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Dolichyl phosphate induces non-bilayer structures, vesicle fusion and transbilayer movement of lipids: a model membrane study

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The effect of dolichol and dolichyl phosphate on fusion between large unilamellar vesicles comprised of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was studied using a fluorescence resonance energy transfer assay. The influence of dolichyl phosphate on the transbilayer movement of DOPC in multilamellar vesicles (MLV) and large unilamellar vesicles (LUV) composed of DOPC and DOPE (1:2) was investigated by using the phosphatidylcholine-specific transfer protein. ^{31}P-NMR and freeze-fracture electron microscopy were employed to study the macroscopic organization of DOPC and DOPE containing model membranes in the absence or presence of dolichyl phosphate. The results indicate that (1) both dolichol and dolichyl phosphate enhance vesicle fusion in a comparable and concentration-dependent way; (2) the amount of exchangeable PC from MLVs is increased by dolichyl phosphate, probably as a result of fusion processes; (3) dolichyl phosphate destabilizes the bilayer organization in MLVs comprised of DOPE and DOPC, resulting in the formation of hexagonal (H_{II}) phase and 'lipidic' particles.

Introduction

During the last decades the occurrence and distribution of dolichols in various tissues have been investigated [1–3]. In liver cells dolichol was found in all intracellular membranes with the highest concentration in lysosomes and Golgi membranes [4–6]. Furthermore, a considerable portion ($\approx 40\%$) of these dolichols is esterified with fatty acids. Up till now no function has been established for both the free and esterified dolichols. Most interestingly (mostly minor) fraction of the dolichols is phosphorylated. These dolichyl phosphates, which are predominantly located in the endoplasmic reticulum [7,8], are generally accepted as being intermediates in the biosynthesis of oligosaccharide chains N-glycosidically linked to proteins [9]. There are a few facts which indicate that dolichyl pyrophosphate-attached sugars and/or oligosaccharides display a transmembrane movement [10–13]. The nucleo-

Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; N-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)dioleoyl phosphatidylethanolamine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SUV, small unilamellar vesicle.

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tide-bound sugars are synthesized in the cytoplasm and the microsomal membrane is not permeable for any charged substances, while the majority, if not all, of the glycoproteins are found on the luminal side of the vesicles. Also, parts of the glycosyl transferases and dolichyl phosphate-bound sugars of the intact microsomal vesicles exhibit sensitivity to appropriate hydrolytic enzymes. These facts suggest that a transfer mechanism from outside to inside has to exist. The molecular mechanism by which this transmembrane transport occurs is not known.

In order to get an insight into the possible functions of these polyprenols, the interaction of dolichol and dolichyl phosphate with phospholipid model membranes has been studied intensively recently. First, calorimetric and fluorescence depolarization studies revealed that dolichol increases the fluidity in both PE and PC bilayers [14,15]. Furthermore, it has been proposed that the neutral dolichols are sandwiched in between two monolayers of a phospholipid bilayer, while the phosphate moiety of the charged dolichol is located at the membrane-water interface [16]. More recently the latter authors also emphasized the utility of deuterium-labeled polyisoprenols in revealing their order and motions in membranes with NMR techniques [17]. Furthermore, the overall bilayer destabilizing effect of dolichol and dolichol derivatives on PE-containing model membranes, thereby leading to hexagonal (H_{II}) phase formation, has been reported [14]. Independently, this effect was discovered and discussed in relation to membrane curvature by Gruner [18].

In a previous study [14], we speculated on the possible relevance of the bilayer destabilizing capacity of dolichols in relation to the function of dolichyl phosphate as a carrier of sugars across the endoplasmic reticulum membrane. In addition, under conditions that dolichol or dolichyl phosphate destabilizes the bilayer structure in PE-PC mixtures, the enzyme mannosyltransferase II, which catalyzes transfer from GDP-mannose to an oligosaccharide-dolicholpyrophosphate intermediate of glycoprotein biosynthesis, is activated [19]. Furthermore, it is proposed that the dolichol-induced formation of non-bilayer configurations in liposomes comprised of PE is accompanied by membrane leakage [20]. In general, non-lamellar lipid organizations have been proposed to be related to functional properties like membrane fusion and the transbilayer movement of phospholipids [21,22]. Therefore, we investigated the effects of dolichol and dolichyl phosphate on the temperature-dependent fusion between large unilamellar vesicles comprised of PC and PE by using a fluorescence resonance energy transfer assay [23]. Furthermore, we present data concerning the dolichyl phosphate-dependent transbilayer movement of PC both in multilamellar and large unilamellar vesicles comprised of PC and PE. In order to relate membrane function and structure, we studied the influence of dolichyl phosphate on the macroscopic organization of comparable lipid systems by using ^{31}P-NMR and freeze-fracture electron microscopy.

The results indicate that (1) dolichol and dolichyl phosphate enhance vesicle fusion, (2) dolichyl phosphate facilitates the transbilayer movement of PC during the fusion process, and (3) these membrane functions are paralleled by the appearance of intermediate non-bilayer structures.

These results are discussed in the light of the dolichyl pyrophosphate sugar translocation across the endoplasmic reticulum membrane and fusion phenomena in secretory cells.

Materials and Methods

Chemicals. Dolichol-20 was isolated from autopsy specimens of human liver [24] and purified using reverse-phase column chromatography. Dolichyl-20 phosphate was prepared by phosphorylation of dolichol-20 by the procedure of Danilow and Chojnacki [25]. [1-^{14}C]Dolichol-20 (spec. act. 30 mCi/mmol) was obtained from Dr. M. Mizuno (Kurray Co. Ltd. Okayama, Japan). [1-^{14}C]Dolichyl-20 phosphate (spec. act. 2.9 mCi/mmol) was prepared by phosphorylation of [1-^{14}C]dolichol-20 [25]. For identification of the polyprenols ^{1}H- and ^{13}C-NMR spectroscopy and mass spectrometry were used. In different TLC solvent systems both dolichol-20 and dolichyl-20 phosphate gave single spots. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was synthesized from DOPC with phospholipase D in the presence of ethanolamine according to Comfurius and Zwaal [26]. DOPC was prepared according to the procedure of Van

Deenen and De Haas [27]. Dioleoyl-*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine (N-Rh-PE) were purchased from Avanti Polar-Lipids Inc. (Birmingham, AL). Egg PC was purified as described recently [28]. [1-^{14}C]DOPC (spec. act. 114 mCi/mmol) and [*cholesteryl*-1,2(n)-^{3}H]cholesteryl hexadecyl ether (spec. act. 46.8 mCi/mmol) were obtained from NEN Research Products (Boston, MA). Phospholipids and dolichol-20 were stored as stock solutions in chloroform and dolichol-20 phosphate in chloroform/methanol (2:1, v/v) at −20°C under nitrogen atmosphere. Phosphatidylcholine transfer protein, purified from bovine liver [29] and stored in 50% glycerol at −20°C (531 μg/ml) was a generous gift of Professor Dr. K.W.A. Wirtz. Chloroform and methanol were distilled before use. Other reagents were of analytical grade.

