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# The mechanisms of lipid–protein rearrangements during viral infection

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**Abstract**

Membrane fusion and fission are important events in living cell functioning. In spite of the great variety of specific cases, all of these phenomena are probably governed by the same physical principles. The first insight into physics of membrane fusion has been achieved through studies on model lipid systems. These results served as a base for subsequent investigations of the mechanisms of biological fusion. The main objective of this brief review is to expose the landmarks on the pathway of these studies and to discuss problems and perspectives.

Fusion is a multistage process that includes transitions between several numbers of the intermediates. It is adopted that in the case of fusion of two planar bilayers, the following stages take place: formation of close inter-membrane contact, appearance of local monolayer bridge called a stalk, expansion of stalk leading to formation of hemifusion diaphragm (HD) and, finally, creation of fusion pore. Note that the stalk is nanoscopic and still an invisible object. However, there are no doubts that some kinds of monolayer bridge exist while its shape and structure, energetic and kinetic properties are unknown.

The main results on the mechanism of biological fusion were obtained on the cells expressing fusion protein of influenza virus, hemagglutinin (HA). However, this system has no M1 and M2 proteins of influenza, which are responsible for the release of the genetic material of the virus into the target cell. An experimental system developed in our laboratory allows to monitor the fusion of single virions with lipid bilayer and detect RNA release as well as the role of M1 and M2 in this process.

Biological fusion is a result of complicated interplay of lipids and special proteins at nanoscopic range. It seems probable that the first function of the proteins is the preparation of a pre-fusion state also known as membrane docking. Redistribution of the energy between proteins and lipids leads to the creation of so-called dimples accumulating bending energy, which facilitates stalk formation. Probably, proteins participate in the subsequent stages of fusion in the course of a set of downhill conformational changes. Unfortunately, the data on the kinetics of these transitions are not available. Therefore, theoretical analysis is limited by a consideration of lipidic subsystem, while proteins participate as boundary conditions or some superimposed constraints. As a result, taking into account lipid tilting and fusion pore compression, low-energy pathway was proposed, leading directly from modified stalk to pore.
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*Keywords:* Lipid–protein; Rearrangement; Viral infection

## 1. Introduction

Each living cell contains enormous amounts of membrane structures—plasma membrane, vesicles, endoplasmic reticulum, Golgi apparatus, etc. This system of membranes is in a state of permanent restructuring, which takes place via membrane fusion and fission. By this way, the cell releases neuromediators and different macromolecules, performs protein transport from Golgi to plasma membrane and captures important substances from outside via endocytosis. These are all examples of useful fusion ensuring proper cell functioning. However, sometimes, a cell can encounter dangerous fusion, such as virus invasion. In spite of the great variety of specific cases of membrane remodeling, they are all probably governed by the same generic principles.

The objective of this short review is to describe studies on the physics of membrane fusion performed by our team at the Frumkin Institute. These studies started from model lipid systems in the mid 1980s. They lay the foundation for subsequent development of quantitative theory of biological fusion. Therefore, after a short description of

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the results concerning lipid bilayer fusion, our exposition will focus on recent data on viral induced fusion obtained during our current work in Moscow in close collaboration with the laboratories of J. Zimmerberg (NICHD, Bethesda, USA) and F. Cohen (Rush Medical College, Chicago, USA).

