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Phospholipid – from Model Membrane to Industrial Application

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The phospholipid bilayer has been shown to be a fundamental, structural element of biological membranes. An attempt is made here to briefly summarize the main physico-chemical properties of phospholipids necessary to understand their functional role in biological membranes. More recently lipid bilayers have become important in several areas of research for which the term «membrane-mimetic chemistry» has been coined. It comprises membrane mediated processes, e.g., membrane fusion, interfacial catalysis, energy conduction and conversion, drug delivery and targeting. In these research areas it is highly desirable to have simple, quick, and inexpensive methods for the production of closed unilamellar lipid vesicles at one's disposal. In the second part of this review it will be shown how good use can be made of our knowledge of the physico-chemical properties of phospholipids to develop such methods. The discussion is focussed on methods of spontaneous vesiculation. In addition to fulfilling the above-mentioned criteria this new methodology has another important advantage: it allows the production of phospholipid vesicles differing widely in properties. Variables are the vesicle size and size distribution, the surface charge density, the bilayer fluidity, permeability, and stability.

1. Packing of Phospholipids in Single Crystals

Phospholipids are a main constituent of biological membranes and as such they have been the subject of extensive research in the past twenty years. They have a high tendency to aggregate to bimolecular layers (bilayers) which apparently present a preferred minimum free energy structure. This is evident from the observation that phospholipids preferentially form bilayers both in the crystalline, anhydrous state and in aqueous dispersions. In the latter form bilayers have been widely used as model membranes and have proven useful in the study of the intrinsic bilayer properties and the interaction of the phospholipid bilayer with ions, small molecules such as drugs or hormons, and macromolecules, particularly peptides and proteins. Fig. 1 shows the single-crystal structure of 2,3-dilauroyl-DL-glycero-1-phosphoethanolamine[1]. The atom numbering and notation for torsion angles is included in this figure. The mode of intermolecular packing of the phospholipid molecules is the classical bilayer. Between adjacent bilayers a layer of acetic acid molecules of crystallisation is intercalated. Prominent features of the molecular packing are: Firstly, the parallel stacking of the hydrocarbon chains which are oriented perpendicular to the bilayer plane (parallel to the bilayer normal). As discussed below the lack of a chain tilt reflects the fact that the molecular area S is determined by 2Σ, the sum of the cross-sections of the two hydrocarbon chains. Structural parameters of some phospholipid single-crystals are summarized in Table 1. The structural notation used here is given in Fig. 2. Apparently the space requirement of the polar group of this molecule or more precisely the projection of the polar group onto the bilayer plane or layer interface (Fig. 2) is less than 2Σ. Secondly, the glycerol group (C1-C2-C3, Fig. 1) is oriented approximately perpendicular to the bilayer plane forming a continuous zig-zag with the fatty acyl chain (γ-chain) esterified to glycerol C3. The initial part of the β-chain is attached to glycerol C2 at a right angle to the glycerol group. Consequently, the β-chain is initially oriented coplanar but makes a 90° bend at C22 to become aligned parallel to the γ-chain (Fig. 1). This kind of parallel chain stacking causes an axial displacement of the two hydrocarbon chains by three methylene groups. Thirdly, the phosphodiester group (torsion angles α_2 and α_3) has a double synclinal *(gauche)* conformation giving rise to an approximately 90° kink at the phosphate group. As a result the orientation of the polar head group of phosphatidylethanolamine is coplanar, i.e., parallel with respect to the bilayer plane (Fig. 1).

The molecular packing of 2,3-dimyristoyl-D-glycero-1-phosphocholine dihydrate in the single-crystal structure is similar to that of phosphatidylethanolamine discussed above (Fig. 3). The molecules pack in a typical bilayer with parallel stacking of the hydrocarbon chains. However, the two independent molecules labeled 1 and 2 (Fig. 3), which exhibit approximate mirror image symmetry with respect to their head group conformation, are mutually displaced in the direction of the layer normal by one zig-zag unit of their hydrocarbon chains (= 2.5 Å). This offset along the layer normal together with an inclined orientation of the polar group allows the bulky head group, phosphorylcholine, to pack in the layer plane at a relatively small molecular area of 38.9 Å^2. This group has an area of about 50 Å^2 when it is oriented parallel to the layer surface as is the case in the single-crystal structure of a lysophosphatidylcholine analogue (Table 1, cf. Fig. 5). The molecular area S = 38.9 Å^2 thus achieved by this special packing mode is almost identical to that of dilauroylphosphatidylethanolamine (S = 38.6 Å^2). There is still a slight mismatch between the molecular area S = 38.9 Å^2 and the sum of the

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NATURALLY OCCURRING AMPHIPHILES

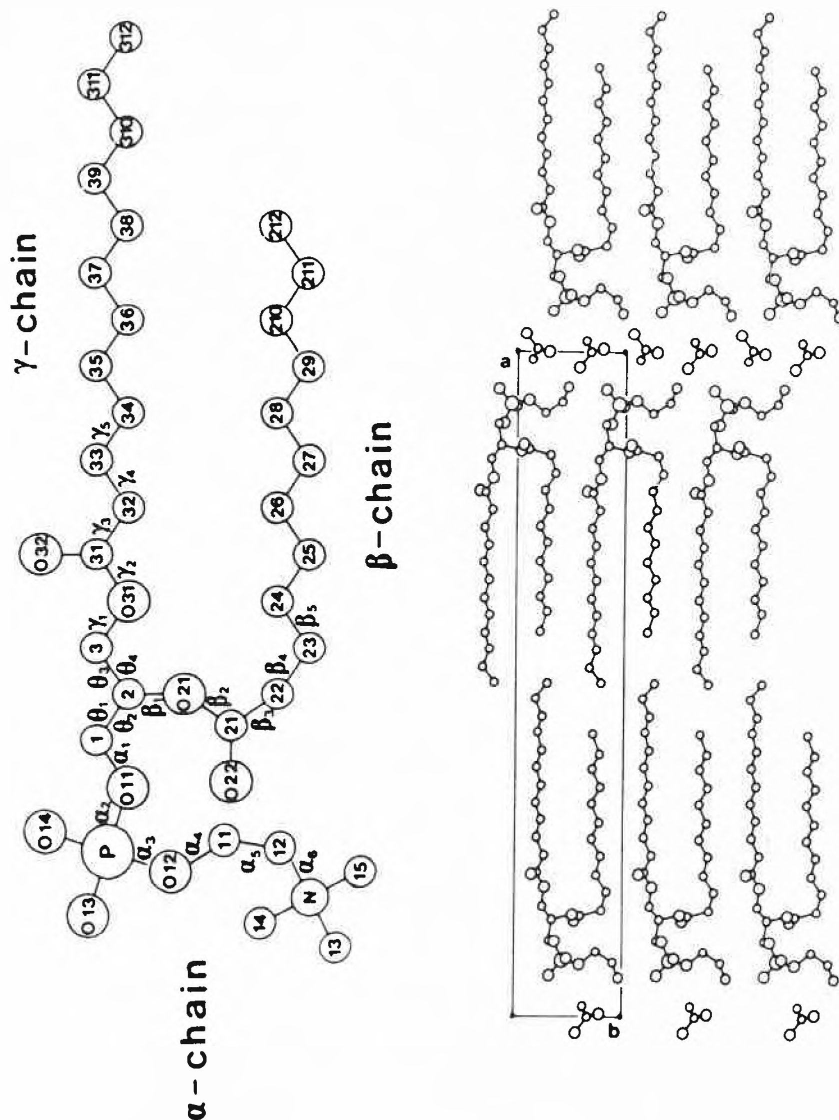

Fig. 1. Molecular packing in the single-crystal structure of 2,3-dilauroyl-DL-glycero-1-phosphoethanolamine–acetic acid[1] *projected onto the a–b plane. The rectangle represents the unit cell containing four phospholipid molecules. The atom numbering and notation for torsion angles is given in the enlarged molecule on the left.*

Table 1. Structural parameters of phospholipid single-crystals [a].

	DLPE	DMPC	PGPE	LPPC
crystal system	monoclinic	monoclinic	monoclinic	monoclinic
space group	$P2_1/c$	$P2_1$	$P2_1/a$	$P2_1/c$
unit cell	a = 47.7 Å	a = 8.72 Å	a = 7.66 Å	a = 24.82 Å
	b = 7.77 Å	b = 8.92 Å	b = 9.08 Å	b = 9.53 Å
	c = 9.95 Å	c = 55.4 Å	c = 37.08 Å	c = 10.94 Å
	β = 92.00°	β = 97.40°	β = 90.2°	β = 99.70°
bilayer thickness (Å)	47.7	54.9		24.5
thickness of the polar region (Å)	7.9	10.4		7.3
molecular area S (Å^2)	38.6	38.9	34.8	52.1
hydrocarbon chain cross-section (Å^2) [b]	19.3	19.0	18.7	19.7
hydrocarbon chain tilt φ [c]	0° (0°)	12° (12.3°)	57.5° (57.5°)	41° (40.9°)
inclination of P–N-dipole towards layer plane	15°	17.27°		7°

[a] The abbreviations used in this table are: DLPE = 2,3-dilauroyl-DL-glycero-1-phosphoethanolamine–acetic acid; DMPC = 2,3-dimyristoyl-D-glycero-1-phosphocholine dihydrate; PGPE = 3-palmitoyl-DL-glycero-1-phosphoethanolamine; LPPC = 3-lauroylpropanediol-1-phosphocholine monohydrate.

