J. Membrane Biol. 67, 165–182 (1982)

The Journal of
Membrane Biology

Topical Review

Electric Field-Induced Cell-to-Cell Fusion

U. Zimmermann and J. Vienken

Arbeitsgruppe Membranforschung am Institut für Medizin, Kernforschungsanlage Jülich GmbH, Postfach 1913, 5170 Jülich, FRG (Germany)

Cell-to-cell fusion has great potential in membrane research, membrane reconstitution and genetic mapping [25, 34, 49]. Furthermore, both somatic hybridization and genetic engineering offer a way of modifying plant cells and, in turn, of improving crops [49, 53]. If lymphocyte cells are fused with a permanent cell line so-called hybridoma cells are produced [32] which are capable of producing monoclonal antibodies of predetermined antigenic specificity. These hybridoma cells (antibody-producing hybrids) can be envisaged as important tools in future clinical diagnosis and therapy as well as in the purification and enrichment of compounds of cell-biological and medical interest [18, 65, 72]. However, despite many promising results and considerable efforts, fusion still seems to be something of an art rather than a precise science.

Phenomenologically, cell-to-cell fusion is achieved presently *in vitro* by chemicals or inactivated virus [2, 30, 31, 33, 34, 48, 64]. Fusion can usually only be achieved by using membrane-disrupting agents and procedures and/or unphysiological conditions (e.g., high Ca^{2+} concentrations, high or low pH values, hypotonic conditions, etc.)

The field of chemically and virus-induced fusion has been excellently reviewed by several authors over the last years [17, 34, 45, 47, 48]. In view of the bewildering array of data on chemically and virus-induced fusion we are obliged to conclude that we are still far from solving the molecular mechanism underlying the fusion process.

Any progress in this field and in the development of novel fusion techniques will have to be evaluated by comparison with the inherent limitations of the current fusion procedures, which are listed below:

1. The optimum fusion conditions have to be empirically derived for each cell system; as a result, they vary considerably from species to species and are partly contradictory [47].
2. The fusion processes between any two cells of different species in a mixed population cannot be followed under the microscope, although this would be of particular interest when producing hybridoma cells.
3. The number of cells to be fused cannot be preselected.
4. The fusion process is not synchronous and very often extends over a long time period (up to 1 hr).
5. During fusion a loss of intracellular substances is generally observed which may be associated with a decrease in the viability of the hybrids.
6. Viability can also be affected by the fusogenic compounds and viruses which are present during the whole fusion process and which are thus able to interact with the total membrane surface in an uncontrolled manner.
7. The yield of hybrids is normally very low; giant cells by fusion of hundreds to thousands of cells in particular cannot be obtained by these methods. An increase in the yield of fused cells very often results in a decreased viability. Thus, it is necessary to determine empirically the right balance between fusion frequency and yield.

This list of disadvantages inherent in the current fusion techniques seems to illustrate the absolute need not only for a new approach, but also for the development of a general concept for fusion in order to relate the phenomenological observations to a few fundamental processes.

Two years ago we reported on a new fusion technique based on the exposure of cells to an alternating, nonuniform electric field of low strength followed by the application of a short electric field pulse of

Key words fusion · electric field · electrical breakdown · hybridoma cells · giant cells

0022-2631/82/0067-0165 $03.60

© 1982 Springer-Verlag New York Inc.

high intensity [81, 85, 88, 90]. Tight membrane contact between at least two cells is achieved by dielectrophoresis generated by the alternating nonuniform electric field while reversible electrical breakdown in the membrane contact zone is achieved by subsequent injection of a field pulse of high intensity. This event results in the fusion of the cells [85, 87].

This field-induced fusion technique avoids most of the disadvantages of the chemically and virus-induced fusion procedures. Current research into this topic has considerably increased the amount of available information and justified the development of a new concept of membrane fusion. It will certainly be necessary to carry out more experiments, but in the meantime an attempt will be made to evoke new ideas which may aid and spur the search for new experiments and concepts in this special area.

Dielectrophoresis

As a result of Brownian motion and the repellent electrostatic forces arising from the net negative charge on the outer membrane surface, cells in a suspension will not come into close membrane contact (with the exception of certain specific interactions in some cells, e.g. *Dictyostelium discoideum* [38]). Close membrane contact is of course one of the prerequisites for fusion. With the field-induced fusion technique close membrane contact is achieved by dielectrophoresis and mutual interaction between cells which have dipoles generated by the field [23, 35, 37, 41, 44, 62]. The term dielectrophoresis should not be confused with electrophoresis. Both terms imply the study of motion. The essential difference is that, unlike electrophoresis, dielectrophoresis is concerned with the motion of neutral particles in a nonuniform field

As illustrated in Fig. 1*a*, the neutral particle becomes polarized in the presence of an electric field, with the positive charge being on the side nearest the cathode and the negative charge of the same magnitude on the opposite side nearest the anode.

In a uniform field the field strength is equal on both sides. Thus, there is no net force to act upon the neutral particle, and motion will not occur. In a nonuniform field, on the other hand, the field strengths on both sides of the particle are unequal, giving rise to a net force which acts on the particle and results in a translational motion towards the region of highest field intensity (Fig. 1*b*) (for exceptions of negative dielectrophoresis, *see* [41, 44]).