## 2. Model systems

The elucidation of the physical mechanism of fusion in a living cell is a challenging goal. The first insight into this phenomenon has been achieved through studies on model systems. D. Gingell and L. Ginsberg played an outstanding role in these attempts, proposing a hypothesis of stalk as a first intermediate in a pathway to complete fusion [1]. Markin and Kozlov transformed this hypothesis into a quantitative theory [2] by using the Helfrich equation for the bending energy of a curved membrane. Experiments performed by Chernomordik et al. [3] confirmed the main predictions of stalk theory. This study has been carried out on a modified system consisting of two planar bilayers (BLM), introduced earlier by Liberman and Nenashev [4] and independently by Neher [5]. It has been demonstrated that fusion proceeds through a number of successive steps (Fig. 1). These five steps include the formation of close contact between two BLMs due to application of the hydrostatic pressure, appearance of stalk, expansion of stalk until the creation of a hemifusion diaphragm (HD) and rupture of HD leading to the formation of the membrane tubule (MT). Note that the last stage manifests completion of the fusion because the merger of lipids and association of initially separated water compartments have taken place. The shape of the membrane tubule is described by a catenoid, which becomes unstable if its lumen radius decreases down to the minimum value. After this occurs, the system returns to its initial state of two planar bilayers. Recent study of the last stage of fusion has demonstrated that the transition to the initial state is more complex (Frolov et al. [6]). After the collapse of the membrane tubule, it transforms into a nanotubule (NT). It was possible to monitor reversible transitions between MT and NT by changing the parameters of the system. The authors hypothesized that this phenomenon resembles a kiss-and-run pattern during exocytosis.

Molecular geometry of lipids in proximal and distant monolayers is a key parameter of stalk theory (Fig. 2). Negative spontaneous curvature of proximal monolayers containing phosphatidiletanolamine (PE) promotes stalk formation, while positive spontaneous curvature (lysophosphatidilcholin, LPC) inhibits monolayer fusion. On the contrary, positive spontaneous curvature of distant monolayers induces the rupture of HD and promotes complete fusion. These predictions of stalk theory were proven not only on BLMs but also in many cases of biological fusion. This finding has shown that stalk theory can be applied, although with the reservations to biological systems.

The initial version of stalk theory is not free of certain drawbacks: (1) Fusing bilayers were assumed to be in close contact, while in the experiment, actual inter-bilayer distance was about 3 nm; (2) the contribution of distant monolayers in free energy was not taken into account; (3) the shape of monolayer stalk and fusion pore was prescribed to be toroidal without any argument. To solve problem (1), Leikin et al. [7] considered thermal fluctuations in the shape of the bilayers providing formation of local contacts. These calculations led to the reasonable values in the rates of fusion. Problem (2) remained unresolved for a relatively long time until Siegel [8] took into account the bending energy of distant monolayers and came up with a huge value for the intermediate corresponding to the transmonolayer contact. Subsequent theoretical studies (see below) have shown that this "energy crisis" was an obvious result of the choice of traditional bending as the sole deformation mode. Moreover, this approach led to the overestimation of the energy of hydrophobic voids. Note that the disregard of these effects in the Markin–Kozlov theory [2] was not crucial for the analysis of the experimental data on BLM–BLM fusion because the bilayers contained small amounts of organic solvent still sufficient for the formation of microlences. It seems probable that fusion sites were settled on the top of microlences. Problem (3) was considered in a recent paper of Markin and Albanesi [9], where it was shown that stalk may have the shape of zero total bending energy. However, the radius of such stalk approaches

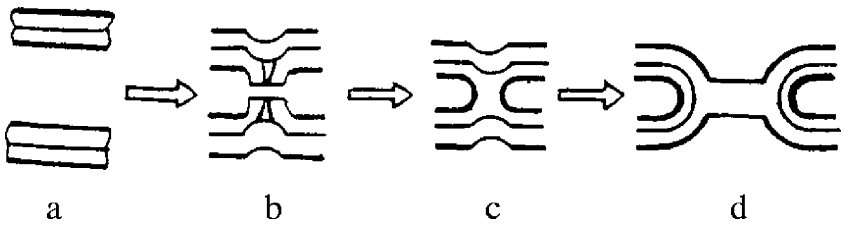


Fig. 1. Stalk mechanism of fusion. (a) Two bilayers in close contact; (b) the creation of semi-stalks; (c) formation of a bridge called a stalk; (d) formation of so-called hemifusion diaphragm (HD). Rupture of HD leads to creation of membrane tube.