[b] The cross section is the projection of the hydrocarbon chain onto a plane oriented perpendicular to the hydrocarbon chain long axis as indicated in Fig. 2.

[c] The angle φ of the hydrocarbon chain tilt is related to S and Σ by $S \cos\varphi = n\Sigma$, where n is the number of hydrocarbon chains per lipid molecule and $n\Sigma$ is therefore the sum of the cross-sections of the hydrocarbon chains. The values in brackets are included for comparison and represent the values calculated from $S \cos\varphi = n\Sigma$ using the S and Σ values in the table.

cross-sections of the hydrocarbon chains 2Σ = 38.0 Å^2 (Table 1). As a consequence, the hydrocarbon chains are slightly tilted with respect to the bilayer normal. The angle of this tilt φ = 12° (cf. Fig. 2 and 3) is given by the equation $S \cos\varphi = n\Sigma$, where n is the number of the hydrocarbon chains per lipid molecule and hence $n\Sigma$ is the sum of the cross-sections of the hydrocarbon chains. As mentioned before, the orientation of the polar group is more inclined with respect to the layer plane compared to dilauroylphosphatidylethanolamine. The angle of inclination of the P–N-dipole towards the layer plane is 15° in phosphatidylethanolamine and 17° and 27° in molecule 1 and 2, respectively, of dimyristoylphosphatidylcholine (Table 1).

The molecular packing of the lysophospholipid, 3-palmitoyl-DL-glycero-1-phosphoethanolamine, in the single-crystal structure is shown in Fig. 4. The molecules pack in the bilayer with extremely tilted hydrocarbon chains. The phosphoethanolamine head group is aligned parallel to the bilayer plane occupying an area S = 34.8 Å^2. The hydrocarbon chains are densely packed with a cross-section of Σ = 18.7 Å^2. Inserting these values in $S \cos\varphi = n\Sigma$ (with n = 1) gives an angle of tilt φ = 57.5°. This large value of φ is close to the maximum value of 60° at which hydrocarbon chains can be tilted in a bilayer. Apparently the marked mismatch between S and $n\Sigma$ leads to the extreme chain tilt observed in this crystal structure. As a consequence, two hydrocarbon chains adjacent in the a-direction of the unit cell are axially displaced by five methylene groups (Fig. 4).

The molecular packing of a lysophosphatidylcholine analogue, 3-lauroylpropanediol-1-phosphocholine monohydrate, in the single-crystal structure is shown in Fig. 5. This lysophospholipid analogue has a propanediol component instead of the glycerol. The lipid molecules pack head-to-tail forming a common hydrocarbon chain matrix. The thickness of this hydrocarbon layer is about half of the bilayer thickness (Table 1). This is due to the interdigitation of the hydrocarbon chains. The orientation of the polar group is parallel to the layer plane occupying an area S = 52.1 Å^2 at the layer surface. In this case S exceeds $n\Sigma$ = 19.7 Å^2 (n = 1) by more than a factor of 2. The equation $S \cos\varphi = n\Sigma$ would predict an angle of chain tilt of φ = 67.8°. However, if the area occupied by the polar group exceeds the cross-section of the hydrocarbon chains by a factor of 2 or more, i.e., if $S > 2n\Sigma$, hydrocarbon chain interdigitation occurs (Fig. 5). As a result of this chain interdigitation, two hydrocarbon chains are accommodated per polar group. Hence, the molecular packing shown in Fig. 5 resembles that of a diacylphospholipid with tilted hydrocarbon chains, except that the thickness of the hydrocarbon chain region is given by the chain length of one rather than two fatty acids. The observed chain tilt of 41° is predicted by the

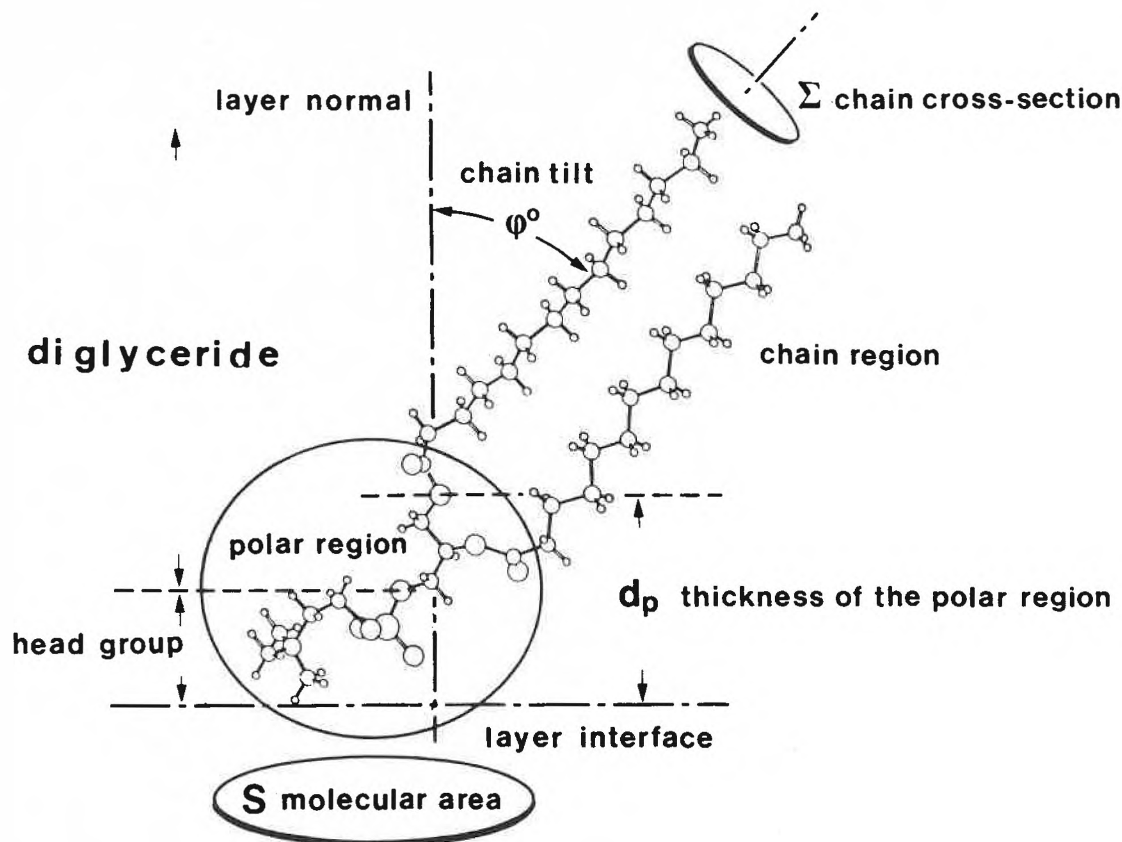

Fig. 2. Structural notation. A schematic drawing of a diacylphosphatidylcholine molecule is used to define various structural regions and parameters. S = molecular area at the layer interface; Σ = cross-section of the hydrocarbon chains perpendicular to the chain axis; φ = angle of tilt between the hydrocarbon chain axis and the bilayer normal. The thickness of the polar region d_p and of the head group projected onto a plane perpendicular to the bilayer plane is indicated by arrows (from [2]).

equation $S \cos \varphi = n \Sigma$ with n = 2, assuming that as the result of interdigitation two hydrocarbon chains are accommodated per lipid polar group. The tilt of the hydrocarbon chains (Fig. 5) apparently results from the residual mismatch of $S = 52.1$ Å^2 and $n \Sigma = 39.4$ Å^2.

The justification for a more detailed discussion of the intermolecular packing and conformation of phospholipids in the crystal is the finding that the motionally averaged conformation of phospholipid in the hydrated, liquid-crystalline state has a number of features in common with the single-crystal structure. This has been the subject of several review articles[2,3]. It is clear from the crystal structure depicted in Figs. 1, 3–5 that the packing mode is determined by the ratio $S/n\Sigma$. There is a corollary in the phase behaviour of phospholipids (see chapter 2). This ratio $S/n\Sigma$ appears to be an important factor in determining the lyotropic phase of phospholipids.

2. The Liquid-Crystalline State

When a phospholipid crystal is heated there is an endothermic transition at a certain temperature, T_c or Krafft point, which is well below the capillary melting point T_m. Hence the resulting liquid-crystalline state is referred to as mesomorphic state

$$\text{crystal} \underset{}{\overset{T_c}{\rightleftharpoons}} \text{liquid-crystal} \underset{}{\overset{T_m}{\rightleftharpoons}} \text{liquid}$$

To a first approximation this transition to the liquid-crystal is a first order transition and characterized by the following processes:

- There is a lateral expansion of the crystal, S increases.
- The bilayer thickness d decreases.
- Heat is taken up and the heat absorbed causes melting of the hydrocarbon chains. The static state of the crystal changes to the highly dynamic state of the liquid-crystal.
- There is an entropy increase which is about half ($\Delta S = 1.1$ entropy units per CH_2) of that accompanying the melting of paraffin ($\Delta S = 2.6$ entropy units per CH_2)[7].
- Water is readily taken up by the crystal hydrating the polar group (Fig. 6).
- The lateral compressibility of the bilayer goes through a maximum at T_c[8].