If the particle is geometrically nonsymmetrical or composed of anisotropic material, polarization may produce a torque acting upon the particle to orientate or align it along the field lines. If the polarity of the electrode arrangement is reversed (*see* Fig. 1*c*), the neutral particle will still move towards the region of highest field intensity; this means that, in contrast to electrophoresis, dielectrophoresis also occurs in an a-c field. As in all polarization processes, the force is proportional to the square of the field strength [23, 41, 44, 54, 55, 62, 63]. Dielectrophoresis (particularly of small cells) usually requires quite divergent fields for a strong effect. While electrophoresis is observable in particles and molecules of any size (atomic size up to macroscopic bodies), dielectrophoresis gives rise to a force which is proportional to the particle volume. Dielectrophoresis is not normally observed in particles with diameters of less than 0.3 μm, because the diffusional forces exceed the dielectrophoretic force (for a rigorous treatment, *see* [41, 44].

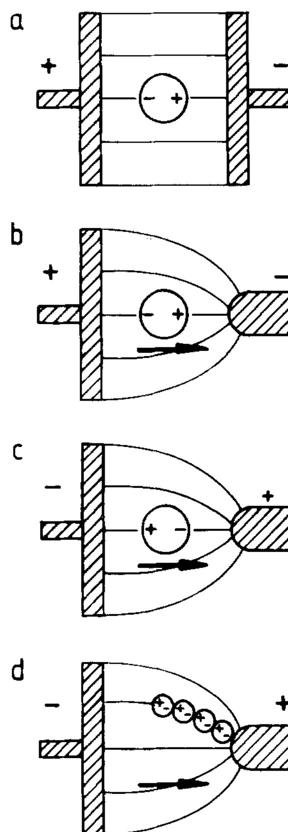

Fig. 1. (*a*) Diagrammatic representation of a neutral particle in a homogeneous electric field: A dipole is induced in the particle in response to the field. Since the electric field intensity is equal on both sides, no net force will be exerted on the particle. (*b*) In an inhomogeneous electric field neutral particles are able to migrate, because the field intensity is not equal on both sides resulting in a net force; so-called dielectrophoresis. (*c*) The direction of dielectrophoresis is independent of the polarity of the field. In an alternating field all particles will move in the direction of higher field strengths (for exceptions *see* [41, 44]). (*d*) If the particles approach during dielectrophoresis they are attracted to each other due to their dipoles, so-called mutual dielectrophoresis. This leads to the formation of "pearl chains" of cells

A further fundamental difference between electrophoresis and dielectrophoresis is that the latter phenomenon requires a substantial difference in the relative permitivities of the particle and the surrounding medium [41, 44, 61]. The dielectric properties of living cells change with increasing frequency. There are three notable changes, termed α-, β- and γ-dispersion in the frequency ranges between 1–10^8 Hz [12, 61]. Conductivities also exhibit changes corresponding to these three dispersions, with the conductivity increasing with increasing frequency. The dielectrophoretic force is thus a function of frequency. The β-dispersion which results from polarization processes in the membrane-bulk interphase (due to Maxwell-Wagner polarization) may play the most important role in dielectrophoretic collection of cells in fusion experiments. β-dispersion is observed in the frequency range between 10^4 to 10^6 Hz. For a rigorous treatment of these passive electrical properties of living cells the reader is referred to the work of Fricke, Cole and Schwan [12, 19, 61].

Mutal Dielectrophoresis

So far possible interactions between cells have been ignored. One cell approaching another polarizable cell, during its movement towards the region of highest field intensity, will encounter an enhancement of the local field divergence and will tend to move towards that cell since the field strength will be higher nearer that cell. As a result, cells in a nonuniform field form chain-like aggregates (so-called pearl chains) under point-to-point membrane contact (*see* Fig. 1*d*, 3*a*). This effect is termed mutual dielectrophoresis [41, 44, 62]. The attraction forces arising from the dipole generation within the cells overcome both the electrostatic repulsion between the apposed membrane surfaces bearing net charges and the repulsive hydration force. The latter force is much greater than electrostatic repulsion at separations less than about 2 nm [51]. The repulsive hydration force is assumed to be a consequence of the work required to remove water from hydrophilic surfaces as they approach. Schwan and his colleagues [54, 63] have developed a general theory to account for the formation of "pearl chains" of spherical and nonspherical particles in a-c fields. The theory is in reasonable agreement with the experimental results and explains the frequency-dependent alignment of ellipsoid cells with their longer axis either transverse or in parallel to the field lines [54, 55, 63].

Membrane Contact

Membrane contact between cells is achieved by dielectrophoresis and mutual interaction in a nonuniform a-c field. The inhomogeneity of the a-c field and, in turn, the force acting on the cells during dielectrophoresis depends among other parameters on the electrode arrangement used. The slight inhomogeneity arising from the electric field between two cylindrical wires arranged in parallel has been shown to be sufficiently high to safely collect cells with a diameter of more than 4 μm (Fig. 2*a*) [85, 87].

For smaller cells and isolated organelles a stronger divergence of the fields is required; this can be achieved by using, e.g., a central wire held coaxially within an outer cylindrical electrode (Fig. 2b). The latter set-up is also required for the intracellular dielectrophoretic collection of organelles, granules and other particles as well as in field-induced exocytosis ([4] and Zimmermann et al., *unpublished results*). Large amounts of fused cells can be obtained by using the electrode arrangement shown in Fig. 2*c* or by means of flow chamber systems (Fig. 2*d*, *e*).

