SEMINAR

LIPID POLYMORPHISM AND THE OCCURRENCE OF NON-BILAYER PHASES IN MODEL AND BIOLOGICAL MEMBRANES*

B. de KRUIJFF, A. J. VERKLEIJ, J. LEUNISSEN-BIJVELT, C. J. A. van ECHTELD, W. J. GERRITSEN, C. MOMBERS, P. C. NOORDAM, J. de GIER and P. R. CULLIS**

Institute of Molecular Biology and Department of Biochemistry, State University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

1. Introduction

In the current models for biological membranes the lipids are organized in a continuous bilayer that forms the semipermeable barrier which selectively separates the cell content from its surroundings and which

● Lecture delivered at la Houches by B. de Kruijff.
● *Deparunent of Biochemistry, University of British Columbia, Vancouver, B.C. V6T 1W5.

R. Balian et al., eds.
Les Houches, Session XXXIII, 1979 – *Membranes et Communication Intercellulaire / Membranes and Intercellular Communication*

acts as a matrix for the more functional membrane proteins. However, these models do not account for two of the basic properties of the lipid part of biological membranes.

(a) The great variety of lipids found in a single membrane is not understood and only one lipid species like unsaturated phosphatidylcholine can satisfy the above requirements. Even more important is the observation that each membrane contains, next to bilayer-forming lipids, significant amounts of lipids that, in isolated form dispersed in buffer, do not adopt a bilayer configuration but organize themselves in different structures. Among those lipids are: unsaturated phosphatidylethanolamines [1–7], monoglucosyldiglycerides (8, 9], phosphatidic acid [10], and cardiolipin [11,12] (in the presence of Ca^{2+}) which prefer the hexagonal H,, phase and phospholipid precursor molecules like lysophospholipids, fatty acids, and diglycerides which prefer micellar or other phases. The presence of these lipids can expect to actively mitigate against the bilayer structure of the membrane.

(b) Many different processes occur in biological membranes in which a part of the lipids has to temporarily leave the bilayer configuration. Clear examples are: membrane fusion (including endo-, exo-, and phagocytosis) and transbilayer movements of membrane lipids (flip-flop).

In this seminar we would like to develop the view that "non-bilayer" lipids are actively involved in these dynamic processes, a hypothesis that will first be tested in model membrane systems.

2. Model systems

The ability of hydrated membrane lipids to adopt a variety of phases in addition to the bilayer phase, has been demonstrated already some twenty years ago. The structural characteristics of these alternative phases have been solved by the extensive X-ray studies of Luzzati and co-workers [13–15]. The recent introduction of ^{31}P NMR in combination with freeze-fracture electron microscopy has made it possible to obtain detailed insight in the polymorphic phase behaviour of membrane lipids. This is illustrated in fig. 4.1 for an aqueous dispersion of phosphatidylethanolamine isolated from hen eggs. At low temperatures (below 25" C) this phospholipid organizes itself in extended bilayers as evidenced by the characteristic asymmetrical ^{31}P NMR spectrum with a high field peak and a low field shoulder [3, 4, 7, 16] and the smooth

