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Properties of Hexadecaprenyl Monophosphate/Dioleoylphosphatidylcholine Vesicular Lipid Bilayers

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Abstract. In our study we investigated hemispherical phospholipid bilayer membranes and phospholipid vesicles made from hexadecaprenyl monophosphate (C_{80}-P), dioleoylphosphatidylocholine (DOPC) and their mixtures by voltammetric and transmission electron microscopy (TEM) techniques. The current-voltage characteristics, the membrane conductance-temperature relationships and the membrane breakdown voltage have been measured for different mixtures of C_{80}-P/DOPC. The membrane hydrophobic thickness and the activation energy of ion migration across the membrane have been determined. Hexadecaprenyl monophosphate decreased in comparison with DOPC bilayers, the membrane conductance, increased the activation energy and the membrane breakdown voltage for the various value of C_{80}-P/DOPC mole ratio, respectively. The TEM micrographs of C_{80}-P, DOPC and C_{80}-P/DOPC lipid vesicles showed several characteristic structures, which have been described. The data indicate that hexadecaprenyl monophosphate modulates the surface curvature of the membranes by the formation of aggregates in liquid-crystalline phospholipid membranes. We suggest that the dynamics and conformation of hexadecaprenyl monophosphate in membranes depend on the transmembrane electrical potential. The electron micrographs indicate that polyprenyl monophosphates with single isoprenyl chains form lipid vesicular bilayers. The thickness of the bilayer, evaluated from the micrographs, was 11 ± 1 nm. This property creates possibility of forming primitive bilayer lipid membranes by long single-chain polyprenyl phosphates in abiotic conditions. It can be the next step in understanding the origin of protocells.

Key words: Phospholipid membrane — Polyprenyl monophosphate — Membrane permeability — Vesicle morphology — Transmission electron microscopy — Prebiotic lipid vesicles

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Introduction

Biological membranes play a central role both in the structure and function of all cells. Membranes basically define compartments and determine the nature of all communication between the inside and outside of the cell and subcellular compartments (Gennis, 1989). The terpenoid theory of the origin of the primitive membranes proposed that dipolyprenyl phosphates and other simple terpenoids might form spontaneously on the surface of a clay or other minerals and self-organize into vesicles (Ourisson & Nakatani, 1994). These vesicles may evolve into progressively more complex units, similar to protocells. A vesicle is not a cell, but the formation of a vesicle is by itself a far-reaching event (Morovitz, 1992). Terpenoids (archeal lipids, hopanoids, carotenoids, sterols, polyisoprenols: dolichols, polyprenols, polyprenyl phosphates, etc.) are involved in the formation or reinforcement of all known biological membranes (Nakatani et al., 1993).

Polyisoprenols are natural products and they are derivatives of a common C_5 isoprene unit. Their phosphoryl derivatives — polyprenyl phosphates and dolichyl phosphates, function mainly as carriers of glycosyl units across membranes in the glycolysation reactions (Hemming, 1983; Van Duijn et al., 1986; Janas & Tien, 1988; Bugg & Brandish, 1994). The occurrence of phosphate esters of polyisoprenols in membrane fractions from prokaryotic and eukaryotic cells is frequently reported (Chojnacki et al., 1987; Bugg & Brandish, 1994; Jankowski et al., 1994; Bach, 1995). Unicellular eukaryotes, fungi, animal and some plant tissues contain phosphoryl deriva-

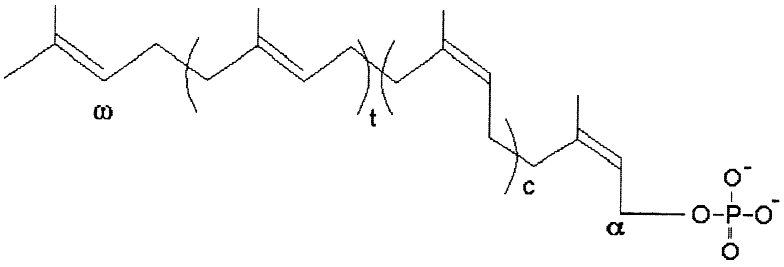

Fig. 1. The structure of hexadecaprenyl monophosphate (C_{80}-P)-$\omega t_2 c_{12}\alpha$P, where ω is an isoprene residue farthest from the estrified hydroxyl group, *t* is a *trans*-isoprene residue, *c* is a *cis*-isoprene residue, α is α-unsaturated, terminal *cis*-isoprene residue and P is the phosphate group.

tives of α-saturated polyisoprenols (dolichyl monophosphates, dolichyl diphosphates) (Jankowski et al., 1994; Bach, 1995). Bacterial membranes and leaves of some plants contain α-unsaturated polyisoprenols (polyprenols) and their phosphoryl derivatives (Chojnacki et al., 1987; Troy, 1992; Świeżewska et al., 1994).

The peptidoglycan layer of bacterial cell walls is biosynthesized using a lipid carrier undecaprenyl diphosphate to assemble and transport the disaccharide-pentapeptide precursor (Troy, 1992; Bugg & Brandish, 1994). Similar lipid-linked cycles are involved in the biosynthesis of bacterial lipopolisaccharides and eukaryotic glycoproteins, the latter involving the structurally related dolichyl phosphate as a lipid carrier. The lengthy polyisoprenoid chain seems an important property for the lipid acceptors, and this probably relates to their ability to fluidize locally the membrane bilayer (Janas et al., 1989; Bugg & Brandish, 1994).

The molecule of hexadecaprenyl monophosphate (C_{80}-P) consists of a hydroxyl group, which is esterified with the phosphate group — a hydrophilic part with negative charges, and a long unsaturated, mainly of poly-*cis*-configuration, isoprenyl chain — a hydrophobic part. This molecule is composed of 16 isoprene units with the structure: $\omega t_2\ c_{12}\alpha P$ where ω is an isoprene residue farthest from the estrified hydroxyl group, *t* is a *trans*-isoprene residue, *c* is a *cis*-isoprene residue, α is α-unsaturated, terminal *cis*-isoprene residue and *P* is the phosphate group (Chojnacki et al., 1995). Long-chain polyprenols isolated from plant photosynthetic tissues of *Spermatophyta* contains di-*trans*, poly-*cis* prenols with the general structure: $\omega t_2 c_n\ \alpha$OH, where the number of isoprene units *n* varies usually from 6 to 30–40, depending on the plant species (Świeżewska et al., 1994). The phosphoryl derivatives of these long-chain poly-*cis*-prenols have been present in small amounts in leaves of many species of *Gymnospermae* and *Angiospermae* (Chojnacki et al., 1987). The polyprenol/phospholipid bilayer has often served as an experimental model of biological membranes (Sunamoto et al., 1983; Lai & Schutzbach, 1984; Vigo et al., 1984; Janas et al., 1986; De Ropp et al., 1987; Janas et al., 1990) due to its similarity to biological membranes.

In the present study we investigated bilayer lipid membranes (BLM) and lipid vesicles made from dioleoylphosphatidylocholine (DOPC), C_{80}-P and C_{80}-P/DOPC mixtures by voltammetric and transmission electron microscopy (TEM) techniques. The current-voltage characteristics, the membrane conductance-temperature relationships, and the membrane breakdown voltage have been measured for DOPC and different mixtures of C_{80}-P/DOPC. The membrane hydrophobic thickness and the activation energy of ion migration across these membranes have been determined. The TEM micrographs of C_{80}-P, DOPC and C_{80}-P/DOPC lipid vesicles showed several characteristic structures.

Materials and Methods

Chemicals

DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) was purchased from Sigma and then additionally purified by using silica acid (Mallincrodt). It gave a single spot on Silica Gel TLC plates (Merck) in chloroform/methanol/water (65:25:4, by vol.) and in chloroform/methanol/acetic acid/water (50:30:8:4, by vol.). Hexadecaprenol (C_{80}) was isolated from leaves of *Picea abies* (Chojnacki et al., 1975) and purified. It gave a single spot on Silica Gel G TLC plates (Merck) in ethyl acetate/toluene (5:95, by vol.) and on RP-18 HP TLC plates (Merck) in acetone. The purity of C_{80} was verified by using an HPLC method (Świeżewska & Chojnacki, 1988). Hexadecaprenyl monophosphate (C_{80}-P) (Fig. 1) was made by chemical phosphorylation of hexadecaprenol based on the method of Danilov et al. (Danilov et al., 1989). After the isolation using DEAE-Sephadex A-25 column (Pharmacia) C_{80}-P gave a single spot on Silica Gel G TLC plates (Merck) in chloroform/methanol/water (65:25:4, by vol.). *n*-Decane and butanol were purchased from Aldrich and Fisher, respectively.

