Phospholipids and membrane transport

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The ability of membrane lipids to adopt nonbilayer configurations suggests dynamic roles for lipids in many functional abilities of biological membranes. In this work evidence supporting the involvement of lipids in three types of membrane transport process is presented and discussed. These transport processes include facilitated transbilayer transport of polar molecules, transport mechanisms involving fusion events, and transport possibilities arising from alternative membrane morphology. In particular it is shown that lipids such as cardiolipin, which adopt the hexagonal H_{II} phase in the presence of Ca^{2+}, may be logically proposed to facilitate Ca^{2+} transport across membranes via an inverted micellar intermediate. Alternatively, in transport processes such as exocytosis the ability of Ca^{2+} to generate membrane instabilities favouring nonbilayer alternatives suggests a crucial role of phospholipid in the fusion event vital to exocytotic release. Finally, nonbilayer lipid structures may be suggested to favour formation of isolated compartments connected by a continuous membrane where lateral diffusion processes can lead to transport. These various possibilities are summarized in a "metamorphic mosaic" model of biological membranes.

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Le pouvoir des lipides membranaires d'adopter une configuration non bilamellaire suggère des rôles dynamiques de ces lipides dans plusieurs fonctions des membranes biologiques. Dans ce travail, nous présentons des preuves confirmant l'implication des lipides dans trois types de processus de transport membranaire. Ces types de transport sont le transport facilité des molécules polaires à travers la double couche, des mécanismes de transport impliquant des étapes de fusion et des possibilités de transport résultant d'une morphologie membranaire différente. Notamment, nous montrons que des lipides comme la cardiolipine, qui adopte la phase hexagonale H_{II} en présence du Ca^{2+}, peuvent logiquement faciliter le transport du Ca^{2+} à travers les membranes via un intermédiaire micellaire retourné. D'autre part, dans les processus de transport comme l'exocytose, le pouvoir du Ca^{2+} de générer des instabilités membranaires favorisant des configurations non bilamellaires, suggère un rôle crucial des phospholipides dans l'étape de fusion vitale à la libération exocytotique. Finalement, les structures lipidiques non bilamellaires pourraient favoriser la formation de compartiments isolés, réliés par une membrane continue où les processus de diffusion latérale peuvent conduire au transport. Ces diverses possibilités sont résumées en un modèle de "mosaïque métaphorique" des membranes biologiques.

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Introduction

Until recently it has been generally assumed that the major, if not the only, role of lipids in biological membranes is to provide a semipermeable barrier between intracellular and extracellular environments. Within the terms of membrane models such as the "fluid mosaic" model (1) active and facilitated transport and other functions vital to cell or organelle viability are mediated by proteins extending into or through the lipid bilayer, with the possibility that local "annular" lipids play some ill-defined regulatory role. However, it now appears that the resulting view of lipids as inert building blocks of biomembranes is incomplete, and that lipids may participate directly in many functional processes (2). Our purpose in this work is to point out some potential roles of phospholipids in certain diverse membrane mediated transport phenomena.

The major fact which gives rise to these possibilities is the observation that all biological membranes appear to contain lipids which adopt non-bilayer phases, particularly the hexagonal (H_{II}) phase, in isolation. In the case of eukaryotic cell membranes a major example is PE, as PE's isolated from the (human) erythrocyte (3), endoplasmic reticulum (4), sarcoplasmic reticulum (E. Mah and M. J. Hope, unpublished), and rat liver mitochondria (5) all exhibit the H_{II} phase at 37°C in the presence of (excess) aqueous buffer. These observations clearly suggest that this major lipid component (commonly constituting 30–40 mol% of the

ABBREVIATIONS: NMR, nuclear magnetic resonance; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; PS, phosphatidylserine; SPM, sphingomyelin; TLC, thin-layer chromatography; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine.

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membrane phospholipid) does not contribute to the integrity or stability of the membrane bilayer.

In this work we present and discuss results which suggest that the ability of lipids to adopt nonbilayer structures may be of importance to three types of membrane transport. These include facilitated transbilayer transport of polar molecules, transport involving membrane fusion phenomena (such as exo- and endo-cytosis) as well as transport processes arising from the different membrane morphology allowed by nonbilayer lipid alternatives.

Materials and methods

The lipids employed in this study were primarily synthesized and (or) isolated in this laboratory, with the exception of cardiolipin (sodium salt) which was obtained from Sigma, St. Louis. PA, PE, and PS were obtained from soya PC employing the base exchange capacity of phospholipase D and carboxymethylcellulose column chromatography (6). PS and PA were converted to their sodium salts as indicated elsewhere (7). PE from egg yolk was obtained by established procedures (8). Lipids from human erythrocyte membranes (PC, PS, SPM, and PE) were isolated as indicated in previous work (9). All lipids employed were more than 99% pure as indicated by TLC.

Phospholipid-dependent Ca^{2+} uptake into chloroform

A procedure similar to that of Tyson *et al.* (10) was employed. Briefly, phospholipid corresponding to 6 μmol of phosphorus was dissolved in 6 mL of chloroform. (The chloroform was obtained as the lower phase in the final step of the Bligh and Dyer (11) procedure.) Subsequently, 4 mL of an aqueous buffer (100 m*M* NaCl, 10 m*M* $CaCl_2$, 10 m*M* Tris–HCl, pH 7.4) was added to the chloroform, followed by the addition of 1 μCi $^{45}CaCl_2$ (1 Ci = 37 GBq) (NEN Canada, 10 Ci/g of Ca) in 10 μL. Ruthenium red (Sigma, 6 μmol), a dye which inhibits Ca^{2+} uptake in mitochondria (12), was added to the aqueous buffer in some cases. The resulting two-phase system was then gently shaken for 3 h at 20°C. Subsequently the $CHCl_3$ and aqueous phases were separated by brief centrifugation (15 min, 1000 rpm) and 100 μL of the chloroform phase transferred to a scintillation vial for counting.

