Biodiversity in biomembranes

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Abstract

All living organisms are made up of cells which are distinguished from the external world by physical boundary called "the cell membranes". A number of important proteins control cell function by interacting with extracellular stimuli. However, the functions and structures of cell membrane molecules such as lipids and carbohydrates still remain unclear. In a biodiversity point of view, some biomembrane properties were investigated in two different aspects.

(1) Branched polyprenyl phosphates (Isoprenyl polyprenyl phosphates)

Several polyprenylated structures have been widely found in sediments. Such structures should derive from alcohols or phospholipids. It is suggested that isoprenyl polyprenyl phosphates could have existed in primitive cell membranes, though still unfound in present biomembranes. Various branched polyprenyl phosphates were synthesized and investigated physical properties of their membranes. Microscopy studies showed that these branched polyprenyl phosphates made vesicles in a pH-dependent manner. In order to evaluate the water permeability of their membranes, osmotic swelling of a suspension of the unilamellar vesicles was measured by stopped flow/light scattering method. It was found that the water permeability of the vesicles changed according to structure and chain length. These results suggested that these branched polyprenyl phosphates probably existed in primitive cell membrane.

(2) Phosphorylated cholesterol

Lipidic part of biomembranes in vertebrate consists of a phospholipid (membrane constituent) and cholesterol (membrane reinforcer). Why was diacylglycerol phosphorylated but not cholesterol in the process of evolution? And does the membrane composed of the mixture of phosphorylated cholesterol and diacylglycerol exist? To find some clues to address these questions, cholesteryl phosphorylcholine (CPC) was synthesized and the physical properties of vesicles were investigated.

At a certain pH and a suitable relative ratio of the mixture of CPC and diacylglycerol, the formation of vesicles was observed. The conditions of pH for making vesicles of CPC/diacylglycerol mixture were restricted rather than that of mixture of a phospholipid and cholesterol.

The water permeability of vesicles made from 1:1 molar mixture of CPC and diacylglycerol is higher than 1:1 molar mixture of phospholipid and cholesterol. However, indeed CPC/diacylglycerol mixture formed a stable vesicle. These results suggested that such cholesteryl phospholipids might be present in the membrane of some organisms hitherto not yet studied. Nowadays biomembranes acquired some properties that make living organisms adapt to various environments hence contributing to extended biodiversity.

(3) "Primitive" membrane toward proto-cell

A possible evolution process from vesicles, spontaneously formed by the self-organization of "primitive" membrane constituents, towards an assembly bearing an outside "wall" has been investigated. First it has been shown that phytyl-pullulan could coat vesicles made of double-chain lipids (2,3-diphytanyl-*sn*-glycero-1-phosphocholine (DphPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)). Cholesteryl-pullulan coated double-chain lipids but not single-chain lipids. There must be a closely matching relation between the size and shape of the membrane constituents and the hydrophobic molecules to be inserted, which would provide a selection mechansim of lipid membrane constituents in the course of biomembrane evolution.

Next, using the above hydrophobized polysaccharides, the molecular recognition between lectins and polysaccharides on the giant vesicle surface was studied. This provides an example in which the self-organization of amphiphiles in water into closed vesicles leads automatically to self-complexification, on the way towards "proto-cells".

(4) Function of cell membranes

Annexins are a family of structurally related proteins that bind to phospholipids and carbohydrates. In this study the some immune property and effects of annexins on Gram-positive bacteria in the immune system pathway were investigated. First, the interaction of annexins with lipoteichoic acids, located surface of Gram-positive bacteria were observed. Then, the effects of annexins on the attachment of macrophages and Gram-positive bacteria were examined. It was indicated that annexins suppress the attachment of *Staphylococcus aureus* to human macrophages. These findings suggest that annexins might act as anti-inflammatory proteins at a cellular level via blocking the pathway of interaction between immune cells and their targets.

General introduction

1. 1 Structure and function of membranes

All living organisms are made up of cells which are separated from the external world by a physical boundary called the "cell membrane". In 1972, J. S. Singer and G. Nicolson proposed a "fluid mosaic model" for the organization of biological membranes (Fig. G-1) [1]. The essence of this model is that membranes are composed principally of lipid bilayers.
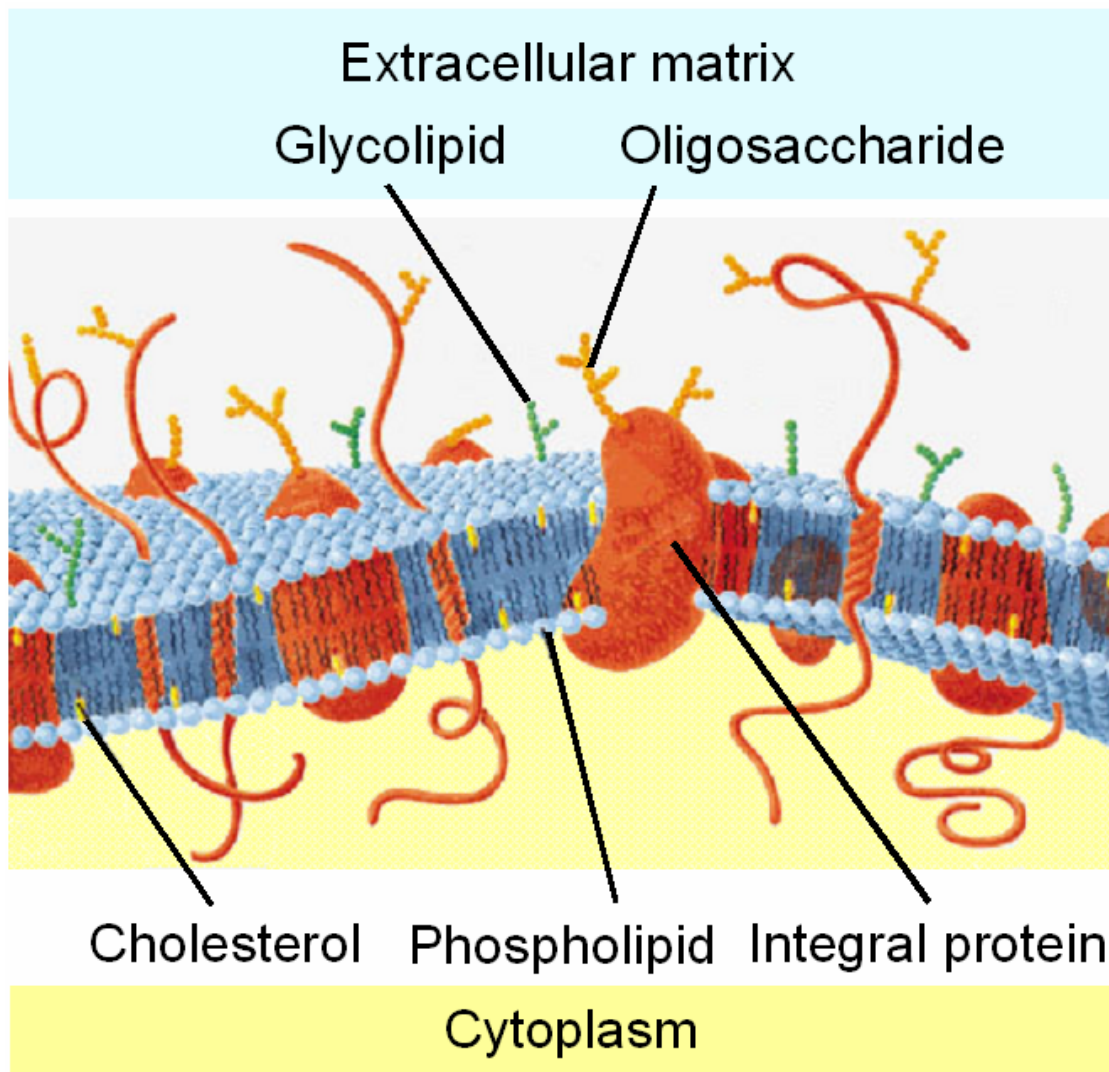

Fig. G-1   Fluid mosaic model of biological membrane
(Singer-Nicolson model) [2]

Numerous proteins are located in the membrane and various polysaccharides exist on the surface of their extra cellular membrane.

Although these lipid bilayers are in only two molecules' thickness, they are highly impermeable to water and to solutes such as ions, sugars and peptides. Proteins located in the membranes carry out several vital functions such as ion or hormonal transport. Extra cellular polysaccharides form the extracellular matrix (ECM), which affects cell adhesion, recognition, differentiation and proliferation.

1. 2 Self-aggregation of lipid

Lipids are fundamental components of membranes. Fig. G-2 shows a typical membrane lipid in vertebrates, with a straight hydrocarbon
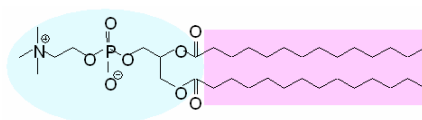
Fig. G-2   Phosphatidylcholine
(a phospholipid)

chain as a hydrophobic part (red) and a polar head-group as a hydrophilic part (blue).   Hydrocarbon chains and polar groups are highly variable, but all membrane lipids possess a critical common structural feature, i.e. they are *amphiphilic molecules*.   Thus, when lipid molecules are mixed in aqueous solution, they spontaneously aggregate into various types of organized structures such as micelles or vesicles, commonly called "lyotropic liquid-crystals".   Or lipid molecules are extracted from aqueous solution as an oil phase (phase separation).

The driving force for the aggregation of lipid molecules in water is "hydrophobic interactions" of the hydrophobic part.   Water molecules form extended networks of hydrogen bonds in the liquid state.   If the

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hydrophobic part of lipid is exposed to water, the hydrogen bonds between water molecules are disturbed at the surface of hydrophobic part. To minimize the loss of hydrogen bonds, water molecules are forced to be restructured around the hydrophobic part. Then hydrophobic parts of lipids contact each other to decrease the total area of the hydrophobic part-water interface. Therefore the self-aggregation of lipid depends on the concentration of lipid in water. Fig. G-3 shows the three main phase types of lipid-water system: the L_α (liquid-crystalline phase), the L_β (gel-like phase) and the L_c (crystal phase). When temperature is below the chain melting transition temperature, L_c phase become into L_β phase by addition of water. While, temperature is higher than the chain melting transition temperature, L_c phase become into L_α phase (biomembrane model) via L_β phase. That is, not only concentration of lipid in water but

L_c (crystal phase) L_β (gel-like phase) L_α (liquid-crystalline phase)
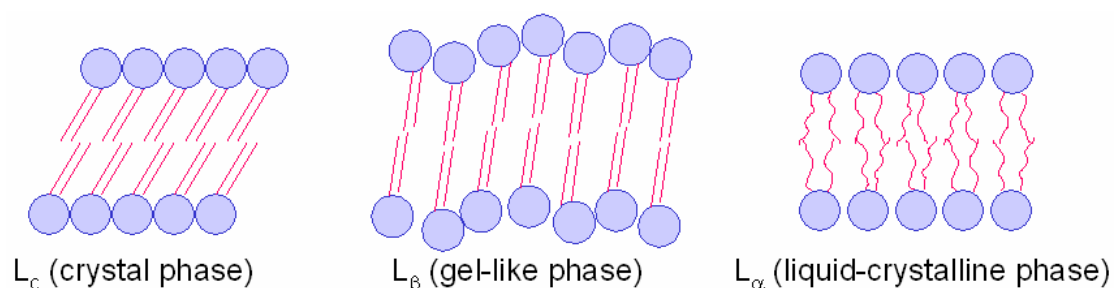
Fig. G-3   The three main types of phase of lipid-water system.

The L_c phase has the chains of each amphiphile "frozen". In L_β phases the head-groups are disordered but the chains show a increased degree of freedom, and they are partially ordered and are essentially confined to the all trans configuration. There is no lateral diffusion. In a liquid-crystalline phase L_α, which is the least ordered of these three phases, movement within the bilayer is not restrained, as the alkyl chains are melted and fluid-like. The hydrocarbon tails are thus able to move. Collisions with neighbouring molecules then occur, since the molecules are able to undergo rapid rotational and translational motions as well as thermally activated lateral diffusion in the bilayer. This phase is used as a biomembrane model.

also temperature is important factor for the formation of vesicles, L$_\alpha$ phase.

On the other hand, Israelachivili et al. figured out one theory about the self-aggregated structures obtained in an aqueous suspension of amphiphiles. They stated that the self-aggregated structures depend on the critical packing parameter P = *v/a$_0$lc* of the amphiphilic molecules (*a$_0$* = the optimal area per molecule at the lipid-water interface; *lc* = chain length; *v* = hydrocarbon volume) [3]. Table G-1 shows the relation between critical packing parameter, critical packing shapes of lipid and the structures of formed aggregates.

Some of self-aggregated structure features are provided in following clause.

Spherical micelle (P < 1/3): The polar head groups are located mostly at the surface and the core has a relatively hydrophobic surrounding. For the formation of micelles, the size of the head group must be larger compared with that of the hydrocarbon chain. Therefore the head group must be located at the surface with a large curvature. Micelles are formed with small number of molecules and sizes are limited about 20 nm in diameter.

Vesicle (P = 1/2-1): Some amphiphiles aggregated into a molecular bilayer. This bilayer encapsulates an aqueous pool inside and
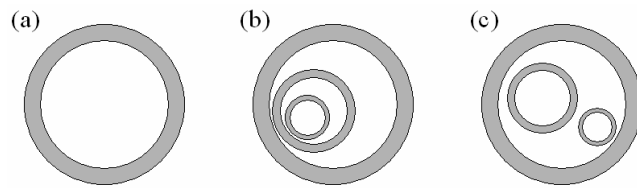
Fig. G-4   Variety of vesicles; uni- (a), multi- (b) and oligo-lamellar vesicles (c)

a physical boundary separates the inside from the outside.   This barrier is highly impermeable.   Vesicles are used as a simple model of biomembranes.   If they are composed of one bilayer sheet, vesicles are called "uni-lamellar vesicles" (Fig. G-4 (a)).   And if they contain several sheets of bilayers, they are called "multi-lamellar vesicles" or "oligo-lamellar vesicles" (Fig. G-4 (b, c)).   The diameter of vesicles ranges from 20 nm to several tens of μm (vesicles are larger than 1 μm are called "giant vesicles").
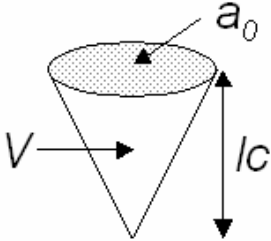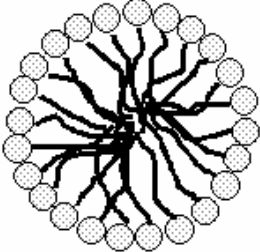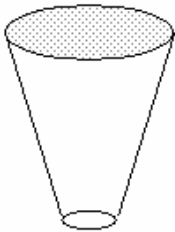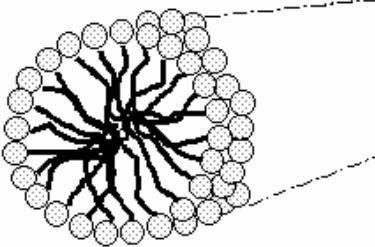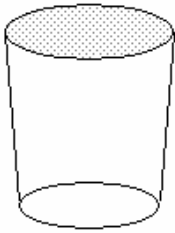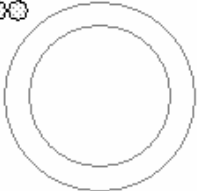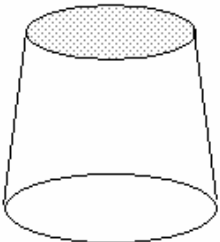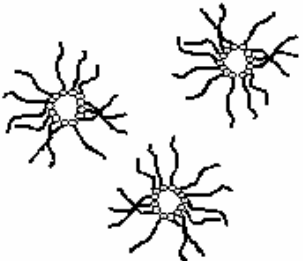
critical packing parameter $v/a_0 l_c$	critical packing shape	structure formed
< 1/3		Spherical micelle
1/3-1/2		cylindrical micelle
1/2-1		lamellar bilayer
>1		inverted micelle

Table G-1   Critical packing parameter, critical packing shapes of amphiphiles and the structures of formed aggregates [3].

1. 3 Membrane components

Living organisms are divided into three distinct kingdoms such as Archaea, Bacteria and Eukarya.   Eukarya, including plants and animals, are the most modern organisms.   The lipidic part of their cell membranes is composed of phospholipids and sterols (Fig. G-5).   Phospholipids are fundamental molecules, forming bilayers.   Sterols[*], a type of terpenoids, are known as membrane reinforcers, because they modulate membrane rigidity and fluidity.   In the case of mammals, cholesterol acts as membrane reinforcer [4, 5], and in the case of plants, sitosterol reinforces membranes [6].
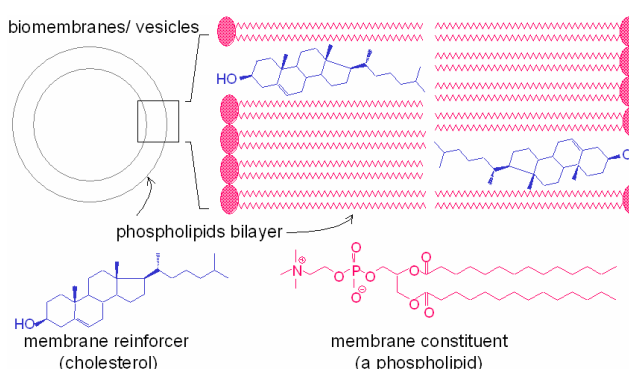
Fig. G-5   Model of eukaryote membrane structure

Some aspects of membranes of other kingdoms are as follows.

-Archaea membranes

Archaea membranes are formed by a bilayer of the di-phytanyl ethers, or by a monolayer of the di-bisphytanyl ethers, a kind of terpenoids (Fig. G-6). Their hydrophobic chains are highly branched and ether-linked ones, which are different from eukaryotic or bacterial ester-linked membrane molecules.

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[*]sterols: Sterols can be thought of as modified terpenes, where methyl groups have been moved or removed, or oxygen atoms added.   Terpenes are a class of hydrocarbons and which are derived from isoprene C_5H_8 (CH_2=C(CH_3)CH=CH_2) units and have the basic formula of multiples of isoprene, i.e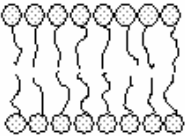., $(C_5H_8)_n$.   The isoprene units can be arranged in a linear way or forming rings.   One can consider the isoprene as one of nature's preferred building blocks.
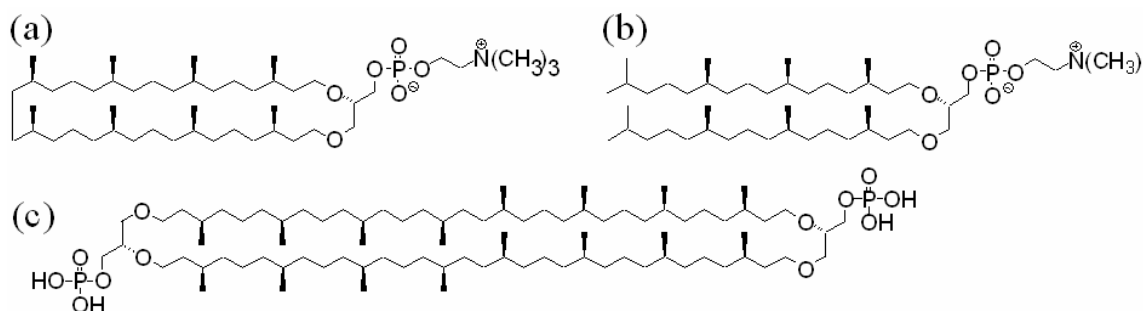
Fig. G-6 Archaea membrane constituents. Di-phytanyl di-ethers (a, b) and di-bisphytanyl tetra-ethers (c) exist in *Methanococcus jannaschii* (a), Halobacterium (b) and Sulfolobus, Thermoplasma, some Methanogenes(c)

-Bacteria membranes

Bacterial membranes are composed of di-acylglyceryl phosphates like eukaryote, while they do not possess sterols. However they have other kinds of terpenoids such as hopanoids or carotenoids (Fig. G-7) [7]. These molecules also act as membranes reinforcers.
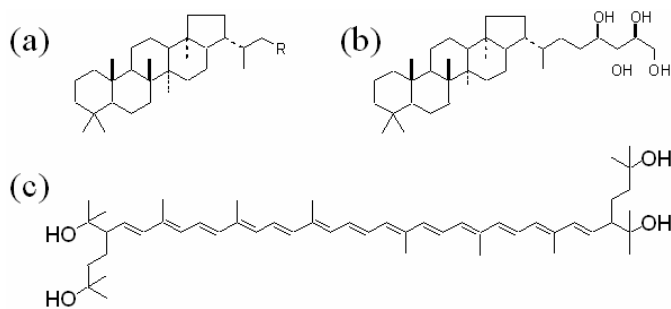
Fig. G-7 Bacteria membrane reinforcers, hopanoid (a), bacteriohopanetetrol (b) and α,ω-dipolar carotenoid (c).

It could be concluded that living organisms have evolved from the original cell to multicellular organisms of three kingdoms, and terpenoids play an important role in membranes of all organisms, in archaea: principal membrane constituents, in bacteria or eukarya: membrane reinforcers [8].

1. 4　Evolution of membrane components

　　Fig. G-8 shows the structure of terpenes found in sediments and their predicted precursors [8].　Although some are already found in extant organisms, others have not yet been identified in present-day organisms. The investigation of the origin of membrane is very important for understanding the origin of life.　Guy Ourisson and Yoichi Nakatani have introduced the "Hypothetical evolution of membrane components and membrane reinforcers" by considering the structure of molecular fossils and present membrane terpenoids, and their biosynthetic pathways (Fig. G-9).　They postulated that primitive membrane could have been the acyclic polyprenols, linked to an appropriate and simple polar head-group like a phosphate group.　Hypotheses of prebiotic synthesis of polyprenol phosphates on a mineral surface have also been introduced (Fig. G-10). The self-aggregation of polyprenol phosphates in water leads to the formation of vesicles, a closed compartment: the inside of the vesicle is water and the outside is also water, and the hydrophobic membrane separates the inside from the outside.　The formation of such organized systems leads automatically to the emergence of novel physicochemical properties, and make it possible to progressively complexify the system. This may well have been an essential aspect of the formation of the first living cells.　A number of researches have been preformed in recent years about the formation of protocells from vesicles [10, 11].
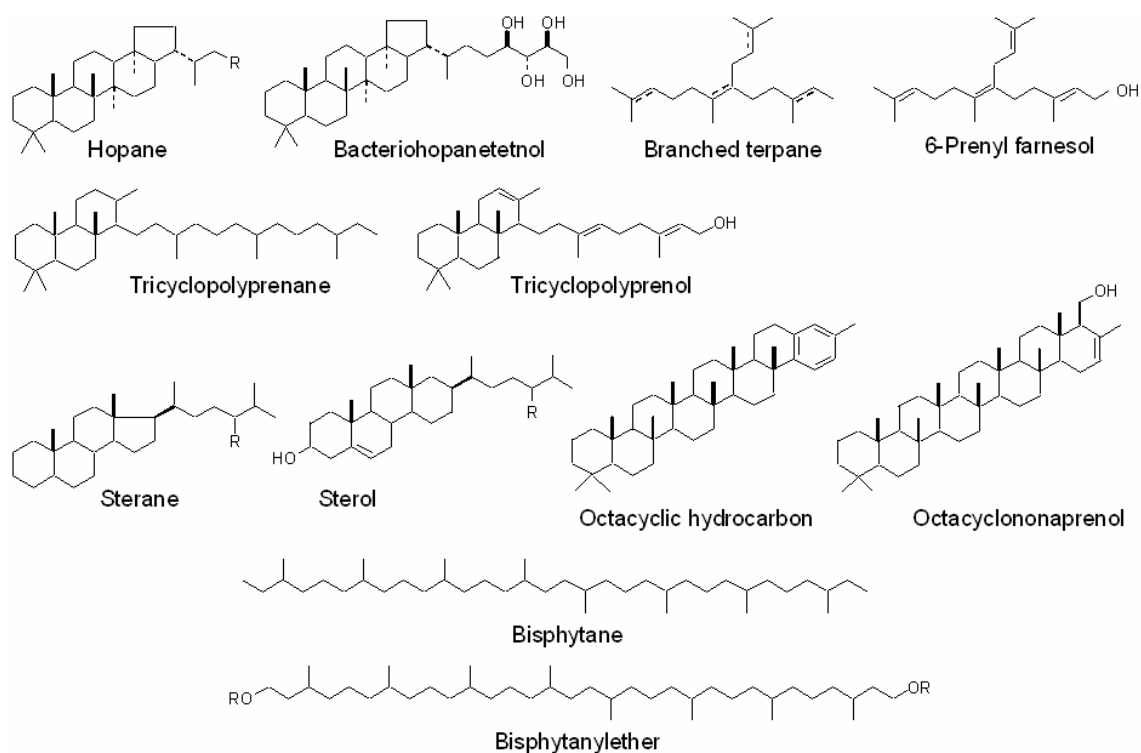
Fig. G-8   Structure of terpenes found in sediment of different geologic
ages and their predicted precursors [8].

