), the area per molecular charge, A_c, in nm^2, and the

other symbols have their usual meaning.

For anionic lipids with an effective molecular area $A_c = 1.2$ nm^2 (corresponding to one charge per four fluid chains in the PC/FA (1 : 2) complex at pH $\equiv$ pK), Eqn. 12 yields the following results: $\Delta pK_{el} = 2.97$ and 1.67, for $J = 0.005$ and 0.1, respectively, all at $T = 338$ K ($\approx 65\,^\circ$C). The corresponding values pertaining to $T = 298$ K are 7–10% smaller. This is only marginally smaller than the estimate (obtained by using Eqn. 9) from the total pK shift characteristic of the carboxyl groups of phosphatidylserine, $\Delta pK_{el} \approx 1.75$ [45]. It should be kept in mind, however, that these result represent the upper limits, because Gouy-Chapman theory is known to overestimate the coulombic surface potential when hydration effects are neglected (see, for example, Ref. 46).

Using $\Delta pK_{el} \approx 1.70$ (cf. previous paragraphs) and the average 'intrinsic' pK value for the carboxylic group from Table II, $pK_a = 4.85$, one obtains the following estimate for the nonelectrostatic pK shift, $\Delta pK_h = \Delta pK_{int} - \Delta pK_{el} \approx 0.95$, at ambient temperature and for $J \approx 0.1$ *.

For titration of the carboxylic groups in phosphatidylcholine/fatty acid mixed systems, the electrostatic and the Born part of the nonelectrostatic pK shift add up, whilst the contribution $\Delta pK_{h,p}$ goes in the other direction: $\Delta pK_{int} = -\Delta pK_{h,p}^{COO^-} + \Delta pK_{h,Born} + \Delta pK_{el}$. The effective shift pertaining to the titration of the phosphate group of phosphatidylcholines is thus given by: $\Delta pK_{int} = -\Delta pK_{h,p}^{PO_4^-} + \Delta pK_{h,Born} - \Delta pK_{el}$, the latter two contributions partly compensating each other.

If we assume, for the sake of simplicity, that the electrostatic contribution to the titration behaviour in both instances is equal, $\Delta pK_{el} = 1.7$, the values $\Delta pK_{h,p}^{COO^-}$ and $\Delta pK_{h,p}^{PO_4^-}$, the local polarities, as well as the effective hydration of these two groups in the DMPC/MA (1 : 2) complexes are seen to be approximately the same. This shows that the fatty acid molecules in mixtures with phosphatidylcholine decrease the overall polarity and ability for water binding of the PC/FA (1 : 2) complexes.

The vicinity of fatty acid molecules also appears to increase the binding affinity of phosphate groups for protons by reducing the competition by hydronium ions for the same binding sites. Whereas pure phosphatidylcholine protonates with $pK_a \ll 1$ (cf. Table I), owing to the high hydrophilicity of its phosphate group and the strong headgroup-proton repulsion, which both yield large, negative $\Delta pK_{h,p}^{PO_4^-}$ values, the pK value of the phosphate group complexed with two carboxylic residues gives a substantially higher $pK_a^{PO_4^-} \approx 1.5$ *. The proximity of phosphatidylcholine headgroups, on the other hand, seems to shift the pK of fatty acid molecules toward lower values compared to that of pure fatty acid-soap bilayers (cf. Table II). This suggests that the shift of the latter is decreased, owing to PC, by $\Delta pK_{h,p}^{COO^-} \approx -0.6$.

Association rates

To date, only the most simple theoretical models have been used to analyse measured data [17,18,35,36] and no attention has been paid to the possible effects of isothermal phase transitions. This latter omission is impermissible, however; the change in slope of the measured curves with fatty acid concentration near 10% FA, which has been interpreted by others as a sign of strong vesicle association, could be due entirely to such phase transitions. This may explain why our curves, measured on a reduced temperature scale, are lacking such a change in slope.

In this work we have used a mass action kinetics model in an attempt to extract molecular parameters from the association data. Fig. 7 indicates that this choice is a reasonable one. The agreement between the calculated curves and the

* Titrating 30% stearic acid in egg phosphatidylcholine monolayers, Bouloussa and co-workers [47] have concluded, by using an indirect extrapolation method, that the limiting pK (FA) value for $\phi_{el} \to 0$ is 6.15, which gives an upper value estimate: $\Delta pK_h \leq 1.3$. Thus, the agreement between the two results is satisfactory. It would probably improve if allowance were made for the effect of nonlamellar phase formation, which is prone to diminish the hydrational pK shift.