The dynamics of the liquid-crystalline state is summarized in Table 2. The motion of the phospholipid molecule is anisotropic and cooperative. Spectroscopic measurements indicate that the molecule undergoes fast rotation about its long axis (or z-axis) with frequencies $\nu \geqslant 10^8$ s^{-1}. Furthermore, there is fast lateral diffusion within the plane of the monolayer with a diffusion coefficient $D = 10^{-8}$ cm^{2}s^{-1}. This corresponds to a mean displacement $\Delta\bar{x}$ in the monolayer of about 1 µm. In contrast to the fast rotation about the z-axis (= bilayer normal, see schematic diagram in Table 2), rotations about the x- or y-axis are negligible. In hydrated, liquid-crystalline phosphatidylcholine bilayers the transverse movement of phospholipid molecules from one layer of the bilayer to the other is practically frozen (Table 2). In addition to the molecular motion summarized in Table 2 there is segmental motion. NMR relaxation time measurements[9] as well as order parameter determination[3] indicate that there is a flexibility gradient along the molecular long axis: the segmental motion increases towards the centre of the bilayer. Order parameter profiles derived from ESR spin labeling[10] and ^{2}H-NMR measurements[11] are shown in Fig. 7. The deuterium order parameter S_{mol} of dipalmitoyl-3-*sn*-phosphatidylcholine at T_c and above T_c as a function of the position of the ^{2}H-labeled C-atom shows little variation up to about C-atom 10. From C-atom 10 to the centre of the bilayer there is a marked decrease in the

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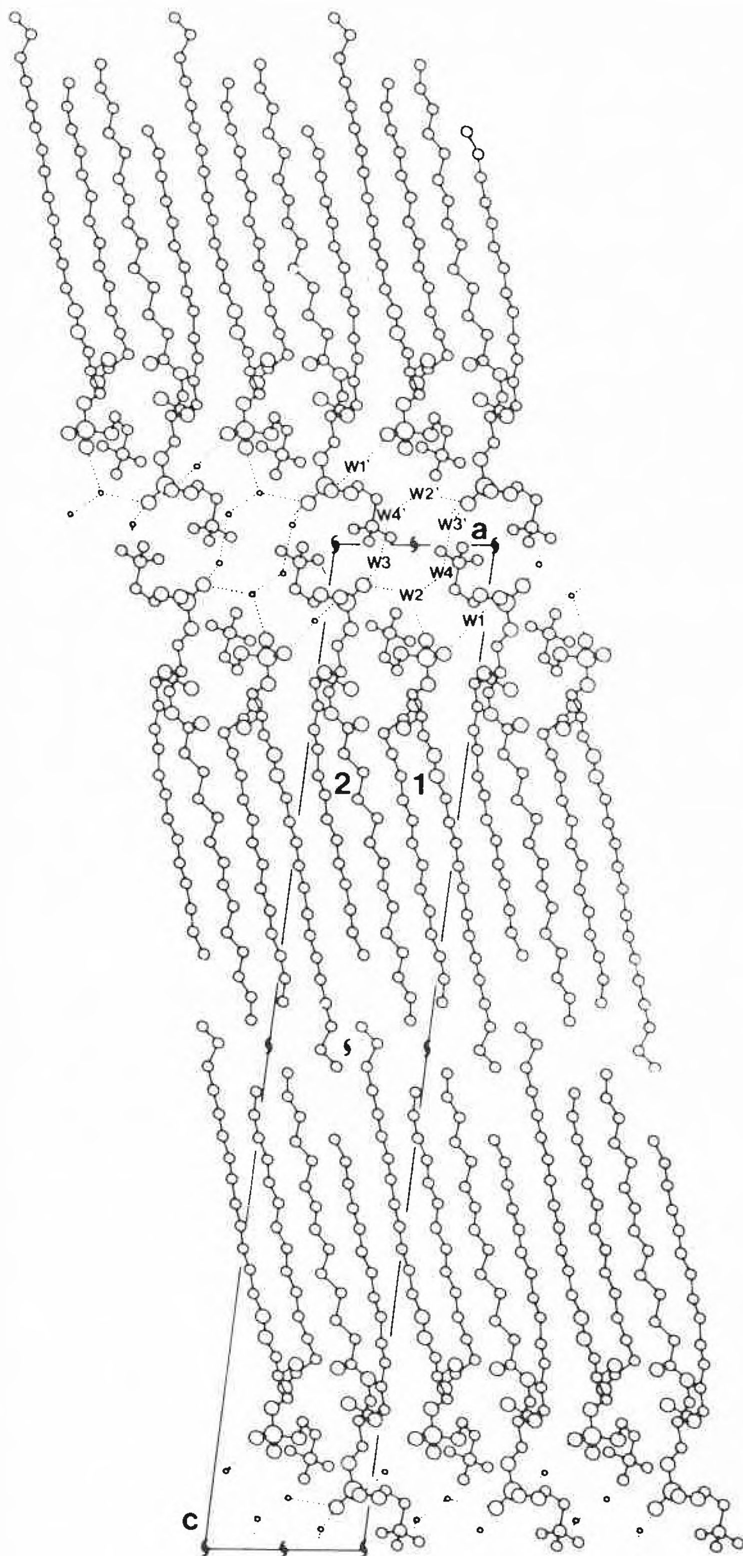

*Fig. 3. Molecular packing in the single-crystal structure of 2,3-dimyristoyl-*D*-glycero-1-phosphocholine dihydrate* [2,4] *projected onto the a–c plane. The two different phospholipid molecules in the asymmetric unit cell (indicated by the parallelogram) are labeled 1 and 2. The position of the water molecules is indicated either by W_1–W_4 or by small open circles. Hydrogen bonding between the water molecules is represented by dotted lines.*

order parameter. These results are contrasted by the ESR order parameter which decreases approximately linearly with the position of the labeled C-atom. The discrepancy between the two order parameter profiles is probably due to a local perturbation of the lipid hydrocarbon chains in the presence of the relatively bulky spin label group. It is worth noting that very similar deuterium order parameters profiles to those shown in Fig. 7 are obtained for hexagonal phospholipid phases (see below, [12]). This indicates that the amplitude of the angular fluctuations of the segmental motion, which essentially arise from rotational isomerisation about C–C bonds (of frequency $\nu > 10^7$ s^{-1}), is similar in lipid bilayers (lamellar phase) and lipid cylinders constituting hexagonal phase I and II.

Phospholipids are known to exhibit polymorphism: both in the crystal as well as in aqueous dispersions they can occur in more than one phase, a phenomenon referred to as thermotropic and lyotropic mesomorphism, respectively. The polymorphism of phospholipids has been reviewed extensively, e.g. [13]. At present a prediction of the phospholipid phase based on theoretical considerations is not possible. However, as mentioned above, the relative space requirement of the polar group and the hydrophobic part of the phospholipid molecule play an important role in determining the phase behaviour. Undoubtedly, the structural properties discussed in chapter 1 bear on the phase behaviour of phospholipid in aqueous dispersion. As a matter of fact, based on the dynamic, molecular shape as for instance represented by the $S/n\ \Sigma$ ratio, rudimentary guidelines for predicting the phospholipid phase have been proposed [14]. This is shown in Fig. 8. Phospholipid molecules with cylindrical shape ($S = n\ \Sigma$) can be readily accommodated in a bilayer and such molecules have been reported to form preferentially smectic (lamellar) phases. Wedge-shaped molecules with $S > n\Sigma$, which are not compatible with planar bilayers, tend to form the hexagonal I phase or micellar solutions. Lipid molecules with $S < n\Sigma$ have the shape of an inverted cone and are supposed to form the inverted hexagonal phase (hexagonal II) or inverted micelles.

3. The Swelling of Phospholipids in Water

Our discussion of aqueous lipid dispersions will be confined to lipid and lipid mixtures forming smectic (lamellar) phases. It has been shown that, when H_2O is added to a phospholipid crystal, the water molecules penetrate the polar group lattice hydrating the phosphate group and other parts of the polar group [15]. The effect of hydration is to decrease the transition temperature T_c. This is shown for the $NH_4^{\oplus}$ salt of dimyristoylphosphatidylserine in Fig. 9. With increasing H_2O-content the transition occurs at progressively lower temperature, reaching a limiting value of 39 °C at a H_2O content of about 45 wt. %. Both below and above the transition temperature, water penetrates readily the polar group lattice and the bilayer swells. This is only true for charged lipid bilayers. Uncharged and zwitterionic bilayers are readily hydrated and dispersed only above their transition temperature. The hydration and swelling of a bilayer is shown

*Fig. 4. Molecular packing in the single-crystal structure of 3-palmitoyl-*DL*-glycero-1-phosphoethanolamine*[6] *projected onto the a–c plane. The rectangle represents the unit cell containing four phospholipid molecules.* D*-and-*L*-enantiomers, which in this projection appear alternately in the a direction, are separated by half a unit cell edge (4.54 Å) in the b direction. In the top layer, a row of* L*-enantiomers is shown. In this layer, the axial displacement of the hydrocarbon chains is indicated by the dashed line. Due to the extreme chain tilt ($\varphi = 57.5°$) the axial displacement amounts to five CH_2-groups.*