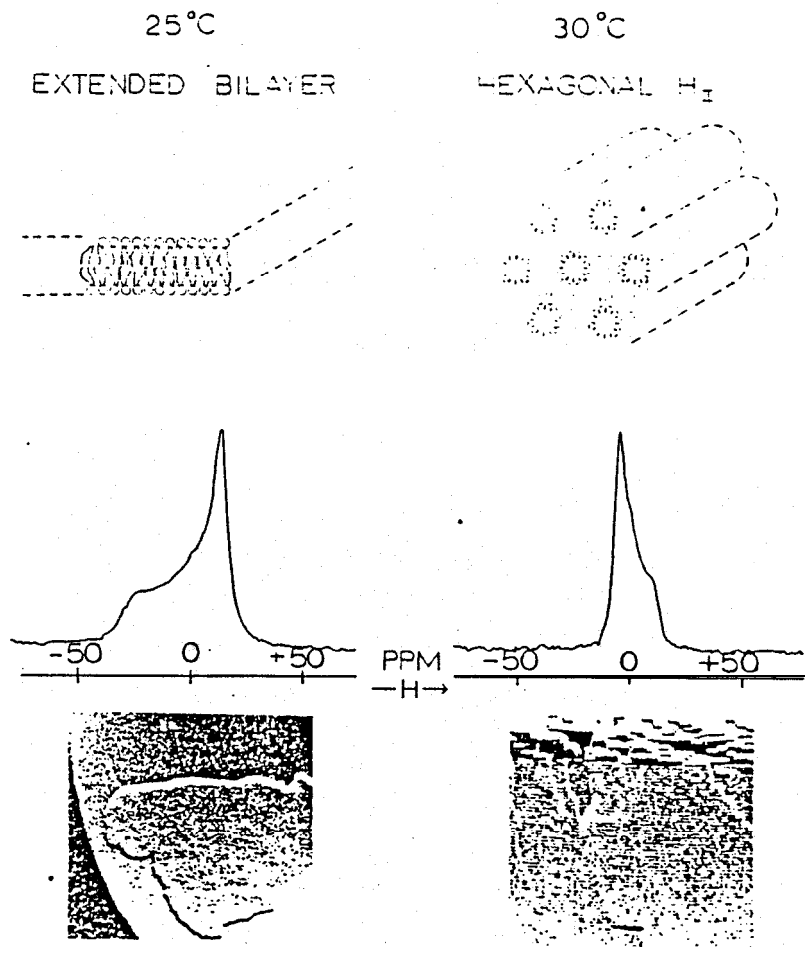

Fig. 4.1. ^{31}P NMR and freeze-fracture detection of bilaycr (25" C) and hexagonal H_{II} phase (30' C) of an aqueous dispersion of egg phosphatidylethanolamine. Magnification of the micrographs about 100 000 ×.

fracture planes as observed by freeze-fracturing. In the temperature interval 25-30" C, a phase change occurs so that this lipid is now organized in the hexagonal H_{II} phase with the spectrum of half the spectral width and a reversed asymmetry [3, 4, 7, 16] and a freeze-frac-

ture appearance of fracture planes being composed of long parallel lines [5]. The temperature of the bilayer-hexagonal transition is very sensitive to the degree of unsaturation of the acyl chains and is higher for more saturated species [7]. At the physiological temperature, naturally occuring phosphatidylethanolamines often prefer the hexagonal phase [7]. Other remarkable features of this transition are its sharpness and its low heat content. The complete transformation form bilayer to hexagonal or vice versa commonly occurs within a 5° C temperature interval for both synthetic and naturally occurring species (which have a heterogeneous fatty acid composition). The heat content of this phase change is an order of magnitude lower than the heat involved in the gel→ liquid crystalline phase transition as is illustrated in the thermogram of an egg phosphatidylethanolamine dispersion (fig. 4.2). The very low energy barrier between macroscopically very different configurations suggests that the acyl chain packing in both phases is similar which was also concluded from magnetic resonance experiments [17,18] and has important consequences for the possible dynamic functional roles of non-bilayer lipid structures in biological membranes.

Phosphatidylcholines have a strong bilayer stabilizing effect on phosphatidylethanolamines [4,6]. In contrast, cholesterol strongly destabi-

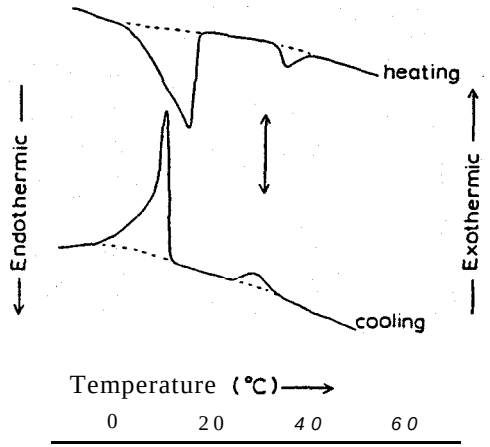

Fig. 4.2. Differential sunning calorimetry suns of an aqueous dispersion of egg phosphatidylethanolamine. The gel → liquid crystalline transition occurs from 0 -18' C. The arrow indicates the temperature of the bilayer → hexagonal transition as detected by ^{31}P NMR.

