Chemistry & Biology
Article

"Primitive" Membrane from Polyprenyl Phosphates and Polyprenyl Alcohols

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We dedicate this work to the memory of Professor Guy Ourisson and Mrs. Marie-Claire Dillenseger.
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DOI 10.1016/j.chembiol.2006.11.017

SUMMARY

Polyprenyl phosphates, as well as polyprenyl alcohols bearing different isopentenyl C_5 units, have been synthesized. The pH range of spontaneous vesicle formation of polyprenyl phosphates with or without polyprenyl alcohols was defined by fluorescence microscopy. A variety of the acyclic or monocyclic polyprenyl phosphates studied formed stable vesicles in water over a wide range of pHs, and the addition of polyprenyl alcohols allowed the vesicle formation of polyprenyl phosphates at higher pHs. Osmotic swelling of a suspension of unilamellar vesicles using the stopped-flow/light-scattering method enabled us to evaluate the water permeability of polyprenyl phosphate vesicles with or without 10 mol% of free polyprenyl alcohol. The addition of many polyprenyl alcohols to polyprenyl phosphate vesicles decreased the water permeability, and some reduced it even more efficiently than cholesterol.

INTRODUCTION

We have earlier observed that terpenoids are universal, necessary constituents of all living organisms and postulated an original scenario for the early formation of membranes and their evolution [1]. It is possible to arrange known membrane terpenoids in a phylogenetic sequence, and a retrograde analysis led us to propose that polyprenyl phosphates might be even more primitive membrane constituents than archaeal membrane lipids (Figure S1; see the Supplemental Data available with this article online). The polyprenyl chains of archaea can be biosynthesized from isopentenol in C_5 increments by a simple alkylation of the double bond [2]. The first step, condensation of isopentenol, was achieved by simple treatment of the monoprenol at room temperature with montmorillonite [3]; repetition of this reaction leads to an expansion from C_{10} to C_{15} and then C_{20}. Selectivity of the lipophilic chain length can be ensured by phase separation, using appropriate polar head groups. We postulated that the simplest possible polar head is a phosphate, as in many other biochemical reactions [4], because of its universal presence in the head groups of membrane lipids.

Based on these observations, we synthesized several phosphate esters containing one or two polyprenyl chains, and we previously demonstrated that they do form vesicles [5, 6], as well as monolayers at the air-water interface [7]. In brief, single-chain polyprenyl phosphates occupy a central position in the postulated phylogenetic sequence of membrane terpenoids. They are biosynthetic intermediates of all terpenoids and alone are capable of forming vesicles [2]. From a prebiotic point of view, Walde et al. have independently studied the vesicle-forming properties of different single straight-chain alkyl phosphates and phosphonates in water. They showed that n-dodecylphosphate spontaneously forms vesicles when the pH of the medium approaches the pKa_1 (about half of the molecules are then monoionic and the other half are completely protonated) [8].

On the other hand, some terpenoids are membrane reinforcers in eukaryotes (cholesterol, other sterols) and in bacteria (hopanoids, α, ω-dihydroxylated carotenoids) [9]. The question then arises as to which components serve as reinforcers of acyclic polyprenyl phosphates in the "primitive" membrane model we proposed. Polyprenyl phosphates might be obtained by phosphorylation of polyprenyl alcohols under prebiotic conditions [10, 11]. We speculated that primitive membranes are composed of a mixture of phosphates and alcohols, and we now show that free alcohols stabilize vesicles containing phosphates.

Here, we first present a more detailed study of the formation of vesicles from a series of synthesized single-chain polyprenyl phosphates in order to analyze different parameters involved in vesicle formation (chain length, degree of unsaturation, acyclic or cyclic chain, pH, and the presence or absence of the free alcohol). We found that polyprenyl phosphates containing 15–30 C atoms form vesicles in a wide pH range. Polyprenyl phosphates containing shorter chains (C_{10}) or longer ones (C_{45}) atoms do not spontaneously form vesicles. Next, we discuss the