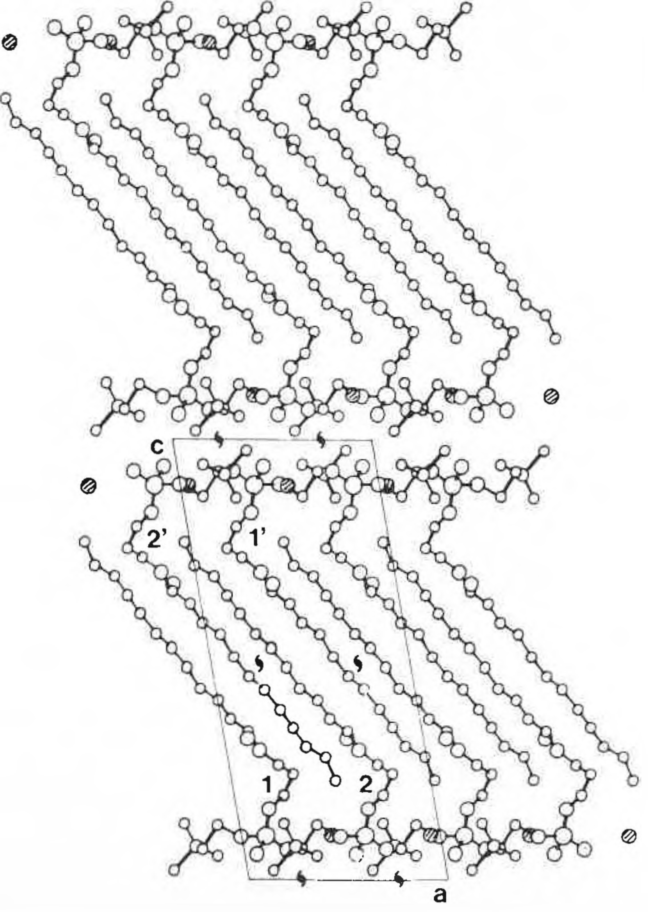

Fig. 5. Molecular packing in the single-crystal structure of 3-lauroylpropanediol-1-phosphocholine monohydrate[2,5] *projected onto the a–c plane. The unit cell indicated by the parallelogram contains four symmetry-related molecules arranged in pairs of conformational enantiomers. Molecules 1 and 2 are mirror images and related to the corresponding molecules on the opposite layer side by a two-fold screw axis (⸙). Water molecules of hydration are represented by the hatched circles.*

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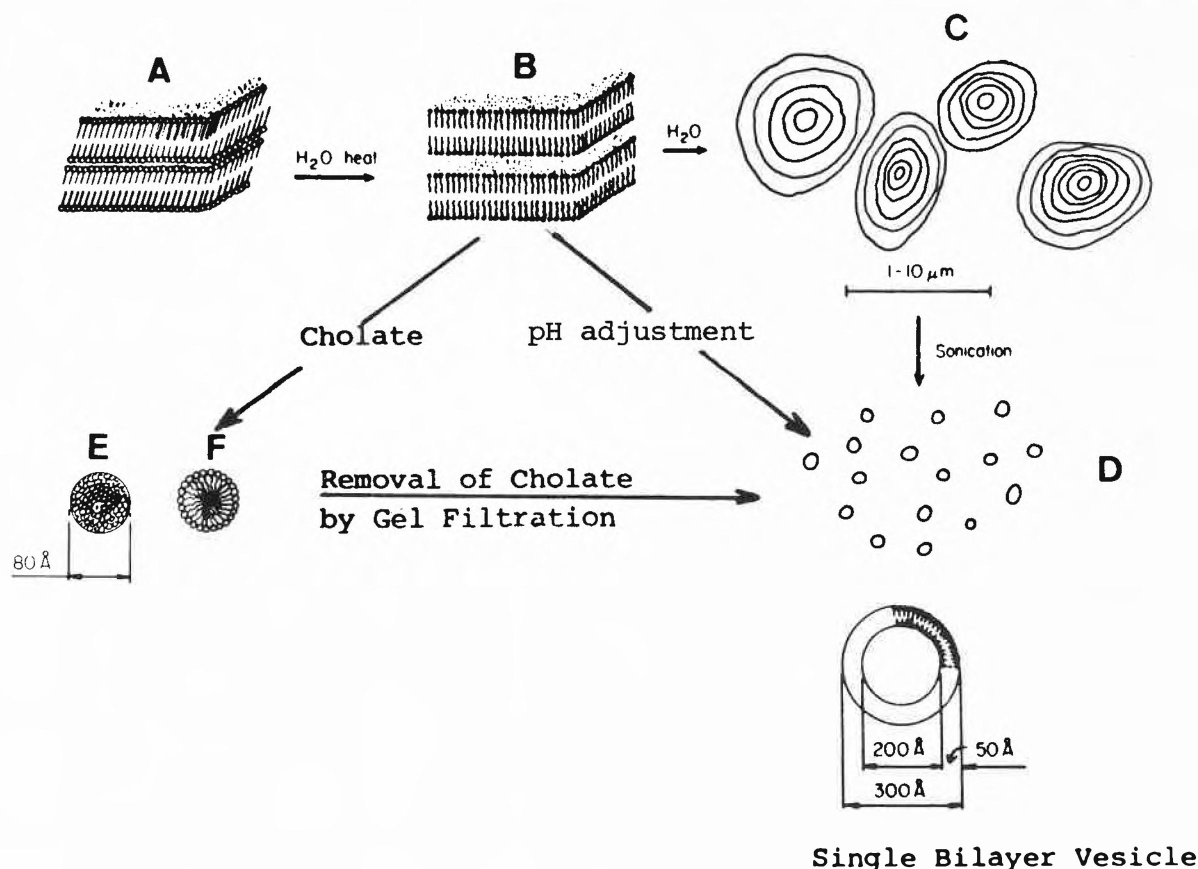

Fig. 6. Schematic drawing showing stacked bilayers of a diacylphosphatidylcholine in the anhydrous crystal (A). The hydrocarbon chains are tilted with respect to the layer normal. At the Krafft point (T_c) the hydrocarbon chains melt and water readily penetrates the polar group lattice. At this temperature (T_c) the transition from the crystal to the liquid-crystal (B) takes place. With increasing H_2O content, water molecules are bound to the phospholipid polar group: 11–12 water molecules per diacylphosphatidylcholine molecule. Water in excess of bound water is intercalated between the bilayers and as a result the bilayers swell. The limited swelling of phosphatidylcholine bilayers is indicated by the fact that when the water content exceeds 23 mol H_2O/mol of phosphatidylcholine, a two phase system is formed (C) consisting of multilamellar particles (liposomes) and excess H_2O. Each line of the particles in C represents one bilayer: the onion-like staking of the fully hydrated, swollen bilayers is obvious. When an aqueous dispersion of these multilamellar particles (liposomes) is subjected to ultrasonication, the bilayers disintegrate to form small unilamellar vesicles (D) which are surrounded by a single bilayer. If sufficient bile salts such as sodium cholate are added to hydrated phosphatidylcholine bilayers (B or C), the multilamellar structures are solubilized to small mixed micelles consisting of phosphatidylcholine and cholate (E, F). Upon removal of the cholate small unilamellar vesicles are formed (D). Removal of the cholate from the mixed micelle can be accomplished by dialysis or gel filtration. Spontaneous vesiculation of bilayers by a transient pH-change is indicated by path B to D. In this case the bilayers in B are either phosphatidic acid or mixtures of this with phosphatidylcholine. Spontaneous vesiculation occurs when the pH of the aqueous lipid dispersion is raised transiently to 10–12 and then returned to neutrality.

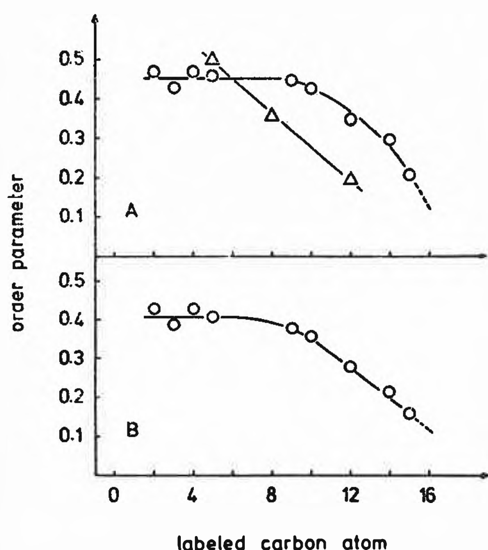

Fig. 7. Order parameter S_{mol} of dipalmitoylphosphatidylcholine bilayers determined by deuterium magnetic resonance as a function of the position of the labeled C-atom. A: (○) ^2H-NMR at 41°C, 48.5 wt.% dipalmitoylphosphatidylcholine, 51.5 wt.% H_2O [3,11]; (△) spin label order parameter for 20 wt.% dipalmitoylphosphatidylcholine dispersion in H_2O [10]. B: (○) ^2H-NMR at 50°C, 48.5 wt.% dipalmitoylphosphatidylcholine, 51.5% H_2O [3,11].

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Table 2. Scheme describing the molecular motion observed in hydrated, liquid-crystalline phospholipid bilayers. The values given are characteristic of diacylphosphadylcholine bilayers.

	frequency (s^{-1})
1. Intramonolayer	
rotation about the long (z) axis	10^8
lateral diffusion	10^7 ($D = 10^{-8}\ cm^2s^{-1}$) ($\Delta\bar{x} = 2\ \mu m$)
protein (rhodopsin)	
rotational } diffusion	$5 \times 10^3 - 5 \times 10^4$
lateral } diffusion	($D = 10^{-10} - 10^{-11}\ cm^2s^{-1}$) ($\Delta\bar{x} = 0.1\ \mu m$)
2. Intermonolayer	
flip-flop about the x- or y-axis	3×10^{-5} (10 h)

	molecular shape	phase
S = n Σ	cylinder	smectic, bilayers
S > n Σ	cone or wedge	hexagonal I micelles ($H_I a$)
S < n Σ	inverted cone	hexagonal II inverted micelles ($H_{II} a$)

Fig. 8. The dynamic molecular shape as an important factor determining the phase behaviour of a lipid (from [14]).

schematically in Fig. 6 (B). The swelling of bilayers is best measured by X-ray low-angle diffraction which yields the lamellar repeat distance d (= sum of the thickness of the bilayer and the water layer intercalated between bilayers).