Formation of Hemispherical Bilayer Lipid Membranes

Bilayer lipid membranes in the form of hemispheres were formed according to the technique described previously (Shagina et al., 1983; Langer, 1986; Janas et al., 1986, 1989) on a Teflon capillary tube in unbuffered (pH 6) aqueous solution of 0.1 M and 0.2 M NaCl (inside and outside, respectively). DOPC and C_{80}-P/DOPC mixtures used for membrane formation were dissolved in *n*-decane/butanol (3:1, v/v) to obtain a concentration of 10 mg of lipid per ml of solvent. The area of the solvent-containing macrovesicular bilayer lipid membrane was about 50 mm^2. The process of bilayer formation was detected both optically, using a low power microscope, and electrically, using the low-level capacitance meter. The experimental setup used for

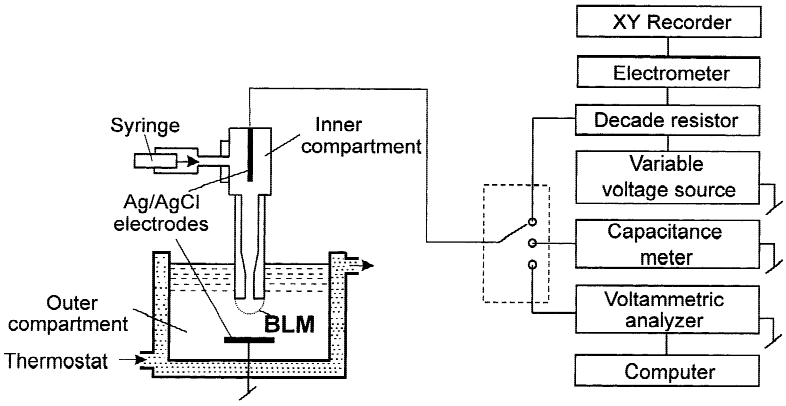

Fig. 2. The experimental setup used for the formation and electrical measurements of macrovesicular bilayer lipid membranes (BLM). For details, *see* text.

the formation and electrical measurements of macrovesicular BLM is presented in Fig. 2.

Electrical Measurements

Silver chloride electrodes were used to detect the electric potentials and the currents. The area of the membrane, *S*, was determined by an optical measurement of membrane dimensions. The temperature, *T* was controlled by water circulating from an external bath. Electrical conductance of the membrane, *G*, was calculated from current-voltage characteristics. To obtain the values of the breakdown voltage, V_B, the applied voltage was increased by a scan rate of 10 mV sec^{-1}. The membrane rupture was reflected by a rapid increase of current (Janas et al., 1989). The activation energy (the temperature-dependent part) of ion migration across the membrane, E_A, was determined from Arrhenius plots of normalized conductance of bilayer lipid membranes (Smith et al., 1984):

$$(ln[(G/C)/(G_o/C_o)]) = (E_A/R)[(1/T) - (1/T_o)] \quad (1)$$

where $ln[(G/C)/(G_o/C_o)]$ is the normalized conductance of the membrane, G_o and C_o are membrane conductance and membrane capacitance, respectively, at temperature T_o, *R* is the gas constant. The normalization of membrane conductance (with respect to the membrane capacitance measured simultaneously) corrects any variations in the bilayer conductance which are due to variations in bilayer thickness or bilayer area. The capacitance of the membrane, *C*, was determined from recorded membrane discharge curves (Janas et al., 1989). Membrane electrical capacitance is related to the membrane hydrophobic thickness, *h*, according to the formula (Tien, 1974):

$$h = \epsilon\epsilon_o S/C \quad (2)$$

where *S* is the membrane surface, $\epsilon_o = 8.85 \times 10^{-14}\ F\ cm^{-1}$. $\epsilon = 2.1$ is the dielectric constant of the membrane interior.

Electron Microscopy of Lipid Vesicles

Lipid vesicles were prepared from DOPC, C_{80}-P or the mixture of C_{80}-P/DOPC (0.01 and 0.2 mole ratio). Small amounts of lipids, mixed in chloroform, were dried under nitrogen at 45°C until a thin film of dry lipid formed on the wall of the test-tube. The lipid suspensions were obtained by addition of water from Millipore water system. The concentration of lipid was 1 mg per ml of water. Lipids were hydrated at 45°C for 24 hr. The vesicle dispersions were obtained by vortexing hydrated lipids using a laboratory vibrator (Janke & Kunkel, IKA Labortechnik VF 2) for 5 min at moderate modes.

A droplet of vesicle dispersion was put on the microscope copper grid (400 mesh) covered previously with Formvar membrane and with a thin evaporated layer of carbon. The samples were dried at room temperature and negatively stained by depositing a drop of 1% aqueous uranyl acetate solution for 1 min, then grids were rinsed in water from Millipore water system. The dried grids were again covered with a thin evaporated layer of carbon. Samples were analyzed in the transmission electron microscope JEOL JEM 1200EX at 80 kV.

Results

The Electrical Measurements of DOPC and C_{80}-P/DOPC Bilayers

The behavior of hexadecaprenyl monophosphate /DOPC membranes as a function of applied potential was studied by performing voltammetric experiments. As presented in Fig. 3, the current-voltage characteristics are symmetric and linear for values of the potential in the range (−20 to +20) mV. The value of the curve slope increases with the increase of the percentage of hexadecaprenyl monophosphate (C_{80}-P) in the bilayers. The effect of hexadecaprenyl monophosphate on membrane-specific conductance of bilayer lipid membranes formed from various mixtures of C_{80}-P/DOPC is illustrated in Fig. 4. The values of the conductance were derived from the *I/V* curves by the least squares fitting. The dependence is presented on a semilogarithmic scale. The value of membrane conductance obtained for DOPC bilayer equals $(4.8 \pm 0.9) \times 10^{-8}$ S cm^{-2} and is in accordance with the value $(4 \pm 1) \times 10^{-8}$ S cm^{-2} reported by Gamble et al. (1982). The values of membrane conductance, in comparison with DOPC membrane, decrease for small concentrations of C_{80}-P in the bilayer (in the range of the mole ratio 0–0.01) with maximal 30-fold drop up to the value of $G_S = (1.67 \pm 0.34) \times 10^{-9}$ S cm^{-2} for the mole ratio equals 0.01. The conductance increases for concentrations of hexadecaprenyl monophosphate higher than 0.01 in comparison with C_{80}-P/DOPC membrane, mole

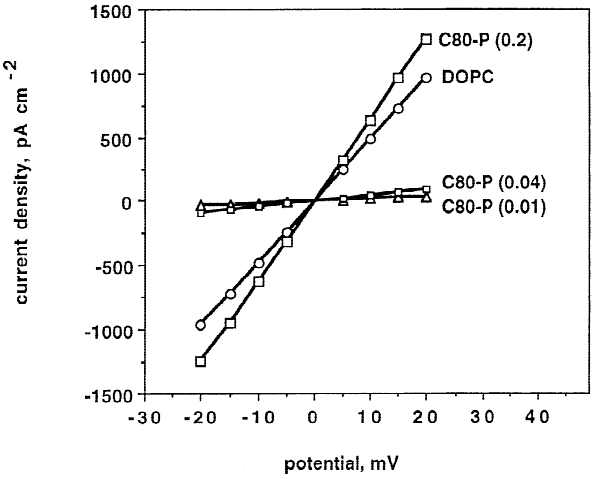

Fig. 3. Current-voltage steady-state characteristics of bilayer lipid membranes for different C_{80}-P/DOPC mole ratios. Experiments were performed at 25 ± 1°C.

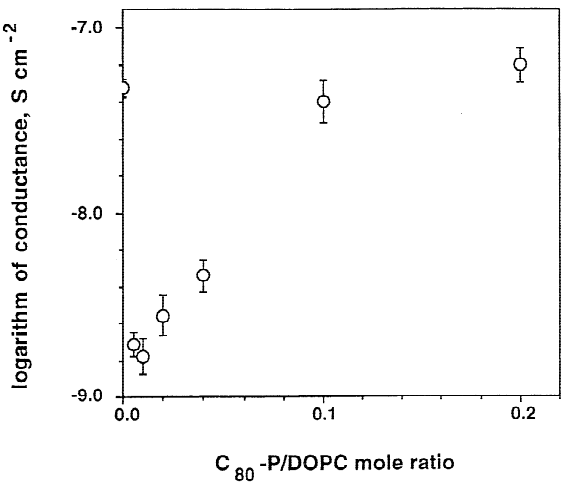

Fig. 4. The ionic conductance versus the C_{80}-P/DOPC mole ratio. The values of membrane conductance were derived from the *I/V* curves by the least-squares fitting. Each point represents the mean value (±SD) obtained from 6 to 8 different macrovesicular bilayer lipid membranes. Experiments were performed at 25 ± 1°C.

ratio 0.01. The maximal rise, over 4-fold, was observed for concentrations 0.2. The normalized conductance of bilayer lipid membranes was measured as a function of temperature in the range of 25–42°C. Typical trends are reported in Fig. 5. An increase of normalized conductance was observed with increasing temperature. The Arrhenius plots were linear, the slope of the curves depending on the percentage of C_{80}-P in the bilayer.

