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Application of ^{1}H and ^{31}P NMR to topological description of a model of biological membrane fusion

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The process of biological membrane fusion can be analysed by topological methods. Mathematical analysis of the fusion process of vesicles indicated two significant facts: the formation of an inner, transient structure (hexagonal phase — H_{II}) and a translocation of some lipids within the membrane. This shift had a vector character and only occurred from the outer to the inner layer. Model membrane composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) was studied. ^{31}P- and ^{1}H-NMR methods were used to describe the process of fusion. ^{31}P-NMR spectra of multilamellar vesicles (MLV) were taken at various temperatures and concentrations of Ca^{2+} ions (natural fusiogenic agent). A ^{31}P-NMR spectrum with the characteristic shape of the H_{II} phase was obtained for the molar Ca^{2+}/PS ratio of 2.0. During the study, ^{1}H-NMR and ^{31}P-NMR spectra for small unilamellar vesicle (SUV), which were dependent on time (concentration of Pr^{3+} ions was constant), were also recorded. The presence of the paramagnetic Pr^{3+} ions permits observation of separate signals from the hydrophilic part of the inner and outer lipid bilayers. The obtained results suggest that in the process of fusion translocation of phospholipid molecules takes place from the outer to the inner layer of the vesicle and size of the vesicles increase. The NMR study has showed that the intermediate state of the fusion process caused by Ca^{2+} ions is the H_{II} phase. The experimental results obtained are in agreement with the topological model as well.

Key words: topology, fusion, vesicle, ^{31}P-NMR, ^{1}H-NMR

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INTRODUCTION

The fusion of membranes is a fundamental cellular process regulating, among others, intercellular transport, neurotransmission, secretion of enzymes and cell penetration by viruses (Jena, 2000). Fusion of biological membranes is a crucial element of transport within the eukaryotic cell, secretion, phagocytosis and over processes involving organellar or plasma membranes. Lipid vesicles formed by the plasma membrane during endocytosis fuse with intracellular membranes; similar vesicles budding off from organelles export their contents outside the cell by fusing with the plasma membrane (Clauguec, 1999; Liu, 1999). These processes mainly involve the endoplasmic reticulum (ER), Golgi apparatus, endosomes, lysosomes, secretion vesicles and the plasma membrane (Mayer, 2001). The last stage of endo- and exocytosis is the docking and fusion of the secretion vesicles with the organelle or plasma membrane, involving cytosolic proteins (Sritharan *et al*., 1998). Over the last two decades substantial progress has been made in biochemical description of factors involved in the docking of secretion vesicles and their fusion. At present, intense research is being conducted to decipher molecular mechanisms of the interactions between the secretion vesicles and the plasma membrane and the role of these interactions in vesicle docking and fusion, e.g., between the proteins of the plasma membrane of neurons and the synaptic vesicle membrane (Sritharan *et al*., 1998).

Hitherto, a number of fusion models have been proposed to describe the process of biological membrane fusion. Numerous authors have suggested that the process of fusion is accompanied by formation of transient forms related to the appearance of the hexagonal phase H_{II} (Cullis & Hope, 1978; Verkleij *et al*., 1978; Verkleij *et al*., 1980; Talmon, 1986; Ellens *et al*., 1989). Current theory states that monolayer curvature leads to the formation of nonlamellar structures prior to the fusion pore formation. Both outer membrane layers exhibit negative curvature whilst both inner membrane layers exhibit positive curvature during the formation of highly curved intermediates (Darkes *et al*., 2002). The model presented in this paper assumes the formation of an intermediate form of vesicle in the H_{II} phase. The model does not assume breaking of the continuity of membranes at the transition stages, which is more energetically favourable (Siegel & Kozlov, 2004; Siegel & Tenchov, 2008). Topology is applied in many areas, including biology, and the model proposed in this paper (with all intermediate stages) is described in terms of topological methods. Objects readily used as models are manifolds. In general, a manifold is an object locally resembling a proper Euclidean space. In practice, manifolds that are applied most often are the one-dimensional ones (curves), two-dimensional ones (surfaces) and tree-dimensional ones