^{31}P NMR determination of phospholipid polymorphism

The polymorphic preferences of aqueous dispersions of phospholipid (as obtained on vortexing a dry lipid film in the presence of aqueous buffer) or biological (chromaffin granule) membrane preparations were monitored employing ^{31}P NMR techniques as discussed elsewhere (2). Briefly, phospholipids in large bilayer structures exhibit broad asymmetric ^{31}P NMR spectra with a low-field shoulder and a high-field peak, which are separated by approximately 40 ppm (see Fig. 1*a*; $R=0$). Hexagonal (H_{II}) phase phospholipids, on the other hand, elicit ^{31}P NMR spectra which have reversed asymmetry compared with the bilayer situation and are narrower by a factor of two (cf. Fig. 1*a*; $R=1$). Finally, lipids in structures allowing isotropic motion (e.g., lipid in small lamellar vesicles, or in nonbilayer configurations such as inverted micelles (13)) show narrow symmetric ^{31}P NMR resonances.

^{31}P NMR spectra were obtained employing a Bruker WP 200 Fourier transform spectrometer operating at 81.0 MHz for ^{31}P and equipped with temperature control and proton decoupling facilities.

Preparation of oriented multilayers

Oriented multilayers of egg yolk PE were prepared (see Refs. 14 and 15) at 20°C by squeezing the fully hydrated egg PE between glass disks (0.8-mm diameter, prepared from microscope cover glass slips) and orientation was checked with a polarizing microscope equipped with a $\lambda/4$ retardation plate. Subsequently, the oriented systems were stacked in a 10-mm NMR tube which also contained a saturated NaCl solution in an open, separate container. The NMR tube was then sealed.

Chromaffin granule release

Chromaffin granules (large granule fraction) were isolated from fresh bovine adrenal glands as indicated elsewhere (Ref. 16 and footnote 1). Spectrophotometric assays for release were performed by monitoring the absorbance at 265 nm according to established procedures (17). Sonicated vesicles were prepared from aqueous dispersions of 50 μmol of "inner monolayer" erythrocyte phospholipid (25 mol% PS, 50 mol% PE, and 12 mol% PC and SPM (18) together with an equimolar amount of cholesterol with respect to total phospholipid) employing a Fisher Sonic Dismembrator tip sonicator at power setting 50 for 5 min which resulted in a clear dispersion. Incubations of chromaffin granules (0.2 μmol phospholipid) in the absence and presence of various amounts of inner monolayer vesicles were performed at 37°C in the manner indicated elsewhere[1].

Results and discussion

(A) Phospholipids and facilitated transbilayer transport

Whereas passive diffusion processes of various molecules across bilayer lipid structures have received detailed attention, the possibility that certain lipids may directly facilitate transbilayer transport has been relatively ignored. We have suggested elsewhere (19) that structures such as intrabilayer "inverted micelles" or "inverted cylinders" (H_{II} phase) could serve as intermediaries in flip-flop processes. This is consistent with the observation that the Ca^{2+} stimulated generation of lipidic particles (which appear to correspond to intrabilayer inverted micelles (13, 20) in cardiolipin–PC systems also results in rapid flip-flop (21).

Here, we specifically address the question as to whether certain lipids can act to facilitate the transport of divalent cations such as Ca^{2+}, and whether such an ability can be correlated with an ability of the divalent cation to induce nonbilayer structure. The possibility that certain lipids have ionophoretic capabilities has been investigated by other workers (see Ref. 10 and references therein), but has not been previously correlated with their structural preferences.

In order that any compound exhibit ionophoretic abilities it must clearly have the capability to form a lipid-soluble complex with the agent to be transported. As a first approximation to the biological situation, therefore, it may be expected that lipids which are able to facilitate the uptake or solvation of a given ion into an organic phase could also act as an ionophore

[1]R. Nayar, M. J. Hope, and P. R. Cullis, submitted.

TABLE 1. Amount of Ca^{2+} taken up into an organic phase (chloroform) in the presence of various phospholipid species

Lipid species	Amount of Ca^{2+} taken up (μmol)
Cardiolipin	4.7
Cardiolipin + ruthenium red (equimolar)	0.48
Phosphatidic acid	10.7
Phosphatidylserine	1.1
Phosphatidylethanolamine	0.02
Phosphatidylcholine	0.36
No lipid	0.00

NOTE: For details of protocol see text. Amount of phospholipid present in all cases corresponds to 6.0 μmol phosphorus. PA, PS, and PE were obtained from soya PC employing the base exchange capacity of phospholipase D (6). PC was obtained from hen eggs.