The relationship between the value of activation energy of ion transport across the membrane, E_A, and the percentage of hexadecaprenyl monophosphate in macrovesicular bilayers is shown in Fig. 6. The values of activation energies were derived from the Arrhenius plots

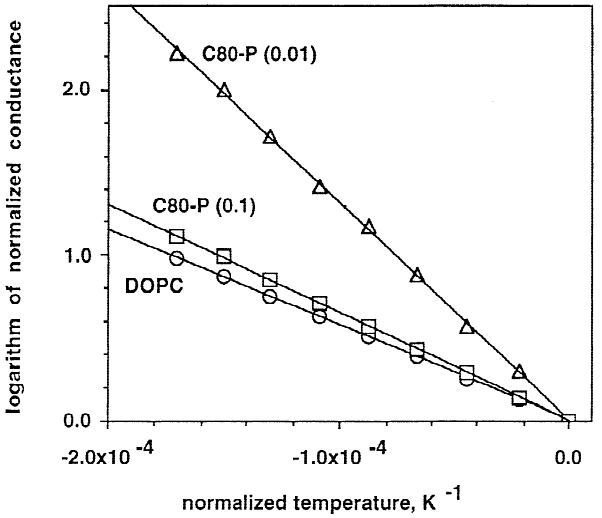

Fig. 5. Arrhenius plots of normalized conductance of macrovesicular bilayer lipid membranes, for different C_{80}-P/DOPC mole ratios. Logarithm of normalized conductance was calculated as: $ln[(G/C)/(G_o/C_o)]$, where G and C represent membrane conductance and membrane capacitance, respectively, for the temperature studied; G_o and C_o are membrane conductance and membrane capacitance, respectively, at 298 K. Normalized temperature was calculated as: temperature^{-1} (K^{-1}) − $(298K)^{-1}$.

by the least squares fitting. For smaller concentrations of hexadecaprenyl monophosphate in the membrane, an increase of E_A was observed in comparison with DOPC bilayers. For higher concentrations of C_{80}-P in the membrane a decrease of the value of E_A was observed in comparison with C_{80}-P/DOPC, mole ratio 0.01. The E_A value increases from (48.2 ± 3.1) kJ mol^{-1} for DOPC bilayers to the maximal value, E_{Amax}, of (109.2 ± 4.0) kJ mol^{-1} for bilayers prepared from the mixture C_{80}-P/DOPC, mole ratio equal to 0.01. The activation energy then decreases to the value of (44.8 ± 2.6) kJ mol^{-1} for the C_{80}-P/DOPC mole ratio equal to 0.2. The effect of hexadecaprenyl monophosphate on the membrane hydrophobic thickness, h, of bilayer lipid membranes formed from various mixtures of DOPC and C_{80}-P is illustrated in Fig. 7. A decrease of the membrane thickness from (5.18 ± 0.07) nm to the value of (5.06 ± 0.10) nm for the relative C_{80}-P concentrations from 0.00 to 0.2 is observed. The variations of the membrane thickness are however within the experimental error range.

Figure 8 illustrates the effect of hexadecaprenyl monophosphate on the breakdown voltage of the membrane, V_B. The membrane electromechanical stability (proportional to the value of V_B) was found to be modulated by the presence of hexadecaprenyl monophosphate in the bilayer lipid membrane. The increase of V_B is observed for small concentrations up to the mole ratio 0.005 of C_{80}-P in the membrane in comparison with DOPC membrane, and then, for higher concentrations of

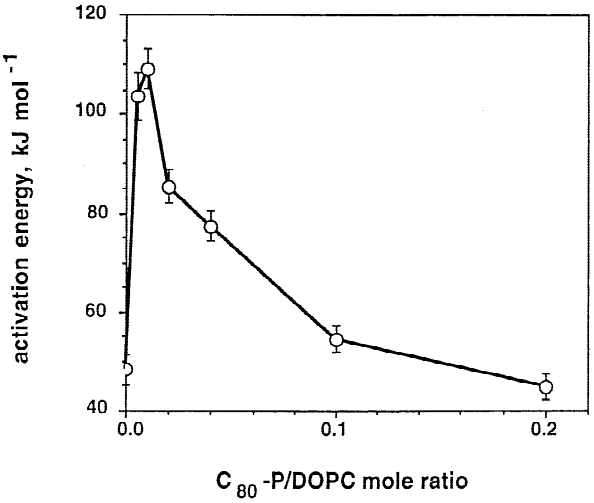

Fig. 6. The activation energy of ion migration versus the C_{80}-P/DOPC mole ratio. The values of activation energies were derived from the Arrhenius plots by the least squares fitting. Each point represents the mean value (±SD) obtained from six to eight different macrovesicular bilayer lipid membranes.

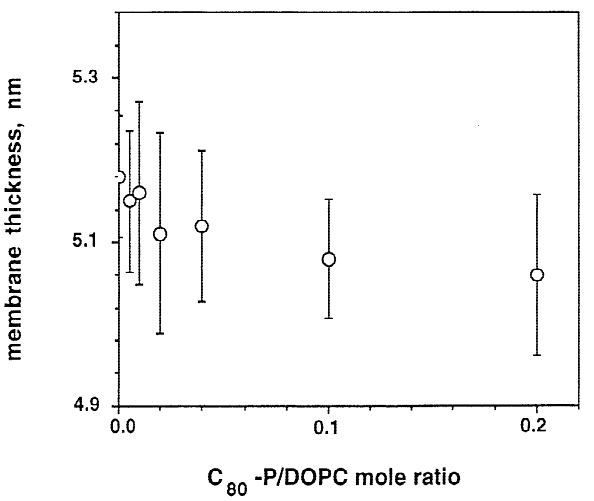

Fig. 7. The membrane hydrophobic thickness versus the C_{80}-P/DOPC mole ratio. Each point represents the mean value (±SD) obtained from 6 to 8 different macrovesicular bilayer lipid membranes. Experiments were performed at 25 ± 1°C.

C_{80}-P in the membrane, a decrease of the value of V_B is observed in comparison with C_{80}-P/DOPC, mole ratio 0.005. The value of membrane breakdown voltage increases from (203 ± 10) mV for DOPC bilayers to the maximal value, V_{Bmax}, of (316 ± 19) mV for the bilayer prepared from the C_{80}-P/DOPC mixture at 0.005 mole ratio. The breakdown voltage then decreases to the minimal value, V_{Bmin}, of (187 ± 18) mV for the bilayer prepared from the C_{80}-P/DOPC mixture at 0.2 mole ratio.

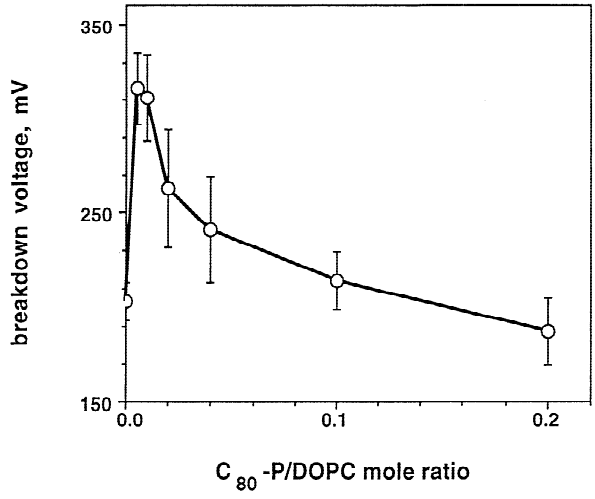

Fig. 8. The membrane breakdown voltage *vs.* the C_{80}-P/DOPC mole ratio. Each point represents the mean value (±SD) obtained from 6 to 8 different macrovesicular bilayer lipid membranes. Experiments were performed at 25 ± 1°C.