Lipid vesicles preparations. Lipids, mixed at the desired ratio in chloroform, were dried by evaporation under reduced pressure. Multilamellar vesicles (MLV) were prepared by dispersing the dried lipids in a buffer containing 100 mM NaCl and 10 mM Tris-acetate (pH7.4). For ^{31}P-NMR experiments the buffer contained 25% $^{2}H_2O$. Small unilamellar vesicles (SUV) were prepared by sonication of multilamellar vesicles under nitrogen at 0°C for 20 min using a Branson tip sonicator (energy output, 50 W). After sonication, the dispersion was centrifuged at 35000 × *g* for 30 min and the supernatant, containing the small unilamellar vesicles, was used for the experiments. Large unilamellar vesicles (LUV) were prepared in a 100 mM NaCl and 10 mM Tris-acetate (pH7.4) solution by the reverse-phase evaporation method of Szoka et al. [30], followed by extrusion through a Durapore membrane (Millipore Millex-HV filter unit; 0.45 μm pore size). Before use the large unilamellar vesicles were stored at 4°C. Lipid phosphorus was assayed according to Böttcher et al. [31].

Incorporation of externally added dolichol or dolichyl phosphate in lipid vesicles. Small unilamellar vesicles comprised of DOPC and DOPE (1:2) (300 nmol/ml) containing 0.5% N-Rh-PE were prepared as described above. After an incubation at 35°C (15 min) 10 μl of tetrahydrofuran, containing various amounts of [1-^{14}C]dolichol-20 or [1-^{14}C]dolichyl-20 phosphate, were added to 1 ml of the vesicle suspension under continuous stirring. After 15 min the vesicle suspension was eluted over a Sepharose 2B column (15 × 1 cm). The vesicles were eluted in the void volume. After measuring the N-Rh-PE fluorescence and radioactivity, the degree of polyprenol incorporation in the vesicles can be calculated. In the absence of vesicles, the dolichol or dolichyl phosphate did not elute from the column under similar conditions.

Nuclear magnetic resonance (NMR). Broad-band proton-decoupled (input power 18 W) ^{31}P-NMR spectra were obtained with a Bruker WH90 spectrometer operating at 36.4 MHz. After preincubation of the samples during 30 min free induction decays were accumulated from 40000 transients (2 h) employing a 8 μs 40° radio-frequency pulse and a 12 kHz sweep width. To increase the signal-to-noise ratio, the accumulated free induction decays were exponentially multiplied prior to Fourier transformation, resulting in a 50 Hz line broadening.

Freeze-fracture electron microscopy. For freeze-fracturing, the samples were quenched at room temperature by plunge-jet freezing using the KF80 Reichert device. No cryoprotectants were used. The frozen samples were subsequently worked up in a Balzer freeze-etch machine according to standard procedures. The replicas were examined in a Philips 301 electron microscope.

Membrane fusion. Membrane fusion was determined by the fluorescence resonance energy transfer assay of Struck et al. [23]. One population of large unilamellar vesicles containing 2 mol% of NBD-PE and 2 mol% of N-Rh-PE (population A) was mixed in a 1:19 ratio with unlabeled large unilamellar vesicles (population B). The decrease of resonance energy transfer between the two fluorescent probes, as a result of vesicle fusion and the concomitant dilution of the fluorescent lipids with the unlabeled membrane, was determined by measuring the increasing NBD-PE fluorescence at 525 nm after excitation at 450 nm. This increase of fluorescence at 525 nm was related quantitatively to that observed for vesicles containing 0.1 mol% NBD-PE and 0.1 mol% N-Rh-PE-(population C). After preincubation at various temperatures during 15 min, the samples were cooled at room

temperature for 20 min. The membrane fusion was then monitored at 20°C in a Perkin-Elmer LS-5 luminescence spectrometer with 10 nm excitation and emission slits equipped with a chart recorder. All measurements were repeated with identical results after another 10 min incubation at 20°C.

Phosphatidylcholine exchange assay. The transfer of [^{14}C[DOPC from large unilamellar vesicles or multilamellar vesicles containing DOPC, DOPE (1 : 2 molar ratio) and various amounts of dolichyl-20 phosphate to small unilamellar vesicles was measured as described previously [33] with some slight modifications. The incubation mixture contained MLV or LUV (2 μmol phospholipid) ($25 \cdot 10^3$ dpm [1-^{14}C]DOPC), SUV (20 μmol phospholipid) (egg PC containing 1.10^6 dpm [^{3}H] cholesteryl hexadecyl ether, as a non-exchangeable marker) and 50 μg PC transfer protein. During incubation the mixture was rotated at 20°C and at time intervals of 30 min samples (1 ml) were withdrawn. The large vesicles in these samples (MLV or LUV) were sedimented at $35\,000 \times g$ for 30 min, washed two times with 1 ml buffer and assayed for ^{14}C/^{3}H radioactivity and lipid phosphorus. The percentage [^{14}C]DOPC remaining in the large vesicles was calculated from ^{14}C radioactivity and phospholipid phosphorus, after correction for small amounts (max. 6%) of contaminating small vesicles, as determined from ^{3}H radioactivity.

