

# Electroporation and Electrofusion of Membranes

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## 1. Introductory remarks

### *1.1. Membrane fusion ensures specific, controlled transfer of molecules in life processes and is important for biotechnology and biomedical research*

Fusion of membranes is a physical process which was of critical importance for the rapid evolution of life on earth and is involved in a variety of life processes today. One could imagine that billions of years ago the mechanical and/or electrical interactions in the ancient ocean mediated close apposition and destabilization of membranes which resulted in their fusion. Fusion of parts of the same membrane led to cell division and fusion of different membranes resulted in cell fusion. Both phenomena led to transfer and exchange of molecules which contributed to the rapid acceleration of the evolution of life on earth. One could further speculate that the 'first' fusion reactions were non-specific and any membranes could fuse provided appropriate conditions, including molecular contact, membrane destabilization and proper composition. Subsequently, specialized proteins appeared which led to the specific fusion of membranes and to controlled transfer of molecules. Today, the specific fusion of membranes seems to be the predominant mode of fusion in biological systems; however, the ability of membranes to fuse non-specifically, e.g., by external electric fields, provides information about the fundamental mechanisms of fusion and is important for biotechnology, medicine and research in biology.

### *1.2. Manifestations of cell fusion were observed nearly two centuries ago, while electrofusion was discovered in the late 1970's*

The first manifestations of a fusion phenomenon were observed almost two centuries ago by the German biologist Johannes Muller (see [1]). He discovered multinucleated giant cells in histological specimen while studying pathological conditions. By the turn of the nineteenth century, the medical literature contained several reports of 'polykaryocytosis' as symptomatic of a variety of diseases, including tuberculosis, variola, varicella, and rubeola. This raised the question whether these giant cells originated from successive mitoses or from fusion of mononucleated cells [2]. Later fusion was clearly established [3] as a mode of their formation, but it was not until the 1960's, when the studies of fusion 'exploded'. During a relatively short period of time a number of interesting fusion phenomena were discovered and characterized by using light microscopy (for history see [1]): (i) viruses can induce formation of giant multinucleated cells (syncytia) [4–6], (ii) during fertilization the acrosomal membrane interdigitates and then coalesces with the egg membranes [7], (iii) mononucleated myoblasts fuse to form myotubes, at least in vitro [8, 9], and (iv) cell hybrids can be formed in vitro by spontaneous cell fusion [10, 11]. The interest in studying fusion

grew in the next decades mainly because of its importance for production of hybrid cells. In the early 1970's polyethylene glycol was introduced as a fusion agent for plant protoplasts [12, 13] and animal cells [14] (see also the chapter of K. Arnold in this book). In the late 1970's groups from Japan and Germany reported that cell fusion can be also induced by external electric fields (electrofusion) [15–18].

### *1.3. Electric fields can induce fusion of a wide variety of membranes*

During the last decade numerous studies have shown that external electric fields can induce fusion of a wide variety of cell and artificial membranes (for review see [19–23]). This experimental observation is a demonstration of an inherent ability of membranes to fuse if appropriate conditions are provided and indicates the existence of properties of membrane systems, related to fusion, which are largely independent of the type of membranes. These properties include membrane stability and adhesion. Membranes are designed by nature to be stable and resist external constraints. They must be destabilized, i.e. they must be forced to change their structure to molecular conformations appropriate for fusion. The destabilized membranes must be at close apposition to allow merging of their lipid matrix. Therefore, understanding of fusion mechanisms requires understanding mechanisms of membrane destabilization and establishment of contact. External direct current (DC) fields can destabilize membranes and induce formation of pores (electroporation) (for recent review see [23]). External alternating current (AC) fields can induce membrane approach and contact predominantly by a process termed dielectrophoresis [24]. In the following chapter, I will focus on membrane fusion induced by electric fields (electrofusion) but I will also discuss electroporation and dielectrophoresis which are closely related to electrofusion.

I first briefly discuss polarization as one of the basic mechanisms of interactions of membranes with electric fields, leading to electroporation, dielectrophoresis and electrofusion, and then summarize observations on electroporation and dielectrophoresis related to electrofusion. In the rest of the chapter, I focus on observations of electrofusion and on the current concepts of its mechanisms. Why electrofusion may be important for understanding biological fusion is also briefly discussed.

## **2. Polarization of membranes underlies their destabilization, adhesion and fusion**

### *2.1. Polarization is due to restricted motion of charges*

External electric fields can induce formation of pores in membranes, move cells by dielectrophoresis and fuse membranes. All these phenomena are based on the same physical process: the polarization of material in electric fields. The polarization of membranes or any other material results from the fundamental interaction of electric fields with charges. Electric fields exert forces on charges which can either move if they are free to do so or accumulate if they are limited in their motion. The free motion of charges depends on the conductivity of the material while the charge

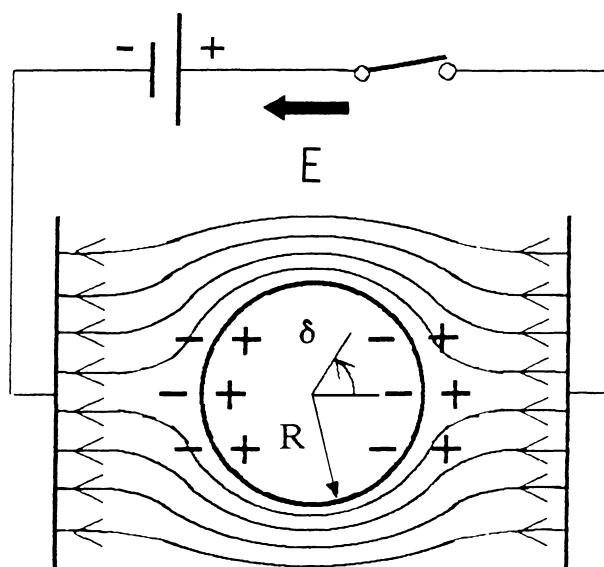


Fig. 1. Membranes restrict motion of charges and lead to cell polarization by external electric fields. The induced transmembrane voltage is maximal at the cell poles and minimal at the equator. Adapted from [37].

redistribution in a limited space is characterized by its polarizability. Figure 1 illustrates schematically how cells can be polarized due to the restricted motion of ions imposed by the plasma membranes.

## 2.2. Interaction of electric fields with polarized membranes induces mechanical stresses

The interaction of the external electric fields with the polarized material results in forces which can then induce motions inside particles or motion of the particles as whole. The motions inside the material can result in structural rearrangements or even mechanical fracture in the material, which for membranes can subsequently lead to their electroporation and electrofusion. The motion of the particles as a whole can occur even in the absence of a net charge, but only in non-uniform electric fields. It is a consequence of the interaction of the redistributed charges which have zero net charge but locally interact with electric fields of different strength, which leads to a net force exerted on the particle. This phenomenon, termed dielectrophoresis [24], can also occur in homogeneous external fields if there are other particles yielding local non-uniformities of the electric field. This leads to mutual attraction of the particles, to their approach and eventually to adhesion.

### 2.3. Forces exerted on polarized membranes can induce structural rearrangements, approach of membranes and their fusion

The magnitude and the type of motion depends on the electric field, the nature and geometry of the material. Membranes have low polarizability (relative dielectric constant about 2) and low conductivity (specific membrane conductance about  $1 \text{ mS/cm}^2$ , which corresponds to a 'bulk' conductivity of about  $1 \text{ nS/cm}$ ), surrounded by a medium of high dielectric constant (about 80) and high conductivity (about  $0.1 \text{ S/cm}$ ). Application of external fields to membrane systems leads to motion of ions in the medium surrounding the membranes, accumulation of charges at the membrane surfaces and membrane polarization. The charges at the membrane surfaces create electric field inside the membrane which is commonly of much higher strength than the field in the surrounding medium. The electric field inside the membrane interacts with the polarized membrane material which results in intramembrane motions and structural rearrangements. At sufficiently high field strengths these events can lead to formation of pores and discharge of the membrane surfaces by ionic currents through the pores. If another membrane is at close apposition, the molecular rearrangements can also result in fusion by mutual diffusion of the lipid molecules which leads to their intermixing and membrane merging. The molecular contact needed for fusion can be enhanced by the mutual attraction of the two polarized membranes. While this qualitative description of the interactions of membranes with electric fields leading to electroporation, mutual approach and fusion probably reflects basic features of those phenomena, the quantitative modeling and understanding of their molecular mechanisms are very complicated and far from clear. In the next two sections I describe basic experimental approaches and observations, as well as attempts for theoretical modeling of electroporation and dielectrophoresis.

## 3. High voltage pulses electroporate membranes

### 3.1. High voltage pulses can permeabilize membranes

In the late 1960's and the early 1970's it was found that application of high voltage direct current (DC) pulses to cell suspensions leads to killing of bacteria and yeasts [25], lysis of erythrocytes and protoplasts [26], release of catecholamines and ATP from chromaffin granules [27] and transcellular ion flow in bacteria [28] (for early electroporation data see [22]). This was originally attributed to electric breakdown of the cell membrane, which implies irreversible rupture of the membrane [26]. Later it was shown that the membrane permeability changes can be transient in nature [27] and that they can be analyzed in terms of reversible dielectric breakdown [29]. The first electroporative gene transfer into living cells with the subsequent actual expression of the foreign gene [30] led to explosive development of the studies on interactions of membranes with high voltage pulses. The term electroporation was introduced by Neumann [30] and presently generally accepted to refer not only to the phenomenon of formation of pores but also to all pore related events caused by exposure of membranes to high field strengths [31]. How actually electropores may look like is shown in fig. 2.

Fig. 2. Left panel: external (E) membrane face of an electropermeabilized human red blood cell frozen at 40 ms after the application of a high voltage pulse. Right panel: protoplasmic (P) membrane face of an electroporated erythrocyte frozen at 220 ms after the pulse. 60,000 $\times$ . Borrowed from [157].

### 3.2. The devices for electroporation are conceptually simple

One of the reasons for the success of the electroporation as a method of choice for gene transfer and studies of membrane behavior in electric fields is the conceptual simplicity of the experimental devices used to induce electroporation. They consist of two electrodes embedded in the cell suspension (see, e.g., fig. 1, where only one cell is shown). The electrodes are connected to a high voltage pulse generator which allows to control the voltage and duration of the electric pulse. The gene DNA or any other water soluble substance, which should be transferred into cells, is in the medium. The pulse application results in formation of pores in the cell membrane. This leads to exchange of molecules between the medium and the cytoplasm by diffusion, electroosmosis or other mechanisms. The membranes resealed after the pulse and the substance of interest is entrapped inside the cells.

### 3.3. The transmembrane voltage induced by the external electric fields is accurately described by a simple formula

Presently there are a wide variety of protocols, electrode configurations, media composition and pulse generators which can be used to electroporate a number of cells (for recent review see [23]). Let us consider a membrane which forms a spherical shell as shown in fig. 1. The application of an external electric field leads to currents of ions, which accumulate at the membrane surfaces and give rise to induced surface potentials on both sides of the membrane. This results in creation of a voltage  $V$  across the membrane. The basic relationship which is mostly used to estimate this transmembrane voltage induced by a rectangular pulse of field strength  $E$  is

$$V = V_m [1 - \exp(-\tau/t_p)], \quad (1)$$

where  $V_m$  is the maximal value of the transmembrane voltage,  $\tau$  is the duration of the pulse and  $t_p$  is the characteristic polarization time. For spherical membranes of radius  $R$ ,  $V_m$  and the charging time constant  $t_p$  are given by [32, 33]

$$V_m = 1.5ER \cos \delta, \quad (2)$$

$$t_p = RC_m(r_i + 0.5r_0), \quad (3)$$

where  $\delta$  is the angle between  $E$  and the radius vector, see fig. 1,  $C_m$  is the membrane capacitance,  $r_i$  and  $r_0$  are resistivities inside and outside the cell, and the membrane conductance was neglected (see also [34]). Applicability of eq. (2) to membrane systems was confirmed by using voltage sensitive dyes [35–37], see fig. 3. For the system, used by Kinoshita and his collaborators [37], the radius of the sea urchin eggs is  $5 \times 10^{-3}$  cm, a typical electric field strength  $E$  is 100 V/cm, the membrane capacitance  $C_m$  is  $1 \mu\text{F}/\text{cm}^2$ , the resistivity of the  $\text{Ca}^{2+}$ -free sea water  $r_0$  is 20 ohm-cm, and the intracellular resistivity  $r_i$  is 200 ohm-cm. The maximal induced transmembrane voltage  $V_m$  as calculated by using eq. (2) is 0.75 V and the charging time  $t_p$

Fig. 3. Induction of surface potential in a sea urchin egg as monitored by a potential sensitive dye. Snapshots were taken with an exposure time of  $0.3 \mu\text{s}$  under a pulsed laser fluorescence microscope at the indicated times after the onset of an external electric field of  $100 \text{ V/cm}$ . Intensity profiles are shown at the bottom. The positive electrode was on the right side of the cell. From [37].

calculated from eq. (3) is  $1 \mu\text{s}$ . The above values are typical for a wide variety of systems.