Analysis of the Lipid Vesicles in the Transmission Electron Microscope

Lipid vesicles prepared from DOPC, C_{80}-P and C_{80}-P/DOPC mixtures (mole ratio 0.01 and 0.2), were analyzed in the transmission electron microscope (TEM). Examples of these vesicles are shown in Figs. 9–12. They were chosen from about 100 micrographs and represent typical tendency of the modification of the vesicle structure. Figure 9 presents typical DOPC dispersion of spherical vesicles of various size in the range from ~30 nm to ~100 nm. Figure 10*a–d* shows lipid vesicles prepared from C_{80}-P/DOPC, mole ratio 0.01. The shape of the vesicle presented in Fig. 10*a* is rather regular with the diameters from 220 to 300 nm. The spherical vesicles are also shown in Fig. 10*b* in the center of the micrograph. The diameters of these regular vesicles are ~100 nm. Some of these vesicles create aggregates composed of two vesicles. Figure 10*c* presents spherical vesicles with diameters from ~30 to ~70 nm (arrows show these structures). Among the spherical vesicles there are some bigger lipid structures with diameters from ~300 to ~600 nm. Membranes of these large spherical structures indicate variable thickness and intensity of contrast by uranyl acetate. Among the regular vesicles and large spherical structures prepared from C_{80}-P/DOPC, mole ratio 0.01 there is an elongated structure (Fig. 10*d*). The shape of the membrane is very marked and the thickness of this membrane is 5 ± 1 nm. The dimensions of this elongated vesicle range from 450 to 1600 nm. The interior of this vesicle is heterogeneous and there are seen light, big shapes. There is a small narrowing of the vesicle in the lower part.

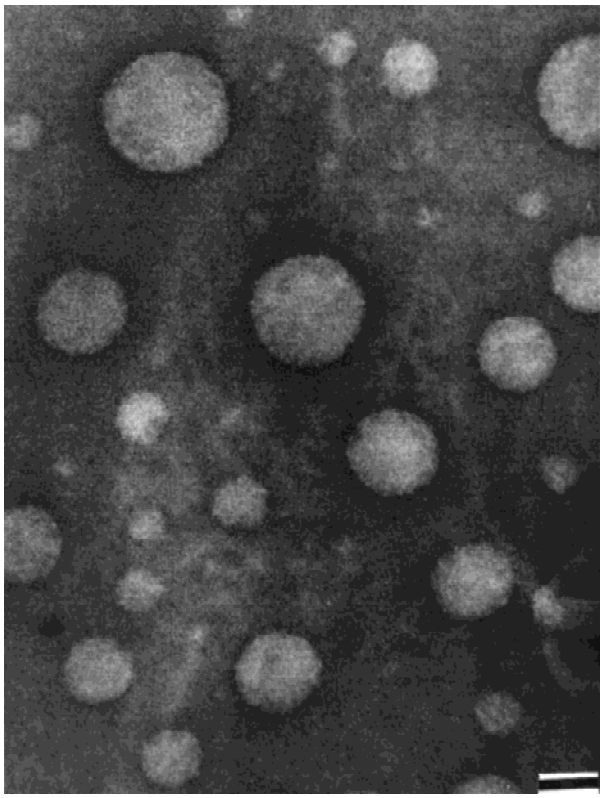

Fig. 9. Transmission electron microscopy micrograph of lipid bilayer structures prepared from DOPC. Magnification ×100,000, the bar represents 50 nm.

A new kind of structure, prepared from C_{80}-P/DOPC mixture, mole ratio 0.2, can be seen in Fig. 11*a–d*. Figure 11*a* shows the large, elongated and bent vesicle (structure 1) and some regular small vesicles (structure 2 and 3). The structure 1 is connected with aggregation of vesicles (structure 4) by the short bridge. One of the vesicles from the aggregation is also connected with the other from this aggregation. The part of structure 1 is similar to the budding structure (arrows show this fragment of the structure). The dimensions of this elongated vesicle range from 120 to 1000 nm. The micrograph presented in Fig. 11*b* shows some regular (structure 1 and 2) the elongated vesicle (structure 3) and some irregular structures (structure 4 and 5). All these structures are connected by bridges (*see* arrows). Figure 11*c* presents the aggregation of three irregular vesicles. There are structures 1, 2 and 3. The dimensions of these vesicles range from 200 to 500 nm. The part of membranes (marked by arrows) of the vesicle 1 seems to be multilayers. The micrograph presented in Fig. 11*d* shows the large, irregular vesicle. This structure seems to be unilamellar and its width is about 500 nm.

Lipid structures prepared from C_{80}-P can be seen in Fig. 12*a–e*. The micrographs indicate that polyprenyl monophosphate forms regular and irregular vesicles with various sizes. The vesicles presented in Fig. 12*a*, marked by arrows, form aggregates. The dimensions of the spherical, regular vesicles presented in Fig. 12*b–c* range from 20 to 100 nm. The width of larger, irregular vesicles is about 200 nm. The large structure (structure 1) presented in Fig. 12*d* seems to be created as the effect of the fusion of three vesicles. Membranes of this structure are grainy. The vesicle situated above this structure (structure 2) is an irregular, budding vesicle.

A specific large structure can be seen in Fig. 12*e*. The dimensions of this vesicle range from 800 to 1100 nm. The lipid bilayer that bounds the vesicle is clearly visible (marked by arrows). The thickness of the bilayer is 11 ± 1 nm. The internal structure is heterogeneous and includes the smaller vesicles. Some of them are regular but others are elongated. Inside one of the small vesicles (structure 1) we can see the fusion of the little vesicles. All of the structures presented in the micrograph Fig. 12*e* resemble the cell with organelles.

The electron micrographs show that lipid vesicles prepared from DOPC are regular whereas lipid vesicles prepared from C_{80}-P or C_{80}-P/DOPC, besides regular structures, form several irregular characteristic structures. It can not be excluded that some of these vesicles can form multilamellar structures.

Discussion

The first report on the formation of spherical bilayer lipid membranes made from polyisoprenols was in 1986 (Janas et al., 1986). The authors investigated, using a voltammetric technique, the properties of spherical lipid bilayers made from dodecaprenol (C_{60})—a nonphosphorylated form of polyisoprenols. The value of the hydrophobic thickness equal to 8.5 nm for these bilayers are in accordance (with regard to the change of the polyprenyl chain length) with the thickness equal to 11 nm of the C_{80}-P bilayer membrane bounding the vesicles, evaluated from the micrographs and reported in our study. These data obtained for lipid bilayers imply that longitudinal dimensions of the folded C_{80}-P and C_{60} chains are 5.5 and 4.25 nm, respectively. These results correspond to both the theoretical results (using the molecular mechanics methods) and the experimental results (using the small-angle X-ray scattering technique) obtained for dolichol-19 (C_{95}) (Murgolo et al., 1989). The folding of the polyisoprenyl chains correlates with the very flexible conformations of these chains resulting from the poly-*cis* configuration. Subsequently, Plobeck et al. (1992) showed, using TEM technique, the formation of spherical vesicles from short-chain dipolyprenyl phosphate: sodium di-farnesyl phosphate ((di-C_{15})-P); however the authors were not able to estimate the thickness of the membrane-bounding the vesicles.