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Abbreviations: H_{II}, hexagonal phase; L_{α}, lamellar phase; NMR, nuclear magnetic resonance; MLV, multilamellar vesicles; $\Delta\sigma$ – splitting; I_{out}/I_{in}, ratio of areas under signal (intensity) assigned to outer/inner layer of vesicle; PC, phosphatidylcholine, L-α-phosphatidylcholine ($C_{42}H_{82}NO_8P$); PE, phosphatidylethanolamine, L-α-phosphatidylethanolamine ($C_{41}H_{78}NO_8P$); PS, phosphatidylserine, L-α-phosphatidylserine ($C_{42}H_{79}NNaO_{10}P$); SUV, small unilamellar vesicle.

(space substitutes). An important task in a theory investigating topological objects is their classification. In the theory of manifolds classification of objects to homeomorphism is an important problem. This problem has been solved for one and two-dimensional manifolds. For classification of two-dimensional manifolds, the surface is divided into simpler components, for each of which a two-dimensional manifold can be constructed. The basic operation applied for this purpose (and in many other situations) is the operation of connect sum of the topological manifolds (Kosniowski, 1980; Jänich, 1984; Janas *et al.*, 1994; Cherezov *et al.*, 2003).

The process of model membrane fusion was observed by nuclear magnetic resonance (NMR). NMR methods, first of all ^{31}P-NMR, are fundamental for the investigation of lipid membrane organisation. Phase transition from the lamellar phase (L_α) to the hexagonal phase in lipid membranes has already been studied by ^{31}P-NMR (Koter *et al.*, 1978; Hammondah *et al.*, 1981; Hui *et al.*, 1981; Sankarom *et al.*, 1989; Boesze-Bottaglia *et al.,* 1992; Epand *et al.*, 1993; Fenske, 1993; Dentino *et al.*, 1995; Domingo *et al.*, 1995; Huang & Epand, 1997; Yeagle *et al.*, 1997; Sánchez-Piñera *et al.*, 1999). The idea is based on results obtained for the 'shape model' proposed by Israelachvili and Mitchell (1975). According to this model lipid molecules are considered as building blocks of different shapes, i.e., they are assumed to be cone-shaped, cylindrical or inverted-cone shaped structures. Depending on the experimental conditions and the membrane composition the interplay of different geometries leads to the formation of micelles, bilayers, or hexagonal phases (Israelachvili & Mitchell, 1975; Wenk & Seelig, 1998).

On ^{31}P NMR spectra of MLVs is possible to conclude the point of $L_\alpha \rightarrow H_{II}$ phase transition because of the temperature dependence of the chemical shift anisotropy ($\Delta\sigma$). As $\Delta\sigma$ depends on the molecular motion and temperature, its temperature depsendence can be used for determination of the phase transition temperatures (Fenske, 1993; Huang & Epand, 1997, Wenk & Seeling, 1998). The temperature at which this phase transition occurs is highly dependent on the hydrocarbon chain composition of the phospholipid and the environment of the bilayer. Therefore, temperature and membrane composition (including the appropriate amount of phosphatidylethanolamine (PE)) allow detection of the point of $L_\alpha \rightarrow H_{II}$ phase transition. Accurate determination of the phase transition dependent on temperature was possible because of the characteristic shape of the ^{31}P-NMR spectrum for the $L_\alpha \rightarrow H_{II}$ phase transition (Verkleij *et al.*, 1980; Hui *et al.*, 1981; Tokutami *et al.*, 1981; Tilcock & Cullis, 1981; Sundler *et al.*, 1981; Ohki, 1982; Hope *et al.*, 1983; Sankarom *et al.*, 1989; Yeagle, 1992; Yeagle, 1997). The temperature of the $L_\alpha \rightarrow H_{II}$ phase transition for studied the MLVs composed of phosphatidylcholine (PC), phosphatidylethanolamine and phosphatidylserine (PS) was established on the basis of previous studies of phase transitions measured by ^{31}P-NMR (Koter *et al.*, 1978; Hammondah *et al.*, 1981; Hui *et al.*, 1981; Sankarom *et al.*, 1989; Boesze-Bottaglia *et al.,* 1992; Epand *et al.*, 1993; Dentino *et al.*, 1995; Huang & Epand, 1997; Yeagle *et al.*, 1997; Wenk & Seeling, 1998; Veiga *et al.*, 1999).