#### 3.4. Intramembrane field strength is much higher than the strength of the applied electric field

The above relationships show that the cell radius is very important parameter in electroporation. Since the transmembrane voltage  $V$  is proportional to the cell radius, cells of larger radii can be electroporated at smaller strengths  $E$  than smaller cells. Another interesting prediction of eq. (2) is that the electric field strength inside the membrane  $E_m$  is much stronger than the applied field  $E$ . An estimate of  $E_m$  ( $E_m = V_m/d$ ,  $d$  being membrane thickness) shows that the membrane amplifies the external field by a factor of  $R/d$  which can be of the order of 100 or more.

It is also seen from eq. (2) that the induced transmembrane voltage depends on the position along the membrane. At the poles, at  $\delta = 0$ , the induced voltage is maximal, while at  $\delta = 90^\circ$  it is equal to zero. This leads to a variation in the pore size and in the number of pores along the cell surface. Since  $V_m$  changes its sign when  $\delta$  equals  $\pi/2$  and  $3\pi/2$ , see fig. 1 and eq. (2), and the inherent preexisting transmembrane voltage  $V_i$  does not, the actual potential which is the sum of both, will be larger on the one half of the spherical membrane than on the other one.

The charging time  $t_p$  is another important parameter in electroporation. It increases with an increase in the resistivities and the cell radius. The pulse duration should be always longer than the charging time in order to get maximal transmembrane voltage.

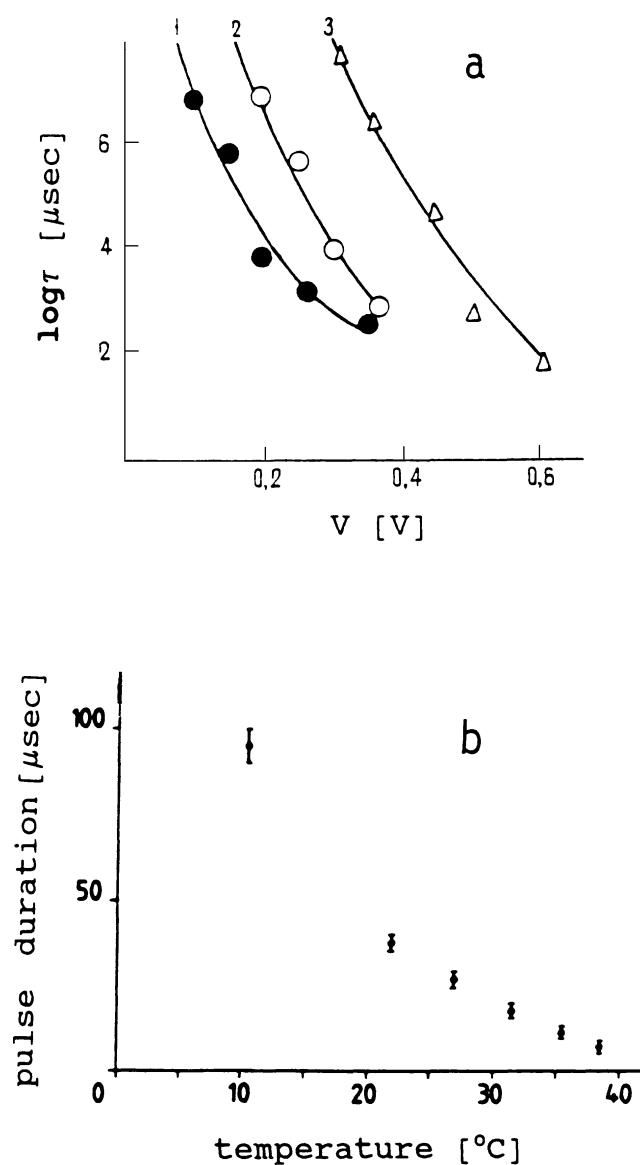


Fig. 4. (a) The duration of the electric pulse  $\tau$  needed to disrupt lipid bilayer membranes decreases with increasing the transmembrane voltage  $V$ . Empty circles indicate bilayers of phosphatidylcholine, solid circles show the data for the same bilayers but in the presence of  $4 \times 10^{-4}$  g/l lysophosphatidylethanolamine, and the empty triangles correspond to bilayers from phosphatidylethanolamine. The continuous lines are a theoretical prediction from an energy-based stochastic approach for electroporation [41]. From [65]. (b) The pulse duration  $\tau$  needed for electroporation decreases with the increasing temperature  $T$ . The transmembrane voltage applied across the pea protoplast membranes was 1.7 V. From [40].

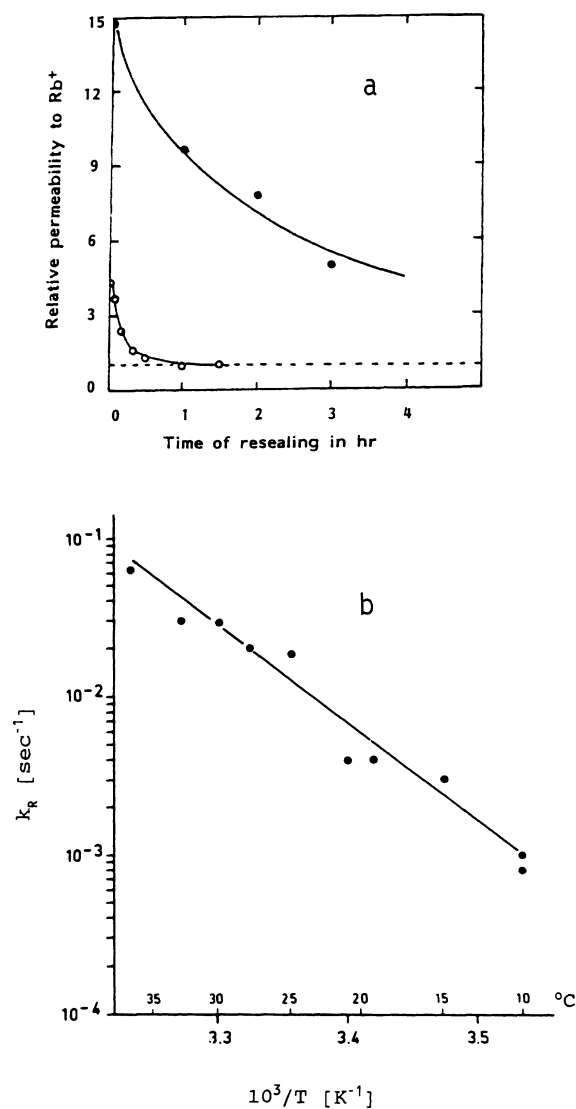


Fig. 5. (a) Restoration of membrane permeability occurs within minutes to hours. (Lower curve) Erythrocytes became leaky to  $Rb^+$ , but not to sucrose, after a treatment with a 4 kV/cm pulse for 2  $\mu s$ . Restoration of the membrane permeability took more than 30 min. (Upper curve) Red blood cells became transiently permeable to sucrose after treatment with high voltage pulses (3.7 kV/cm) for longer periods of time (20  $\mu s$ ). Restoration of the membrane permeability against  $Rb^+$  took more than 20 hr. From [45]. (b) The rate of pore resealing increases with an increase in temperature. Human erythrocytes were subjected to electrical pulse ( $E = 6$  kV/cm,  $t = 40$   $\mu s$ ) and their permeabilities were measured at different times after the pulse. Then the rate constants of resealing,  $k_R$ , were calculated and plotted as function of the temperature. From the slope of the Arrhenius plot a mean value of  $28.1 \pm 1.8$  kcal/mole was calculated for the activation energy. From [44].

### *3.5. Membranes are electroporated when the transmembrane voltage exceeds a threshold value*

Electroporation is a threshold phenomenon. Substances can be exchanged through electroporated membranes only if the transmembrane voltage exceeds a certain critical value which for cells is in the range from several hundreds mV to 1–2 V [22, 37–40]. The threshold voltage depends on the pulse duration [39–41]. An increase in the pulse duration needed for electroporation leads to a decrease in the critical voltage (fig. 4a). The pulse duration needed for electroporation at constant transmembrane voltage decreases with an increase in temperature (fig. 4b). The activation energy for the data shown in fig. 4b is 15 kcal/mol. Once the transmembrane voltage reaches its critical value, the poration takes place within microseconds [37, 38, 42]. Longer electric pulses produce larger pores [43]. The fast recovery of cell membranes after poration occurs within milliseconds while the complete recovery may last for seconds, minutes [35, 37, 44] or even hours [45] (fig. 5a). The reseal of electropores in lipid domains also may take seconds [36, 46]. The rate of pore reseal increases with increasing temperature (fig. 5b). The activation energy for the reseal (28 kcal/mol [44]) is somewhat higher than that for the formation of pores. Pulses of very long duration or/and high voltage can lead to irreversible membrane destabilization with subsequent membrane fragmentation. Loading of cells with water soluble molecules vary widely within the cell population (fig. 6), which may indicate variations of membrane properties and/or size between individual cells.

### *3.6. Transfer of molecules by electroporation is asymmetric*

Permeation of ions and fluorescence dyes through the porated membranes have often be found to be asymmetric [47–50]. In some cases, e.g., in sea urchin eggs the dye and  $\text{Ca}^{2+}$  permeation was much higher on the negative-electrode side, whereas the dye burst was seen mainly on the positive side in liposomes [37]. It was suggested that the inherent physiological potential is added to the induced transmembrane potential and this is the cause of the asymmetric formation of pores [48]. This explanation, however, can not be applied to the sea urchin eggs where the higher potential is on the positive side. The electroosmotic flow can also cause asymmetric transfer of molecules due to the negative surface charge of the plasma membranes [49, 50]. This explains the motion of molecules from the negative toward the positive electrode but can not explain the observations that molecule transfer was observed long after the removal of the electric field. Further studies are needed for elucidation of the mechanisms of the asymmetric electroporation.

### *3.7. The threshold voltage of electroporation decreases with an increase in intramembrane mechanical stresses (membrane tension)*

An interesting observation which can provide clues for the mechanism of electroporation is that the critical voltage needed for poration of cell membranes decreases with an increase in the membrane tension controlled by osmotic forces [51, 52]. Needham and Hochmuth [53] developed a new experimental approach based on micropipette technique which allows to set precisely the membrane tension and measure

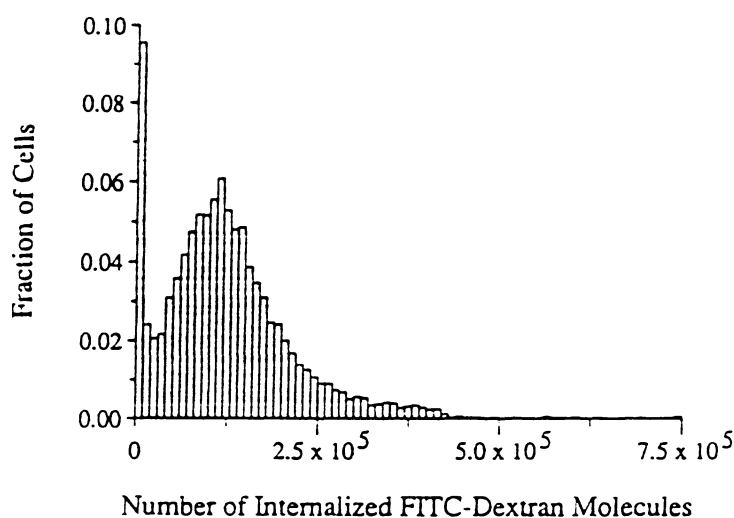


Fig. 6. Population distribution of the number of FITC-dextran molecules taken up by cells subjected to a single  $50 \mu\text{s}$  pulse of  $8 \text{ kV/cm}$  strength. The average of this distribution corresponds to  $1.4 \times 10^5$  molecules of FITC-dextran. The wide distribution around this mean value is clearly indicated by the graphical display. From [158].

the critical voltage of membrane permeabilization. They found that for lipid vesicles the square of the critical transmembrane voltage is linearly related to the membrane tension (fig. 7). At zero membrane tension, the critical membrane voltage increased from 1.1 to 1.8 V with an increase in cholesterol content. These results indicate that the electric fields induce stresses in the membranes, which are equivalent to stresses induced by external mechanical pressures as those in the micropipettes and that those stresses depend on the lipid composition, especially on the presence of cholesterol. How stresses lead to formation of pores in membranes, however, is a question which remains to be answered.