lizes the bilayer structure in unsaturated phosphatidylethanolamine containing systems [4, 6, 7]. The bilayer-hexagonal transition can also be isothermally induced by the addition of Ca^{2+}, both in pure systems like cardiolipin [I 1,12] and phosphatidic acid [10], as well as in phosphatidylethanolamine-phosphatidylserine mixtures [19]. As a most interesting intermediate between the bilayer and hexagonal H,, phase a new phase is observed which is characteristic by a narrow symmetrical ^{31}P NMR resonance indicating isotropic motional averaging. This is illustrated in fig. 4.3 for a dioleoylphosphatidylethanolamine-dioleoylphosphatidylcholine-cholesterol (3: 1: 2) mixture in which, by a temperature increase, the isotropic phase was induced [9]. Freeze-fracturing of this sample shows the presence of numerous particles and pits of a uniform size of approximately 100 Å which are associated with the lipid bilayer [9]. ^{31}P NMR and freeze-fracturing evidence for lipidic particles was further obtained in phosphatidylcholine-cardiolipin (Ca^{2+}) and phosphatidylcholine–monoglucosyldiglycetide mixtures (9, 20]. As a tentative model for these lipidic particles we proposed [9,20] the inverted micelle sandwiched between the monolayers of the lipid bilayer (see fig. 4.4d). Tumbling of the micelle and lateral diffusion of the lipids in the micelle will provide the isotropic motion detected in the ^{31}P NMR experiments. The additional possibility that rapid exchange may occur between the inverted micellar and surrounding bilayer lipids (fig. 4.4) adds a new dimension to the dynamics of lipids in membranes.

An important question in relation to the possible occurence of non-bilayer phases in biological membranes is whether such structures can be found in the hydrated total lipid extracts of these membranes. In the case of the human erythrocyte, the total lipids organize themselves in a bilayer at 37" C [21,22] despite the presence of 30 mol % of phosphatidylethanolamine which, in isolated form above 10° C. adopts the hexagonal H,, phase [7] and therefore clearly demonstrates the bilayer's stabilizing capacity of the other membrane phospholipids. However, very recent work suggests that this situation is more exceptional than common. In fig. 4.5 the ^{31}P NMR and freeze-fracturing results of the total lipids of *E. coli* are presented at the growth temperature. It is now obvious that mainly the hexagonal H,, and an isotropic phases are observed. This latter phase might again be of an inverted micellar nature since particles and pits are found on the fracture planes. Very similar results are found for the total lipids of the photoreceptor [23] and the inner mitochondrial membrane [24].