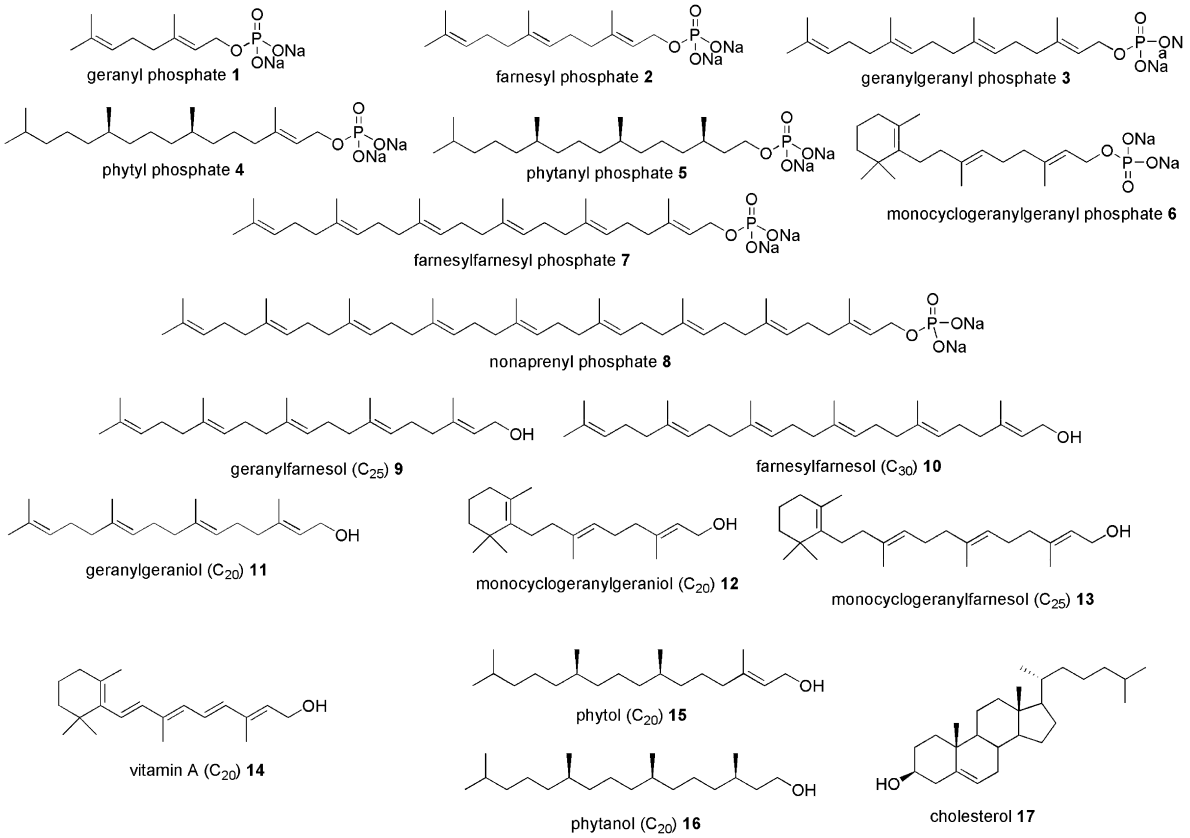

Figure 1. Structure of the Polyprenyl Phosphates and Terpenic Alcohols Tested

formation of vesicles from the mixed systems composed of a single-chain polyprenyl phosphate and a free polyprenyl alcohol as a function of pH. We also describe the reinforcing effect of polyprenyl alcohols on polyprenyl phosphate membranes, by using a stopped-flow/light-scattering method to evaluate the water permeability [12]. In addition, we demonstrate that the water permeability of some polyprenyl phosphate vesicles can be even more efficiently reduced by some polyprenyl alcohols than by cholesterol. These data indicate that polyprenyl alcohols are suitable reinforcers of polyprenyl phosphate membranes.

RESULTS AND DISCUSSION

Synthesis of Polyprenyl Phosphates and Polyprenyl Alcohols

We synthesized a series of monopolyprenyl phosphates as sodium salts: farnesol **2**, geranylgeraniol **3**, phytol **4**, phytanol **5**, mono-cyclogeranylgeraniol **6**, farnesylfarnesol **7**, and a series of polyprenols: geranylfarnesol **9**, farnesylfarnesol **10**, monocyclogeranylgeraniol **12**, monocyclogeranylfarnesol **13** (Supplemental Data). Vitamin A **14** and cholesterol **17** are commercially available. Nonaprenyl phosphate **8**, geranylgeraniol **11**, phytol **15**, and phytanol **16** were gifts from Nishin Flour Milling Co. (Figure 1).