### 3.8. *The electromechanical models of electroporation describe membrane rupture as mediated by intramembrane stresses induced by the field*

Two main theoretical approaches were developed to describe electroporation – electromechanical and energetic. They originated from theories in the physics of condensed matter and physical chemistry of thin liquid films. The electromechanical approach considers membranes as elastic [51, 53–55] or viscoelastic bodies [56, 57] and applies the principles of elasticity and of physical chemistry of thin liquid films. The basic conclusion from this approach is the existence of a critical transmembrane voltage above which the membrane is unstable and eventually ruptures. The derived value for the critical voltage is in good agreement with the experimental data. One problem with the initial development of this approach [51, 54] was that at the critical voltage the membrane thickness should be reduced by about 40%. For an incompressible membrane this implies that the membrane surface area is capable of an extraordinary expansion which is not confirmed by data [53]. Two different solutions

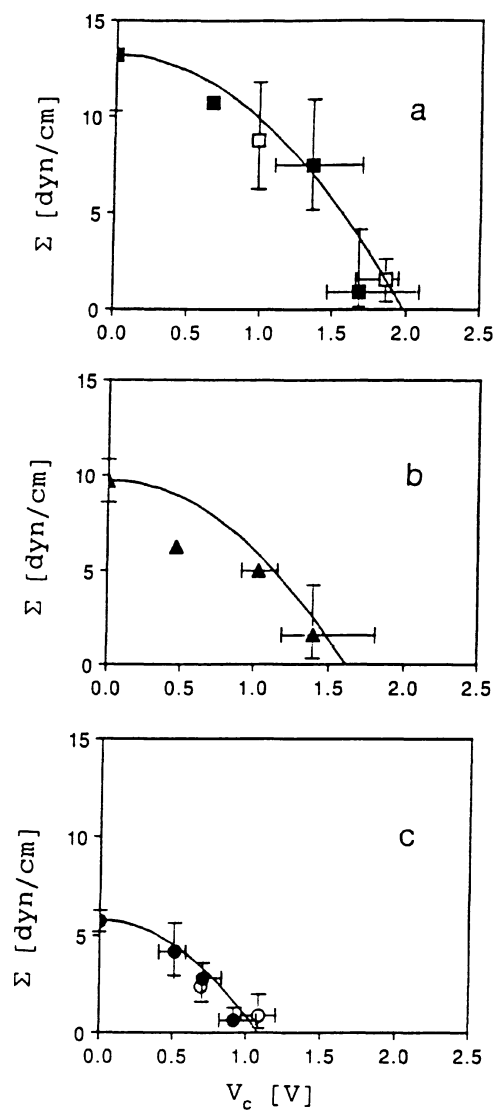


Fig. 7. Critical voltages for membrane permeabilization  $V_c$  as function of applied membrane tension  $\Sigma$ . (a) vesicles composed of a mixture of stearylphosphatidylcholine (SOPC) and cholesterol (1 : 1) containing small amounts (5%) of charged lipid, dioleoylphosphatidylglycerol (DOPG), in conducting solutions (10 mM NaCl plus sucrose/glucose) (filled symbols); vesicles from SOPC:cholesterol (1 : 1) in solutions of low conductivity (1 mM NaCl plus glucose) (open symbols), (b) vesicles from erythrocyte lipids in conducting solutions, (c) vesicles from SOPC containing small amounts of DOPG in conductive solutions (filled symbols); vesicles from SOPC in solutions of low conductivity (open symbols). The solid curves are plots of an equation based on the electromechanical model of membrane rupture. From [53].

to this problem were suggested. In one of them the surface tension of the two sur-

faces of the membrane was explicitly taken into account [56] and the rupture of the membrane was considered to be due to the instability of thermal fluctuations of the membrane surface shape represented as a superposition of surface waves. This led to the prediction that the average membrane thickness may not be changed significantly but the membrane can be ruptured locally by the growing surface shape instabilities. Another solution of the problem of large membrane thicknesses change was the following. The membrane tension [53] or the mechanical stresses in the membrane [55] induced by the external electric field were calculated and it was postulated that the membrane ruptures at certain critical tension which corresponds to small membrane area changes. This approach does not predict theoretically the critical voltage but rather postulates its existence as an inherent property of the membrane which can be measured accurately. In principle this property could be calculated by using the theories of fracture mechanics or other theories of material failure.

While ingenious and inherently correct the later approach does not allow to theoretically predict the dependence of the critical voltage on the pulse duration. An approach based on the viscoelastic behavior of the membranes was suggested to explain this dependence [56]. According to the viscoelastic model of membrane electroporation, the external electric field leads to growth of unstable undulations of the membrane surface with a rate proportional to the membrane viscosity. The membrane ruptures when the amplitude of the fastest growing perturbation becomes equal to the membrane thickness. The predictions of the viscoelastic model and its generalizations [58] are in good agreement with data for cell membranes [39, 40] and bilayer lipid membranes [41]. The calculated concentration of pores is also close to that observed for erythrocyte membranes by Chang, which is  $8/\mu\text{m}^2$  [59]. It must be pointed out that models based on surface shape instabilities could describe only initial stages of pore formation. Whether those pores expand and rupture irreversibly the membranes cannot be predicted by these models, at least at this stage of their development.

### *3.9. The energy-based approaches describe formation and expansion of pores as overcoming energy barriers*

A conceptually different approach for describing the formation and expansion of pores induced by electric fields is based on energy considerations [41, 60–66]. It was assumed that the free energy of the pores is a sum of two components – one is due to the surface energy and the other one is due to the pore edge energy (line tension). While the surface energy component tends to expand the pores, the edge energy component tends to close them. When the pore radius exceeds a critical value equal to the ratio of the surface energy density to the pore edge energy, the pore expands spontaneously until the membrane ruptures. Pores with radii smaller than the critical one have to overcome an energy barrier in order to increase their radii above the critical one. This takes time which is inversely proportional to the Boltzmann's factor  $\exp(-E_a/kT)$ , where  $E_a$  is the height of the energy barrier,  $k$  is Boltzmann's constant and  $T$  is the absolute temperature. The application of external electric fields leads to a decrease in the pore energy due to the polarization of water in the pore which is higher than that of the membrane material. This

decreases the energy barrier with a factor which is proportional to the square of the transmembrane voltage. The decrease of the energy barrier leads to shorter periods of time needed to overcome that barrier and reach the critical pore radius. The stochastic mechanism of pore expansion by overcoming energy barriers can describe a number of experimental data for bilayer lipid membranes (for review see [64–66]). However, like the electromechanical model it is phenomenological and does not allow to distinguish between hydrophobic and hydrophilic pores or other structures. It is assumed that the pores allowing transfer of molecules are hydrophilic pores, which originate from hydrophobic pores. The details of the mechanism of conversion to hydrophilic pores is unknown.

It seems reasonable to assume that the formation of hydrophilic pores results from the growth of unstable shape undulations of the membrane surfaces and partially by conversion of hydrophobic pores. The creation of pores by the electric field does not preclude the possibility that preexisting pores can serve as ‘nucleation’ sites for formation of larger pores [67]. The relative contribution of each of these two mechanisms of initial formation of pores depends on the phase state of the membranes and the pulse strength.

Several other approaches for describing the formation of pores and the kinetics of electroporation were suggested [34, 62, 63, 68]. While some of these models suggest plausible molecular rearrangements during electroporation, rigorous experimental prove is lacking.

The above considerations mainly apply to the formation and expansion of pores in lipid membranes. This does not exclude the possibility for pore formation through integral proteins or at the lipid-protein interface [44, 68] (fig. 8). How actually the pores are formed is not clear yet.

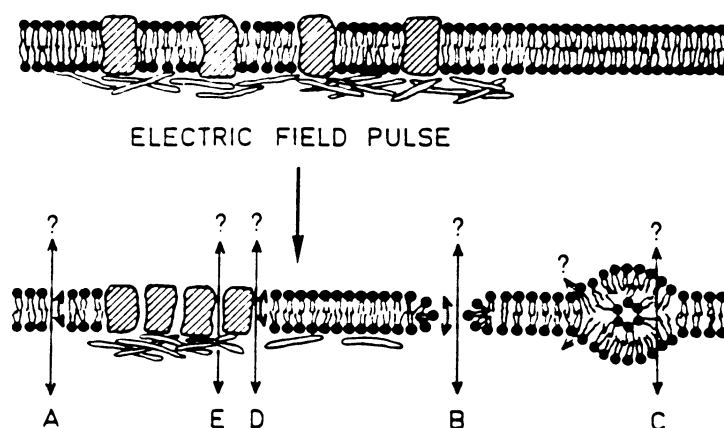


Fig. 8. Schematic view of the various types of membrane alterations and defects following electroporation. The scheme illustrates the concept that the field pulse might perturb integral and/or peripheral (membrane skeletal) proteins and thereby affect the organization of the lipid domains. Leaks ( $\updownarrow$ ) and flip sites ( $\circ$ ) could be located in (A) hydrophobic pores, (B) hydrophilic pores, (C) nonlamellar phases, or (D) areas of a mismatch between lipid and integral proteins. Leaks could also be formed between aggregated proteins (E). From [44].

#### **4. Mutual attraction and adhesion of the cells in AC fields is due to dielectrophoresis**

##### *4.1. Formation of 'pearl chains' of cells in AC fields is due to dielectrophoresis*

Nearly one century ago, Kerr observed electric field-mediated formation of 'pearl chains' linking many suspended particles (mentioned by O'Konski [69]). Liebensy observed the same phenomenon with erythrocytes [70]. Figure 9 shows an example of aligned cells in electric fields. Pearl chain formation can be considered as a special case of the movement of particles in non-homogeneous fields because one particle distorts the field acting on the other and vice versa. Movement of cells will take place if the gradient of the square of the electric field strength does not vanish (fig. 10) and if the force is strong enough to overcome the thermal motion. The motion of particles in non-homogeneous fields was termed dielectrophoresis [24]. Krasny-Ergen [71] applied the principal of minimal potential energy to study theoretically the pearl chain formation. Later this effect was investigated thoroughly by Schwan and coworkers [72, 73] and Pohl [24].

Fig. 9. Formation of pearl chains by dielectrophoresis of mouse leukemic lymphoblasts as observed by phase contrast. The applied AC field was of 0.8 kV/cm strength and 100 kHz frequency. From [117].

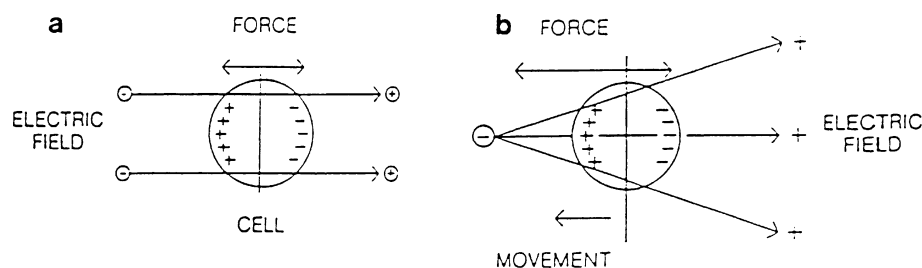


Fig. 10. Charge separation and force on a cell in (a) a homogeneous and (b) an inhomogeneous electric field. From [159].

#### 4.2. The dielectrophoretic force exerted on individual cells can be accurately measured

The dielectrophoretic force  $F$  acting on a spherical particle of radius  $R$  in an inhomogeneous field of strength  $E$  can be represented as

$$\vec{F} = 2\pi R^3 \epsilon_0 K_e \nabla E^2 \quad (4)$$

where  $\epsilon_0$  is the permittivity of free space and  $K_e$  is an effective net polarizability of the cell.

