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LIPID POLYMORPHISM AND THE FUNCTIONAL ROLES OF LIPIDS IN BIOLOGICAL MEMBRANES

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Contents

I. Introduction

The reasons for the great variety of lipids found in biological membranes, and the relations between lipid composition and membrane function pose major unsolved problems in membrane biology. Perhaps the only major functional role of lipids which may be regarded as firmly established involves the bilayer structure of biological membranes. The observations that biological membranes contain regions of bilayer structure [1], and that model systems consisting of naturally occurring and synthetic phospholipids often spontaneously adopt such a configuration on hydration, provide strong evidence that the lipid component is responsible for the basic biomembrane structure. The fact remains however, that a single phospholipid species such as phosphatidylcholine could

satisfy such structural requirements. In this context, the observation that a typical mammalian cell membrane contains one hundred or more distinctly different lipids implicitly suggests that lipids play other functional roles. In this review we shall indicate alternative functional roles arising from a property of lipids which has not received the serious attention it deserves in recent years, namely the ability of lipids to adopt non-bilayer configurations in addition to the bilayer phase. This ability implies a view of biological membranes which differs from previous models such as the fluid mosaic model of Singer and Nicolson [2] or the earlier unit membrane model [3]. It is therefore appropriate to briefly review the possible functional roles of lipids in terms of these earlier models so that the requirement for an alternative approach becomes apparent.

IA. Functional roles of lipids in the fluid mosaic model of membranes

Within the constrictions of the fluid mosaic model, it is implicit (although not explicitly stated) that the lipid component assumes a closed bilayer structure, thus realizing both a structural matrix with which functional proteins may be associated as well as an internal environment which may be regulated and controlled. The major advance of the fluid mosaic hypothesis over previous contenders was that it included an ability of the membrane components to diffuse laterally in the plane of the membrane (due to the now well documented fluidity of the lipid matrix) as well as postulating an 'interruption' of this matrix by integral proteins which penetrate into or through the bilayer. Lipid diversity can then be rationalized on the basis that integral protein function may be modulated by the local fluidity of the bilayer matrix and/or by the detailed composition of the annular lipids at the protein-lipid interface. Such proposals are supported by observations that certain integral proteins require a fluid (liquid-crystalline) phospholipid environment for function [4,5], and that such function is inhibited in the presence of gel-state lipids. It may then be suggested that gel-state lipids are available to membrane protein in vivo, and regulate function according to their proximity and abundance. Alternatively, the presence of particular lipid species in the annulus may be important, either to provide an environment of appropriate viscosity or to effect conformational changes vital to function by binding tightly to the protein.

Certain difficulties with this rationale for lipid diversity become apparent on consideration of two features of biological membranes and reconstituted systems. First, gel-state lipids do not appear to be present in most biological membranes (particularly those of eukaryotic cells) as the unsaturated nature of most naturally occurring lipids results in hydrocarbon transitions which occur well below physiological temperatures (see, for example, data obtained for erythrocyte membrane lipids) [6,7]. Further in more metabolically active membranes (such as the inner mitochondrial and endoplasmic reticulum membranes [8,9]) where possible regulatory roles of lipids would be expected to be more obviously expressed, an increased rather than a decreased unsaturation of the lipids is observed. Finally, the observations that membrane proteins usually depress hydrocarbon transition temperatures [10,11] and that integral proteins preferentially partition into fluid regions [12–14] imply that gel-state lipids may not be available for regulatory roles even if present in the membrane.

Secondly, in spite of intensive effort, there is little firm evidence that integral membrane proteins require specific lipids for activity. The sarcoplasmic reticulum ATPase, for example, functions well when reconstituted in the presence of various phospholipids [4,15] (and even in an environment provided by pure detergent [16]) and similar indica-

tions of nonspecific lipid requirements are obtained for cytochrome oxidase [17,18], the $(Na^+ + K^+)$-ATPase from kidney [19], as well as the $(Ca^{2+} + Mg^{2+})$-ATPase from human erythrocytes [20]. Further, evidence exists for rapid exchange between annular lipid and bulk lipid [21], an observation inconsistent with the presence of bound lipid vital to function. This latter observation is at variance with evidence presented for relatively immobilized boundary lipid in reconstituted cytochrome oxidase systems [22], but more recent work is fully consistent with rapid exchange between annular and bulk bilayer lipid [23,24]. Thus, while the possibility remains that the local fluidity or lipid composition may affect protein function, the evidence supporting such proposals as a general rationale for the variety of lipids in biomembranes must be regarded as inconclusive.

In addition to these difficulties, there are certain conceptual problems involved in assuming a constant bilayer structure for the lipid component. As our understanding of the membrane-mediated processes progresses, it is becoming increasingly clear that a variety of functional capacities are difficult to reconcile with an inviolate bilayer structure. This includes such basic processes as cell fusion, exo- and endocytosis, transbilayer movements of lipids ('flip-flop'), facilitated transport as well as protein insertion and orientation. In this context the need for a reappraisal of the potential structures and associated functions of lipids becomes apparent. We hope that this review makes it clear that consideration of the polymorphic capabilities of lipids can lead to a better understanding of the molecular mechanisms of such processes.

IB. Lipid polymorphism: Historical perspective

The ability of hydrated lipids to adopt a variety of phases in addition to the bilayer phase is well documented with a literature extending over the last twenty years. Particularly significant contributions to this research area have been made by Luzzatti and coworkers [25–27] employing X-ray techniques to solve in detail the structural characteristics of these alternatives. This work has laid the foundation for most of the possibilities we discuss here. In addition, these [28] and other [29,30] investigators immediately recognized the possibility that these non-bilayer structures may be related to biomembrane structure and function. The reemergence of these somewhat neglected ideas, cast in a different form, arises from a variety of factors. First, as detailed above, the shortcomings of currently available models of membranes are becoming increasingly obvious. Second, and of equal importance, the battery of techniques available to study lipid polymorphism have recently been significantly extended by the the introduction of NMR (particularly ^{31}P NMR) methodology, which may be usefully applied to both model and biological membrane systems. In addition, the increasing sophistication of freeze-fracture techniques has also provided a complementary independent method for directly visualizing macromolecular structures assumed by lipids. Third, due to improvements in lipid isolation and synthesis, well defined model systems are now available which allow less ambiguous assessments of the potential properties of lipids in biological membranes. Taken together, these improvements in technique and experimental systems have resulted in observations which imply that non-bilayer structures may occur in biological membranes, thus allowing new possibilities for the dynamic participation of lipids in functional processes.