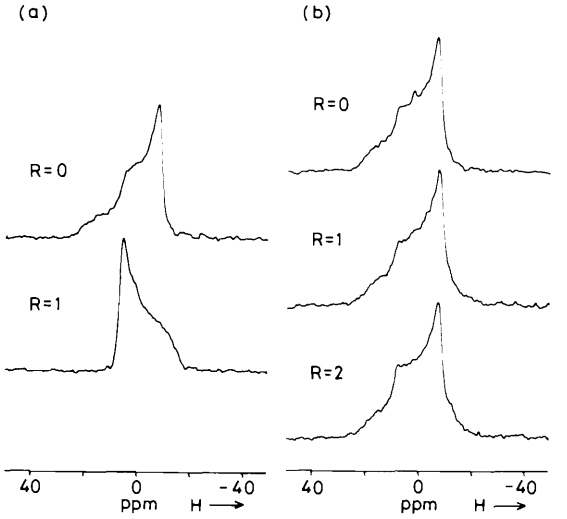

FIG. 1. The 81.0-MHz ^{31}P NMR spectra at 30°C arising from (*a*) an aqueous dispersion of 25 μmol cardiolipin and (*b*) an aqueous dispersion of 25 μmol cardiolipin and equimolar ruthenium red in the presence of varying amounts of Ca^{2+}. The ratio *R* refers to the molar ratio of Ca^{2+} to cardiolipin. The aqueous buffer employed contained 100 m*M* NaCl, 10 m*M* Tris–HCl (pH 7.4), and 2 m*M* EDTA (with or without ruthenium red), and the lipid was dispersed by vortex mixing in 0.7 mL of this buffer in a 10-mm NMR sample tube. Ca^{2+} was added as aliquots of a 0.1 *M* stock $CaCl_2$ solution. Accumulated free induction decays were obtained from up to 500 transients employing an 11-μs 90° radiofrequency pulse, a 20-kHz sweep width, and gated proton decoupling, with an interpulse time of 0.8 s. An exponential multiplication corresponding to 50-Hz line broadening was applied prior to Fourier transformation.

for that ion in vivo. Although the choice of the organic phase is somewhat arbitrary it is logical to choose one with a dielectric constant close to that expected in the membrane interior, provided by the fatty acid environment. Oleic acid has a dielectric constant of 2.5 (22), and thus chloroform, which has a dielectric constant of 4.8 (22) is a reasonable choice.

We therefore assayed the phospholipid facilitated uptake of Ca^{2+} from an aqueous phase to chloroform following the procedure indicated in Materials and methods and the results obtained for various phospholipid species are summarized in Table 1. These results are in agreement with those of Tyson *et al.* (10) and show clearly that cardiolipin and PA effectively sequester Ca^{2+} into the organic phase, whereas cardiolipin in the presence of ruthenium red as well as PS, PC, and PE are relatively ineffective.

The ^{31}P NMR spectra obtained for the cardiolipin system in the presence and absence of Ca^{2+} are summarized in Fig. 1. In agreement with previous results (2) the addition of Ca^{2+} to cardiolipin dispersions triggers a bilayer to hexagonal (H_{II}) transition. It is of interest to note, however, that the presence of equimolar ruthenium red (with respect to phospholipid) inhibits the ability of equimolar Ca^{2+} to trigger H_{II} phase formation, and that only a small increase in the H_{II} component occurs even at Ca^{2+}/cardiolipin ratios of 2.0. This behaviour may be contrasted to the effects of Ca^{2+} on phosphatidylserine liposomes, which results in precipitation of the lipid and ^{31}P NMR spectra characteristic of anhydrous phospholipids in the "rigid-lattice" (no motion) situation, as indicated elsewhere (7). X-ray studies (23) indicate that the Ca^{2+} PS salt has a lamellar structure. Ca^{2+} addition to PC and PE did not affect the ^{31}P NMR spectrum.

Given that unsaturated PA has been demonstrated employing X-ray techniques to adopt the H_{II} configuration in the presence of Mg^{2+} (24), the results presented here show a remarkable correlation between the ability of divalent cations to induce the inverted hexagonal (H_{II}) phase for aqueous lipid dispersions and the ability of such lipids to facilitate uptake of Ca^{2+} into an organic phase. The structure of the lipid–Ca^{2+} complexes in the organic phase is not known; however, NMR studies indicate that lipids in water–organic solvent systems are, in general, organized in inverted micelles in the organic phase (25). This is supported by the observation that more Ca^{2+} is sequestered into the chloroform than is required to obtain an electroneutral complex (see Table 1). In short, these results are clearly consistent with the possibility that divalent cations such as Ca^{2+} can form complexes with certain membrane lipids which could be soluble in the nonpolar hydrocarbon core of a biological membrane. This would certainly be expected to facilitate the transbilayer transport of such agents.

This possibility is further supported by freeze-fracture studies of model systems containing cardiolipin. As indicated above, Ca^{2+} induces lipidic particle formation in such systems and the appearance of lipidic particles (inverted micelles) is accompanied by an increased permeability to divalent cations (21).

These data are of particular interest with regard to the inner mitochondrial membrane, which actively

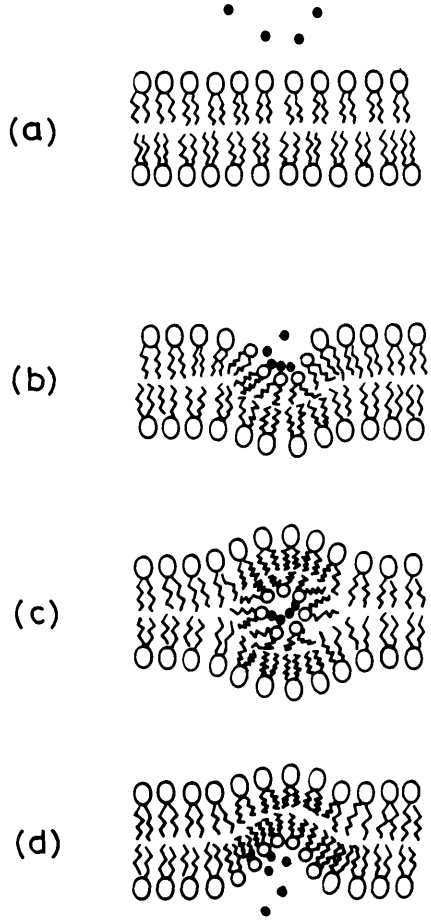

FIG. 2. A model of facilitated transport of Ca^{2+} (or other divalent cations) via formation of an intermediate intrabilayer inverted micellar cation–phospholipid complex (*c*). The headgroups of the charged phospholipid (e.g. cardiolipin) interacting with the cation are depicted as being smaller in order to indicate a reduction in the area per phospholipid molecule in the headgroup region arising from reduced interheadgroup electrostatic repulsion in the presence of the cation (see Ref. 2).

transports (accumulates) Ca^{2+} (26), a process which is specifically inhibited by ruthenium red (12). Obviously this would be consistent with a role of cardiolipin as a Ca^{2+} carrier during at least part of the uptake process. A potential carrier mechanism is indicated in Fig. 2, which proceeds via an intermediary intrabilayer inverted micellar Ca^{2+}–phospholipid complex. This model suggests that in association with Ca^{2+}, these phospholipids would also be transported across the membrane. We have recently proposed a similar mechanism to explain the translocation of cytochrome *c* across cardiolipin containing bilayers (27).