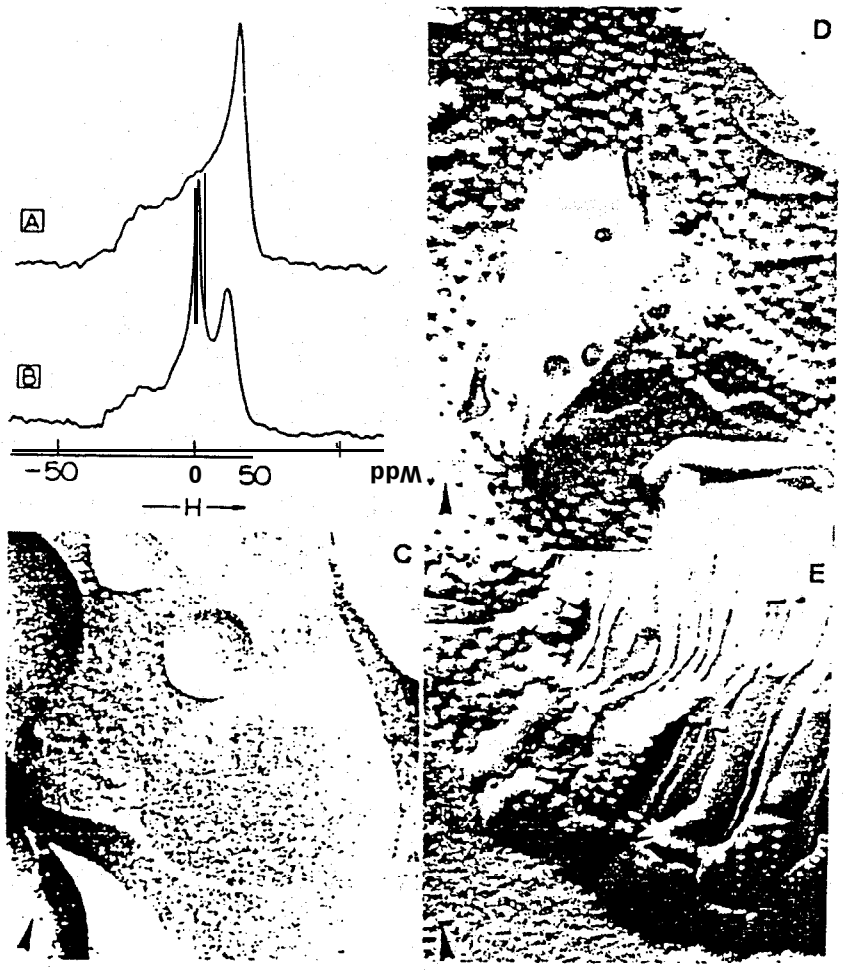

Fig. 4.3. ^{31}P NMR and freeze-fracturing of an aqueous dispersion of 18 : 1_c/18 : 1_c-phosphatidylethanolamine: 18 : 1_c/18 : 1_c-phosphatidylcholine : cholesterol (3: 1: 2). (a) ^{31}P NMR and (c) freeze-fracturing of the sample at 10° C. (b) ^{31}P NMR and (d,e) freeze-fracturing of the sample at 10° C after being heated for 5 min at 60° C. Magnification of the micrographs about 1000 000×.