Microscopic Studies of Vesicle Formation

Spontaneous vesicle formation depends on the relative sizes of the hydrophilic and hydrophobic parts in the molecule. According to Israelachvili et al. [13], vesicle formation of lipids depends on the critical packing parameter $P = v/a_0 l_c$ (a_0 = the optimal area per molecule at the lipid-water interface; l_c = chain length; v = hydrocarbon volume), and the value P must lie between 1/2 and 1. The surface area a_0 depends on the charge repulsion of the phosphate polar head and increases according to the following order: diacid < monoanion < dianion.

Vesicle Formation with Polyprenyl Phosphates

First, we studied the spontaneous vesicle formation of polyprenyl phosphates alone as a function of pH at 25°C with different parameters by optical or confocal microscopy using Nile Red as a selective fluorescent probe.

Different structural parameters of polyprenyl phosphate have been studied:

Polyprenyl Chain Length—Entries 1, 2, 3, and 4; Table 1. Geranylgeranyl phosphate **3** forms vesicles in alkaline conditions (until pH 8.6), but vesicle formation was observed for farnesyl phosphate **2** only at a pH lower than 5.7. If the phosphate moiety bears two negative charges, electrostatic head-group repulsions increase, the apparent head-group size increases, and highly curved micelles may be formed. Geranylgeranyl phosphate **3** has a longer

Table 1. Vesicle Formation as a Function of the pH from a Mixture of Phosphate—2, 3, 4, 5, and 6—with or without 10 mol% of Alcohol—9, 10, 11, 12, 13, and 16—in Water

Entry	Phosphates	10 mol% of Polyprenyl Alcohols	Range of pH
1	**2**	—	1.9–5.7
2	**3**	—	2.2–8.6
3	**4**	—	2.2–9.6
4	**5**	—	2.9–10.7
5	**6**	—	3.4–5.0
6	**3**	**9**	3.8–9.2
7	**3**	**10**	4.0–9.7
8	**3**	**11**	3.8–13.0
9	**3**	**12**	3.4–12.4
10	**3**	**13**	6.3–12.2
11	**4**	**9**	4.0–9.7
12	**4**	**12**	4.0–13.0
13	**5**	**13**	3.7–9.7
14	**5**	**16**	4.0–10.7

chain (C_{20}) than farnesyl phosphate **2** (C_{15}) and a more favorable lipophilicity/hydrophilicity ratio, which ensures spontaneous vesicle formation at higher pHs for geranylgeranyl phosphate **3**. Potentiometric titration (Table S1) of a vesicle suspension of farnesyl phosphate **2** indicated pKa_1 = 2.2 and pKa_2 = 6.8. Since farnesyl phosphate **2** forms vesicles in the range pH 1.9–5.7, the presence of the mono-anion of farnesyl phosphate is important for vesicle formation. The possible intermolecular hydrogen bonding in the head group area of farnesyl phosphate **2** could stabilize the vesicles formed [8, 14]. The importance of the presence of the mono-anion to form vesicles is also observed in the case of geranylgeranyl phosphate (pKa_1 = 2.4; pKa_2 = 6.5): vesicle formation (pH 2.2–8.6).

Extensive studies of farnesylfarnesyl phosphate **7** (C_{30}) were not possible, as the amount available was too small and spontaneous vesicle formation of this phosphate has been observed only at pH 8.2. Neither nonaprenyl phosphate **8** (C_{45}) nor geranyl phosphate **1** (C_{10}) formed vesicles at any of the tested pHs (2.0–11.0), as judged by optical microscopy. Thus, farnesyl phosphate **2** is the smallest polyprenyl phosphate (C_{15}) that forms the vesicles, and the upper limit is between C_{30} and C_{40}.

Degree of Unsaturation. Although phytyl phosphate **4** and phytanyl phosphate **5** have the same number of carbon atoms (C_{20}) as geranylgeranyl phosphate **3**, they form vesicles in more alkaline conditions, up to pH 9.6 and pH 10.7, respectively. To explain this observation, we calculated the volume and length of geranylgeraniol **11**, phytol **15**, and phytanol **16** by the PM3 MM method. The PM3 MM Hamiltonian was used with precise key words (opt; freq, volume), with the GAUSSIAN03 program package (Table 2) [15]. The calculation performed on this analogous series revealed that the volume of the hydrophobic part of the phytyl phosphate **4** and phytanyl phosphate **5** increases by almost 15% in comparison with geranylgeranyl phosphate **3**. This implies that the volume of their hydrophilic head group can balance the volume of the hydrophobic part even at higher pHs. According to their pKa values (Table S1), we can again observe the importance of the mono-anion form for vesicle formation in the case of phytyl phosphate **4** and phytanyl phosphate **5**.