The cell polarizability  $K_e$  was measured for a wide variety of cells by using several different approaches [24, 74–77]. The dynamic method for measuring  $K_e$  is based on measuring the rate of motion of single cells in a cylindrically symmetrical system and calculating the force  $F$  by using the Stokes formula [74, 78]. The basic advantage of this method is that it allows accurate measurement of  $K_e$  for individual cells. It was found that  $K_e$  depends on the solution conductivity [24, 79], the frequency of the electric field [24, 77, 80], calcium ions [79], the type of cells [24, 76, 77] and surface charge [81]. A typical value for pea protoplasts (radius 15  $\mu\text{m}$ ) at solution conductivity 0.47 mS/m and frequency 1 MHz is  $K_e = 110$ . One of the basic conclusions is that in the radio frequency range most of the living cells behave as highly conductive spheres and the dielectrophoretic force can be estimated by assuming that the cell polarizability  $K_e$  is of the order of the relative permittivity of the surrounding medium (for water solutions of the order of 10 to 100).

#### 4.3. The intercellular attraction force increases with decreasing the intermembrane separation and is proportional to the square of the field intensity

Similar considerations are valid for the interaction of two cells in AC fields. The interaction force in the radio frequency range can be estimated by assuming that the cells behave as highly conductive spheres. The calculations are, however, very complicated and can be evaluated only numerically [82]. Their evaluation showed very good agreement with the experimental data [82] (fig. 11). The data for the force of attraction in the low frequency (60 Hz) [83] and the radio-frequency (0.1–10 MHz)

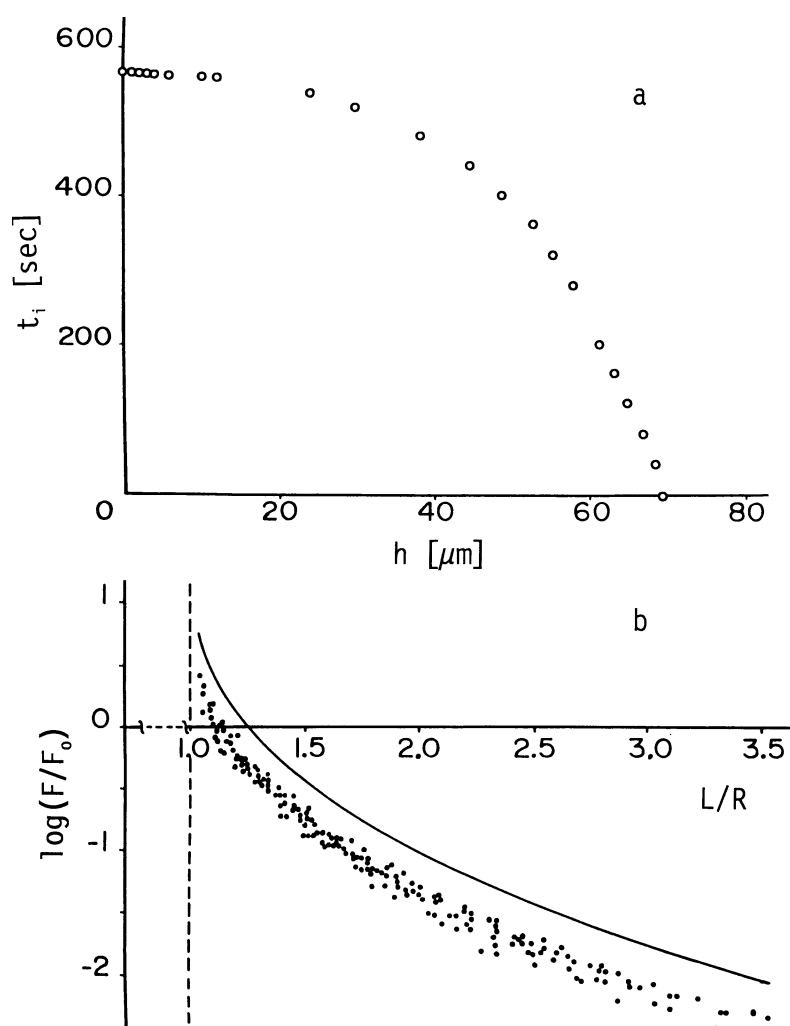


Fig. 11. (a) Dependence of the intermembrane separation  $h$  on time  $t_i = t_c - t$  during mutual dielectrophoresis of cells,  $t_c$  is the time required to make contact ( $t = t_c$  at  $h = 0$ ); (b) Force  $F$  of mutual attraction as a function of the dimensionless separation  $L/R = 1 + h/2R$  calculated from the experimental data in the range of 1 to 10 MHz (points) and theoretical prediction for highly conductive spheres (continuous line).  $F_0 = \epsilon_0 \epsilon_r E^2 R^2$ ,  $\epsilon_r$  is the relative permittivity. From [82].

range [82] was obtained from the measured rates of approach of the cells. It was found that i) the force of mutual attraction increases strongly with decreasing the separation distance, ii) the membranes deform at close apposition to form almost plane-parallel surfaces, and iii) the force of attraction is proportional to the square of the electric field strength (fig. 12).

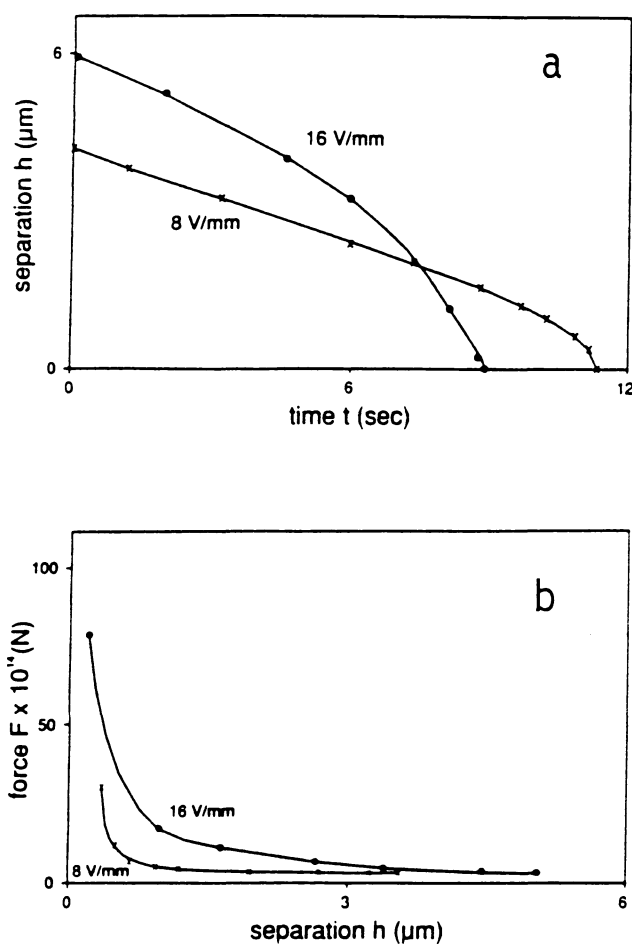


Fig. 12. (a) Dependence of separation  $h$  on time  $t$  for erythrocyte ghosts in 60 Hz AC fields for two different field strengths, 8 V/mm and 16 V/mm; (b) Calculated force of attraction  $F$  as function of separation. From [83].

#### 4.4. The attraction of cells induced by AC fields can affect fusion efficiency

The equilibrium separation between the membranes is determined by the balance of the dielectrophoretic force and the repulsive intermembrane forces (hydration, electrostatic, steric). Therefore any increase in the AC field strength leads to a decrease in the intermembrane separation. Another important parameter is the contact area between the membranes. Any increase in the contact area leads to a higher probability of fusion. The increase in the force of attraction induced by the external AC field results in an increase of the contact area and the membrane tension. Therefore, increasing the intensity of the AC fields can enhance fusion by increasing the probability for fusion. Very high AC field strengths, however, may result in

heat generation and turbulent flows, which decrease the fusion efficiency. Hence, an optimal AC field strength exists at which the fusion efficiency is maximal.

After removing the AC field, the cells commonly separate, driven by Brownian motion. This indicates that AC fields lead to reversible cell aggregation and are not sufficient to overcome the energy barriers preventing adhesion and fusion of membranes. In several instances, however, it was observed that AC fields can induce permanent adhesion of red blood cells [74, 78, 84]. This was originally attributed to fusion, but later was found to be tight adhesion or in the best case probably semi-fusion. Therefore, electric fields of higher intensity should be applied to induce real fusion. They should, however, be applied for short periods of time to avoid heat generation and turbulent flows, which can disrupt the cell aggregates. How membranes can be fused by such high voltage pulses is discussed next.

## 5. Electroporative pulses induce fusion of adhered membranes

### 5.1. *Electrofusion is induced at the same threshold voltages as electroporation*

In 1979 Senda et al. published a paper describing an interesting observation [15]. When an electric field was applied by microelectrodes to two plant protoplasts, brought at close contact by using a micromanipulator, the cells underwent morphological changes until they formed a single fusion product as observed by light microscopy. At about the same time three other research groups fused cells by high voltage electric pulses but using different approaches to bring the cells at close contact. The Berg's group used polyethylene glycol (PEG) to aggregate cells and then stimulated yeast protoplast fusion by electric field [17]. The evidence for fusion was genetic because the pulses led to formation of viable hybrids which grew on minimal media to form prototrophic colonies. Neumann et al. [16] achieved cell agglutination by rolling the cell suspension in plastic tubes, while Zimmermann and Scheurich [18] used AC fields to bring plant protoplasts at close approach by dielectrophoresis. Figure 13 shows an example of electrofusion as observed by a fluorescent dye redistribution assay [85].

The common and striking feature of these observations of electrofusion was that the magnitude of the transmembrane voltage needed for fusion was about the same (of the order of 1 V) as that for electroporation, while electrofusion was insensitive to the way the cells were brought into contact. Later it was demonstrated that not only the absolute value of the transmembrane voltage correlates with that needed for electroporation, but also the entire functional dependence of the pulse voltage on its duration is the same (fig. 14) [40]. These observations led to conclusions important both for the design of protocols for efficient fusion and for understanding its mechanisms.

### 5.2. *Devices for electrofusion are very similar to those for electroporation*

Since electrofusion is so similar to electroporation with respect to the characteristics of the electric pulses, the devices used for electrofusion are very similar to those

Fig. 13. Electrofusion of erythrocyte ghosts as demonstrated by a fluorescence dye redistribution assay [85]. One cell, originally labeled with DiI, is surrounded by two originally unlabeled cells which are not visible under fluorescence. The labeled cell does not show any indication of dye transfer before the pulse (79 : 24 : 74). The high-voltage electric pulse (strength 0.3 kV/mm, duration 0.9 ms) is applied at 79 : 25 : 44. The first appearance of fluorescence as small 'horns' on the originally unlabeled cells can be seen about 4 sec later (at 79 : 29 : 04). The horns increase in length (79 : 32 : 89) until reaching the end of the originally unlabeled membranes (79 : 40 : 56) and the state of uniform fluorescence brightness at 80 : 05 : 15. The width of the alphanumeric is 50  $\mu\text{m}$ . From [85].

used for electroporation. The main difference is in the way the cells are brought at close contact. A wide variety of approaches has been used to induce cell contact, including mechanical by utilizing micromanipulators and centrifugation; chemical, by utilizing PEG and other agglutinating substances, and electric, by using AC fields. The cells can also make contact spontaneously or the contact can be achieved by increasing the cell concentration. One of the most used and convenient way to induce cell adhesion is by dielectrophoresis [24, 86]. In the rest of this chapter I discuss

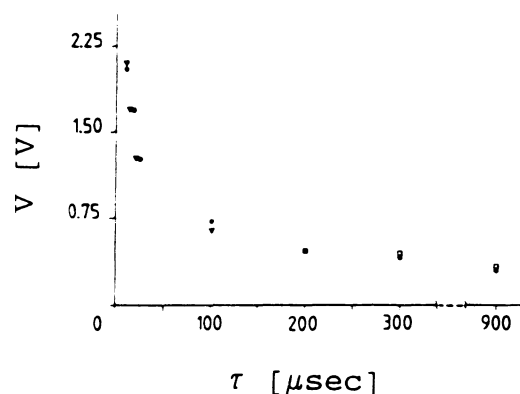


Fig. 14. Correlation between the threshold transmembrane voltage  $V$  of electrofusion (triangles), of electroporation (circles) and of cell destruction (squares) as function of the pulse duration  $\tau$ . The square of the threshold voltage is inversely proportional to the pulse duration in agreement with the fluctuation wave mechanism of electroporation. From [40].

predominantly electrofusion of cells brought at close approach by dielectrophoresis not only because it is widely used but also because it allows fine regulation of the separation between membranes.