Results

^{31}P-NMR and freeze-fracture electron microscopy

In order to possibly relate membrane functions like fusion and phospholipid transbilayer movement with the occurrence of non-bilayer phospholipid structures, we have studied first the influence of dolichyl-20 phosphate on the macroscopic organization of hydrated PE/PC samples. We have selected a mixture in which DOPE and DOPC are present in a molar ratio of 2 : 1, since previous studies have shown that in this mixture the phospholipids organize in bilayers up till 40°C, while above this temperature the hexagonal (H_{II}) phase preferring DOPE (bilayer to H_{II} phase transition of pure DOPE ≈ 10°C [39]) can induce structural transitions [34].

Fig. 1A shows the ^{31}P-NMR spectra of an aqueous dispersion of DOPE-DOPC (2 : 1) recorded at different temperatures. Between 20°C and 60°C an axially symmetric powder pattern with a low-field shoulder and a high-field peak (separated by approximately 40 ppm) is observed, typical for phospholipids in extended bilayers [35,36]. At 70°C a sharp signal is superimposed at the resonance position of phospholipids undergoing rapid isotropic motion ($\tau_c < 10^{-5}$ s). When the sample is cooled down to 20°C, the spectrum reveals the same lineshape as was observed at 70°C indicative for strong hysteresis which is typical for PC-PE mixtures [37]. Employing freeze-fracture electron microscopy we are able to visualize the membrane structure of the sample described above. Fig. 2A shows the freeze-fracture electron micrograph of the aqueous mixture containing DOPE and DOPC (2 : 1) quenched at 20°C. In agreement with the ^{31}P-NMR bilayer lineshape, this picture reveals smooth fracture faces of alternating bilayers typical for multilamellar systems. Fig. 2B represents the same sample at 20°C after being heated to 70°C and subsequently cooled back. An interwoven network of lipid fracture planes can be observed, comparable with previous data concerning Ca-cardiolipin complexes [38].

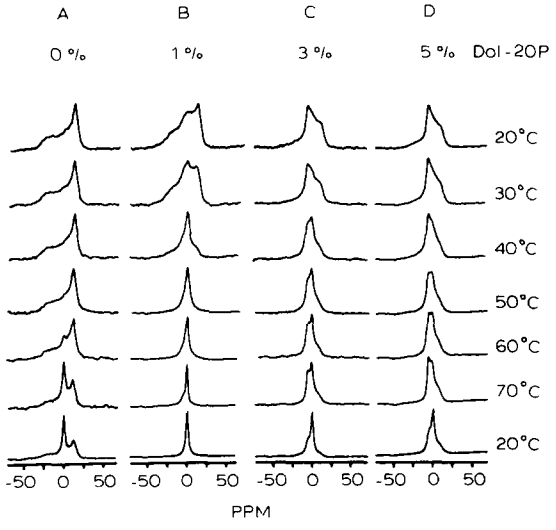

Fig. 1. Proton-decoupled 36.4 MHz ^{31}P-NMR spectra obtained from aqueous dispersions of DOPE-DOPC (2 : 1) (A), containing 1% (B), 3% (C) and 5% (D) dolichyl-20 phosphate at 20, 30, 40, 50, 60, 70 and 20°C successively.