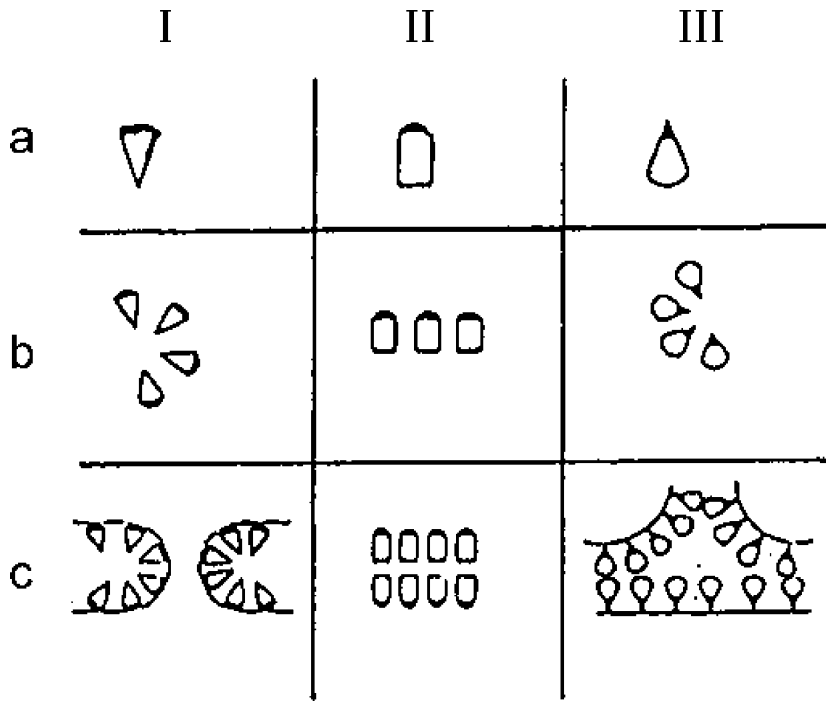


Fig. 2. Shape of lipid molecules (a) and the preferable structure of monolayer (b) and bilayer (c).The first column corresponds to the cones, say LPC; the second to cylinders, f.e. PC, and the last to inverted cones, f.e. PE. It is clear that cones are pore formers while inverted cones are stalk formers.

infinity, which restricts these results by planar lipid membranes.

## 3. Biological systems: experimental approaches

### *3.1. Influenza virus*

Fusion of biological objects is mediated by special proteins. In the case of exocytosis, the fusion machine is composed of several different proteins, while the influenza virus has only one fusion protein, hemagglutinin (HA). The structure of the ectodomain of HA and its conformational transitions after acidification (pH 7–5) are known in detail. Therefore, the influenza virus became the best model for the investigation of the mechanism of fusion. The most important part of the viral particle—ribonucleoprotein (RNP)—is packed in a double envelope (Fig. 3a). The first envelope consists of polymerized M1 proteins and the second is made from lipids. The last one contains M2 proteins and trimers of HA molecules. M2 proteins play the role of proton channels regulating pH within the virion. The HA molecule consists of two subunits—HA1 and HA2. The first one mediates the initial contact with the target membrane, while HA2 drives the fusion. At pH 7, all trimers are perpendicular to the surface of viral membrane. Each HA molecule, which is anchored into the lipid envelope by transmembrane domain, contains a short fusion peptide buried in a hydrophobic "pocket" of HA. After acidification, fusion peptide is released from the pocket and penetrates into the target membrane (Fig. 3b [10]). At pH 7, the HA molecule is similar to a compressed spring. If several HA molecules form a rosette and act in a cooperative way, then the energy released by HA after acidification is sufficient to bring together the membranes and bend them into a "dimple". The theory of a lipid dimple formation in the HA rosette was developed by Kozlov and Chernomordik [11]. According to this theory, fusion proteins create a torque, which bends the lipid patch and transforms HA energy into elastic energy of the curved "hat" of the dimple. Recent electrophysiological data and electron microscopy have shown (Frolov et al. [12]) that dimples indeed appear at HAb2–RBC contact after acidification. Although the precise mechanism of dimpling is still not entirely clear, it seems reasonable to believe that the key role in this process belongs to protein–lipid interactions.