II. Lipid polymorphism and experimental techniques

The bilayer arrangement of hydrated lipids is only one of a great variety of phases available. Alternative configurations include the hexagonal H_I and H_{II} phases, the micellar phase as well as those phases exhibiting cubic or rhombic structures. For detailed descriptions of the characteristic dimensions and symmetries of these latter configurations the reader is referred to Refs. 25–28. The micellar, bilayer and hexagonal H_{II} arrangements are indicated in Fig. 1. The X-ray technique is certainly the classical technique for the characterization of these macroscopic structures adopted by hydrated lipid systems. As with any other technique, however, it does have certain limitations, particularly when more than one phase is present in a given lipid system. In such situations it is often difficult to detect the occurrence and amount of the less predominant phase. These problems are exacerbated for biological membranes by the need to obtain a stack of closely opposed membranes. It is therefore fortunate that phosphorus nuclear magnetic

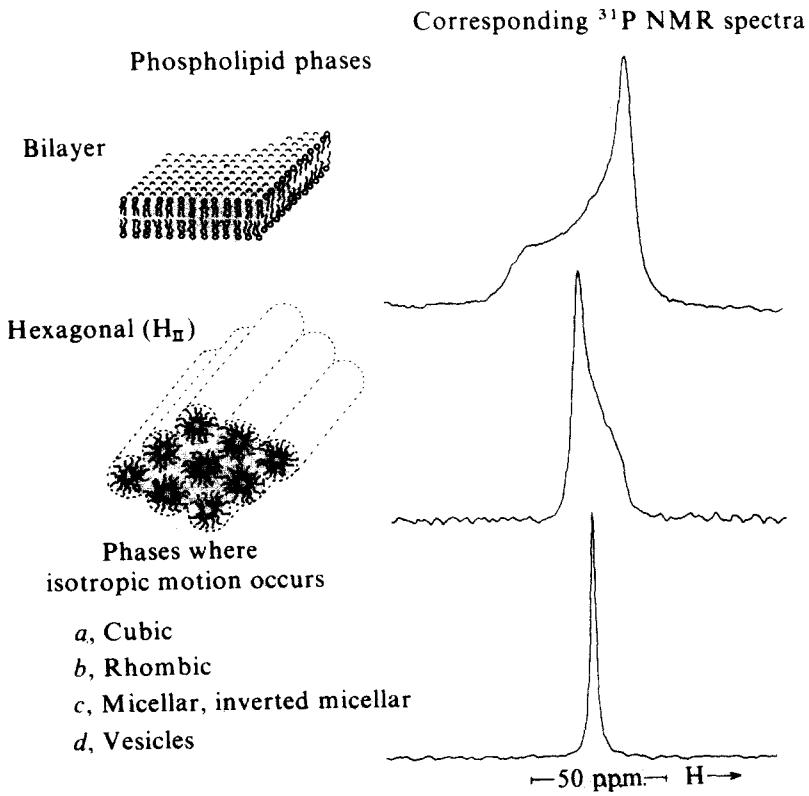

Fig. 1. Polymorphic phases available to hydrated liquid crystalline phospholipids and corresponding (36.4 MHz) ^{31}P NMR spectra. The bilayer spectrum was obtained from aqueous dispersions of egg yolk phosphatidylcholine, whereas the hexagonal (H_{II}) phase spectrum was obtained from (naturally occurring) soya bean phosphatidylethanolamine. The 'isotropic motion' ^{31}P NMR spectrum was obtained from a mixture of 85 mol% soya phosphatidylethanolamine and 15 mol% egg yolk phosphatidylcholine. All preparations were hydrated in 10 mM Tris-acetic acid ($p^2H = 7.0$) and 2 mM EDTA. The spectra were obtained at 30°C in the presence of broad band proton decoupling. For further details see Ref. 33. Reproduced with permission from Ref. 81.

resonance (^{31}P NMR) techniques which have recently been introduced [32,33] remove to some extent the above mentioned problems.

The use of ^{31}P NMR to detect lipid polymorphims rests on three factors. First, the lipid phosphorus exhibits a large chemical shift anisotropy, which for large (radius $\geqslant$2000 Å) liquid-crystalline bilayer systems is only partially averaged by the restricted modes of motion available, which consists primarily of rapid rotation of the molecules about their long axis [32–35]. In the presence of proton decoupling, this results in a characteristic broad spectrum with a low field shoulder and high field peak, which are separated by $\Delta\sigma_{CSA}^{EFF} \approx -40$ ppm. A typical 'bilayer' spectrum is illustrated in Fig. 1 (a). Second, with the possible exception of phosphatidic acid [32], all glycerol-based phospholipids (including phosphatidylcholine [35,38], phosphatidylethanolamine [32, 33,39], phosphatidylserine [34,40,41], phosphatidylglycerol [32,34] and phosphatidylinositol [32]) as well as the most abundant mammalian phosphosphingolipid, sphingomyelin [42], have similar values of $\Delta\sigma_{CSA}^{EFF}$ resulting in almost equivalent lineshapes for these different species when in the liquidcrystalline bilayer configuration. Thus in mixed lipid systems, including biological membranes, effectively all the endogeneous phospholipids contribute to a composite bilayer lineshape if they are in the bilayer phase. The third factor involves the ability of lipids to undergo lateral diffusion. In large bilayer structures, such as hand-shaken liposomes or biological membranes, the ability lipids to diffuse laterally does not produce an effective motional averaging mechanism as reorientation due to such processes is not fast on the NMR timescale (10^{-5} s). This is in contrast to the situation with small sonicated vesicles, where the lateral diffusion of the lipid around the vesicle and vesicle tumbling produce line-narrowing effects [43]. However, lipids in the hexagonal (H_{II}) phase do experience additional motional averaging as compared to those in large bilayer structures because motional averaging due to lateral diffusion around the small (~20 Å diameter) aqueous channels occurs. As indicated elsewhere [32,33,38], this results in characteristic ^{31}P NMR lineshapes which have reversed asymmetry compared to the bilayer spectra and are narrower by a factor of two. Finally, lipids in inverted micellar configurations (or other phases such as the cubic or rhombic) allow effectively isotropic motion to occur, as lateral diffusion results in averaging over all orientations, leading to a narrow, symmetric ^{31}P NMR spectrum. A summary of the lineshapes observed is presented in Fig. 1.

It is obvious that the interpretation of the ^{31}P NMR spectra relies heavily on previous X-ray determinations of phospholipid phases. In this sense it is an extrapolative technique, where characteristic ^{31}P NMR spectra are associated with phases characterized by X-ray or other techniques in simple model systems, and subsequently applied to more complex systems where the classical techniques are not as straightforward to apply.

III. Lipid polymorphism: Model systems

The predilection of unsaturated phosphatidylethanolamines for non-bilayer configurations has been recognized for some time. Early X-ray studies by Reiss-Husson [44] on mixed lipid systems and by Rand et al. [45] on chromatographically pure naturally occurring phosphatidylethanolamine indicates a particular preference for the hexagonal (H_{II}) arrangement. This finding has recently been more closely investigated employing ^{31}P NMR techniques [33] for various naturally occurring and synthetic phosphatidylethanolamines in the presence of excess water, and the influence of such factors as temperature, fatty acid composition, pH and ionic strenght characterized. Two particularly

important features have emerged. First, in addition to a hydrocarbon phase transition, these unsaturated phosphatidylethanolamines also exhibit a bilayer to hexagonal (H_{II}) polymorphic phase transition as the temperature is increased, which occurs within 10°C of the high temperature end of the gel-liquid crystalline transition. Thus the bilayer to hexagonal (H_{II}) transition temperature (T_{BH}) is sensitive to the fatty acid composition, occurring for example, in the region of −10°C for the polyunsaturated phosphatidylethanolamine derived from soya beans [32,34] and in the region of 54°C for a more saturated species obtained from *Escherichia coli* [33]. Further, this polymorphic phase transition is remarkably abrupt, and complete transformations from bilayer to H_{II} configurations or vice-versa commonly occur over a 5°C temperature interval. It is also of interest that the bilayer-hexagonal transitions of naturally occurring species of phosphatidylethanolamine (which have a heterogeneous fatty acid composition) occurs over a temperature interval which is similar to that observed for synthetic species with a homogeneous fatty acid composition. This contrasts with the gel-liquid crystalline transition, which is markedly broader in naturally occurring (see, for example, Ref. 46), as opposed to synthetic [47] lipid systems.