Ca^{2+} ions were used in the study as the fusogenic agent. Calcium ions are involved in many life processes and are found in all living cells. They are known to be involved in metabolism, proliferation, cell adhesion, learning and memory formation (Ramonet *et al.*, 2002). An increase in the Ca^{2+} concentration is necessary for many physiological functions, including the fusion of membranes, which is related to secretion of neurotransmitters, enzymes and hormones (Potoff *et al.*, 2008). Phospholipids, e.g. phosphatidylserine, take part in the process of membrane fusion dependent on Ca^{2+} ions (Jacobson & Papahadjopoulos, 1975; Tokutami *et al.*, 1981; Uster & Deamer, 1981; Ohki, 1982; Hope *et al.*, 1983; Bentz & Düzgünes, 1985; Deleers *et al.*, 1986; Papahadjopoulos *et al.*, 1990; Huang & Epand, 1997). Intense study of model membranes has shown that divalent cations can induce fusion of membranes containing PS and PE, probably by charge neutralisation, local dehydration or induction of local defects in lipid packing (Burger, 2000; Potoff *et al.*, 2008). The composition of the model membranes studied in this work was carefully chosen by taking earlier reports into consideration (Verkleij *et al.*, 1980; Düzgünes *et al.*, 1981; Sundler *et al.*, 1981; Tilcock & Cullis, 1981; Hope *et al.*, 1983; Holland *et al.*, 1996).

First, the concentration of Ca^{2+} ions which should be added to MLVs suspension to bring about the $L_\alpha \rightarrow H_{II}$ phase transition was established on the basis of the characteristic shape of the ^{31}P-NMR spectrum for the $L_\alpha \rightarrow H_{II}$ phase transition. Then, ^{31}P-NMR spectra were taken at various concentrations of Ca^{2+} ions for MLVs with the same compositions and at a temperature that was below the $L_\alpha \rightarrow H_{II}$ phase transition temperature. A ^{31}P-NMR spectrum with the characteristic shape of the H_{II} phase was obtained for the molar Ca^{2+}/PS ratio of 2.0.

During the study, ^{1}H-NMR and ^{31}P-NMR spectra for small unilamellar vesicles (SUV), which were dependent on time (concentration of Pr^{3+} ions was constant), were also recorded. The presence of the paramagnetic Pr^{3+} ions permits observation of separate signals from the hydrophilic part of the inner and outer lipid bilayers because of the changes in the chemical shift of the signals (Hunt & Tipping, 1978; Hunt & Jawaharlal, 1980; Hunt & Jones, 1983). The praseodymium ions form unstable associates with oxygen atoms in the phosphate group and increase their coordination number (Jones & Hunt, 1985; Kaszuba & Hunt, 1990; Gabrielska & Gruszecki, 1996). As a consequence, the chemical environment of protons from the analysed compounds changes as does the dipolar interaction of protons with unpaired electrons of the metal, which results in changes in the chemical shift but with the coupling constants remaining unchanged. The chemical shift depends on the metal–proton distance and decreases with its increase (Jones & Hunt, 1985; Kaszuba & Hunt, 1990). Interesting signals are obtained in the ^{1}H-NMR spectrum from protons of choline methyl groups in individual layers of the vesicles; in the ^{31}P-NMR spectrum, in turn, signals come from phosphorus atoms located in the hydrophilic part of phospholipids with differentiation into individual lipid layers of the model membrane. The process of obtaining separate signals from the outer and the inner layers on the NMR spectrum, with the use of paramagnetic ions, is called splitting (Kaszuba & Hunt, 1990; Jeżowska *et al.*, 1994; Gabrielska & Gruszecki, 1996; Kuczera *et al.*, 1997). The idea of splitting, defined as the distance between the signals assigned to the choline or phosphate groups from the inner and the outer layers of the vesicle membrane, and the ratio of intensity of these signals have been used earlier by Hunt and co-workers (Hunt & Tipping,1978; Hunt & Jones, 1983; Jones & Hunt, 1985).