Hexadecaprenyl monophosphate molecules consist

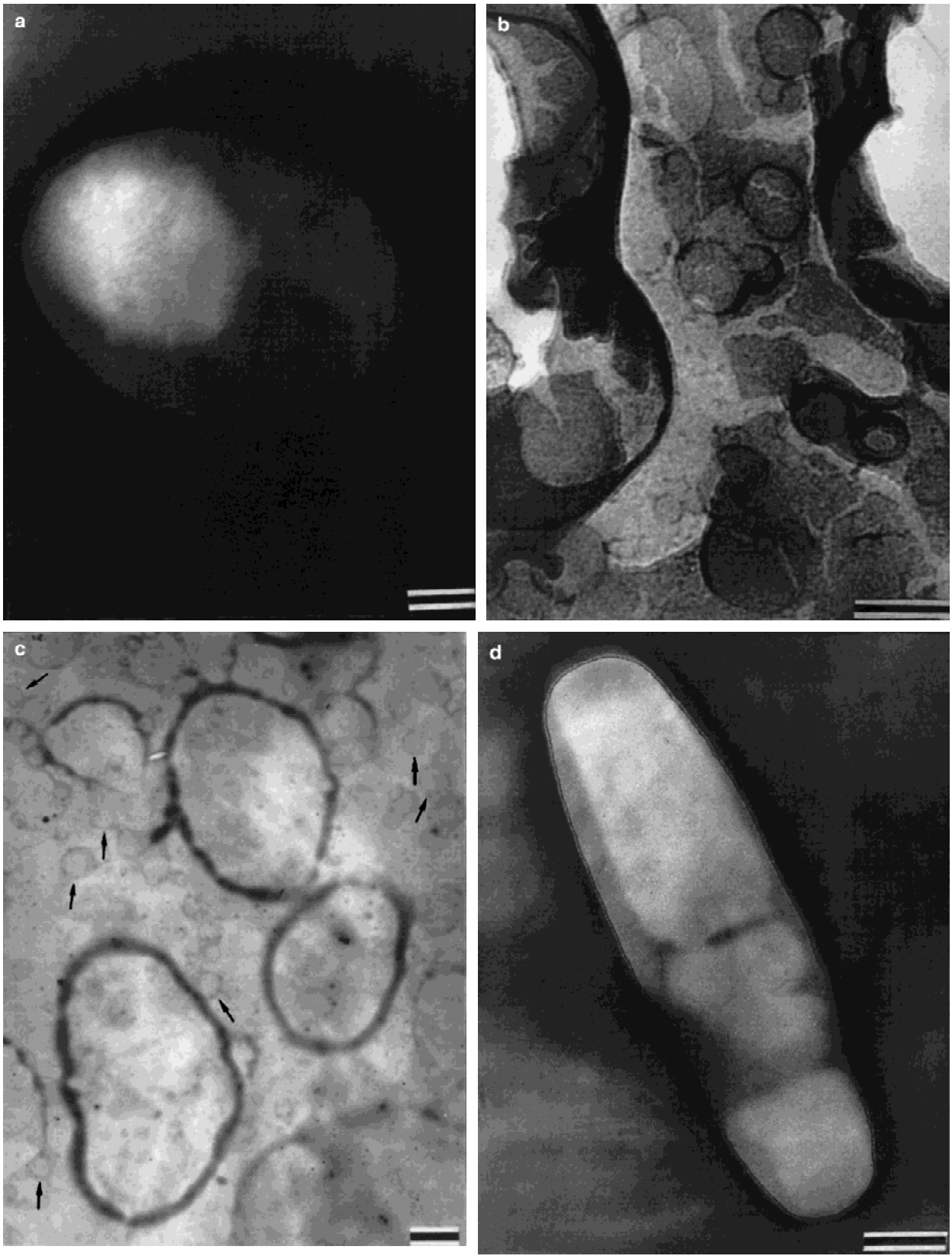

Fig. 10. Transmission electron microscopy micrographs of lipid bilayer structures prepared from C_{80}-P/DOPC, mole ratio 0.01. Magnification and scale bar: (*a*) × 150,000, 50 nm; (*b*) × 20,000, 200 nm; (*c*) × 5,000, 1,000 nm; (*d*) × 25,000, 200 nm.

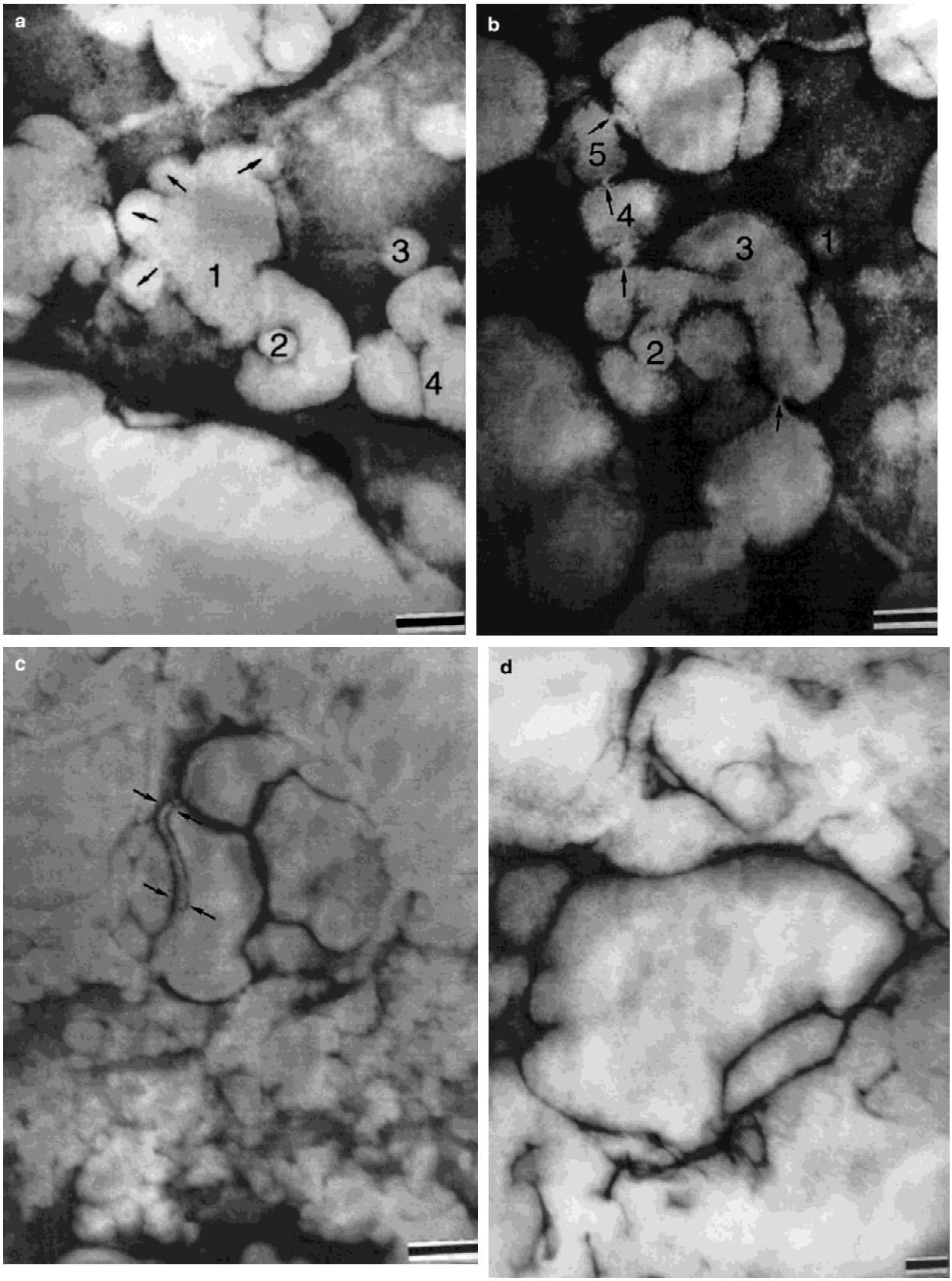

Fig. 11. Transmission electron microscopy micrographs of lipid bilayer structures prepared from C_{80}-P/DOPC, mole ratio 0.2. Magnification and scale bar: (*a*) × 40,000, 200 nm; (*b*) ×40,000, 200 nm; (*c*) × 40,000, 200 nm; (*d*) × 50,000, 100 nm.

of a long unsaturated, mainly poly-*cis* configuration isoprenoid chain with the phosphate group bonded to the unsaturated α-isoprene residue. The *cis* geometry enables the chain to be more compact and fold into a shorter length than poly-*trans* isoprenoids. Hexadecaprenyl monophosphate molecules seem to orient in the membrane with their phosphate headgroup at the hydrophilic interface. Similar properties were experimentally detected by McCloskey and Troy (1980), Valtersson et al. (1985), and De Ropp et al. (1987) in the case of lipid bilayers modified by dolichyl phosphate. Our investigations show that the lipid bilayers modified by hexadecaprenyl monophosphate (C_{80}-P) modulate electrical, transport and mechanical properties of these membranes. The conformational changes of a negatively charged lipid molecule can be induced electrostatically, by changing membrane surface charge density (Vogel & Stockburger, 1983). It seems that changes of modified membranes are mainly caused by a negative electrical charge of the C_{80}-P phosphate groups. All the studied membranes were in the liquid-crystalline state, because the phase transition temperature for dioleoylphosphatidylocholine bilayers is below −20°C (Small, 1986) and hydrocarbon chain of hexadecaprenyl monophosphate contains several double bounds in *cis* conformation (Chojnacki et al., 1995). No phase transition of α-saturated polyprenol/DOPC bilayers could be detected between 12 and 82°C (Valtersson et al. 1985).