As regards the swelling of smectic lipid phases in H_2O, two different classes of lipids can be differentiated: (i) neutral and zwitterionic lipids showing no or limited swelling with increasing H_2O-content, and (ii) charged lipids exhibiting continuous swelling in H_2O. Examples of the different swelling behaviour are shown in Fig. 10a.

The $NH_4^{\oplus}$ salt of dimyristoylphosphatidylserine which bears one net negative charge at neutral pH is an example of the second class. When dispersed in H_2O, the lamellar repeat distance d increases continuously with increasing water content both below and above the transition temperature T_c. In contrast, the lamellar repeat distance d of the protonated form of dimyristoylphosphatidylserine at pH 2 is invariant with H_2O-content. Under these conditions the phosphatidylserine is zwitterionic and does not swell at all. The value of

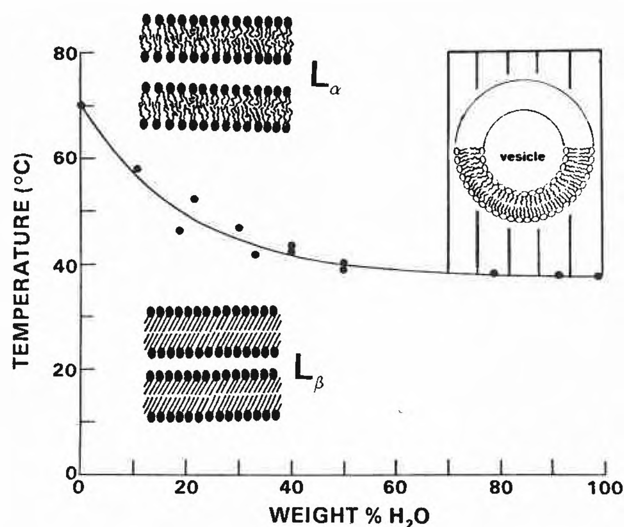


Fig. 9. Phase diagram of the $NH_4^\oplus$ salt of dimyristoylphosphatidylserine in H_2O. The solid line represents the gel-to-liquid-crystal transition temperature as determined by differential scanning calorimetry. The hatched area on the right represents the region where the transition from fully-swollen multilamellar particles (liposomes) to large unilamellar vesicles of diameter > 0.1 μm takes place (from [21]).

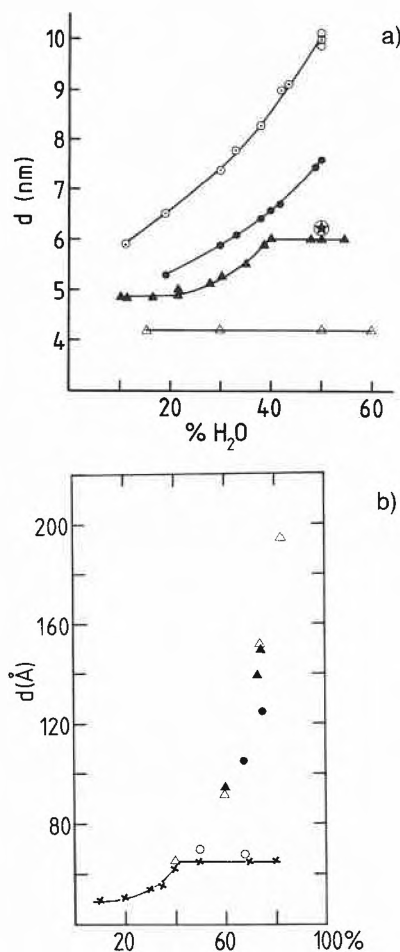


Fig. 10. (a) Swelling of phospholipid bilayers in H_2O. The lamellar repeat distance d determined by X-ray diffraction is plotted as a function of H_2O content (wt.%) for the $NH_4^\oplus$ salt of dimyristoylphosphatidylserine at 20°C (○), a temperature well below the gel-to-liquid-crystal phase transition temperature (T_c) which is at 39°C; for the same phospholipid in the liquid-crystalline state at 50°C (●). The data point at 50 wt.% H_2O (⊛) gives the lamellar repeat distance of $NH_4^\oplus$-dimyristoylphosphatidylserine in 0.5 M NaCl. At pH ≈ 2 where dimyristoylphosphatidylserine is protonated and hence zwitterionic no swelling is observed (△). The limited swelling of dimyristoylphosphatidylcholine in the liquid-crystalline state at 39°C is represented by ▲ (from [21]). – (b) Lamellar repeat distance d as a function of the water content (wt.%) for egg phosphatidylcholine (×) and mixtures consisting of this phospholipid and either a positively or negatively charged detergent; for egg phosphatidylcholine containing 0.6 wt.% cetyltrimethylammonium bromide (CTAB) (○), egg phosphatidylcholine containing 0.9 wt.% CTAB (●), egg phosphatidylcholine containing 1.5 wt.% CTAB (△); for egg phosphatidylcholine containing 3.5 wt.% sodium oleate (▲) (from [18]).

$d = 42.0 \pm 0.5$ Å is identical to that of the crystal of protonated dimyristoylphosphatidylserine. Zwitterionic dimyristoylphosphatidylcholine shows limited swelling in H_2O: d increases with increasing water content reaching an upper limit of $d \approx 60$ Å at about 40% H_2O (Fig. 10a). Above this water content there is a two-phase system as shown in the schematic drawing in Fig. 6 (C). It consists of fully hydrated and swollen multilamellar dimyristoylphosphatidylcholine particles or liposomes and excess H_2O.

The thermodynamically stable structure of zwitterionic phospholipids in excess H_2O is therefore the multilamellar liposome. This conclusion is generally true for uncharged and zwitterionic lipids forming smectic phases in excess H_2O. The question of the thermodynamically stable structure of charged lipids such as phosphatidylserines in excess H_2O is of considerable practical importance. At water contents in excess of 50% (Fig. 10a) the sharp low-angle X-ray reflections become diffuse due to stacking disorders. As a result the lamellar repeat distance d cannot be derived any longer. However, from freeze-fracture electron microscopy it is clear that the swelling of the bilayers continues at these water contents.

Evidence from small-angle X-ray scattering and freeze-fracture electron microscopy shows that at water contents in excess of about 80% the large, fully-swollen multilamellar liposomes disintegrate and large unilamellar vesicles (LUV) are formed. We conclude that the thermodynamically stable structure in the excess water region of the phase diagram of charged lipids is the unilamellar vesicle, i.e., a lipid particle consisting of a solvent-filled cavity that is surrounded by a single, closed lipid bilayer. Hence, if excess H_2O or salt solution of moderate ionic strength ($I < 0.2$) are added to a dry, smectic film of a charged diacylphospholipid deposited on the glass wall of a flask, unilamellar vesicles will form spontaneously. This process is referred to as *spontaneous vesiculation*. The dispersion formed is polydisperse consisting of unilamellar vesicles with diameters ranging from 0.1 to several μm; however, a large fraction (≈ 90%) of the vesicles have a diameter between 0.1 and 2 μm. The vesicle size analysis of such dispersions is carried out using a combination of gel filtration, dynamic light scattering, and freeze-fracture electron microscopy.

It should be emphasized that spontaneous vesiculation only occurs when water or salt solutions of moderate ionic strength are added to smectic films of charged diacylphospholipids. It has been shown [16] that the continuous swelling of $NH_4^\oplus$-dimyristoylphosphatidylserine with increasing water content is reversed upon the addition of NaCl. With increasing NaCl-concentration a linear reduction in the lamellar repeat distance d occurs. This is due to $Na^\oplus$ shielding the negative charges on the phosphatidylserine bilayer. As a result, the bilayer separation shrinks by water being extruded from the interbilayer space. For instance, in 0.5 M NaCl the lamellar repeat distance d of $NH_4^\oplus$-dimyristoylphosphatidylserine is reduced to a value close to that observed for zwitterionic phosphatidylcholine (see ⊗ in Fig. 10a).

In the presence of excess NaCl, charged lipid bilayers behave therefore like zwitter-

ionic phospholipids exhibiting limited swelling in H_2O. The nature of the counter ion is unimportant; similar effects as with $Na^{\oplus}$ are observed with other cations[17,18] indicating that the effect is purely electrostatic.

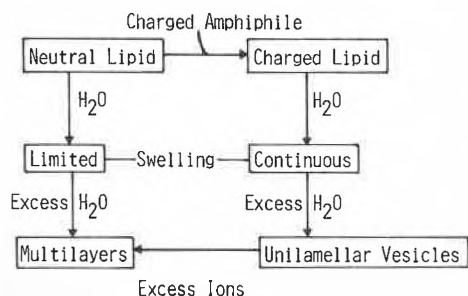
4. Spontaneous Vesiculation

The surface charge density of natural and synthetic phosphatidylserines varies between about 23 and 36 μC/cm² depending on the nature of the fatty acyl chains. *Luzzati* et al.[18] made an observation which is important from a practical point of view. By doping egg phosphatidylcholine with only a few percent of a positively or negatively charged detergent (amphiphile) continuous swelling is induced: the limited swelling characteristic of zwitterionic phosphatidylcholine dispersions is changed to that characteristic of charged phospholipid bilayers.