An electrofusion device consists of a generator and a chamber. The generator typically has three main components: 1) high voltage generator for DC pulses of short duration, 2) AC generator for inducing dielectrophoresis, and 3) electronic switch which switches the DC and AC fields. The DC pulses can be either exponentially decaying, squared or radio frequency modulated. The AC field is commonly in the MHz range, but low frequency electric fields (60 Hz) were also used. The generator output is connected to the working chamber which has two electrodes, commonly made from platinum. The size and configuration of the electrodes vary widely. The simplest configuration consists of two plane-parallel electrodes embedded in the cell suspension. In this case the cell concentration should be relatively high. The separation between the cells should be of the order of their diameters or less to allow for efficient mutual dielectrophoresis to occur. For diluted cell suspensions cylindrical or spherical electrodes may be more appropriate. Those electrodes create non-homogeneous fields which move the cells leading to an increase of the cell concentration near the electrodes (if the dielectrophoresis is positive which is the common case for biological cells). Excellent reviews for different electrode configurations and generators can be found in the book of Chang et al. [23].

### 5.3. Long-lived fusogenic states exist after membrane electroporation

The classic protocol for inducing fusion is to bring the cells at close approach (e.g., by dielectrophoresis) first and then to apply the high voltage DC pulse. It was, however, occasionally observed that cells still can be fused if the pulse is applied first and then the cells are brought at contact [87, 88]. In 1986 Sowers' and Teissie's groups studied thoroughly this phenomenon and found that erythrocyte ghosts [89] and Chinese

hamster ovary cells [90] can be fused by the pulse-first protocol. The efficiency of fusion is, however, somewhat lower than that for the contact-first protocol [91] and multiple pulses must be applied [92]. These observations led to the concept of existence of long-lived fusogenic states [89] or transient permeant structures [90]. The life-time of these states is in the range of seconds to minutes and therefore pores surrounded solely by lipid molecules in liquid-crystalline state are unlikely candidates for such states because their life-time is much shorter. In cell membranes, however, proteins could be involved or lipid domains in solid state or both. In support of this hypothesis are the observations that the long-lived fusogenic state is laterally immobile within the time scale of the experiments which is of the order of minutes [90, 92]. A  $^{31}\text{NMR}$  study of the membrane phospholipid organization in Chinese hamster ovary cells following electroporation suggests that the organization of the polar heads is altered [93]. This can lead to an increase in the surface hydrophobicity, which was observed experimentally for plant protoplasts [94]. The increase in the surface hydrophobicity can result in a decrease of the repulsive hydration forces, enhanced membrane contact and fusion [95, 96].

#### 5.4. Fusion is localized and results in formation of intracellular vesicles

While the nature of the long-lived fusogenic state remains unclear, morphological and kinetic studies revealed other important characteristics of the electrofusion process. It was frequently observed that fusion of large cells was accompanied by formation of intracellular vesicles [97]. Probably intracellular vesicles are also formed during

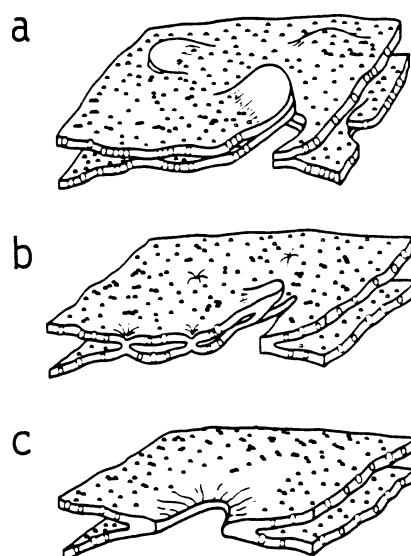


Fig. 16. An ultrastructural model of electrofusion. (a) Within 100 ms of pulse application, the aqueous layer between the membranes is perturbed and localized membrane contacts are formed. The membranes are also perturbed. (b) By about 2 sec membrane continuity is established. (c) Within 10 sec following pulse application, permanent lumina are formed. From [98].

Fig. 15. Formation of intracellular vesicles after electrofusion of erythrocyte ghosts induced with a single pulse ( $E = 0.4$  kV/cm, decay half-time = 1.2 ms) as revealed by thin section electron microscopy. Left panel: two stable diaphragm fusion zones shared between three erythrocyte ghosts in a pearl chain (oriented left-right); Middle and right panels: two other representative examples of stable planar diaphragm fusion zones indicating periodicity and heterogeneity in fusion pore diameters. From [100].

fusion of smaller cells but the limit of resolution of the light microscopy does not allow to identify them when their diameters is smaller than  $0.2\text{--}1\ \mu\text{m}$ . Electron microscopy studies provided further evidence that fusion is initiated locally [98, 99] and involves small areas (probably nm or tens of nms). The initial fusion sites then can expand resulting in formation of intracellular vesicles or other structures with

a characteristic periodic pattern [100] (fig. 15). This led to the proposition of an ultrastructural model of electrofusion by Stenger and Hui [98] (fig. 16). According to this model, the membranes are at close apposition (about 15 nm) before the pulse application. The DC pulse leads to their local destabilization. The destabilized areas make local contact and fuse. It is interesting to note that periodic structures leading to local contacts were observed when erythrocytes were adhered by polyionic macromolecules [101, 102]. Therefore one might speculate that local contacts are due not only to the existence of local perturbation (destabilization) of the membranes but they can arise during the subsequent approach of the membranes. A mechanism based on the instability and growth of membrane undulations, represented as a superposition of waves, was suggested to explain these and other observations [57, 103]. This mechanism is discussed in more details later.

It should also be noted that the observation of intracellular vesicles following fusion suggests an explanation how the excess membrane is removed after fusion if the total volume of the fused cells is kept constant. Probably fusion initiates at different points in the intermembrane contact area including its perimeter. The expansion of the locally fused membranes then leads to formation of vesicles inside the cell which removes the constraint imposed by the incompressibility of the membranes.

### *5.5. The cell cytoskeleton is reorganized during electrofusion*

One might think that not only the incompressibility of the membranes but also the intracellular filamentous proteins, known as cytoskeleton, can impose constraints impeding the morphological changes leading to rounding of the cells and formation of single fusion products consisting of two or more cells. By utilizing immunofluorescence microscopy it was shown that in Chinese hamster ovary cells the microfilaments were not affected by the DC pulses, but the microtubules disappeared during the first minutes after the pulse and then reformed during the subsequent incubation [104]. In another type of cells (CV-1), however, all three components of the cytoskeleton, microtubules, microfilaments and intermediate filaments underwent significant reorganization during electrofusion [105]. The microtubules played an active role in the nuclear movement during cell fusion [105] (fig. 17). Another observation supports the notion for the role of the cytoskeleton in fusion and raises the question for the limits of light microscopy in detection of fusion. I found that while at room temperature fusion of erythrocyte ghosts, as indicated by a fluorescent dye redistribution assay, commonly does not lead to rounding of the fused cells and formation of giant cells, the exposure of the ghosts at 37°C and higher temperatures leads to formation of significant number of giant cells (unpublished). A more extensive study [100] indicated that the disruption of the spectrin network, which can be considered as a rudimentary cytoskeleton, leads to formation of giant cells. The occurrence of fusion and lack of morphological changes observable under the light microscope for erythrocyte ghosts with intact spectrin network shows that in many cases fusion may not be detected by utilizing only light microscopy. Whether cytoskeleton affects the very act of membrane fusion remains to be elucidated.

Fig. 17. Distribution of microtubules in cells at various stages of electrofusion. (a) Before electric treatment, (b) after 5 min, (c) 15 min, (d,e) 30 min, (f) 2 h, (g,h) 3 h. Scale bar = 25  $\mu\text{m}$ . From [105].

### 5.6. Assays based on fluorescence dyes allow accurate measurement of fusion kinetics

The development of fusion assays based on fluorescence dyes provided more accurate monitoring of fusion than the light microscopy and led to the possibility to measure the kinetics of membrane electrofusion in real time. There are two different approaches utilizing fluorescent dyes. The first one is based on the redistribution of the dye from a labeled membrane to an originally unlabeled membrane observed by fluorescence microscopy. The second one relies on formation of fluorescence complexes upon mixing of the fusing membranes. The dye redistribution assay was used both with membrane soluble (commonly DiI) and water soluble (commonly FITC-dextran) dyes [85, 87], while the fluorescent complex formation assay was used utilizing the water soluble terbium (Tb) ions and dipicolinic acid (DPA) which upon mixing form the fluorescent Tb-DPA complex [99, 106]. The Tb/DPA assay allows continuous monitoring of fusion in cell suspensions by a spectrofluorometer, while the dye redistribution assay monitors fusion of single cells. By combining the dye redistribution assay with a videomicroscope the spatial and temporal development of the fluorescence in the originally unlabeled cells can be precisely monitored. This allowed to find and extensively characterized the lag times (delays) following the application of the fusogenic pulse. Because the delays are an important source of information about the life-time of intermediates in fusion leading to understanding its kinetic mechanisms I next discuss delays in fusion in more details.

### 5.7. Fusion occurs after a lag time (delay) following the application of the fusogenic trigger

The delay is the time period between the triggering of fusion and the membrane coalescence [107]. From an experimental point of view the delay is the lag time between the application of the trigger of fusion and the first indication of fusion. Measuring delays requires answering several questions. When is exactly the trigger applied? What is the response time of the measuring system? What is the minimal increase in the signal which should be assumed as an indication of fusion? In electrofusion the moment of trigger application can be precisely located in time. The time resolution of videomicroscopy systems is of the order of tens of milliseconds. For assays based on fluorescent dyes redistribution the first indication of fusion is the increase in the fluorescence in the originally unlabeled membrane near the membrane contact due to the diffusion of the dye. This time is also of the order of tens of milliseconds. Therefore, delays in electrofusion can be measured by assays, based on fluorescent dyes redistribution monitored by videomicroscopy, with an accuracy of the order of tens of milliseconds.

Delays in fusion vary widely for different systems. They can be less than 5 ms for the neurotransmitter release [108] or minutes and hours for cell fusion induced by the HIV-1 envelope proteins [109–112]. For planar-planar bilayer fusion [113] the lifetimes of the events leading to fusion ('waiting time' for fusion) are in the range of seconds to minutes. Even for the same system, but for different experimental conditions, the delays can vary by orders of magnitude. For instance, delays in viral fusion are in the range of milliseconds to minutes [114–116].

5.8. *Delays in electrofusion decrease with an increase in the field strength and are proportional to the solution viscosity*

A systematic study of delays in electrofusion of erythrocyte ghosts [85] showed that they decreased from 4 to 0.3 s with an increase in (i) the pulse strength from 0.25 to 1 kV/mm (fig. 18a), (ii) the pulse duration in the range 0.073–1.8 ms, and (iii) the dielectrophoretic force which brings the membranes at close apposition before triggering fusion. They increased proportionally to the increase in the solution viscosity (fig. 18b). The delays decreased 2–3 times with an increase in temperature from 21 to 37°C. The Arrhenius plot yielded straight lines. The calculated activation energy, 17 kcal/mol, does not depend on the pulse strength [85] and is similar to that found for the fusion yield of mouse lymphoma cells [117] and electroporation [40].

The data for delays in electrofusion of erythrocyte ghosts [85] can be described by an empirical formula [107]

$$\text{Delay} = C\mu \exp(E_a/R_gT), \quad (5)$$

where  $C$  is a constant which does not depend on the activation energy  $E_a$  and the solution viscosity  $\mu$ ,  $R_g$  is the gas constant and  $T$  the absolute temperature. The constant  $C$  is inversely proportional to the driving force of the fusion reaction and proportional to the system resistance to fusion. Therefore, it depends on all the factors which determine the driving force and the fusion resistance. The activation energy does not depend on the strength of the fusogen. Typical values of  $C$  are in the range 10–1000 Pa<sup>-1</sup> and of the activation energy  $E_a$  is 10–30 kcal/mol. A more refined theoretical analysis of the delays in fusion is presented elsewhere [118].