Dielectrophoresis and pearl-chain formation usually have to be performed in virtually nonconductive solution (conductivity less than $10^{-4}\ \Omega\ cm^{-1}$), if the cells are examined and fused with the electrode arrangement described above. Otherwise, the presence of electrolytes creates problems of heating which result in turbulences and the disruption of pearl-chain formation and the fusion process.

If flow chamber systems analogous to particle analyzers (Coulter Counter) are used and equipped with an efficient cooling device, dielectrophoresis and fusion can in principle also be carried out in the presence of electrolyte ([43, 80] and Zimmermann et al., *in preparation*). Mannitol, sorbitol, glucose, sucrose or histidine are used as nonelectrolyte solutions [87]. Experiments with plant protoplasts, mammalian cells, and vesicles have shown that these solutions have no severe side-effects on membrane integrity or on the viability of the cells if the incubation time does not exceed 1 hr at room temperature. However, the whole procedure including dielectrophoresis and fusion does not normally exceed 15 min. In any case, the flow chamber systems which allow both the presence of small amounts of electrolyte (Ca^{2+}, K^+ and Na^+) during fusion and the production of fused cells on a large scale may be superior to the equipment shown in Fig. 2*a* (however, *see below*).

For cells suspended in nonelectrolyte solution the frequency range for positive dielectrophoretic collection should be higher than 10 kHz. Below 1–10 kHz, electrolysis is frequently observed (because of the low conductivity of the solution), which is associated with adverse side-effects on dielectrophoretic collection, membrane fusion and cell viability.

The optimum choice of frequency for dielectrophoretic collection in the frequency range between

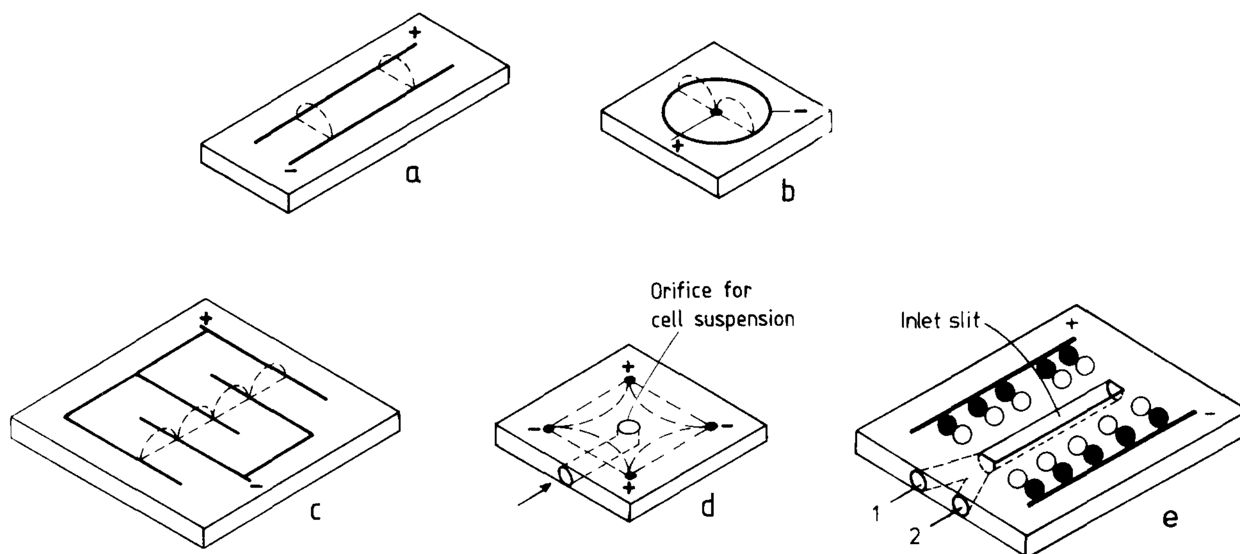

Fig. 2. Set-up, used for electrically induced fusion (field-lines of the electric field: ——). (*a*) Two cylindrical platinum wires are mounted in parallel on a slide. The gap between the two electrodes has to be matched to the size of the cells or vesicles to be fused. (*b*) Stronger divergence of the electric field can be achieved with a set-up in which a central wire is surrounded by an outer cylindrical electrode. (*c*) Electrode arrangement for the production of large amounts of fused cells. (*d*) Flow chamber system in which the cells enter centrally the gap between four electrodes, arranged crosswise. Cells move to the region of highest field intensity between the individual electrodes due to dielectrophoresis. (*e*) Flow chamber system for the production of cell hybrids of different species in high yield. Two highly diluted cell suspensions are successively sucked through the slit between the electrodes. The formation of cell pairs, consisting of species 1 (●) and species 2 (○) is favored

10 kHz and 80 MHz depends both on the frequency dependence of the dielectrophoretic force and on the rotation frequency of a given species. Crane and Pohl [15] reported that statistical rotation of a few cells in an a-c field occurs over the whole frequency range. By using careful experimental arrangements, Zimmermann et al. [89] were able to demonstrate that every species exhibits a narrow frequency range in which all cells rotate (20 to 40 kHz for plant protoplasts, 80 to 100 kHz for red blood cells, 180 to 200 kHz for yeast cells, etc). The optimum frequency range for rotation of a given species can be changed by treating the cells with enzymes or by the addition of chemicals. Preliminary experiments performed in the frequency range between 10 and 100 MHz have shown that rotation of cells also occurs at several distinct frequencies in this frequency range (Zimmermann, Hub, Pilwat). This is expected from the mechanism underlying the generation of a dipole within the cell membrane (polarization, dipole orientation of phospholipids and probably also proteins, and interaction with natural dipoles within the membrane which may be associated with carrier transport [89]).