The second important feature of the bilayer-H_{II} transition exhibited by phosphatidylethanolamines is a very low enthalpy associated with this dramatic structural rearrangement. This is indicated by the calorimetric behaviour of egg phosphatidylethanolamine illustrated in Fig. 2 (a), where only a small enthalpy change is visible on proceeding from the bilayer to the hexagonal H_{II} phase. Such characteristics are even more marked

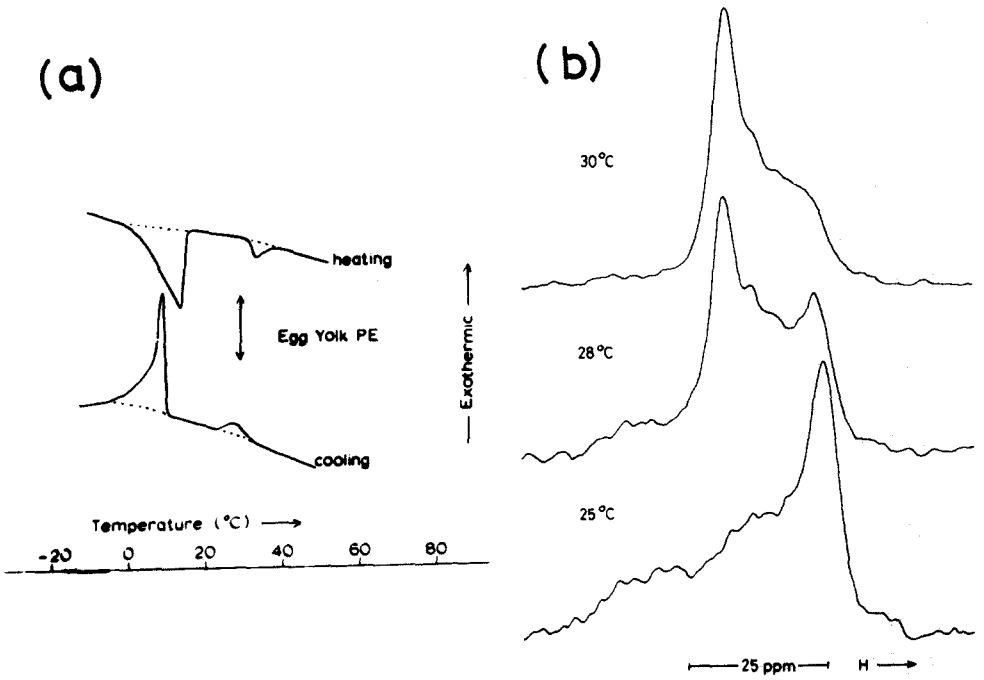

Fig. 2. (a) Calorimetric scans of aqueous dispersion of egg yolk phosphatodylethanolamine. A heating and cooling rate of 5°C/min was employed. The double headed arrow indicates the temperature of the bilayer to hexagonal (H_{II}) polymorphic phase transition as detected by ^{31}P NMR. (b) 36.4 MHz ^{31}P NMR spectra of the same aqueous dispersions of egg yolk phosphatidylethanolamine employed in (a). Broad band proton decoupling was employed. Reproduced with permission from Ref. 7.

for synthetic phosphatidylethanolamine with homogeneous fatty acid composition where, in the case of the dioleoyl species for example, the bilayer-H_{II} transition is not detected by calorimetric techniques [48]. These observations have two important implications in that they suggest a very low energy barrier for transitions between bilayer and non-bilayer configurations, and also indicate that the acyl chains are not markedly more disordered in the H_{II} phase than in the bilayer phase. The latter point is consistent with label studies employing NMR [49] and ESR [50] techniques, which also indicate little change in order parameters between bilayer or non-bilayer lipids.

The preference of unsaturated phosphatidylethanolamines for the hexagonal (H_{II}) arrangement has important implications. By way of example, phosphatidylethanolamine isolated from the erythrocyte membrane adopts the H_{II} phase at temperatures above 10°C, as illustrated in Fig. 3. Thus, at physiological temperatures this component, which comprises 30% of the membrane phospholipid [51], will act not to ensure membrane bilayer integrity, but rather will actively mitigate agains such structure. Thus one is immediately faced with a direct challenge to current views of membrane lipid function, as it is rather difficult to reconcile the view that the major function of lipids is to provide a semi-permeable bilayer matrix with the fact that a large component of the lipid would

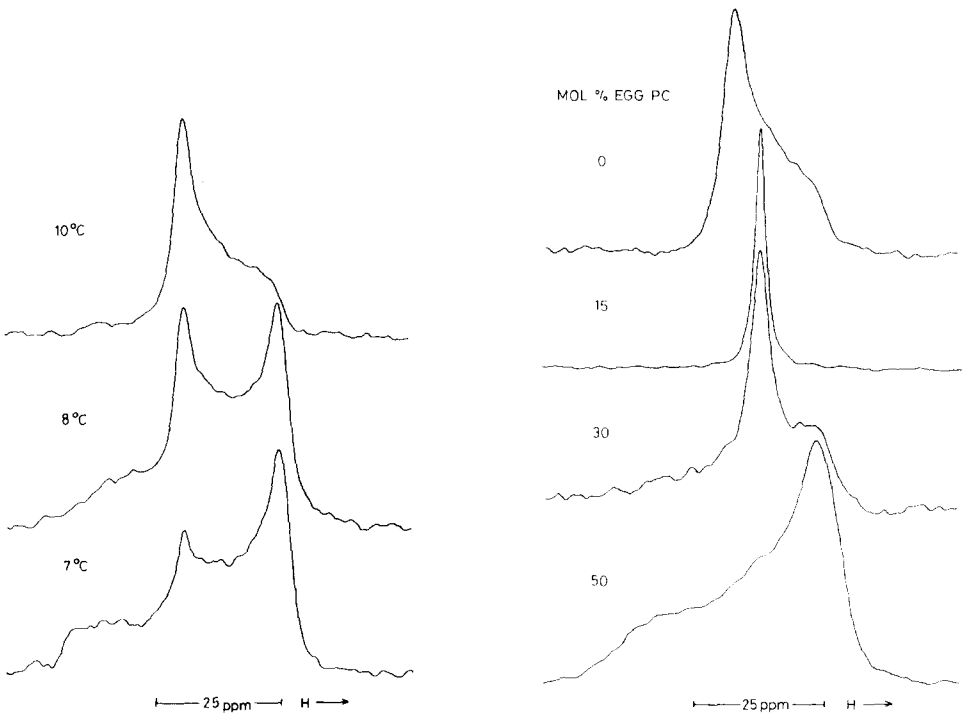

Fig. 3. 36.4 MHz ^{31}P NMR spectra of aqueous dispersion of human erythrocyte phosphatidylethanolamine dispersed in 25 mM Tris-acetic acid (p^2H = 7.0) and 2 mM EDTA. These spectra were obtained employing broad band proton decoupling. Reproduced with permission from Ref. 7.