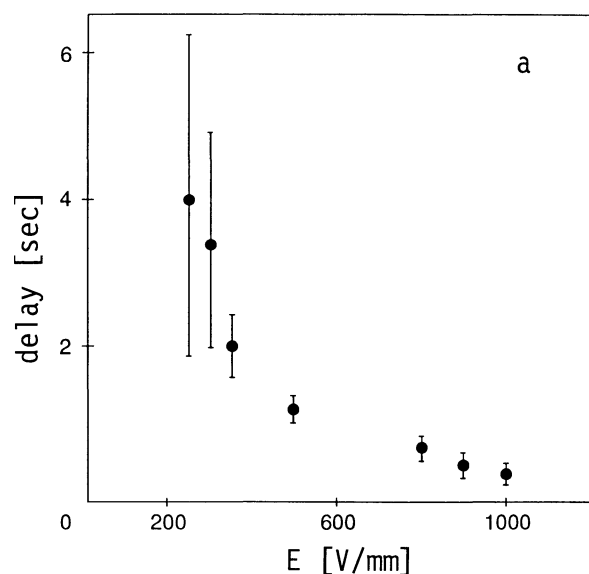


Fig. 18(a). The delay in electrofusion of erythrocyte ghosts decreases with increasing the pulse strength. From [85].

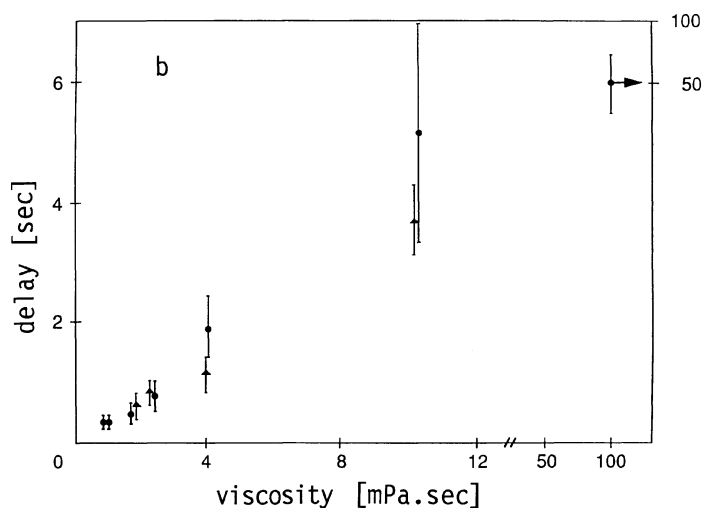


Fig. 18(b). The delay in electrofusion is proportional to the solution viscosity. From [85].

### 5.9. Rates of fusion can provide information for the time course of membrane merging and fusion pore expansion

While the delays reflect the life-time of the intermediates before the actual intermixing of the membrane components, the rates of fusion provide information for the subsequent stages of the fusion process. Rates of fusion are commonly measured by taking the derivatives of the fluorescence changes with respect to time. For individual cells they reflect the rate of diffusion of the dye through the intermembrane junction after membrane merging. There are two limiting cases: 1) the dye transfer through the intermembrane junction is fast compared to the lateral diffusion in the originally unlabeled membrane and 2) the transfer through the intermembrane junction is slower than the diffusion.

In the first case the rate of dye transfer is entirely determined by the diffusion coefficient of the dye in the originally unlabeled membrane and the membrane geometry. This is the case, e.g., shown in fig. 19, borrowed from the study of electrofusion of erythrocyte ghosts (see also fig. 13) [85]. The plot of the square of the distance between the diffusion front and the membrane contact zone versus time is linear. The slope gives  $4D$ ,  $D$  being the diffusion coefficient, which for this particular case is equal to  $0.55 \times 10^{-8} \text{ cm}^2/\text{s}$ . This is one method of measuring the lateral diffusion of fluorescent dyes. Therefore in this case the increase in fluorescence in the initial stage of the membrane transfer is not a measure of rate of fusion. It only shows that the rate of dye transfer through intermembrane junctions is faster than the rate of diffusion. After the diffusion front reaches the far end of the cell, the fluorescence intensity continues to increase until reaching the final equilibrium state. This stage also depends on the diffusion. The case of fast dye transfer through intermembrane junctions occurs when the total length of membrane contact by junctions is larger than the contact perimeter. For example, if the intermembrane

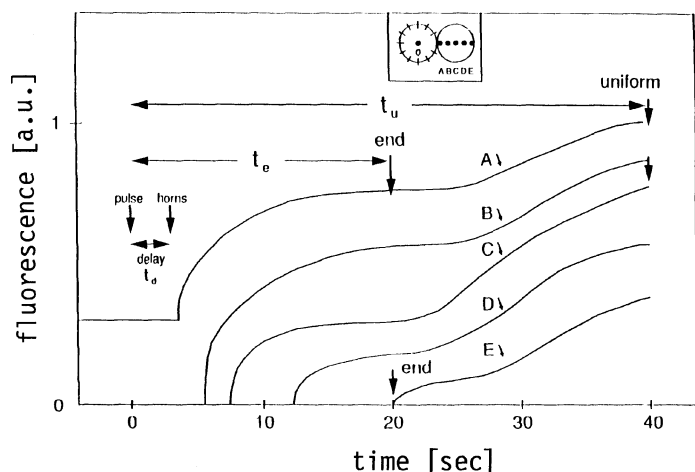


Fig. 19. An example of kinetics of dye redistribution to an originally unlabeled membrane following application of a high voltage pulse. The space locations of the points of measurement are shown in the inset. The distances from the contact with the labeled membrane are (in  $\mu\text{m}$ ) A, 0 (the contact); B, 1.7; C, 3.3 (the center of the erythrocyte ghost); D, 5; and E, 6.6 (the far end of the membrane). The fluorescence intensity is normalized to that at the center of the labeled membrane (point O in the inset). The fluorescence intensity at the membrane contact (point A in the inset) is higher than zero even before the pulse because of the close proximity of the labeled membrane. The increase in fluorescence begins after a delay at a point in time that coincides with a characteristic appearance of the fluorescence as 'horns' (see fig. 13). The dye diffuses until reaching the far ends of the membranes (after time  $t_e$ ) and an almost uniform redistribution (after time  $t_u$ ). From [85].

junctions are channels, fast dye transfer occurs when the product of the number of channels and their average perimeter is larger than the perimeter of the membrane contact.

When the rate of dye transfer through the fusion junction is lower than the diffusion rate, the increase of fluorescence is uniform. It depends only on the time and the properties of the fusion junctions. The rate of increase reflects the number and size of the fusion junctions and their change with time. When the number and size of the fusion junctions are constant, the fluorescence increase should be linear with time in the beginning of the dye transfer. Any differences from the linear relationship could be attributed to changes of the size and number of the fusion junctions, including creation of new or closing of 'old' junctions. Therefore, fluorescence changes after delays may contain important information about intermediates in fusion and their evolution with time. Unfortunately, this important process has not been quantitated yet.

For a population of cells, the rate of fusion can be defined in at least two ways. Either it is the number of cells fused per unit time or it is the total fluorescence change per unit time. In the first case fusion rates can be measured simply by counting the number of cells fused as a function of time. Evidently, in this case the rate of fusion is given by the individual differences in the lag times (delays). It will be equal to the number of fused cells which have delays within a time interval divided by that time interval.

The spectrofluorimetric assays measure rates of fusion by monitoring the fluorescence increase over time [106]. In this case, in addition to the contribution of variations in delays, there will be also an effect of the rate of dye transfer to the unlabeled cells. For instance, the water soluble dye can be transferred through pores by electroosmosis within ms [50]. Therefore, when using contents mixing assays the measured rates of fluorescence increase can be higher than the actual rates of fusion. This can be the reason for the high rates and lack of delays in the kinetic curves obtained by using water soluble dyes [99, 106].

*5.10. Fusion yields and delays are related but may reflect different properties of the fusing membranes*

Fusion yields are commonly defined as the maximal number of fused labeled cells normalized to the total number of labeled cells [87]. At that time the fusion rate is zero. Sometimes, for convenience fusion yield can be considered as a function of time. Then it reflects the number of cells so far fused until a given time. The rate of fusion at that time is the derivative of the fusion yield with respect to time. Further I discuss only the maximal fusion yield.

For a population of cells, the change of the parameters of the system, which lead to decrease of the delay, cause commonly an increase in fusion yields. For example, in electrofusion of erythrocyte ghosts, the increase in pulse strength and duration, decreases the delay and increases the fusion yield [85]. This means that under stronger pulses more cells acquire the property to fuse. Those which would have fused anyway do it faster. Several experimental results, however, indicate that while fusion yields and delays are related they may reflect different physical parameters [85]:

- 1) Under strong pulses practically all cells fuse, i.e. the fusion yield is near 100% and further increase in the pulse strength does not lead to an increase in the fusion yield. The delay can be increased, however, several fold with a further increase of the pulse strength.
- 2) For weak pulses, the delay does not change significantly. The fusion yield, however, increases several fold with the increase of the pulse strength.
- 3) The fusion yield changes significantly, while the delay does not, in buffers of different ionic strength.

With respect to the effect of ionic strength on fusion yield, there are inconsistencies between several reports. Fibroblasts fused with higher yield in solutions of higher ionic strength [119], erythrocyte ghosts show a maximum at 20–30 mM phosphate buffer saline, while chinese hamster ovary cells showed lower fusion yields in high ionic strength solutions [120, 121]. Similar inconsistencies exist for the effect of calcium ions. Mouse leukemic lymphoblasts (L5178Y) required  $\text{Ca}^{2+}$  for their fusion [117], while chinese hamster ovary cells electrofusion was reduced by increasing calcium ion concentration in the range from 0 to 1 mM [120]. The important role of calcium ions in biological systems, for fusion and the phase state of membranes has been known for years, but what is the molecular mechanism of its action on fusion is still unknown [122]. More refined studies of the effect of  $\text{Ca}^{2+}$  on electrofusion may provide critical information needed for understanding its role in fusion.

## 6. Kinetic pathways of membrane fusion resemble coalescence in colloid systems

In what follows I present a current understanding of the kinetic mechanisms of electrofusion. I begin with a brief description of very simple systems – fusion of monolayers; then consider fusion of lipid bilayers, and finally I present a plausible qualitative description how cell membranes may fuse.

### 6.1. 'Fusion' of lipid monolayers on liquid surfaces is by diffusion or intermixing driven by surface pressure gradients

Bubbles (or drops) in foams (or emulsions) are inherently unstable and tend to coalesce ('fuse'). The driving force for the fusion reaction is the higher free energy of the individual bubbles than the fused bubbles. Assuming that the final volume of two fused bubbles of equal radii,  $R$ , and surface tensions,  $\sigma$ , is equal to the sum of the volumes of the individual bubbles, the surface free energy is  $(2 - 2^{2/3})4\pi\sigma R^2$  times larger than that after fusion. The large excess in free energy leads to rapid fusion of the bubbles. The fusion rate, however, decreases with increasing the initial separation between the bubbles, the viscosity of the liquid and the density of the monolayers from surface active substances, particularly lipids, which cover the bubble surfaces. At high monolayer densities fusion may never occur in spite of the gain in free energy. Numerous experimental and theoretical studies have shown that there are two major types of resistance to coagulation and coalescence (in particular, fusion) in colloid systems – hydrodynamic and energetic (for review see, e.g., the books of Scheludko [123], and Dukhin, Rudev and Dimitrov [124]).

The hydrodynamic resistance is due predominantly to the viscosity of the liquid between the bubble surfaces and determines the rate of the so-called 'fast coalescence'. In the case of 'pure' (without energy barriers) fast coalescence, e.g., bubbles in the absence of surfactants, the driving force of the fusion reaction  $F = -dG/dh$  ( $G$  being the free energy and  $h$  being the reaction coordinate, e.g., separation between the bubbles) is always positive. The overall rate of fusion is solely determined by the balance of the driving force and the viscous resistance of the liquid layer between the bubbles. The fusion rate decreases with an increase in viscosity and a decrease in surface tension.

The energy barriers are due to repulsive forces between the bubble surfaces and/or to structural reorganizations of the lipid molecules during the very act of fusion. They lead to the so-called 'slow coalescence'. The fusion rate in this case is proportional to the probability of overcoming the energy barriers by thermal fluctuations. An example are bubbles with surfaces covered by lipid monolayers of high density. External factors, e.g., electric fields, can be needed to decrease the energy barriers in order to induce fusion. After overcoming the energy barriers, the system can reach the final equilibrium state by a kinetic pathway determined by its hydrodynamic resistance.