Hopanes and bacteriohopanetetnol are found respectively in sediments and in many bacteria and cyanobacteria. Branched terpanes, which might be derived from 6-prenyl farnesol, were isolated from many sediments. Tricyclopolyprenanes are found in many sediments, which might be derived from tricyclopolyprenol, are found in many sediments. Octacyclic hydrocarbons, which might be derived from octacyclononaprenol, are found in some sediments. Steranes are frequently found in sediments, and they probably arose in the sediment by maturation from sterols. Bisphytane was isolated from several sediments. Their significance was a puzzle until the first bisphytanyl-phopholipids were found in thermophilic archaea. These molecular fossils are valuable for what they teach us of the range of membrane constituents of older organisms.
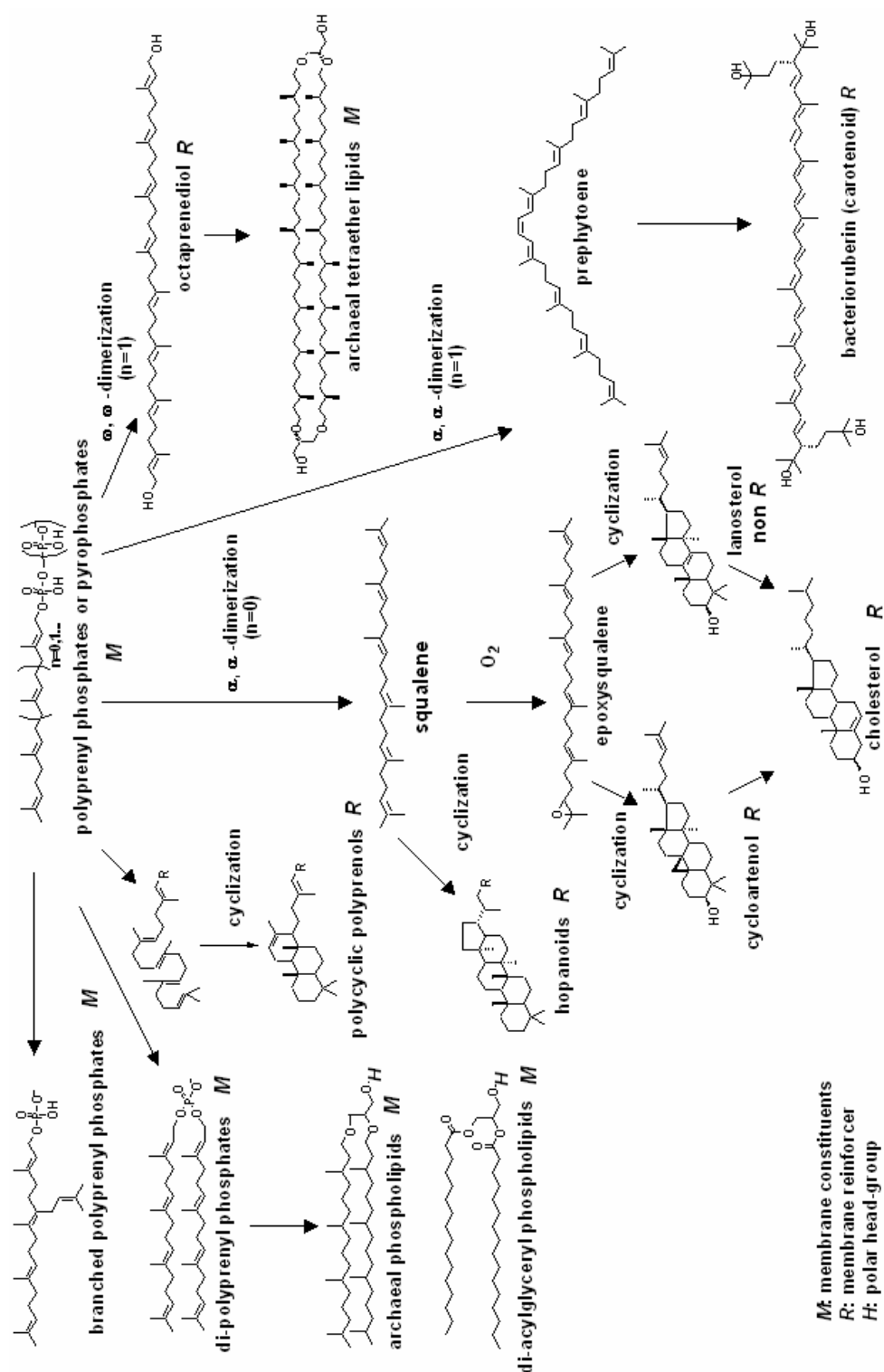
Fig. G-9   Hypothetical evolution of membrane components and membrane reinforcers [8].

The scheme presents a proposal for how several modern-day membrane components arose from polyprenyl phosphates. Di-acylglyceryl phospholipids are shown for comparison.
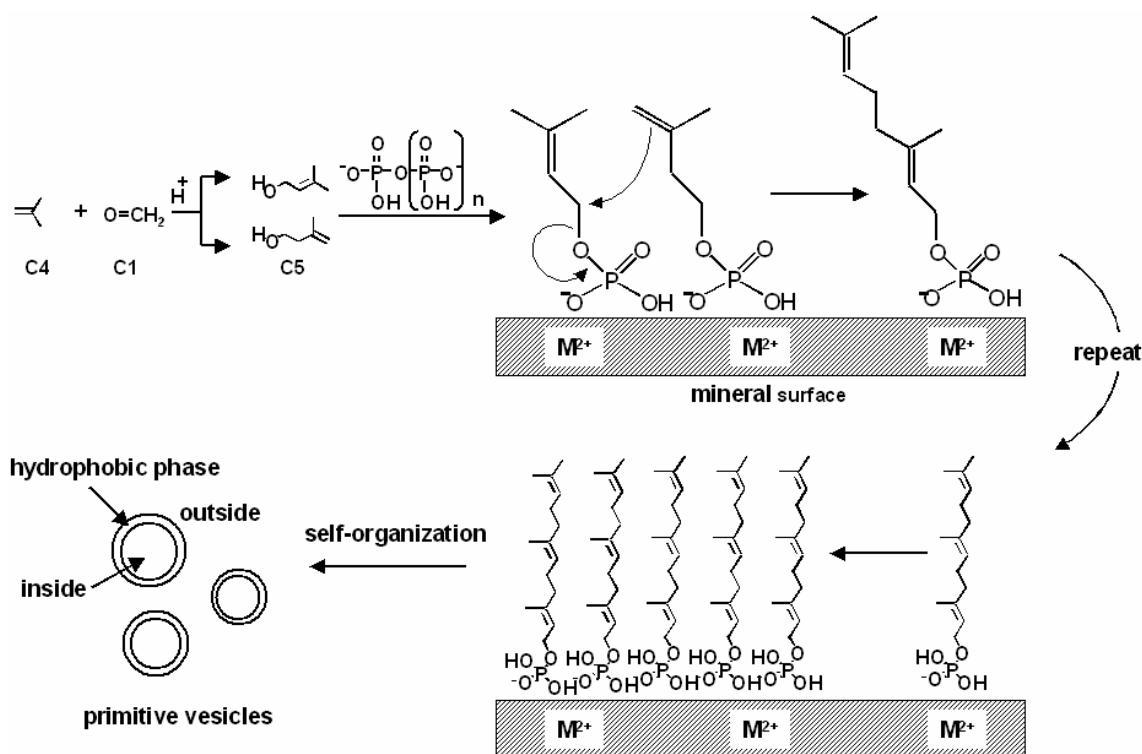
Fig. G-10   Possible prebiotic synthesis of polyprenol phosphate on a mineral surface, forming primitive vesicles. [8, 12]

Formaldehyde (C_1) occured in the prebiotic environment. Isobutene (C_4) was also present in the prebiotic environment. Formaldehyde and isobutene formed C_5 units. C_5-alcohols were phosphorylated by polyphosphoric acid, which was produced by volcanoes. Prenyl phosphate units were attached to the mineral surface by electrostatic forces. Two C_5 units condensed to form a C_{10} unit (geranyl phosphate), and this sequential head-to-tail elongation of C_5 units formed progressively longer polyprenyl phosphates. Prenyl groups condensed until critical concentration has been reached and they became able to form vesicles.

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1. 5 Acquired biological function of membranes: Cell recognition

As written in section 1.1, all living organisms are cellular and each cell is surrounded by a membrane, which separates the inside from the external world. In the evolution process, cell membranes have acquired several biological functions to communicate with the external world. Cells express diverse molecules on their surfaces such as polysaccharide epitopes and protein receptors. These are specific to each cell. For example, when cells are diseased, diseased cells frequently alter polysaccharide expression patterns in comparison with healthy cells. Infectious pathogen-microorganisms such as bacteria have certain common molecular patterns (PAMPs[*]: pathogen-associated molecular patterns) on the surface of cell wall, these do not exist in animals. Due to surface molecules differences between bacteria and animal, animal cell can recognize bacteria as foreign substances and remove them. Therefore recognition processes of foreign substances are the basis for host defense pathway. In order to understand host defense mechanism, screening of polysaccharide-protein interaction is important.

1. 6 Polysaccharide-binding proteins: Annexins

Annexins are a family of structurally related proteins with polysaccharide-binding properties [13, 14]. Annexins are composed of 4 (or 8 in the case of annexin A6) highly α-helical repeated domains called

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[*]PAMPs: PAMPs contain the peptidoglycan of gram-positive bacteria such as lipoteichoic acids; the lipopolysaccharide (LPS, also called endotoxin) of gram-negative bacteria; double-stranded RNA of some viruses of both plants and animals, and so on.

"core domain", each of which is around 70 amino acids in length.   Each annexins has an N-terminal domain with specific length and sequence. For example, as shown in Fig. G-11, annexin A1 (N-terminal; 40 amino acid residues, molecular weight; 38 kDa) has a longer N-terminal domain than annexin A4 (N-terminal; 14 amino acid residues, molecular weight; 34 kDa).

Several annexins exist in Eukaryotes (Table G-2), 12 annexin subfamilies have been found in vertebrates (A1-A11, A13) until recently. Since annexins were identified as phospholipase A_2 inhibitors [15], evidences of annexins as inflammation modulators have been widely provided.   In 1992, it was first discovered that p33/41 has binding affinity for polysaccharide.   p33/41 was purified from bovine kidney by using two-step affinity chromatograph on heparin and fetuin columns.   Later p33/41 was identified as annexin A4 by amino acid sequence analysis and cDNA cloning [13].   Annexin A5 also binds to polysaccharide such as heparin, heparin sulfate in a calcium-dependent manner [14].   These polysaccharide ligands of annexins are found on the lumen side of secretary compartment or on the outside of cells, indicating that annexins could play important roles in ECM.   However, no study to clarify the polysaccharide binding activities of the annexins has been reported up to now.
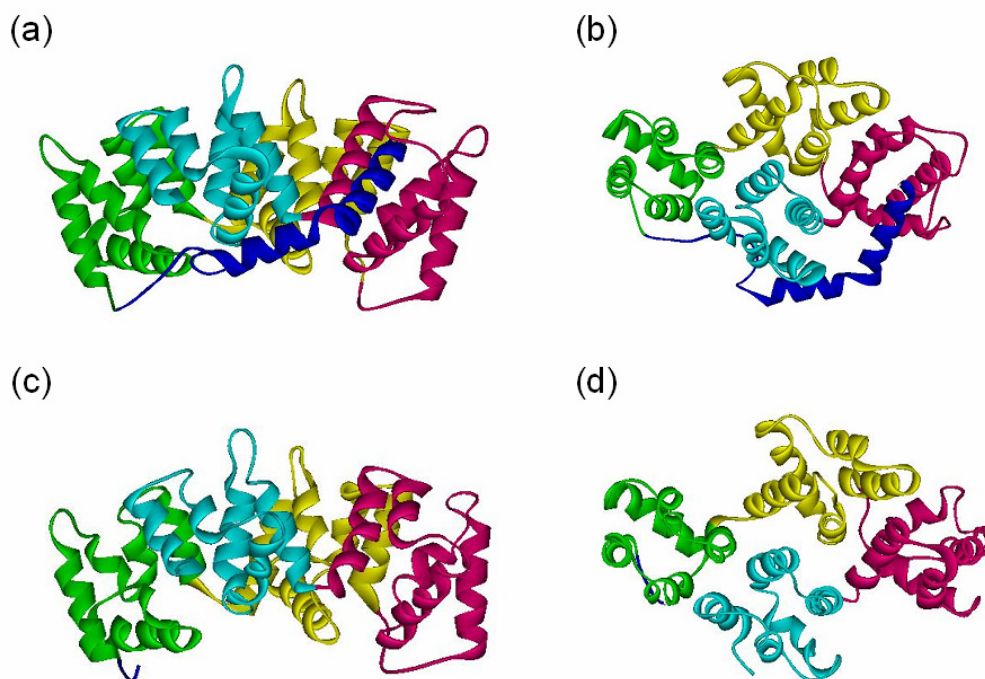
Fig. G-11   Structural comparison between annexin A1 (a, b) and A4 (c, d),   (program DS viewer pro).

Ribbon diagrams of the crystal structure of (a, b) recombinant porcine annexin A1 (PDB-code 1MH6) and (c, d) bovine annexin A4 lacking first 9 amino acid residues (PDB-code 1AOW) in the absence of calcium ions [16, 17].   (a, c) are side view, (b, d) are top view.   N-terminal domains are indicated in blue.   Core domains are composed of four sub-domains colored in green (repeat 1), yellow (repeat 2), pink (repeat 3) and light blue (repeat 4).   These two annexins have the similar structural feature, such as the slightly curved disc (a convex surface), except N-terminal domain.   Since calcium ion and membrane binding sites are located within the core domain, most of the family members bind to phospholipids in the presence of calcium ions.   N-terminal domain mediates regulatory interactions with protein ligands, regulates the annexin-membrane association and calcium sensitively [18-21].

A. human annexins plus cognate orthologs

Name	Synonyms/Former name(s)	Human gene / Non-human gene symbol	
annexin A1	liposortin 1, annexin I	ANXA1	Anxa1
annexin A2	calpactin 1, annexin II	ANXA2	Anxa2
annexin A3	annexin III	ANXA3	Anxa3
annexin A4	annexin IV	ANXA4	Anxa4
annexin A5	annexin V	ANXA5	Anxa5
annexin A6	annexin VI	ANXA6	Anxa6
annexin A7	synexin, annexin VII	ANXA7	Anxa7
annexin A8	annexin VIII	ANXA8	Anxa8
annexin A9	annexin XXXI	ANXA9	Anxa9
annexin A10		ANXA10	Anxa10
annexin A11	annexin XI	ANXA11	Anxa11
annexin A12	*unassigned*		
annexin A13	annexin XIII	ANXA13	Anxa13

B. animal annexins without human orthologs

Name	Organism/Former name	Gene symbol
annexin B9	3 species of insect, annexin IX	Anxb9
annexin B10	4 species of insect, annexin X	Anxb10
annexin B11	1 species of insect, annexin	Anxb11
annexin B12	*Cnidaria*, annexin XII	Anxb12
	3 species of flatworms, 5 annexins	
	10 species of roundworms, 5 annexins	
	(including *C. elegans* annexins XV-XVII, XXX)	

C. fungi/molds and closerelatives

Name	Organism/Former name	Gene symbol
annexin C1	*Dictyostelium* and *Neurospora* annexin XIV	Anxc1
annexin C2-C5	4 species of fungi/ molds/alveolates	Anxc2-c5

D. plants

Name	Organism/Former name	Gene symbol
annexin D1-D25	35 species including annexin XVIII and annexins XXII-XXIX	Anxd1-d25

E. protists

Name	Organism/Former name	Gene symbol
annexin E1	*Giardia* annexin XXI	Anxe1
annexin E2	*Giardia* annexin XIX	Anxe2
annexin E3	*Giardia* annexin XX	Anxe3

Table G-2   The new annexin nomenclature [21].

The five major annexin groups (A-E) are shown.   The vertebrate annexins (A1-A13) are unlikely to be widely represented in invertebrate species.   The oldest of this group, namely, annexins A7, A11, and A13, are possible exceptions, and an annexin A11 ortholog has been described in the mollusk *Aplysia*.   Within the groupB, the *Caenorhabditis elegans* annexins have yet to be assigned numbers.   In the groupC, the *Dictyostelium* annexin VII (synexin), is now established as being orthologous to the *Neurospora* annexin.

1. 7　Aims of this work

Over the past 35 years a large variety of terpenes has been found in sedimental organic matter. The structure of many of these "fossils" made it possible to deduce the probable structures of their "living" precursors. Some of those precursors are found in extant organisms, whereas others are still unknown. More than 200 hopane derivatives had been first isolated from extracts of sediments, and their ubiquitous presence called for general interpretation, because hopanes had been identified only in scattered plants. Later, bacteriohopanetetrol and other hopane derivatives have been found in many bacteria and cyanobacteria and these novel biohopanoids are probably the origin of the geohopanoids. The functional equivalence of bacteriohopanetetrol to cholesterol is demonstrated by biophysical methods. The former plays the same role in Bacteria as cholesterol does in Eukarya, that is, it is a membrane reinforcer. Another example of "biodiversity in biomembranes" is clearly observed in the difference of membrane constituents in acteria/eukarya (di-acyl phospholipids) and in Archaea (di- or tetra-etherphospholipids). Are there other examples?

Recently, several highly branched polyprenylated hydrocarbons have been widely found in sediments, and such molecules might be derived from their corresponding alcohols or phospholipids. It is suggested that polyprenyl-substituted polyprenyl phosphates could exist in primitive membranes, even though they have not yet been found in present biomembranes. In the first part of this work (Chapter 1), the vesicle formation of synthesized isoprenylated polyprenyl phosphates in various pH buffers has been studied. The physical properties of their membranes

were also investigated. These studies could permit to discuss whether these phosphates might be possible primitive membrane constituents, and suggest they might.

Presently, the lipidic part of eukaryotic membranes is principally composed of di-acyl phospholipids and sterols. The study described in Chapter 2 was motivated by the question "why was phosphorylated diacylglycerol but not phosphorylated cholesterol involved in the process of evolution?" or "do membranes composed of a mixture of phosphorylated cholesterol and diacyl glycerol exist in some organisms not yet studied?". "Can they exist at all?" Cholesteryl phosphate (CP), sitosteryl phosphate (SP) and cholesteryl phosphorylcholine (CPC) were synthesized. By comparing the physical properties of CP, SP or CPC/diacylglycerol vesicles with vesicle made of biomembrane constituents, their biomembrane properties have been discussed from a biodiversity point of view.

The self-organization of amphiphiles in water into closed vesicles could lead to self-complexification toward "proto-cells". In Chapter 3, it was studied how lipophilic polysaccharide molecules could coat the primitive membranes. Using florescence optical microscopy; pullulan, a polysaccharide (MW about 55 kDa) bearing hydrophobic phytyl chains (as model of a "primitive" membrane lipid moiety) or cholesteryl moieties (as model of a eukaryotic membrane component) was used. It would lead to an assembly reminiscent of the cell wall of microorganisms, and it does. In the second step, to investigate how complexification of the membrane

surface could occur to acquire a higher function to communicate with the outside world, the molecular recognition between lectins (fluorescence labeled concanavalin A and annexin V) and polysaccharides on a giant vesicle surface was studied using the hydrophobized polysaccharides. Spontaneous binding was observed under optical microscopy. This method could be useful for an easy assay of binding between polysaccharides and proteins.

Cell membranes have a number of important functions such as specific recognition of foreign molecules. Annexins are present in ECM. Recent studies have shown that annexins bind to lipid A of Gram-negative bacteria and suppress cellular responses to endotoxin, This result suggests that annexins function as modulators of anti-inflammation via recognition of foreign substance. The final part of this thesis (Chapter 4) deals with the interaction between annexins and foreign substances. This study was conducted with the aim to understand the biological function of annexins derived by polysaccharide-binding activities. At first, the binding properties of annexins for various exogenous substances were investigated. And then, the physiological functions of annexins, mainly in the immune system, were studied. The results obtained suggest that annexins might act as anti-inflammatory proteins by modulating the pathway of interaction between immune cells and their targets.

Chapter 1

Membrane properties of branched polyprenyl phosphates, postulated as primitive membrane constituents

Introduction

Life is cellular and the physical boundary separates the living organism from the outside world by a thin lipidic membrane resulting from the self-assembly of amphiphilic molecules. We have first observed that terpenoids are universal constituents of all living organisms : some terpenoids are membrane reinforcers in Eukaryotes (cholesterol in animals or phytosterols in plants) and in Bacteria (hopanoids, $\alpha,\omega-$dihydroxylated carotenoids), whereas C_{20} or C_{40} polyprenyl lipids are structural membrane constituents in Archaea [21]. We have then postulated an original scenario about the early formation of membranes and their evolution: it was possible to arrange the membrane terpenoids in a phylogenetic sequence (Fig. G-10), and a retrograde analysis has led us to conceive that *polyprenyl phosphates* might have been primitive membrane constituents [8]. Indeed, we have synthesized several phosphate esters containing one or two polyprenyl chains, and have demonstrated that single- or double-chain polyprenyl phosphates do form vesicles [22, 23]. Furthermore, we have postulated that the highly branched isoprenoid alkanes and alkenes which are distributed widely and abundantly in many sediments, may have been derived from branched polyprenyl phosphates potentially present in the biomembranes of some primitive organisms [24, 25]. These polyprenyl-branched polyprenyl phosphates could result from a simple alkylation of non-substituted polyprenyl phosphates. The recent isolation of the branched isoprenoid hydrocarbons from diatoms also suggests that the corresponding alcohols or phosphates must still exist on Earth [26].

We have recently synthesized a series of 2- or 6-(poly)prenyl-substituted polyprenyl phosphates and found that these higher branched polyprenyl phosphates form vesicles in water in a certain pH domain [27- 29].

Here, we first present a more detailed study on the formation of vesicles from a series of synthesized 6-(poly)prenyl-substituted polyprenyl phosphates in order to analyze different parameters influencing the formation of vesicles (substituted-chain length, position of the double bonds, and pH). Nine of the branched polyprenyl phosphates (**1a-1c**, **2a-2c**, **3a-3c**), containing C_{20} to C_{30} atoms (Fig. 2), form vesicles in a certain pH range; the lipophilicity/hydrophilicity ratio is as expected an important factor. Then, we study the water permeability through membranes of these branched polyprenyl phosphate vesicles by use of our stopped-flow/light-scattering method [7]. We demonstrate that these highly branched polyprenyl phosphates can more effectively reduce the water permeability than the non-substituted polyprenyl phosphates **4a** and **4b**. Not only do they form membranes like the simple polyprenyl phosphates, but they are even more efficient.

Results & discussion

1. Microscopy observation

Spontaneous vesicle formation is influenced by the relative sizes of the hydrophilic and hydrophobic parts in the molecule.   Israelachivili et al. proposed that the vesicle formation depends on the critical packing parameter P = v/a_0lc (a_0 = the optimal area per molecule at the lipid-water interface; lc = chain length; v = hydrocarbon volume), the value of which must be between 1/2 and 1 for vesicle formation [3].   It was also predicted that when the value of P decreases below 1/3, spherical micelles are formed (Table G-1).

The variation of pH in the medium brings about changes in the space requirement of the ionized and hydrated phosphate head-groups, and the surface area a_0 increases in the following order: diacid < monoanion < dianion.   Therefore,   vesicle   formation   of   the   nine 6-(poly)prenyl-substituted polyprenyl phosphates in water was studied in the function of pH of the medium.

Table 1-1 shows the domain of vesicle formation of the nine branched polyprenyl phosphate/water systems.   The structural features of the nine branched polyprenyl phosphates were as follows: all compounds are alkylated at position 6 in the C_{15} main chain.   The phosphates **1a**, **2a** and **3a** (Fig. 1-1) are alkylated by a prenyl group (C_5), phosphates **1b**, **2b** and **3b** are alkylated by a geranyl group (C_{10}) and phosphates **1c**, **2c** and **3c** are alkylated by a farnesyl group (C_{15}).   Groups A, B and C are classified by the position of the double bond located near the middle of the main

chain; A: position 5, B: position 6, C: position 7.   Vesicle formation was checked mainly by differential interference contrast microscopy.   In most cases (+ mark in Table 1-1), multilamellar vesicles of various layer thicknesses and shapes were spontaneously formed, and their sizes (1-20 μm) are large enough to be seen by optical microscopy (Fig. 1-4).   In the case of the phosphates **1a** and **3a** (both at pH 8.85) small particles undergoing a Brownian motion were observed, and they were identified as small vesicles (10-150 nm) by electron microscopy.

As shown in Table 1-1, the branched polyprenyl phosphates formed vesicles in a pH-dependent manner.   An experimental potentiometric titration for phosphate **1b,** obtained between pH = 2.70 and 11.00 is presented in Fig. 1-5.   The processing of the potentiometric data leads to the determination of two dissociation constants ($pK_1 = 4.59 \pm 0.03$, $pK_2 = 8.43 \pm 0.04$).   The distribution diagram of the protonated species of phosphate **1b** at a concentration of 6×10^{-4} M is presented in Fig. 1-6. These results imply that a certain ratio of monoanion/dianion is required for the vesicle formation of phosphate **1b**.

In addition, it was indicated that longer chain compounds require higher pH values for the formation of vesicles.   For example, the phosphate **1a** ($n = 0$, C_{20}) gave vesicles from pH 4.5 to 8.85, the phosphate **1b** ($n = 1$, C_{25}) and the phosphate **1c** ($n = 2$, C_{30}) gave vesicles from pH 7.0 and pH 7.86, respectively.   The C_{20} phosphates have a shorter chain than the C_{25} and C_{30} phosphates, so that the lipophilicity/hydrophilicity ratio of C_{20} favours spontaneous vesicle even in lower pHs.