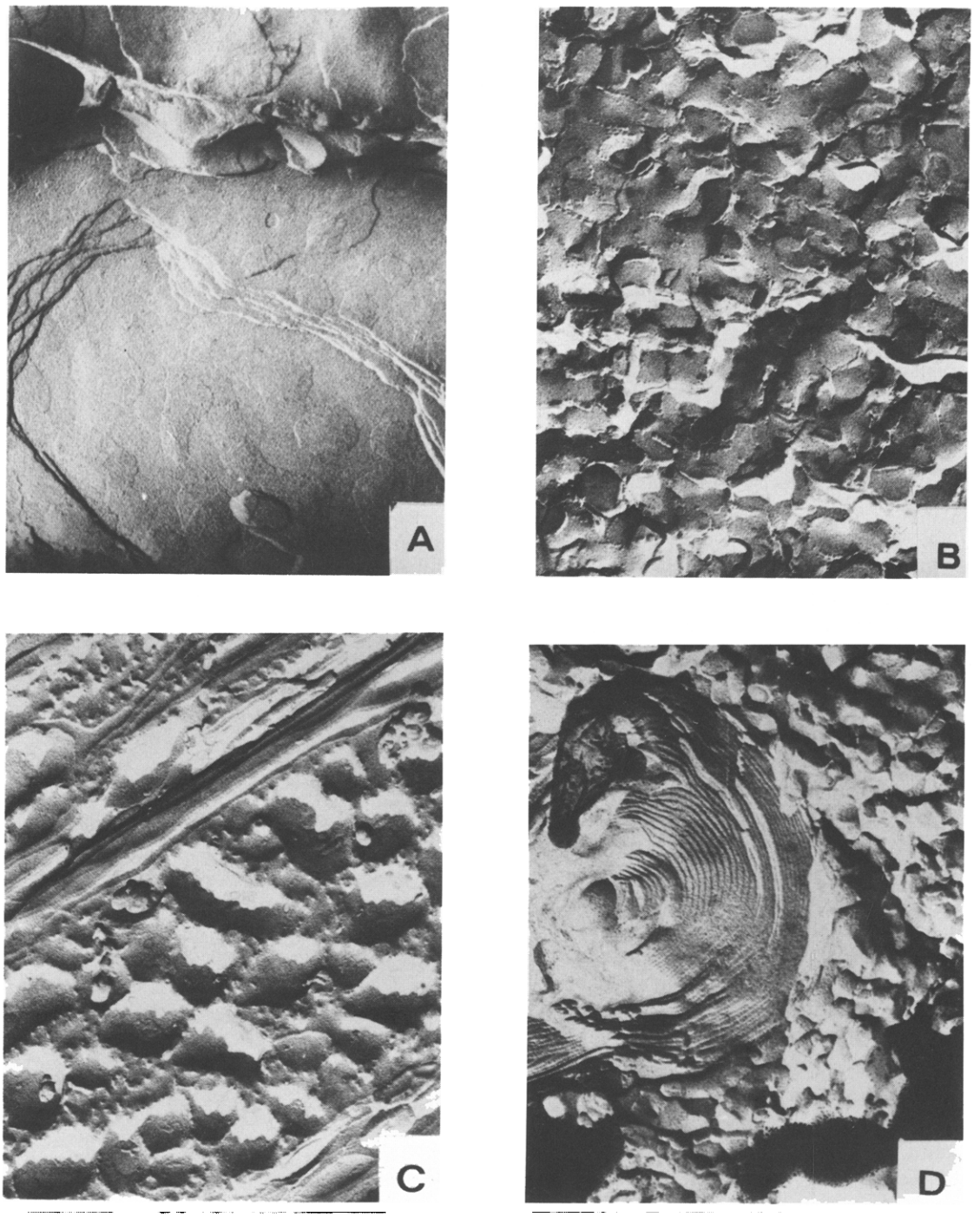

Fig. 2. Freeze-fracture electron microscopy pictures of hydrated samples consisting of DOPE-DOPC (2:1). A: quenched from 20°C; magnification × 57000. B: quenched from 20°C after heating to 70°C; magnification × 57000. C: containing 1 mol% dolichyl-20 phosphate; quenched from 20°C after heating to 70°C; magnification × 100000. D: containing 3 mol% dolichyl-20 phosphate; quenched from 20°C; magnification × 57000.

Furthermore, at the edges of the fracture planes rows of 'lipidic' particles and corresponding pits can be seen. These lipidic particles are suggested to represent inverted micelles either embedded in between two monolayers or present at a contact side between two bilayers (for a review, see Ref. 22). Such a macroscopic organization of phospholipids is in agreement with the observed isotropic peak in the corresponding ^{31}P-NMR spectrum [39].

Incorporation of up till 5 mol% dolichyl-20 phosphate in the hydrated DOPE-DOPC (2:1) mixture leads to dramatic changes in the phase behavior. At 20°C the presence of 3 or 5 mol% dolichyl phosphate predominantly results in hexagonal (H_{II}) phase formation (Fig. 1C and 1D). In a ^{31}P-NMR spectrum the hexagonal (H_{II}) phase is characterized by a 50% reduction of linewidth and a reversed asymmetry in comparison with a bilayer type of spectrum. This is typical for phospholipids undergoing rapid rotation ($\tau_c < 10^{-5}$ s) around an axis perpendicular to the long axis of the molecule [40]. Fig. 2D shows the electron micrograph of a DOPE-DOPC (2:1) sample in which 3 mol% dolichyl phosphate is incorporated at 20°C. In good agreement with the ^{31}P-NMR data hexagonally organized tubes can be observed and, to a minor extent, some smooth fracture faces. With increasing temperature two effects can be seen. First, in the temperature range between 20 and 40°C the hexagonal (H_{II}) phase tends to dominate over the bilayer component in samples containing 3 or 5 mol% dolichyl phosphate (Fig. 1C and 1D). Second, above 40°C the spectra change gradually and become more isotropic. This latter effect is most accentuated in the sample containing only 1 mol% dolichyl phosphate (compare Figs. 1B, 1C and 1D). In addition, also the DOPE-DOPC (2:1) samples containing 1–5 mol% dolichyl phosphate show a profound hysteresis. After heat treatment at 70°C, freeze-fracture replicas of the hydrated mixture containing 1 mol% dolichyl phosphate show circularly arranged lipidic particles, vulcano like protrusions, ridges and fissures at 20°C (Fig. 2C). These lipidic features are probably involved in the formation of a 'honeycomb' type of structure in which inverted micelles and extended inverted micelles (H_{II} tube) are located at the nexus of intersecting bilayers in the multilamellar system [22].

External addition of 5 mol% dolichyl phosphate in tetrahydrofuran to a hydrated mixture comprised of DOPE and DOPC (2:1) also leads to similar changes in the macroscopic organization of the lipids. As measured by ^{31}P-NMR, already at 20°C the spectrum shows a coexistence of bilayer, hexagonal (H_{II}) and 'isotropic' phases (Fig. 3). With increasing temperature the isotropic component in the spectra tends to dominate. As a comparison, these spectra are very similar to those obtained when 3 mol% dolichyl phosphate is incorporated in the same phospholipid mixture (Fig.

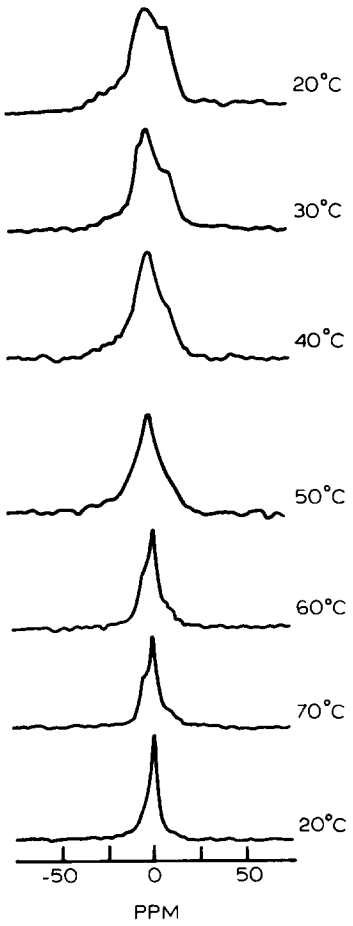

Fig. 3. Proton-decoupled 36.4 MHz ^{31}P-NMR spectra obtained from an aqueous dispersion of DOPE-DOPC (2:1) after external addition of 5 mol% dolichyl-20 phosphate in tetrahydrofuran. 100 μl of tetrahydrofuran (containing the polyprenols) was added to 20 ml of the phospholipid dispersion under stirring at 20°C. After 15 min the liposomes were centrifuged (35000 × g; 4°C; 20 min.) and resuspended in 1 ml of buffer.

3 and Fig. 1C), suggesting that the externally added dolichyl phosphate is only partly incorporated (see next section). In a control experiment addition of a comparable amount of tetrahydrofuran does not effect the ^{31}P-NMR characteristics of a hydrated DOPE-DOPC (2 : 1) mixture (data not shown), demonstrating that the structural changes are due to the dolichyl phosphate.