MATERIALS AND METHODS

Egg L-α-phosphatidylcholine (PC) ($C_{42}H_{82}NO_8P$), egg L-α-phosphatidylethanolamine (PE) ($C_{41}H_{78}NO_8P$) and

brain L-α-phosphatidylserine sodium salt (PS) ($C_{42}H_{79}NNaO_{10}P$) were from Avanti Polar Lipids, USA and were more than 99% purity. Their fatty acid composition was as follows: PC 14:0 0.12%, 16:0 32.7%, 16:1 1.1%, 18:0 12.3%, 18:1 32.0%, 18:2 17.1%, 20:2 0.2%, 20:3 0.3%, 20:4 2.7% and 1.0% unknown; PE 16:0 17.3%, 16:1 0.5%, 18:0 24.2%, 18:1 18.1%, 18:2 14.0%, 20:2 0.2%, 20:3 0.3%, 20:4 16.0%, 22:6 4.2% and 5.2% unknown. No details concerning PS fatty acid composition was available. Usually, both fatty acid residues in PS are 18 carbons with saturated chains or monounsaturated chain.

Model membranes and NMR

Published procedure were used to obtain MLVs and SUVs (Jeżowska *et al.*, 1994; Gabrielska & Gruszecki, 1996; Kuczera *et al.*, 1997; Wenk &Seeling, 1998; Timoszyk & Janas, 2003; Timoszyk *et al.*, 2004). Briefly, a mixture of lipids (0.56 PE:0.28 PS:0.16 PC) was dissolved in chloroform and dried under a stream of nitrogen. The mixture was further dried in vacuum for 30 minutes. The thin lipid film was dispersed by vortexing in D_2O to give MLVs. The suspension was sonicated for 45 min with a 20 kHz sonicator with a titanium probe to give SUVs.

The vesicle suspension was transferred into 5-mm NMR tubes. The ^{1}H-NMR and ^{31}P-NMR spectra were recorded on an Avance Bruker DRX 500 MHz Spectrometer operating in the Fourier transform mode. The ^{1}H-NMR spectra were obtained at 500.133 MHz and the ^{31}P-NMR ones were collected with proton decoupled mode at 161 MHz.

Topological concepts

The following topological concepts were used for determination of the mode of transformation of the membranes or subcellular structures.

Topological space is a pair (X, τ), where X is a set in which a family of open subsets τ, known as open sets (sets with no boundaries), has been defined. The family of open subsets has the following properties:

1. an empty set and the whole space are open sets,
2. an intersection of two open sets is an open set,
3. a sum of an arbitrary number of open sets is an open set.

The family t of subsets of the space X meeting the above conditions is called the topology on set X, while the conditions it meets are called the axioms of the topology.

Thus, the topology is a family of open sets (Kosniowski, 1980). One of the operations on spaces is the mapping (f: $X \rightarrow Y$) of topological spaces into each other known as homeomorphism. This transformation is bijective and continuous and the inverse transformation (f^{-1}: $Y \rightarrow X$) is also continuous. The two topological spaces X and Y, for which homeomorphism exists, are called homeomorphic. The topological space can be imagined as a plastic object made of wax or rubber, while a homeomorphism is the operation transforming one space (X) into the other (Y) with no gluing of any points (as it is a bijection) and no breaking (it is continuous and the inverse operation is also continuous). Another operation is the connect sum of manifolds ($X\#Y$) also used for the classification of two-dimensional manifolds (Kosniowski, 1980; Jänich, 1984).

For 2D surfaces the idea of the connect sum is simple and suggestive. From one space denoted as X_1 we cut out a circle ($X_1 \backslash K_1 = OX_1$) and we do the same from another space denoted as X_2 ($X_2 \backslash K_2 = OX_2$). The surfaces are glued along the boundaries of the cuts, which are homeomorphic with the circles. In this way a new 2D manifold is obtained, which is a connect sum of the manifolds X_1 and X_2, and which is denoted as $X\#Y = OX_1 \cup_{\varphi} OX_2$ (Jänich, 1984). It has been proved that having three fundamental surfaces: a sphere, a torus and a projective plane, it is possible to construct any 2D manifold, compact and with no boundary.