(B) Fusion and membrane transport

In a recent review, Morré *et al.* (28) have summarized the persuasive evidence supporting the "membrane flow" concept as related to membrane biogenesis, turnover and extracellular release of various products. Membrane fusion plays a critical role in this model, either in the budding off of transport vesicles (such as coated vesicles (29)) from one compartment, which subsequently release their contents on fusion with the membrane of a second compartment, or in the transport of membrane constituents from the endoplasmic reticulum to other membranes via vesicular structures. We have presented elsewhere (30, 31) evidence to suggest that nonbilayer lipid structures such as the hexagonal H_{II} or inverted micelle configurations play important intermediary roles during fusion events. In this section we investigate two related aspects of lipid polymorphism which give insight into first the mechanism of bilayer to hexagonal (H_{II}) transitions and secondly into mechanisms of extracellular release via exocytosis.

On the mechanism of bilayer–H_{II} transitions

As shown in previous work (2, 3) unsaturated PE's undergo an abrupt transition from a bilayer structure to the long "inverted" cylinders of the hexagonal (H_{II}) phase as the temperature is increased through a characteristic value (T_{BH}) which is sensitive to the degree of unsaturation. To gain further information on the mechanism involved we employed (egg) PE oriented between glass plates (for details of sample preparation see Materials and methods). As has been well discussed elsewhere (2, 3) the characteristic asymmetric "bilayer" or "hexagonal (H_{II})" ^{31}P NMR spectra of nonoriented systems arises from the chemical shift anisotropy experienced by the phospholipid phosphorus. This results in a chemical shift of the ^{31}P NMR spectrum from a particular phospholipid which is sensitive to the orientation (with respect to the magnetic field) of the bilayer or the H_{II} phase cylinder in which it resides. In particular, phospholipids in a bilayer organization exhibit orientation dependent chemical shifts according to (19, 32)

$$[1] \quad \Delta\sigma(\theta') = \sigma_0 - \tfrac{2}{3}\,\Delta\sigma_{CSA}{}^{EFF}(3\cos^2\theta - 1)/2$$

where σ_0 is the chemical shift (in ppm) in the presence of rapid isotropic motional averaging (e.g., for small vesicle systems), $\Delta\sigma_{CSA}{}^{EFF}$ is the separation between the low-field shoulder and high-field peak of the unoriented "bilayer" ^{31}P NMR line shapes (usually in the region of −40 ppm), and θ is the angle between the magnetic field and the perpendicular to the plane of the bilayer. Alternatively, it is straightforward to show (19, 32) that for phospholipids in the hexagonal configuration, the chemical shift of phospholipids in a particular cylinder is given by

$$[2] \quad \Delta\sigma(\theta') = \sigma_0 + \tfrac{1}{3}\,\Delta\sigma_{CSA}{}^{EFF}(3\cos^2\theta' - 1)/2$$

where all the symbols are as defined for Eq. 1, with the exception that θ' is now the angle between the magnetic field and the axis of the cylinder. These equations allow a simple prediction. Bilayer phospholipids ori-

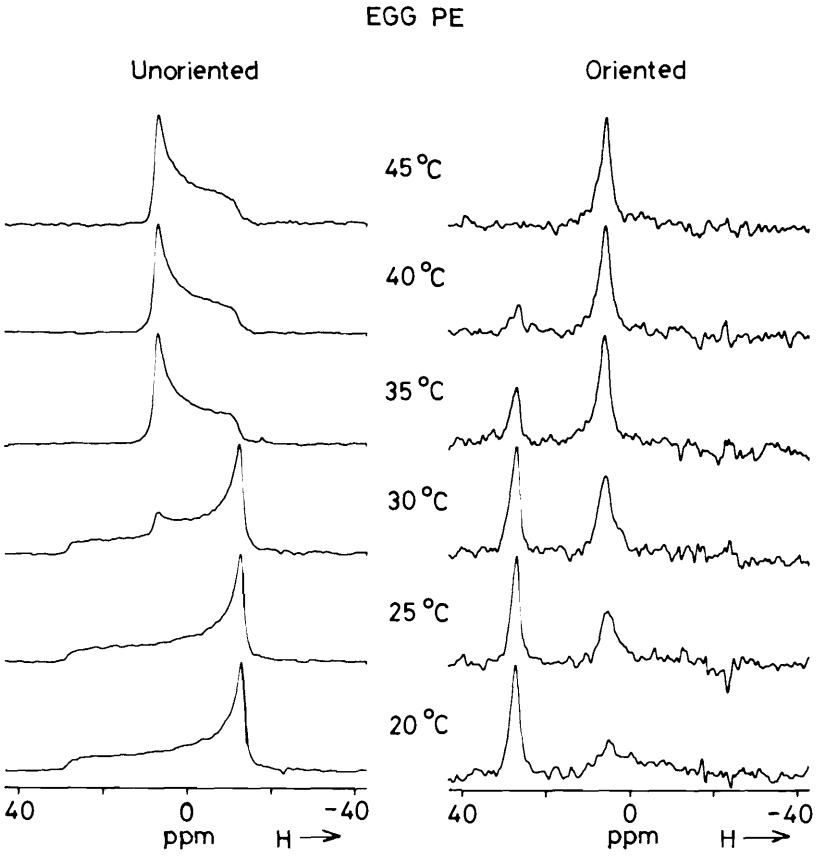

FIG. 3. The 81.0-MHz ^{31}P NMR spectra at various temperatures arising from (unoriented) egg PE aqueous dispersions and hydrated egg PE oriented between glass plates. The unoriented systems were treated in the same manner as indicated in the legend of Fig. 1. The oriented systems were prepared as indicated in Methods, and spectra obtained from up to 5000 transients employing the same conditions as for Fig. 1. The ^{2}H "lock" was not employed.