The results obtained from ^{31}P-NMR and freeze-fracture electron microscopy studies strongly suggest that dolichyl phosphate destabilizes the bilayer organization of the DOPE-DOPC (2 : 1) mixture in a concentration-dependent way, resulting in the formation of lipidic particles and hexagonal (H_{II}) phase.

Vesicle fusion experiments

We next investigated the influence of polyprenols on the fusogenic properties of large unilamellar vesicles comprised of DOPE and DOPC as a function of both temperature and PE content.

Fig. 4A shows the temperature dependency of lipid mixing for large unilamellar vesicles comprised of DOPE and DOPC in various ratios in the absence of dolichyl phosphate. Before each fluorescence measurement, the vesicles were incubated at the relevant temperature for 15 min. In the sample with a DOPE-DOPC molar ratio of 1 : 2, no increase in fluorescence at 525 nm could be observed below 50°C, indicating that there is no spontaneous fusion. Above this temperature a very small increase of the fluorescence can be detected, indicative for a low degree of vesicle fusion. By increasing the DOPE-DOPC ratio two effects can be seen. First, the temperature at which lipid mixing starts decreases proportionally and, second, at a given temperature the extent of fusion increases. For example, in the sample with a DOPE-DOPC molar ratio of 2 : 1, fusion starts already at room temperature. These results indicate that spontaneous fusion of these phospholipid vesicles is dependent on both the PE content and the temperature.

Fig. 4B shows the influence of 5 mol% dolichyl phosphate on the fusogenic properties of comparable DOPE-DOPC mixtures. This effect is represented by both a decrease of the critical fusion temperatures for the various PE-PC ratios and an increase in the extent of fusion for the different samples at all relevant temperatures. Furthermore, as can be seen in Fig. 5, the vesicle fusion is strongly dependent on the dolichyl phosphate concentration. Incorporation of up till 5 mol% dolichyl phosphate in DOPE-DOPC (2 : 1) LUVs gradually

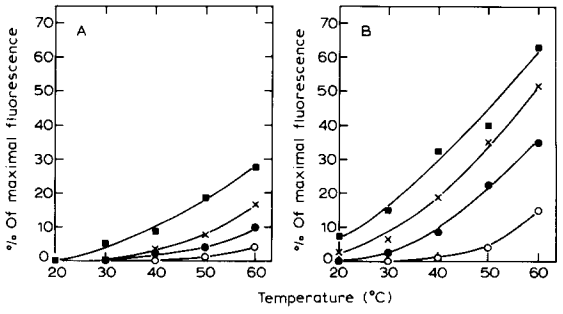

Fig. 4. Fusion between LUVs in the absence (A) and presence (B) of 5 mol% dolichyl-20 phosphate. ○, DOPE-DOPC (1 : 2); ●, DOPE-DOPC (1 : 1); ×, DOPE-DOPC (3 : 2); ■, DOPE-DOPC (2 : 1). Fusion was measured by the resonance energy transfer assay. The degree of vesicle fusion is expressed as the percentage of maximal NBD fluorescence and plotted as a function of temperature.

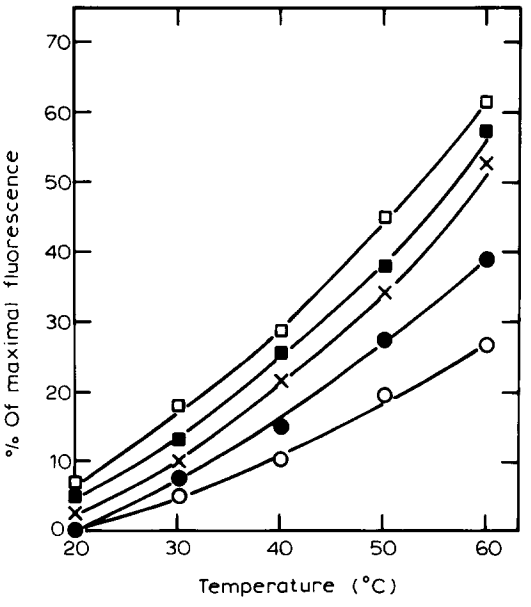

Fig. 5. Fusion between LUVs comprised of DOPE and DOPC (2 : 1) containing 0% (○), 1% (●), 2% (×), 3% (■), and 5% (□) dolichyl-20 phosphate. Membrane fusion is expressed as a function of temperature versus the percentage of maximal NBD fluorescence.

enhances the fusion over the temperature range between 20 and 60°C.

Fusion experiments have been performed too on large unilamellar vesicles containing different amounts of dolichol-20. This phosphate-free parent compound also has been shown to possess a strong bilayer destabilizing capacity in DOPE-DOPC (2 : 1) mixtures [14]. Fig. 6 shows the temperature-dependent fusion of large unilamellar vesicles comprised of DOPE and DOPC (2 : 1 molar ratio) in the absence and presence of 2 and 5 mol% dolichol-20. Apparently, dolichol-20 stimulates vesicle fusion in a comparable way as dolichyl-20 phosphate.

The effects of dolichol and dolichyl phosphate on vesicle fusion, as described above, have been studied by using model membranes in which these polyprenols were preincorporated already. It is interesting to know whether also external addition of dolichol or dolichyl phosphate to large unilamellar phospholipid vesicles can induce membrane fusion. Fig. 7A shows the lipid mixing characteristics of DOPE-DOPC (2 : 1) large unilamellar vesicles after addition of up to 30 mol% (based upon phospholipid phosphorus) dolichyl-20 phosphate in tetrahydrofuran. Already the addition of 2 mol% dolichyl phosphate leads to a dramatic stimulation of the vesicle fusion at 35°C. Addition of more than 2 mol% dolichyl phosphate does not further enhance the fusion. When dolichol-20 is added to DOPE-DOPC (2 : 1) large unilamellar vesicles, the fusion is stimulated in a comparable way (Fig. 7B).