To understand kinetic mechanisms of fusion one needs to know how the interplay between the fusion driving force and the system resistance determines the kinetic pathways leading to fusion. It is reasonable to assume that the system utilizes the

*fastest* pathway to reach equilibrium. Thirty years ago, Scheludko (see in [123]) suggested that the most rapid kinetic pathway leading to fast coalescence is the growth of thermal fluctuations of the shape of the interacting surfaces, represented as a superposition of surface fluctuation waves. The fluctuation wave mechanism of bubble and drop coalescence was further developed by Ivanov, Scheludko and their collaborators [125, 126] to include the viscous resistance of the liquid film as a whole and the physical properties of the surfactants. Ivanov and Dimitrov [126] found that the viscosity of the monolayer and the surface diffusion of its molecules [127] are critical for the rate of growth of the fluctuation waves and succeeded to explain why the critical separation at which the bubbles fuse is affected by the monolayer density, a phenomenon which was observed in a variety of foam and emulsion systems.

Further studies of the dynamic properties of lipid monolayers have shown that in addition to the surface diffusion, there is another process by which the lipid molecules can move very rapidly under the action of differences in surface pressures arising from differences in the monolayer densities; this is the so-called Marangoni effect (see, e.g., [128]). The Marangoni effect leads to much faster lateral motion of the lipid molecules than the surface diffusion and is strongly affected by the type of lipid molecules, their escape into the bulk of the liquid, the liquid viscosity and the presence of protein molecules [128–130]. For example, for dipalmitoyllecithin monolayers the Marangoni ‘diffusion’ coefficient was measured to be in the range of 350 to 3400 cm<sup>2</sup>/s [128], which is about 9 orders of magnitude higher than the respective surface diffusion coefficient. However, since the Marangoni effect depends on the thickness of the liquid support, for approaching bubbles at separations of about 10 nm it can result in ‘only’ 10<sup>3</sup>-fold faster rate of lipid molecules motion than the diffusion. Another interesting phenomenon is the monolayer collapse. It was shown that the kinetics of monolayer collapse can be described by a Maxwell type of viscoelastic body [131]. The escape of the lipid molecules in the third dimension under high pressures may lead to fusion with another monolayers if they are at close approach.

For very high monolayer densities, the energy barriers determine the life-time of the film separating the bubbles. The process of rupture of this film, consisting of two adhered monolayers, was elegantly described by Derjaguin and his collaborators [132–134], who calculated the energy barriers by using the concept of linear tension (edge energy). It must be emphasized that Derjaguin [134] considered bilayer membranes which are metastable because they are under tension, and that the tension is the driving force for their rupture. Therefore, liposomes may not be described by this approach, because in most cases they are not under tension. For the current concepts of thin film dynamics and instability see [135, 136].

The lessons to be learned from the studies of these ‘simple’ systems are: 1) there are two major kinetic mechanisms for the fusion process to develop – (i) growth of surface shape instabilities and (ii) overcoming energy barriers, 2) in both cases the properties of the thin liquid layer between the surfaces, and those of the monolayers are critical for the kinetics of fusion, and 3) the fusion *per se* is diffusion of the lipid molecules and Marangoni effect, and therefore is determined by the type of the lipid molecules and the interactions with their environment.

## 6.2. Fusion of bilayers requires overcoming the intramembrane attraction

While fusion of lipid monolayers is ‘simply’ intermixing of their molecules driven by concentration and surface pressure gradients, i.e. by lateral diffusion and Marangoni effect, fusion of lipid bilayers could be more complicated because of their three-dimensional structure (for a current view on the structure, dynamics and conformations of bilayers see the reviews of Sackmann [137] and Lipowsky [138]). The strong mutual attraction of the two leaflets in the bilayer leads to restrictions in the ability of the outer leaflets to intermix. Therefore formation of intermediate structures may be needed to overcome the energy barrier due to the internal bilayer interactions [139]. While this fundamental difference between monolayers and bilayers could lead to different molecular mechanisms, the major kinetic pathways of fusion of monolayers and bilayers may be similar. The liquid layer between the approaching surfaces and the surface properties of monolayers and bilayers are essentially the same. Even the Van der Waals forces and the bending rigidity, which depend on the thickness of the bilayer, may not differ significantly, provided that the physico-chemical environment is the same. These similarities indicate that the kinetics of the intermembrane interactions leading to fusion might be qualitatively the same as for monolayers covering bubble (or drop) surfaces. The fusion *per se* ultimately requires intermixing of the lipid molecules and therefore should involve ‘monolayer type’ of lateral diffusion and Marangoni effect. Based on these arguments and the similarity of the phenomenological equations which describe the mechanics of monolayers and bilayers, I proposed that fusion of bilayer membranes may follow similar kinetic pathways as fusion of monolayers in colloid systems, and that the knowledge gained in colloid and surface chemistry should be used to describe some features of fusion of bilayer and cell membranes [57, 103, 140–142].

The overall fusion process could be divided into two major processes. The first one is the ‘adhesion’ step. It leads to establishment of close molecular contact. The second one is the ‘destabilization’ step. It results in overcoming the internal resistance of the bilayers to fuse due to their intramembrane interactions. While the adhesion step is commonly followed by the destabilization step, fusion could also occur if the membranes are first destabilized and then adhered, albeit at lower efficiency.

There are two major kinetic pathways which can lead to ultimate molecular contact (fig. 20) [142]. The first one is by a continuous, gradual, approach of the two membranes (fig. 20; 1). It commonly occurs when the attractive intermembrane forces are not very strong and the system reaches equilibrium without development of instabilities of the liquid layer between the membranes. This pathway can lead to formation of a single bilayer by ‘semi-fusion’ of the outer leaflets of the interacting bilayers (fig. 20; 1a) [113, 143], formation of a flat liquid layer between the two bilayers (fig. 20; 1b) or initiate an expansion of the liquid layer between the bilayers (fig. 20; 1, 2). The second major kinetic pathway is by a ‘discontinuous’ decrease of the separation between the two bilayers due to the growth of unstable fluctuations of the membrane shape (fig. 20; 2). It occurs when the driving force is large, e.g., after application of high voltage electric pulses. While the average thickness of the liquid

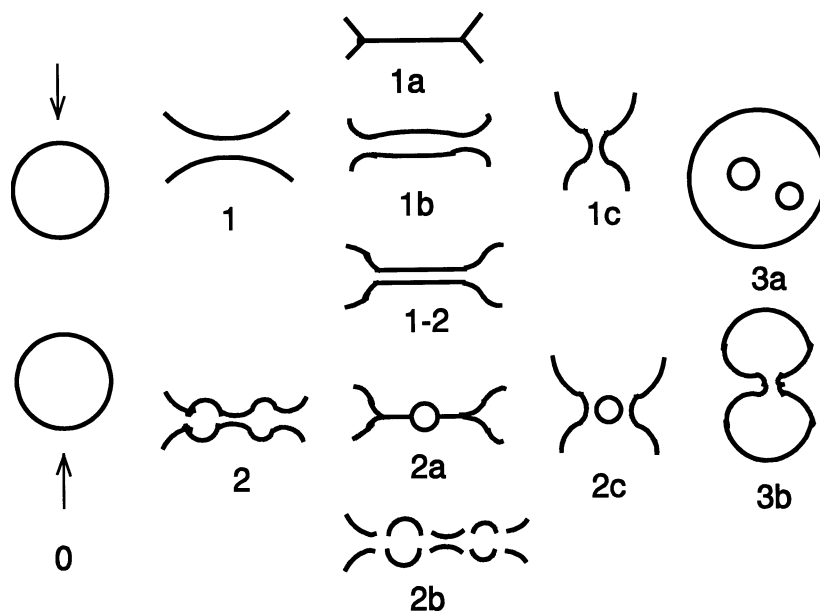


Fig. 20. A sketch of kinetic pathways of electrofusion of membranes. (0) Two membranes approach each other under the action of a driving force due to intermembrane attraction or external fields, e.g., electric. (1) The membranes approach each other by gradually changing their shapes to almost flat membranes which surround a liquid layer of almost uniform thickness. (2) Fast localized membrane approach due to unstable shape undulations. (1a) A single bilayer membrane formed by 'semi-fusion' of two membranes which can expand to reach an equilibrium state. (1b) Non-uniform 'non-wavy' liquid layer between approaching membranes. (1-2) A liquid layer between membranes which has a uniform thickness. (2a) Localized 'semi-fusion' of membranes and formation of 'lenses'. (2b) Destabilization of the membranes and formation of pores. (1c, 2c) Fusion of membranes and formation of intracellular vesicles or other structures. (3a,b) Post-membrane fusion phenomena leading to final fusion products. From [142].

layer between the membranes does not significantly change with time, the growth of the membrane shape instabilities results in the establishment of local contacts where the separation between the membranes is very small (fig. 20; 2a, 2b). These local contacts can either expand to reach a final equilibrium state (fig. 20; 1a; 1, 2) or they can lead to destabilization of the membranes (fig. 20; 2b).

The membrane destabilization can be induced by the molecular interactions at the local contact, especially when the two membranes are under tension, or by external forces arising, e.g., from electric fields. In the first case the merging of the membranes obviously follows their destabilization or occurs simultaneously. When external electric pulses are applied and the membranes are not at molecular contact, the membranes could be destabilized (fig. 20; 2b), but fuse only after making contact. Depending on the strength and duration of the electric pulse and the separation between the membranes, these membranes could fuse during the pulse or after the pulse. In any case fusion of the membranes could result in formation of small vesicles (fig. 20; 2c) or other membrane formations (fig. 20; 1c). The final stage of

the fusion process is the expansion of the fusion pore (fig. 20; 1c; 2c), commonly driven by the membrane tension, until reaching the final equilibrium shape of the fused membranes. The fused membranes can surround a rounded cell (fig. 20; 3a) or the fusion product can preserve the overall morphology of the cells before fusion (fig. 20; 3b).

Theoretical formulas were derived to describe the kinetics of each of these kinetic pathways [57, 141]. Without going into mathematical details, I will discuss several estimates which allow to predict the conditions under which each of the stages could occur and estimate its rate. The time to reach contact by a continuous approach of the membranes (fig. 20; 1) increases with increasing the liquid viscosity and the total area of contact, and with decreasing the driving force and the separation between the membranes. The same is valid for the discontinuous membrane approach (fig. 20; 2), except that the contact area in this case should be substituted by the area of the fastest growing undulation (wave), which is of the order of the square of its characteristic length (wave length). Because of the dependence of that area on the intermembrane forces and other physical parameters and the non-linear character of the functional relationships between those parameters, the characteristic time of the wave growth could be much shorter than the rate of approach of the membranes as a whole. An estimate of the ratio of the two characteristic times (of continuous and discontinuous membrane approach) leads to the formula  $F/h\Sigma$ , where  $F$  is the force which drives the membrane approach,  $h$  the intermembrane separation and  $\Sigma$  the membrane tension. For  $F = 10^{-6}$  dyn,  $h = 10$  nm and  $\Sigma = 1$  mdyn/cm, this ratio is of the order of 1000. This means that under those conditions it is 1000-fold faster to establish local contact by unstable fluctuational waves than to gradually approach the membranes. Therefore, in this case the kinetic pathway is by discontinuous approach (fig. 20; 2). For liquid viscosity equal to 0.01 P the time of this type of approach for the above example is 10 s. Similar considerations apply for tension free membranes where the time of localized approach is determined by the bending rigidity. In this case ( $\Sigma = 0$ ) another formula is valid where  $\Sigma$  should be replaced by a dimensional factor proportional to the bending rigidity  $K$ .

The local membrane contact could expand by a rate which is determined by the liquid viscosity, membrane tension, contact angle and the thickness of the liquid layer between the membranes [140, 141, 144]. This rate can be very low which could lead to very long times of establishment of uniform liquid layer between the adhering membranes. Similar theoretical considerations and formulas apply for expansion of membranes consisting of one bilayer surrounded by two monolayers of bilayers (fig. 20; 1a, 2a) [140]. The rate of expansion in all these cases,  $U$ , can be estimated by the formula:  $U = h\Sigma/4\mu l$ , where  $h$  is the thickness of the mobile layer between the two monolayers (or bilayers), e.g., the solvent in the case of supported bilayer lipid membranes,  $\mu$  is its viscosity,  $\Sigma$  is the tension of the two interacting monolayers (or bilayers), which drives the expansion, and  $l$  is the length of the perimeter of contacting monolayers (or bilayers) along which the tension is applied. This formula predicts times of expansion of the order of seconds for supported bilayer lipid membranes. More sophisticated formulas are presented in

[140, 144]. For solvent free membranes, where  $h$  is zero or very small, expansion may not occur or may be very slow.