The following theorem is true:

Each 2D manifold that is compact and has no boundary is homeomorphic either with a sphere or with a connect sum of a certain number of toruses or a connect sum of a certain number of projection planes.

The operation of the connect sum can be also defined for an n-dimensional manifold. Let X_1 and X_2 be manifolds of the same dimension n. From the inside of manifold X_i, $i = 1, 2$; a ball K^n_i is chosen. The surroundings OX_i of the K^n_i ball cross section and the manifolds X_i are determined. Then the ball K^n_i is removed and the manifold $X_i \backslash K^n_i$ is obtained. The operation of the connect sum of manifolds X_1 and X_2 (denoted as $X_1 \# X_2$) is obtained by gluing manifold $X_1 \backslash K^n_1$ to $X_2 \backslash K^n_2$ by a certain homeomorphism h, identifying the surroundings OX_i, where $i = 1, 2$. The connect sum of two manifolds is thus obtained by cutting out of each of them a certain ball, and gluing the subspaces obtained along the boundary of the removed balls (Kosniowski, 1980; Jänich, 1984).

The operation of the connect sum is also used for classification of manifolds of higher dimensions, although no such unique construction as for 2D surfaces is possible in this case; however, certain partial results can be obtained. Besides the operation of the connect sum, the sphere surface that is a special case of topological diversity was used in our mathematical model.

RESULTS

The topological model that uses the operation of the connect sum assumes a two-phase process of fusion of membrane vesicles — it starts with the outer layers and then precedes to the inner layers of membrane vesicles (Janas *et al.*, 1994). Figure 1(1) presents the initial stage of the fusion process, in which the outer and inner layers of membrane vesicles are modeled through X_i and Y_i concentric spheres, respectively, where $i = 1, 2$. Interacting vesicles approach each other and it results in a deformation of the outer layers of the membranes. Because of such deformation, convex and concave parts can be discerned. For the both vesicles interacting, the common elements of these parts are spheres (OX_1 and OX_2) (Janas *et al.*, 1994).

The surroundings of convex (OX_1^i, OX_2^i) and concave (OX_1^e, OX_2^e) parts, which are elements important for construction of the connect sum, can be marked for these spheres. Furthermore, fusion of the outer layers of membranes occurs that is topologically modeled by the $(X_1 \# X_2)_e$ structure formed as a result of the operation of the connect sum X_1 and X_2 (Fig. 1(2)). The formation of the hexagonal phase is modeled by an inner element that is topologically called the $(X_1 \# X_2)_i$ structure formed as a result of the operation of the connect sum of outer layers of the membrane vesicles. The topological model of the fusion of membrane vesicles explicitly indicates formation of the H_{II} phase (Fig. 1(2)). It results from the operation of the connect sum as defined earlier. With proper homeomorphism (mapping φ_1 or φ_2), points of convex — OX_1^e and OX_1^e and concave part surroundings

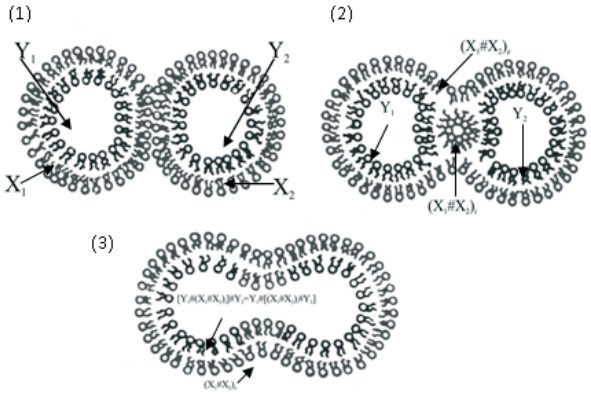

Figure 1. Mathematical model of the fusion process.
(1) Vesicles prior to fusion: X_1, X_2 — manifolds modelling outer layers, Y_1, Y_2 — manifolds modelling inner layers. **(2)** State after fusion of outer layers; $(X_1\#X_2)_i$ — manifold modelling H_{II} phase $(X_1\#X_2)_e$ — manifold modelling outer layer of vesicle after fusion. **(3)** Final stage of fusion. Two concentric spheres are two topological manifolds modelling outer layer $(X_1\#X_2)_e$ and inner layer $(Y_1\#(X_1\#X_2)_i)\#Y_2 = Y_1\#((X_1\#X_2)_i\#Y_2)$ of vesicle formed after fusion.