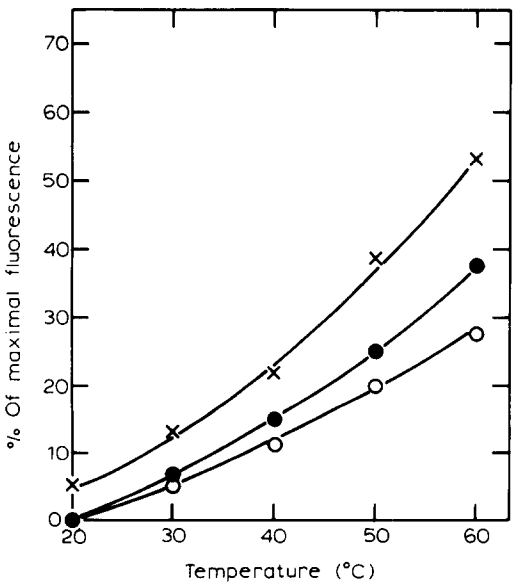

Fig. 6. Vesicle fusion between DOPE-DOPC (2:1) LUVs containing 0% (○), 2% (●) and 5% (×) dolichol-20. The percentage of maximal NBD-PE fluorescence, representing the degree of fusion, is plotted as a function of temperature.

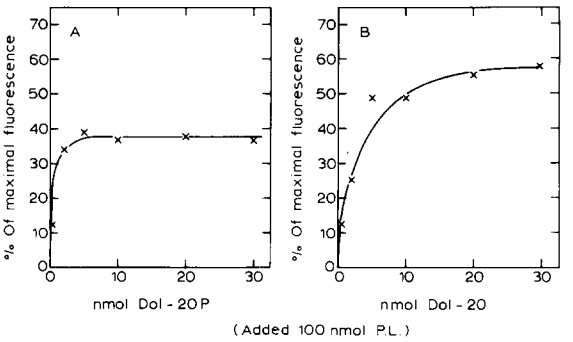

Fig. 7. DOPE-DOPC (2:1) LUVs fusion at 35°C after external addition of dolichyl-20 phosphate (A) and dolichol-20 (B). Dolichol-20 or dolichyl-20 phosphate were dissolved in tetrahydrofuran and aliquots of this solution (2–10 μl) were added to the vesicles suspension in a total volume of 2 ml under stirring at 35°C. After 15 min of incubation the fluorescence was monitored. The vesicle fusion is expressed as the percentage of maximal NBD fluorescence at 525 nm and plotted as a function of the amount of dolichol-20 (phosphate) added.

Since it has been proposed previously [22,42] that non-lamellar lipid structures could be involved in membrane fusion processes, we also studied the resulting macroscopic lipid structure by using freeze-fracture electron microscopy under conditions that dolichyl phosphate induced vesicle fusion as observed via lipid mixing measurements. In the control experiment, predominantly unilamellar vesicles can be visualized (Fig. 8A). External addition of 5 mol% dolichyl phosphate at 35°C to large unilamellar vesicles comprised of DOPC and DOPE (1 : 2 molar ratio) results in several morphological changes. Most of the lipid is present in aggregated multilamellar structures. On the fracture faces features can be seen (Fig. 8B) such as ridges and fissures next to vulcano-like protrusions and pits, complementary to lipidic particles, which, it has been suggested, are intermediates in membrane fusion [22]. The maximal degree of vesicle fusion is significantly higher for

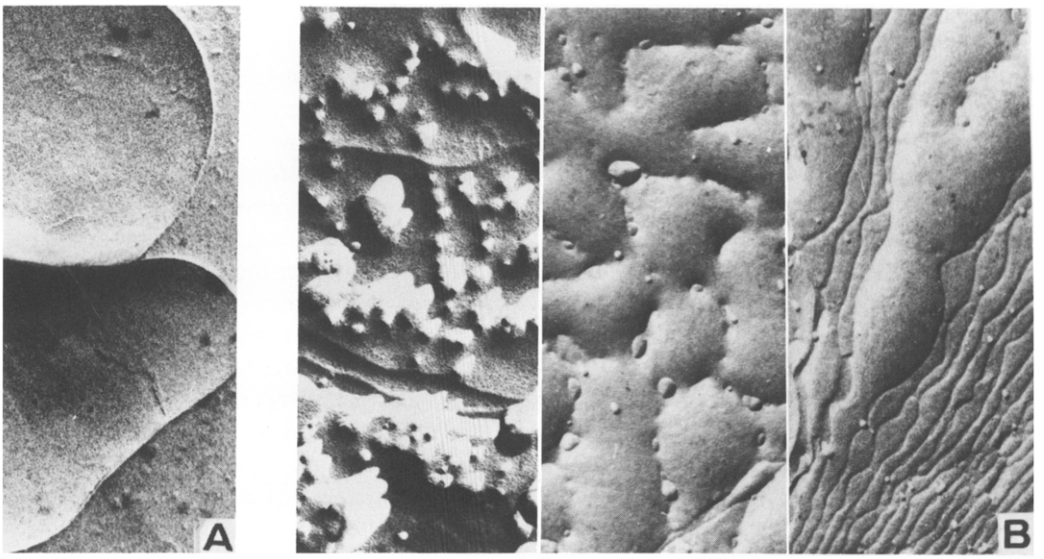

Fig. 8. Freeze-fracture electron microscopy pictures (magnification × 30000). A: large unilamellar vesicles consisting of DOPE and DOPC (2:1) after addition of 1% (by volume) tetrahydrofuran. B: after addition of 5 mol% dolichyl phosphate in tetrahydrofuran (1% by volume) to DOPE-DOPC (2:1) LUVs.

dolichol as compared to dolichyl phosphate. After addition of 5 mol% dolichol-20 at 35°C the lipid mixing reaches a two-times higher level as compared with the result obtained when 5 mol% dolichol-20 is preincorporated in the same phospholipid mixture (for a comparison, see Figs. 6 and 7B). The addition of a comparable amount of tetrahydrofuran to the DOPE-DOPC (2:1) large unilamellar vesicles does not cause any change in the fluorescence (data not shown). The difference between the fusogenic effects of externally added dolichol-20 and dolichyl-20 phosphate is possibly due to differences in the extent to which the two polyprenols are incorporated in the membrane. Therefore, we determined the degree of incorporation of both dolichol and dolichyl phosphate after external addition to DOPC-DOPE (1:2) vesicles. As can be seen in Fig. 9, the more hydrophobic parent polyprenol, dolichol-20, is to a larger extent incorporated in the vesicles than the phosphorylated form. The higher degree of fusion induced by the external addition of both dolichol-20 and dolichyl-20 phosphate as compared to the preincorporated polyprenols can be the result of temporary local higher outer monolayer concentrations, resulting in bilayer destabilization possibly in combination with increased vesicle aggregation. Finally, the possibility of an additional effect of the unincorporated polyprenols on the vesicle fusion can not be excluded completely.