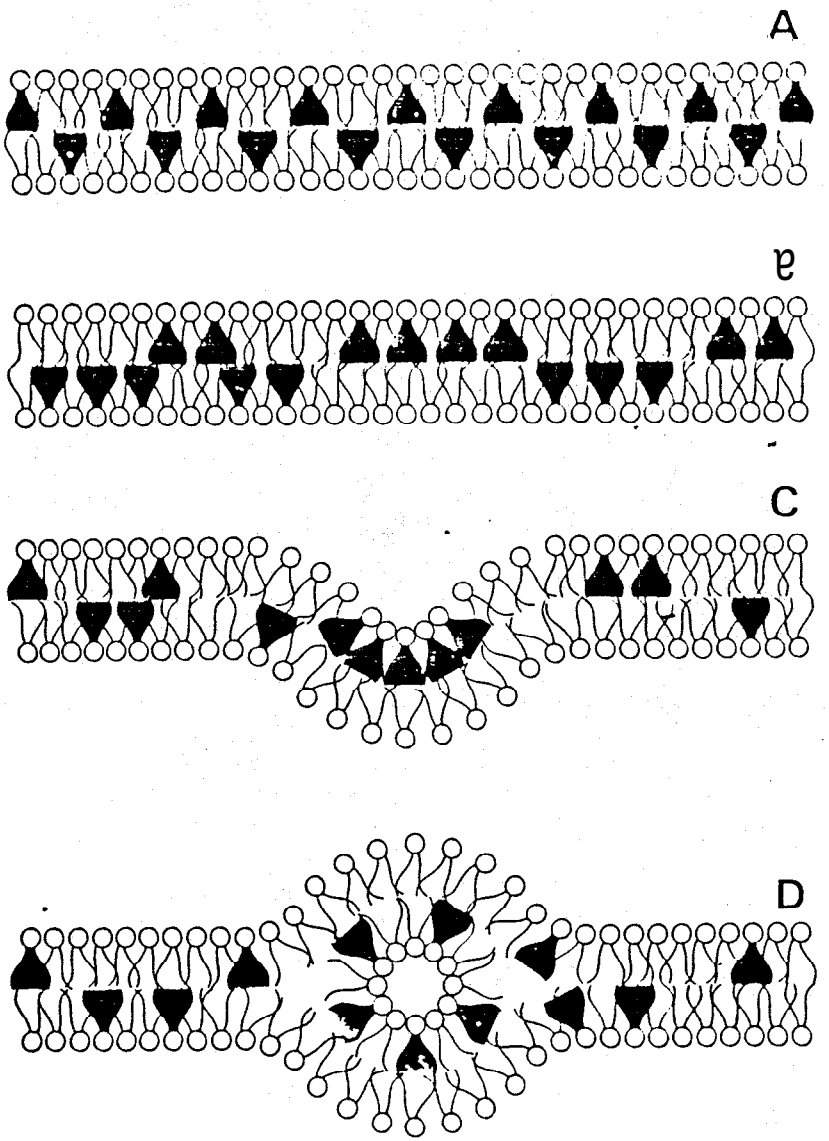

Fig. 4.4. Dynamic formation of inverted micelles in a lipid bilayer. In a lipid bilayer (a) composed of lipid molecules with an overall cylindrical shape (e.g. phosphatidylcholine) and molecules with a wedge shape (e.g. phosphatidylethanolamine indicated as dark molecules), statistically local high concentrations of these latter molecules (b) will cause an inward curvature of the lipid bilayer (c) reversibly leading to the formation of inverted micelles (d).

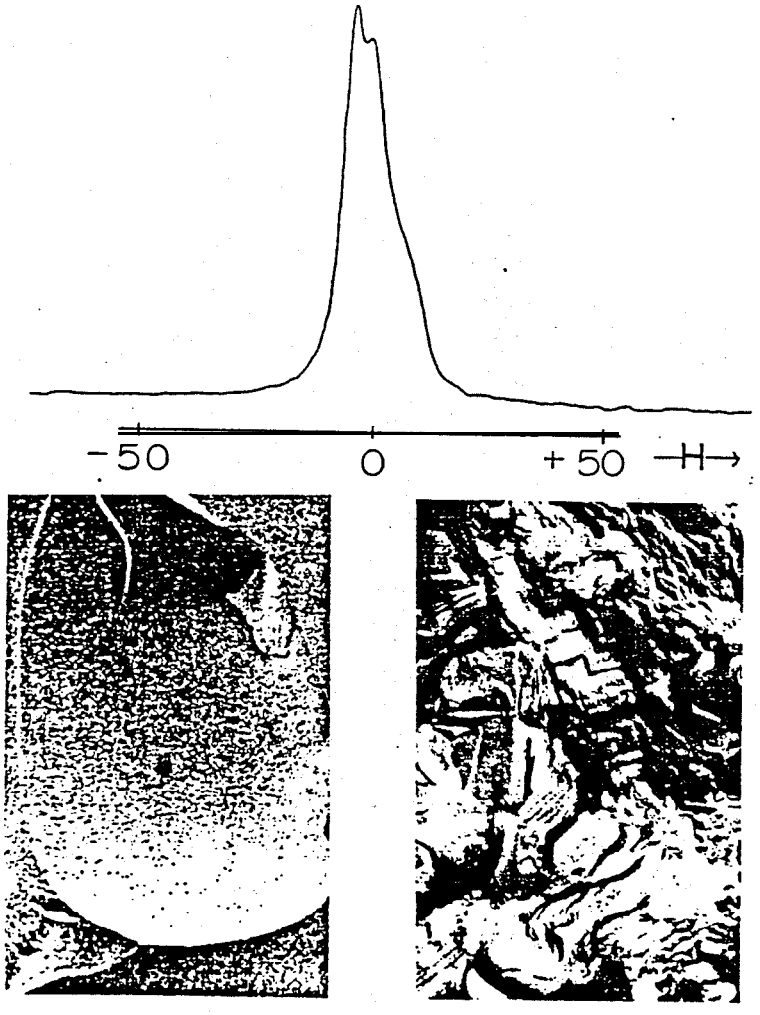

Fig. 4.5. ^{31}PNMR and freeze-fracturing of the total lipids of E. *coli* at 37° C. The micrographs (final magnification about 100 000×) show lipid bilayers associated with lipidic particles and the hexagonal H_{II} phase.