Table 2. Calculated Molecular Volume and Molecular Length of Polyprenyl Alcohols Geranylgeraniol 11, Phytol 15, and Phytanol 16 by GAUSSIAN03 Method Using PM3 MM

Polyprenyl Alcohol	Volume[a] (cm^3/mol)	Length[b] (Å)
11	269.331	18.059
15	308.076	18.483
16	308.380	18.059

[a] Total volume of polyprenyl alcohol.
[b] Total length of polyprenyl alcohol.

Presence of One Ring. Monocyclogeranylgeranyl phosphate **6** spontaneously forms vesicles in buffers in a narrow range of pH, between 3.4 and 5.0 (Figure S2), although the length of this compound is approximately the same as that of farnesyl phosphate **2**, which forms vesicles between pH 1.9 and pH 5.7. The presence of the cyclohexenyl ring appears to perturb the coherence with surrounding molecules.

Next, we studied the effect of different concentrations of polyprenyl alcohols in polyprenyl phosphates on the vesicle formation as a function of pH:

*Geranylgeranyl Phosphate **3** + x mol% Geranylgeraniol **11***. Addition of 10 mol% of geranylgeraniol **11** to geranylgeranyl phosphate **3** remarkably shifted the range of the vesicle formation toward higher pHs (3.8–13.0) compared with phosphate alone (pH 2.2–8.6) (Table 3). The presence of alcohol **11** in the phosphate membrane increases the ratio of the hydrophobic volume to the hydrophilic volume in the system, and vesicle formation is facilitated at higher pHs. Increasing the ratio of geranylgeraniol **11** to geranylgeranyl phosphate **3** disturbed vesicle formation in acidic conditions. A similar effect of alcohols had been reported in the formation of vesicles from fatty acids, suggesting the formation of stable hydrogen bond networks between carboxylic acid and alcohol [14].

*Geranylgeranyl Phosphate **3** + Polyprenyl Alcohol.* In Table 1 (entries 6, 7, 8, 9 and 10), vesicle formation as a function of pH was compared in the presence of 10 mol% of geranylfarnesol **9** (C_{25}) or farnesylfarnesol **10** (C_{30}) in geranylgeranyl phosphate **3** membranes. The pH range of vesicle formation is narrower for the alcohols **9** (pH 3.8–9.2) and **10** (pH 4.0–9.7) than for alcohol **11** (pH 3.8–13.0). A more compact packing may be achieved in the membrane with phosphate **3**/alcohol **11**, since their hydrophobic parts have the same structure.

Monocyclogeranylgeraniol **12** (C_{20}) at 10 mol% permits vesicle formation of geranylgeranyl phosphate **3** in more

Table 3. Vesicle Formation as a Function of the pH from a Mixture of Geranylgeranyl Phosphate 3 and x mol% of Geranylgeraniol 11 in Water

Entry	x mol% of Geranylgeraniol	Range of the pH
1	0	2.2–8.6
2	10	3.8–13.0
3	20	6.8–13.2
4	30	7.5–13.2
5	40	9.8–13.2
6	50	12.6–13.2

basic conditions (pH 3.4–12.4), with a small destabilization effect in acidic conditions, whereas monocyclogeranylfarnesol **13** (C_{25}) at 10 mol% is more strongly destabilized in acidic conditions (pH 6.3–12.2).

From these results, we conclude that if the structure of the alcohol is similar to that of the phosphate, vesicles form in a wider range of pHs [14]. The presence of one ring shifted the range of vesicle formation to a higher pH.

*Phytyl Phosphate **4** + 10 mol% Polyprenyl Alcohol.* Phytyl phosphate **4** has a larger hydrophobic volume than geranylgeranyl phosphate **3** (Table 2). Thus, vesicle formation of phytyl phosphate **4** (pH 2.2–9.6) can occur at higher pHs than with geranylgeranyl phosphate **3** (pH 2.2–8.6).

The presence of geranylfarnesol **9** at 10 mol% does not significantly improve vesicle formation of phytyl phosphate **4** at basic pHs (pH 4.0–9.7) and causes a strong destabilization in acidic pHs. On the contrary, monocyclogeranylgeraniol **12** at 10 mol% in phytyl phosphate **4** favors, remarkably, the formation of vesicles at higher pHs (pH 4.0–13.0). Again, the presence of one ring shifts the range of vesicle formation toward more basic pHs (Table 1, entries 11 and 12).