The destabilization of the membranes may occur either by the fluctuation wave mechanism or by overcoming energy barriers or by both mechanisms as discussed before (sections 3.8 and 3.9). It leads to formation of pores, defects or other structures. The shape of the lipid molecules can affect the stability of those structures [113].

The interaction of two pores or a pore and a membrane may lead to intermixing of the lipid molecules and to their fusion. The interaction between the two membranes can contribute to their destabilization due to an increase in the intramembrane electric field strength. External electric fields can destabilize the membrane-membrane contact much faster than the liquid layer between the membranes because of the greater transmembrane voltage across the membranes [58]. Therefore one might expect that, depending on the electric field strength, the adhered membranes can be either first destabilized and then they interact to fuse, or they may form a close (molecular contact) and then fuse. Probably in most cases the membranes are first destabilized and then they fuse. This is supported by theoretical considerations indicating that after formation of pores the rate of local membrane approach may increase significantly due to their permeabilization [145] and the increase in the force of local attraction due to electric currents [146]. Direct experimental evidence supporting this conclusion is, however, lacking.

The intermixing of the membranes, i.e. the very act of fusion, is very fast. An estimate, based on the lateral diffusion of lipids leads to times  $\tau_f = l_D^2/4D$  of the order of microseconds if the characteristic distance  $l_D$  is about 5 nm and the diffusion coefficient  $D$  is of the order of  $10^{-8}$  cm<sup>2</sup>/s. Surface pressure differences between the two bilayers (Marangoni effect) can lead to even shorter times of intermixing.

The intermixing of lipids from the two interacting membranes leads to formation of fusion junctions, which can transform into fusion pores. The fusion pores can either expand (fig. 20; 2c), stabilize (fig. 20; 3b) or in rare cases close. For liquid crystalline membranes under tension the expansion of the fusion pores can be relatively fast process with a rate proportional to the membrane tension  $\Sigma$  and inversely proportional to the liquid viscosity  $\mu$  and the radius of curvature of the membranes  $R_c$  [142]. The rate of expansion could be also affected by the membrane viscosity. Numerical estimates of the characteristic time of expansion  $\tau_e$ , based on the formula  $\tau_e = \mu R_c / \Sigma$  [142], give a reasonable value of 10 ms when  $\mu = 0.01$  P,  $\Sigma = 0.01$  dyn/cm and  $R_c = 0.1$  mm. When the membranes are not under tension, the driving force for the fusion pore expansion is zero or very small. The time of expansion therefore could be very long. High membrane viscosities may also increase the expansion times. Under conditions favoring very long times of expansion, the fusion pores may stabilize or even close thus returning the system back to the initial stage of two separate membranes.

The duration of the fusion process as a whole is a sum of the durations of each stage. While the times for membrane destabilization and intermixing are very short, the initial (adhesion) and latest (expansion) stages can be very long and therefore rate determining. This indicates one of the fundamental problems for studying mechanisms of fusion. Fusion itself is very rapid and localized while the 'background' processes (adhesion and expansion) are slow and involve large parts of the membranes.

### 6.3. *The molecular mechanism of bilayer fusion is unknown*

Because of the very small amount of membrane lipid involved in fusion pores and their very short life-times direct measurement of the intermediate structures in fusion of lipid bilayers is very difficult if not impossible. Some progress was made by using NMR for fusion of membranes in lipid suspensions [147–149]. Whether this is relevant to fusion of lipid bilayers remains to be seen. The lack of direct experimental measurements of lipid bilayer fusion hinders the understanding of the molecular mechanisms of fusion. In contrast, there are several theoretical models which try to calculate intermediate structures requiring minimal activation energy for their formation [139, 150, 151]. These models are helpful in the design of new experiments for better understanding of the molecular rearrangements during fusion.

### 6.4. *Fusion of cell membranes can be qualitatively described but the molecular rearrangements remain unclear*

Unlike lipid bilayers, cell membranes are highly heterogeneous, contain significant amounts of proteins, glycoproteins and glycolipids, are surrounded by surface layers of adsorbed molecules, and are attached to the cytoskeleton (for a current view on the structure and dynamics of cell membranes and nice cartoons, see the review of Sackmann [137]). The high level of complexity of cell membranes leads to difficulties in modeling fusion. Nevertheless, phenomenological models could be still helpful for understanding certain aspects of electrofusion.

The following picture for the kinetic pathways of cell electrofusion emerges from the comparison of the experimental data with the theoretical models. The cells, held at close apposition by dielectrophoresis or other interactions, are unable to fuse because of the inherent stability of the membranes and the repulsive forces between them. The application of high voltage pulses leads to at least two effects: 1) further approach of the membranes [152, 153] and 2) their destabilization. The membrane destabilization and formation of pores or other structures enhances the local approach of the membranes by (i) providing the driving force due to the high free energy state, which can reduce or eliminate the repulsive intermembrane forces and (ii) decreasing the local hydrodynamic resistance which increases the rate of local approach. The excess free energy of the pores (or defects, or unknown structures) can be either dissipated as thermal motion without formation of fusion junction or can be utilized in the rearrangements of the lipid molecules leading to fusion. Efficient fusion occurs when the membranes are at close approach or brought rapidly at close approach after the pulse before the free energy of the (activated) ‘fusogenic state’ has been dissipated. On the one hand, membrane proteins can enhance the fusion reaction if they stabilize the state of high free energy by not allowing rapid dissipation of energy, increase membrane binding and/or provide an appropriate environment for the structural rearrangements of the lipid molecules. On the other hand, they can decrease the efficiency of the fusion reaction by creating additional (mainly steric) resistance to local membrane approach. Which effect will dominate depends on the specific lipid and protein composition and on the phase state of the membranes.

The high free energy of the activated membranes is probably localized in electropores. Two pores apposing each other are the most efficient kinetic pathway of

fusion because the driving force is high and the hydrodynamic resistance is small. Such pores are also predominantly formed when the membranes are at close apposition. This does not exclude the possibility, however, that pores in one of the membranes can destabilize locally the opposing membrane albeit at low efficiency. This is probably the case when the two membranes are brought in contact after the pulse, because the geometric probability for two pores being apposing each other is very low due to the low pore concentration in the membranes. This may be one of the causes for the low efficiency of fusion in this case.

Because of the high free energy of the membranes after the pulse the liquid film between them can be destabilized, which can lead to a rapid localized approach until reaching molecular contact. Thus the molecular contact may occur during the pulse if the duration of the pulse is longer than the time needed to make contact. If the approach is slow, the membrane contact may occur after the pulse. The occurrence of molecular contacts does not necessarily lead to merging of the two membranes. Energy barriers for the molecular rearrangements of the lipid molecules from a pore (or another) configuration to a fusion junction should be overcome to initiate fusion. The height of these barriers is of the same magnitude as that for lateral mobility of lipid molecules and therefore one might speculate that the lipid rearrangements kinetically resemble the lateral lipid diffusion in membranes. Energy barriers may also exist before the occurrence of molecular contact if the free energy of the pores is not sufficient to overcome the repulsive intermembrane forces.

Fusion of membranes occurs after overcoming those energy barriers and leads to local continuity of the membranes, i.e. to fusion junctions. The formation of fusion junctions may lead or may not lead to continuity of the cell cytoplasm, i.e. to the formation of a fusion pore. Depending on the structure of the fusion intermediates, fusion pores may form simultaneously with formation of fusion junctions, may form later or may never form. Since for cell membranes the molecular structure of the intermediates is not known and it is difficult, if not impossible, to describe this structure theoretically, one might only speculate how and when formation of fusion pores occurs.

The fusion pores can stabilize, reverse to the initial prefusion state ('flickering') or expand. The expansion of fusion pores may lead to 'fusion' with other pores, rounding of the fused cells and eventually formation of giant cells (syncytia), when many cells fuse. Alternatively, the cells may retain their shape if the fusion pores do not expand, because of constraints arising, e.g., from the cytoskeleton or other factors which remain to be elucidated.

## **7. Fusion in life processes involves specialized proteins but kinetically can be similar to electrofusion**

Electrofusion provides a convenient way to study fundamental mechanisms of membrane fusion, which may help to understand the fusion mechanisms in biological systems. While the major requirements for electrofusion and biological fusion (close contact, destabilization, overcoming energy barriers and appropriate structural rearrangements) are the same, fusion in biological systems is tightly controlled by specific

protein interactions and full understanding of its mechanisms requires understanding the structure and conformational changes of the fusion proteins.

The biological fusion process which is best understood in terms of molecular structure and conformational changes of fusion proteins is viral fusion [154, 155]. Viral fusion is caused by proteins which undergo conformational changes to expose hydrophobic peptides after activation by low pH or interaction with receptor molecules. The exposure of the hydrophobic fusion peptide to the water environment results in an increase in the free energy of the peptide and its immediate environment possibly including the surfaces of the interacting membranes. This leads to a high free energy state which could be similar to that of a fusogenic electropore in electrofusion. Therefore, the kinetic pathways of viral fusion after activation of the viral proteins could be similar to those of electrofusion. The existence of a high energy state of the viral fusion protein leads to local mutual approach of the lipid bilayers and destabilization of the membranes and of the liquid layer between them. The lipid matrix of the membrane then undergoes localized structural rearrangements resulting in lipid intermixing. An indication that this stage of viral fusion is also similar to that in electrofusion is the magnitude of the activation energy which in both cases is of the order of the activation energy of the lateral mobility of the lipid molecules (15–25 kcal/mol) [85, 156]. After the formation of fusion junctions and pores, they can either expand and lead to formation of giant cells or stabilize and preserve the morphology of aggregated cells [85, 100].

The comparison between electrofusion and virus fusion may lead to the discovery of new important parameters affecting the kinetics of viral fusion. For example, by analogy with electrofusion one might predict that delays in viral fusion should increase with increasing the viscosity of the liquid between the membranes. This was confirmed experimentally for fusion of Sendai virus [118]. A detailed comparison between electrofusion and viral fusion is made elsewhere [107].

The above considerations could be applied to a wide variety of biological processes involving fusion, including exo- and endocytosis, fertilization, cell division, intracellular transport and myoblast formation.

## **8. New approaches are needed to understand molecular mechanisms of fusion**

Electrofusion is a multistep process, which requires close apposition of the membranes, their destabilization and appropriate structural rearrangements of the lipid matrix. While the kinetic mechanisms of fusion could be understood in terms of phenomenological models, the molecular mechanisms are unknown and new approaches are needed for their elucidation. Understanding mechanisms of electrofusion is not only of practical importance for its optimization but also may help in understanding fundamental mechanisms of fusion in biological systems.

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#### List of symbols

- AC alternating current
- $C$  proportionality constant in eq. (5)
- $C_m$  membrane capacitance
- $d$  membrane thickness
- $D$  diffusion coefficient
- DC direct current
- DiI 1,1'-dihexadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate
- $E$  electric field strength
- $E_a$  activation energy
- $F$  driving force
- $F_0 = \varepsilon_0 E_r E^2 R^2$
- FITC fluorescein isothiocyanate
- $G$  free energy of intermembrane interaction
- $h$  separation between surfaces including membrane surfaces
- $k$  Boltzmann's constant
- $k_R$  rate constant of pore resealing
- $K$  membrane rigidity
- $K_e$  effective net cell polarizability
- $l$  length of perimeter of contacting monolayers or bilayers
- $l_D$  characteristic diffusion length in fusion
- $L = h/2 + R$
- $r_i$  intracellular electric resistivity
- $r_0$  extracellular electric resistivity
- $R$  radius of cells or other spherical particles
- $R_c$  curvature radius of membranes
- $R_g$  gas constant
- $t$  time
- $t_c$  time required to make membrane contact
- $t_e$  time needed for a fluorescence dye to diffuse to the far ends of originally unlabeled membranes after fusion
- $t_p$  charging time
- $t_u$  time needed to reach an uniform redistribution of fluorescence dyes after membrane fusion
- $T$  absolute temperature
- $U$  rate of expansion of membrane contact
- $V$  transmembrane voltage

- $V_c$  critical transmembrane voltage needed for electroporation  
 $V_i$  inherent transmembrane voltage (at  $E = 0$ )  
 $V_m$  maximal transmembrane voltage  
 $\delta$  angle between radius vector and electric field vector  
 $\varepsilon_0$  permittivity of free space  
 $E_r$  relative permittivity  
 $\mu$  liquid viscosity  
 $\Sigma$  membrane tension  
 $\sigma$  surface tension  
 $\tau$  pulse duration  
 $\tau_e$  characteristic time of fusion pore expansion  
 $\tau_f$  characteristic fusion time

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