Continuous swelling is observed for egg phosphatidylcholine doped with a few percent of either positively charged cetyltrimethylammonium bromide (CTAB) or negatively charged sodium oleate (Fig. 10b)[18]. The surface charge density of these lipid mixtures is 1–2 μC/cm², less than 1/10 of that of pure phosphatidylserines. From the continuous swelling in H_2O of these egg phosphatidylcholine-detergent mixtures it can be expected by analogy with charged phosphatidylserine that these lipid mixtures will undergo spontaneous vesiculation in excess H_2O. This is borne out by experiments showing that indeed LUV are formed when excess H_2O is added to films made of these lipid mixtures.

The swelling in H_2O of lipid and lipid mixtures as discussed above may be summarized in Scheme 1: Neutral (zwitterionic) and charged lipids or lipid mixtures differ in their swelling behaviour and in the thermodynamically stable structure present in excess H_2O. By doping neutral lipids with a charged amphiphile, the resulting lipid mixtures behave like charged lipids. On the other hand, in excess ion concentrations charged lipids exhibit swelling properties typical for neutral lipids.

Scheme 1



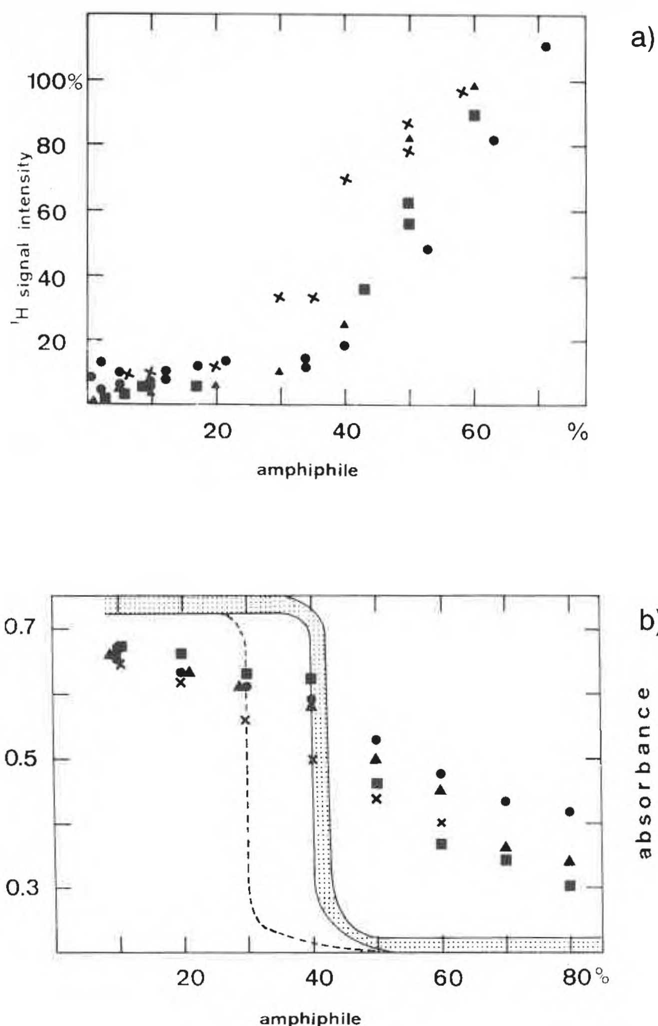


Fig. 11. (a) Signal intensities of the ¹H-NMR spectra recorded from mixed disperisons of egg phosphatidylcholine and a positively charged detergent (amphiphile) as a function of the detergent content in %. Mixed lipid dispersions at 10 mg total lipid/mL ²H₂O, pH 5–6, consist of egg phosphatidylcholine and a positively charged detergent: dodecyltrimethylammonium bromide (■), tetradecyltrimethylammonium bromide (▲), cetyltrimethylammonium bromide (●), cetylpyridinium chloride (×). ¹N-NMR spectra were recorded at 360 MHz and the intensity of the hydrocarbon chain signal was measured and plotted as % of the total lipid present in the aqueous dispersion (for details see ref.[21,22]. *– (b) The ESR order parameter S_{33} of the aqueous mixed lipid dispersions described in (a) as a function of the detergent (amphiphile) content. The meaning of the symbols is as above. The order parameter S_{33} of 5-doxylstearic acid incorporated in the mixed bilayers is shown. The dashed line represents the turbidity expressed in abitrary units of mixed bilayers containing cetylpyridinium chloride as measured by the absorbance at 340 nm. The stippled area encloses all turbidity curves measured for all the other mixed lipid dispersions (for details consult ref.*[21,22]*).*

The effect of the addition of small amounts of CTAB to egg phosphatidylcholine is shown in Fig. 10b and has been already discussed. Fig. 11 shows the effect of admixing increasing quantities of this detergent and other positively charged detergents to egg phosphatidylcholine bilayers dispersed in excess H_2O. The effect of increasing concentrations of these positively charged detergents (amphiphiles) on the ¹H-NMR signal intensity of the lipid spectrum is shown in Fig. 11a.

¹H-NMR is used here routinely to differentiate between large and small unilamellar lipid vesicles. The method is based on the observation that only small unilamellar vesicles (SUV) and micelles with a diameter $\lesssim 0.1$ μm contribute to the high-resolution spectrum while large lipid aggregates (diameter > 0.1 μm) such as multilamellar liposomes and LUV do not give high-resolution NMR spectra[19]. The signal intensity of the NMR spectrum is a good measure of the amount of lipid present as SUV or micelles. Up to amphiphile contents of 30% the fraction of lipid giving rise to a high-resolution spectrum is less than about 15% (Fig. 11a); an exception is cetylpyridinium chloride which is apparently more effective than the other detergents. At am-

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phiphile contents > 30% there is a sigmoidal increase in the ^{1}H-signal intensity which is accompanied by an abrupt drop in the turbidity of the dispersions (cf. Fig. 11). Visually the dispersions clarify at amphiphile contents ≳ 40%. Included in Fig. 11b is the ESR order parameter S_{33}. Above ≈ 40% amphiphile and coinciding with the clarification of the dispersion there is a discontinuity in S_{33}. In excess detergent the S_{33} values are characteristic of lipid micelles.

From the NMR and ESR evidence presented in Fig. 11 together with electron microscopy of freeze-fractured samples of the same lipid mixtures as investigated for Fig. 11, the following picture emerges: As expected from the continuous swelling behaviour of egg phosphatidylcholine-CTAB mixtures, this mixture and the other charged lipid mixtures (cf. Fig. 11) undergo spontaneous vesiculation when H_2O is added to their dry lipid film and a dispersion is produced by hand-shaking. Up to amphiphile concentrations, at which the sigmoidal increase in ^{1}H-NMR signal intensity is observed (Fig. 11), the vesiculation leads to mainly LUV and some oligomeric large vesicles. The latter are LUV that contain smaller unilamellar vesicles entrapped in their internal, aqueous cavity. With increasing amphiphile content the fraction of SUV grows at the expense of the LUV. At amphiphile concentrations where the sigmoidal increase in the NMR signal intensity occurs, the SUV with a diameter < 0.1 µm is the most stable structure. In excess amphiphile concentrations (≳ 50%), where the ^{1}H-signal intensity is 100%, NMR and ESR evidence indicate that besides SUV mixed micelles consisting of phospholipid and detergent are present; furthermore, with increasing detergent content (above ≈ 50%) the micelle content appears to increase at the expense of SUV.

It is important to stress that the behaviour of charged lipid dispersions consisting of zwitterionic phosphatidylcholine and a positively charged detergent as shown in Fig. 11 is a general one. Many structurally different detergents have been tested as to their effect on the phase behaviour of phosphatidylcholine. We can conclude that the behaviour discussed above (cf. Fig. 11) is characteristic of detergents with a single hydrocarbon chain of 14–18 C-atoms. Three component systems consisting of zwitterionic phosphatidylcholine, a positively or negatively charged detergent, and excess H_2O undergo spontaneous vesiculation over a wide range of phospholipid/detergent ratios. Within this range there is a composition or a range of compositions, though narrow it may be, where the SUV with a diameter < 0.1 µm is the predominant structure. It is clear from Fig. 11 and from other experiments not described here that the detergent concentrations at which the sigmoidal rise in ^{1}H-signal intensity is observed depends on the chemical structure of the detergent. There appears to be a gradual transition from unilamellar vesicles to mixed micelles, the concentration range at which this occurs also depends on the nature of the detergent. The mixed micelle apparently prevails in the excess detergent region of the phase diagram (Fig. 11).

The phase behaviour shown in Fig. 11 is contrasted by that of pure diacylphospholipids bearing one (net) negative charge such as phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, phosphatidic acid at neutral pH (cf. Fig. 12). As described above, negatively charged diacylphospholipids also undergo spontaneous vesiculation; however, the resulting dispersion is polydisperse consisting of LUV with a diameter ranging from about 0.1 µm to several µm. SUV are not produced under these cicrumstances. This is also true for phospholipid mixtures consisting of zwitterionic phosphatidylcholine and one of the negatively charged diacylphospholipids.