*Phytanyl Phosphate **5** + 10 mol% Polyprenyl Alcohol.* The combination of the very hydrophobic phytanyl group [16] and its phosphate moiety **5** produces very stable vesicles (pH 2.9–10.7) (Table 1, entries 4, 13, and 14). The addition of 10 mol% of phytanol **16** caused no large change in the range of vesicle formation (pH 4.0–10.7). However, the presence of monocyclogeranylfarnesol **13** at 10 mol% destabilizes vesicle formation at both acidic and basic pHs (pH 3.7–9.7).

Water Permeability

In order to evaluate the reinforcing effect of polyprenyl alcohols, the water permeability of polyprenyl phosphate vesicles with or without 10 mol% of free alcohol was studied. Osmotic swelling of a suspension of unilamellar vesicles of homogeneous size was measured using the stopped-flow/light-scattering method, following previously described procedures [12].

Since farnesyl phosphate **2** (C_{15}) did not form stable vesicles at pH 4.0 at 21°C, we studied water permeability with the C_{20} phosphates. Initially, we characterized the water permeability of the polyprenyl phosphates alone under different experimental conditions.

Table 4. Water Permeability of Unilamellar Vesicles of Phosphates—3, 4, 5—at 15°C ± 0.1°C and pH 5.81 Measured by the Stopped-Flow/Light-Scattering Method

Entry	Phosphates	Diameter[a] (nm)	k[b] (s^{-1})	$t_{1/2}$[c] (ms)
1	**3**	180 ± 16	34.8 ± 0.7	19.9 ± 1.0
2	**4**	187 ± 31	9.8 ± 0.7	70.7 ± 4.9
3	**5**	190 ± 43	6.4 ± 0.8	108.3 ± 7.7

[a] Average diameter ± standard deviation.
[b] Average rate constant ± standard deviation.
[c] Average $t_{1/2}$ ± standard deviation.

Effect of the Temperature. A suspension of unilamellar vesicles of phytyl phosphate **4** was prepared, and the water permeability was measured at different temperatures (15°C, 21°C, 28°C, and 37°C) and pH 5.81. The fluidity of the membrane increased with temperature, which facilitates water effusion (Figure S3).

Effect of Unsaturation. Vesicles of geranylgeranyl phosphate **3**, phytyl phosphate **4**, and phytanyl phosphate **5** were prepared, and their water permeability was measured at 15°C and pH 5.81 (Table 4). The water permeability of phytyl phosphate **4** vesicles and that of phytanyl phosphate **5** vesicles are, respectively, 3.5 times and 5 times lower than for geranylgeranyl phosphate **3** vesicles (entries 1, 2, and 3, Table 4). The membranes of phosphates **4** and **5** are thus more compact than those of phosphate **3**.

We could not compare the effects of branched phosphates with those of nonmethylated ones. With dodecylphosphate (phase-transition temperature: 2.3°C), Walde et al. observed stable vesicle formation at pH 1.4–3.0 and crystallization at pH 3 and 9 [8]. However, we observed that the presence of 150 mM NaCl buffer (used in our stopped-flow experiments) or 200 mM citric acid-NaH_2PO_4 buffer left the phosphate in a crystalline state, although vesicle formation was observed at pH 2.6 in pure water. As the phase-transition temperature of hexadecylphosphate is about 40°C, we were unable to conduct stopped-flow experiments, which were conducted at 15°C for branched phosphates.

Next, we studied a mixture of phosphate and 10 mol% of free alcohol (Figure 1) in order to compare the effect of these alcohols on the water permeability measured at 15°C and at pH 5.81 with different parameters of the alcohols.

Geranylgeranyl Phosphate 3 + 10 mol% Polyprenyl Alcohol

Effect of Chain Length. The combination of geranylgeraniol **11** (C_{20}) and geranylgeranyl phosphate **3** (C_{20}), which have the same hydrophobic structures, increased the water permeability of vesicles (entry 1, Table 5). On the contrary, the addition of geranylfarnesol **9** (C_{25}) and farnesylfarnesol **10** (C_{30}) decreased the water permeability of vesicles (entries 2 and 3, Table 5). These data imply that the length of the added polyprenyl alcohol plays an important role in the

Table 5. Water Permeability of Unilamellar Vesicles Obtained from a Mixture of Geranylgeranyl Phosphate 3 and 10 mol% of Alcohol at 15°C ± 0.1°C Measured by the Stopped-Flow/Light-Scattering Method