* It is worth recalling that Klausner et al. [4], on the basis of fluorescence polarization studies, have concluded that saturated fatty acids do not alter the bilayer interior but have a significant ordering effect on the interfacial region.

experimental data is sufficiently good to suggest also that under our experimental conditions vesicle dissociation is unimportant. Further confirmation of this is the close harmony between the association rate values given in Figs. 2 and 6 and the data of fig. 7, which is nearly quantitative. This all suggests that our C_a values are trustworthy and justifies further theoretical analysis as a means of gaining insight into the molecular origin of the interactions governing vesicle–vesicle association in such systems.

For this purpose, a model must be chosen for the calculation of the surface potential and vesicle–vesicle interaction potential. In this work we have decided to confine our attention to three possible sources of this interaction. (The steric (fluctuation) potential [48,49] under our experimental conditions may be neglected.)

Firstly, the electrostatic repulsion potential has been approximated with the result of Gouy-Chapman diffuse double layer theory:

$$U_{el}(d) \approx [4RT\pi r_v^2 \lambda \sigma_{el,ef} \phi_{el}(\sigma_{el,ef})] / [e\epsilon\epsilon_0(2r_v + d)] \exp(-d/\lambda) \quad (13)$$

Secondly, the van der Waals attraction potential has been evaluated from the approximate result [50]:

$$U_{vdW}(d) \approx -(H_A N_A/6)\{[2(d/r_v) + (d/r_v)^2]^{-1} + 2[1 + d/r_v + (d/r_v)^2/4]^{-1} + \ln(1 - [1 + d/r_v + (d/r_v)^2]^{-1})\} \quad (14)$$

And thirdly, the hydration interaction potential has been estimated from the result analogous to Eqn. 13 [44,40]:

$$U_h(d) \approx [4RT\pi r_v^2 \xi \sigma_{p,ef} \phi_h(\sigma_{p,ef})] / [\epsilon_0 e_w(2r_v + d)(1 + r_v/\xi)] \exp(-d/\xi) \quad (15)$$

The parameters $\lambda = 0.304T/298c^{\frac{1}{2}}$ nm, ξ, σ_{el}, σ_p, and $H_A \approx 6 \cdot 10^{-21}$ J are the coulombic (Debye) and hydration force screening lengths, the net and local excess surface charge density, and the Hamaker constant, respectively. Here we have used the estimates $\xi = 2$ nm and $e_w = 3.5 \cdot 10^{-20}$ A · s. The electrostatic surface potential can be approximated with $\phi_{el}(\sigma) \approx (2kT/e) \sinh^{-1} e\sigma\lambda/2kT\epsilon\epsilon_0$) and the hydration-induced part of the surface potential is given approximately by $\phi_h(\sigma) \approx (-2kT/e_w) \sinh^{-1}(e_w\sigma\xi/2kT\epsilon_\infty\epsilon_0)$, where $\epsilon_\infty = 5$; the effective surface charge densities may taken in the first approximation to be $\sigma_{el,ef} = [\sigma_{el} + \sigma_p(\xi/\lambda)^2(\epsilon/\epsilon_\infty - 1)]$ and $\sigma_{p,ef} = (\sigma_{el} + \sigma_p)$ [43]. As before, the parameters d and r_v represent the intervesicle separation and radius, respectively. The temperature dependence of the interaction potential has been considered only implicitly.

From Eqns. 13–15 the vesicle–vesicle association rates can be calculated and compared with experiments; by using Eqns. 7 and 13–15 together with the relation $U_{ex} = U_{th} \equiv U_{el} + U_h + U_{vdW}$, the molecular parameters of interaction can be then deduced or their assumed values checked.

We have performed this calculation in terms of the fatty acid concentration, which determines the effective interfacial potential, as an independent variable (Table I). If hydration effects are neglected, the value of the apparent potential varies with the effective separation used in the equations, d_{ekv}, and always decreases with temperature. Because of the large discrepancy between the measured and calculated data, however, fatty acids in mixtures with phosphatidylcholine must consequently be concluded to affect not only the electrostatic but also the nonelectrostatic (i.e., hydrational and structural) properties of the system.

Such a conclusion is vindicated by Tables III and IV. The former gives the equivalent distance of separation between PC/FA (1 : 2) mixed vesicles as a function of pH and ionic strength, calculated using four different approximations to the interaction potential. The latter presents the corresponding apparent collision efficiencies and the experimental and calculated values for the rate of association between such vesicles at 65 °C. When Gouy-Chapman electrostatic double-layer theory is used, irrespective of whether linear (LGC) or nonlinear (GC), the calculated apparent distance of interaction varies widely; if the (noncoulombic) surface hydration effects are included, within the framework of the nonlocal electrostatic model [51,52] (LNLE and NLE), for example, this variability is lessened. But even in the latter case, no perfect constancy is achieved – irrespective of