Since there is no evidence as yet for the existence of a hexagonal H_{II} phase lipid in biological membranes the "isotropic phase" is of particular interest, as experiments on different membranes do suggest lipid structures allowing such isotropic motion.

3. Biological membranes

One of the most closely characterized biological membranes is that of the human etythrocyte which exhibits ^{31}P NMR spectra (arising from at least 97% of the endogenous phospholipids [21, 22], which are fully consistent with virtually all the lipids being organized in a lipid bilayer (see fig. 4.6a). This bilayer is very stable and must, next to the membrane phospholipids, also be determined by cholesterol and/or membrane proteins because extensive phosphoiipid degradation by various phospholipases, producing high amounts of non-bilayer lipids like lysophospholipids, diglycerides, and ceramides, does not affect the bilayer structure [25]. This stability might be related to the low metabolic activity of the membrane, the long life span of the erythrocyte, and its exposure to large mechanical stresses during its passage through blood capillaries.

Support for the existence of non-bilayer lipid structures in intact biological membranes have recently been obtained for the metabolically very active endoplasmic reticulum membranes of rat, beef, and rabbit liver. Two laboratories [26, 27] have independently reported that the phospholipids in microsomes (isolated vesiculated fragments of the endoplasmic reticulum) give rise at 37" C to ^{31}P NMR line shapes (see fig. 4.6b) indicating isotropic motion of the lipids within the membrane. Most interestingly and in full agreement with the results on model membranes discussed in the previous section, at lower temperatures (below 30" C) an increasing fraction of the signal has the normal bilayer shape (fig. 4.6c). Strong ^{31}P NMR evidence has been obtained that this temperature-dependent phase change also occurs in the endoplasmic reticulum of intact rat liver [28]. These results would be consistent with the existence of inverted micellar or (short) cylindrical arrangements of lipid inside the bilayer. Furthermore, the results strongly indicate rapid exchange of bulk bilayer lipids with such structures. In addition it can be speculated that membrane proteins (possibly cytochrome P_{450} as suggested by Stier et al. [27]) actively encourage these manifestations of non-biiayer lipids as aqueous dispersions of the extracted lipids do show the normal "bilayer" spectra [26, 27].

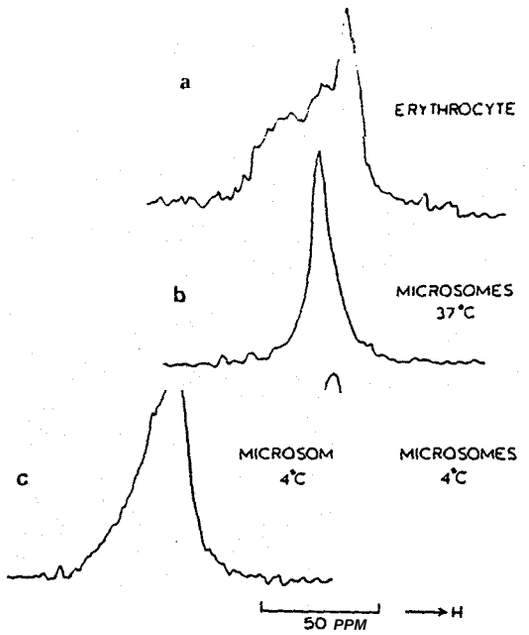

Fig. 4.6. ^{31}P NMR spectra of various biological membranes.

The sarcoplasmic reticulum membrane, although to a lesser extent than the endoplasmic reticulum membrane, also gives rise to ^{31}P NMR spectra indicating isotropic motion of (part) of the membrane phospholipids [29]. It is remarkable that similar findings were obtained already some eight years ago by Davis and Inesi [30], who concluded on the basis of ^{1}H NMR studies that some 20% of the endogenous sarcoplasmic reticulum lipid enjoyed isotropic motion on the NMR time scale.