Entry	Polyprenyl Alcohols	Diameter[a] (nm)	k[b] (s^{-1})	$t_{1/2}$[c] (ms)
1	**11**	179 ± 24	117.22 ± 3.48	5.9 ± 0.2
2	**9**	185 ± 23	13.06 ± 0 .34	53.0 ± 1.3
3	**10**	200 ± 35	2.67 ± 0.10	260.0 ± 10.0
4	**12**	187 ± 26	41.6 ± 2.10	16.6 ± 1.9
5	**13**	190 ± 21	2.76 ± 0.08	251.1 ± 5.6
6	**14**	183 ± 39	13.5 ± 0.40	51.3 ± 1.3
7	**15**	165 ± 24	2.96 ± 0.09	234.0 ± 7.1
8	**16**	192 ± 48	3.03 ± 0.13	228.0 ± 0.8
9	**17**	162 ± 35	3.60 ± 0.10	192.5 ± 7.6

[a] Average diameter ± standard deviation.
[b] Average rate constant ± standard deviation.
[c] Average $t_{1/2}$ ± standard deviation.

Table 6. Water Permeability of Unilamellar Vesicles Obtained from a Mixture of Phytyl Phosphate 4 and 10 mol% of Alcohols at 15°C ± 0.1°C Measured by the Stopped-Flow/Light-Scattering Method

Entry	Polyprenyl Alcohols	Diameter[a] (nm)	k[b] (s^{-1})	$t_{1/2}$[c] (ms)
1	**11**	198 ± 34	2.90 ± 0.31	239.0 ± 21.5
2	**9**	181 ± 17	3.10 ± 0.44	223.5 ± 24.6
3	**10**	203 ± 40	2.50 ± 0.41	277.3 ± 37.2
4	**12**	193 ± 47	3.10 ± 0.33	223.5 ± 22.2
5	**13**	200 ± 65	1.98 ± 0.24	350.0 ± 28.3
6	**14**	182 ± 20	4.70 ± 0.31	147.4 ± 8.80
7	**17**	183 ± 20	2.29 ± 0.11	302.7 ± 15.0

[a] Average diameter ± standard deviation.
[b] Average rate constant ± standard deviation.
[c] Average $t_{1/2}$ ± standard deviation.

organization of the system. Possibly, polyprenyl alcohols bearing a longer chain than the corresponding phosphate are incorporated into both leaflets of the bilayer in an interdigitated manner. This would improve the van der Waals interactions and the compactness of the membrane [17].

Effect of Cyclic Moieties. The addition of monocyclogeranylfarnesol **13** (C_{25}) bearing the same length of hydrophobic chain as geranylgeraniol **11** (C_{20}) decreases the water permeability of geranylgeranyl phosphate **3** vesicles (entry 5, Table 5). The presence of one ring led to a decrease of the water permeability, probably because the incorporation of these more rigid molecules improves the order of the membrane. In the same way, cholesterol **17**, a well-known ubiquitous membrane reinforcer in animals [18–21], stabilizes the geranylgeranyl phosphate **3** membranes. However, monocyclogeranylfarnesol **13** (C_{25}), as well as acyclic molecules such as farnesylfarnesol **10** (C_{30}), phytol **15** (C_{20}), and phytanol **16** (C_{20}), are better reinforcers than cholesterol **17** (entries 3, 5 7, 8, and 9, Table 5). This suggests that the degree of membrane reinforcement depends on the precise structural relationship between the membrane constituent and the additive.

Degree of Unsaturation. In the series geranylgeraniol **11**, phytol **15**, and phytanol **16**, unsaturation in the molecule led to a dramatic change in the water permeability of the geranylgeranyl phosphate **3** vesicles. The addition of phytol **15** or phytanol **16** strongly decreased the water permeability of geranylgeranyl phosphate **3** vesicles (entries 7 and 8, Table 5), whereas addition of geranylgeraniol **11** increased it (entry 1, Table 5).

Monocyclogeranylgeraniol **12** (C_{20}) increased the water permeability of geranylgeranyl phosphate **3** vesicles, whereas vitamin A **14**, which has approximately the same length of hydrophobic chain as the former compound, decreased the water permeability (entry 6). This effect may be attributed to the presence of the conjugated double bonds in vitamin A **14**, which increase the rigidity of the molecule.