On the other hand, among the three phosphate C_{20} isomers **1a**, **2a** and

3a, the phosphate **2a** could form vesicles at pH 3.1, but not the phosphates **1a** and **3a**. To explain this observation, we calculated the volume of the hydrophobic part and the distance between C(11) and the phosphorus atom (L(C(11)-P)) in the main chain of the phosphates **1a**, **2a** and **3a** (Table 1-2). Fig. 1-7 shows the most stable conformation of the phosphates **1a**, **2a** and **3a**. The volume of the hydrophobic part of their most stable conformation is in all cases about 254 Å^3 and therefore there is no significant difference in the lipophilicity/hydrophilicity ratio. However, L(C(11)-P) of the phosphate **2a** is longer than those of the phosphates **1a** and **3a** (Table 1-2). The probability of existence of each conformer is presented in Fig. 1-8, in which the energy levels are on the vertical axis and L(C(11)-P) is on the horizontal axis. As shown in Fig. 1-8, in the case of phosphate **2a** (cross-hatched circle), most of the values of L(C(11)-P) are distributed around 11 Å at any energy level (Ave. 11.46 ± 0.38 Å). On the other hand, in the case of phosphate **1a** (dotted circle) and **3a** (closed black circle), the value of L(C(11)-P) can vary from 5 to 10 Å (phosphate **1a**, Ave. 9.14 ± 1.44Å; phosphate **3a**, Ave. 8.83 ± 1.23 Å). These results imply that the successive saturated carbons (C(7)-C(8)-C(9) of phosphate **1a** and C(4)-C(5)-C(6) of phosphate **3a**) may induce flexibility of the main chain, resulting in shorter L(C(11)-P) as well as larger standard deviation values. On the contrary, phosphate **2a** might be more rigid due to the lack of such a flexible moiety (Table 1-2, Fig. 1-8). That is, the phosphate **2a** is much more straight than the others, and therefore the attractive cooperative van der Waals forces between surrounding molecules in vesicles made of the phosphate **2a** could be larger than those of the phosphates **1a** and **3a**.

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Additionally, the significantly small standard deviation value of the phosphate **2a**, that is, the virtually constant L(C(11)-P), might be also an important factor for the spontaneous formation of vesicles. These factors might explain the stable vesicle formation at pH 3.1 of the phosphate **2a**, which interestingly contains a "terpene backbone".

The fact that only small vesicles (10-150 nm) were obtained for the phosphate **1a** and **3a** at pH 8.85 suggests that the lipophilicity/hydrophilicity ratio would not be favorable at pHs higher than 8.85 to make vesicles, and only spherical micelles could be formed [30].

The microscopic observation thus allowed us to determine the pH range of the spontaneous vesicle formation of the nine branched polyprenyl phosphates. We have shown that these polyprenyl phosphates can form vesicles in a wide range of pH. The length and the double bond position in the main chain of these phosphates are important factors for the vesicle formation. These results are in accordance with the theory of Israelachivili et al. about the ratio of hydrophobic/hydrophilic parts in the molecule for the formation of vesicles [3].

2. Water permeability of vesicles

To evaluate the robustness of the vesicles of the branched polyprenyl phosphates, osmotic swelling of a solution of unilamellar vesicles was measured by using our stopped-flow/light-scattering method [7]. The half-time of vesicle swelling is smaller when membrane permeability increases. We have studied the effect of different structural parameters: length of the branched chain and position of the middle double bond, and

we have compared the results with those of the polyprenyl phosphates themselves.

2-1. Effect of the branched chain length

Table 1-3 shows that vesicles made from C_{20} chains (phosphates **1a** and **2a**) are more permeable among each group.   Due to the shorter chain length of phosphates **1a** and **2a**, the attractive cooperative van der Waals forces in vesicles are the weakest among each group.   Thus the vesicles were packed less densely, and as a result their vesicles were more permeable.   The vesicles of C_{30} (phosphate **1c**) were more permeable than those of C_{25} (phosphates **1b**).   Thus the branched chain length of polyprenyl phosphates C_{25} seems to be optimal to form tight vesicles.

The water permeability of the phosphates **2b** and **3b** was not measured, because they formed multilamellar vesicles even after 20 filtrations of the vesicle suspensions through polycarbonate membranes.

2-2. Effect of the position of the middle double bond

We then compared the kinetics ($t_{1/2}$ in ms) of two C_{20} isomers (phosphates **1a** and **2a**) and three C_{30} isomers (phosphates **1c**, **2c** and **3c**). Extensive studies of the phosphate **3a** were not possible due to the small amount available.   The $t_{1/2}$ of the phosphate **2a** vesicles is about 3 times higher than that of the phosphate **1a** vesicles, and the $t_{1/2}$ of the phosphate **2c** and **3c** vesicles is more than 2 times higher than those of the phosphate **1c**.   This suggests that the phosphates **1a** and **1c** are respectively more fluid than their isomers bearing the same chain atoms.

2-3. Comparison with non-substituted polyprenyl phosphate

Farnesyl phosphate **4a** in C_{15} and geranylgeranyl phosphate **4b** in C_{20} were postulated as "primitive" membrane constituents [8]. The phosphate **4a** forms vesicles at pHs between 1.9 and 5.7 under natural swelling conditions from its thin film [31], but the measurement of the water permeability of vesicles made of this phosphate **4a** at pH 4.00 was not possible due to their instability in our experimental conditions. The phosphate **4b** forms vesicles at pHs between 2.0 and 8.6 under natural swelling conditions from its thin film [31], but vesicles formed at pH 8.5 were not stable enough under our experimental conditions. Therefore the value of the water permeability obtained at pH 5.81 was used for the comparison [31]. The water permeability of non-substituted geranylgeranyl phosphate **4b** is higher than that of geranyl-branched C_{25} phosphate or farnesyl-branched C_{30} phosphate (Table 1-3). This implies that these substitutions make their vesicles more stable against mechanical stress. Polyprenyl-branched polyprenyl phosphates could be obtained by a simple alkylation of non-substituted polyprenyl phosphates in prebiotic conditions. The results obtained imply that polyprenyl-substitution could be one step of the evolution of biomembranes.

Group	Phosphate	Number of C atoms (Main and side chains)	pH value of buffer [b]								
			3.1	4.0	4.5	5.3	6.1	7.0	7.86	8.5	8.85
A	1a	20 (15 + 5)	–	–	+	+	+	+	+	+	+
	1b	25 (15 + 10)	–	–	–	–	–	+	+	+	+
	1c	30 (15 + 15)	–	–	–	–	–	–	+	+	+
B	2a	20 (15 + 5)	+	+	+	+	+	+	+	+	+
	2b	25 (15 + 10)	–	–	–	–	–	+	+	+	+
	2c	30 (15 + 15)	–	–	–	–	–	+	+	+	+
C	3a	20 (15 + 5)	–	–	+	+	+	+	+	+	+
	3b	25 (15 + 10)	–	–	–	–	–	+	+	+	+
	3c	30 (15 + 15)	–	–	–	–	–	+	+	+	+

Table 1-1: Spontaneous vesicle formation of the 6-(poly)prenyl-substituted polyprenyl phosphates **1a-1c, 2a-2c** and **3a-3c** at various pH values[a]

[a] Observed by differential interference contrast microscopy at 24 °C. [b] pH 3.1, 4.0, 4.5, 5.3 and 6.1: sodium acetate-acetic acid; pH 7.0: Na_2HPO_4-NaH_2PO_4; pH 7.86: Tris-HCl; pH 8.5 and 8.85: borate. +: vesicle formation; −: no vesicle formation.

Polyprenyl phosphate	Volume [a] (Å^3)	L(C(11)-P) [b] (Å)	Weighted average of L(C(11)-P) [c] (Å)
1a	254.90	10.04	9.14 ± 1.44
2a	254.39	11.43	11.46 ± 0.38
3a	254.70	8.38	8.83 ± 1.23

Table 1-2: Calculated molecular volume and distance between C(11) and the phosphorus atom of the 6-(poly)prenyl-substituted polyprenyl phosphates **1a**, **2a** and **3a**.

[a] total hydrophobic volume of polyprenyl chains in the most stable conformation

[b] distance between C(11) and the phosphorus atom in the main chain in the most stable conformation

[c] weighted average ± standard deviation of distance between C(11) and the phosphorus atom in the main chain taking into account the population relevance of each conformer (relative energy range from minimal to 5 kcal/mol)

Group	Phosphate	Temperature (°C)	pH	Diameter [a] (nm)	k [b] (s^{-1})	$t_{1/2}$ [c] (ms)
	1a	16.0	8.50	184 ± 7	104 ± 21	6.7 ± 1.3
A	**1b**	16.1	8.50	205 ± 10	7.7 ± 1.5	90 ± 18
	1c	16.1	8.50	209 ± 11	12.3 ± 0.5	57 ± 2.3
B	**2a**	14.5	8.50	199 ± 10	35.7 ± 18	19.4 ± 1.0
	2c	14.6	8.50	211 ± 11	5.4 ± 1.0	128 ± 24
C	**3c**	14.7	8.50	205 ± 11	4.9 ± 0.6	141 ± 17
Reference	**4b**[d]	15.0	5.81	186 ± 16	34.8 ± 0.7	19.9 ± 1.0

Table 1-3: Water permeability of unilamellar vesicles of 6-(poly)prenyl-substituted polyprenyl phosphates **1a–1c**, **2a**, **2c** and **3c,** and geranylgeranyl phosphate **4b** measured by stopped-flow/light-scattering method.

[a] Average diameter ± standard deviation. [b] Average rate constant ± standard deviation. [c] Average $t_{1/2}$ ± standard deviation. [d] Values from ref. [31].
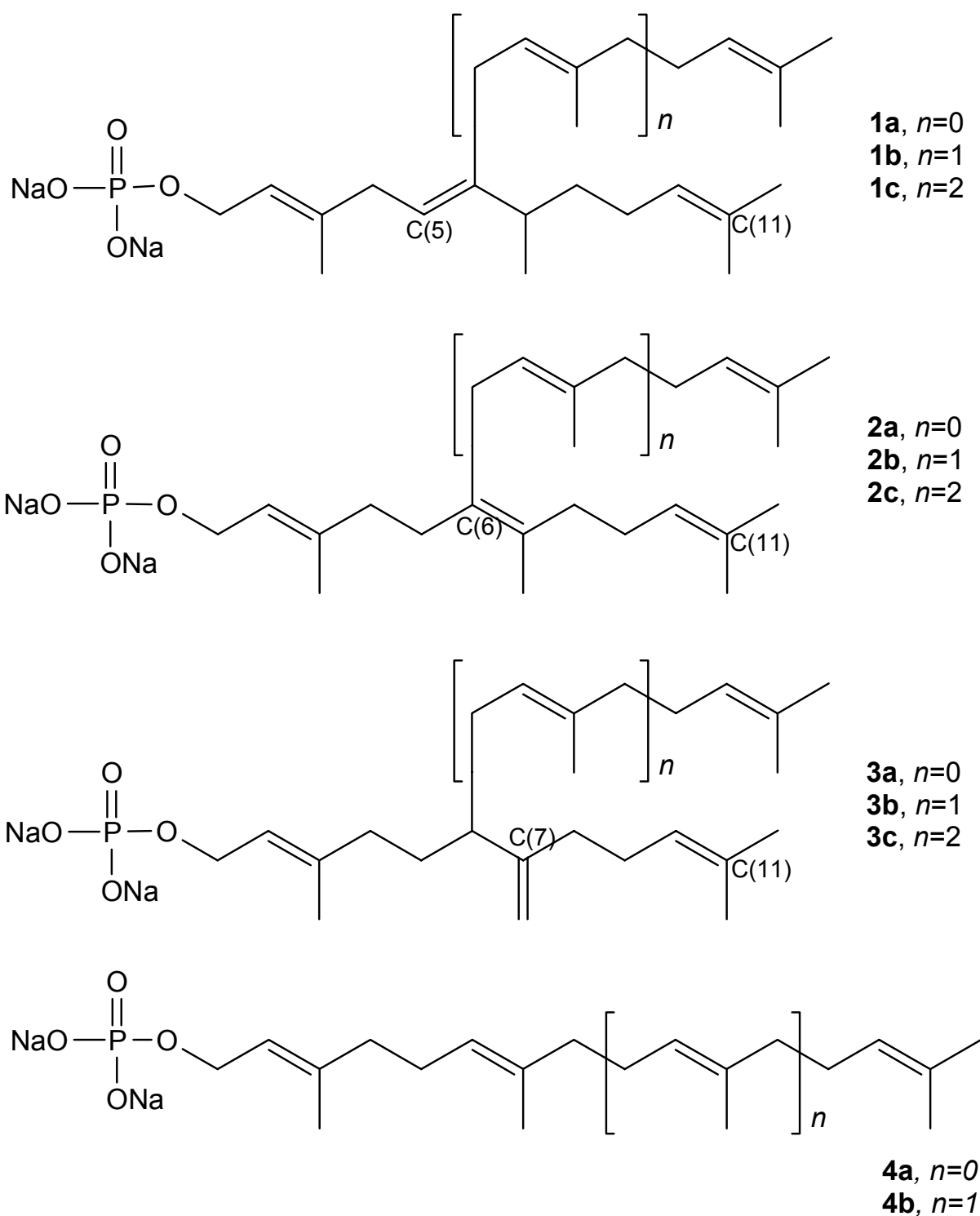
Fig. 1-1   Structures of synthesized 6-(poly)prenyl-substituted polyprenyl phosphates **1a-1b**, **2a-2c**, **3a-3c**, farnesyl phosphate **4a** and geranylgeranyl phosphate **4b**.
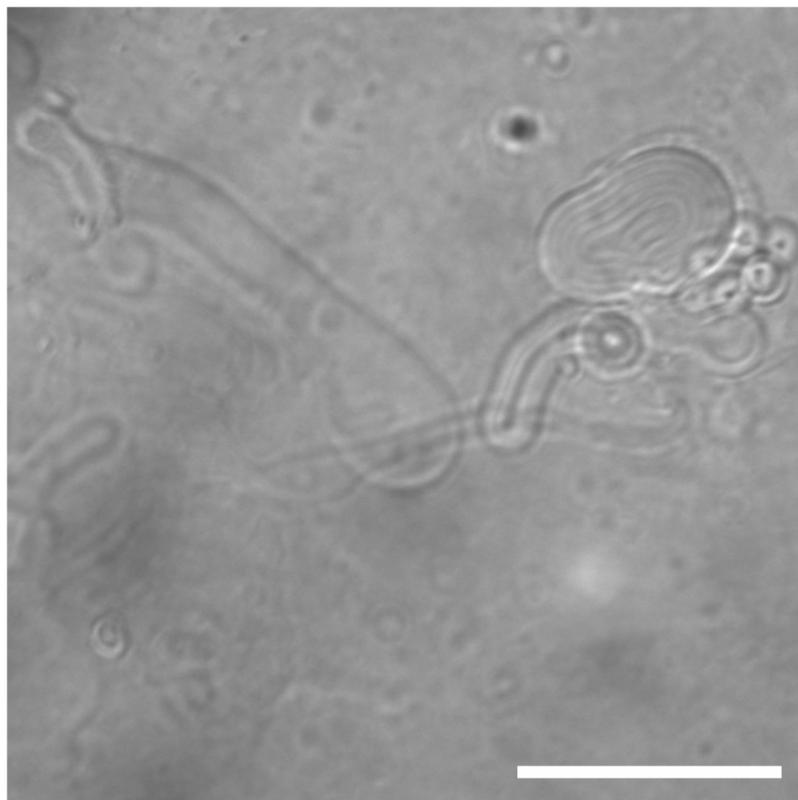
Fig. 1-2　Differential interference contrast microscope images of the giant vesicles of phosphate **1a** at pH 7.0.　The bar represents 10 μm.
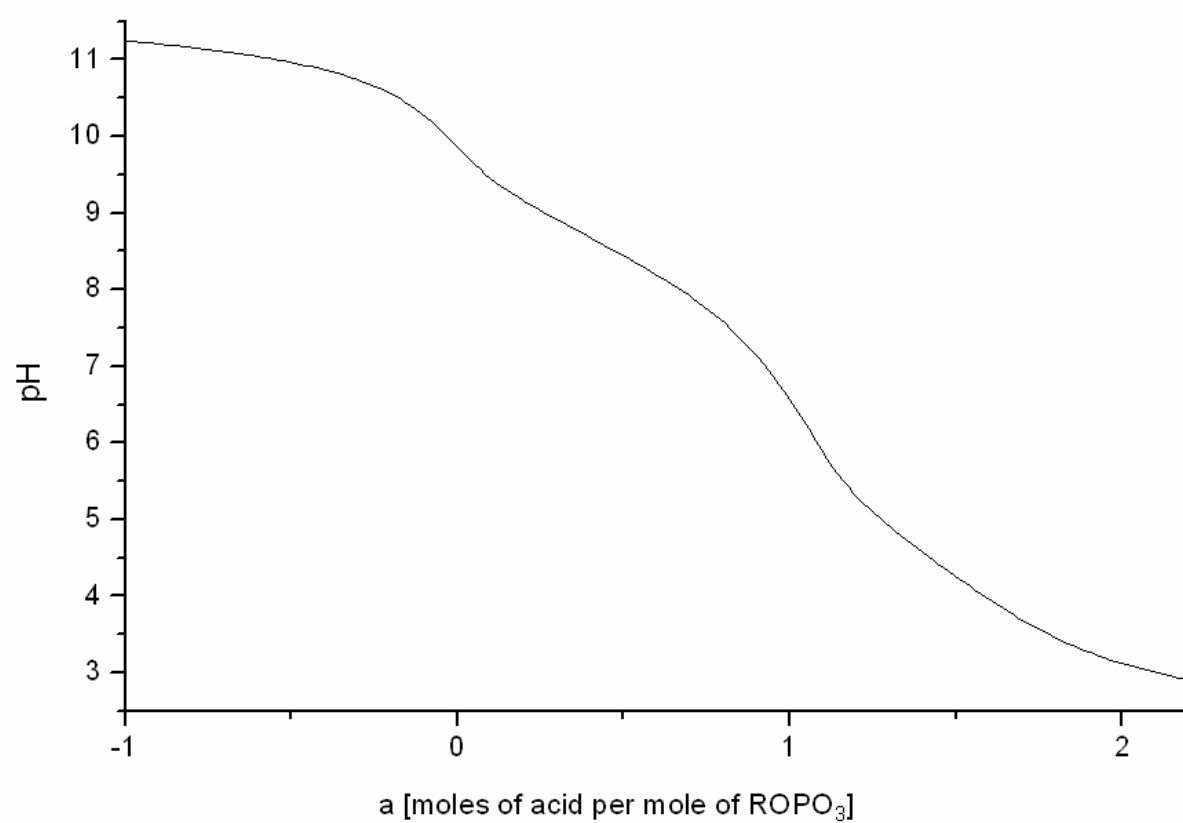
Fig. 1-3　Titration of vesicles of phosphate **1b**.　Negative values of a (moles of acid per mole of ROPO$_3$) correspond to the titration of NaOH.
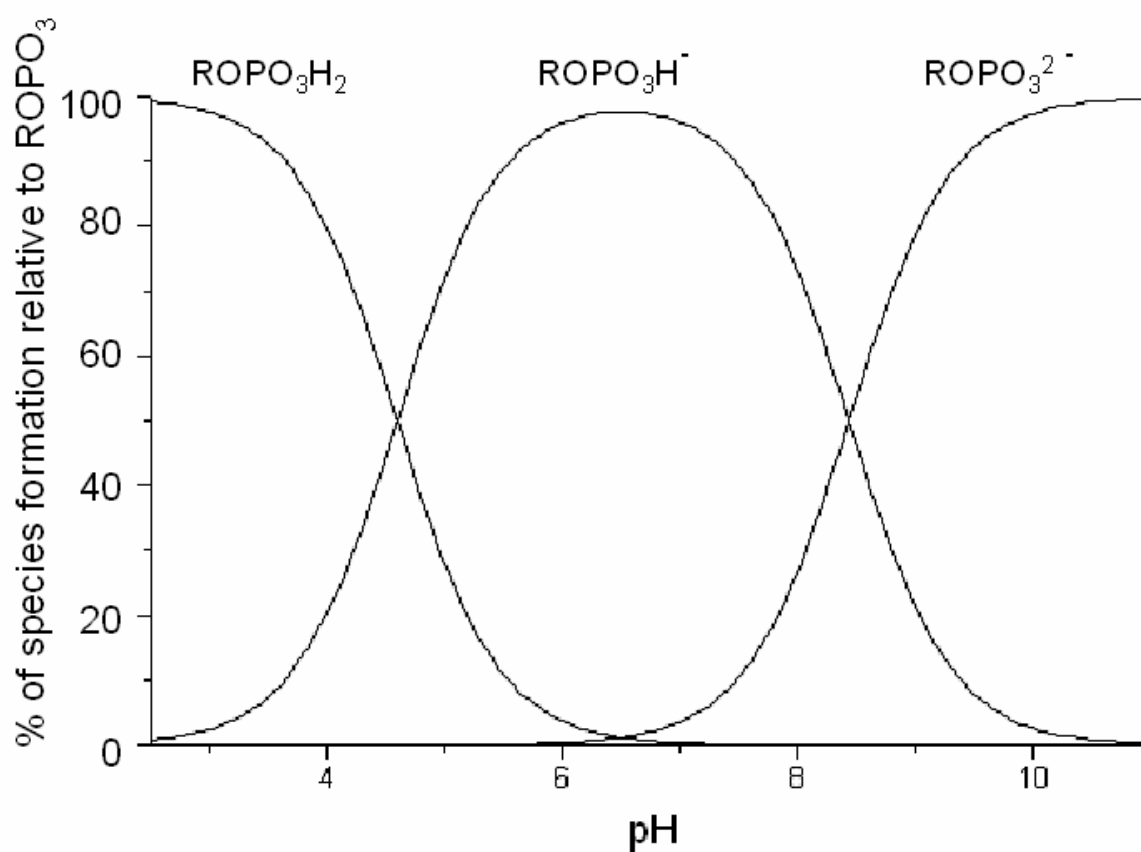
Fig. 1-4   Distribution diagram of the protonated species of polyprenylated polyprenyl phosphate **1b** as a function of pH. $[P]_{total} = 6 \times 10^{-4}$M, I = 0.1 M NaCl, T = (25.0 ± 0.2)°C.
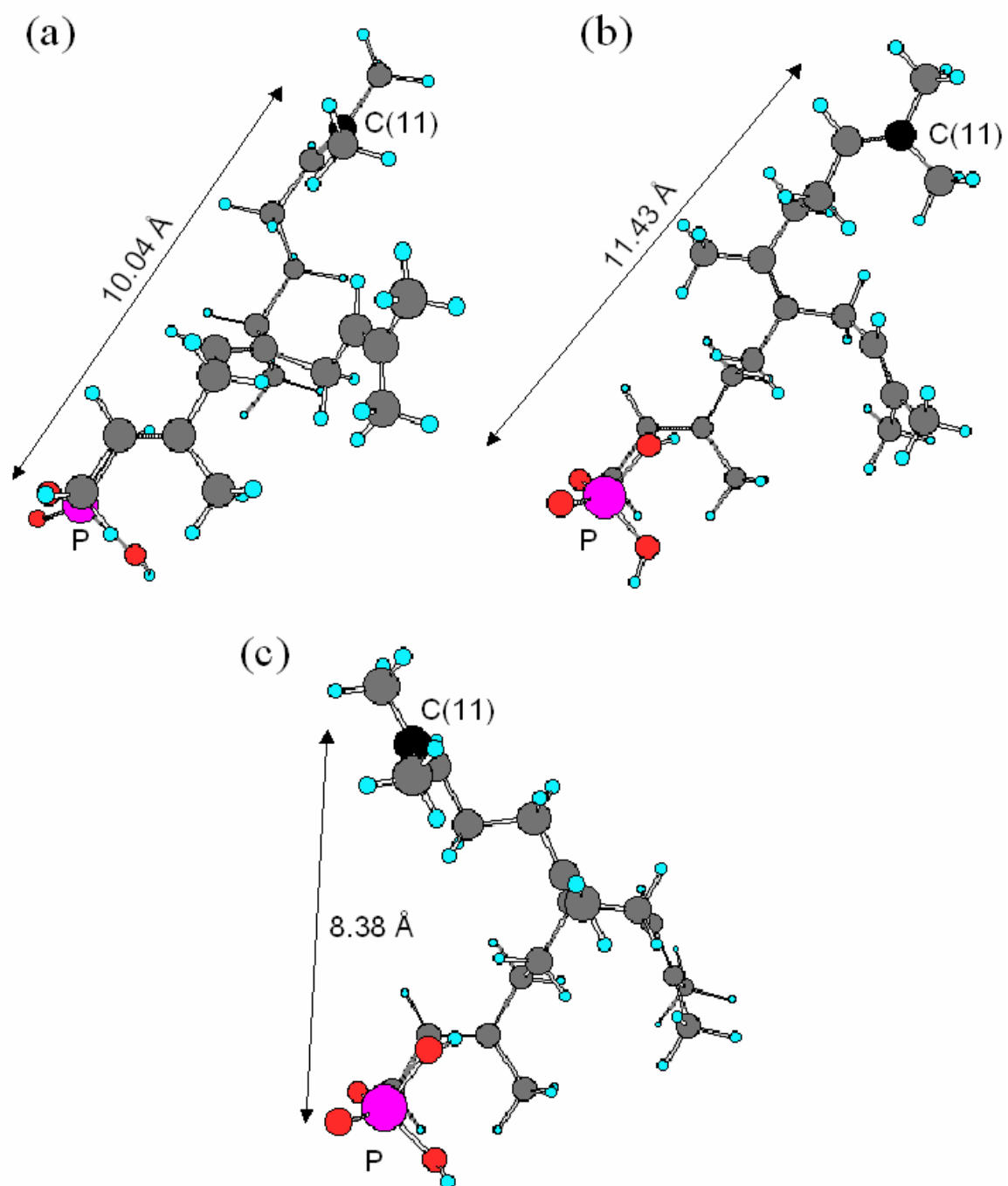
Fig. 1-5   Structure of the most stable conformation of phosphates **1a** (a),