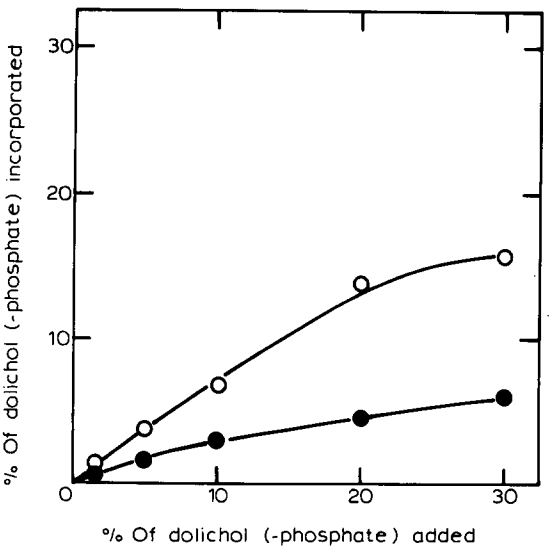

Fig. 9. Incorporation of externally added dolichol-20 (○) and dolichyl-20 phosphate (●) in vesicles comprised of DOPC and DOPE (1:2) at 35°C. For experimental details, see Materials and Methods.

Exchange of phosphatidylcholine

Using the phosphatidylcholine-specific transfer protein we have tried to gain insight into the possible relation between the bilayer destabilizing effect of dolichyl phosphate and the transbilayer motion of the PC in model membrane systems. For this purpose we have measured the exchangeability of PC from both multilamellar and large unilamellar vesicles consisting of DOPE and DOPC (2 : 1 molar ratio) and varying amounts of dolichyl phosphate. Fig. 10A shows the transfer of [^{14}C]DOPC from multilamellar vesicles containing 0, 1, 3 and 5 mol% dolichyl phosphate, respectively, as measured at 20°C. In the absence of dolichyl phosphate about 30% of the PC is available for exchange.

Incorporation of up to 5 mol% dolichyl phosphate does not enhance the exchangeability of PC at 20°C. However, after heat treatment (70°C, 1 h) of the multilamellar vesicles, the transfer of PC at 20°C is dependent on the presence of dolichyl phosphate (Fig. 10B). In the absence of the polyprenol again 30% of the [^{14}C]PC molecules can be transferred to the acceptor vesicles. Incorporation of dolichyl phosphate gradually increases the amount of PC available for exchange. When 5 mol% dolichyl phosphate is present in the multilamellar vesicles, 63% of the PC molecules can be exchanged.

These PC-exchange experiments have been performed too on large unilamellar vesicles comprised of DOPE and DOPC (2 : 1, molar ratio). As shown in Fig. 11, in the absence or presence of 1 mol% dolichyl phosphate about 40% of the [^{14}C]DOPC can be exchanged by the transfer protein at 20°C. This number of PC molecules available for exchange is lower than the expected minimal value of 50% for the amount of PC present in the outer monolayer of large unilamellar vesicles. However, during exchange the distribution of [^{14}C]DOPC over the donor and acceptor vesicles reaches equilibrium under which condition another 5% of the PC molecules seems unexchangeable. Furthermore, the possibility of the presence of a small fraction of multilamellar vesicles can not be excluded. When the concentration of dolichyl phosphate reaches 5 mol%, 66% of the PC molecules can be exchanged. This result indicates that dolichyl phosphate also enhances the exchangeability of PC from these vesicles in a concentration-dependent way, most likely representing an induction of the transbilayer movement of PC.

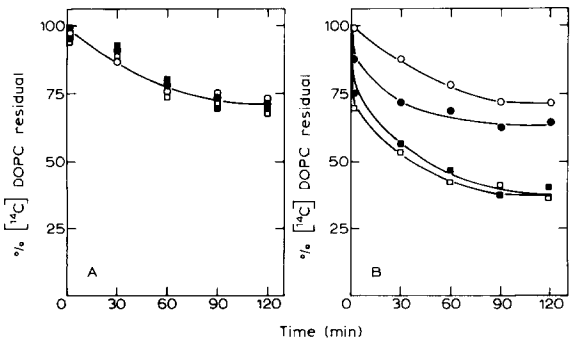

Fig. 10. Transfer protein-mediated exchange of ^{14}C-labeled DOPC between MLVs of DOPC-DOPE (1 : 2) containing 0% (○), 1% (●), 3% (■) or 5% (□) dolichyl-20 phosphate and SUVs at 20°C (A) and at 20°C after being heated up to 70°C during 1 h (B).

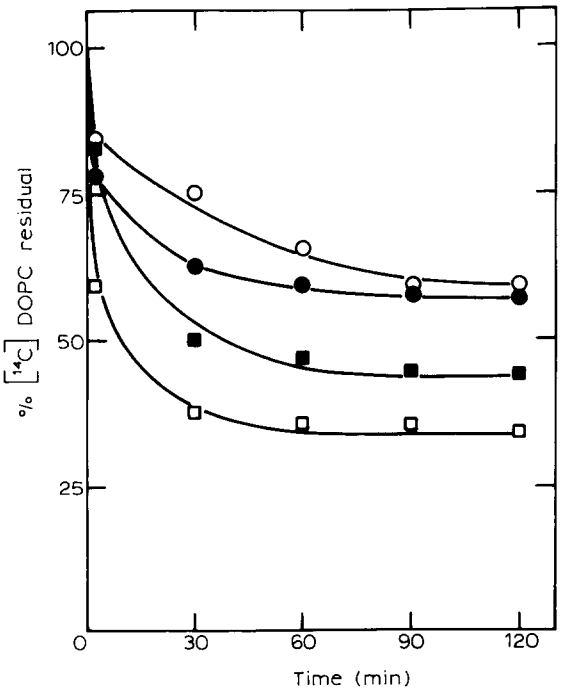

Fig. 11. Exchange of [^{14}C]DOPC from LUVs, comprised of DOPC and DOPE (1 : 2) at 20°C in the presence of 0% (○), 1% (●), 3% (■) or 5% (□) dolichyl-20 phosphate, to SUVs.