Virus entry into the cell starts with endocytosis. As a result, the virus is found in the acidic endosome, which activates the HA molecules and induces fusion and then the release of RNP into the cytoplasm (Fig. 3c). It is obvious that it is extremely difficult to study the fusion of viral particles with an endosomal membrane in vivo. Therefore, it was necessary to work out the experimental systems and devices adequate for the monitoring of HA-induced fusion in vitro.

Fusion of any objects implies a merger of the membranes and enclosed water solutions. To study this process, monitoring of the redistribution of the lipid-soluble and water-soluble fluorescent dyes is usually performed. Thus, it is possible to distinguish between complete fusion and

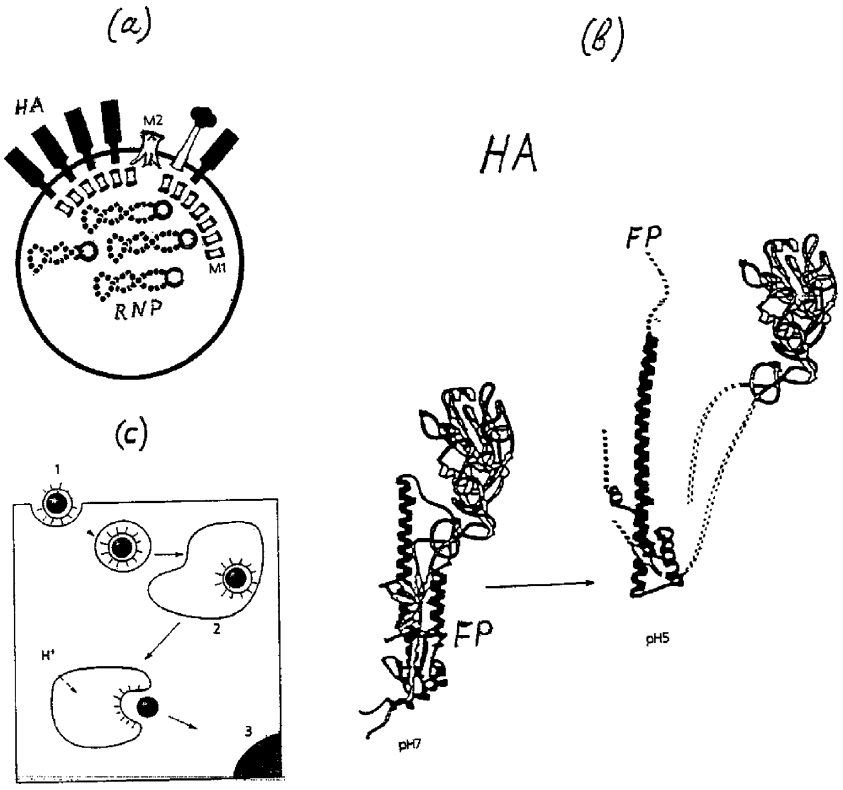


Fig. 3. Influenza virus (a), fusion protein, HA (b) and virion's pathway to a nucleus (c).

hemifusion, when only proximal monolayers merge. This approach is in use in the experiments with the suspension of viral particles and cells or lipid vesicles. However, such systems are not appropriate for studying molecular mechanisms of fusion. These problems could be, in principle, approached with such advanced techniques as patch clamp and fluorescent microscopy of single cells. However, viral particles are too small (0.1–0.2 μm) to be studied with traditional microelectrode methods. The real breakthrough happened after the invention of HA-expressing cells, which can be considered as a good model of viral particles suitable for the microelectrode measurements. Simultaneous monitoring of the electric current and fluorescence during fusion of a single red blood cell (RBC) and influenza virus had shown that the pore opens before visible lipid flux [13]. This finding was considered by Tse et al. [13] as a proof of the proteinous nature of the initial fusion pore. However, the subsequent measurements of L. Chernomordik et al. had demonstrated that the addition of LPC to proximal monolayers inhibits currents but does not influence lipid merger [14]. Therefore, it seems probable that in biological fusion, as well as in model systems, the next stage after dimpling is monolayer fusion with stalk formation and expansion. To explain the delay of lipid flux in normal conditions, L. Chernomordik et al. hypothesized that the HA rosette forms a fence, which is non-permeable to lipid molecules. It is obvious that this suggestion still needs to be proven.