2a (b) and **3a** (c) and their distance between C(11) and phosphorus atoms.
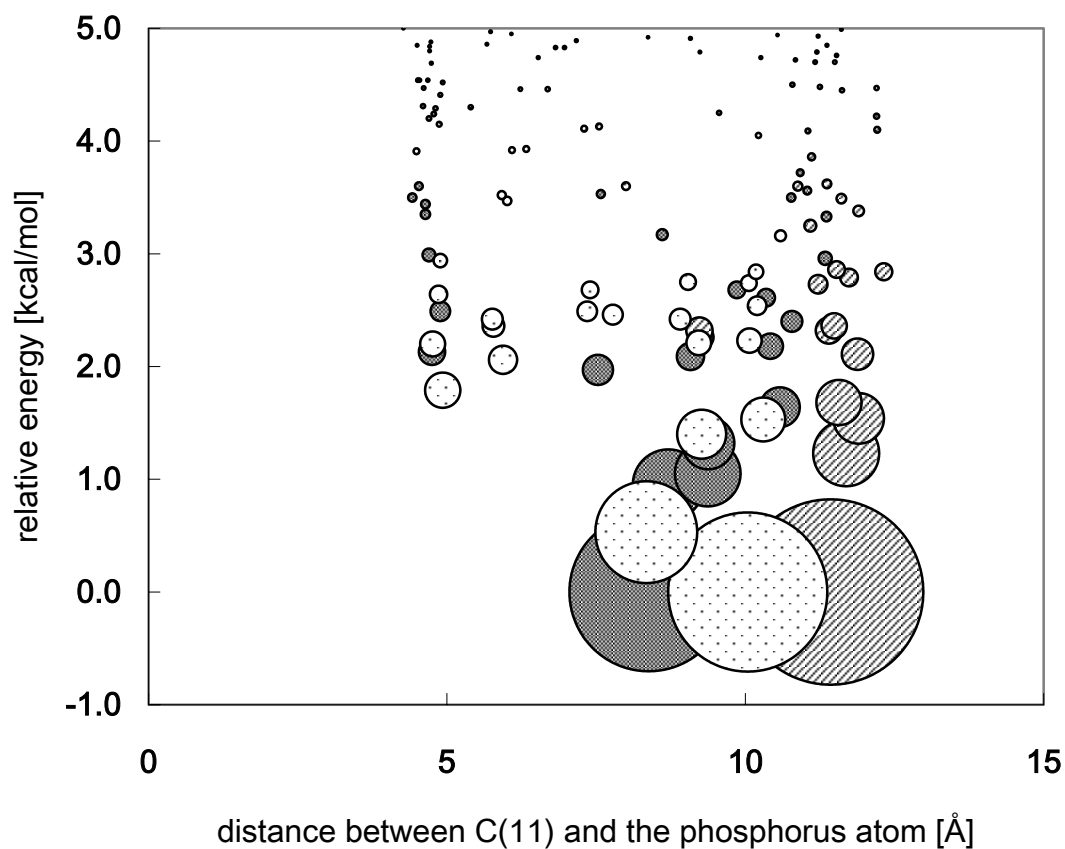
Fig. 1-6　Diagram of the energy levels for phosphate **1a**, **2a** and **3a**.　The stable conformation of phosphates was calculated by conformational energy calculation.　The vertical axis indicates the relative energy of conformers (values are expressed as 0 of the minimal energy in a range from minimal to 5 kcal/mol).　The horizontal axis indicates the distance between C(11) and the phosphorus atom (in Å).　The diameter of the circles is expressed as the percent of the existing probability of each conformer: phosphates **1a, 2a** and **3a** are respectively presented in dotted circles, cross-hatched circles and closed black circles.

Conclusion

We have first shown that polyprenyl-branched polyprenyl phosphates do form vesicles in a pH-dependent manner and that a certain ratio of monoanion/dianion is required for the formation of vesicles. The volume of the hydrophobic part and the distance between C(11) and the phosphorus atom in the main chain were calculated. The double bond position in the middle chain affected the chain length and its flexibility. It was implied that the terpene backbone, maintaining a virtually constant distance of main chain in most of conformers, might be important factor for the formation of vesicles. From the results of water permeability of vesicles, it was indicated that the branched chain length and the position of the middle double bond affected the water permeability of the polyprenyl-branched polyprenyl phosphate vesicles. We found that C_{25} polyprenyl phosphate isomers are more favorable to form stable vesicles than the C_{20} and C_{30} homologs. The position of the middle double bond at C(7) in the polyprenyl-branched polyprenyl phosphates is more suitable for the formation of stable vesicles than the position at C(5) or C(6).

These 6-(poly)prenyl-substituted polyprenyl phosphates thus could make stable vesicles in a physiological pH, and C_{25} and C_{30} phosphates showed lower water permeability than non-branched geranylgeranyl phosphate. We could therefore suggest these amphiphiles might be possible primitive biomembrane constituents, one step advanced from non-substituted polyprenyl phosphate in a hypothetical phylogenetic tree [8].

Materials & methods

General

Isoprenyl-branched polyprenyl phosphates [29] were obtained from Prof. Nagano, Ochanomizu University.

1. Microscopic observation

The substances were dissolved in chloroform/methanol (1:1, v/v). A solution was mounted on the glass slide (0.17 mm thick) and then dried at room temperature. Before the solution was applied, the glass slide was circumscribed with a hydrophobic PAP-pen (Daido Sangyo Co., Tokyo). To hydrate the lipid film remaining on the glass, buffer was added (pH 3.1, 4.0, 4.5, 5.3 and 6.1: sodium acetate-acetic acid; pH 7.0: Na_2HPO_4-NaH_2PO_4; pH 7.86: Tris-HCl; pH 8.5 and 8.85: borate) to reach a final concentration of 0.3 mg/ml for the optical microscopic observation or 2.5 mg/ml for the electron microscopic observation. Before use, the buffers were prepared with MilliQ water and filtered through 0.22 μm filters (Millex GS, Millipore, Bedford, MA) to limit dust.

The sample was observed by differential interference contrast microscopy: Axiovert 135, 63 x /1.40 plan Achromat Oil DIC objective, x 2.5 insertion lens (Carl Zeiss, Thornwood, NY), light sources: halogen lamp, video system: CCD camera (C2400-75H) and image processor (Argus 20), Hamamatsu Photonics (Hamamatsu).

2. Conformational analysis

The exhaustive searches of low-energy conformers of the flexible phosphates **1**, **4** and **7** were performed with CONFLEX program [32, 33] on a CACheTM system using the MM2 force field for energy minimization [34, 35] followed by semi-empirical molecular orbital calculations (PM3 and then PM3-COSMO) [36] of the resulting conformers using the Hamiltonian implemented in MOPAC 6.0 [37]. CACheTM is a registered trademark of Oxford Molecular Inc.

3. pKa measurement

1) Preparation

All the solutions were prepared in distilled water. Water was further purified by passing through a mixed bed of ion-exchanger (Bioblock Scientific R3-83002, M3-83006) and activated carbon (Bioblock Scientific ORC-83005) and de-oxygenated by CO_2- and O_2-free argon (Sigma Oxiclear cartridge). The stock solution was prepared using an AG 245 Mettler Toledo analytical balance (precision 0.01 mg).

HCl ($\sim 10^{-1}$ M) solution was titrated by NaOH (10^{-1} M, Carlo Erba, Titrisol Normex) with phenolphthalein (Prolabo, purum) as an indicator. Carbonate-free NaOH solution ($\sim 10^{-1}$ M) was prepared by dissolving solid product (SDS, p.a.) and standardized by titration with potassium hydrogen phthalate (Fluka, puriss. p.a.) to the phenolphtalein end point.

The solution of the phosphate **1b**, ($\sim 4.5 \times 10^{-3}$ M) was prepared in carbonate-free NaOH solution and titrated by HCl.

2) Potentiometric Titrations

The acidic solution of polyprenylated polyprenyl phosphate was titrated by carbonate-free NaOH. Potentiometric titrations were performed using an automatic titrator system DMS 716 Titrino (Metrohm) with a combined glass electrodes (Metrohm 6.0234.100, Long Life) filled with 0.1 M NaCl (Fluka, p.a.) in water. The ionic strength was fixed at $I =$ 0.1 M with NaCl (Merck, suprapur). The combined glass electrode was calibrated as a hydrogen concentration probe by titrating known amounts of CO_2-free NaOH with HCl solutions [38]. The cell was thermostated at 25.0 ± 0.2 °C by the flow of a Haake FJ thermostat. A stream of argon, pre-saturated with water vapour, was passed over the surface of the solution. The potentiometric data (about 140 points for each titration) were refined with the Hyperquad 2000 program [38]. The successive protonation constants were calculated from the cumulative constants determined with the program. The uncertainties in the log K values were estimated as 3σ, where σ is the standard deviation. The distribution curves of the protonation constants of ligand as a function of pH were calculated using the Haltafall program [38].

4. Stopped-flow/light-scattering

1) Preparation of vesicles

Phosphates (10 mg) were dissolved in 1 ml of chloroform/methanol (1:1, v/v). The solvents were removed by evaporation under vacuum. Subsequently, the dry films were hydrated by addition of 10 ml buffer (10 mM Tris-HCl, pH 7.86 or 8.50, 150 mM NaCl), and vesicle suspensions

was obtained. To obtain unilamellar vesicles, the vesicle suspensions were subjected to water bath sonication for 10 min, followed by the freeze-thaw procedures described in a preceding paper [7]. And then the vesicle suspension was filtered through polycarbonate filters (Nucleopore, UK, 1000 nm, 800 nm, 400 nm and 200 nm filters) in an extruder (Lipex Biomembranes Inc., Canada) and the diameter size was analyzed by photon-correlation spectroscopy on a Coulter-Counter N4MD instrument [7]. After several filtrations, when the size of unilamellar vesicles became approximately 200 nm in diameter, the vesicle suspension was used for stopped-flow/light-scattering assays. It was checked that these vesicle samples were stable for one day at room temperature by comparison of the vesicle size just after their preparation and one day later. The samples which were freshly prepared on the same day were used for the stopped-flow/light-scattering experiments.

2) Measurements by stopped-flow/light-scattering methods

The unilamellar vesicle suspensions containing 150 mM NaCl were rapidly mixed with the same volume of hypoosmolar NaCl free buffer (10 mM Tris-HCl, pH 7.86) in the stopped-flow/light-scattering instrument (Biosequential SX-18 MV stopped-flow ASVD spectrofluorimeter, Applied Photophysics, UK) at 17.6 ± 0.3 ºC. The variation of scattered light intensity vs. time upon osmotic shock was followed at the fixed wavelength of 400 nm (slit width = 3 nm). The data obtained were analyzed using a Bio-Kine Analysis V 3.14 software (Bio-Logic, Mundelein, IL).

Chapter 2

A novel type of membrane based on cholesteryl phosphocholine, cholesteryl phosphate or sitosteryl phosphate and dimyristoylglycerol

Introduction

The lipidic part of eukaryotic membranes is principally composed of phospholipids and sterols such as cholesterol or sitosterol. Phospholipids are derivatives of glycerol doubly esterified by straight-chain fatty acids and furthermore linked to a phosphate-containing highly polar head-group. Cholesterol is an essential reinforcer of mammalian membranes, without which the self-organization of phospholipid molecules does not give physically strong vesicles. The hydroxyl group of cholesterol is situated at the lipid-water interface, its molecular dimensions approach closely those of phospholipid molecules in its stretched form and its hydrophobic tail is localized in the middle of the membrane [4, 39]. Thus, cholesterol reinforces the lipid bilayer by cooperative attractive van der Waals forces [5]. Sitosterol is one of the major sterols in plants. It reinforces membranes of soybean phospholipids very efficiently [6].

Questions arise: why were diacylglycerol phosphoryl derivatives but not cholesteryl phosphocholine involved in the process of evolution? Or are there some organisms (not yet studied) whose lipid membranes are composed of cholesteryl phospholipids/diacylglycerol or cholesterol? Preliminary results with a mixture of cholesteryl phosphate (CP) and dimyristoylglycerol (1,2-dimyristoyl-*rac*-glycerol, DMG) have been reported in a preceding paper [40]. In the present study, to find some clues for those questions, phosphoryl sterols were synthesized, such as CP, sitosteryl phosphate (SP) and cholesteryl phosphocholine (CPC), amphiphilic molecules, and membrane properties were investigated on the

following systems: (1) CP, SP or CPC alone, (2) mixtures of CP, SP or CPC with DMG, cholesterol or sitosterol. For these studies, fluorescence microscopy, differential scanning calorimetry, small angle X-ray scattering and stopped-flow/light scattering methods were used.

Results & discussion

1. Synthesis

Cholesteryl phosphate (CP) **1** and sitosteryl phosphate (SP) **2**: SP was prepared according to the procedure for the synthesis of CP previously described (Scheme 2-1) [40]. Cholesterol or sitosterol was phosphorylated by phosphorus oxychloride, followed by hydrolysis of the phosphodichloridate, in which distilled water was used instead of aqueous sodium hydroxide adopted in the previous report [40], to obtain the pyridinium salts **1** or **2**. The compound obtained was purified by crystallization from dioxane and subsequent washing with diethyl ether to afford cholesteryl phosphate pyridinium salt (yield: 64 %) and sitosteryl phosphate pyridinium salt (yield: 29 %).

Cholesteryl phosphocholine (CPC) **3**: The synthesis of CPC was achieved by following a procedure previously reported for the synthesis of diacylphosphatidylcholines (Scheme 2-2) [41, 42]. Cholesterol was first allowed to react with 2-chloro-1,3,2-dioxaphospholane-2-oxide **7**, to obtain the cholesteryl cyclic phosphate **8**. Then, the cyclic phosphate ring was opened with anhydrous trimethylamine in an autoclave. Purification was carried out by silica gel chromatography, and crystallization from methanol/acetone to afford **3** (yield: 22 %).

2. Microscopy observation

Phospholipids like 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)

observed by optical microscopy, form giant vesicles on simple contact of their thin films with buffers (pH 3.10-11.3).   This is also the case with a mixture of DMPC and cholesterol even with very small concentration of cholesterol and at any pH tested (pH 5.80, 7.86 and 9.30) (Fig. 2-2(f)).   However, the molar ratio of DMPC/cholesterol in the vesicles formed was not analyzed.   It could differ from that in the film on the glass.   It was reported that a 2:1 cholesterol:phospholipid association is possible under certain conditions and a 1:1 association is favoured in most circumustances [5].   On the contrary, CP, SP, CPC or non-phosphoryl diacyl lipid DMG alone did not give any vesicles or tubular structures by themselves at any pH studied.   Thus it was examined whether CP, SP or CPC could form vesicles by adding DMG.   At a certain pH and a suitable relative molar ratio of the mixture of CP/DMG, SP/DMG or CPC/DMG, the formation of vesicles large enough to be seen by optical microscopy (d > 0.2 μm) was indeed observed.   The presence of smaller vesicles by electron microscopy was not checked.   Under the conditions indicated in Fig. 2-2, it could be observed in a few minutes, even without any shaking, the formation of closed vesicles or tubules from the solid surface of the above mixtures.   These structures were more easily detected after addition of a small amount of the lipophilic fluorescent dye Nile Red (Fig. 2-3).   These vesicles are of various layer thicknesses, shapes and sizes.   These observations revealed that these phosphoryl sterols could build membrane systems different from the usual ones by addition of non-phosphoryl acyl lipids.   However, the pH range for producing vesicles from the mixture of phosphoryl sterols and DMG is more restricted than that of the natural

membrane systems, made of mixtures of a phospholipid and cholesterol. In a comparison of three systems: CP/DMG (Fig. 2-2 (a)), CPC/DMG (Fig. 2-2 (c)) and SP/DMG (Fig. 2-2 (b)), the first two systems formed vesicles at any pH (pH 5.80, 7.86 and 9.30) tested, but for the SP/DMG system, vesicle formation was not observed pH 5.80.   The hydrophobic part of SP is larger than that of CP or CPC, since the side chain of the former is branched with an ethyl group at C_{24}.   This may explain why, at pH 5.80 in the SP/DMG system, the ratio of the hydrophobic moiety to the hydrophilic one is apparently unfavourable for the formation of vesicles [3].   At pH 9.30, the vesicle formation was observed in a wider range of molar ratios of the CP/DMG system (Fig. 2-2 (a)) than that of the CPC/DMG one (Fig. 2-2 (c)).   However, the situation is inversed at pH 5.80.

Next, the vesicle formation of CP, SP or CPC mixed with sterols was investigated.   CPC mixed with cholesterol (Fig. 2-2 (e)) formed vesicles at pH 5.80, 7.86 and 9.30 at a suitable relative molar ratio of CPC/cholesterol.   However, CP mixed with cholesterol (Fig. 2-2 (d)) formed vesicles only at pH 9.30 (not at pH 5.80 and 7.86).   The pH value influences the space requirement of the ionized and hydrated phosphate head-group of CP in the following order: diacid < monoanion < dianion. At pH 9.30, the predominant dianion form of CP might compensate the increase of the hydrophobic volume, as a result of incorporation of cholesterol molecules in the system.   Furthermore, the electrostatic repulsion between neighbouring head-groups becomes large.   This repulsion might also make it possible to incorporate cholesterol molecules into vesicles.   On the other hand, SP mixed with sitosterol did not form

vesicles at any pH and at any relative molar ratio studied.   To form vesicles, a good packing of molecules is important.   As was already mentioned, the presence of a branched ethyl group on the side chain of SP could disturb compactness with surrounding molecules.

3. Differential scanning calorimetry (DSC) and small angle X-ray scattering (SAXS)

The thermotropic behavior of aqueous dispersions of CP, SP or CPC mixed with DMG was analyzed by differential scanning calorimetry (DSC). The clear phase transition peak of CP or SP mixed with 40 mol% DMG in aqueous buffer (pH 7.40) was respectively detected at 36.5 ℃ and 35.8 ℃ (Fig. 2-4 (a, b)).   On the other hand, a mixture of CPC and DMG (30, 50 and 80 mol%) did not show any signal.   This implies that such mixtures are in an amorphous state, namely having no ordered arrangement, as they did not show a clear peak.

Next, to identify the phase structures of DMG or CP alone, or CP mixed with DMG, small angle X-ray scattering (SAXS) was used at two different temperatures, at lower and higher temperatures than the phase transition temperature of the mixture of CP and 40 mol% DMG mentioned above.   As shown in Fig. 2-5 (a), the SAXS patterns for DMG measured at 25 °C and 45 °C are pratically indistinguishable, i. e., both gave three sharp diffraction peaks at 4.32, 2.16, 1.44 nm (in a ratio 1: 1/2: 1/3 typical for a lamellar structure).   This indicated that DMG forms a lamellar structure, most presumably a gel-like phase (L_β).   The SAXS pattern for CP measured at 25 °C and 45 °C gave only a single broad peak at about

3.92 nm.　　Due to the very limited number of peaks observed, identification of the phase structure of CP is unable (Fig. 2-5 (b)).　　Finally, the SAXS for CP mixed with 40 mol% DMG was examined.　　As shown in Fig. 2-5 (c), at 25 ºC, the SAXS pattern for the mixture of CP and 40 mol% DMG gave one peak at 4.05 nm.　　At 45 ºC the SAXS pattern gave also one peak at 4.32 nm.　　It is not possible to identify the detailed phase structure from only one peak.　　However, this observation suggests that the structure of CP/DMG mixture was different from that of DMG or CP alone.　　Additionally it could be said that when the temperature was raised from 25 ºC to 45 ºC, the d value was expanded.　　This finding supports the result of above DSC measurement and for the CP/DMG mixture a phase transition might occur between 25 ºC and 45 ºC.

4. Water permeability of the vesicles

To evaluate the water permeability of the vesicles made from CPC with two concentrations (50 mol% and 80 mol%) of DMG, the osmotic swelling of the unilamellar vesicles was studied by using the stopped-flow/light-scattering method.　　If the membrane becomes more compact, the water permeability is reduced [7].　　The results are summarized in Table 2-1.　　They show that when the CPC ratio in the vesicles increased from 20 mol% to 50 mol%, the water permeability became lower.　　This could be due to condensation effect of the steroid component in hydrophobic regions, which would increase the attractive cooperative van der Waals forces.　　Furthermore the difference in water permeability between vesicles made of DMPC + 50 mol% cholesterol and

CPC + 50 mol% DMG was investigated. We recall that both vesicles consist of the same molecular composition in both hydrophobic and hydrophilic parts. It was shown that DMPC + 50 mol% cholesterol vesicles were more impermeable.

The functions of cholesterol in the biomembrane, especially in phospholipid bilayers, have been widely reported, e.g. reduction of the mean molecular area of phospholipid, decrease of the degree of mobility of the acyl chains of the phospholipid, and increased impermeability of bilayers [7, 43]. The specific structure of cholesterol, such as the rigid steroid ring and the 3β-hydroxyl group is necessary for these physical functions. The 3β-hydroxyl group functions as a proton donor and forms a hydrogen bond with the carbonyl group of phospholipid [44]. And additionally it could form hydrogen bond with the phosphate group of phospholipid. The cooperative attractive van der Waals forces of the skeleton with the chains of the phospholipid can then operate. Therefore cholesterol stabilizes the phospholipid bilayer.

In the contrast, CPC is a cholesterol derivative, and the hydrophilic 3β-hydroxyl group of cholesterol is substituted by phosphocholine. Hence DMG could not make a hydrogen bond with CPC. The lack of hydrogen bond between CPC and DMG might explain the difference of water permeability between the above two systems: CPC/DMG and DMPC/cholesterol.

Composition	Diameter [a]	k [b]	$t_{1/2}$ [c]
	(nm)	(s^{-1})	(ms)
CPC + 80 mol % DMG	193 ± 10	1.34 ± 0.09	517 ± 35
CPC + 50 mol% DMG	202 ± 10	0.89 ± 0.16	780 ± 140
DMPC + 50 mol% cholesterol	208 ± 10	0.39 ± 0.02	1780 ± 90

Table 2-1: Water permeability of unilamellar vesicle of CPC/DMG or DMPC/cholesterol at 17.6 ± 0.3 ºC measured by stopped-flow/light-scattering method.