– OX_1^i and OX_2^i are defined. Therefore, we finally have: $(X_1\#X_2)_e = OX_1^e \underset{\varphi_1}{\cup} OX_2^e$ and $(X_1\#X_2)_i = OX_1^i \underset{\varphi_2}{\cup} OX_2^i$. Figure 1(3) presents a final effect of the fusion process that is called the $Y_1\#(X_1\#X_2)_i\#Y_2$ structure, also formed as a result of the operation of the connect sum in the topological model. In this phase, there is a connection between the inner layers of membrane vesicles and the $(X_1\#X_2)_i$ element formed after the fusion of the outer layers (Janas *et al.*, 1994).

Figure 2(1) presents the ^{31}P-NMR spectrum of MLVs consisting of PE:PS:PC as a function of temperature in the absence of Ca^{2+}ions. The temperature dependencies describe the $L_a \rightarrow H_{II}$ phase transition.

A shape spectrum characteristic for the H_{II} phase was observed for MLVs (Fig. 2(1)) (Koter *et al.*, 1978; Hammondah *et al.*, 1981; Hui *et al.*, 1981; Sankarom *et al.*, 1989; Boesze-Bottaglia *et al.,* 1992; Epand *et al.*, 1993; Dentino *et al.*, 1995; Yeagle *et al.*, 1997). The temperature of phase transition was in the range of 310 K–313 K.

Figure 2(2) presents the ^{31}P-NMR spectrum of MLVs consisting of PE:PS:PC *versus* the concentration of Ca^{2+} ions given (as the molar ratio to PS) at 283 K. The polymorphism of such vesicles is sensitive to the presence of Ca^{2+} ions. The shape of the ^{31}P-NMR spectrum characteristic of the hexagonal phase induced by Ca^{2+} ions is obtained for the Ca^{2+}/PS molar ratio of 2.0 (Fig. 2(2)).

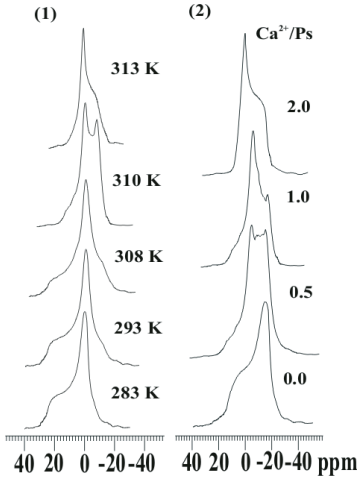

Figure 2. ^{31}P-NMR spectra of MLVs:
(1) *versus* temperature in absence of Ca^{2+}ions; **(2)** *versus* molar ratio of Ca^{2+}/PS (at 283 K).

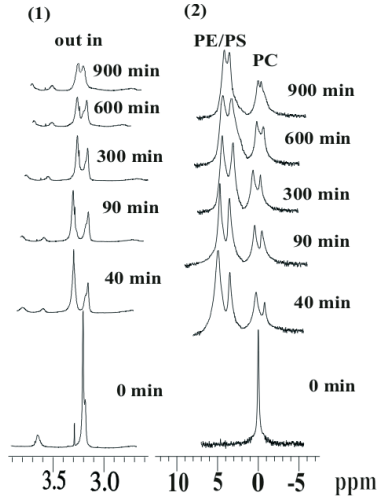

Figure 3. NMR spectra of SUVs at 283 K.
Time dependence of (1) ^{1}H-NMR and (2) ^{31}P-NMR spectra of SUVs consisting of PE:PS:PC, after addition of 5.0 mM Pr^{3+} ions (in presence Ca^{2+}/PS molar ratio of 2.0). (in) = signal from inner layer of vesicle, (out) = signal from outer layer of vesicle, PE/PS signal from phosphorus atoms of phosphatidylethanolamine and phosphatidylserine, PC signal from phosphorus atoms of phosphatidylcholine.