### 3.2. Fusion of a single viral particle with BLM

The HA-expressing cell differs from a real virus in many ways. Indeed, lipid composition and density of HA are different. Additionally, HA-expressing cells do not contain M1 and M2 proteins. The last feature is the most important because M1 and M2 proteins play a significant role in RNP release and fusion pore evolution. Up to now, it looked like the small size of the viral particles is an obstacle for monitoring of the fusion on the level of a single virion.

However, an innovative technique has been lately developed in our laboratory, which allows detecting fusion of a single virion with BLM (Maksaev et al. [15]). The device consists of a horizontal BLM installed between upper and lower perfusible compartments containing electrolyte solution (Fig. 4).Viral particles are injected in the upper compartment with pH 7 and adsorb on BLM. Then a micropipette containing solution with pH 5 is pressed to BLM arresting few virions on a lipid patch. Viral particles are loaded with lipid-soluble fluorescent dye in a self-quenching concentration. Low pH in the pipette induces membrane fusion, and dye molecules diffuse into BLM. Dye redistribution could be detected through the appearance of the fluorescence. Simultaneously, fluctuating electric current, flowing through the fusion pore and possible conductive pathways in viral membrane was observed (Fig. 5). Initially, it was assumed that these conducting pathways are going through the M2 channels in the viral

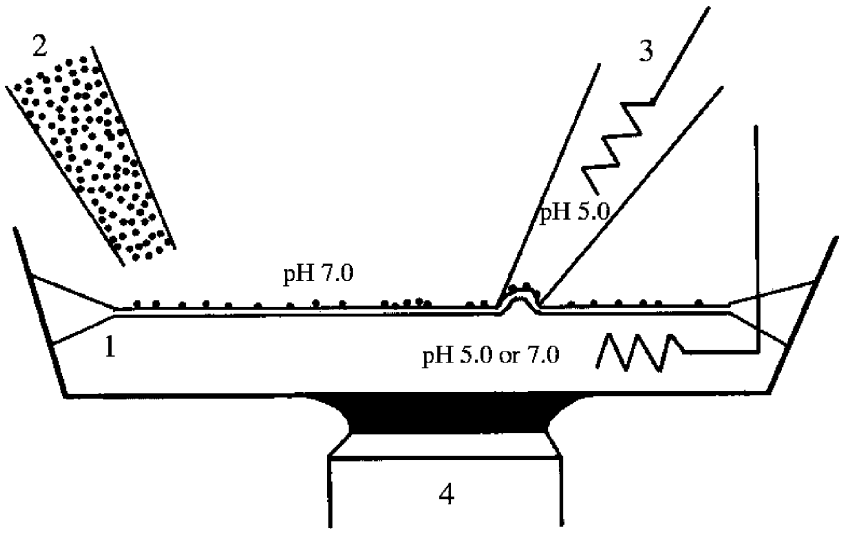


Fig. 4. A model system developed to study fusion of virions with BLM: (1) BLM, (2) a pipette, used for injection of virus suspension, pH 7.4, (3) the second pipette of ~ 1 μm in diameter, pH 4.8, (4) an inverted fluorescent microscope.