There is also evidence that in the inner mitochondhal membrane a significant fraction of the phosphatidylcholine experiences isotropic motion possibly due to non-bilayer alternatives; this is implicit in the ^{2}H NMR results of Arvidson et al. [31]. These results are substantiated to some extent by recent ^{31}P NMR experiments [24] but must be treated with care in view of the observation that, for intact mitochondria at 37" C. changes in the functional state (P/O ratio) and the amount of "isotropic" ^{31}P NMR signal occurs within minutes of incubation at 37"

C [24]. For osmiophilic bodies from pig lung some 5% of the phospholipids undergo isotropic motion which was suggested to be due to apolar proteins (32).

4. Functional aspects of non-bilayer phases in membranes

Although the functional implications of the occurence of non-bilayer phases are obvious in many areas of membrane biology as discussed elsewhere [33], strong supportive evidence is only available in two cases, e.g. membrane fusion and lipid flip-flop.

Membrane fusion. Regardless of whether fusion is mediated by protein, lipid. or another agent, during some stage of the fusion process part of the lipids will have to leave the bilayer configuration. This was clearly demonstrated in the case of human erythrocyte ghosts where, upon treatment with fusogenic agents like fatty acids, the presence of the hexagonal H_{II} phase could be detected [34]. Furthermore, unilamellar phosphatidylcholine-cardiolipin (1:1) vesicles fuse upon the addition of Ca^{2+} in which the process is accompanied by the appearance of lipidic particles at the fusion interface [35].

Lipid flip-fop. It has been suggested that transient formation of intrabilayer inverted lipid-structures, like those shown in fig. 4.4, provide a mechanism for flip-flop processes resulting in redistribution of lipids across the bilayer [4]. Support for this hypothesis comes from measurements on microsomal membranes. At 37" C the phospholipids in these membranes undergo isotropic motion and have a fast flip-flop [36, 37]. At 4" C, mainly bilayer structure is observed, and the rate of phosphatidylcholine flip-flop appears to be drastically reduced [37]. *In* the sarcoplasmic membrane also isotropic motion of part of the phospholipids occurs and rapid transbilayer movements of lysophosphatidylcholine [29] and part of the phosphatidylcholine pool have been observed [38].

Other membrane systems in which rapid flip-flop occurs (e.g. certain bacterial membranes [39]) have lipid compositions which are also consistent with the occurence of non-bilayer structures. In fact, the data on the total lipids of *E. coli* (fig. 4.5) and the observation that in isolated *E. coli* inner membranes part of the phospholipids undergo isotropic motion [40] strongly support this hypothesis. However. it should be realized that alternative flip-flop mechanisms including protein mediated processes [41, 42) might be operating in membranes as well.