The difference in behaviour of charged detergents with a single long hydrocarbon chain and charged diacylphospholipids with two long hydrocarbon chains is shown schematically in Fig. 12. Charged diacylphospholipids with one electronic charge per two hydrocarbon chains, either in pure form or mixed with zwitterionic phosphatidylcholine at a content ≳ 10%, vesiculate spontaneously to form LUV of diameter > 0.1 µm. With lipid mixtures consisting of zwitterionic phosphatidylcholine and a charged lipid the requirement for spontaneous vesiculation is the charge density to exceed the threshold value of about 1–2 µC/cm^2. Single-chain detergents with one electronic charge per hydrocarbon chain induce vesiculation with the formation of either LUV or SUV depending on the detergent content

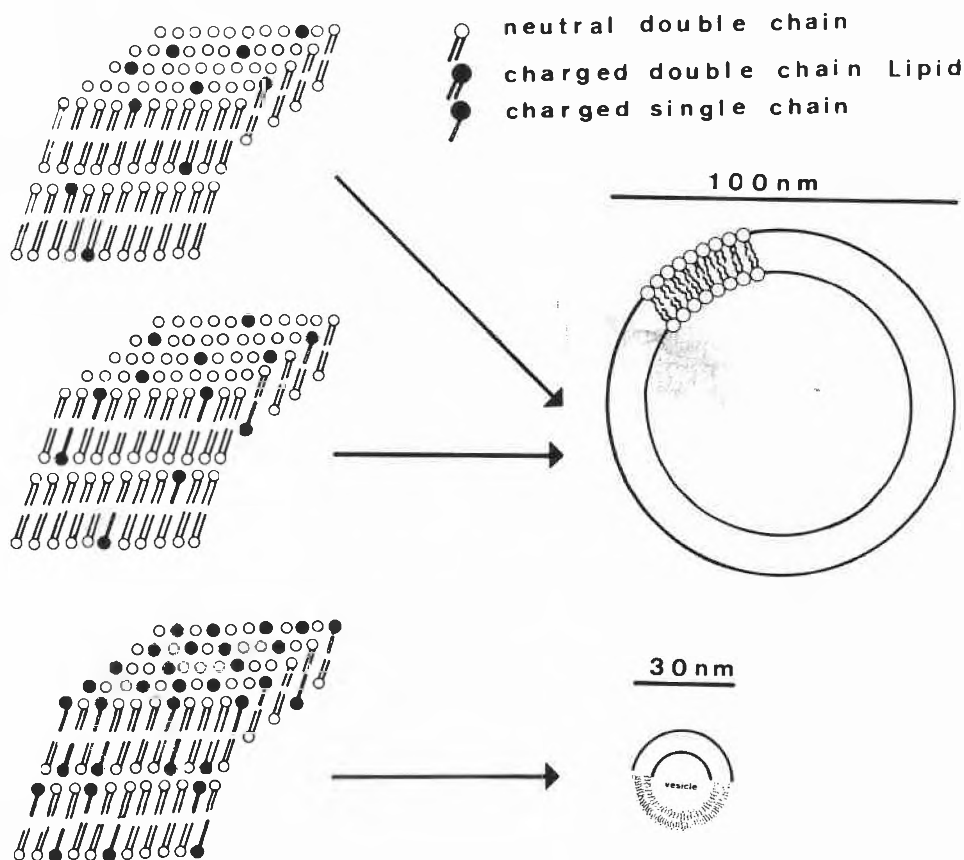

Fig. 12. Schematic diagram (not to scale) showing spontaneous vesiculation induced by either negatively charged diacylphospholipids or charged detergents with a single, long hydrocarbon chain. The diagram indicates that spontaneous vesiculation occurs if H_2O is added to dry mixed films of bilayers consisting of zwitterionic phosphatidylcholine and either a charged diacylphospholipid or a charged single-chain detergent. The only requirement for spontaneous vesiculation to occur is that the surface charge density of these bilayers has to exceed a certain threshold value of about 1–2 µC/cm^2. The difference in the effect of negatively charged diacylphospholipids and of charged, single-chain detergents should become clear. The former, either in pure form or mixed with zwitterionic posphatidylcholine, vesiculate spontaneously forming large unilamellar vesicles of diameter > 0.1 µm (top) while mixtures of charged, single-chain detergents with zwitterionic phosphatidylcholine vesiculate spontaneously forming either large unilamellar vesicles (middle) or small unilamellar vesicles (bottom). The size of unilamellar vesicles depends on the detergent content as discussed in the text.

(Fig. 12). In excess detergent, mixed micelles are formed.

It should be clear from the above discussion that by selecting a certain detergent and a certain detergent/phosphatidylcholine ratio the surface charge density of the bilayer will be determined; this in turn determines the physico-chemical properties of the resulting lipid dispersion such as vesicle size and bilayer properties, for instance bilayer fluidity and permeability. By the proper choice of the detergent and detergent composition it should be possible to vary the physico-chemical properties of the resulting lipid dispersion systematically and to produce vesicles with predetermined properties.

Based on the qualitative rules derived from the phase behaviour of charged, single-chain and double-chain lipids (cf. Fig. 12), a method for the spontaneous formation of SUV with a diameter of 20–50 nm has been developed[20]. The method uses diacylphosphatidic acid or mixtures of this charged phospholipid with zwitterionic phosphatidylcholine or other lipids provided a smectic (lamellar) phase is formed in excess H_2O. The method involves a transient pH-change to pH 11–12 rendering the primary phosphate group of phosphatidic acid fully ionized. Phosphatidic acid bears one negative charge at neutral pH, but at pH > 11 the phosphate group can be shown to be fully ionized. Under these conditions the phosphatidic acid molecule carries two charges per two hydrocarbon chains and it will act basically as a charged single-chain detergent. As predicted by the rudimentary guidelines developed above, the transient pH-change induces spontaneous vesiculation with the formation of SUV of diameter of 20–50 nm[20]. This is shown in the schematic diagram of Fig. 6. ^{31}P-NMR evidence for the formation of SUV is given in Fig. 13. When the pH is readjusted to neutrality, the small vesicles formed are retained. If mixtures of egg phosphatidic acid and zwitterionic phosphatidylcholine are subjected to the pH-change, the fraction of SUV formed can be shown to depend on the lipid composition. The fraction of SUV increases approximately linearly with phosphatidic acid content. If, however, a mixed phosphatidic acid/phosphatidylcholine film is dispersed in H_2O of neutral pH the spontaneous vesiculation leads to LUV. The effects of phosphatidic acid as a vesiculation inducing agent are qualitatively in agreement with the rule summarized in Fig. 12.

The methods of spontaneous vesiculation discussed above are simple and quick. The classical methods for the production of SUV are included in the schematic diagram of Fig. 6. They involve sonication of large multilamellar liposomes or LUV (path C→D, Fig. 6). An alternative path is the solubilization of phospholipid bilayers by bile salts such as for instance sodium cholate. Upon removal of the detergent by standard techniques such as gel filtration or dialysis, SUV of a narrow size

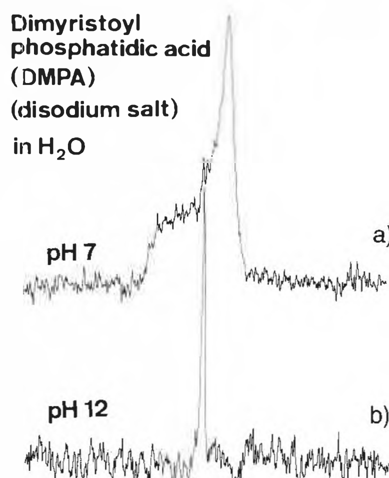

Fig. 13. Spontaneous vesiculation of dimyristoylphosphatidic acid (as the disodium salt) in H_2O induced by a transient pH-change to 12. Proton-decoupled powder ^{31}P-NMR spectra of the aqueous phospholipid dispersion (100 mg/mL = 0.157 M) at pH 7 (a) and pH 12 (b). The ^{31}P-NMR powder spectrum in (a) is typical for liquid-crystalline bilayers as present for instance in large multilamellar phospholipid particles (liposomes) or large unilamellar vesicles (LUV); the molecular and segmental motion in the liquid-crystal lead to an axially symmetric chemical shielding tensor. The chemical shielding anisotropy $\Delta\delta$ is given by the edges of the spectrum in (a). In small lipid aggregates such as small unilamellar vesicles (SUV) of diameter < 0.1 μm or lipid micelles Brownian tumbling of the lipid aggregate and lateral diffusion of the molecule around the vesicle produce further line-narrowing. As a result the powder pattern collapses to a single line (b).

distribution are formed (path B→F→D, Fig. 6). In contrast to these classical methods the technique of spontaneous vesiculation presented here has the advantage that it does not require any elaborate laboratory equipment and it avoids tedious procedures such as sonication, dialysis, or gel filtration. It is therefore an economical way of producing vesicles and may lend itself to industrial application.

Recently lipid bilayer vesicles have become important in membrane mediated processes for which the term «membrane-mimetic chemistry» has been coined. It involves processes such as membrane fusion, catalysis of surface reactions, energy transduction and conversion, and drug delivery. In this field of application simple, quick, and economical methods for the preparation of lipid bilayer vesicles are highly desirable. The methods of spontaneous vesiculation described in the present report certainly fulfill a number of requirements and their potential and usefulness in membrane-mimetic chemistry will undoubtedly be tested in the future.