membrane. To test this idea, M2 channels were blocked by an inhibitor of M2 channels, amantadine. Indeed, the electric current fluctuations were substantially suppressed after amantadine treatment. However, this experiment does not prove that the M2 channels are the main current pathways. Actually, blocking of M2 channels results also in closing gates for protons. This means that the pH within the virus remains at 7, the same as it is in the lower compartment. Therefore, M1 envelope in this experiment remains in the intact polymerized state, while it probably ruptures in usual fusion assay in the absence of amantadine and, consequently, pH 5 within the virus. To check possible influence of the M1 envelope on the electric pattern, the same measurements were done at pH 5 in the lower compartment [16]. It was shown that in this case, amantadine had only minor influence on the electric activity. This means that M2 channels are not the main current pathways. It seems likely that rupture of M1 envelope results in the appearance of some conductive defects in lipid envelope of viral particle. The best way to prove this statement is to

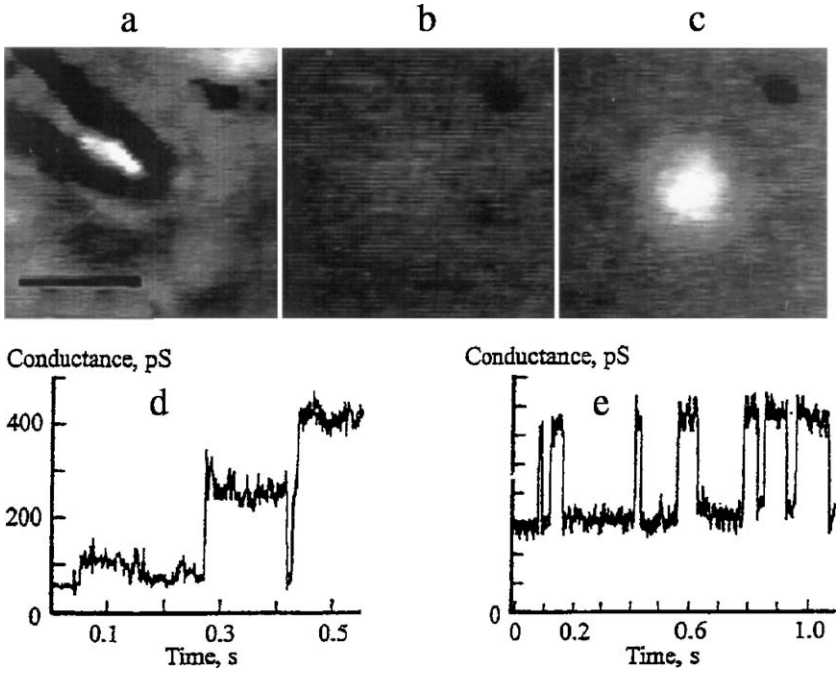


Fig. 5. Fusion of viral particles with a small membrane region localized with a micropipette: (a) microphotograph of a micropipette at the background of the membrane, phase contrast; (b) a fluorescent microphotograph of the same pipette after establishment of the gigaohmic contact with the membrane; (c) the same, 1 s later; (d), (e) electric activity registered on the membrane region localized by the micropipette with buffer solution, pH 4.8.

detect RNP release depending on the pH value in lower compartment. The problem is, however, rather complicated because a number of released RNP complexes after fusion may be very low. Our estimations show that this value is about 10–30 RNP particles in lower compartment. Using PCR technique, we detected RNA release in 50% of cases, where fusion took place in the absence of amantadine and pH 5 in both compartments. Therefore, the results of these experiments demonstrate that acidification of the solution, including intra-virion space, induce not only merger of lipid membranes but also rupture of the M1 envelope and RNP release.