Fig. 4. 36.4 MHz ^{31}P NMR spectra of aqueous dispersions of mixtures of soya phosphatidylethanolamine and egg phosphatidylcholine. The amount present is expressed as a percentage of the total phospholipid. Other conditions as for Fig. 3. Reproduced with permission from Ref. 33.

rather not assume such a phase. One is led to consider alternative functional roles for such lipids which we discuss in Section VI.

Given that biological membranes such as that of the erythrocyte do, in fact, exhibit largely bilayer structures (witness the 'bilayer' ^{31}P NMR lineshapes obtained for erythrocyte ghosts [52]) however, and that this structure appears dictated by the lipid component (as indicated by the bilayer arrangement of model membrane systems composed of extracted lipids [52]) it is clear that at least some endogeneous lipid does play a structural 'bilayer stabilizing' role. Phosphatidylcholines are logical contenders for such a role, in view of their preference for bilayer structure in model liposomal systems as indicated by the bilayer ^{31}P NMR lineshapes obtained [35–38]. This behaviour appears to be independent of the fatty acid composition or other biologically relevant variables [35]. The ability of phosphatidylcholine to stabilize the bilayer configuration may be conveniently examined by monitoring the phase behaviour of initially non-bilayer systems (such as soya bean phosphatidylethanolamine) in the presence of increasing amounts of the bilayer species, and an example of such experiments is given in Fig. 4. This figure shows that the addition of more than 30 mol% egg yolk phosphatidylcholine to soya bean phosphatidylethanolamine induces the bilayer phase for the bulk of the endogeneous phospholipids, and at equimolar concentrations, the bilayer phase alone is observed. Similar results are obtained employing synthetic saturated and unsaturated liquid crystalline phosphatidylcholines [53] as well as (bovine brain) sphingomyelin [42] and give strong circumstantial support to the proposal that a major, if not the major, functional role of phosphatidylcholines and sphingomyelin in biological membranes is to stabilize the bilayer lipid configuration.

A remarkable and unexpected feature of these results, however, is the appearance of an intermediary phase, characterized by a narrow symmetric ^{31}P NMR lineshape indicating isotropic motional averaging, at intermediate (e.g. 15 mol%) phosphatidylcholine contents. As indicated in the previous section, a variety of structures available to phospholipids could give rise to such spectra, including vesicles, micelles, or lipids in inverted micellar, cubic or rhombic phases. Although the former possibilities (vesicles, micelles) can be eliminated by the observation that the systems giving rise to these signals consist of large visible aggregates of lipid suspended in the aqueous phase, the ^{31}P NMR technique alone cannot discriminate between the other alternatives. As we shall emphasize later in this work, however, the structure of this 'isotropic' phase (or phases) is of major interest, for whereas there is as yet no evidence for the existence of H_{II} phase lipid in biological membranes, narrow ^{31}P NMR signals indicating isotropic motional averaging have been observed.

Returning to the influence of other lipid species on membrane bilayer stability, the effects of cholesterol are of particular interest. On the basis of the well characterized ability of cholesterol to condense phosphatidylcholine monolayers together with the ability to reduce the permeability of corresponding bilayer liposomal systems [54] it may be suspected that cholesterol acts to stabilize bilayer structure in vivo. In the case of phosphatidylcholines there is certainly no evidence to the contrary, as saturated and unsaturated phosphatidylcholine in the presence of equimolar concentrations of cholesterol exhibit bilayer ^{31}P NMR spectra [35]. However, the influence of cholesterol on mixed systems containing unsaturated phosphatidylethanolamines where bilayer structure has been stabilized by the presence of phosphatidylcholine is quite remarkable [33,53]. In such systems containing saturated (16 : 0/16 : 0) phosphatidylcholine, equimolar cholesterol acts to stabilize the bilayer, whereas the bilayer structure of similar systems

containing unsaturated phosphatidylcholines is positively disrupted by the presence of cholesterol, which promotes formation of the H_{II} phase. Such disruption is not, however, observed in analogous bilayer systems stabilized by the presence of sphingomyelin [42], which has led to the suggestion that a role of sphingomyelin in vivo may be to preserve bilayer structure in the face of high cholesterol contents.

Acidic (negatively charged) phospholipids also exhibit an ability to assume non-bilayer configurations, particularly in response to the presence of divalent cations such as Ca^{2+}. By way of example, X-ray studies have shown that in the absence of Ca^{2+}, cardiolipin (a major component of the inner mitochondrial membrane) assumes the bilayer phase [55]. The introduction of equimolar concentrations of Ca^{2+}, however, causes the lipid to adopt the hexagonal (H_{II}) phase, a finding which is supported by ^{31}P NMR [56] and freeze-fracture [56,57] experiments. In addition, the ^{31}P NMR results show that cardiolipin proceeds from the bilayer to H_{II} arrangements via an intermediary phase characterized by isotropic motional averaging, which is observable at intermediate Ca^{2+} concentrations [56]. As in the case of soya bean phosphatidylethanolamine/egg yolk phosphatidylcholine systems, the structure of this intermediary is not well characterized, although freeze-fracture results would be consistent with an inverted micellar lipid arrangement [56,58]. Unsaturated phosphatidic acid has also been shown to adopt the H_{II} phase in the presence of Ca^{2+} [59], and thus the behaviour of cardiolipin is not an isolated phenomenon.

It may be argued, however, that the behaviour of these charged lipid species is not relevant to the behaviour of the majority of biological membranes, in that cardiolipin and phosphatidic acid are usually only minority components. It is in this context, therefore, that the behaviour of systems containing phosphatidylserine, which is the major charged lipid species of eukaryotic cell membranes, is particularly interesting. In the absence of Ca^{2+} unsaturated phosphatidylserine adopts the bilayer configuration in excess water [41]. The introduction of equimolar (with respect to charge) amounts of Ca^{2+} to phosphatidylserine systems results in precipitation of the lipid dispersion and formation of so-called cochleate lipid structures [60]. In these structures the motion in the phosphate region is severely restricted, as indicated by rigid lattice (no motion) ^{31}P NMR lineshapes obtained [41] and an order of magnitude increase in the spin-lattice relaxation time T_1 [41]. This certainly indicates a strong and specific Ca^{2+}-phosphatidylserine interaction. (Similar Ca^{2+}-dependent freeze fracture morphology [61] and ^{31}P NMR characteristics [32] have been observed for phosphatidylglycerol, the major acidic phospholipid of prokaryotic cell membranes.)

In mixed lipid systems, phosphatidylserine can stabilize bilayer structure in much the same manner as phosphatidylcholine, inducing bilayer structure for egg phosphatidylethanolamine at 37°C (which prefers the H_{II} phase above 30°C) at about 20 mol% [62]. The subsequent addition of Ca^{2+}, however, results in a triggering of H_{II} phase formation as illustrated in Fig. 5. This behaviour may be attributed to Ca^{2+}-induced lateral segregation of the phosphatidylserine component (as is observed in phosphatidylserine/phosphatidylcholine systems [63] or to an altered 'shape' of Ca^{2+}-phosphatidylserine complexes formed [62]. Either effect could reduce or remove the bilayer stabilizing capacity of the phosphatidylserine, allowing the preference of the phosphatidylethanolamine component for the H_{II} configuration to predominate. This ability of Ca^{2+} to trigger formation of non-bilayer lipid structures is potentially most important, and has possible relevance to the behaviour of the erythrocyte when high intracellular levels of Ca^{2+} are obtained.