From a macroscopic view, rotation occurs because of dipole-dipole interactions between two adjacent cells arranged at a certain angle to each other [26]. Optimum rotation occurs when the two cells are arranged at a 45° angle. Cell chains which were set up in parallel to the field lines at a certain frequency (say 2 MHz for plant protoplasts) very often topple over in the 45° position, if the frequency of the a-c field is switched to the optimum frequency (e.g. 20–40 kHz for plant protoplasts). In consequence higher field strengths are needed at these specific frequencies to orientate the cell chains in the direction of the field lines. In general, thereby the field strength at which breakdown of the cell membrane occurs is exceeded leading to irreversible destruction and bursting of the cells.

Rotation obviously disrupts the orientation parallel to the field lines and the formation of cell chains with tight membrane contact between the cells.

The field strength of the a-c field is the third parameter which must be carefully controlled in dielectrophoretic collection and, in turn, in the establishment of tight membrane contact. On the one hand, the dielectrophoretic force has to exceed the diffusional forces arising from concentration profiles of the cells in the suspensions in the presence of the a-c field. On the other hand, pearl-chain formation has to be performed at relatively low voltages in order to shield the cell interior from high field strengths and to avoid breakdown [26, 61, 87].

However, at low electric field strengths the contact area between two adjacent spherical cells may be rather small. Suitable field strengths for the formation of "pearl chains" are in the range of 100 to 200 V/cm.

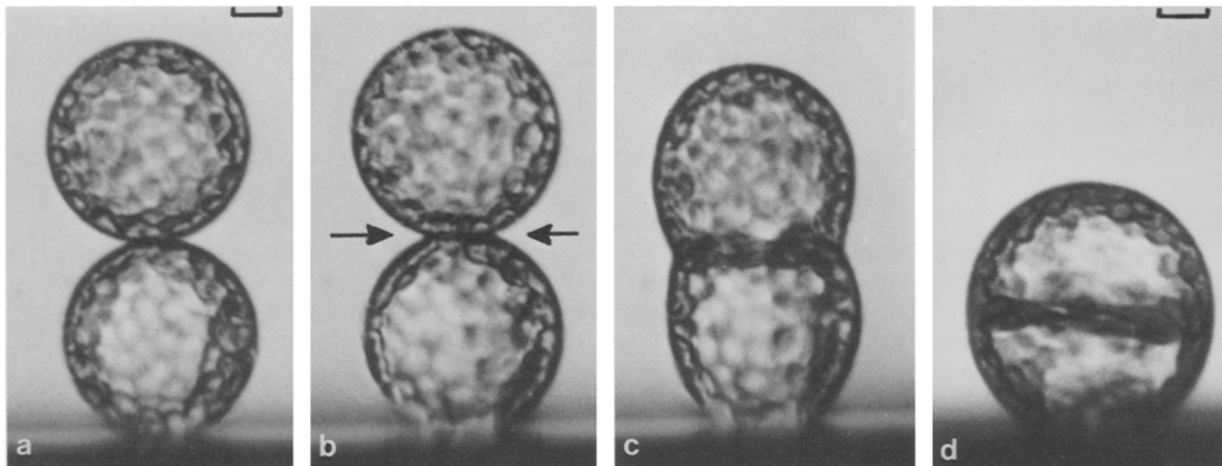

Fig. 3. Fusion of mesophyll protoplasts of *Avena sativa*. (*a*) Cells suspended at high dilution in a 0.5 M mannitol solution were collected dielectrophoretically (frequency of the a-c field 500 kHz, field strength 200 V/cm) under formation of point-to-point contact between two adjacent cells. Interference contrast micrographs. (bar: 10 μm). (*b*) Flattening of the cells in the area of membrane contact was achieved by increasing the field intensity of the a-c field. (*c, d*) Fusion was induced by an electric field pulse of 600 V/cm and 15 μsec duration. Time course of fusion, taken after 10 sec (*c*) and 30 sec (*d*). (bar: 10 μm)

In order to initiate cell fusion, closer membrane contact has to be established by increasing field strength of the a-c field for a very short time (just prior to application of the breakdown pulse). The optimum conditions for the electric field-induced fusion process are achieved when the membranes of two adjacent cells flatten out on coming into contact with each, thus forming a relatively large zone of contact (*see* Fig. 3*b*). Much higher field strengths result in stretching and deformation of the cells, budding and vesicle formation, particularly if the field strength of the a-c field exceeds the breakdown voltage of the membrane [20, 58]. At the same time, streaming of the cytoplasm and the organelles is observed (*unpublished results*) very similar to the phenomenon of cyclosis seen in plant cells and slime molds [3, 29, 66, 67].