## 4. Theoretical approaches

Protein-mediated fusion is a multistage process, which includes dimple formation followed by stalk and, finally, fusion pore creation. We proposed new intermediates, considered kinetics of all stages and obtained low-energy pathway solving " energy crisis" in fusion (Fig. 6 [17]). The thermodynamics of dimple formation was described elsewhere [11]. Let us discuss kinetics of stalk formation on the tips of two opposing dimples. When the distance between the two tips becomes very small (about a few angstroms), a strong repulsive hydration force prevents the dimples from coming closer together (see Rand and Parsegian [18]). A balance between repulsive hydration force and the force generated by the fusion proteins determines the equilibrium distance between the tips of the two dimples (Fig. 6-1). Using the formalism developed previously [7] we estimated the equilibrium inter-membrane distance as 1 nm. Transient lateral shift of polar head groups of lipids yields in small hydrophobic patches at the tips of the two membranes. Because hydrophobic surfaces attract each other, *cis*-leaflets can merge to create the stalk. We calculated the energy barrier that separates neighboring dimples from becoming a stalk by considering the simultaneous fluctuation of the inter-membrane distance and the radius of a hydrophobic patch. The merger of the *cis*-monolayers follows from the saddle-shaped topology of the energy surface. The height of the energy barrier for the favored pathway was estimated as 37 kT. Based on adopted value for the pre-exponential factor, we came to a reasonable figure for the waiting time of stalk formation ($\sim$ 1s).

At the point of stalk formation, the *trans*-leaflets have not yet approached each other, but the energy cost by the void is negligible (Fig. 6-2). With stalk expansion, the void

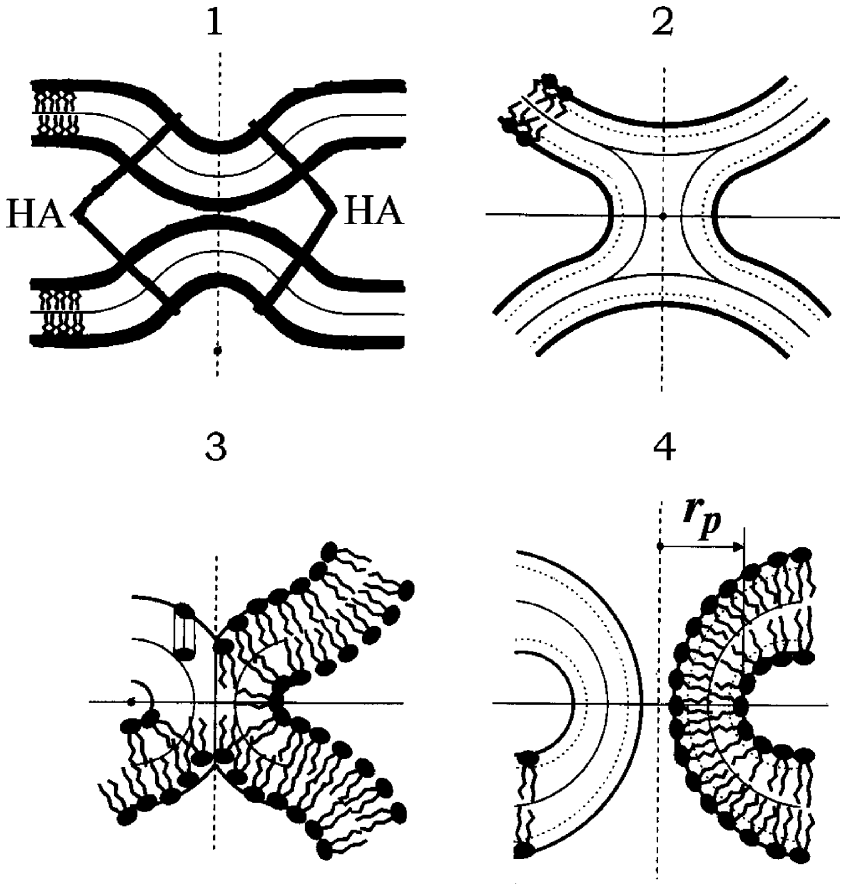


Fig. 6. Intermediate structures of membrane fusion: (1) dimples created by HA-rosette, (2) traditional stalk, (3) modified stalk made by tilted lipids, (4) fusion pre-pore with compressed nearest monolayers.