References

[1.] F. Reiss-Husson, J. Mol. Biol. 25 (1967) 363.
[2] R. P. Rand. D. 0. Tinker and P. G. Fast. Chem. Phys. Lipids 6 (1971) 33.
[3] P. R. Cullis and B. de Kruijff, Biochim. Biophys. Acta 436 (1976) 523.
[4] P. R. Cullis and B. de Kruijff. Biochim. Biophys. Acta 507 (1978) 207.
[5] P. W. M. van Dijck. B. de Kruijff, L. L. hf. van Deenen. J. de Gicr and R A. Demel, Biochim. Biophys. Acta 455 (1976) 576.
[6] P. R. Cullis. P. W. M. van Dijck. B. de Kruijff and J. de Gicr, Biochim. Biophys. Acta 513 (1978) 21.
[7] P. R. Cullis, and B. de Kruijff, Biochim. Biophys. Acta 513 (1978) 31.
[8] Å. Wieslander, J. Ulmius, G. Lindblom and K. Fontell, Biochim. Biophys. Acta 512 (1978) 241.
[9] B. de Kruijff, A. J. Verkleij, C. J. A. van Echteld, W. J. Gerritsen, C. Mombers, P. C. Noordam and J. de Gier, Biochim. Biophys. Acta 555 (1979) 200.
[10] D. Papahadjopoubs, W. J. Vail, W. A. Pangborn and G. Poste, Biochim. Biophys. Acta 448 (1976) 265.
[11] R. P. Rand and S. Sengupta, Biochim. Biophys. Acta 513 (1972) 11.
[12] P. R. Cullis, A. J. Verkleij and P. H. J. Th. Ververgaert, Biochim. Biophys. Acta 513 (1978) Il.
[13] V. Luzzatti and F. Husson, J. Cell Biol. 12 (1962) 207.
[14] V. Luzzatti, T. Gulik-Krzywichi and A. Tardieu, Nature 218 (1968) 1031.
[15] V. Luzzatti and A. Tardieu, Ann. Rev. Phys. Chem. 25 (1974) 79.
[16] J. Seelig, Biochim. Biophys. Acta 515 (1978) 105.
[17] B. Mely, J. Charvolin and P. Keller. Chem. Phys. Lipids 15 (1975) 161.
[18] J. Seelig and H. Limacher. Mol. Cryst. Liq. Cryst. 25 (1974) 105.
[19] P. R. Cullis and A. J. Verkleij, Biochim. Biophys. Acta 552 (1979) 545.
[20] A. J. Verkleij, C. Mombers, J. Leunissen-Bijvelt and P. H. J. Th. Ververgaert, Nature 279 (1979) 162.
[21] P. R. Cullis, FEES. Lett. 70 (1976) 223.
[22] P. R. Cullis and Cb. Grathwohl, Biochim. Biophys. Acta 471 (1977) 213.
[23] W. J. de Grip, E. H. S. Drenthe, C. J. A. van Echteld, B. de Kruijff and A. J. Verkleij, Biochim. Biophys. Acta 558 (1979) 330.
[24] Authors unpublished results.
[25] G. Vermeer, B. de Kruijff, J. Op den Kamp and L. L. M. van Deenen, Biochim. Biophys. Acta 596 (1980) 1.
[26] B. de Kruijff, A. M. H. P. van den Besselaar, P. R. Cullis, H. van den Bosch and L. L. M. van Deenen, Biocbim. Biophys. Acta 514 (1978) 1.
1271 A. Stier, S. A. E. Finch and B. Bösterling, FEBS. Lett. 91 (1978) 109.
[28] B. de Kruijff and A. Rietveld, Biochim. Biophp. Acta (1979) submitted
[29] A. M. H. P. van den Besselaar, B. de Kruijff, H. van den Bosch and L. L. M. van Deenen, Biochim. Biophys. Acta 555 (1979) 193.
[30] D. G. Davis and G. Inesi, Biochim. Biophys. Acta 241 (1971) 1.
[31] G. Arvidson, G. Lindblon and T. Drakenberg, FEES. Lett. 54 (1975) 249.
[32] C. Grathwohl. G. E. Newman. P. 1. R. Phizacherley and M. H. Town. Biochim. Biophys. Acta 552 (1979) 509.
[33] P. R. Cullis and B. de Kruijff, Biochim. Biophys. Acta 559 (1979) 399.
[34] P. R. Cullis and M. J. Hope. Nature 271 (1978) 672.

[35] A. J. Verkleij. C. Mombers, W. J. Gerritsen, J. Leunissen-Bijvelt and P. R. Cullis, Biochim. Biophys. Acta 555 (1979) 358.
[36] D. B. Zilversmit and M. E. Hughes, Biochim. Biophys. Acta 469 (1977) 99.
[37] A. M. H. P. van den Besselaar, B. de Kruijff, H. van den Bosch and L. L. M. van Deenen, Biochim. Biophys. Acta 510 (1978) 242.
[38] B. de Kruijff. A. M. H. P. van den Besselaar, H. van den Bosch and L L. M. van Deenen, Biochim. Biophys. Acta 555 (1979) 181.
[39] J. E. Rothman and J. Lenard, Science I95 (1977) 743.
[40] E. Burnell, L. van Alphen, B. de Kruijff and A. J. Verklcij. Biochim. Biophys. Acta (1979) submitted.
[41] E. J. J. van Zoelen, L. L. M. van Deenen and B. de Kruijff, Biochim. Biophys. Acta 597 (1980) 492.
[42] B. de Kruijff, E. J. J. van Zoelen and L. L. hf. van Decncn. Biochim. Biophys. Acu 509 (1978) 537.