With respect to the electrical properties, the measurements showed that hexadecaprenyl monophosphate increases the activation energy for ion migration and the membrane breakdown voltage. Furthermore the presence of hexadecaprenyl monophosphate in phospholipid bilayer results in changes of the slope of *I/V* curves and also in a decrease of membrane-specific conductance. The membrane-specific conductance (Fig. 4) increases above the C_{80}-P/DOPC, mole ratio 0.01, although values of conductance of modified membranes are smaller than the value of conductance of DOPC bilayers. In the case of undecaprenyl phosphate (C_{55}-P) results were similar and a minimum of membrane conductance was obtained for undecaprenyl phosphate/DOPC bilayers, mole ratio 0.01 and then increased (Janas & Janas, 1995). The results presented in this paper are in agreement with the results of Vigo et al. (1984) for dipalmitoylphosphatidylcholine (DPPC) liposomes modified by dolichyl phosphate. By using a fluorescence depolarization technique they noted that above the transition temperature of DPPC, dolichyl phosphate, contrary to dolichol, acts to stiffen the bilayer more compactly. Similar to our results they found that the rigidifying effect decreased as the concentration of dolichyl phosphate was increased. Therefore the decrease of membrane conductance at smaller C_{80}-P concentrations can result from a restriction of the movement of DOPC molecules in bilayers. The following increase in membrane conductance can arise from a creation of ionic pathways in the boundary region of C_{80}-P-aggregates. McCloskey and Troy (1980) noted that neutral spin-labeled α-saturated polyprenyl phosphate-dolichyl phosphate (Dol-P), did not aggregate at concentrations smaller than 0.03. Vigo et al. (1984) and Valtersson et al. (1985) investigated the effect of Dol-P on the thermotropic phase transition of phosphatidylcholine bilayers. They concluded that Dol-P molecules, at relative concentrations equal to 2.5% or higher (Valtersson et al., 1985) and relative concentrations higher than 1% (Vigo et al., 1984), segregate from the rest of the lipids forming aggregates in the bilayer. Therefore the heterogeneous shapes of vesicles prepared from the mixture C_{80}-P/DOPC at molar ratio 0.2 (Fig. 11*a–d*) can be formed by non-uniform dispersions of C_{80}-P and DOPC.

In our case the aggregation of hexadecaprenyl monophosphate seems to occur at higher relative concentrations than 1%. The aggregation leads to domain formation with phosphate groups in the hydrophilic part of the membrane. In contrast to the behavior of phosphopolyisoprenols, the aggregation of spin-labeled polyisoprenols in phosphatidylcholine membranes was observed even at relative concentrations not exceeding 0.005 (McCloskey & Troy, 1980). These aggregates can modulate the permeability and stability of polyisoprenol-phospholipid membranes. Due to the electrostatic repulsion between the phosphate groups, the aggregation of polyprenyl phosphate seems to occur only partially and at higher concentration. As shown by Lai & Schutzbach (1984) and Schutzbach & Jensen (1989) dolichol promoted the leakage of membranes in liposomes composed of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) but not liposomes composed only of PC. Dolichol and Dol-P destabilized unsaturated PE containing bilayer structures and promoted hexagonal II phase formation (Valtersson et al., 1985).

Differences in properties of modified bilayers for various concentrations of C_{80}-P in membranes have been confirmed also in the case of investigations of the activation energy of ion migration across the membrane, the breakdown voltage and the membrane thickness. The dependence of the activation energy on the percentage of C_{80}-P in the bilayer (Fig. 6) has a maximum equal to (109 ± 4) kJ mol^{-1} at C_{80}-P/DOPC mole ratio 0.01. This maximum is at the same concentration as the minimum of membrane conductance (Fig. 4) and is close to the value of the activation energy for undecaprenyl phosphate/DOPC bilayers equal to (97 ± 4) kJ mol^{-1} (Janas & Janas, 1995). The observed increase in the breakdown voltage can also be related to transmembrane pores produced by the long-chain polyprenyl phosphate aggregates. As analyzed in the paper of Smith et al. (1984) the increase of the activation energy is related to the decrease

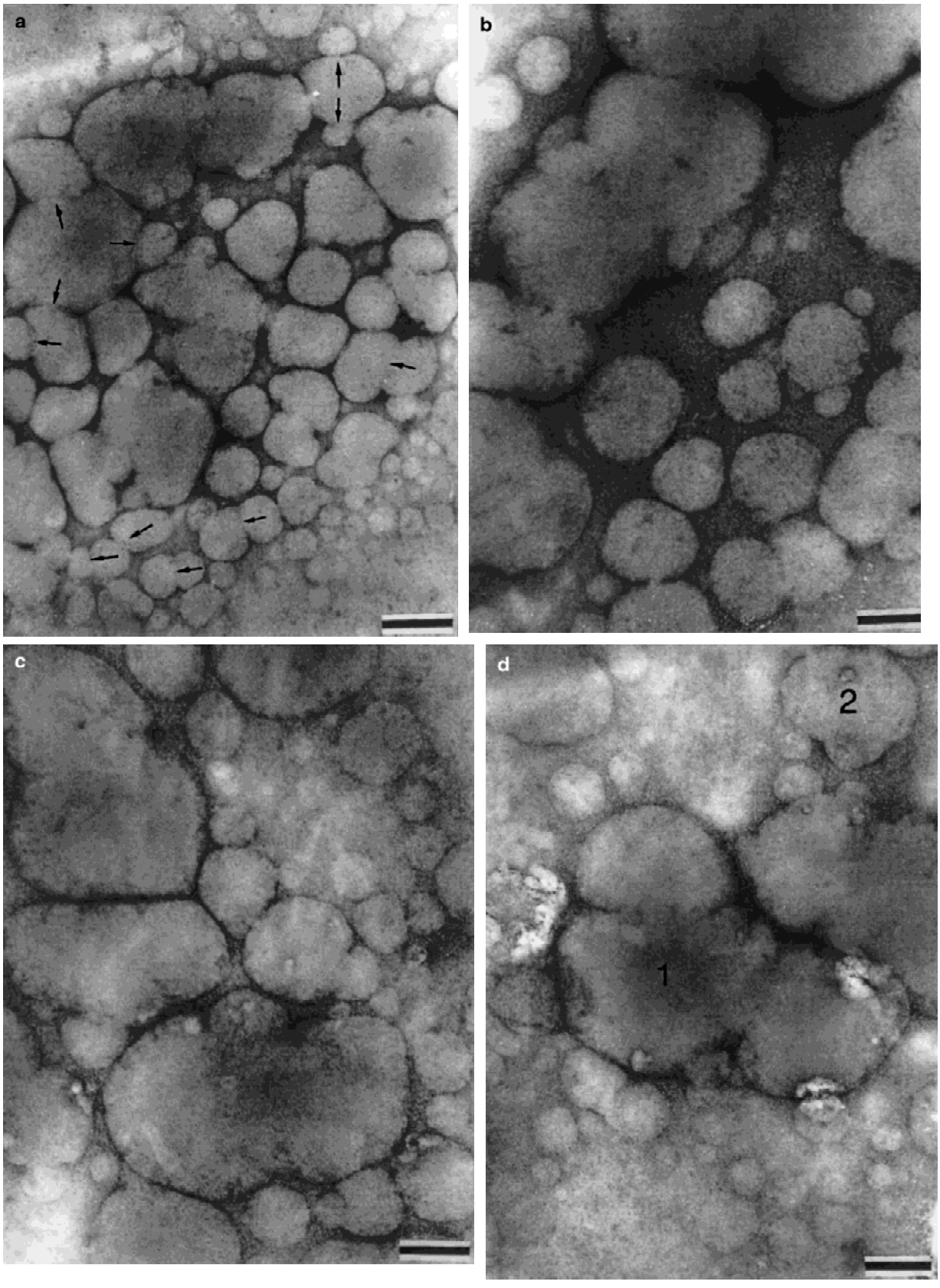

Fig. 12. Transmission electron microscopy micrographs of lipid bilayer structures prepared from C_{80}-P. Magnification and scale bar: (*a*) × 40,000, 200 nm; (*b*) × 75,000, 100 nm; (*c*) × 75,000, 100 nm; (*d*) 75,000, 100 nm; (*e*) × 30,000, 200 nm.

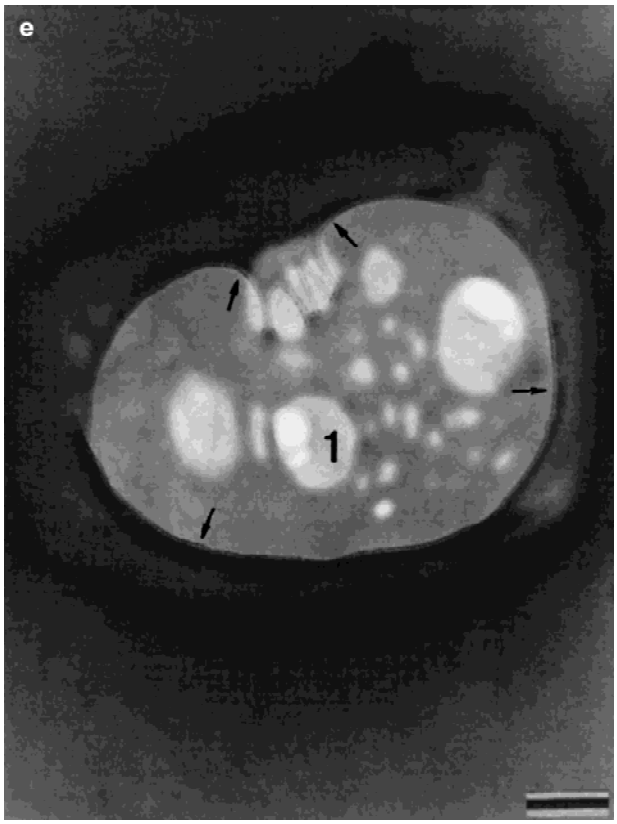

Fig. 12. *Continued.*

of the radius of the transmembrane pore. For the value of the activation energy that equals 18 kJ/mole the authors estimated the minimum pore radius about 1 nm. For C_{80}-P/DOPC bilayers, with the activation energy for ion transport about 6-fold bigger (for the relative concentrations 0.005 and 0.01), the minimal pore radius can be estimated in the range of 0.16 to 0.17 nm. The modulation of the activation energy by the polyprenyl phosphate can therefore result from the formation of these pores. The structure of these pores can be similar to the hexagonal II structures of Dol and Dol-P in phosphatidylethanolamine vesicle dispersion as suggested by Lai and Schutzbach (1984) and Valtersson et al. (1985). Although the ion permeability of the naked bilayers may be of little physiological consequence for prokaryotic or eukaryotic cells, it is crucial for understanding the function of protocells containing no proteins.