ented so that $\theta = 0$ (i.e., with the magnetic field perpendicular to the plane of the bilayer) will give rise to a ^{31}P NMR peak at $-\frac{1}{2}\Delta\sigma_{CSA}{}^{EFF}$ (i.e., at 27 ppm for $\Delta\sigma_{CSA}{}^{EFF} = -40$ ppm). If these lipids then undergo a bilayer to H_{II} transition, however, and the long cylinders run *parallel* to the bilayers from which they are formed, a new resonance will be observed with a chemical shift of $-\frac{1}{2}\Delta\sigma_{CSA}{}^{EFF}$, or 7 ppm for $\Delta\sigma_{CSA}{}^{EFF} = -40$ ppm.

This prediction was tested employing oriented (egg) PE systems. As noted in Fig. 3*a* and elsewhere (3) for unoriented egg PE dispersions this PE undergoes a bilayer to H_{II} transition as the temperature is increased through 30°C. In the case of the oriented PE, this transition is observed as the disappearance of a line at ~27 ppm and the appearance of a resonance at the 7-ppm chemical shift position. This shows that the H_{II} cylinders run parallel to the plane of the bilayers from which they are formed, and is consistent with a characterization of the bilayer-hexagonal (H_{II}) polymorphic phase transition occurring as an *inter*bilayer event, as depicted in Fig. 4. Such behaviour is also consistent with a role of inverted (H_{II} phase) lipid cylinders as intermediaries in fusion events (30), which is also an interbilayer, as opposed to an intrabilayer, process.

Bilayer–H_{II} transitions and mechanisms of exocytotic release

The importance of intermembrane rather than intramembrane events on proceeding from the bilayer to the hexagonal H_{II} configuration is also expressed by the lipid requirements for release of chromaffin granule contents. In a recent work (see footnote 1) we have shown that PE–PS (3:1) sonicated vesicles, which adopt the hexagonal (H_{II}) phase in the presence of Ca^{2+}, can act as adjuncts for Ca^{2+} stimulated release of chromaffin granule contents. During this process it appears that fusion of the PE–PS vesicles with the granule membrane occurs on addition of Ca^{2+}, leading to a loss of membrane integrity and release of granule contents.

We have employed these and related (9) observations to propose a mechanism of the Ca^{2+} stimulated exocytotic release of chromaffin granule contents in

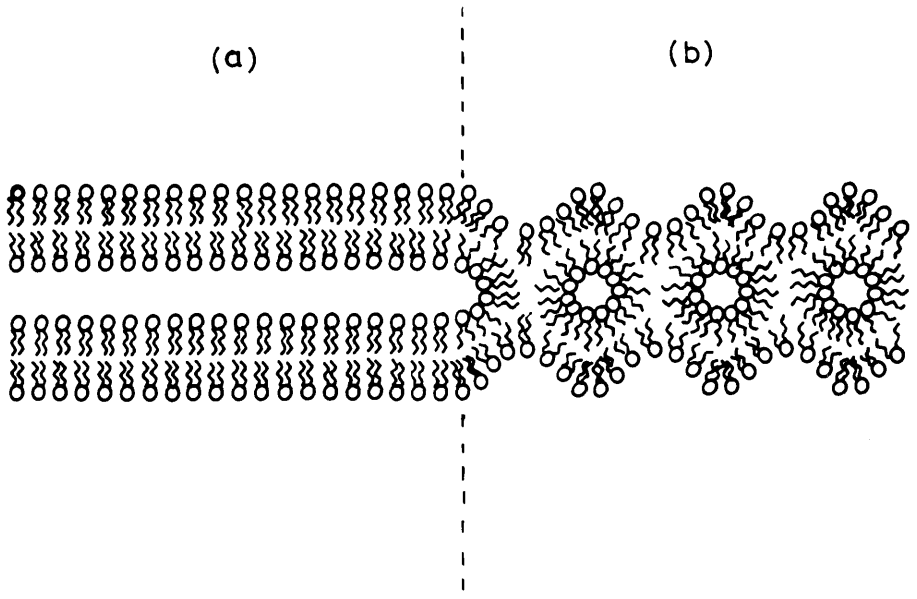

FIG. 4. Proposed mechanism of the bilayer–hexagonal (H_{II}) polymorphic phase transition for two closely apposed bilayers (*a*). In this model the long cylinders characteristic of the H_{II} phase form from the two innermost monolayers (*b*) which results in an orientation of the axis of the cylinder which is parallel to the plane of the bilayers from which it is formed.

vivo (see footnote 1). In this model we suggest that Ca^{2+} can lead to destabilization of the inner monolayer of the plasma membrane, leading to enhanced fusion between the plasma membrane and closely apposed chromaffin granules.