A useful extension of the methods of spontaneous vesiculation is the one described recently[21]. Many pharmacologically active compounds are amphiphilic, surface active with one or more groups that are ionized or at least partially ionized at neutral pH. These compounds behave then like detergents and will have a tendency to orient at lipid-water interfaces. From our discussion of spontaneous vesiculation, a mixture of phosphatidylcholine and amphiphilic drug can be expected to behave similarly to phosphatidylcholine/charged detergent mixtures. This is borne out by the following experiments. The phase behaviour of mixtures of phosphatidylcholine and a pharmacologically active compound is depicted in Fig. 14 and 15 showing the effect of chlorpromazine and gramicidin S·2HCl as two representative examples of amphiphilic, surface active drugs.

The effect of chlorpromazine on the smectic (lamellar) phase of egg phosphatidylcholine (EPC) is shown in Fig. 14. At drug/phospholipid mole ratios < 1 the ^{1}H-NMR signal intensity of egg phosphatidylcholine is less than ≈ 20% of the expected value. Above a mole ratio of ≈ 1 the ^{1}H-signal intensity from both phospholipid and drug increases simultaneously reaching 100% at a mole ratio of ≈ 2. This indicates that the lipid particles present under these conditions have a diameter < 0.1 μm. Freeze-fracture electron microscopy together with other NMR techniques using paramagnetic shift and broadening reagents (data not presented) confirm the conclusion drawn from the ^{1}H-signal intensity measurement. Mixtures of egg phosphatidylcholine and chlorpromazine undergo spontaneous vesiculation when H_2O is added to a dry mixed film of these two compounds. Up to mole ratios of ≈ 1 the vesiculation leads to mainly LUV and oligomeric large vesicles. At higher drug/lipid mole ratios the fraction of lipid present as SUV (with a diameter < 0.1 μm) increases: in the compositional range where the sigmoidal increase in ^{1}H-signal intensity occurs the SUV is the most stable structure. In excess chlorpromazine (mole ratio > 2) mixed micelles consisting of egg phosphatidylcholine and drug are formed.

As an example of a pharmacologically active peptide the effect of gramicidin S·2 HCl on egg phosphatidylcholine (EPC) is shown in Fig. 15. Gramicidin S·2 HCl is an amphiphilic, surface active, cyclic decapeptide containing two positively charged ornithins. It can be shown by surface-potential measurement that gramicidin S·2 HCl orients at the phosphatidylcholine-H_2O interface imparting a net positive charge to the lipid surface. A comparison of Fig. 14 and Fig. 15 shows that mixtures of phosphatidylcholine and gramicidin S·2 HCl behave qualitatively similar to those of phosphatidylcholine and chlorpromazine. The main difference is in the drug/phospholipid mole ratio at which the sigmoidal increase in ^{1}H-signal inten-

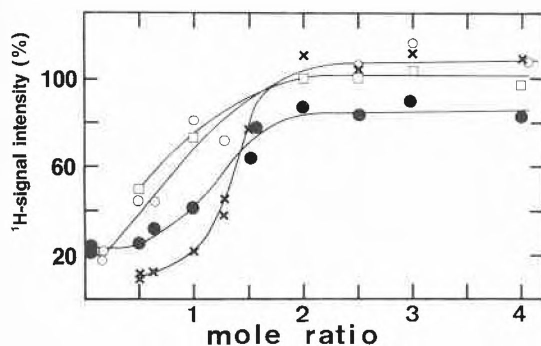

Fig. 14. Signal intensities of high resolution ^1H-NMR spectra recorded from mixed chlorpromazine/EPC dispersions in ^2H_2O as a function of the chlorpromazine/EPC mole ratio. Mixed dispersions of chlorpromazine and egg phosphatidylcholine (EPC) in ^2H_2O were prepared at 10 mg/mL, pH 6–7, as described in [21]. Signal intensities were measured for the CH_2-signal of the phospholipid hydrocarbon chains (×) and the $-N(CH_3)_3$ choline group (●), the aromatic (○) and the $-N(CH_3)_2$ protons (□) of chlorpromazine.

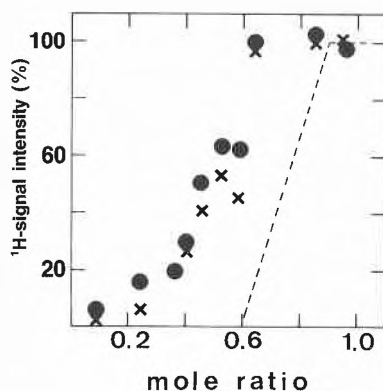

Fig. 15. Signal intensities of the high-resolution ^1H-NMR spectra recorded from mixed gramicidin S·2HCl/EPC dispersions in ^2H_2O as a function of the gramicidin S·2 HCl/EPC mole ratio. Mixed dispersions in ^2H_2O of egg phosphatidylcholine (EPC, 10 mg/mL) containing increasing quantities of gramicidin S·2 HCl were prepared as described in [21]. Intensities were measured for the CH_2-signal (×) and the $-N(CH_3)_3$ choline group (●). The dotted line represents the transmittance (%) of the sample.

sity occurs. What has been argued in discussing Fig. 14 is also valid for Fig. 15.

We have tested a number of amphiphilic drugs forming smectic (lamellar) phases when mixed with phosphatidylcholine. It can be concluded that the behaviour depicted in Fig. 14 and 15 is representative. Provided the surface charge density generated by the surface-bound drug exceeds the threshold value of about 1–2 μC/cm^2 the three-component system consisting of phosphatidylcholine, amphiphilic drug, and water undergoes spontaneous vesiculation. The amphiphilic drug apparently behaves like a charged, single-chain detergent. The parallel behaviour of the three-component systems phosphatidylcholine/charged detergent/H_2O and phosphatidylcholine/charged drug/H_2O is obvious. The nature of the vesicles formed by spontaneous vesiculation depends on the amphiphile/lipid mole ratio where amphiphile can be either a charged detergent or drug. The method of spontaneous vesiculation which is based on the presence of an amphiphilic drug may have important implications in terms of drug encapsulation and delivery. In this case the drug itself oriented at the lipid-water interface is utilized to induce spontaneous vesiculation and possibly to produce the vehicle proper for its delivery.

5. Concluding Remarks and Outlook

The discussion of the molecular packing of phospholipids in single-crystals proved useful for the understanding of phospholipid phase behaviour. It is shown how this understanding coupled with the knowledge of other physico-chemical properties of phospholipids, particularly their swelling behaviour, can be utilized to develop methods of spontaneous vesiculation discussed in the last part of this paper. Spontaneous vesiculation has been defined as the process of formation of unilamellar vesicles upon the addition of H_2O to a dried smectic (lamellar) lipid film deposited on the glass wall of a vessel. The vesicles form spontaneously implying that no external forces of dispersing phospholipid bilayers such as for instance ultrasonication are applied to the system.

It is anticipated that lipid bilayer vesicles will become increasingly important in a number of research areas and that this research will eventually lead to industrial applications. These research areas may be summarized under the collective term of «membrane-mimetic chemistry» comprising membrane mediated processes, e.g., processes such as cell-cell interactions, membrane fusion, catalysis of surface reactions, energy transduction and conversion, drug delivery and targeting to name only a few. Simple, quick, and inexpensive methods for the production of bilayer vesicles such as the methods of spontaneous vesiculation discussed in this review article are therefore highly desirable. These methods apparently fulfill another important requirement: they are versatile in the sense that they allow the production of bilayer vesicles differing widely in physico-chemical properties. Properties which should be variable at will are the vesicle size, size distribution, number of lamellae, surface charge density, bilayer fluidity, permeability, and stability. It should be mentioned at this point that besides bilayer vesicles other lipid phases and structures are useful and suitable media for the study of membrane-mediated processes; these include normal and reversed lipid micelles, lipid monolayers, and black lipid films. The more recent introduction of synthetic surfactants forming smectic, lamellar phases in H_2O represents an important extension of liposome technology. This field of endeavor has moved now into the realm of synthetic organic chemistry from which important contributions can be expected in the future. Several research groups have been engaged in the synthesis of surfactants that pack readily in bilayers and form therefore smectic (lamellar) phases in H_2O. These surfactants may contain polymerizable groups, such as diacetylene, methacryl, styryl, or vinyl groups. Polymerization is readily induced by exposure to ultraviolet light, gamma irradiation, or by a free radical mechanism. Depending on the location of the polymerizable group, vesicles can be either «zipped up» at their surface or within the hydrophobic layer. This approach holds great promise as to the possibility of controlling the vesicle properties and varying these properties in a controlled way. Polymerized vesicles have been shown to be stable for months; furthermore the bilayer fluidity can be readily adjusted to any desirable level and in turn the bilayer permeability or the release of compounds entrapped in their internal cavity. The control of the physico-chemical properties of lipid (surfactant) vesicles as well as the variation of these properties in a controlled fashion are certainly a prerequisite for the successful use of vesicles in membrane-mimetic chemistry. There is good reason to believe that in the near future bilayer vesicles of greater sophistication will be developed. These will be made up of bilayers containing glycolipids, proteins, and glycoproteins in addition to common phospholipids. It is hoped that this new generation of sophisticated membrane vesicles will be better suited than the classical lipid vesicles for the study of complex processes such as targeted drug delivery and energy conversion.

As to the industrial application of liposomes, a number of technical problems remain to be solved. It is hoped that fundamental and applied research will contribute to sorting out these problems. At present it is difficult to predict what role, if any, liposomes will play in industry and a fair assessment of their industrial potential seems to me still premature.

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