Phytyl Phosphate 4 + 10 mol% Polyprenyl Alcohol

Effect of Chain Length. For the different acyclic polyprenyl alcohols (geranylgeraniol [C_{20}] **11**, geranylfarnesol [C_{25}] **9**, and farnesylfarnesol [C_{30}] **10**), the length of the hydrophobic part barely influenced water permeability (entries 1, 2, and 3, Table 6). However, in the case of monocyclopolyprenyl alcohols **12** (C_{20}) and **13** (C_{25}), the alcohol bearing the longer chain significantly improved the barrier property of the membrane (entries 4 and 5, Table 6).

Effect of Cyclic Moieties. Monocyclogeranylfarnesol **13**, which has approximately the same total length as geranylgeraniol **11**, diminished more the water permeability of phytyl phosphate **4** vesicles (entries 1 and 5, Table 6). The presence of the cyclohexenyl moiety, which makes the membrane more rigid, may explain this decrease in water permeability. Similarly, cholesterol **17** also diminished the water permeability (entry 7, Table 6).

Conclusion

We provide evidence here that free polyprenyl alcohols can play the role of reinforcers in ''primitive'' vesicles formed from polyprenyl phosphates.

First, microscopic observations have allowed us to determine the pH range of spontaneous vesicle formation with polyprenyl phosphates, with or without polyprenyl alcohols. Acyclic or monocyclic polyprenyl phosphates were shown to form stable vesicles in water over a wide range of pHs. The length of the carbon chain and the degree of unsaturation were found to be important factors for vesicle formation of polyprenyl phosphates as a function of pH.

Moreover, the addition of polyprenyl alcohols allowed vesicle formation of polyprenyl phosphate at higher pHs by increasing the ratio of the hydrophobic-to-hydrophilic volume in the system. The addition of a polyprenyl alcohol

bearing a longer chain than the polyprenyl phosphate did not significantly change the pH range of vesicle formation.

These results show that a better incorporation could be achieved when the size and shape of the alcohol are close to those of the polyprenyl phosphate forming the membranes.

We then showed that polyprenyl alcohols are indeed, in most cases, reinforcers of polyprenyl phosphate membranes. We have further demonstrated that some polyprenyl alcohols are better reinforcers in certain polyprenyl phosphate membranes than cholesterol. This conclusion was obtained using a stopped-flow/light-scattering method to measure water permeability.

We also studied different parameters that could affect water permeability of vesicles made of polyprenyl phosphates or polyprenyl phosphate/polyprenyl alcohol. Water permeability increased with temperature. A decrease in the degree of unsaturation of polyprenyl phosphates, all of approximately the same length, tended to stabilize the membrane by enhancing the cohesion of the hydrophobic part, and therefore it decreases the water permeability. Finally, different structural parameters of polyprenyl alcohols, such as the presence of rings, the degree of unsaturation, as well as the hydrophobic length, were found to play a very important role in the organization and compactness of the membrane.

SIGNIFICANCE

We had earlier postulated and then confirmed using physico-chemical methods that hopanoids or dipolar-carotenoids act as membrane reinforcers in prokaryotes, as cholesterol does in eukaryotes. We now hypothesized that free polyprenyl alcohols might reinforce "primitive" membranes of vesicles made of polyprenyl phosphates. In the present paper, we first determined the range of the hydrophobic length in polyprenyl phosphates that enables them to form vesicles: those containing 15–30 C atoms form vesicles. We then demonstrated that the lipophilicity/hydrophobicity ratio is important for vesicle formation in a variety of polyprenyl phosphate/polyprenyl alcohol/water systems. In these systems, some polyprenyl alcohols reduce the water permeability of vesicles, even more efficiently than cholesterol, when the size and shape of the membrane constituents and the hydrophobic molecules to be inserted are closely matched. This would provide a mechanism for selecting lipid membrane constituents during the course of biomembrane evolution. These results reinforce our hypothesis that polyprenyl alcohols might have been reinforcers of "primitive" membranes made of polyprenyl phosphates.

EXPERIMENTAL PROCEDURES

Synthesis

Synthesis and complete procedure of polyprenyl phosphates and polyprenols are provided in Supplemental Data.