This model is clearly speculative, not least because the inner monolayer composition (and structural preferences) of the adrenal cell plasma membrane are unknown. However, in a previous work we have shown that the inner monolayer lipids of the erythrocyte membrane (the only membrane for which the inner and outer monolayer lipid compositions are well characterized (18)) partially adopts the hexagonal (H_{II}) phase in the presence of Ca^{2+} (9). It would obviously strengthen our thesis if this mixture of phospholipids could also induce chromaffin granule release in the same manner as the PE–PS system. That this is the case is illustrated in Fig. 5. As shown in Fig. 5*b*, the addition of Ca^{2+} to the small sonicated "inner monolayer" vesicles (which give a narrow symmetric resonance, Fig. 5*a*) results in formation of an appreciable H_{II} phase component (which is also accompanied by precipitation of the vesicles). The ability of these vesicles to act as adjuncts for Ca^{2+} stimulated release of the granule contents is shown in Fig. 5*b* where total release is observed for a ratio of exogenous ("inner monolayer") phospholipids to endogenous (chromaffin granule) phospholipids of 8.0. It may therefore be suggested that if the inner monolayer of the adrenal cell plasma membrane has a similar lipid composition to that of the erythrocyte the presence of Ca^{2+} would be expected to preferentially stimulate granule – plasma membrane fusion, vital to the exocytotic event.

(C) Transport within alternative membrane structures

The results presented here and elsewhere (2) clearly indicate that the ability of lipids to adopt nonbilayer configurations allows new dynamic roles of lipids in membrane function that were not previously suspected. This ability also suggests new alternatives for membrane structure and organization, which is an aspect we have not addressed previously. To present these alternatives in a coherent manner we first discuss results obtained in certain model systems which have played an important role in our thinking.

The model systems in question are those containing mixtures of bilayer and hexagonal (H_{II}) phase lipids. These systems, which are in certain ways more valid models for biological membranes than those containing purely bilayer lipids, exhibit unique features. For the purpose of this discussion we will mainly confine our remarks to the (unsaturated) PE–PC–cholesterol model system. When such lipid mixtures are hydrated and maintained at or near temperatures such that the PE component prefers the bilayer organization, pure bilayer systems are formed (Refs. 13, 19, 33, and 34). However, on heating to temperatures where the PE prefers the H_{II} phase, a narrow ^{31}P NMR peak appears, which can become the dominant spectral feature (see, for example, Fig. 6 of Ref. 33). Remarkably, however, this behaviour is often not reversible, for on cooling to the starting temperature the narrow

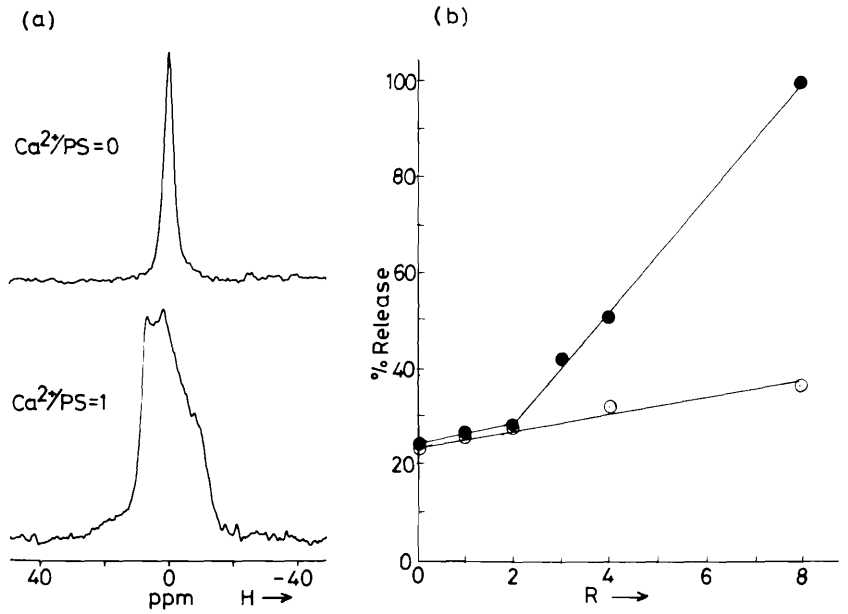

FIG. 5. (*a*) The 81.0-MHz ^{31}P NMR spectra at 37°C arising from sonicated vesicles prepared from 40 μmol of "inner monolayer" erythrocyte phospholipids (25 mol% PS, 50 mol% PE, 12 mol% PC, and 12 mol% sphingomyelin; equimolar cholesterol) in the absence and presence of 10 μmol Ca^{2+}. Data collection and manipulation was the same as indicated in the legend of Fig. 1. The Ca^{2+} was added as 100 μL of a 0.1 *M* $CaCl_2$ stock solution, causing immediate precipitation of the vesicle suspension. (*b*) Release of chromaffin granule contents as assayed by spectrophotometric techniques (see footnote 1) after 15 min incubation of chromaffin granules (1.5 mg protein, 50 μL of large granule fraction) in the presence of "inner monolayer" vesicles (total volume 0.4 mL) at 37°C. After the incubation this mixture was diluted to 4 mL and centrifuged (12 000 × *g*, 10 min, 0°C), and the supernatant assayed for released ATP by reading the absorbance at 265 nm. Controls employing Ca^{2+} alone (10 m*M*) did not induce release above the background level. ●, after 10 min of incubation $CaCl_2$ was introduced to arrive at a 5 m*M* Ca^{2+} concentration, and incubation was then continued for 5 min; ○, no $CaCl_2$ added. The ratio *R* refers to the molar ratio of exogeneous "inner monolayer" vesicular phospholipid to endogeneous chromaffin granule phospholipid. For other details see text and footnote 1.

resonance can remain the major, indeed often the only, ^{31}P NMR response. In conjunction with this behaviour, freeze-fracture studies (34) reveal the presence of "lipidic particles" (35) after the heating–cooling cycle in DOPC–DOPE–cholesterol (Refs. 13 and 34), systems which are *not* present in the original preparation. Lipid in these lipidic particles, interpreted as intrabilayer inverted micelles, would be expected to give rise to narrow high resolution ^{31}P NMR spectra. However, as noted elsewhere (13) the amount of lipid in these intramembranous structures is always much less than the amount of lipid giving rise to "isotropic" ^{31}P NMR spectra, leading one to suspect that other sources of motional averaging exist.