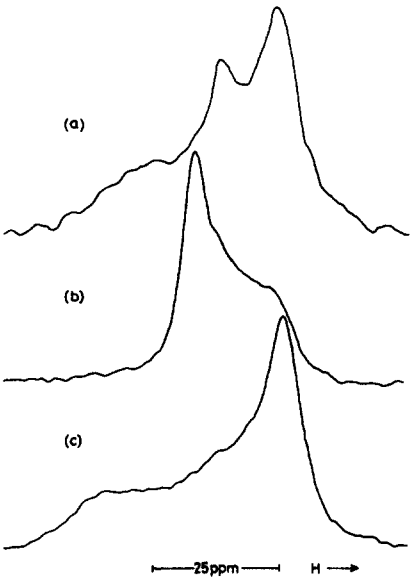

Fig. 5. 36.4 MHz ^{31}P NMR spectra of an aqueous dispersion of 20 mol% bovine brain phosphatidylserine and 80 mol% egg yolk phosphatidylethanolamine at 37°C: (a) in the absence of Ca^{2+} or dibucaine; (b) in the presence of Ca^{2+} (Ca^{2+}/phosphatidylserine ratio of 0.5 (mol/mol)); (c) as (b) plus dibucaine (dibucaine/phosphatidylserine ratio 1.0 (mol/mol)). The aqueous dispersion contained 50 mM Tris-acetic acid (p^2H = 7.2) and 300 mM NaCl. Other conditions are as described in Ref. 62. Reproduced with permission from Ref. 62.

It is also of interest to note that this Ca^{2+} induced triggering of H_{II} phase formation can be reversed by agents, such as local anaesthetics, which displace Ca^{2+} from membranes [62] as indicated by the effects of dibucaine (Fig. 5 (c)).

The data discussed to this point clearly establish that the hexagonal (H_{II}) phase is a ubiquitous lipid configuration. However, it is also clear that this phase would not be expected to play a major role in biological membranes as it is difficult to envisage such structures maintaining the permeability barrier vital to cellular integrity. It is in this sense that the phase (or phases) previously noted as intermediaries between bilayer and H_{II} arrangements, become topical. A particularly interesting indication of the structures that may be present has been obtained employing freeze-fracture techniques on cardiolipin [56] and cardiolipin/phosphatidylcholine [65,66] systems. The addition of Ca^{2+} to such systems results (see Fig. 6) in the formation of lipid structures visualized as (complementary) particles and pits on the freeze-fracture micrographs which have been interpreted as inverted micellar lipid structures sandwiched between the two monolayers of the lipid bilayer [65]. The presence of these Ca^{2+}-induced 'lipidic particles' is also reflected in ^{31}P NMR studies [66], where narrow 'high resolution' NMR signals are observed for a portion of the phospholipids. Further, similar ^{31}P NMR and freeze-fracture features have been observed for a variety of other model membrane systems, including phosphatidylcholine mixed with monoglucosyldiglyceride or phosphatidylethanolamine (in the presence of cholesterol) [66], as summarized in Fig. 6. It is most interesting that such structures can also be detected in aqueous dispersions of the total lipid extracts derived from the inner mitochondrial [66], *E. coli* [67], and rod outer segment [68]

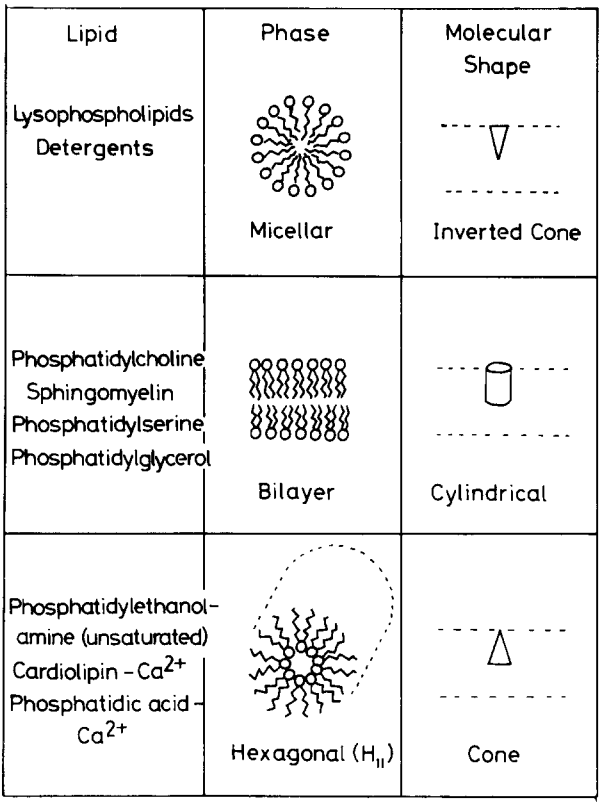

Fig. 7. Polymorphic phases and corresponding dynamic molecular shapes of component lipids.

which assume a more cylindrical shape would be most easily accommodated in the familiar bilayer phase.

These proposals have received much experimental support, most of which is implicit in the results discussed in the previous section. With regard to the polar region, for example, the smaller headgroup of phosphatidylethanolamine (as compared to phosphatidylcholine) as well as the possibility of intermolecular hydrogen bonding [72] would be expected to result in a reduced area per molecule at the lipid-water interface, thus producing a cone shaped molecule compatible with the H_{II} phase often observed for these phospholipids. Alternatively, in the acyl chain region, increased unsaturation may be expected to lead to a more pronounced cone shape, a suggestion fully compatible with the requirement for a minimal degree of unsaturation for H_{II} phase phosphatidylethanolamines [39] as well as the observation of lower bilayer-H_{II} transition temperatures as the number of unsaturated bonds is increased [39]. Further, increasing the amplitude of the thermal motion of the acyl chains by increasing the temperature again leads to cone shapes compatible with H_{II} structure, as indicated by the bilayer to hexagonal H_{II} transitions observed for both pure phosphatidylethanolamines [39] and mixed lipid systems [33,53] as the temperature is raised. Finally, the ability of cholesterol to induce H_{II} phase formation in certain mixed lipid systems [33,53] would also be consistent with a cone shape of cholesterol as indicated by other studies [73,74].

These molecular shape considerations can also be extended to acidic phospholipids for

Fig. 6. Freeze-fracture micrographs of lipidic particles in cardiolipin/phosphatidylcholine (1 : 1) – Ca^{2+} systems (CARD/PC), monoglucosyl diglyceride/phosphatidylcholine (1 : 1) systems (MGDG/PC), and dioleoyl phosphatidylethanolamine/dioleoyl phosphatidylcholine/cholesterol (3 : 1 : 2) systems (PE/PC/CHOL). Magnification, 150 000×.

membranes. Finally, these structures are visible on the fracture faces of cardiolipin/phosphatidylcholine vesicles [69] undergoing Ca^{2+}-induced fusion, and often appear to be localized at the fusion interface.

There are two aspects of these lipidic particles which will be receiving detailed attention in the future. First, there is a possibility that the intra-bilayer lipids may experience exchange between the inverted micellar and surrounding bilayer environments. Clearly, such an ability would add a new dimension to lipid dynamics and function. Second, as discussed by Verkleij and Ververgaert [70] these results offer alternative interpretations of particles observed in freeze-fracture studies of biological membranes, which have been previously been assumed to originate solely from integral membrane protein.