Hexadecaprenyl monophosphate in bilayers seems to decrease the bilayer thickness for C_{80}-P/DOPC, mole ratio ranging from 0.00 to 0.2. The change in the membrane hydrophobic thickness can be regarded as an indicator of the change in the phospholipid membrane fluidity caused by other amphiphilic molecules (Turner & Oldfield, 1979). The local regulation of the bilayer thickness can be connected with the function of microdomains. In the disordered boundary region of microdomains formed by the phosphorylated derivative of hexadecaprenol molecules, ion pathways, facilitating the ion transport can be formed.

Hexadecaprenyl monophosphate increases the value of the breakdown voltage of DOPC bilayers. As shown in Fig. 8, the maximal increase over 50% in comparison with DOPC membranes, was observed for the C_{80}-P relative concentrations 0.005 to 0.01. Similar values of the breakdown voltage were obtained for C_{55}-P/DOPC bilayers (Janas & Janas, 1995). In our experiments, the studied polyprenyl phosphate increases the electromechanical stability (proportional to the value of the breakdown voltage) of the membranes, which reflects the stabilization effect of polyprenol on the phosphatidylcholine bilayers in the presence of transmembrane electrical potential. Contrary to the behavior of C_{80}-P, polyprenyl alcohol increases the membrane-specific conductance, and all values of conductance of modified membranes are higher than the value of conductance of DOPC bilayers, decrease activation energy of ion migration, breakdown voltage and membrane thickness (Janas et al., 1989).

As reported by Plobeck et al. (1992), lipid vesicles, formed from short-chain dipolyprenyl phosphates, could not be obtained by hydration of a lipid film, but only by ultrasonication with subsequent filtration. In our preparation of vesicles from long-chain mono-polyprenyl phosphates (C_{80}-P), the application of the method of hydration of a lipid film was successful. Therefore the conditions of our experiment seem to be more similar to natural conditions of the prebiotic synthesis of polyprenyl phosphate molecules, forming primitive vesicles. The dispersion of vesicles prepared this way from DOPC, C_{80}-P or C_{80}-P/DOPC mixtures were investigated by transmission electron microscopy. Analysis of lipid vesicles in TEM indicates the changes of the membrane elasticity for liposomal membranes modified by hexadecaprenyl monophosphate, respectively, to the concentrations of C_{80}-P in bilayers. In the case of C_{80}-P/DOPC dispersions, both spherical and nonspherical vesicles were observed. There were multibudding structures, elongated vesicles, and fused vesicles. Vesicles were smooth or rough. The membrane texture of rough vesicles was grainy. On the basis of these micrographs we infer that hexadecaprenyl monophosphate changes the membrane fluidity and elasticity and modulates the surface curvature of modified lipid membranes, especially for relative concentrations of C_{80}-P in the membrane higher than 0.03. It seems unlikely that the polyprenyl phosphate molecules segregate out of some membranes and form lipid globules because the polar head-group of C_{80}-P can interact with the DOPC polar head-group (possessing both a negative charge localized in the phosphate group and a positive charge localized in the choline group) through the charge-dipole type of in-

teraction. The variety of obtained structures supports the terpenoid theory of the origin of cellular life (Ourisson & Nakatani, 1994). Ourisson and Nakatani in their paper described the synthesis of sodium di-polyprenyl phosphates. The molecule of this compound consists of two isoprenyl chains, whereas the molecule of hexadecaprenyl phosphate used in our experiment consists of the single isoprenyl chain. It seems more probable that these "primitive" membranes were formed from more simple molecules — single-chain polyprenyl phosphates. Eukaryotic membranes usually consist of a bilayer of cholesterol and molecules of diacyl phospholipid with two, but acyl chains. Bacterial membranes often contain hopanoids instead of cholesterol (Ourisson & Rohmer, 1992) and in the case of archaebacterial membranes lack *n*-acyl lipids and instead consist of polyprenic diphytanylglyceryl phospholipids — molecules without double bonds, and other similar polyterpenyl ethers (De Rosa et al., 1986). The occurrence of single-polyprenyl phosphates in membrane fractions from prokaryotic and eukaryotic cells is well documented (Chojnacki et al., 1987; Bugg & Brandish, 1994; Jankowski et al., 1994; Bach, 1995). Pozzi and coworkers (Pozzi et al., 1996) formed closed vesicles from short single-chain polyprenyl phosphates: sodium farnesyl phosphate (C_{15}-P) and sodium geranylgeranyl phosphate (C_{20}-P). The authors, in their paper, presented a copy of a video of the differential interference contrast of a multivesicular structure of sodium geranylgeranyl phosphate with diameters from 1400 to 15,000 nm but no lipid bilayers have been demonstrated The authors suggested that the membranes seem to be thick because of the diffraction-limited resolution of the optical system. In the case of our analysis, the electron micrographs indicate that polyprenyl monophosphates with single isoprenyl chains form lipid vesicles bounded by bilayers with the thickness of 11 ± 1 nm. The molecules of C_{15}-P and C_{20}-P were composed of 3–4 isoprene units, whereas the molecule of long-chain hexadecaprenyl phosphate is composed of 16 isoprene units. Molecules of prokaryotic and eukaryotic polyisoprenols and their phosphoryl derivatives consist of isoprenyl chains composed usually of 6 −20 isoprene units (E. Swieżewska, 1999; *personal communication*). The length of the isoprenyl chain of the compound examined by us corresponds to the lengths of isoprenyl chains of polyisoprenols and polyisoprenyl phosphates existing in nature. In the case of long-chain prenyl phosphates, the van der Waals interactions, which stabilize the membrane hydrophobic layer, are stronger than for short-chain prenyl phosphates. These stronger interactions can lead to the easier (energetically preferable) formation of lipid bilayers.

Ourisson and Nakatani (1994) suggested that the simpler sodium monoprenyl phosphates could form vesicles when combined with suitable amounts of the free polyprenols. In contrast to their suggestion Pozzi et al. (1996) obtained large multivesicular structures of short-chain polyprenyl phosphates without free polyprenols. In addition, in our experiments the electron micrographs indicate that polyprenyl long-chain phosphates can form small vesicles. Therefore these molecules could have been precursors of modern phospholipids. In the process of the molecular origin of life similar vesicles could evolve into protocells. The effect of hexadecaprenyl monophosphate on the surface curvature of the membranes can result from its molecular shape with a considerably hydrophobic part in comparison with the small hydrophilic part with the phosphate group. The variety of structures of phosphatidylcholine vesicles has been reported by Klösgen et al. (1997). They demonstrated the existence of superstructures of phospholipid bilayers. The existence of the superstructure can be regarded as evidence that lipids may directly influence the activity of biological membranes by changing their fluid-crystalline properties. Some functions of the phosphate ester derivatives of long-chain polyprenols in bilayers are connected with modification of some properties of the membranes. The obtained data of voltammetric investigations and analysis by the transmission electron microscopy show that electrical, mechanical and transport properties of lipid membrane change under the influence of hexadecaprenyl monophosphate. The results indicate that hexadecaprenyl monophosphate can modulate the surface curvature of the membranes by the formation of microdomains. The properties of modified membranes can result from the presence of the negative charges in the hydrophilic part of hexadecaprenyl monophosphate molecules and are modulated by the concentration of this compound in membranes. We suggest that the dynamics and conformation of the phosphate ester derivatives of long-chain polyprenols in membranes depend on the transmembrane electrical potential. The indication of the formation of vesicular bilayers by C_{80}-P creates the possibility of forming primitive bilayer lipid membranes by long single-chain polyprenyl phosphates in abiotic conditions. It can be the next step in understanding the origin of protocells.