The ^{31}P NMR results are consistent with phospholipid in lamellar structures of 1000 Å (1 Å = 0.1 nm) radius or less, where lateral diffusion processes alone can result in isotropic averaging (36). However, these structures cannot be independent closed vesicles, as the corresponding lipid dispersions occur as large, visible globules of lipid, which can be isolated by low speed centrifugation, suggesting that the small structures are integral subunits of a macroscopic entity.

The correlation of two events noted in freeze-fracture studies of these and related systems leads us to propose a logical alternative. Briefly, it is noted that the lipidic particles observed in PE–PC–cholesterol systems are often arranged in organized rows (Refs. 13 and 34), and that lipidic particles occur at the fusion interface in fusing vesicular systems composed of these (20) and other (31) phospholipids. This suggests that the lipidic particles observed in the freeze-fracture of the multilamellar model systems actually correspond to regions of fusion between adjacent bilayers. In turn, this leads to the "honeycomb" model depicted in Fig. 6, where it is proposed that heating the PE–PC–cholesterol multilamellar liposomes to a temperature above the T_{BH} of the PE component promotes interbilayer fusion (cf. section B) with associated lipidic particle formation which results in the additional formation of interstitial regions where the lipids remain in a bilayer organization. If these interstitial regions are small enough (e.g., radius of 1000 Å or less) the component phospholipids will enjoy isotropic averaging and exhibit narrow ^{31}P NMR spectra as observed experimentally. This model is also fully consistent with the permeability behaviour and trapped volume observed for PE–PC–cholesterol systems (see Ref. 34).

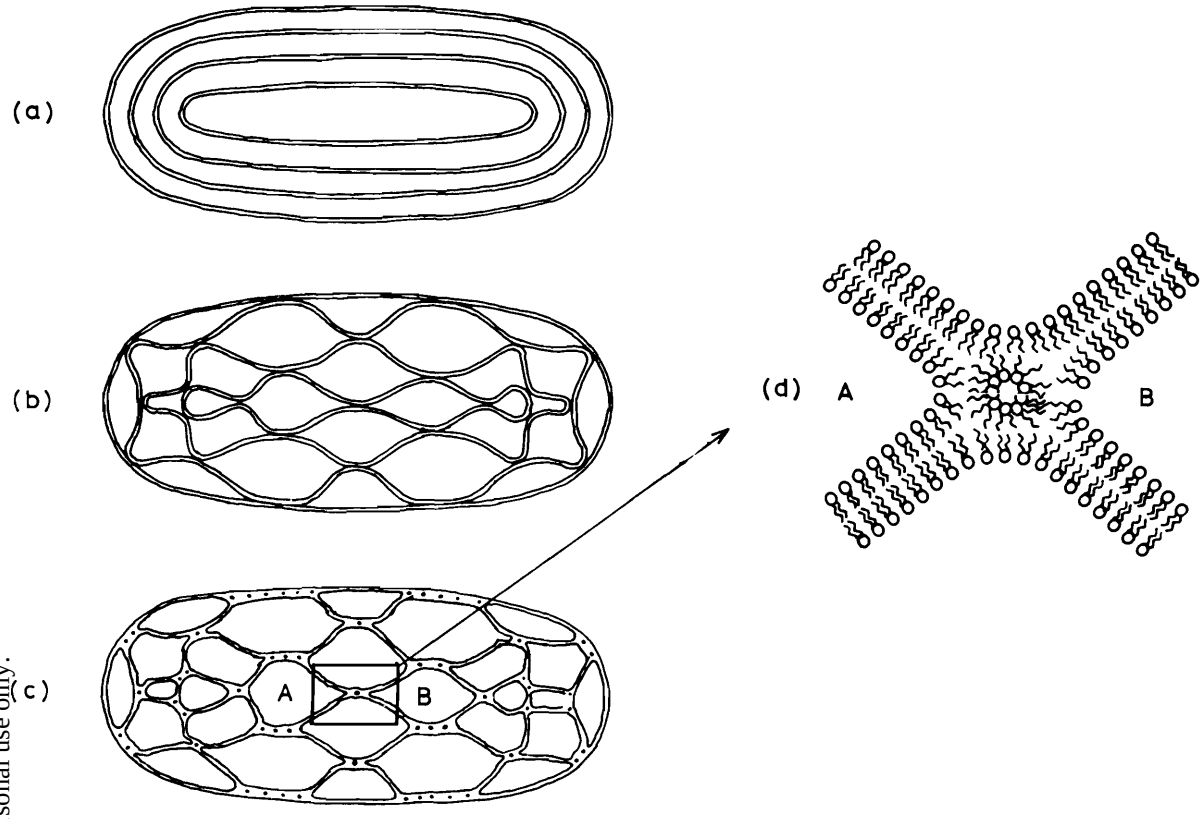

FIG. 6. Mechanism of formation of a "honeycomb" structure from multilamellar PE–PC–cholesterol systems and other systems containing mixtures of "bilayer" and hexagonal (H_{II}) phase lipids. In 6*a* the usual depiction of multilamellar liposomes is given; however it is clear that in general regions will exist where the layers are in close apposition. A stylized version of this is given in 6*b*. In 6*c* it is postulated that these regions will undergo (partial) fusion with associated formation of lipidic particles at the interface. Compartmentalization in a continuous membrane structure then results, as indicated for compartments A and B in the expanded diagram of part *d*.