The number of cells to be fused can be controlled through several parameters. At low suspension densities aggregates consisting of no more than two cells are readily obtained. Two-cell aggregates are also obtained if the divergence of the field and the field strength is not too large. In this case, the dielectrophoretic movement is slow, and the probability that more than two to three cells will become attached to each other is very low. Fusion between a number of cells can be achieved in two different ways. Either large electrode gaps are used (combined with higher divergent fields and higher field strength) in order to achieve long "pearl chains" or the suspension densities are raised so that many pearl chains are formed in parallel to each other (Fig. 4*a*). Under these conditions lateral fusion between cells in different adjacent pearl chains can take place (*see below*). The formation of large fused cells (giant cells) can also be achieved by subjecting the cells to fusion in the presence of low concentrations of enzymes (*see below*).

In order to fuse cells of different origin the following procedure has to be followed: first, a dilute suspension of cells of one species is sucked between the electrodes in the presence of a slightly divergent field of low intensity. Then, cells of the second species are injected between the two electrodes, again using low suspension densities.

A suitable arrangement for fusion of two cells from different species is shown in Fig. 2*e*. Between the two parallel electrodes an orifice is drilled into the microslide (length corresponding to that of the electrodes, diameter slightly higher than that of the cells). The orifice is connected with two reservoirs containing the respective cell suspension. A diluted suspension of the first species is allowed to enter and a monolayer of cells is formed on both electrode surfaces. The second species is then introduced resulting in cell pairs consisting of species "1" and species "2". This method gives a 60–80% yield of hybrids.

Electrical Breakdown

Reversible electrical breakdown in the zone of membrane contact is the primary process responsible for the initiation of fusion. In order to understand the underlying processes in the membrane, it is necessary to consider some important properties of electrical breakdown. A number of reviews concerning this sub-