IV. Dynamic shapes of lipids and polymorphic phase behaviour

It is useful to introduce a naive but instructive rationale for the polymorphic phase behaviour of membrane lipids, which postulates that the preference of a lipid species for a given structure reflects the dynamic molecular shape assumed by the individual components. Other authors [71] have invoked similar considerations to rationalize the behaviour of particular lipid systems. Briefly, as indicated in Fig. 7, lipids assuming the hexagonal (H_{II}) phase may be considered to exhibit a 'cone' shape, where the polar headgroup region is at the smaller end of the cone. Alternatively, lysophospholipids may be suggested to display an 'inverted cone' shape where the cross-sectional area of the polar region is larger than that subtended towards the end of the acyl chain. This shape would be compatible with the micellar phase adopted by these lipids. Finally, lipids

which the area per molecule at the lipid-water interface is sensitive to the net charge in the polar region [75]. In the case of cardiolipin isolated from mitochondria the relatively small headgroup associated with four (usually very unsaturated [76]) acyl chains would be expected to result in a cone-shaped molecule compatible with the H_{II} phase structure. Thus, the observation of bilayer structure in the absence of divalent cations [55,56] suggests that charge repulsion effects increase the effective area per molecule in the polar region. This possibility is fully consistent with the previously mentioned ability of Ca^{2+} to induce H_{II} phase structure, a process which appears to occur via charge neutralization [56]. Similarly, at pH values above 5, unsaturated phosphatidylserine adopts the bilayer phase, but at pH = 2.5 (below the pK of the carboxyl group) the hexagonal H_{II} phase is observed [41], which may again be attributed to reduced charge repulsion effects.

A final point concerning a need for diversity in the molecular shapes of lipid constituents, which does not involve the formation of alternative lipid structures, concerns the lipid composition in the region of integral protein. As pointed out by Israelachvili [77], such proteins may also have varying shapes in the bilayer, requiring cone or inverted cone lipids to provide optional packing and sealing at the protein-lipid interface. These speculations are consistent with recent observations [78] that reconstituted glycophorin-dioleoyl phosphatidylcholine membranes require the addition of small quantities of cone shaped lipid in order to render the membrane impermeable to shift reagents. It is obvious that these considerations provide a somewhat different picture of boundary lipid than is currently popular.

V. Non-bilayer lipid structures and biological membranes

The investigations on model membrane systems detailed here clearly establish that lipids in biological membranes cannot be presumed, a priori, to be in a bilayer configuration. It is therefore important to establish first whether non-bilayer lipid structures do occur in biological membranes, and secondly to establish what functional requirements they may satisfy.

Perhaps the most closely characterized biological membrane is that of the human erythrocyte (ghost), which exhibits ^{31}P NMR spectra (arising from at least 97% of the endogeneous phospholipids [21]) which are fully consistent with the vast majority of the lipid being in the bilayer configuration, as indicated in Fig. 8 (a). This bilayer appears to be unusually stable, as extensive phospholipid degradation (employing various phospholipases) to produce non-bilayer lipids such as lysophospholipids, diglycerides and ceramides does not induce appreciable non-bilayer structure [79]. This stability, which may arise in part from the influence of membrane protein, may be related to the long life span of the erythrocyte and its ability to undergo extensive deformation without lysis during flow through narrow blood vessels. It is interesting, however, that the introduction of membrane active agents such as oleic acid (a so-called 'fusogen' [80]) can cause a wholesale disruption of bilayer structure, promoting formation of the hexagonal (H_{II}) phase as indicated in Fig. 6 (b). This behaviour has been used to suggest the involvement of non-bilayer phases as intermediates during fusion events [81] as will be discussed in the following section.

The occurrence of lipid experiencing isotropic motion in intact biological membranes has recently been observed for the endoplasmic reticulum membrane derived from rat, bovine and rabbit liver. Two laboratories [82,83] have independently reported that microsomal preparations (isolated vesicular fragments of the endoplasmic reticulum) give rise to ^{31}P NMR spectra (see Fig. 8 (c)) indicating isotropic motion for some fraction of

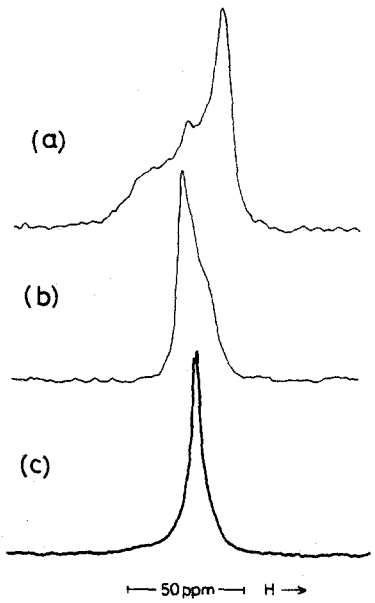

Fig. 8. 36.5 MHz ^{31}P NMR spectra obtained at 37°C from (a) 150 mg (dry weight) erythrocyte (ghost) membranes hydrated in an aqueous buffer containing 0.12 M NaCl, 6 mM KCl, 5 mM $Mg_2SO_4 \cdot 7\,H_2O$, 2 mM $CaCl_2 \cdot 2\,H_2O$ and 20 mM Tricine (pH 6.8); (b) as (a) but incubated in the presence of 50 mg oleic acid, resulting in an oleic acid/phospholipid ratio of 2.4. For details see Ref. 81. (c) Rat liver microsomes; for details see Ref. 82.

the endogeneous phospholipids at 37°C, which would be consistent with the occurrence of inverted micellar or (short) cylindrical H_{II} arrangements of lipid inside the bilayer. Further, these results also suggest that membrane lipids experience rapid exchange between these structures and bulk bilayer lipid. It is interesting that at lower temperatures (below 30°C) an increasing fraction of the lipid contributes to the normal 'bilayer' ^{31}P NMR spectra, which corresponds to effects often observed in model systems as the temperature is lowered (see previous section). ^{31}P NMR evidence is now available which indicates that this temperature dependent phase change also occurs in the endoplasmic reticulum of intact rat liver [84]. Finally, it would appear that membrane protein (possibly cytochrome *P*-450 as suggested by Stier et al. [83]) actively encourages isotropic motion as aqueous dispersions of the extracted microsomal lipids show normal bilayer ^{31}P NMR spectra at 37°C [82]. As this isotropic motion does not arise from microsomal tumbling [82,83] it is tempting to ascribe it to non-bilayer lipid structures. Unfortunately, the possibility that lateral diffusion in the bilayer of these small systems produces the observed averaging cannot be excluded.

Similar indications of isotropic motion for a portion of the endogeneous phospholipids as indicated by ^{31}P NMR are obtained for the related sarcoplasmic reticulum membrane [85]. Further, it may be noted that similar results had been obtained some eight years ago by Davis and Inesi [86] who concluded on the basis of ^{1}H NMR studies that some 20% of the sarcoplasmic reticulum lipid experienced isotropic motion on the NMR timescale [86].

Evidence that a significant fraction of lipids also experience isotropic motion in the inner mitochondrial membrane is implicit in the ^{2}H NMR results of Arvidson et al. [87]. These results, which are substantiated to some extent by recent unpublished work in the

author's laboratories, are particularly intriguing in the light of the ongoing controversy concerning the structure of the inner mitochondrial membrane [88]. Finally, in osmiophilic bodies from porcine lung about 5% of the phospholipid undergoes isotropic motion which has been suggested to be due to the influence of apolar proteins [89].