Figure 3(1) shows the ^{1}H-NMR spectrum of SUVs consisting of PE:PS:PC at 283 K in the bottom curve in presence of 2.0 Ca^{2+}/PS molar ratio and the following spectra display the time changes after an addition of Pr^{3+} ions to 5.0 mM. Splitting of the signal from protons of the choline groups occurs because of the presence of the Pr^{3+} ions. According to the data from Fig. 3(1), the signal on the right-hand side (in) comes from the protons of the choline groups of the inner layer of vesicles, whereas the signal on the left-hand side (out) from those in the outer layer of vesicles.

The intensity of the signal is proportional to the number of atoms of a given chemical group from which the signal comes from. Therefore, if the signal (in) has a lower intensity than the signal (out), there are less phosphatidylcholine molecules in the inner layer than in the outer layer of the vesicle.

Figure 3(2) shows the ^{31}P-NMR spectra of SUVs at 283 K in the bottom curve in presence of 2.0 Ca^{2+}/PS molar ratio and the following spectra show the time changes after an addition of Pr^{3+} ions to 5.0 mM. The ^{31}P-NMR spectrum, displays splitting of the signal from phosphorus atoms located in the inner layers of the vesicle after the addition of paramagnetic ions. On top of that, splitting of the signal into bands corresponding to individual phospholipid molecules forming the membrane is also observed.

The diversification of the chemical shift of the signal is related to the fact that the phosphorus atoms are located in various chemical surroundings.

DISCUSSION

According to a number of authors, some types of nonlamellar membrane structures can assume intermediate forms in the process of fusion (Cullis & Hope, 1978; Verkleij *et al.*, 1978; Verkleij *et al.*, 1980; Talmon, 1986;

Ellens *et al.*, 1989; Siegel & Epand, 2000; Cherezov *et al.*, 2003; Siegel & Kozlov, 2004). Mathematical analysis of the fusion process of vesicles indicated two significant facts. Firstly, the formation of an inner, transient structure of the model that was denoted as $(X_1\#X_2)_i$. This structure is a result of the operation of the connect sum, the key idea behind the topological model, which visualizes formation of the hexagonal phase. The formation of the H_{II} phase was incorporated in the models proposed by Ellens *et al.* (1989) and Cheresov *et al.* (2003). According to those models, formation of the hexagonal phase during the fusion is energetically favorable.

Secondly, a translocation of some lipids within the membrane was observed based on the topological analysis. This shift had a vector character and only occurred from the outer to the inner layer (Fig. 1(3)) (Janas *et al.*, 1994). The formation of structure denoted as $Y_1\#(X_1\#X_2)_i\#Y_2$, which determines the topological model, is the evidence of one-way shift of lipids within the membrane during the fusion.

The temperature of the ^{31}P-NMR studies of MLVs demonstrated that the $L_a \rightarrow H_{II}$ phase transition could be determined on the basis of changes in the shape of the ^{31}P-NMR spectra depending on the lipid bilayer structures, i.e. the phases and vesicle size. The ^{31}P-NMR spectrum characteristic of MLVs shows a broadened signal assigned to the anisotropy of motion of the lipid molecules captured in the multilayered structure of the vesicle and the signal assigned to the isotropic motion of some lipids (Fig. 1(1), Fig. 2(1)) (Fenske, 1993; Huang & Epand, 1997; Timoszyk *et al.*, 2004). After recording the ^{31}P-NMR spectrum of the shape characteristic of MLVs in the H_{II} phase, a similar experiment was performed as a function of Ca^{2+} concentration (Fig. 2(2)) (Fenske, 1993; Domingo *et al.*, 1995; Huang & Epand, 1997). The shapes of the spectra indicated that a chemical shift anisotropy can be used for observation of the effect of Ca^{2+} ions on the $L_a \rightarrow H_{II}$ phase transition (Fig. 2(1)). This experiment proves that during fusion caused by Ca^{2+} ions, the intermediate state is the H_{II} phase, which is in agreement with the topological model (Fig. 1(2)).