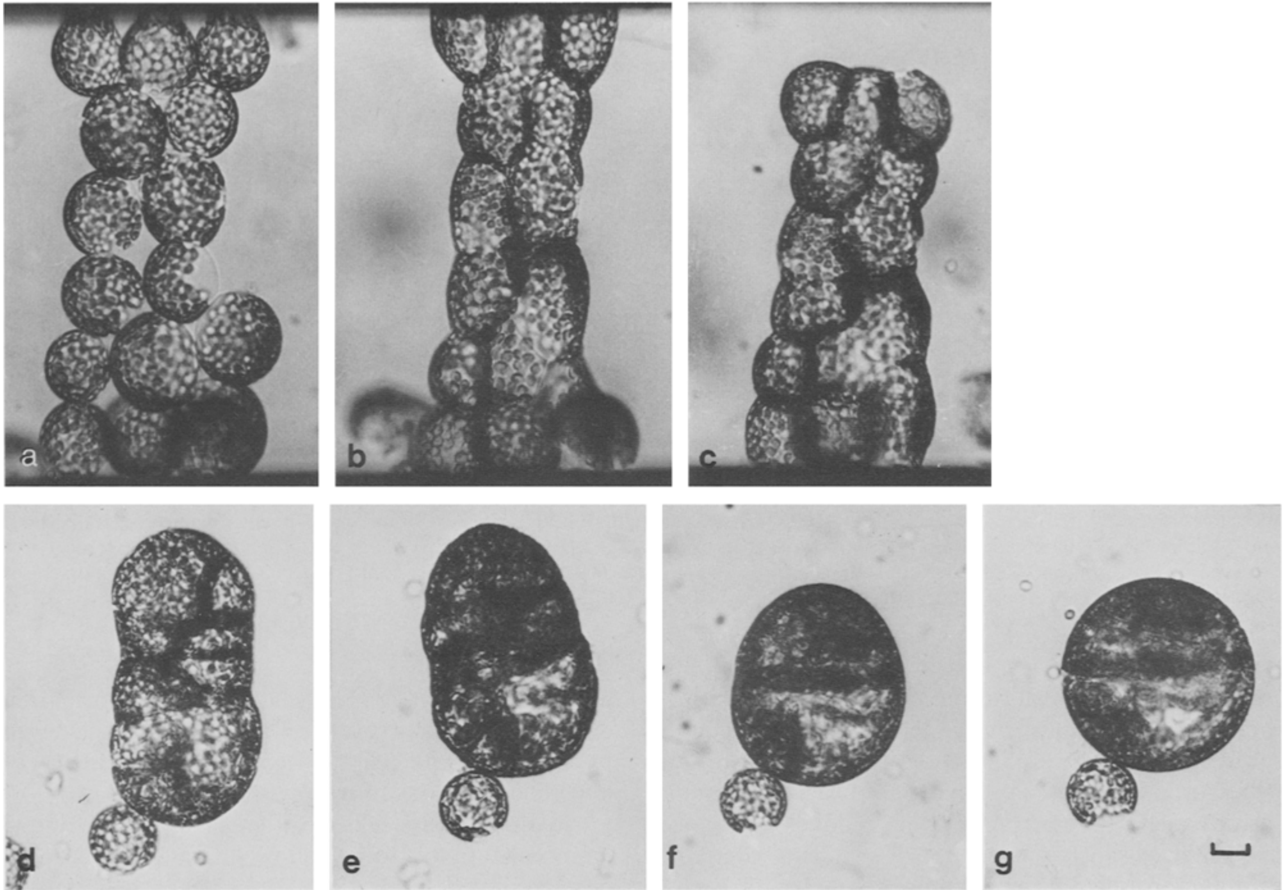

Fig. 4. (*a*) Using large electrode-gaps and higher suspension densities many pearl chains of mesophyll protoplasts of *Avena sativa* were arranged in parallel by dielectrophoresis. Field conditions as in Fig. 3. (*b*) Vertical as well as lateral fusion between adjacent cells was achieved by the injection of a supracritical electric field pulse of 600 V/cm and 20 μsec duration. (cell radius of *Avena* protoplasts on average 17–20 μm, critical field strength 400 V/cm.) (*c*–*g*) Immediately after the field pulse the a-c field was switched off. Rounding up of the fused cells was accelerated by the addition of small amounts of $CaCl_2$. During the induced fusion process, micrographs were taken in time intervals of 1 (*c*), 10 (*d*), 30 (*e*) 50 (*f*) and 60 (*g*) min after field pulse application. (bar: 20 μm)

ject matter have recently been published [4, 76, 82, 87, 88].

Most of our knowledge concerning reversible electrical breakdown has been derived either from experiments performed on cells suspended in electrolyte solution or by the insertion of microelectrodes in giant algal cells, eggs and squid axons [6, 14, 22, 82, 87, 88]. Electrical breakdown measurements on planar artificial lipid bilayer membranes have also made a considerable contribution to our understanding of electrical breakdown [5, 8–10].

Generally speaking, if a cell membrane or a planar lipid bilayer membrane is polarized very rapidly (say, in less than 10 to 100 μsec) to a very high voltage, an electrical breakdown of the membrane is observed which is associated with a dramatic reversible increase in conductivity and permeability. The membrane voltage drops to very low values because of the high conductance state of the membrane. The phenomenon of electrical breakdown should not be confused with the well-known phenomenon of mechanical, irreversible destruction of the membrane [5, 87, 88]. The effects induced in a membrane by electrical breakdown are completely reversible; i.e., after a certain time interval the original membrane resistance and impermeability are restored. If the field strength exceeds the critical field strength required to reach the breakdown voltage by a factor of 2 to 6 (depending on the species) or if the exposure time (pulse length) of the membrane to the field becomes too long (say milliseconds to seconds) the electrical breakdown phenomenon goes over into the mechanical, irreversible one [8, 78]. Most importantly, in the context described here, the breakdown voltage is dependent on pulse length and temperature [5, 8, 9, 14, 78].

While the breakdown voltage of a single cell membrane or an artificial lipid bilayer membrane is independent of the pulse length for pulse lengths below

1 µsec and of the order of about 1 V, the breakdown voltage decreases by a factor of about 2 towards higher pulse lengths (exposure times) and reaches a constant value of about 0.4 to 0.5 V in the 10 to 100 µsec range. The range in which the pulse length dependence of the breakdown voltage is observed varies from cell to cell, and this relationship has to be taken into consideration when cells of different origins are fused.

The breakdown voltage of a membrane also decreases towards higher temperatures, normally by a factor of 2 between 3° and 25 °C [5, 14]. The subsequent resealing process is also temperature-dependent as shown by measurements on artificial planar lipid bilayer membranes made up of oxidized cholesterol [10]. While the resealing process of the lipid bilayer membrane is complete within 20 µsec at 2 °C, the same process requires only 2 µsec at 20 °C.

In cell membranes breakdown usually occurs at the lipid-protein junction or in the proteins [87, 88]. The recovery from field effects induced in proteins takes much longer. At low temperatures, the field-induced permeability increase of a lipid-protein membrane is still maintained, even 30 min after application of the breakdown pulse; and even at higher temperatures, the resealing time is still in the order of several minutes [88].

Most of the available electrical breakdown data are consistent with the view that the membrane is compressed locally due to the high field intensity [13, 74, 75].

It is possible to show theoretically that breakdown occurs when a certain critical membrane thickness is reached (approximately 10–20% compression of the original thickness). Breakdown occurs because the electric compressive forces change more rapidly in response to the changing membrane thickness than the elastic restoring forces within the membrane [13]. The maximum voltage which can be set up across the membrane depends on the elastic compressive modulus (perpendicular to the membrane plane), on the relative dielectric constant and on the unstressed thickness of the membrane area in which breakdown is about to occur [13, 74, 75). There exists experimental evidence that the key assumptions of the electromechanical model for the interpretation of breakdown are correct. In particular, the model proposes that precompression of the membrane by pressure gradients (turgor pressure) or hydrostatic pressures leads to a decrease in the breakdown voltage. This prediction could be verified experimentally for giant cells of *Valonia utricularis* [77]. In addition, it has been possible to demonstrate in human erythrocytes that above a critical hydrostatic pressure of about 600 bar the resting membrane potential is sufficiently high to induce reversible breakdown of the membrane as predicted by the theory [83]. More recently, Benz and Zimmermann [11] and Zimmermann et al. [79] have presented evidence for the existence of mobile charges within the membrane of *V. utricularis* by measurement and analysis of voltage relaxation curves in the low field range. Measurements of the translocation rate of these mobile charges as a function of an increasing pressure gradient (turgor pressure) have revealed that the translocation rate increases with increasing pressure. On the basis of analogous experiments on planar lipid bilayer membranes [7], the results were interpreted in terms of a pressure-mediated compression of the membrane. The value of the elastic compressive modulus deduced from these experiments was in excellent agreement with the value deduced from the breakdown experiments, even though a completely different experimental and theoretical approach was used. We therefore believe that the phenomenon of membrane compressibility has been established beyond reasonable doubt.