TABLE III

SEPARATION DISTANCES

Equivalent distances of separation (nm) (defined by $C_a = C_{a,th}(d_{eqv})$, with $C_{a,th}(d)$ given by Eqn. 6 and interaction potentials given by Eqns. 14–16) for the interaction between PC/FA (1:2) mixed vesicles in suspension, calculated from the data of Table IV by using Eqns. 14–16. To calculate the electrostatic surface potential the following approximations were used: GC: Gouy-Chapman electrostatic double layer theory; LGC: linear Gouy-Chapman electrostatic double layer theory; NLE: nonlocal electrostatic theory; LNLE: linearized nonlocal electrostatic theory.

pH:	1	2	3	4	5	6	7	8	9	10
$J = 0.005$										
GC	(27.6)	22.5	8.0	0.0	0.0	11.7	23.6	29.1	(30.1)	(30.1)
LGC	(34.5)	26.1	8.3	0.0	0.0	12.7	29.1	38.1	(39.9)	(40.0)
NLE	(27.7)	23.4	15.4	12.0	12.1	16.1	24.4	29.4	(30.4)	(30.4)
LNLE	(34.6)	27.4	16.8	12.9	13.1	18.2	30.3	38.6	(40.4)	(40.5)
$J = 0.1$										
GC	(4.5)	3.9	0.0	0.0	0.0	2.0	4.4			
LGC	(5.2)	4.3	0.0	0.0	0.0	2.2	5.2			
NLE	(4.6)	4.9	5.1	5.1	5.0	5.1	5.6			
LNLE	(5.4)	5.7	6.0	5.9	5.9	6.1	7.0			

which form of Eqn. 6 is used – indicative of the approximate nature of the version of the equation (final line of Eqn. 6) used in this calculation.

Even if more sophisticated models are used for the correction describing viscous effects, i.e., for the ratio (D^{∞}/D), the situation is not much better; quantitatively similar results can be obtained in both limits if the lower integration limit is adjusted somewhat (by approx. 5–30%). Also the use of other common spatial dependence functions, e.g., a replacement of the exponent in Eqn. 6 (last line) by the alternative expression $\log[1 + \exp(-d/\lambda)]$, brings no improvement. It must therefore be concluded that further theoretical works will have to be undertaken before reliable analysis of the colloidal properties of these lipid mixtures is possible.

Interfacial potential

The (electrostatic) surface potential of lipid

TABLE IV

PARAMETERS OF INTERVESICLE ASSOCIATION

Apparent collision efficiency, $k_{c,ef}$ [a], experimental values for the rate of association between phosphatidylcholine/fatty acid (1:2) mixed vesicles, C_a [b], and theoretical estimates for the rate of association, $C_{a,th}$ [c] (both C_a values in $dm^3 \cdot mol^{-1} \cdot s^{-1}$), at 65 °C as a function of the suspension pH. [e]

pH:	1	2	3	4	5	6	7	8	9	10
$J = 0.005$										
$k_{c,ef}$ [a] $\times 10^3$	(2.3)	10	5.6	1.6	1.2	0.7	0.18	0.04	(0.02)	(0.02)
C_a [b] $\times 10^{-7}$	(2.0)	8.6	4.8	1.4	1.0	0.6	0.15	0.037	(0.02)	(0.014)
$C_{a,th}$ [c] $\times 10^{-7}$	$\ll 0.01$	$\ll 0.01$	$\ll 0.01$	9.35	1.02	$\ll 0.01$	$\ll 0.01$	$\ll 0.01$	$\ll 0.01$	$\ll 0.01$
$J = 0.1$										
$k_{c,ef}$ [a] $\times 10^3$	(0.7)	3.5	7	5.2	3.3	1.2	0.12			
C_a [b] $\times 10^{-7}$	(0.6)	3	6	4.4	2.8	1.0	1.0			
$C_{a,th}$ [d] $\times 10^{-7}$	9.70	4.44	3.21	3.06	2.80	1.24	0.0015			

[a] Defined as $k_{c,ef} \equiv C_a/C_c$, where C_c is given by Eqn. 6.

[b] Calculated on the basis of the measured time dependencies of sample turbidity by means of Eqn. 5.

[c] Determined from Eqn. 6 by integration, with 10.7 nm as the lower limit of integration, which gives a theoretical fit to the data at pH 5.

[d] Evaluated from Eqn. 6 using 4.135 nm as the lower limit of integration.

[e] All values in parentheses were obtained by graphic extrapolation on the basis of experimental data.