Discussion

In this paper, we have presented data concerning the influence of dolichyl-20 phosphate on (1) the fusion between large unilamellar vesicles comprised of DOPC and DOPE, (2) the exchangeability of DOPC from comparable hydrated phospholipid mixtures and (3) the macroscopic organization of these phospholipids in multilamellar vesicle systems.

With respect to vesicle fusion, three parameters are of importance. First, the degree of vesicle fusion, as determined by the fluorescence resonance energy transfer assay, is dependent on the DOPC-DOPE ratio. In general, at a given temperature, increasing amounts of DOPE stimulate the spontaneous fusion [41,42,43]. Second, for all the relevant DOPC-DOPE ratios, the vesicle fusion increases with increasing temperature. Third, most interestingly, incorporation of up to 5 mol% dolichyl-20 phosphate in DOPC-DOPE LUVs strongly enhances the vesicle fusion in a concentration-dependent way. Apparently, these three factors also effect the macroscopic organization of the phospholipids in multilamellar vesicles. The temperature- and concentration-dependent bilayer destabilizing effect of PEs in hydrated mixtures with PCs has been reported and discussed previously [44,45]. Furthermore, now we have shown by using ^{31}P-NMR and freeze-fracture electron microscopy that dolichyl-20 phosphate induces nonlamellar structures in fully hydrated samples comprised of DOPE and DOPC (2 : 1). In a previous study [14], a comparable bilayer-destabilizing capacity of the related polyprenol, dolichol-20, was observed. In addition, dolichol-20 enhances the vesicle fusion to the same extent as dolichyl-20 phosphate. The fusogenic capacity of dolichol and dolichyl phosphate is expressed too in data concerning the externally added and subsequently incorporated polyprenols in large unilamellar DOPC-DOPE vesicles. In addition, also external addition of dolichyl phosphate results in a pronounced bilayer destabilization in multilamellar DOPC-DOPE (1 : 2) systems. Therefore we suggest that these polyprenols induce fusion between DOPC-DOPE large unilamellar vesicles by their pronounced bilayer-destabilizing character. The involvement of non-bilayer structures in membrane fusion has been proposed and discussed in the past frequently [41,42,46,47]. The molecular mechanism involved in vesicle fusion and in particular the questions concerning the kinetics of fusion and the molecular structure of possible fusion intermediates is difficult to extrapolate from the data presented in this paper, since the fusion and structural experiments had to be performed using different conditions and model systems for technical reasons.

Furthermore we have presented data concerning the exchangeability of DOPC from MLVs and LUVs comprised of DOPC and DOPE (1 : 2) in the absence or presence of dolichyl-20 phosphate by using the PC-specific transfer protein. At 20 °C about 30% of the PC molecules can be exchanged from multilamellar vesicles. In analogy with a previous study [33], this number most likely represents the PC molecules present in the outer monolayer of the MLVs. Apparently, the incorporation of up to 5 mol% dolichyl phosphate does not influence the size of the exchangeable PC pool. This is very interesting, since ^{31}P-NMR and freeze-fracture electron microscopy data showed that the phospholipids are predominantly organized in the hexagonal (H_{II}) phase at 20 °C, suggesting a lack of transfer of PC from the interior of the hexagonal (H_{II}) phase to its surrounding outermost monolayer. This observation requires additional investigations in the near future using more simple systems. On the other hand, after heat-treatment of the MLVs (70 °C, 1 h) the exchangeability of DOPC at 20 °C is clearly dependent on the presence of dolichyl phosphate. When 3 or 5 mol% of dolichyl phosphate is incorporated, 63% of the total PC pool is available for exchange under these conditions. Since this value can not exclusively represent the proportion of PC molecules in the outer monolayer, we conclude that dolichyl phosphate enhances the PC exchange between the outer monolayer and the inner shells of the MLVs. This must involve facilitated transbilayer movement which can be related to the induction of intermediate non-bilayer structures by dolichyl phosphate. As has been shown with ^{31}P-NMR heat treatment and subsequent cooling of dolichyl phosphate containing MLVs (DOPC-DOPE = 1 : 2) result in the formation of a stable 'isotropic' phase. By using freeze-fracture electron

microscopy this 'isotropic' phase has been visualized as circular arrangements of lipidic particles, most likely involved in the formation of a 'honeycomb' type of structure [22,48]. As has been suggested previously [33,49], the presence of lipidic particles at the nexus of intersecting bilayers induces a dynamic state which allows transbilayer movement of PC.

Incorporation of dolichyl phosphate in LUVs comprised of DOPC and DOPE (1 : 2) increases the exchangeability of DOPC already at 20°C. Apparently, there seems to be a discrepancy between the exchangeability of DOPC from MLVs and LUVs in the presence of dolichyl phosphate at 20°C. However, it is interesting to note that a parallel can be drawn between the dolichyl phosphate-induced fusion between DOPC-DOPE (1 : 2) LUVs at 20°C and the size of the exchangeable DOPC pool. A similar observation was obtained by Hoekstra and Martin [50] for the 2,4,6-trinitrobenzenesulfonic acid accessibility of the H_{II} phase preferring DOPE, but not of bilayer-forming DPPE, after Ca^{2+}-induced fusion between PS-PE vesicles. Therefore we propose that the fusion between large unilamellar vesicles results in a structure which allows transbilayer movement of phospholipids, most likely involving intermediate-inverted non-lamellar structures.

Finally, extrapolation of the presented results to the biological situation suggests that dolichol could be involved in membrane fusion in secretory cells and organelles participating in transport, especially since these functions require membrane fusion processes and their dolichol contents are very high [4–6]. Furthermore, the bilayer-destabilizing capacity of dolichyl phosphate could facilitate the translocation of dolichyl pyrophosphate oligosaccharides across the very dynamic endoplasmic reticulum membrane.

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