[a] Average diameter $\pm$ standard deviation. [b] Average rate constant $\pm$ standard deviation. [c] Average $t_{1/2} \pm$ standard deviation.
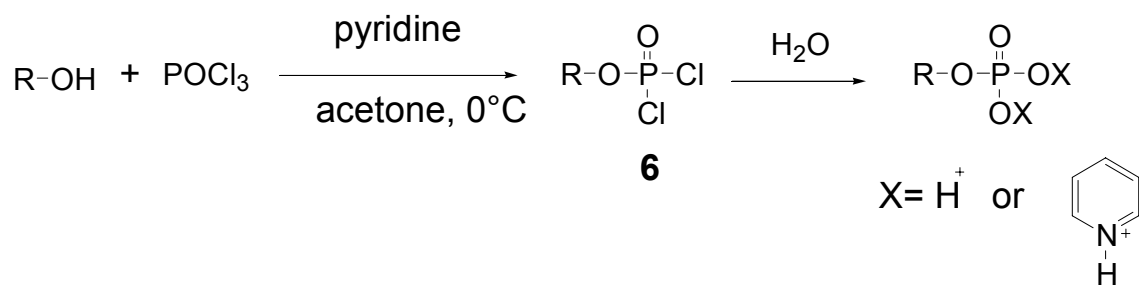
Scheme 2-1: Synthesis of cholesteryl phosphate pyridinium salt or sitosteryl phosphate pyridinium salt; R-OH = cholesterol or sitosterol.
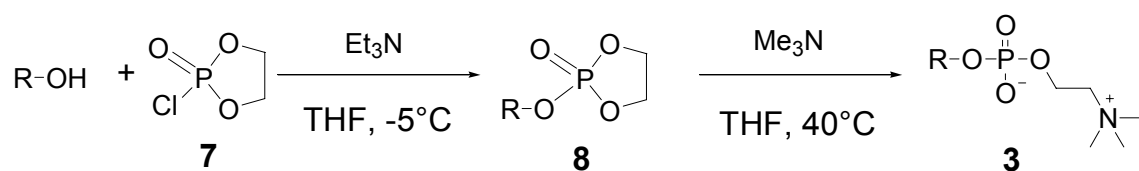
Scheme 2-2: Synthesis of cholesteryl phosphorylcholine **3**; R-OH = cholesterol.
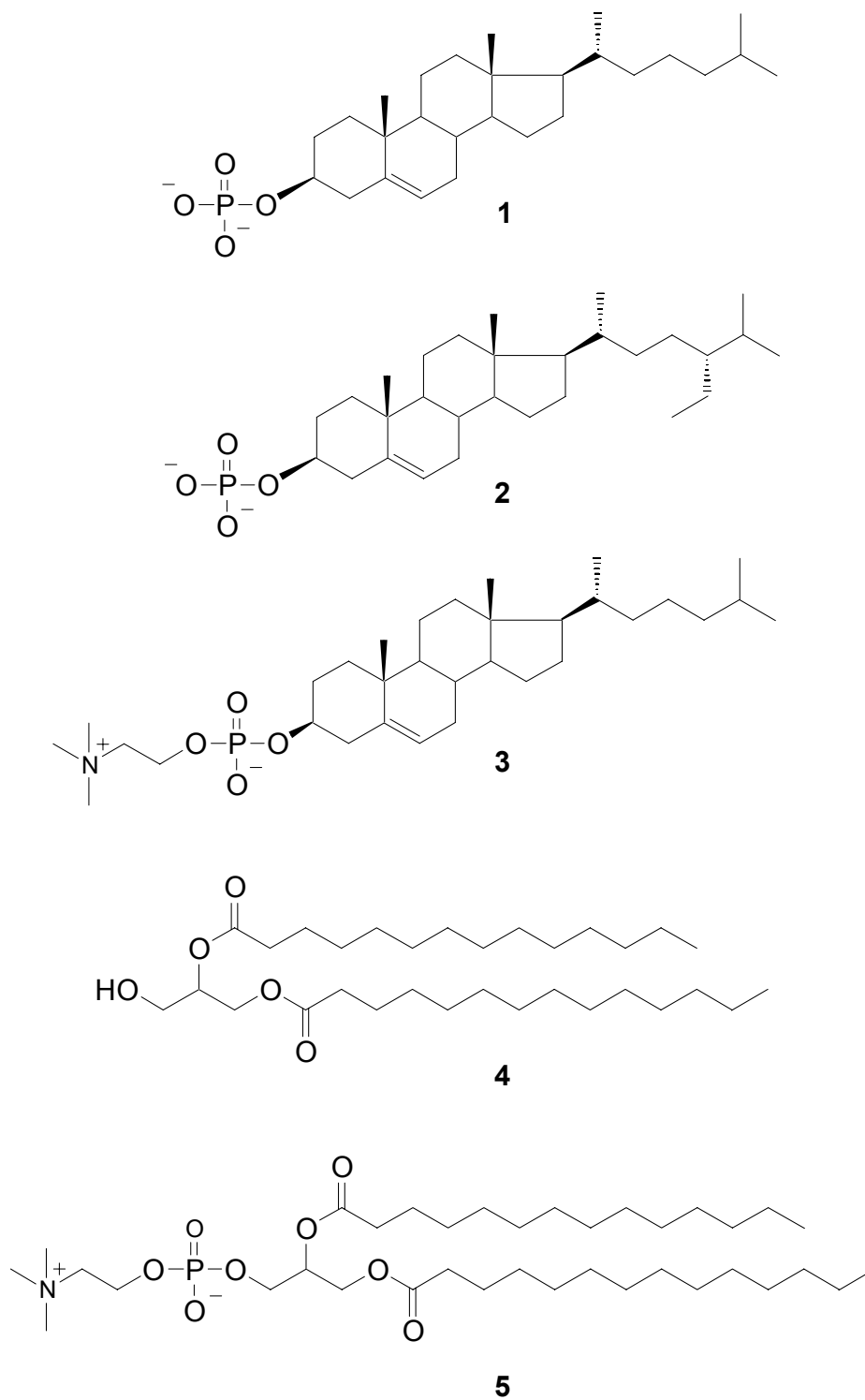
Fig. 2-1   Molecular structure of cholesteryl phosphate **1**, sitosteryl phosphate **2**, cholesteryl phosphorylcholine **3**, dimyristoylglycerol **4**, and L-α-dimyristoyl phosphatidylcholine **5**.
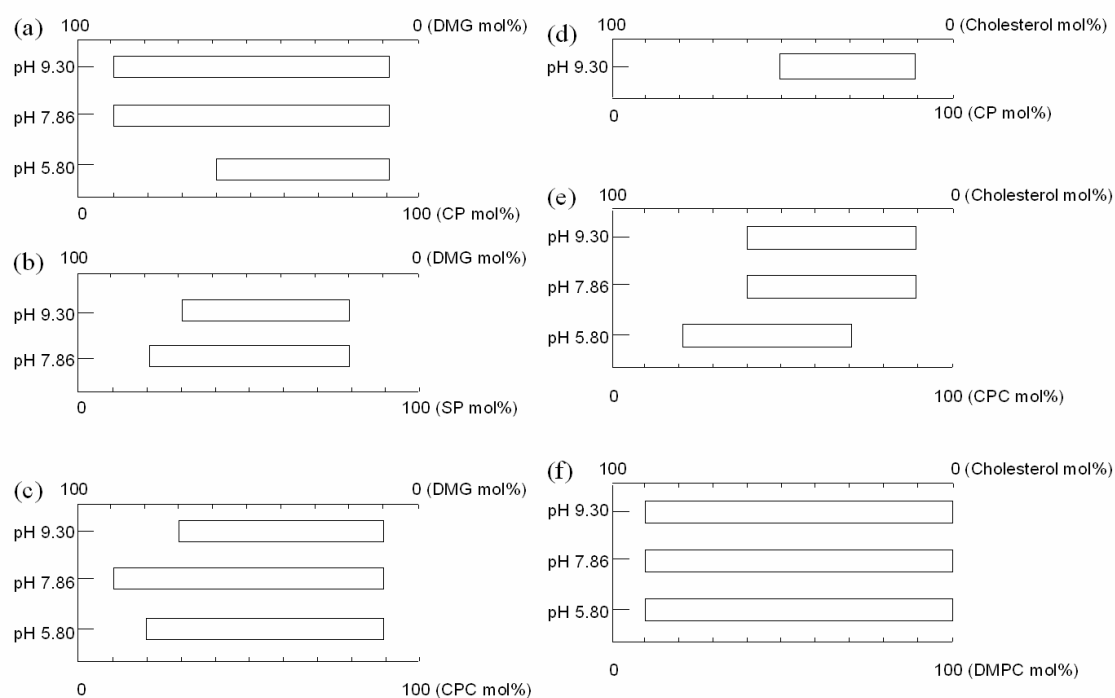
Fig. 2-2 Vesicle formation at three different pHs 5.80, 7.86 and 9.30 from a binary mixture of, on the one hand, cholesteryl phosphate (CP), sitosteryl phosphate (SP) or cholesteryl phosphocholine (CPC) and on the other hand dimyristoylglycerol (DMG) or cholesterol; (a): CP/DMG mixture, (b): SP/DMG mixture, (c): CPC/DMG mixture, (d): CP/cholesterol mixture, (e): CPC/cholesterol mixture, (f): 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)/cholesterol mixture. The boxes define the % range permitting observation of vesicles by fluorescence microscopy.

57
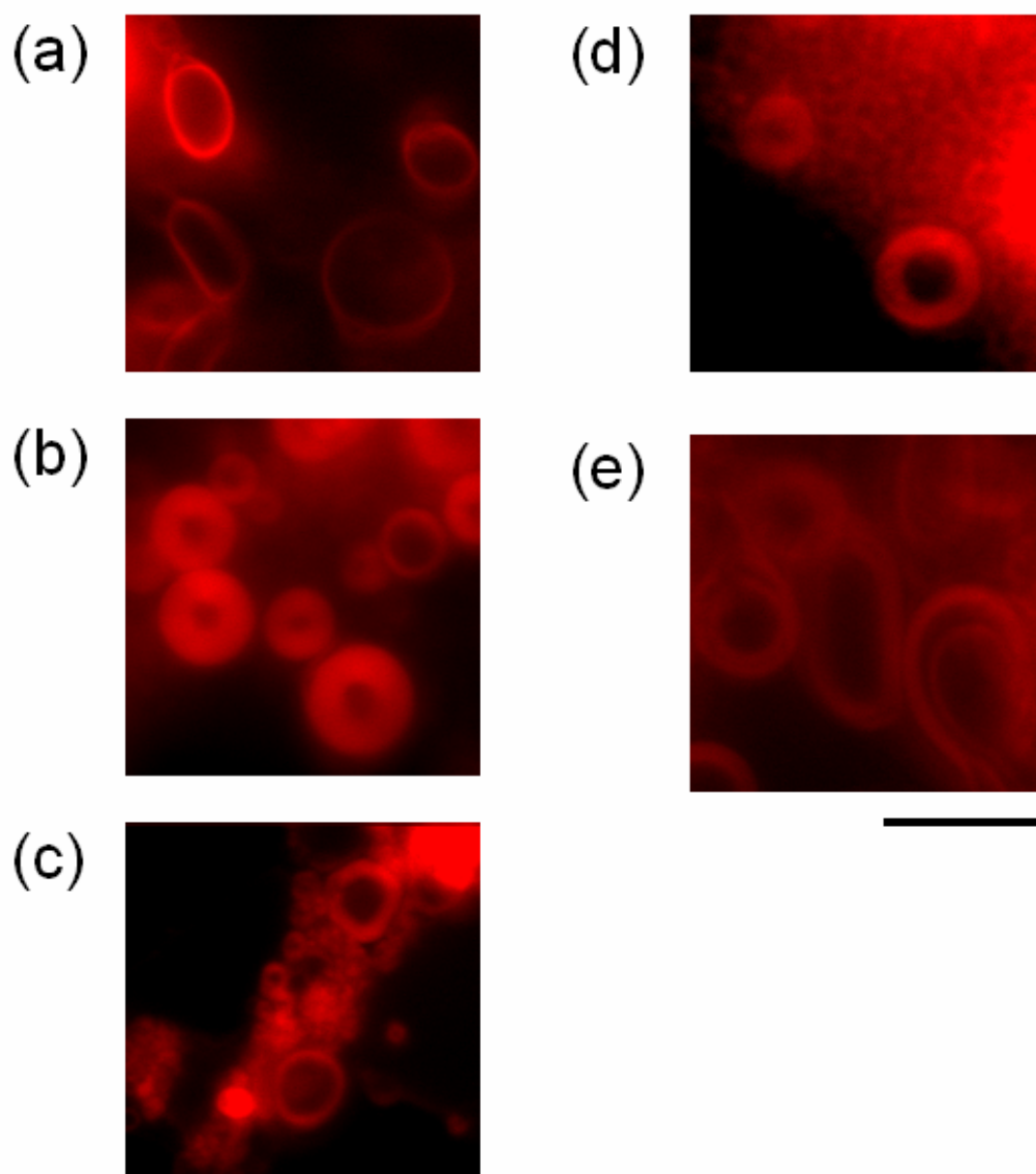
Fig. 2-3 Fluorescence microscopic images of giant vesicles. Vesicle formation from lipid mixture/aqueous systems: cholesteryl phosphate (a), sitosteryl phosphate (b) or cholesteryl phosphorylcholine (c) was mixed with 50% molar dimyristoylglycerol at pH 7.86, cholesteryl phosphate (d) or cholesteryl phosphorylcholine (e) was mixed with 60% molar cholesterol at pH 9.30. The bar represents 10 μm.
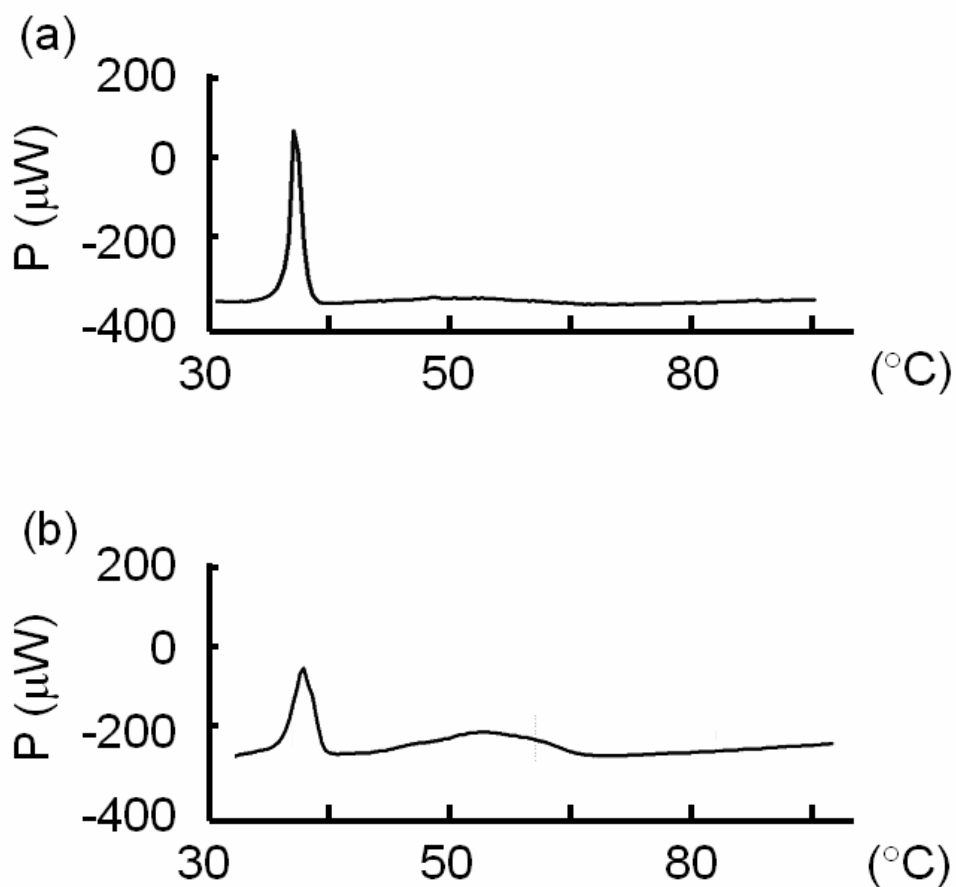
Fig. 2-4   Differential scanning calorimetry measurement of cholesteryl phosphate mixed with 40 mol% dimyristoylglycerol at pH 7.40 (a) and sitosteryl phosphate mixed with 40 mol% dimyristoylglycerol at pH 7.40 (b).   Heating rate: 1°C/min.
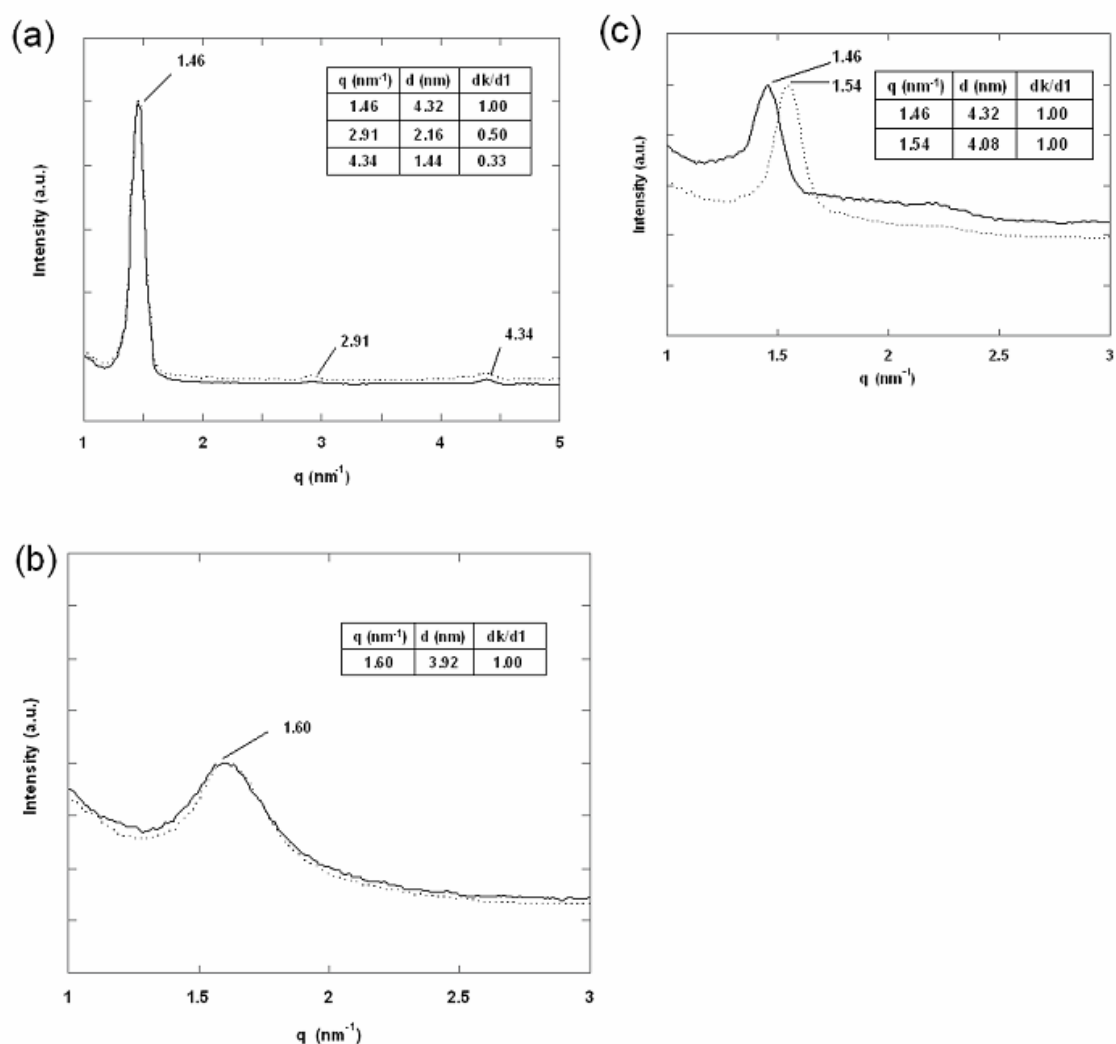
Fig. 2-5   Small angle X-ray scattering (SAXS) profiles: (a) dimyristoylglycerol (DMG) and (b) cholesteryl phosphate (CP) in 50 mM glycine-NaOH (pH 9.30) measured at 25 °C (dotted line) and 45 °C (solid line).   (c) CP mixed with 40 mol% DMG (c) in 100 mM Na$_2$HPO$_4$-NaH$_2$PO$_4$ (pH 7.40) measured at 25 °C (dotted line) and 45 °C (solid line).   q = (4π.sinθ)/λ is the scattering vector, where 2θ is the scattering angle.

Conclusion

At a certain pH and a suitable relative ratio of the mixture of cholesteryl phosphate (CP)/dimyristoylglycerol (DMG), sitosteryl phosphate (SP)/DMG, cholesteryl phosphocholine (CPC)/DMG, CP/cholesterol or CPC/cholesterol formed vesicles. In the case of the mixture of the CP/cholesterol, the vesicle formation was observed only at a higher pH. In the system of SP and sitosterol, no vesicle formation was observed. These results imply that CPC could be a more suitable phosphorylated sterol than CP and SP to build membrane systems different from the usual ones. These results suggest that such cholesteryl phospholipids might be present in the membrane of some organisms hitherto not yet studied; they could also have escaped identification in some "classical" organisms. The fact that they display the membrane-forming properties described here would justify a direct check for their possible presence in natural phospholipid mixtures. However, a mixture of dimyristoyl phosphatidylcholine/cholesterol forms vesicles at any pH and at any relative molar ratio. The pH range for making vesicles of CPC/DMG was rather narrower than that observed for a natural membrane system: a phospholipid/cholesterol mixture. And nowadays biomembranes, which are composed of phospholipids and sterols, acquired such properties that make living organisms can adapt to various environments hence contributing to extended biodiversity.

Materials & methods

General

DMPC, POPC, DMG and 2-chloro-1,3,2-dioxaphospholane-2-oxide were purchased from Sigma (St. Louis, MO). Cholesterol and phosphorus oxychloride was purchased from Fluka (Buchs) and phosphorus oxychloride was distilled before use. Autoclave reactions were carried out in a high-pressure laboratory steel autoclave Model I (Roth, Karlsruhe). Reactions were carried out under an argon atmosphere using flame-dried glassware with magnetic stirring and degassed solvents. THF was distilled from Na/benzophenone. Pyridine and triethylamine were dried and distilled over calcium hydride. Acetone was distilled over potassium carbonate. Column chromatography was carried out on silica gel 60 (70-230 mesh, Merck, La Jolla, CA). ^{1}H NMR at 300 MHz, ^{13}C NMR at 75 MHz and ^{31}P NMR spectra at 121 MHz, were recorded with Bruker AVANCE 300 spectrometers. Chemical shifts (δ) in ppm with respect to CD_3OD as internal standards for ^{1}H and ^{13}C NMR, and to H_3PO_4 as external standard for ^{31}P NMR. Significant ^{1}H NMR data are tabulated in the following order: chemical shift (δ) expressed in ppm, multiplicity (s, singlet; d, doublet; m, multiplet). Mass spectra were recorded on a micro time-of-flight (TOF) spectrometer using atmospheric pressure chemical ionization (APCI) methods for compound **1** and **2**, and electrospray ionization (ESI) method for compound **3** in methanol + 0.1% formic acid. HPLC was carried out using Polaris C18 column (Varian, Inc., CA).

1-1. Synthesis of cholesteryl phosphate (CP) 1

To a solution of cholesterol (5 g, 12.9 mmol) in dry pyridine (50 ml), distilled phosphorus oxychloride (1.2 ml) in dry acetone (25 ml) was added at 0 °C under argon. Precipitation was observed immediately and the mixture was vigorously stirred for 2 hours at room temperature. The mixture was filtered and the resulting solid (cholesteryl phosphochloridate **6**) was washed with cold dry acetone, then hydrolyzed by distilled water (150 ml) under reflux overnight. All solutions were evaporated and bumping was avoided by continuous addition of THF. The residues were crystallized twice from dioxane, followed by washing with diethyl ether (twice 25 ml) to afford 4.23 g (yield: 64%) of cholesteryl phosphate **1** in the form of the monoacid/monopyridinium salt. ^{1}H NMR (300 MHz, CD$_3$OD): 0.65 (*s*, 3H), 0.81 (*d, J* = 6.6 Hz, 3H), 0.83 (*d, J* = 6.6 Hz, 3H), 0.88 (*d, J* = 6.5 Hz, 3H), 0.96 (*s*, 3H), 4.01-4.09 (*m*, 2H), 5.31-5.36 (*m*, 1H); ^{13}C NMR (75 MHz, CD$_3$OD): 11.6, 18.4, 19.0, 20.2, 22.2, 22.5, 23.7, 24.1, 27.8, 28.1, 29.4, 29.5, 31.8, 35.7, 36.1, 36.3, 36.9, 39.4, 39.7, 39.9, 42.2, 50.0, 56.1, 56.6, 77.1, 122.4, 139.7: ^{31}P NMR (300 MHz, CD$_3$OD): 0.63; MS (APCI): 465.31 [M + H$^+$].

1-2. Synthesis of sitosteryl phosphate (SP) 2

Sitosteryl phosphate **2** was prepared in the same way as cholesteryl phosphate. Briefly, to a solution of sitosterol (8.66 g, 20.9 mmol) in dry pyridine (86 ml), phosphorus oxychloride distilled (1.95 ml) in dry acetone (42 ml) was added at 0 °C under argon. The procedure described above for the synthesis of the phosphate **1** is then followed. Sitosteryl phosphate

2 in the monoacid/monopyridinium salt was obtained in 3.54 g (yield: 29 %).

0.80; ^{1}H NMR (300 MHz, CD$_3$OD): 0.64 (*s*, 3H), 0.77 (*d, J* = 6.6 Hz, 3H), 0.81 (*d, J* = 7.2 Hz, 3H), 0.88 (*d, J* = 6.2 Hz, 3H), 0.97 (*s*, 3H), 4.00-4.06 (*m*, 1H), 5.31-5.35 (*m*, 1H); ^{13}C NMR (75 MHz, CD$_3$OD): 11.6, 11.7, 18.6, 18.8, 19.1, 19.6 20.9, 22.9, 24.1, 26.0, 28.1, 29.0, 29.5, 31.7, 31.8, 33.8, 36.1, 36.3, 36.9, 39.6, 39.9, 42.2, 45.7, 50.0, 56.0, 56.6, 77.0, 122.4, 139.8: ^{31}P NMR (121 MHz, CD$_3$OD); MS (APCI): 493.1 [M + H$^+$].

1-3. Synthesis of cholesteryl phosphocholine (CPC) 3

To a solution of cholesterol (6 g, 16 mmol) in dry THF (26 ml), 2-chloro-1,3,2-dioxaphospholane-2-oxide **7** (1.5 ml, 16 mmol) was added dropwise with stirring at −5 °C under argon, followed by addition of triethylamine (2 ml).   After 4 hours of stirring at room temperature, the reaction mixture was filtered to remove the ammonium salt, and the filtrate was evaporated to obtain a white solid of cholesteryl cyclic phosphate **8**. In an autoclave, the cyclic phosphate **8** (6.56 g, 14 mmol), liquid trimethylamine (3.3 ml) and THF (18 ml) were stirred for 24 hours at 40 °C. The resulting mixture was dissolved in chloroform/methanol (2:1).   After removal of the all solvents in vacuum, the residue was purified by silica gel chromatography.   Unreacted materials were eluted with a mixture of dichloromethane/methanol (95:5; 50:50), then compound **3** was collected by using a mixture of dichloromethane/methanol/water (2.5:7:0.5).   After evaporation of the solvents in vacuo, the residue was crystallized from methanol/acetone   to   afford   2.0   g   (yield: 22%)   of   cholesteryl

phosphocholine **3** as a white solid. Purity was determined by HPLC analysis to be over 95%. ^{1}H NMR (300 MHz, CD$_3$OD): 3.21 (*s*, 9H), 3.60-3.64 (*m*, 2H), 3.94-4.02 (*m*, 1H), 4.21-4.25 (*m*, 2H), 5.36-5.39 (*m*, 1H); ^{13}C NMR (75 MHz, CD$_3$OD): 53.3, 58.8, 66.0, 75.7, 121.7, 140.2: ^{31}P NMR (121 MHz, CD$_3$OD): 0.24; MS (Electrospray, methanol + 0.1% formic acid): 552.44 [M + H^{+}].