We believe that the ability of lipids to adopt structures such as those indicated in Fig. 6 has potentially important implications for membrane morphology and associated transport capabilities in vivo. In particular, such structures suggest the possibility that separate, isolated compartments may be connected by a continuous membrane structure (Fig. 6*d*). This would allow unique opportunities for interorganelle communication and transport, and provides an understanding of how membrane-bound systems could maintain compartmentalization while being part of a continuous membrane structure. The importance of understanding such organization is far from academic, as fairly conclusive evidence has been reported (28) which is consistent with a continuity of the endoplasmic reticulum with the nuclear membrane, the outer mitochondrial membrane and the Golgi apparatus for example. With regard to transport, within the scheme presented in Figs. 6*c* and 6*d*, proteins and lipids associated with the outer monolayer of either compartment A or B could be transported to the membrane of the other compartment by lateral diffusion processes without entering the aqueous phase. It may be noted that interorganelle junctions consistent with those possibilities have been observed by Morré and co-workers (Refs. 28 and 37).

(D) Concluding remarks: the metamorphic mosaic model

The data presented here and in previous work clearly point to important roles of lipids in membrane transport, and other cell functions, all of which stem from the nonbilayer structures available to lipids. By way of summary, we present in Fig. 7 a membrane model incorporating most of these possibilities which we term the "metamorphic mosaic" model of biological membranes. While some aspects of this model are obviously highly speculative they are not inconsistent with the physical properties of the lipid component of biomembranes. In closing, the polymorphic capabilities of

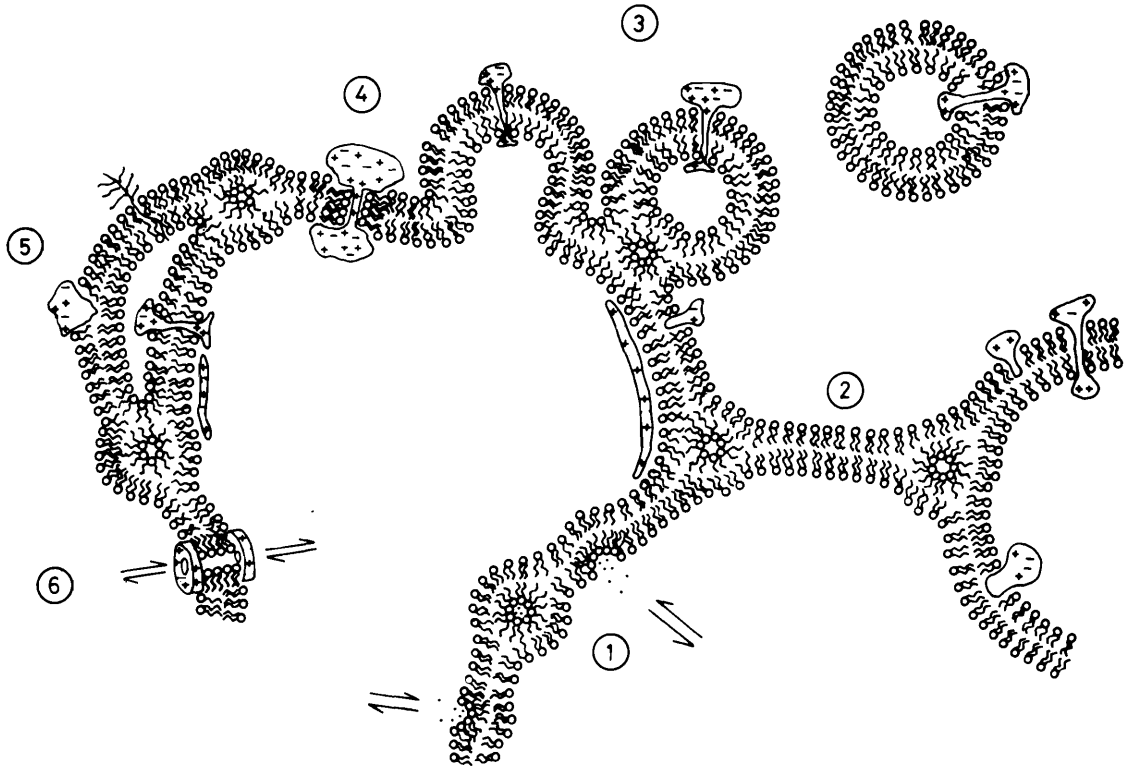

FIG. 7. A metamorphic mosaic model of biological membranes illustrating various structures and processes suggested by the ability of lipids to assume nonbilayer configurations. In part 1 transbilayer transport of polar molecules (e.g., divalent cations) is facilitated by intermediary formation of inverted micelles (see Fig. 2), whereas part 2 indicates membrane continuity between membrane bound compartments (cf. Fig. 6). In section 3 a process of budding off of a membrane bound vesicle is illustrated, as discussed elsewhere (2). The protein in region 4 is shown to assume a transmembrane configuration *without* the requirement for an apolar sequence of amino acids. The protein penetrates the membrane through a (short) cylinder of phospholipid. In the region 5 compartmentalization is depicted within a continuous membrane system, whereas part 6 indicates possibilities of transmembrane transport where hexagonal (H_{II}) phase lipids form an aqueous pore through the membrane. This lipid configuration is stabilized in an orientation perpendicular to the plane of the surrounding bilayer by doughnut shaped proteins with hydrophilic and hydrophobic sides, which could also serve as selectivity filters.

lipids, as expressed in Fig. 7, certainly provide a new, and to our minds most exciting, framework for the understanding of membrane mediated events.

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