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References

Bach, T.J. 1995. Some New Aspects of Isoprenoid Biosynthesis in Plants — A Review. *Lipids* **30:**191–202

Bugg, T.D.H., Brandish, P.E. 1994. From peptidoglycan to glycoproteins: Common features of lipid-linked oligosaccharide biosynthesis. *FEMS Microbiol. Lett.* **119:**255–262

Chojnacki, T., Jankowski, W., Mankowski, T., Sasak, W. 1975. Preparative separation of naturally occurring mixtures of polyprenols on hydroxyalkoxypropyl-sephadex. *Anal. Biochem.* **69:**114–119

Chojnacki, T., Świeżewska, E., Vogtman, T. 1987. Polyprenols from Plants — Structural Analogues of Mammalian Dolichols. *Chemica Scripta* **27:**209–214

Chojnacki, T. 1995. Dolichols Polyprenols and Derivatives, Collection of Polyprenols. pp 17–23. Polish Academy of Sciences, Warsaw

Crowley, J.M. 1973. Electrical breakdown of bimolecular lipid membranes as an electromechanical instability. *Biophys. J.* **13:**711–724

Danilov, L.L., Druzhinina, T., Kalinchuk, N.A., Maltsev, S.S., Shibayev, V.N. 1989. Polyprenyl phosphates: synthesis and structure-activity relationship for a biosynthetic system of *Salmonella anatum* O-specific polysaccharide. *Chem. Phys. Lipids* **51:**191–203

De Ropp, J.S., Knudsen, M.J., Troy, F.A. 1987. ^{2}H NMR investigation of the dynamics and conformation of polyisoprenols in model membranes. *Chemica Scripta* **27:**101–108

De Rosa, M., Gambacorta, A., Gliozzi, A. 1986. Structure, biosynthesis and physico-chemical properties of archaebacterial lipids. *Microbiol. Rev.* **50:**70–80

Gamble, F., Robello, M., Usai, C., Marchetti, C. 1982. Properties of ionic transport through phospholipid-glycolipid artificial bilayers. *Biochim. Biophys. Acta* **693:**165

Gennis, R.B. 1989. Biomembranes. Molecular Structure and Function. Springer-Verlag. New York

Hemming, F.W. 1983. Biosynthesis of Dolichols and Related Compounds. *In:* Biosynthesis of Isoprenoid Compounds. J.W. Porter and S.L. Spurgeon, editors. pp. 305–354. John Wiley, New York

Janas, T., Kuczera, J., Chojnacki, T., Krajewska-Rychlik, I. 1986. Properties of lecithin-dodecaprenol macrovesicular bilayer membranes. *Chem. Phys. Lipids.* **39:**347–355

Janas, T., Tien, H.T. 1988. Influence of dolichyl phosphate on permeability and stability of bilayer lipid membranes. *Biochim. Biophys. Acta* **939:**624–628

Janas, T., Kuczera, J., Chojnacki, T. 1989. Voltammetric analysis of polyisoprenoid-containing bilayer lipid membranes. *Chem. Phys. Lipids* **51:**227–238

Janas, T., Kuczera, J., Chojnacki, T. 1990. Voltage-dependent behavior of dolichyl phosphate-phosphatidylcholine bilayer lipid membranes. *Chem. Phys. Lipids* **52:**151–155

Janas, T., Janas, T. 1995. Interaction of undecaprenyl phosphate with phospholipid bilayers. *Chem. Phys. Lipids* **77:**89–97

Jankowski, W.J., Świeżewska, E., Sasak, W., Chojnacki, T. 1994. Occurrence of Polyprenols and Dolichols in Plants. *J. Plant Physiol.* **143:**448–452

Klösgen, B., Helfrich, W. 1997. Cryo-transmission electron microscopy of a superstructure of fluid dioleoylphosphatidylcholine (DOPC) membranes. *Biophys. J.* **73:**3016–3029

Lai, C.-S., Schutzbach, J. 1984. Dolichol induces membrane leakage of liposomes composed of PE and PC. *FEBS Lett.* **169:**279–282

Langner, M. Gutowicz, J., Gomułkiewicz, J. 1986. Modification of some properties of spherical lipid membranes induced by the association with fructos-1,6-bisphosphate aldolase. *FEBS Lett.* **196:**251–254

McCloskey, M.A., Troy, F.A. 1980. Paramagnetic isoprenoid carrier lipids. 2. Dispersion and dynamics in lipid membranes. *Biochemistry* **19:**2061–2066

Morovitz, H.J. 1992. Beginnings of Cellular Life. Yale University Press, New Haven

Murgolo, N.J., Patel, A., Stivala, S.S., Wong, T.K. 1989. The conformation of Dolichol. *Biochemistry* **28:**253–260

Nakatani, Y., Plobeck, N., Eifler, S., Brisson, A., Ourisson, G. 1993. Polyprenyl phosphates: do they form vesicles like natural phospholipids? *Phosphorus, Sulfur, and Silicon* **77:**125–128

Ourisson, G., Rohmer, M. 1992. The biohopanoids. *Accounts Chem. Res.* **25:**403–408

Ourisson, G., Nakatani, Y. 1994. The terpenoid theory of the origin of cellular life: the evolution of terpenoids to cholesterol. *Chem. Biol.* **1:**11–23

Plobeck, N., Eifler, S., Brisson, A., Nakatani, Y., Ourisson, G. 1992. Sodium di-polyprenyl phosphates form "primitive" membranes. *Tetrahedron letters* **36:**5249–5252

Pozzi, G., Birault, V., Werner, B., Dannenmuller, O., Nakatani, Y., Ourisson, G., Terakawa, S. 1996. Single-chain polyprenyl phosphates form "primitive" membranes. *Angev. Chem. Int. Ed. Engl.* **35:**177–180

Schagina, L.V., Grinfeldt, A.E., Lev, A.A. 1983. Concentration dependence of bidirectional flux ratio as a characteristic of transmembrane ion transporting mechanism. *J. Membrane Biol.* **73:**203–216

Schutzbach, J.S., Jensen, J.W. 1989. Bilayer membrane destabilization induced by dolichylphosphate. *Chem. Phys. Lipids* **51:**213–218

Smith, J.R., Laver, D.R., Coster, H.G.L. 1984. The conductance of lecithin bilayers: the dependence upon temperature. *Chem. Phys. Lipids.* **34:**227–236

Sunamoto, J., Iwamoto, K., Tezuka, T., Kadosaki, K., Kondo, H. 1983. Liposomal membranes. XIV. Fusion of liposomal membranes induced by polyisoprenoids as monitored by fluorescence quenching method. *Chem. Phys. Lipids* **33:**127–133

Small, D.M. 1986. The Physical Chemistry of Lipids. p. 629. Plenum Press, New York

Świeżewska, E., Sasak, W., Mańkowski, T., Jankowski, W., Vogtman, T., Krajewska, I., Hertel, J., Skoczylas, E., Chojnacki, T. 1994. The search for plant polyprenols. *Acta Biochim. Polon.* **41:**221–260

Świeżewska, E., Chojnacki, T. 1988. Long-chain polyprenols in Gymnosperm plants. *Acta Biochim. Polon.* **35:**131–147

Tien, H.T. 1974. Bilayer Lipid Membranes (BLM). Theory and Practice, Marcel Dekker, New York

Troy, F.A. 1992. Polysialylation: from bacterial to brains. *Glycobiology* **2:**5–23

Turner, G.C., Oldfield, E. 1979. Effect of the local anaesthetic on hydrocarbon chain order in membranes. *Nature* **277:**669–670

Valtersson, C., Van Duyn, G., Verkleij, A.J., Chojnacki, T., de Kruijff B., Dallner, G. 1985. The influence of dolichol, dolichol esters, and dolichyl phosphate on phospholipid polymorphism and fluidity in model membranes. *J. Biol. Chem.* **260:**2742–2751

Van Duijn, G., Valtersson, C., Chojnacki, T., Verkleij, A.J., Dallner, G., De Kruijff, B. 1986. Dolichyl phosphate induces non-bilayer structures, vesicle fusion and transbilayer movement of lipid: a model membrane study. *Biochim. Biophys. Acta* **861:**211–223

Vigo, C., Grossman, S.H., Drost-Hansen, W. 1984. Interaction of dolichol and dolichyl phosphate with phospholipid bilayers. *Biochim. Biophys. Acta* **774:**221–226

Vogel, H., Stockburger, M. 1983. Electrostatically induced change of the conformational order of charged lipid membranes. *Chem. Phys. Lipids* **32:**91–103