2. Microscopic observation

Nile Red (ex/em: 559/640 nm) was added (2 mol%) to solution of DMPC, CP, SP, CPC or mixture of CP, SP or CPC with DMG in chloroform/methanol, and then the sample was mounted on a glass slide (0.17 mm thick). Before the solution was applied, the glass slide was circumscribed with a hydrophobic PAP-pen (Daido Sangyo Co., Tokyo). After drying the solvents at room temperature, the lipid film on the glass was hydrated by the addition of 50 mM citric acid-Na$_2$HPO$_4$ (pH 5.80), 50 mM Tris-HCl (pH 7.86) or 12.5 mM borate-NaOH (pH 9.30) buffer to reach a final concentration of 2.5 mg/ml. The buffers had been previously filtered through 0.22 μm filters (Millex GS, Millipore, Bedford, MA) to remove dust. The sample was observed by differential interference contrast microscopy: Axiovert 135, 63 x /1.40 plan Achromat Oil DIC objective, × 2.5 insertion lens (Carl Zeiss, Thornwood, NY), light sources: halogen lamp and Hg laser, Filter sets 09 (excitation BP 450–490, FT 510, emission LP 515), video system: CCD camera (C2400-75H) and image processor (Argus 20), Hamamatsu Photonics (Hamamatsu).

3. Differential scanning calorimetry (DSC)

The high sensitivity calorimetric scans were performed on a DASM-4 microcalorimeter, usually at the rate of 1 ºC/min (heating).   CP, SP or CPC was mixed with 40 mol% DMG (total weight: 5 mg) and then dissolved in a 300 μl of chloroform/methanol (1:1, v/v).   The solvents were evaporated under reduced pressure at room temperature, and the resulting film was dried under vacuum.   Subsequently, the substances were hydrated by addition of 2 ml buffer (100 mM Na_2HPO_4-NaH_2PO_4, pH 7.40) and sonicated 1 h in a water bath (Sonorex RK 100H, Bandelin electronic, Berlin).

4. Small angle X-ray scattering (SAXS)

The phase structures were examined by a small-angle X-ray scattering (SAXS) technique, where Ni-filtered CuKα radiation (wave length = 0.154 nm) generated by a Rigaku RU-200 X-ray generator (40 kV, 100 mA) with a double pinhole collimator (0.5 mm Φ × 0.3 mm Φ) was employed.   The lipid/buffer samples with an excess buffer solution (50 mM glycine-NaOH, pH 9.30 and 100 mM Na_2HPO_4-NaH_2PO_4, pH 7.40) were prepared.   To ensure equilibrium, the lipid/buffer samples were incubated at least 15 - 20 hours at each temperature before the SAXS measurements were performed.   The sample temperature was controlled with a Mettler FP82HT hot-stage within an accuracy of ± 0.5 °C.

5. Stopped-flow/light-scattering method

1) Vesicle preparation

CPC was mixed with 50 or 80 % molar DMG, and DMPC was mixed with 50 % molar cholesterol to make a total weight of 30 mg, and each mixture was dissolved in 1 ml of chloroform/methanol (1:1, v/v). The following procedures were conducted as described in the previous chapter (see Chapter 1 for more detail).

The preparation of the vesicles made from the mixture of CP or SP with 50 mol% DMG was also tried. However, after the freeze-thaw procedures, precipitation took place for these samples and the water permeability of their vesicles was not possible.

Chapter 3

Molecular recognition on giant vesicles : coating of vesicles with a polysaccharide bearing phytyl chains or cholesteryl moieties

Introduction

Biomembranes often are surrounded by a cell wall, selectively recognizing exterior molecules. The aim of our study is to find a possible pathway how "primitive" membranes could evolved towards a cell wall-like structure. We have postulated that polyprenyl phosphates could be phylogenetic precursors of archaeal lipids [8]. In collaboration with Sunamoto's group, we have previously shown that vesicles made of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) could be coated by a polysaccharide, pullulan, once substituted by a cholesteryl moiety (cholesteryl-pullulan) and that vesicles made of phytyl phosphate or 2,3-diphytanyl-*sn*-glycero-1-phosphocholine (DphPC) could be coated by pullulan, once substituted by a phytyl chain (phytyl-pullulan) [45, 46]. In Nature, polyprenyl chains, such as undecaprenyl chain or dolichol chain, anchoring in membranes, play an important role in the synthesis of peptidoglycans or polysaccharides, by locating it on the surface of membranes [47, 48]. We now study the coating of vesicles made of different types of lipids cited below by the above hydrophobized pullulans, to which was covalently attached a fluorescent tag, to make it possible to observe the coated vesicles by optical fluorescence microscopy. Giant vesicles (5 μm and more) were prepared from phytanyl phosphate, phytyl phosphate (as examples of "primitive" membrane lipids), DphPC (as an example of archaeal membrane lipid), and POPC (as an example of eukaryotic membrane lipid) (Fig. 3-1). We have studied the binding of lectins such as concanavalin A (Con A) or annexin V to the surface of

vesicles already coated by the hydrophobized polysaccharides. The results obtained show that spontaneous molecular recognition does occur successively on giant vesicles and this might be a possible complexification pathway of biomembranes [11, 49].

Results & discussion

1. Coating of preformed vesicles with cholesteryl-pullulan

Our previous study showed that preformed vesicles made of phytyl phosphate or 2,3-diphytanyl-*sn*-glycero-1-phosphocholine (DphPC) would not be coated with cholesteryl-pullulan [46]: the cholesteryl moiety does not insert spontaneously into the lipophilic parts of these membranes, due to the mismatch of molecular shape between cholesterol and phytol or phytanol.　As a further step, we have now attempted coating preformed vesicles made of phytanyl phosphate (Fig. 3-2 (a)), DphPC (Fig. 3-2 (b)) or POPC (Fig. 3-2 (c)) with fluorescein iso-thiocyanate (FITC)-cholesteryl-pullulan.　Fig. 3-2 (left) shows preformed giant vesicles observed under phase-contrast microscopy, and images of Fig. 3-2 (b, c) (right) show the corresponding vesicles stained with the green fluorescence color of FITC.　This indicates that these giant vesicles were coated by the polysaccharide; the cholesteryl moiety being inserted into membranes [50].　The preformed vesicles of POPC, a eukaryotic membrane constituent, were treated with FITC-cholesteryl-pullulan (Fig. 3-2 (c)), and shown to be coated, as we had reported earlier [46].　On the other hand, as reported earlier, cholesteryl-pullulan did not coat vesicles of DphPC, after 30 min incubation [46].　However we have now shown that FITC-cholesteryl-pullulan could coat DphPC vesicles after overnight incubation as shown in Fig. 3-2 (b).　This implies that the insertion of the cholesteryl moiety in the DphPC vesicles (Archaeal lipid) is not impossible, but much slower than its insertion into POPC vesicles, which can be coated

within 30 min.   On the other hand, FITC-cholesteryl-pullulan could not coat phytanyl phosphate vesicles even after overnight incubation (Fig. 3-2 (a)).   Therefore, these results suggest that cholesterol could not insert itself satisfactorily into membranes made of single-chain lipids, which were speculated as primitive membrane constituents.

2. Coating of preformed vesicles with phytyl-pullulan

Our previous study had demonstrated the coating with phytyl-pullulan of preformed vesicles made of phytyl phosphate or DphPC [46].   We examined whether phytyl-pullulan could coat DphPC or POPC vesicles.   In this study, to detect the non-fluorescence labeled phytyl-pullulan coating of the surface of vesicles, we have employed a polysaccharide-binding protein.   Fluorescence labeled Concanavalin A (Alexa Fluor 647-Con A) was used to detect phytyl-pullulan coating on the surface of vesicles.   Con A is a well known lectin that has ligand specificity for α-glucopyranosyl or α-mannopyranosyl residues in the presence of calcium ions [51].   Since pullulan is composed of three repeated glucose units, if phytyl-pullulan coated vesicles, we should be able to detect the red color on the vesicle surface, due to the binding of Con A to pullulan.   As a negative control, when a sample was incubated without phytyl-pullulan, the fluorescence of Con A was not detected on the surface of vesicles (data not shown).   As shown in Fig. 3-3 (a), a clear fluorescence due to Con A was observed on the surface of DphPC vesicles. A similar observation was made with POPC vesicles (Fig. 3-3 (b)).   In Nature, some analogous examples were found.   For example, murein, a

major peptidoglycan of Archaea, is synthesized on the surface of membranes, anchored by an undecaprenyl group [47]. Dolichol phosphate, bearing 16-20 isoprenyl units, is located in the endoplasmic reticulum membrane with its phosphate terminus on the cytosolic face; oligosaccharides are synthesized from this compound by a series of enzymatic transfer reactions of sugar units on the membrane surface [48]. Here, we see again examples of the shape-matching relation required between the membrane constituents and the hydrophobic molecules to be inserted.

Next we wondered whether phytyl phosphate and POPC could make vesicles together or separately. For this purpose, we investigated the coating of vesicles made of a 1:1 molar mixture of phytyl phosphate and POPC by FITC-cholesteryl-pullulan. Microscopic observation revealed that all vesicles were stained by FITC-cholesteryl-pullulan (Fig. 3-4). This indicates that phytyl phosphate and POPC could make mixed vesicles and that these vesicles allow cholesterol to enter into their membranes even in the presence of the phytyl moiety.

3. Binding assay between various proteins and various polysaccharides

To confirm the above results and to detect the localization of pullulan and Con A, we studied the coating of vesicles with FITC-cholesteryl-pullulan and with Alexa Fluor 647-Con A. Fig. 3-5 shows the doubly stained vesicles of DphPC or POPC with the green fluorescence color of FITC and the red fluorescence color of Alexa Fluor 647-Con A, resulting in an orange appearance in overlay images. These

observations indicate the clear co-localization of pullulan and Con A on the surface of giant vesicles.

We used this method for the investigation of the recognition of lectins by polysaccharides coating the surface of giant vesicles.   Here we used some lectins such as Con A, GS-II (from *Griffonia simplicifolia*) and annexin V [52, 53].   First, as a negative control, we confirmed that these lectins could not bind to DphPC and POPC vesicles, which are not pre-coated with polysaccharides.   The results were shown in Fig. 3-6 and were summarized in Table 3-1; Con A and annexins V could bind to DphPC and POPC vesicles (one example: see Fig. 3-6 (b)), which are pre-coated by phytyl-pullulan or cholesteryl-pullulan-COOH.   These bindings could be detected after 2 hours incubation.   On the other hand, GS-II could not bind to any vesicles (one example: see Fig. 3-6 (a)); GS-II is known to bind to terminal, non-reducing α- or β-*N*-acetyl-D-glucosaminyl residues [54, 55].

	DphPC		POPC	
	phytyl-pullulan	cholesteryl-pullulan-COOH	phytyl-pullulan	cholesteryl-pullulan-COOH
Con A	+	+	+	+
GS-II	−	−	−	−
Annexin V	+	+	+	+

Table 3-1:   Recognition by concanavalin A (Con A), GS-II or annexin V of 2,3-diphytanyl-*sn*-glycero-1-phosphocholine (DphPC) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles pre-coated with phytyl-pullulan or cholesteryl-pullulan-COOH.

Fig. 3-1   Chemical structure of molecules used in this study.

(a) phytanyl phosphate, (b) 2,3-diphytanyl-*sn*-glycero-1-phosphocholine (DphPC), (c) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), (d) phytyl-pullulan or cholesteryl-pullulan, (e) cholesteryl-pullulan-COOH
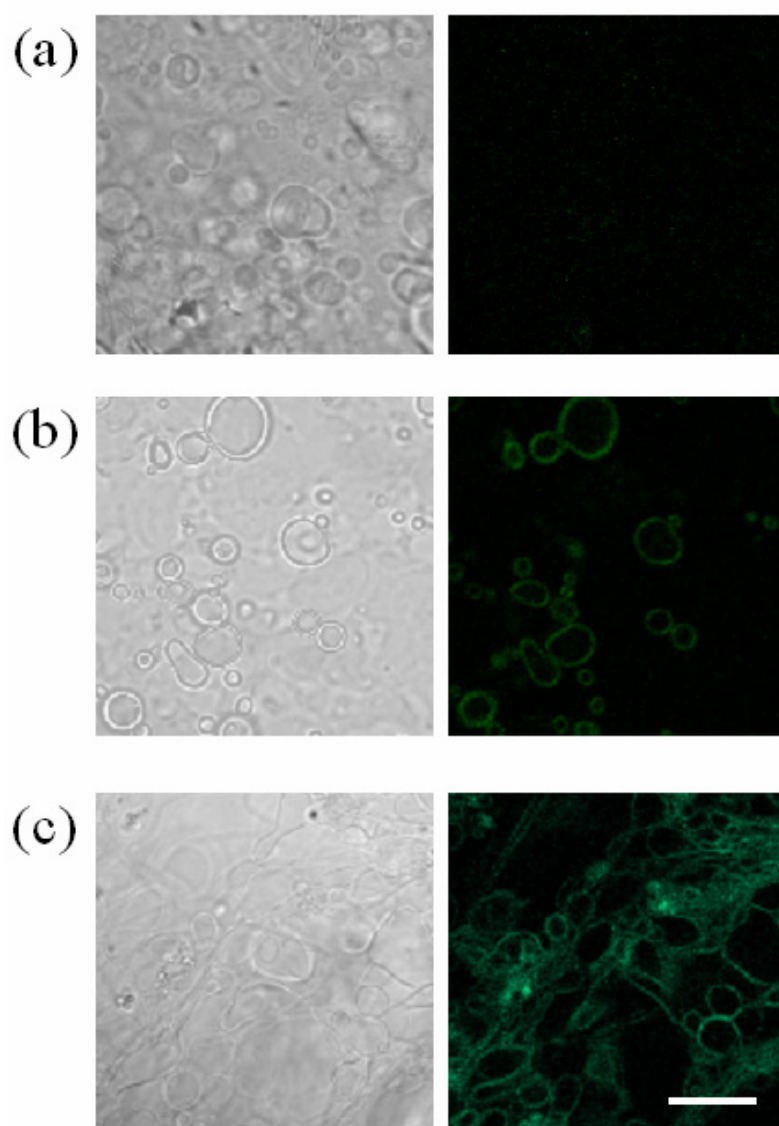
Fig. 3-2　Fluorescence microscopy images of giant vesicles.　Preformed vesicles of phytanyl phosphate (a), 2,3-diphytanyl-*sn*-glycero-1-phosphocholine (b), or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (c) were incubated overnight with FITC–labeled cholesteryl pullulan.　The images on the left show vesicles observed by phase-contrast microscopy and the images on the right were obtained by fluorescence microscopy.　The bar represents 20 μm.
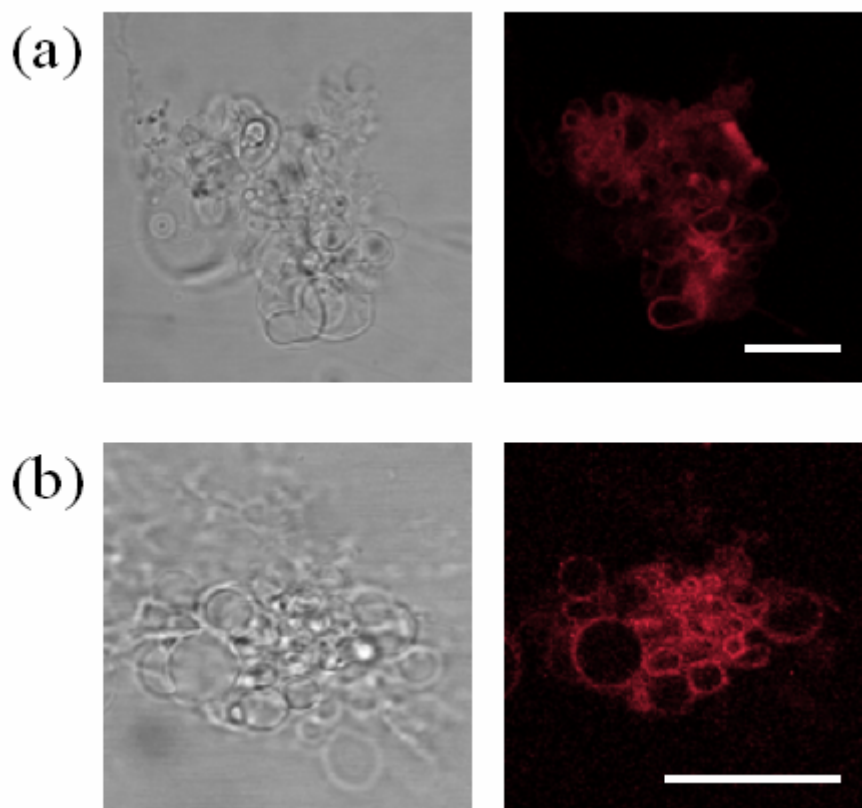
Fig. 3-3   Fluorescence microscopy images of giant vesicles.   Preformed vesicles of 2,3-diphytanyl-*sn*-glycero-1-phosphocholine (a), or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (b) were incubated for 2 h with phytyl-pullulan and then for overnight with Alexa Fluor 647-Concanavalin A.   The images on the left show vesicles observed by phase-contrast microscopy and the images on the right were obtained by fluorescence microscopy.   The bar represents 20 μm.
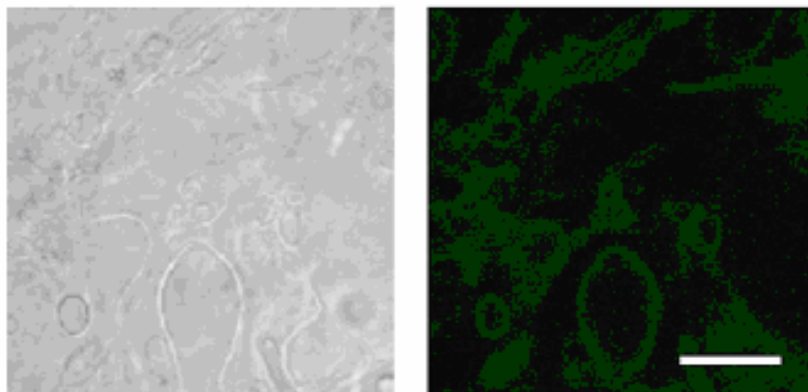
Fig. 3-4   Fluorescence microscopic images of giant vesicles of 1:1 molar mixture of phytyl phosphate and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC).   A thin lipid film of a 1:1 molar mixture of phytyl phosphate and POPC was swollen with FITC-cholesteryl-pullulan.   The images on the left show vesicles observed by phase-contrast microscopy and the images on the right were obtained by fluorescence microscopy.   The bar represents 20 μm.
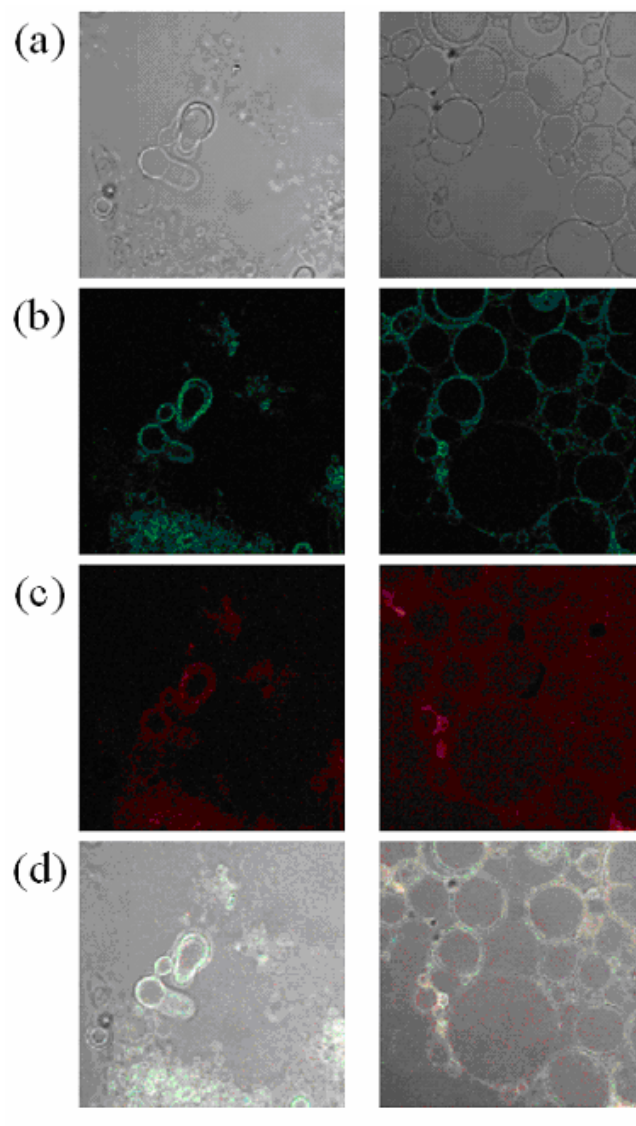
Fig. 3-5 Double fluorescence stain images of giant vesicles of 2,3-diphytanyl-*sn*-glycero-1-phosphocholine (DphPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). Preformed vesicles of DphPC (left) or POPC (right) were incubated overnight with FITC-cholesteryl-pullulan, followed by the addition of Alexa Fluor 647-Concanavalin A. (a): images of giant vesicles by phase-contrast microscopy; (b): images obtained by fluorescence microscopy with the FITC; (c): images obtained with Alexa Fluor 647 fluorescence; (d): overlay images of (a), (b) and (c). The bar represents 20 μm.
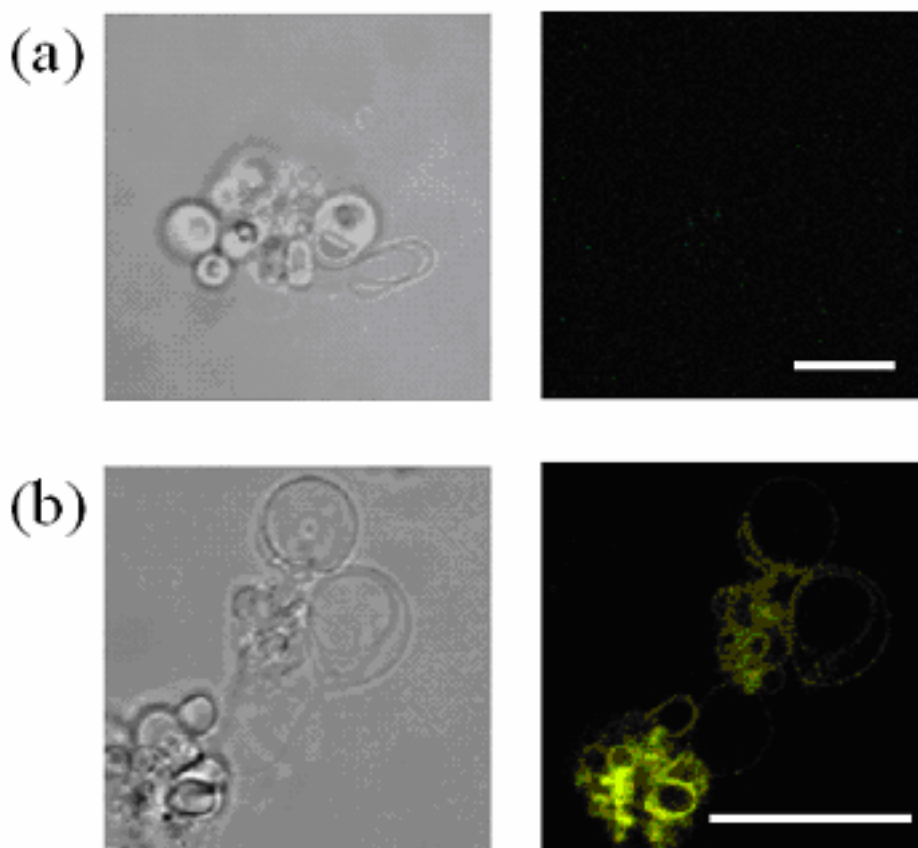
Fig. 3-6   Fluorescence microscopy images of giant vesicles. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine   (POPC)   vesicles incubated overnight with phytyl-pullulan followed by the addition of Alexa Fluor 488-GS-II (a) or Cy3.18-annexin V (b).   The images on the left show vesicles observed by phase-contrast microscopy and the images on the right were obtained by fluorescence microscopy.   The bar represents 20 μm.
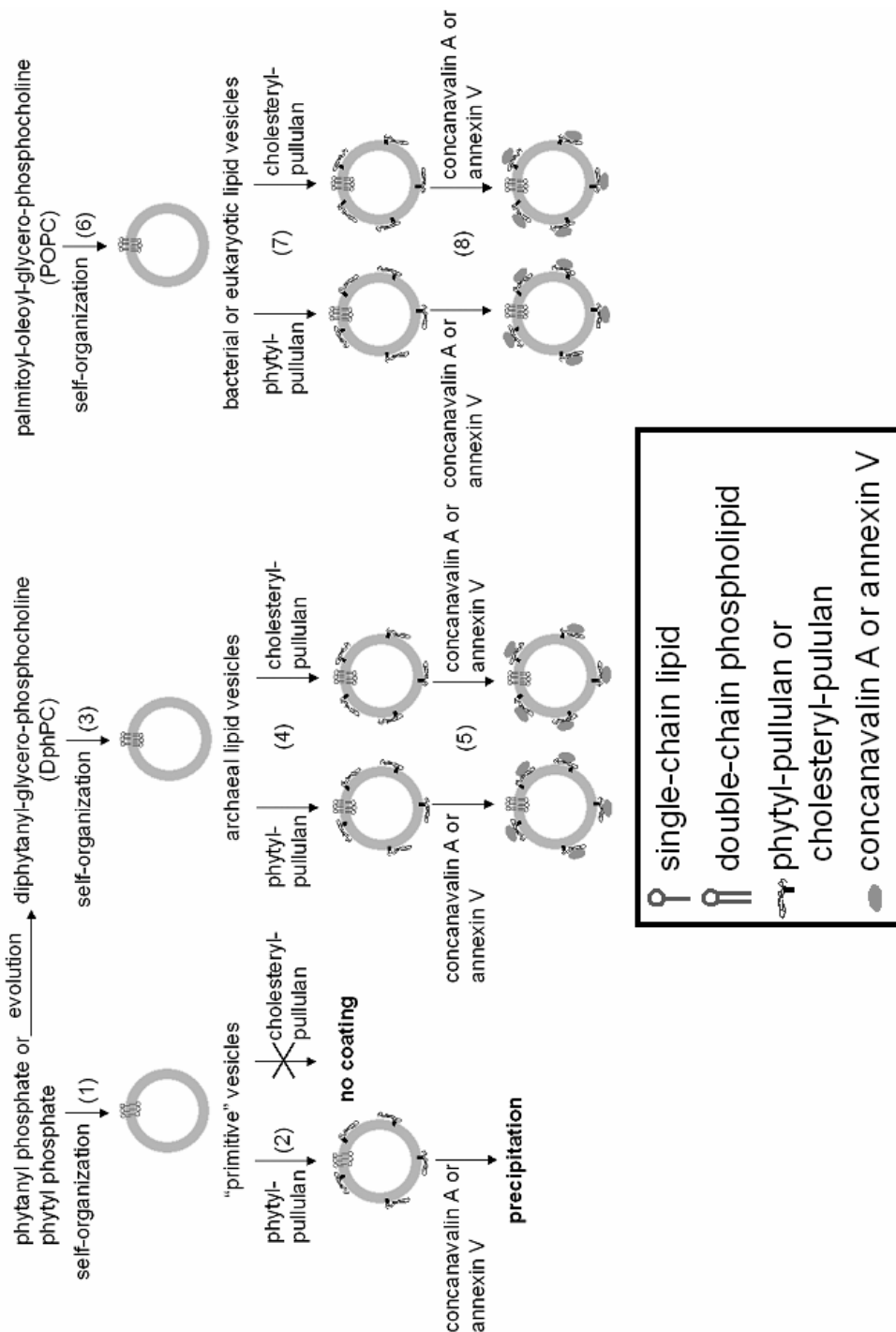
Scheme 3-1: Schematic representation of self-organization of amphiphiles into vesicles, followed by coating of their surface with cholesteryl-pullulan or phytyl-pullulan, leading to an assembly analogous to a cell wall. These structures possess binding affinity for lectins. The x-indication shows no coating of vesicles.