It is assumed that the local electromechanical compression of the membrane leads to breakdown by the formation of pores. The pore density has been estimated to be $10^7/cm^2$ from measurements on planar lipid bilayer membranes [5], with the radius of a single pore estimated to be in the order of 3 nm [10]. A similar value was deduced from breakdown experiments on suspended human red blood cells which were exposed to a field pulse by discharging a high voltage capacitor [87].

The creation of pores which may persist for some time (µsec to min) depending on the location of the breakdown area causes a considerable increase in the membrane permeability. This, in turn, brings about an exchange of intracellular and extracellular components by diffusion. The size of the molecules capable of passing through the membrane depends on the field strength and on the duration of the field pulse [70, 76, 88]. The larger the molecules (and the greater the net charge), the greater the field strength and the longer the exposure times of the field pulse required. At high field strengths, the permeability of the membrane may increase to the point where particles (and molecules) with the size of genes are able to penetrate the membrane. In this way, it has been possible to entrap bacteria in plant protoplasts and to transfer plasmids into permanent cell lines (*unpublished results*). If the external field strength far exceeds the critical field strength required for breakdown, electrical breakdown will go over into the irreversible destruction of the cells (*see above*). Likewise, if the pulse lengths exceed 1 to 5 µsec at higher field strengths, irreversible mechanical breakdown is observed in the membranes of permanent cell lines with

the exception of human erythrocytes [87, 88]. The latter will withstand field durations of up to 50 μsec without any visible deterioration of the membrane functions. Obviously, the electric field and, in turn, the high current densities in the conductive cell interior, as well as the associated osmotic processes create adverse side effects in the organelles and in the cytoplasm, once breakdown has occurred. Since breakdown occurs within 10 nsec [8] it is immediately obvious that the exposure time of the cells to the electric field pulse is a critical factor.

The increase in the permeability of the cell membrane of a given cell size in response to increasing field strength can be explained theoretically on the basis of the integrated Laplace equation ([28], derived for spherical cells):

$$V_c = \frac{3}{2} \cdot a \cdot E_c \cos\vartheta \quad (1)$$

where V_c is the breakdown voltage which is independent of volume [80], a is the radius of the cell and E_c is the critical field strength. ϑ is the angle between a given membrane site and the field direction.

Equation (1) demonstrates that the field strength required for the induction of breakdown depends on both the radius and the angle of a given membrane site with respect to the field direction. If we consider any given cell, the breakdown voltage of the membrane in response to ever-increasing field intensity is first reached at those membrane sites oriented in field direction ($\vartheta = 0°$, $\cos\vartheta = 1$). Towards higher field strengths, membrane sites at an angle of more than 0° with respect to the field direction are also subjected to breakdown. The voltage across membrane sites located at an angle of 90° with respect to the field direction is zero because the cosine ϑ term is zero. On the basis of the pore model we can therefore conclude that more and more pores are created over the whole membrane surface with increasing field strength. At the same time, the diameter of the pores also increases because of the higher field intensity. This possibility of changing the permeability of defined membrane areas by way of the field strength is of paramount importance for lateral cell fusion of hundreds to thousands of cells arranged in many parallel pearl chains by dielectrophoresis.

Fusion

In contrast to the field pulse applications to cell suspensions, the cells are oriented with respect to the field lines under dielectrophoretic conditions. In the light of the foregoing considerations, the breakdown voltage will first be reached in the membrane contact zone in response to a field pulse of sufficient intensity. Breakdown causes a few pores to be generated in the apposed cell membranes so that a channel is formed between the two cells, through which mass transport can take place ([87]; *see also* [27]). Thus, exchange of materials between the cells and the external medium, as observed in suspended cells, can only take place in those two cells at the end of the pearl chain, provided that the field strength of the pulse is just critical or slightly supercritical. Thus, the loss of intracellular material is minimized. Fusion is generally observed between cells within a pearl chain, when a field pulse of 2 to 50 μsec (depending on the species) is applied and when the field strength is 1.5 to 2 times higher than the critical field strength normally required for breakdown in the membrane sites oriented in field direction. The requirement of a slightly higher field strength could suggest that a large number of pores have to be created in order to initiate the fusion process between two cells. Assuming that the contact zone is a circular area with a diameter of 3 μm and assuming further that the field-created pore density is of the same order as that determined for artificial lipid bilayer membranes, one can easily calculate that only 3 to 10 pores are generated by the field within the contact zone, if only the critical field strength is applied. However, a calculation of the number of pores and of the exact area in which the pores are generated by the breakdown pulse cannot be given at the present time, because Eq (1) was derived for spherical cells. Under fusion conditions, however, the cells must be flattened in the membrane contact zone, because as pointed out above, a point-to-point contact of the membranes is not sufficient to induce fusion.