TABLE V

SURFACE POTENTIALS

Effective potential of interaction between phosphatidylcholine/fatty acid (1 : 2) mixed vesicles, $U(d)$, [a] apparent electrostatic surface potential, ϕ_0, and various theoretical estimates for the electrostatic potential of lipid vesicles, $\phi_{el}(\sigma_{el}, \sigma_p)$, as a function of the suspension pH.

pH	$U(d)$ [a] (kJ)	$\phi_0(d = 2.5)$ [b] (mV)	$\phi_0(d = 30)$ [b] (mV)	ϕ_{el} [c] (mV)	$\phi_{h,el}(\sigma_{el}, \sigma_p = 0)$ [d] (mV)	$\phi_{h,el}(\sigma_{el}, \sigma_p = 0.2)$ [d] (mV)
$J = 0.005$						
1	(17.0)	(13.6)	(291.8)	105.1	556.04	615.22
2	12.9	11.8	254.1	71.8	370.56	624.06
3	14.5	12.5	269.6	20.2	95.31	603.09
4	18.1	14.0	300.4	2.7	12.44	595.76
5	18.9	14.3	307.1	4.7	21.73	597.98
6	20.4	14.8	319.1	34.8	169.16	622.44
7	24.2	16.2	347.7	91.8	482.02	713.17
8	28.4	17.5	376.8	125.3	668.54	817.96
9	(30.0)	(18.0)	(387.0)	132.3	708.06	845.84
10	(31.0)	(18.3)	(393.5)	133.1	712.60	849.16
$J = 0.1$			$\phi_0(d = 5)$ [b]			
1	(20.4)	(37.0)	(120.8)	67.1	541.90	566.36
2	15.9	32.6	106.6	50.3	445.70	601.21
3	13.9	30.5	99.8	13.9	191.58	629.23
4	14.7	31.4	102.8	1.9	32.32	634.52
5	16	32.8	107.1	3.3	55.61	638.27
6	18.9	35.6	116.2	25.5	288.34	665.00
7	25.3	41.2	134.7	69.8	557.31	757.16

[a] Calculated from Eqn. 8 and data of Table IV.

[b] Obtained by assuming that vesicles interact over a distance, d, using Eqn. 6, and extrapolating back to the lipid/solution interface.

[c] Calculated from Gouy-Chapman theory by assuming $pK_{COO^-} = 7.5$ and $pK_{PO_4^-} = 1.5$ with $\sigma_{el} = e/0.7 \times \{[K_{COO^-}/(K_{COO^-} + 10^{pH})] + 0.5[10^{pH}/(K_{PO_4^-} + 10^{pH})]\}$ nm^2.

[d] Calculated within the framework of nonlocal electrostatic theory with $\xi = 0.2$ nm, $\epsilon_\infty = 5$, $e_w = 3.5 \cdot 10^{-20}$ A·s and $\sigma_p = 0.2 \times \{[10^{pH}/(K_{COO^-} + 10^{pH})] + [10^{pH}/(K_{PO_4^-} + 10^{pH})]\}$ A·s·m^{-2}.

vesicles is frequently calculated from experimental vesicle association data, via an effective interaction potential between two vesicles. Such a procedure is likely to yield erroneous results, however, as can be seen from Table V.

To estimate the interfacial potential from the effective potential of the vesicle–vesicle interaction (which is per se only approximate), assumption must be made about the effective separation over which this interaction takes place. Commonly, it is taken that $d_{ekv} = 2.5$ nm [53]. Applied to our experimental data this gives electrostatic surface potentials which are far too low, even in comparison with a purely coulombic electrostatic potential, ϕ_{el}. If a larger separation distance is used, $d = 30$ nm, for example, larger estimates for the interfacial potential are obtained; an optimization with regard to the parameter d can even provide agreement with some data points. Under no circumstances, however, can classical electrostatics explain: (a) the strong repulsion between electroneutral PC/FA (1 : 2) vesicles, underlying the high interaction potential values around pH 4; and (b) the decrease in this potential below pH 3.5.

In our view, the most natural way of approaching this problem is to consider the effects of interfacial hydration. If, to begin with, solely the hydration associated with the net coulombic surface charge is considered, i.e., $\phi_{h,el}(\sigma_{el}, \sigma_p = 0)$ is assumed, the situation is rectified with respect to the first of the above-mentioned two issues. In order to explain the second point, allowance must additionally be made for the noncoulombic interfacial hydration, i.e., it must be assumed that $\phi_{h,el} = \phi_{h,el}(\sigma_{el}, \sigma_p \neq 0)$. This improves the agree-

ment between theory and experiment considerably and provides one plausible explanation, in terms of a decrease in the parameter σ_p at lower pH, for the diminished intervesicle repulsion in acidic suspensions. Unfortunately, even if the surface polarity effects are accounted for, the accuracy of the model underlying Eqn. 6 is still not quantitative; however, it is sufficiently good to prove that the hydration phenomena are of paramount importance in such systems.

Acknowledgements

This study has been supported financially by the Deutsche Forschungsgemeinschaft (SFB 102/D14) and S.E.R.C. (GR/C/95428).

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