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Conclusion

We now summarize the results obtained in this paper and our previous short communications in Scheme 3-1 [45, 46]. We observed the spontaneous formation of vesicles from "primitive" single-chain lipids (step 1 in the Scheme 3-1); the vesicles could then be coated by phytyl-pullulan, leading to a cell wall-like structure; however, they are not stable in the presence of lectins, and their development stopped here. On the contrary, once the formation of double-chain lipid vesicles occurs (steps 3 and 6), the coating of the vesicles by phytyl- or cholesteryl-pullulan followed (steps 4 and 7), leading to a cell wall-like structure; lectins then bind to the polysaccharide on the surface of the vesicles (steps 5 and 8). This is analogous to the coating of membranes of Archæa, Bacteria and Eukarya by polysaccharides. Our findings thus show a clear advantage of double-chain lipid vesicles over single-chain lipid ones, which might justify the evolution from single-chain to double-chain lipids.

Materials & methods

General

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and Cy3.18-annexin V (ex/em: 554/568) were purchased from Sigma (St. Louis, MO). 5-Dodecanoylaminofluorescein (D109, ex/em: 496/516), Alexa Fluor conjugates of lectin GS-II from *Griffonia simplicifolia* (Alexa Fluor 488-GS-II, ex/em: 495/519) and Alexa Fluor 647-Concanavalin A (Con A) (ex/em: 650/668) were obtained from Molecular Probes (Eugene, OR). Fluorescein isothiocyanate (FITC) (ex/em: 494/519)-cholesteryl-pullulan, phytyl-pullulan, 2, 3-diphytanyl-*sn*-glycero-1-phosphocholine (DphPC) and phytanyl phosphate were synthesized as previous report [46]. Cholesteryl-pullulan-COOH (21.7 carboxylic acid groups per 100 glucose units) was given by Professor K. Akiyoshi (Tokyo medical and dental University). These compound structures were showed in Fig. 3-1.

1. Vesicle preparation

Preformed vesicle suspensions were prepared as follows. All prepared buffers were filtered through 0.22 μm filters (Millex GS, Millipore, Bedford, MA) to remove dust. One mg of POPC, DphPC or phytanyl phosphate was dissolved in 200 μl of chloroform/methanol (1:1, v/v). To make a thin dry film of the lipid, the chloroform and methanol were evaporated. Then, buffer solution (Buffer A: 50 mM Tris-HCl, pH 7.86, 5 mM CaCl$_2$, 150 mM NaCl) was added to reach a final concentration of 5 mg/ml and incubated for 30 min at room temperature. The buffer A

was used for all subsequent procedures of coating experiments with POPC, DphPC or phytanyl phosphate vesicles. And vesicle suspensions were obtained by natural swelling.

The spontaneous vesicle formations of all samples were observed by optical microscopy.

2. Coating of preformed vesicle with cholesteryl-pullulan

FITC-cholesteryl-pullulan (2 mg) was suspended in 1 ml of chloroform/methanol (1:1, v/v) and sonicated in the dark. The FITC-cholesteryl-pullulan solution (50 μl) was moved onto a glass tube and solvents were dried. Then, to hydrate the solid remaining on the glass tube surface, 50 μl of solution containing vesicle suspension was added. Sample was incubated at room temperature for 30 min or overnight in the dark. The coating of preformed phytanyl phosphate, DphPC or POPC vesicles with FITC-cholesteryl-pullulan was checked by using confocal microscope.

3. Detection of coating of preformed vesicle with phytyl-pullulan

The coating experiments of preformed vesicles with phytyl-pullulan were performed using the same procedure described above for the experiment with FITC-cholesteryl-pullulan. Briefly, preformed vesicle suspension was added to the glass tube, which surface had been covered with a thin film of phytyl-pullulan, and the suspension was incubated for 2 h at room temperature. To this suspension, the buffer containing 1 mg/ml Alexa Fluor 647-Con A (50 μl) was then added and incubated for overnight

at room temperature.   As a control, sample incubated without phytyl-pullulan was prepared.   The localization of Con A was analyzed by confocal microscopy.

4. Coating of mixture of phytanyl phosphate and POPC vesicles with FITC-cholesteryl-pullulan

The 1:1 molar mixture of phytanyl phosphate and POPC (total weight: 2.5 mg) were dissolved in a 1 ml of chloroform/methanol (1:1, v/v). This solution (10 μl) was dropped on the slide glass, and solvents were evaporated to obtain a thin lipid film, which was then swollen with 10 μl of buffer (50 mM Tris-HCl, pH 7.86) containing FITC-cholesteryl-pullulan (2 mg/ml) for 30 min at room temperature in the dark.

5. Interaction between polysaccharide moiety of vesicle surface and proteins

The experiments for the interaction between lectin and polysaccharide moiety was performed using the same procedure described above for the experiment of detection of coating of preformed vesicle with phytyl-pullulan.   Briefly, 25 μl of preformed vesicle suspension of POPC or DphPC was added to the glass tube, which surface had been covered with a thin film of phytyl-pullulan or cholesteryl-pullulan-COOH (50 μg). The suspension was incubated for 2 h at room temperature.   To this suspension, the buffer containing Alexa Fluor 647-Con A (1 mg/ml), Alexa Fluor 488-GS-II (1 mg/ml) or FITC-annexin V (0.04 mg/ml) was then added and incubated for 1, 2, 3 h or overnight at room temperature in the

dark.   As a control, sample incubated without phytyl pullulan or cholesteryl-pullulan-COOH was prepared.   The localization of Con A or annexin V was analyzed using confocal microscopy.

6. Confocal microscopic observation

Confocal images were acquired with an inverted Zeiss LSM 510 using the 488 nm argon laser or the 543nm, 633 nm HeNe laser line. Alexa Fluor 647 emission fluorescence image was detected using a LP 635 nm emission filter.   Cy3.18 emission fluorescence image was detected using a BP530-600 nm filter.   FITC, Alexa Fluor 488 and 5-dodecanoylaminofluorescein emission fluorescence image was detected a BP505-530 nm filter.   A negative control sample was observed in the same condition of laser scanning setup.

Chapter 4

Annexins A1 and A4 inhibit *Staphylococcus aureus* attachment to human macrophages

Introduction

Annexins are a family of structurally related proteins that bind to phospholipids and carbohydrates in the presence of calcium ions [12-14]. They are widely distributed in mammals bodies, extracellular annexins have been found in lung [56-61], plasma [62-68], intestine [69], bile [70], and prostatic secretions [71], where infectious agents must be cleared by an efficient host defense system. Moreover the concentration of annexins in extracellular fluids is remarkably increased during various diseases and by treatment with glucocorticoid, which is one of anti-inflammatory hormone [60-63]. For these reasons it has been proposed that annexins might play a role in immune system.

Since annexins were identified as phospholipase A_2 inhibitors [14], the evidences of annexins as modulators of inflammation have been widely provided. Annexin A1 contributes to the resolution of inflammation through the regulation of interleukin-6 and tumor necrosis factor-α, both of which are considered to be major inflammatory cytokines [72]. Annexin I reduced neutrophil and monocyte infiltration in several animal models [73-75]. In addition, cell-surface receptors for annexins on immune cells such as monocytes, macrophages and neutrophils have also been found [76-78]. Therefore annexins potentially participate in immunological process at multiple levels from cellular to systemic, although the mechanism still remains unclear. More recent studies have shown that annexins A1 and A2 themselves bind to lipid A of Gram-negative bacteria and suppress cellular responses to endotoxin [79], suggesting that annexins

function as modulators of anti-inflammation via recognition of foreign substance.

These lines of evidence have led us to hypothesize that annexins might also have some anti-inflammatory property for Gram-positive bacteria stimuli in host defense. In this study, to gain insight into anti-inflammatory cellular mechanisms of annexins against Gram-positive bacteria, the interaction of annexins A1 and A4, which are known to be especially abundant in lung and blood, with lipoteichoic acids and Gram-positive bacteria have been investigated. In addition the effects of annexins A1 and A4 on attachment of macrophages and Gram-positive bacteria are reported.

Results & discussion

1. Binding of annexins to lipoteichoic acid

Annexins are calcium-dependent phospholipids and carbohydrates binding proteins with anti-inflammatory properties [12-14]. Previous studies have revealed that annexins bind to lipid A, which is present in the envelope of Gram-negative bacteria and cause significant inflammation [79]. Lipid A is a phosphoglycolipid that consists of an acyl chain and phophorylated dihexamine head group. Therefore it has been suggested that annexins bind to lipid A in a manner similar to that for phospholipids. And the direct binding of annexins to lipid A suppressed inflammation responses, these results indicate annexins have anti-inflammatory properties for Gram-negative bacteria [79]. Besides, our study investigates the possibility of annexins binding ability to lipoteichoic acids, which is the cell wall substance of most Gram-positive bacteria. Lipoteichoic acid is composed of a hydrophobic diacylglycerol membrane anchor and hydrophilic group, and not one of lipids. However solid phase assay shown in Fig. 4-1 indicated that the annexin A1 and GST-annexin A4 bound to all lipoteichoic acids derived from various types of Gram-positive bacteria (*Streptococcus mutans (S. mutans), Enterococcus faecalis (E. faecalis), Bacillus subtilis (B. subtilis)* or *Staphylococcus aureus* (*S. aureus*)) in a concentration-dependent manner, while the GST domain protein used as a control of GST-annexin A4 did not bind to any type of lipoteichoic acids. These results indicate that annexins have ligand specificities toward lipoteichoic acids derived from various kinds of

Gram-positive bacteria in the presence of calcium ion.   Since the binding intensities are based on the number of biotin molecules coupled to lipoteichoic acids, the comparison of these values between different bacteria can not be performed.   In the same bacteria, however, it is sure that the amount of annexin A4 that bound to lipoteichoic acid was higher than that of annexin A1.   Lipid A and lipoteichoic acids are candidates to be recognized as foreign agents by the host.   Because most of non-self recognition proteins involved in innate immunity recognize various pathogen-associated molecular patterns on foreign substances, such as lipid A and lipoteichoic acids [80].   Therefore, annexins might bind these foreign substances via recognition of a molecular pattern common in various pathogens and function in immune systems.   Indeed, it has been observed that annexin A5 interact to one of the innate immunity C-type lectin surfactant protein A [81], though its function in immune system has yet to be established [58, 59].

2. Binding of annexins to S. aureus

In order to further study the interaction between annexins and Gram-positive bacteria, bioparticles derived from *S. aureus* were incubated with annexins under various conditions and then subjected to SDS-PAGE. As shown in Fig. 4-2, both GST-annexin A1 and *S. aureus* were co-precipitated in the presence of calcium ion (lane 2) but not in the absence of calcium ion (lane 4).   When GST-annexin A1 and *S. aureus* were incubated with lipoteichoic acid derived from *S. aureus*, GST-annexin A1 was not co-precipitated with *S. aureus*.   A similar observation was

made with GST-annexin A4.   In contrast, the control GST domain protein was detected in supernatant (lane 2, 4, 6), indicating that GST domain protein did not bind to *S. aureus* under any condition.

These results indicated that GST-annexins A1 and A4 were able to distinctly bind to whole *S. aureus* in a calcium-dependent manner, and the bindings were inhibited by lipoteichoic acid, which is the second major cell wall component of Gram-positive bacteria.   In other words, annexins might bind to *S. aureus* via interaction with lipoteichoic acid.

3. *Effect of annexins on the attachment of FITC-S. aureus to PMA-treated THP-1 cells (human macrophages)*

The role of annexins on Gram-positive bacterial recognition from macrophage was investigated by the flow cytometric analysis.   Since PMA-treated THP-1 cells (human macrophages) with FITC-*S. aureus* was incubated at 0°C, the results from the flow cytometric analysis, shown in Fig. 4-3, reflect the attachment of FITC-*S. aureus* to PMA-treated THP-1 cells.   The mean fluorescence channel of flow cytometry is expressed as the percentage of attachment relative to the maximum fluorescence (control).   In the presence of the GST domain, the attachment of FITC-*S. aureus* and PMA-treated THP-1 cells reached the same level as in the control.   On the other hand, the incubation with GST-annexins A1 and A4, the attachment of FITC-*S. aureus* and PMA-treated THP-1 cells were significantly decreased by 73.9% and 61.0%, respectively.

These results indicated that annexins A1 and A4 suppress the

attachment of *S. aureus* to PMA-treated THP-1 cells (human macrophages). The macrophages phagocytose apoptotic cells by recognizing phosphatidylserine which is exposed to apoptosis cell surface as an 'eat-me' signal [82, 83]. It has been previously suggested that masking of phosphatidylserine by annexin A5, which exhibit high-affinity binding to it, inhibit phagocytosis of apoptotic cells by immune cells [84, 85]. Therefore annexins might be able to inhibit the interaction between the immune cells and their targets, e.g. bacteria and apoptotic cells, by masking surface molecules such as lipoteichoic acid or phosphatidylserine, or by interacting with immune cells. Indeed, some reports indicate the presence of cell surface receptors for annexins on immune cells such as monocytes, macrophages and neutrophils [75-78].
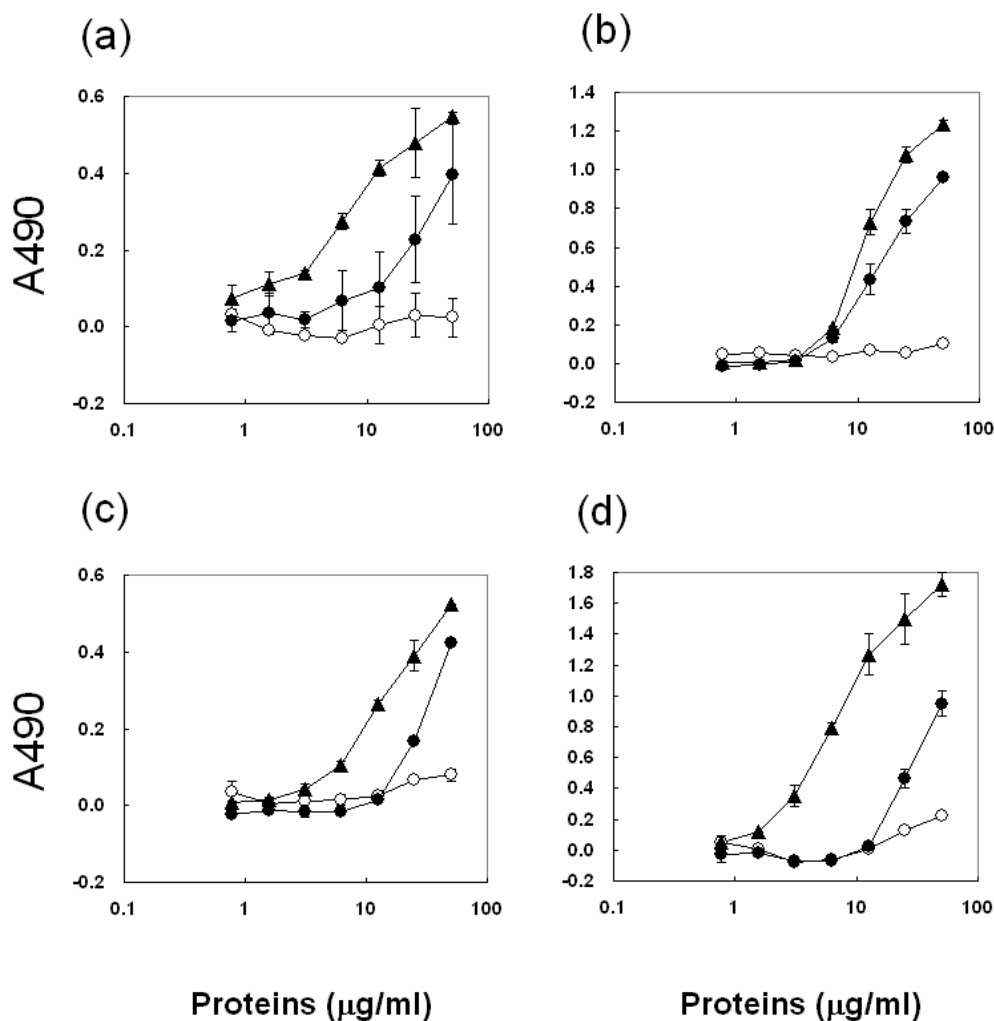
Fig. 4-1　Binding assay of biotin-labeled lipoteichoic acid to annexin A1 or GST-annexin A4.

Annexin A1 (triangles), GST (open circles) or GST-annexin A4 (closed circles) (0.78-50 μg/ml) was immobilized on microtiter plates and then incubated with biotin-labeled lipoteichoic acid from *S. mutans* (a), *E. faecalis* (b), *B. subtilis* (c) or *S. aureus* (d).　Bound biotin-labeled lipoteichoic acid was detected using HRP-avidin.　Results are expressed as means ± SD.
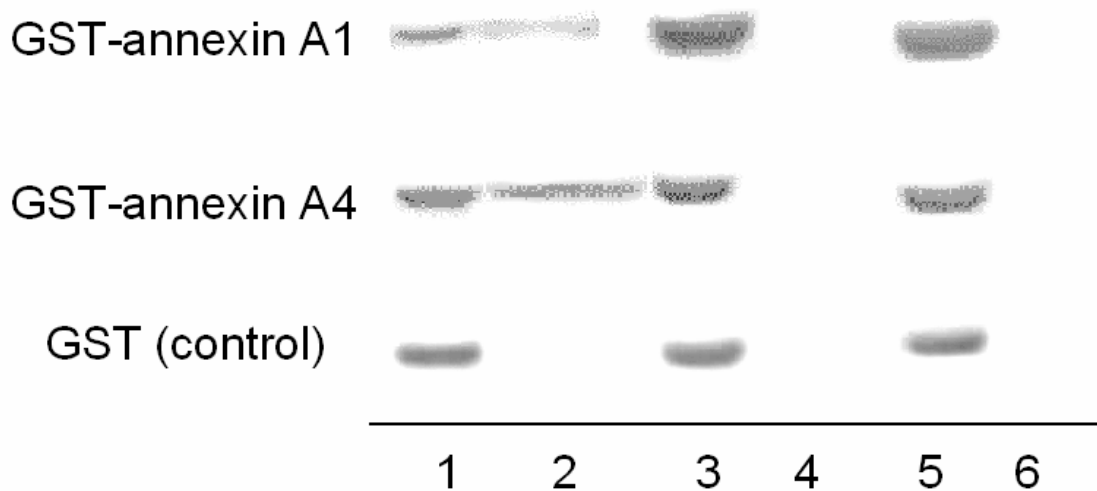
Fig. 4-2   Binding of GST-annexins A1 and A4 to *S. aureus.*

Binding assays were performed using *S. aureus* (1.5×10^8 cells) incubated with GST, GST-annexin A1 or A4 in the presence of $CaCl_2$ (lane 1, 2), EDTA (lane 3, 4) or lipoteichoic acid from *S. aureus* (lane 5, 6).   After centrifugation at 2000 *g* for 1min, each supernatant (lane 1, 3, 5) and precipitate (lane 2, 4, 6) was analyzed by SDS-PAGE, followed by CBB staining.
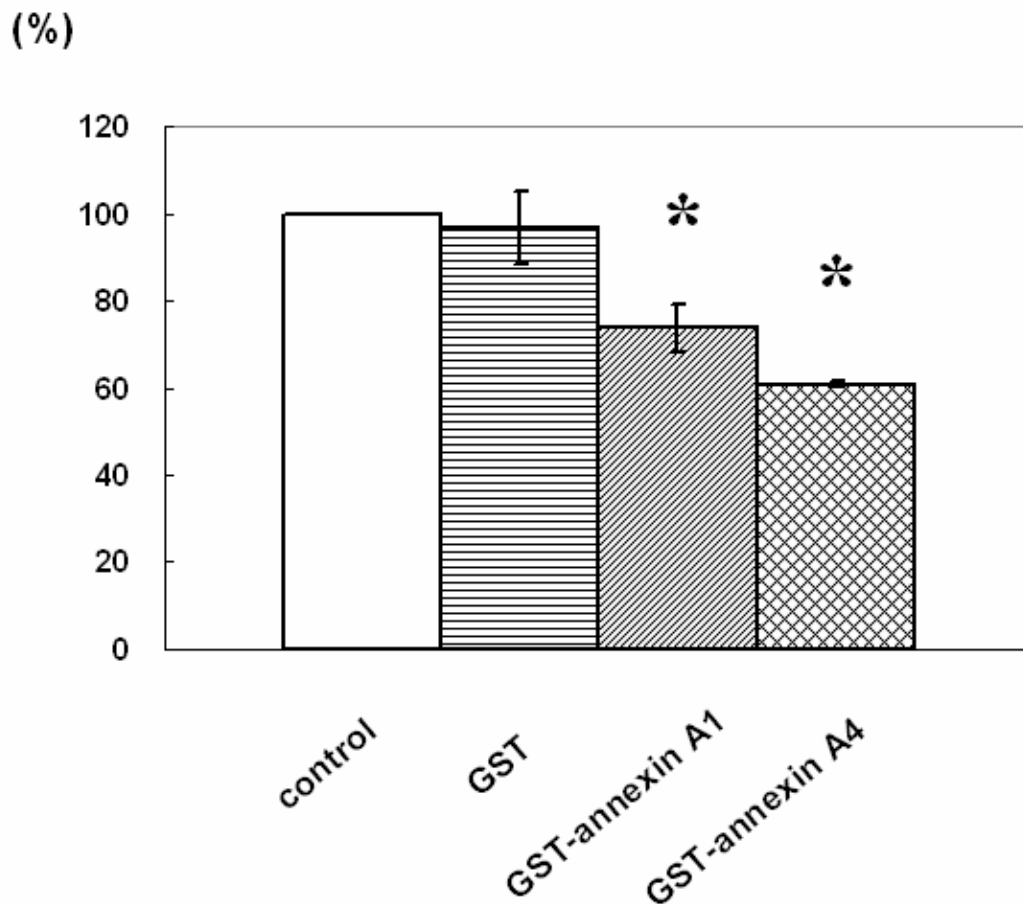
Fig. 4-3　Attachment of FITC- *S. aureus* to PMA-treated THP-1 cells.
THP-1 cells were differentiated by treatment with PMA as described above.
After scraping, FITC-labeled *S. aureus* was added alone (control, open columns) or along with 10 μg/ml GST (horizontally hatched columns), GST-annexin A1 (diagonally hatched columns) or A4 (cross-hatched columns) for 20 min.　The attachments of FITC-labeled *S. aureus* to PMA-treated THP-1 cells were detected by flow cytometric analysis. Data are expressed as percent of the maximum fluorescence (control) and as means ± SE.　Statistic significance was analyzed by 1-tailed Student's *t*-test (*$P < 0.05$).