In order to observe the process of fusion first we added Ca^{2+} ions and then Pr^{3+} ions, thus allowing differentiation of signals assigned to the protons from choline groups of the outer and the inner layers of the membrane (Fig. 3(1)) (Janas *et al.*, 2001; Timoszyk & Janas, 2003). The splitting between the signals representing the two layers of the vesicle was found to decrease with time. Initially the Pr^{3+} ions are only outside the vesicle so the splitting is the greatest (Fig. 3(1)). During fusion, the Pr^{3+} ions get captured inside the vesicles and the splitting decreases (following spectra) (Kuczera *et al.*, 1997; Hunt & Jones, 1983; Timoszyk & Janas, 2003). A similar relation was observed for the ^{31}P-NMR spectra of SUVs, showing two distinct signals assigned to PE/PS and PC (Fig. 3(2)). The ^{31}P-NMR study of SUVs demonstrated that observing the changes of splitting between assigned signals was possible. During the process of fusion splitting decreases. This relation is true for signals coming from phosphorus atoms from PE/PS molecules and PC molecules. It means that Pr^{3+} ions are only outside the vesicles before the fusion but during this process they are getting inside the vesicles with PE, PS and PC molecules as associates. This decreases of the splitting. It proves that the translocation of phospholipids takes place during fusion, as postulated in the topological model (Fig. 1(3)).

However, a decrease of the splitting is not enough to prove the topological model. The experiment should also show the direction of the translocation of the phospholipid molecules. A study of the intensity signal could indicate the direction of the translocation. The ratio of areas under the signal assigned to the outer layer to that assigned to the inner layer (I_{out}/I_{in}) was found to change during the experiment. A similar observation was made by Hunt and co-workers (Hunt & Tipping, 1978; Hunt & Jones, 1983; Jones & Hunt, 1985). The ratio of I_{out}/I_{in} is proportional to the number of choline groups or phosphate groups in the outer and the inner layers. It is obvious that the number of lipid molecules in the outer layer is greater than that in the inner layer, so for SUVs the ratio I_{out}/I_{in} is greater than 1. The smaller the vesicles the lower the number of lipid molecules that can fit the inner layer of the vesicle so the higher the ratio of I_{out}/I_{in} (Jones & Hunt, 1985). Therefore, this ratio indirectly informs about the size of the vesicles as well. The ^{1}H-NMR spectra of SUVs evidence a decrease of the ratio I_{out}/I_{in}, which means that the size of the vesicles increases, mainly as a result of vesicle fusion and that the number of choline groups in the outer layer of the vesicle decreases and in the inner layer - increases (Fig. 3(1)). The relation of I_{out} to I_{in} shows that translocation of lipids from the outer layer of the vesicle to its inner layer is taking place during fusion. This relation is also in agreement with the topological model of fusion presented (Fig. 1(3)). The ratio I_{out}/I_{in} for the vesicles studied never reaches values lower than or equal to 1, which is a value characteristic of MLVs, cubic or H_{II} phase (Hunt & Tipping, 1978; Hunt & Jones, 1983; Jones & Hunt, 1985). The above-mentioned relation can be detected independently for the signals assigned to PE/PS and PC in the ^{31}P-NMR specta (Fig. 3(2)). The ratio I_{out}/I_{in} decreases during fusion for both kinds of observed signals (PE/PS and PC). It means that the vesicle size increases and that the translocation of phospholipid molecules from the outer layer to the inner layer of fusing vesicles occurs. Thus, this phenomenon involves PC as well as PE/PS molecules. Of course, it cannot be excluded that some of the lipid molecules undergo translocation as a result of the flip-flop process.

The obtained results suggest that in the process of fusion translocation of phospholipid molecules takes place from the outer to the inner layer of the vesicle. The decrease in the ratio of areas under the curves corresponding to signals assigned to the outer and inner layers (I_{out}/I_{in}) indicates an increasing size of the vesicles. These results indirectly reflect the occurrence of fusion. The NMR study has showed that the intermediate state of the fusion process caused by Ca^{2+} ions is the H_{II} phase. The experimental results obtained are in agreement with the topological model as well.

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