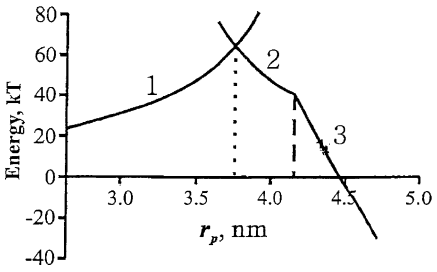


Fig. 7. Free energy of a modified stalk (1), a pre-pore (2) and a fusion pore (3).

becomes significant if bending is considered as a sole deformation mode. However, lipids in the membranes can move in the direction perpendicular to the neutral surface. Hydrophobic attraction between the separated *trans*-monolayers would facilitate these out-of-plane lipid displacements. Through such deformations, the *trans*-monolayers can contact each other yielding the modified stalk without creating significant voids (Fig. 6-3). In addition, formation of a stalk on curved dimple requires much less energy compared with the case of planar membranes. Considering a direct transition from the modified stalk to a fusion pore we introduced a second intermediate designated as compressed pre-pore (Fig. 6-4). As a result, we obtained the low-energy pathway of membrane fusion (Fig. 7). Independently, Kozlovsky and Kozlov [19] came to the similar results by rigorously considering tilt deformations in planar lipid bilayers.

Once formed, fusion pore rapidly enlarges to semi-stable conductance level. A freedom of a fusion pore for the expansion is limited, to some extent, by the M1 envelope and HA rosette. However, after the M1 envelope rupture, the influence of the M1 monomers on pore enlargement is not significant. The strict constrains superimposed by the HA rosette on membrane shape during the first stages of fusion become weaker after pore formation. We assumed that a fusion pore preserves toroidal geometry, but its length and width are free for the changes described by standard dynamic equations of motion. Calculations show that fusion pore quickly achieves semi-stable dimensions, which correspond to energy minimum located in a canyon between energy barriers [20]. The height of the barrier preventing pore expansion diminishes along the dimensions of length and width. The bottom of the canyon slopes gently downward along increasing length. As a consequence, theoretical fusion pore slowly lengthens and widens as the dimensions migrate along the bottom of the canyon, until the barrier vanishes and the pore rapidly enlarges. The dynamics of growth is sensitive to tension, spontaneous curvature, bending elasticity and mobilities. This sensitivity can account for the quantitative differences in pore evolution observed in two different experimental systems: HA-expressing cells fusing to planar bilayer membranes and beige mouse mast cell degranulation. We conclude that the mechanics of membranes could cause the phenomenon of stage-wise growth of fusion pore.

Time constants of pore movement along the length (slow one) and width (fast one) are very different. Therefore, we considered the pore motion in radii space only keeping constant the pore length. We assumed that the two fusing membranes are under different tensions. Pore dynamics and lipid flow through the pore were calculated using Lagrange equations with dissipation caused by intra- and intermonolayer friction [21]. These calculations show that the energy barrier that restrains pore enlargement depends only on the sum of tensions; a difference in tension between the fusing membranes is irrelevant. In contrast, lipid flux through the fusion pore depends on the tension difference but is independent of the sum. Thus, pore growth is not affected by tension-driven lipid flux from one membrane to the other. The calculations explain how increases in tension through osmotic swelling of vesicles cause enlargement of pores between the vesicles and planar bilayer membranes. In a similar fashion, swelling of secretory granules following fusion in biological systems could promote pore enlargement during exocytosis. The calculations also show that pore expansion can be caused by pore lengthening; lengthening may be facilitated by fusion proteins.

## 5. Conclusions

Twenty-five years passed from the time when D. Gingell and L. Ginsberg proposed stalk as a first intermediate in membrane fusion pathway. Since that impressive breakthrough, physics of the membrane fusion made a big progress. Small part of this way is described in this paper. Today in the agenda is the elaboration of the new experimental approaches able to study lipid–protein interactions leading to topological nanoscopic restructuring. This problem is inseparable from the development of new theoretical methods, including molecular dynamics. They will be based on the consideration of the fundamental forces acting between single lipid and protein molecules: electrostatic, van der Waals and hydrophobic.

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