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Conclusion

It is crucial for host defense to maintain a fine balance between an effective inflammatory response and tissue integrity. In response to an inflammatory stimulus, inflammatory cytokines and anti-inflammatory cytokines are released. When glucocortioids are released, the concentration of annexins in blood rises [60-63] and externalization of annexins on the cell surface is induced [86]. And there are many reports showing that annexins suppress the expression of inflammatory cytokines and induce that of anti-inflammatory cytokines [72]. Therefore, annexins are considered as host defense proteins acting anti-inflammatory at systemic inflammation [14, 72, 86]. The present study demonstrates that annexins bind directly to Gram-positive bacteria. And then annexins suppressed the attachment of Gram-positive bacteria to human macrophages. These findings suggest that annexins might act as anti-inflammatory protein at cellular level via blocking the pathway of interaction between immune cells and their targets.

Materials &methods

1. Preparation of recombinant annexins A1 and A4

The total RNA was extracted from HT29 (a human colon cancer cell line) by the method using guanidine-thiocyanate and cesium chloride [87]. The cDNAs were generated with oligo (dT)-primers by using recombinant MMLV reverse transcriptase. Human annexin A1 cDNA encoding complete open reading frame (ORF) was amplified by a PCR method with synthesized primers based on the sequence reported by Wallner *et al.* [88]. The PCR-amplified human annexin A1 cDNA was subcloned into pGEX-5X with *Bam*HI and *Xho*I restriction enzymes. A full length cDNA encoding human annexin A4 was derived from HT29, previously reported by Satoh *et al.* [89]. The sample was subcloned into pGEX-3X with *Bam*HI and *Eco*RI restriction enzymes, respectively. The expression vectors, pGEX-3X and pGEX-5X were obtained from Amersham Pharmacia Biotech, (Uppsala, Sweden). Recombinant annexins A1 and A4 were expressed as glutathione *S*-transferase (GST) fusion proteins in *E. coli* HB101. Expression, purification and enzymatic digestion of the recombinant GST-annexins A1 and A4 were performed as previously described [90].

Untagged annexin A1 was also prepared by treatment of GST-annexin A1 with factor Xa. The untagged annexin A1 thus obtained was recognized by a monoclonal antibody directed against the N-terminal portion (N-19) of human annexin A1 (Santa Cruz Biotechnology, Santa Cruz, CA).

2. Coupling of biotin hydrazide to lipoteichoic acids

Lipoteichoic acids were labeled with biotin using biotin-hydrazide (Pierce, Rockford, IL). Two mg of lipoteichoic acid from *S. mutans, E. faecalis*, *B. subtilis* or *S. aureus* (Sigma St. Louis, MO) were added to 2 ml of 3.3 mM $NaIO_4$/0.1 M NaOAc and subjected to periodate oxidation for 1 h at room temperature in the dark. To stop the oxidation, glycerol was added to reach a final concentration of 15 mM, and incubation continued for 5 min at 0°C and then 10 mg/ml biotin hydrazide dissolved in dimethylsulfoxide, which was prepared just before use, was added to allow reaction for 2 h at room temperature. The excess biotin hydrazide was removed by ultrafiltration through an Ultrafree-4 Centrifugal Filter Unit (Millipore, Bedford, MA). To purify lipoteichoic acids, the solution was separated with gel filtration on a NAP-5 column pre-packed with Sephadex G-25 (Amersham Pharmacia Biotech). Absorbance of each fraction from 200 nm to 300 nm was measured, and lipoteichoic acid-rich fractions were collected. The fractions were blotted onto a PVDF membrane and stained with toluidine blue or further incubated with HRP-avidin and 4-chloro-1-naphthol. The fractions, which were positive for both the toluidine-blue staining and biotin detection, were used as biotin-labeled lipoteichoic acids for the following assays.

3. Binding of annexins A1 and A4 to lipoteichoic acid

A 96-well microtiter plate (Immulon1; Dynatech Laboratories, Chantilly, VA) was first coated with varying concentrations of GST, annexin A1 or GST-annexin A4 and then incubated overnight at 4 °C. For

interactions between annexin A1 and lipoteichoic acids, untagged annexin A1 prepared as described above was used. The wells were washed three times with 10 mM Tris-HCl, pH 7.6, 150 mM NaCl (TBS) and then blocking of unoccupied sites was achieved by using TBS containing 3% BSA for 2 h at room temperature. Subsequently, the blocking solution was removed and biotin-labeled lipoteichoic acid was incubated in the presence of 1 mM $CaCl_2$ for 2 h at room temperature. All subsequent incubations and washing solutions used TBS contained 1 mM $CaCl_2$. The wells were washed three times as described above. Bound biotin-labeled lipoteichoic acid was analyzed using HRP-avidin (1:1000 dilution in 1% BSA/TBS). The wells were washed, and the color was developed by the addition of 200 μl 0.04% (w/v) *o*-phenylenediamine and 0.01% (v/v) H_2O_2 in citrate-phosphate buffer (pH 5.0). Color development was stopped by addition of 4 M H_2SO_4. The absorbance of each well was measured at 490 nm.

4. Binding of annexins A1 and A4 to S. aureus bioparticles

S. aureus bioparticles were obtained from Molecular Probes (Eugene, OR). *S. aureus* (1.5×10^8 cells/500 μl) were suspended in TBS containing 1 mM $CaCl_2$ or 1 mM EDTA, and centrifuged at 2000 *g* for 1 min. All subsequent incubation and washing solutions contained 1 mM $CaCl_2$ or 1 mM EDTA. The pellet was added to 2.5 μg GST (control), GST-annexin A1 or A4, in the presence or absence of 7 μg lipoteichoic acid from *S. aureus* to make a total volume of 20 μl and then incubated for 2 h at 4°C. After centrifuging at 2000 *g* for 1 min, the supernatant was saved and the

pellet was washed three times with 500 μl TBS.　　The supernatant and the pellet were subjected to SDS-PAGE and bound GST, GST-annexins A1 and A4 were visualized by Coomassie brilliant blue (CBB) staining.

5. *Assay of attachment of S. aureus to PMA-treated THP-1 cells*

THP-1 cells were maintained in RPMI 1640 medium containing 10% FCS.　　To induce differentiation, the cells (5×10^5 cells/ml) were incubated in 160 nM PMA 10% FCS-RPMI 1640 for 3 days at 37°C.　　PMA-treated THP-1 cells (human macrophages) were washed with PBS three times to remove undifferentiated cells [91].　　The adherent cells were removed with a cell scraper and used to assay attachment.　　In the presence of 10% FCS-RPMI 1640, 1.5×10^5 PMA-treated THP-1 cells and 2.5×10^7 FITC-labeled *S.aureus* bioparticles (Molecular Probes) were incubated in 10% FCS-RPMI 1640 in the absence (control) or presence of 10 μg GST, GST-annexins A1 and A4 diluted to a final volume of 1 ml in a tube.　　The tubes were incubated for 20 min at 0°C to detect the attachment of FITC-*S. aureus* and PMA-treated THP-1 cells, and then cells were analyzed in a flow cytometer with an excitation wavelength of 488 nm.

General conclusion

Guy Ourisson and Yoichi Nakatani have postulated an original scenario about the early formation of membranes and their evolution: it was possible to arrange the membrane terpenoids in a phylogenetic sequence [8]. Polyprenyl phosphates might have been primitive membrane constituents and they are the precursors of all membrane terpenoids present in the phylogenetic tree. On the other hand, highly branched isoprenoid alkanes were recently found in many sediments, and they may have been derived from the corresponding branched polyprenyl phosphates present in biomembranes in primitive organisms. In the first chapter of thesis, it was shown that sysnthetic polyprenyl phosphates, bearing a branched isoprenyl chain at the C(6) position, formed stable vesicles at physiological pHs. It was also shown that the water permeability of these vesicles was lower than for vesicles made of geranylgeranyl phosphate vesicles (non-branched polyprenyl phosphate). These isoprenyl-branched polyprenyl phosphates might have been obtained in prebiotic conditions from non-substituted polyprenyl phosphate by a simple alkylation. These results confirm that isoprenyl-branched polyprenyl phosphates might be possible primitive biomembrane constituents, one step advanced from non-substituted polyprenyl phosphates such as geranylgeranyl phosphate.

The lipidic part of eukaryotic membranes is principally composed of phospholipids and sterols such as cholesterol (an essential mammalian sterol) or sitosterol (a major sterol in plants). The role of these sterols is

membrane mechanical reinforcement.   Questions arise: "why were diacylglycerol phosphoryl derivatives but not cholesteryl phosphocholine involved in the course of evolution?" or "are there some organisms (not yet studied) whose lipid membranes are composed of cholesteryl phospholipids/diacylglycerol or cholesterol?".   In the second chapter of this thesis, to find some clues for those questions, amphiphilic phosphorylated sterols (cholesteryl phosphate (CP), sitosteryl phosphate (SP) and cholesteryl phosphorylcholine (CPC)) were synthesized and their membrane properties were studied.   Although CP, SP and CPC could not form any vesicles by themselves, they could form vesicles by addition of diacylglycerol or cholesterol.   These results suggest that such phosphorylated sterols might be present in the membranes of some organisms.   On the other hand, the pH range of vesicle formation of phosphorylated sterols/diacylglycerol or sterols mixture was narrower than that observed for a natural eukaryotic membrane system: a phospholipid/sterol mixture.   Therefore, nowadays biomembranes acquired such properties that living organisms can adapt to various environments, hence contributing to extended biodiversity.

There are three major kingdoms of living organisms: Bacteria, Eukarya and Archaea.   Membranes of Bacteria and Eukarya are made of di-acyl phospholipids, whereas the cell membrane lipids of Archaea, di-ether or tetra-etherpolyprenyl phospholipids, are markedly different.   It was speculated that long chain fatty acids might be phylogenetic precursors of di-acyl phospholipids and polyprenyl phosphates could be precursors of

archaeal lipids.   In the third chapter of thesis, it was studied how external lipophilic polysaccharide molecules could coat the "primitive" membranes and the complexification of the membrane surface could acquire a higher function to communicate with the outside world.   For this purpose, it was studied whether giant vesicles (5 μm or more) made of single chain lipids (polyprenyl phosphates, oleic acid) or double chain lipids (eukaryotic di-acyl phospholipid, archaeal di-ether phospholipid) could be coated by a polysaccharide (pullulan, MW about 55 kDa), bearing hydrophobic phytyl chains (phytyl-pullulan) or cholesteryl moieties (cholesteryl-pullulan).   To the polysaccharide was also attached a fluorescent tag, to make it possible to observe coated vesicles with optical microscopy.   The results show that there is a "match-mismatch" relation between the membrane constituents and the hydrophobic molecules to be inserted.   For example, phytyl-pullulan could coat membranes made of single chain lipids or double chain lipids, whereas cholesteryl-pullulan could not coat membranes made of single chain lipids.   This shows that the cholesteryl moiety could not be incorporated in the membrane.   This implies a possible mechanism of selectivity of membrane lipid components in the course of evolution.   Then, using the hydrophobized polysaccharides, the spontaneous molecular recognitions between external lectins and polysaccharides on giant vesicle surface were studied.   It was demonstrated that concanavalin A or annexin V spontaneously bound with pullulan on giant vesicles made of the above lipids.   This method made it possible to directly observe recognition between polysaccharides and lectins, and it could be useful for the initial assay of binding between

polysaccharides and proteins.

Cell membranes have a number of important roles such as specific recognition of external molecules. Recent studies have shown that annexins bind to lipid A of Gram-negative bacteria and suppress cellular responses to endotoxin. This result suggests that annexins function as modulators of anti-inflammation via recognition of foreign substance. In the fourth chapter of thesis, the interaction between annexins and lipoteichoic acid, which are located surface of Gram-positive bacteria, were revealed. Then, the interaction of annexins with *Staphylococcus aureus* (Gram-positive bacteria) was observed. This result implies that annexins could interact not only with Gram-negative bacteria but also with Gram-positive bacteria. Then, it was shown that when *Staphylococcus aureus* was attached with annexins, the attachment of *Staphylococcus aureus* to human macrophages was suppressed. These findings suggest that annexins might act as anti-inflammatory protein via modulating the pathway of interaction between immune cells and their targets.

All living organisms from small bacteria to human have a common cellular system. Cells perhaps have begun with closed vesicles made of the self-assembly of simple amphiphilic molecules, in which the membrane might function only as a physical boundary separating the cell from the external world. Once vesicles were formed, the self-complexification of this system could be automatically carried out: vectorial properties (small vesicles), extraction of lipidic molecules, their specific orientation in bilayers, increased concentration and orientation of amphiphilic molecules,

coating of vesicles with a carbohydrate wrapping, analogous to the bacterial cell wall, entrapment of DNA molecules, followed by the transcription and the translation, etc. In short, self-organization of amphiphiles in water into closed vesicles could lead automatically to self-complexification into "proto-cells". Fossil records suggested the life has started more than 3 billion years ago. As living organisms developed in wide regions of the earth in a variety of conditions, their membrane constituents evolved diversely in the past 3 billion years. Today, cell membranes have become more complex by incorporating of glycoproteins, glycolipids and their surfaces are covered with a variety of polysaccharides. These molecules allow a cell to communicate with the external world and sustain cellular homeostasis. It can be said that biomembranes are evolving from the beginning of life up to now and show a biodiversity in their components. In this process, the biomembranes might have acquired lipid selectivity in their components. Therefore, by focusing on the evolution of cell membrane components such as isoprenyl-branched polyprenyl phosphates, which are postulated as possible primitive biomembrane constituents, or phosphorylated sterols, which are speculated as alternative membrane molecules, we could obtain many valuable insights into the properties of both primitive and present membrane constituents and reinforcers. Moreover, investigation of the interaction between proteins and polysaccharides could also provide an important step toward a better understanding of higher membrane functions.

List of Abbreviations

APCI	atmospheric pressure chemical ionization
BSA	bovine serum albumin
B. subtilis	*Bacillus subtilis*
CBB	coomassie brilliant blue
Con A	concanavalin A
CP	cholesteryl phosphate
CPC	cholesteryl phosphorylcholine
D109	5-dodecanoylaminofluorescein
DMG	1,2-dimyristoylglycerol
DMPC	dimyristoyl phosphatidylcholine
DphPC	2,3-diphytanyl-*sn*-glycero-1-phosphocholine
ECM	extracellular matrix
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
E. faecalis	*Enterococcus faecalis*
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
GST	glutathione *S*-transferase
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein iso-thiocyanate
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
NMR	nuclear magnetic resonance spectroscopy
MS	mass spectra
MW	molecular weight
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen-associated molecular patterns
PBS	phosphate-buffered saline

PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
PVDF	polyvinylidene difluoride
S. aureus	*Staphylococcus aureus*
SAXS	small angle X-ray scattering
SDS	sodium dodecyl sulfate
S. mutans	*Streptococcus mutans*
SP	sitosteryl phosphate
SPR	surface plasmon resonance
TBS	10 mM Tris-HCl, pH 7.6, 150 mM NaCl
THF	tetra-hydrofuran
TOF	time-of-flight

Acknowledgements

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Mari GOTOH

2nd March 2006, Tokyo

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論文要旨

　全ての生物は細胞を基本単位とし細胞膜という物理的境界によって外界から区別されている。そこには外部からの刺激に応じ細胞機能を制御する重要なタンパク質群が存在している。しかしながら、この細胞において重要な脂質分子や細胞外マトリックスの主成分である糖鎖の構造と性質に関しては未知な部分が多く残されている。そこで、生物の多様性の観点から生体膜に関する研究を行った。

（1）分岐ポリプレニルリン酸　（イソプレニル置換ポリプレニルリン酸）

　イソプレニル基置換ポリイソプレノイドは、広く堆積物から見出され、これらの構造はアルコールあるいはリン脂質に由来していると考えられる。したがって、現在の生体膜からは見出されていないが、原始膜成分として"イソプレニル置換ポリプレニルリン酸"が存在していたことが推定される。

　合成されたさまざまな分岐ポリプレニルリン酸が形成する膜の物理学的性質を調べた。光学顕微鏡による観察より、分岐ポリプレニルリン酸が pH 依存的にベシクルを形成することが示された。また、分岐ポリプレニルリン酸の形成する一枚膜ベシクルの水の透過性を評価するために、ベシクルの浸透圧膨張を stopped-flow/light-scattering method により調べた。その結果、水の透過性は分子構造と鎖長によって変化することが明らかになった。これらの結果は、イソプレニル基置換ポリプレニルリン酸が、実際に太古の生体膜として存在していた可能性を支持する。

（2）リン酸化コレステロール

　真核生物、原核生物、古細菌の生体膜は膜構築成分(両親媒性分子で自発的に自己集合し、膜構造を持つベシクルを形成)と膜補強成分(膜を補強し流動性を制御する)から構成されている。しかしながら、なぜコレステロールではなくジアシルグリセロールがリン酸化されたのであろうか？また、あるいはリン酸化コレステロールとジアシルグリセロールとの組み合わせの膜が存在したのだろうか？これらの謎を解明していくために、リン酸化コレステロール（コレステリルホスフォコリン:CPC)を合成し、それらが構築する膜の物理的性質について調べた。

　CPC とジアシルグリセロール(ジミリストイルグリセロール:DMG)の混合物は適切な混合率、pH 条件下で自己集合し膜を形成することが明らかになった。この CPC/DMG からなる膜はリン脂質(ホスファチジルコリン)/コレステロールよりも限られた pH 条件下でのみ形成された。また、CPC/DMG (1:1)から形成されるベシクルの水の透過性はリン脂質(ホスファチジルコリン)/コレステロール (1:1)から形成されるベシクルよりも高かったが、CPC/DMG は安定な膜を形成していた。これらの結果から、CPC と DMG を膜成分とする生物が見出される可能性は否定できない。また現在、見出されている生体膜組成は生物がさまざまな外部環境に適応し、生物多様性を可能にする特性を獲得していると推察された。

（3） 原始的ベシクルから細胞膜へ

”原始”膜構成分子の自己集合によって自然に形成されるベシクルから細胞壁獲得にいたる進化の過程を追及した。まず、二本鎖脂質(DphPC(2,3-diphytanyl-*sn*-glycero-1-phosphocholine), POPC(1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine))のベシクルが phyryl-pullulan に被覆されることを示した。cholesteryl-pullulan は二本鎖脂質のベシクルを被覆したが一本鎖脂質ベシクルは被覆しなかった。これより膜構成成分と膜に挿入される疎水性分子の大きさ、形の間に適切な関係が必要なことが推測される、またこれは進化の過程における膜構成分子の選択機構の存在を示す。

次にこの疎水化多糖を使用して、巨大ベシクルの表面におけるレクチンと多糖の分子認識について研究した。これは、両親性分子が水の中で自己集合しベシクルを作り、自然に”原始細胞”へと向かう自己複雑化を引き起こす例となる。

（4） 細胞膜高次機能について

アネキシン(Anx)ファミリータンパク質はリン脂質や細胞外マトリックス糖鎖に結合する。近年、細胞外 Anx は大腸菌の細胞表面の抗原分子リピドAにも結合することが報告された。このことから、本研究では Anx が免疫機構の一端を担っているとの仮説をたて研究を行った。まず Anx の異物認識能の有無を検討するため、生体内に侵入するグラム陽性菌が細胞膜表層にもつ成分(リポテイコ酸)との結合試験を行った。その結果、Anx は各種グラム陽性菌由来のリポテイコ酸に結合し、さらに黄色ブドウ球菌(グラム陽性菌)自体にも結合することが示された。続いて食細胞による非自己への接着に与える Anx の影響を調べた。その結果、Anx は食細胞の異物への接着を抑制していることが明らかになった。

以上の結果から、Anx は異物に対し広い結合能を持つことで自然免疫に寄与し、食細胞の過剰な免疫反応が引き起こすアレルギーなどの炎症を抑制する抗炎症作用を有すると考えられる。

Résumé

Tous les organismes vivant sont fait de cellules qui sont séparés du monde extérieur par une barrière, la membrane cellulaire. Un nombre important de protéine contrôle la fonction cellulaire en interagissant avec les stimuli extracellulaires. Cependant, les fonctions et structures des molécules de la membrane cellulaire tel que les lipides et les sucres ne sont toujours pas entièrement déterminées. Du point de vue de la biodiversité, quelques propriétés des biomembranes ont été étudiées sous deux aspects différents.

(1) les phosphates de polyprényles ramifiés

Plusieurs composés polyterpanique ont été abondamment trouvés dans les sédiments, et de telles structures auraient pour origine des alcools ou des phospholipides. Bien qu'ils n'aient toujours pas été trouvés dans les biomembranes actuelles, les phosphates de polyprényle ramifié auraient pu exister dans les membranes primitives. Plusieurs phosphates de polyprényle ramifié ont été synthétisés et nous avons effectué les études physico-chimiques de leurs propriétés membranaires. Les études microscopiques ont montré que les phosphates de polyprényle ramifié forment des vésicules en fonction du pH. Afin d'évaluer la perméabilité membranaire à l'eau de ces membranes, le gonflement osmotique d'une suspension unilamellaire de vésicules a été mesuré par la méthode de la diffusion de la lumière en flux à écoulement bloqué. Nous avons montré que la perméabilité à l'eau dépend étroitement de la structure et de la

longueur de chaîne. Ces observations suggèrent que les phosphates de polyprényle ramifié pourraient être des constituants membranaires primitifs des membranes cellulaires.

(2) Cholestérol phosphorylé

Les membranes des vertébrés sont constitués de deux sortes de lipides : les constituants membranaires phosphorylés et les renforçateurs membranaires non phosphorylés comme le cholestérol. Pourquoi le cholestérol n'a pas été phosphorylé au cours de l'évolution des membranes ? Les membranes composées de diacylglycérol non phosphorylé et de cholestérol phosphorylé existent-elles ? Afin de répondre à ces questions, le cholestérol phosphocholine (CPC) a été synthétisé et ses propriétés physico-chimiques membranaires étudiées.

Nous avons observé la formation de vésicules stables par microscopie optique d'un mélange approprié de CPC et de diacylglycérol à différents pH. Cependant, le rapport molaire entre le phospholipide et l'alcool permettant la formation de vésicules est plus étroit pour le mélange CPC/diacylglycérol que pour le mélange diacylglycérophospholipide/cholestérol. De plus, la perméabilité à l'eau des vésicules d'un mélange de CPC et de diacylglycérol dans un rapport molaire 1 : 1 est plus élevé que le mélange de diacylglycérophospholipide et de cholestérol dans le même rapport molaire 1 : 1. Les membranes des organismes vivants ont des propriétés d'adaptation à différents environnements contribuant ainsi à l'extension de la biodiversité. Ces résultats suggèrent donc que le cholestérol phosphorylés pourrait être présent dans les membranes de certains organismes qui n'ont pas encore été

étudiés.

(3) Des membranes « primitives » vers les proto-cellules

Un processus d'évolution possible de vésicules formées par des constituants membranaires « primitifs » est le recouvrement de la membrane externe par un assemblage moléculaire pouvant former un « mur ». En premier lieu, nous avons montré que le phytyl-pullulan pouvait recouvrir les vésicules de lipides à double chaînes (2,3-diphytanyl-sn-glycero-1-phosphocholine (DphPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)). Le cholestéryl-pullulan recouvre les lipides à double chaîne mais pas les lipides à une seule chaîne. Un mécanisme de sélection existe probablement entre la taille et la forme des constituants membranaires et les molécules hydrophobiques à insérer.

Ensuite, l'utilisation des polysaccharides hydrophobes a permis la reconnaissance moléculaire entre les lectines et les polysaccharides sur la surface de vésicules géantes, ceci fournit un exemple de la complexification des membranes primitives vers les « proto-cellules ».

(4) Fonction des membranes cellulaires.

Les annexines sont une famille des protéines qui se lient aux phospholipides et aux carbohydrates. Dans cette étude, les propriétés immunologiques et les effets de l'annexines sur les bactéries Gram-positive dans le système immunitaire ont été étudiés. Premièrement, l'interaction des annexines avec l'acide lipotéichoique, localisé sur la surface des bactéries Gram-positive, de *Staphylococcus aureus* (bactérie Gram-positive) a été observée. Deuxièmement, les effets de l'annexines sur

l'attachement des macrophages et des bactéries Gram-positive ont été examinés. Les résultats ont montré que les annexines supprimaient l'attachement de *Staphylococcus aureus* sur les macrophages humains. Cette découverte suggère que les annexines peuvent agir comme protéine anti-inflammatoire au niveau cellulaire en bloquant la voie d'interaction entre les cellules immunitaires et leurs cibles.