Before leaving this section, however, a word of caution is in order. First, in metabolically active systems such as intact mitochondria significant increases in 'isotropic' ^{31}P NMR components and changes in functional state (e.g. respiratory control) occur within minutes of incubation at 37°C (Cullis, P.R. and de Kruijff, B., unpublished results). Therefore care must be taken to ensure that the effects observed are characteristic of viable systems. Second, the elucidation of phospholipid structure in biological membranes via ^{31}P NMR is often significantly complicated by the presence of non-phospholipid phosphorus. In most membranes phospholipids are the major phosphorus-containing compounds, but some membranes such as bacterial membranes often contain large amounts of phosphorus in compounds which can account for up to 70% of the ^{31}P NMR signals detected. These can give rise to 'isotropic' signals which makes an unambiguous interpretation in terms of membrane structure very difficult. Third, when phenomena giving rise to isotropic phospholipid motion are observed, NMR techniques alone cannot discriminate between sources such as lateral diffusion in highly curved bilayers and non-bilayer lipid structures as the origin of this additional motion.

VI. Functional roles of lipids

The potential range of functional roles of lipids in biological membranes is greatly increased by the availability of non-bilayer alternatives, particularly intra-bilayer inverted micellar and/or short cylindrical segments (H_{II} configuration). Structural roles related to maintaining bilayer integrity may then be assigned to lipids such as phosphatidylcholines and sphingomyelins, whereas lipids adopting non-bilayer phases in isolation or in response to the presence of agents such as Ca^{2+} may be suggested to facilitate processes requiring non-bilayer intermediates.

Among many potential candidates two fundamental abilities of biological membranes which appear likely to employ 'non-bilayer' lipids are membrane fusion phenomena (including related processes such as exo- and endocytosis) and transbilayer transport processes (including lipid 'flip-flop' and facilitates transport). We discuss these two areas in turn.

VIA. Membrane fusion

A major stimulus for investigations of the properties of non-bilayer lipid came from the straightforward observation that it is difficult, if not impossible, to rationalize cell fusion events with an inviolate bilayer structure of the lipid component. At some stage in the fusion event, irrespective of whether fusion is mediated by protein or lipid, a portion of the lipid must experience a departure from bilayer structure. It was with this precept in mind that studies were performed on the erythrocyte (ghost) membrane to investigate whether lipid-soluble agents ('fusogens' [80]) which induce cell fusion between erythrocytes in vitro promote fusion by facilitating the formation of non-bilayer intermediates [81]. The observation that membrane concentrations of fusogen sufficient to induce fusion between erythrocytes were also sufficient to induce the H_{II} phase in a portion of the isolated (ghost) membrane (see Fig. 8 and Ref. 81) was then employed to suggest a mechanism of membrane fusion where the intermediate structure consisted of lipid cylinders characteristic of the H_{II} phase [8]. The fact that many lipid species can adopt

or induce such configurations further suggested that this may be a general mechanism of fusion in vivo.

Subsequent experiments [69] suggest that at least in some systems a description of the intermediate structures as inverted micelles is more likely to be correct. These experiments were suggested by the well documented requirement for Ca^{2+} for fusion events in vivo [90] in association with the ability of Ca^{2+} to induce non-bilayer structure in certain lipid systems such as cardiolipin as indicated in Section III. Thus, in vesicles composed of an equimolar mixture of beef heart cardiolipin and egg yolk phosphatidylcholine it was demonstrated that Ca^{2+} induces fusion, and, even more to the point, that these fusion events are associated with formation of inverted micellar lipid structures at the fusion interface [69]. It may therefore be suggested that the requirement for Ca^{2+} for fusion in vivo arises from its ability to engender appropriate non-bilayer intermediates for fusion to proceed via the model indicated in Ref. 81.

It should be recognized that fusion events are ubiquitous events in membranes, and are not necessarily confined to formation of polykarocytes. A particularly interesting extension of the fusion model detailed here applies to the behaviour of the erythrocyte on ATP depletion. Pronounced morphological changes are observed [91] and membrane bound vesicles are 'blebbed off' [92], events that appear to be related to higher intracellular concentrations of Ca^{2+}. These events may be correlated with the asymmetrical distributions of phospholipid across the erythrocyte membrane [93] and, in particular, with the effects of Ca^{2+} on the lipids of the inner monolayer. This monolayer has a lipid composition (49% phosphatidylethanolamine, 25% phosphatidylserine and 12% of both phosphatidylcholine and sphingomyelin [93]) which suggests a certain instability, given the preference of the phosphatidylethanolamine component for the H_{II} configuration at physiological temperatures [39]. The additional observation that Ca^{2+} can remove the bilayer stabilizing capacity of the phosphatidylserine component [94] further implies that this instability is likely to be expressed when the intracellular levels of Ca^{2+} are raised. It may therefore be speculated that a certain portion of the inner monolayer lipids may adopt intra-membrane inverted micellar or cylindrical (H_{II}) configurations in the presence of Ca^{2+}, which would serve to reduce the area of the inner monolayer and produce the observed morphological changes. More importantly, however, the instability of the inner monolayer allows a detailed molecular model of the 'blebbing off' process to be proposed, as indicated in Fig. 9. This model may also be suggested to apply in general to processes of exo and endocytosis in biological membranes.

VIB. Transbilayer transport

Phospholipid asymmetry and transbilayer movements of lipid are subjects of considerable interest of late [95]. In particular, the process whereby lipids move from one monolayer (of a biomembrane) to the other is difficult to understand at the molecular level, particularly if bilayer structure is always maintained. We have suggested [33] that transitory formation of intrabilayer inverted lipid structures provides a mechanism for flip-flop processes resulting in redistribution of lipids across the bilayer, as indicated in Fig. 10. Two features of this model are of interest. First, the inverted micellar intermediary structure is drawn approximately to scale with respect to the thickness of the bilayer, and it is clear that no impossible topological problems are presented by such structures. Second, given the apparent low activation energies required for lipid rearrangements from the bilayer to H_{II} phases, it is quite conceivable that the lipid in the intrabilayer structures is in exchange with surrounding bilayer lipid on either side of the membrane.

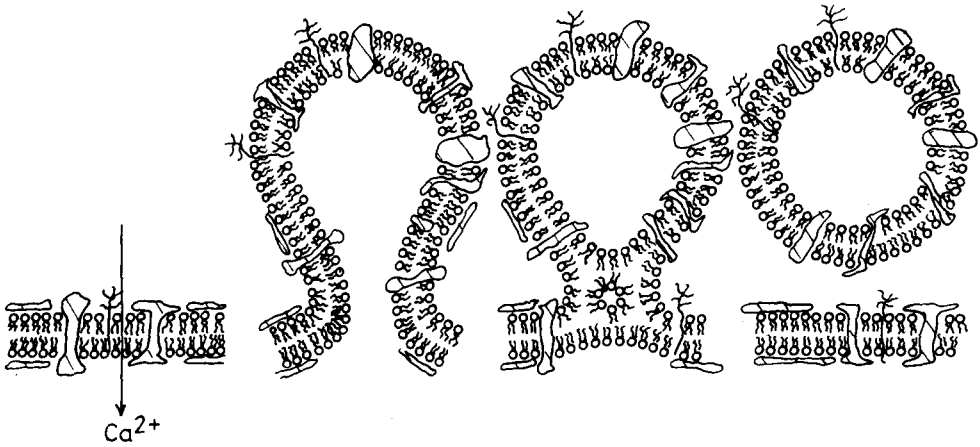

Fig. 9. Model of the 'blebbing off' process observed for erythrocyte membranes (see text, Section VIA). This model may also be suggested to apply in general to processes of exocytosis and endocytosis in biological membranes. The shaded areas represent integral membrane proteins (glycophorin, band 3, etc.) and extrinsic protein.