Microscopic Observation

Preparation of Samples

A typical procedure was as follows: a mixture of lipid (3 mg) with or without 10 mol% of terpenic alcohol was dissolved in 3 ml of a 1:1 (v/v) mixture of chloroform and methanol. An aliquot (5 μl) of the solution was dropped on a coverglass (0.17 mm thick). After 10 min of drying at room temperature, the lamellar solid remaining on the slide was brought into focus, and 50 μl of a buffer at 25°C was added. Aqueous buffers were prepared with MilliQ water (citric acid-Na_2HPO_4 buffer [pH 2.6–7.6], Na_2HPO_4-NaH_2PO_4 buffer [pH 7.8–8.0], glycine-NaOH buffer [pH 8.6–10.6], Na_2HPO_4-NaOH buffer [pH 11.0–11.9], or NaCl-NaOH buffer [pH 12.0–13.0]). Samples were unsealed. Vesicles were observed to grow from the edges of the solid.

Differential Interference Contrast Microscopy

The sample was observed by differential interference contrast microscopy: Axiovert 135, 63×/1.40 Plan Achromat Oil DIC objective, ×2.5 insertion lens (light sources: Hg and halogen lamps) (Carl Zeiss). Video system: CCD camera (C 2400-75H) and image processor (Argus 20) (Hamamatsu Photonics).

Fluorescence Microscopy

To a mixture of lipid with or without 10 mol% of terpenic alcohol (total: 3 mg) dissolved in 300 μl of methanol/chloroform (1:1, v/v), 5 mol% of Nile Red (λ_{ex} = 559 nm, λ_{em} = 640 nm) was added. The sample was handled as described above and was observed by differential interference contrast optical microscopy using the fluorescence mode.

Water Permeability

Variation of scattered light intensity (I) versus time (t) upon osmotic shock thermostated at T = 15.0°C ± 0.1°C (MT/2 Lauda thermostat) was followed at the fixed wavelength of 400 nm (entrance and exit slits width = 2 mm) on a Biosequential DX-17MV stopped-flow ASVD spectrofluorimeter (2 mm path length cuvette; Applied Photophysics). Analysis of data was performed with Bio-Kine Analysis V 3.14 software (Bio-Logic). The vesicle dispersions were subjected to the osmotic shock 60 min after their preparation (the preparation and fitting of the vesicles solutions are provided in the Supplemental Data). The stability of the samples was checked by comparison of the average size of the vesicles just after their preparation and their average size 5 hr later. A sample was deemed stable if it remained monodispersed and the average size of the vesicles was constant. An aliquot of vesicle dispersions prepared with buffer A (citric acid-Na_2HPO_4 buffer, 150 mM NaCl [pH 5.81]) was rapidly mixed with hypomolar buffer B (citric acid-Na_2HPO_4 buffer, 0 mM NaCl [pH 5.81]) in the stopped-flow instrument. Each experimental curve (1000 points) was obtained by monitoring the change in scattered light intensity (I) following the rapid mixing ($t \leq 3$ ms) of equal volumes (100 μl) of sample and hypotonic buffer. Typically 10–15 injections provided independent experimental kinetic curves that were superimposed, averaged, and numerically treated by the Biokine software, which uses a factor analysis method and a Simplex algorithm. The results of 10–15 runs of experiments were then averaged: the corresponding k values and standard deviations are given in tables. The values of the first-order rate constants k determined for the theoretical exponential model measure the H_2O permeability of the vesicles.

pKa Titration

Potentiometric titrations were performed using an automatic titrator system DMS 716 Titrino (Metrohm) with 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), and the cell was thermostated at 25.0°C ± 0.2°C by the flow of a Haake FJ thermostat (the procedures for the preparation of solutions are provided in the Supplemental Data).

Supplemental Data

The supplemental data includes Supplemental Figures S1, S2, and S3 and Table S1; the synthetic procedure and analysis of polyprenyl phosphates and polyprenols; the complete procedure for the

stopped-flow analysis; and the complete procedure for the pKa titration. They are available at http://www.chembiol.com/cgi/content/full/14/3/313/DC1/.

ACKNOWLEDGMENTS

S.S. and N.R. were funded by doctoral fellowships from the Ministry of Higher Education and Research (France) and "Les Amis des Sciences." E.G.K. thanks the European Community for a Marie Curie Intra-European Fellowship. We thank Dr. Michiko Atsumi and Ochanomizu University for the calculation of molecular volume and length of polyprenyl alcohols. We thank Prof. T. Eguchi, Tokyo Institute of Technology, Dr. M. Tsuji, Nisshin Flour Milling, and Dr. T. Takigawa, Kurare, for gifts of phytanol, phytol, all-*trans*-geranylgeraniol, and all-*trans*-nonaprenyl phosphate.

Received: August 7, 2006
Revised: November 20, 2006
Accepted: November 20, 2006
Published: March 23, 2007

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