Such possibilities are consistent with the ^{31}P NMR characteristics of various membranes discussed in the previous section and the measured rates of transbilayer movements of lipids in these systems. In the case of the endoplasmic reticulum membrane, for example, at 37°C the ^{31}P NMR results indicate isotropic motion of the phospholipids [82,96] which have also been demonstrated to experience rapid 'flip-flip' [96,97]. Further, at 4°C where mainly bilayer structure is observed the rate of transbilayer movement of phosphatidylcholine appears dramatically decreased [96]. Alternatively, in the sarcoplasmic reticulum membrane part of the phospholipids experience isotropic motion and again rapid transbilayer movement of lysophosphatidylcholine [85] and part of the phosphatidylcholine pool is observed [98]. Other biological membranes in which rapid flip-flop occurs (e.g. certain bacterial membranes [95]) have lipid compositions consistent with the occurrence of non-bilayer structures. In fact, the aqueous dispersions of the total lipid extract of *E. coli* exhibit ^{31}P NMR spectra at 37°C which demonstrate isotropic motion of part of the phospholipids [67]. An intrabilayer inverted micellar origin of these features is indicated by the observation of 'lipidic particles' in the lipid extract system employing freeze-fracture techniques [67]. It is of interest to compare these observations with the behaviour of the erythrocyte membrane for which bilayer ^{31}P NMR spectra are observed and where phospholipid flip-flop is indeed very slow [99].

It should be realized however that alternative flip-flop mechanisms might be possible, particularly in view of the following observations. First, fast transbilayer flip-flop occurs at the gel-liquid crystalline phase transition [100]. Second, glycophorin enhances the flip-flop rate of lysophosphatidylcholine [101] and phosphatidylcholine [102] by two orders of magnitude in phosphatidylcholine bilayers. Alternatively, an asymmetric perturbation of the bilayer leading to an imbalance in surface pressures between the two monolayers can induce fast flip-flop of phosphatidylchloline [103] and phosphatidic acid [104]. Finally, cholesterol moves rapidly across (vesicular) bilayer membranes composed of phosphatidylcholine [105,106]. An association of non-bilayer phases with these rapid flip-flop processes has not been demonstrated, and would appear unlikely in view of the lipid composition of these various systems.

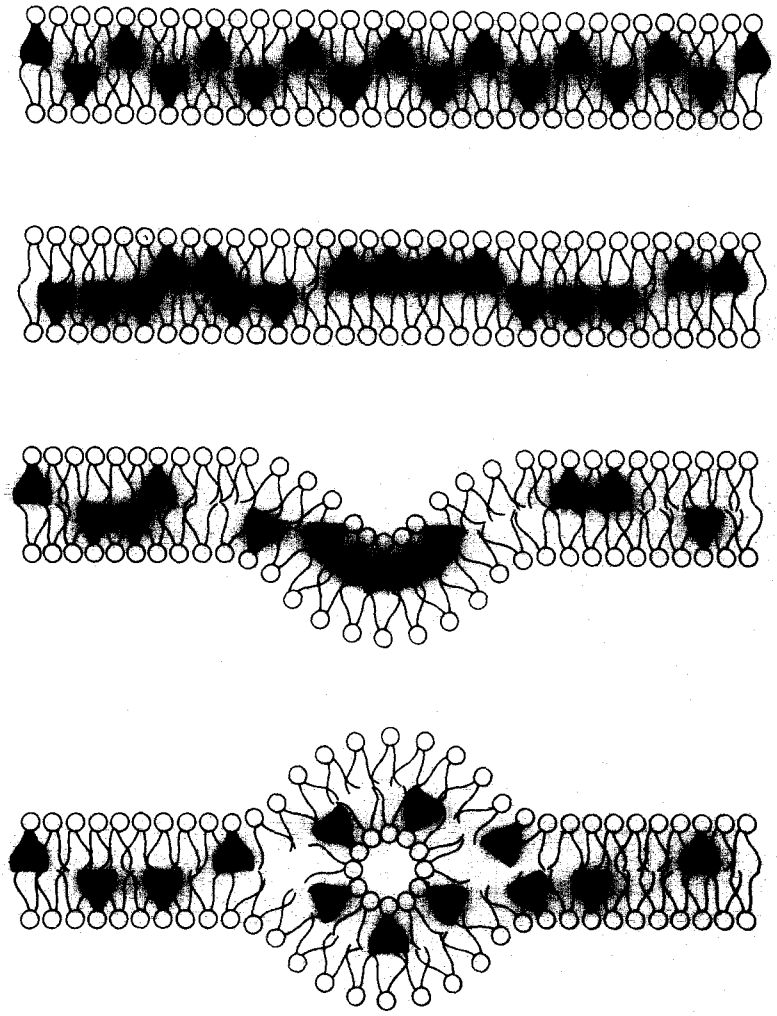

Fig. 10. Dynamic formation of inverted micelles in a lipid bilayer which may result in redistribution of membrane lipid across the bilayer.

In addition to the transbilayer transport of membrane lipids, a transport mechanism such as that of Fig. 10 may also be related to facilitated transport of water-soluble molecules across the membrane. A general characteristic of any carrier system must be an ability to form a lipid soluble complex with the agent to be transported – a demand which would be satisfied for polar molecules trapped in the aqueous compartment of the intra-bilayer structure. Formation of this non-bilayer intermediate could be stimulated or modulated by the polar molecule itself, or by membrane protein. By way of example, Ca^{2+} can stimulate formation of intra-membrane inverted micellar structures in cardiolipin-phosphatidylcholine membranes [65,66], and cardiolipin has Ca^{2+} ionophore capabilities in other model systems [107]. Further, net transport may be envisaged to occur if the lipid carrier is able to return to its original monolayer to initiate another transport cycle. These or similar mechanisms may be related to the ability of the inner mitochondrial membrane to sequester Ca^{2+} in vitro [108].

In addition, a role of H_{II} lipids as channel formers through membranes is directly suggested by the characteristic 20 Å aqueous pore running through the constituent lipid cylinders, as has been proposed by Luzzatti et al. [28]. The major difficulty with such possibilities is that least energy considerations would appear to preclude an orientation of such a cylinder perpendicular to the plane of the surrounding bilayer, and it is necessary to postulate a role of proteins to stabilize this arrangement [109]. Although this is by no means inconceivable, there is presently no supporting evidence for such a hypothesis.

VII. Concluding remarks

The ability of endogeneous membrane lipids to adopt non-bilayer configurations clearly provides a variety of possibilities for the direct involvement of lipids in many functional abilities of biological membranes. The fact that these alternative structures may be sensitive to factors such as Ca^{2+} concentration, local lipid composition and the presence of membrane protein further implies a satisfying number of mechanisms for the isothermal regulation and control of associated functions. Finally, within the strictures of this 'metamorphic mosaic' model of biological membranes, a new rationale for lipid diversity emerges which indicates a requirement for lipids with diverse dynamic shapes.

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