Furthermore, it is not possible at the present time to rule out a number of other factors which may introduce considerable uncertainty into the calculation of the actual membrane potential difference at a given field intensity. Among other things, the calculations are based on the assumption that the field is homogeneous, which is indeed the case for the regions between the two electrodes, but does not apply to the areas close to the electrodes. More importantly, the Laplace equation is derived on the assumption that the equilibrium potential is set up across the membrane in response to the electric field. This is certainly true in the case of electrical breakdown studies on suspended cells in electrolyte solutions, because the time constant of the charging process of the membrane is about 50 nsec. In the presence of nonelectrolytes, on the other hand, the time constant may be in the order of a few microseconds because of the low conductivity of the external solution [28]. Given that the duration of the applied breakdown pulse resulting in fusion is of the same order of magnitude the equilibrium potential will al-

most certainly not be achieved under these conditions. The solution of the Laplace equation for the membrane potential in response to an alternating field of varying frequency has recently been given by Holzapfel et al. [26],

$$U_m(t)=\frac{3}{2}\cdot\frac{E_o\cdot a\cdot\cos\vartheta}{1+(\omega\tau)^2}(\cos\omega\tau+\omega\tau\cdot\sin\omega\tau) \quad (2)$$

with the amplitude

$$U_m^o=\frac{3}{2}\cdot\frac{E_o\cdot a}{1+(\omega\tau)^2}$$

where ω is the angular frequency, τ the time constant of the charging process of the membrane, and a the radius of the cell. If we assume that a pulse length of about 10 µsec corresponds to a frequency of about 10^5 Hz we can estimate that only 70% of the equilibrium potential is established across the membrane during the d-c field pulse application.

The relationship between the breakdown voltage and the pulse length (*see above*) introduces a further uncertainty into the calculation of the membrane area where the breakdown voltage is reached. So far, it has not been possible to measure this relationship for small cells. It is thus conceivable that the breakdown voltage declines to values of 0.5 V for pulse lengths of more than 20 µsec.

On the other hand, the actual membrane potential could be higher because the d-c field pulse is applied in addition to the a-c field used for dielectrophoresis. The actual voltage across the membrane during the breakdown event thus depends on the phase of the a-c field during which the single d-c pulse is injected. In the light of these uncertainties it is extremely difficult to estimate the true field strength in the vicinity of a cell. These aspects certainly merit detailed theoretical consideration and experimental investigation in the future. A knowledge of the exact field strength distribution between the two electrodes, the time constant of the charging process and the pulse length dependence of the breakdown voltage would help considerably in the accurate prediction of the conditions for cell-to-cell fusion and for fusion of liposomes with cells.

Plant Protoplasts and Vacuoles

As indicated in Fig. 3 which shows mesophyll cell protoplasts of *Avena sativa,* fusion is initiated as soon as breakdown has occurred [86]. As usual [24], the protoplasts were suspended in a mannitol solution (0.5 M) at pH 7 in the absence of calcium (ignoring the presence of trace contamination). A further example of fusion is given in Fig. 4. In this case many pearl chains of cells arranged in parallel were subjected to fusion.

As soon as the intermingling of the cell membranes becomes visible under microscope, the dielectrophoretic voltage is gradually reduced to zero. Otherwise a fused tube is obtained which may bridge the gap between the electrodes and will not turn into a spherical cell [69, 85]. The fusion process takes 0.5 to 5 min on average and is completed with the formation of a spherical cell. The time required for the entire process under these conditions depends on the number of cells subjected to fusion. In the case of many cells (Fig. 4) fusion and rounding up took about 40 to 60 min. The time between the initiation of fusion by the breakdown pulse and the formation of a spherical cell also depends on the time required for the fusion of the vacuoles inside the protoplasts. Fusion between vacuoles is usually observed 20 to 60 sec after the initiation of fusion between the plasmamembranes of two adhered cells. It appears that fusion between vacuoles can occur spontaneously if the two vacuoles come into close contact inside the fused cell aggregate. However, it is also possible that the field strength of the d-c pulse is sufficiently high to induce breakdown in the tonoplast membrane also, because these compartments occupy nearly 70% of the total cell volume and thus have a radius similar to that of the whole cell [80]. If this is indeed the case, we have to assume that the resealing time of the vacuolar membrane is longer than a couple of minutes. The latter explanation cannot be ruled out at the present time because spontaneous fusion between vacuoles is a rather rare event. On the other hand, using isolated vacuoles prepared from mesophyll protoplasts of *Kalanchoë daigremontiana* leaves, Vienken et al. [69] have shown that fusion can be initiated by electric field pulses as well. The electric field-induced fusion process of vacuoles is very rapid and is virtually complete after a couple of seconds.

Fusion between protoplasts of different species, but also between different cells of the same species (e.g. fusion between stomatal protoplasts and mesophyll protoplasts of *V. faba*) is also reported in the literature [59, 69, 86, 87]. We can thus conclude that this technique works efficiently as far as the fusion of plant protoplasts is concerned.

Erythrocytes

While plant protoplasts and vacuoles, prepared enzymatically from plant tissues, can be subjected to electric field-induced fusion without any further chemical treatment, a slightly modified procedure has to be applied for mammalian cells or eggs (*see below*). Only very low yields of fused cells are obtained when un-

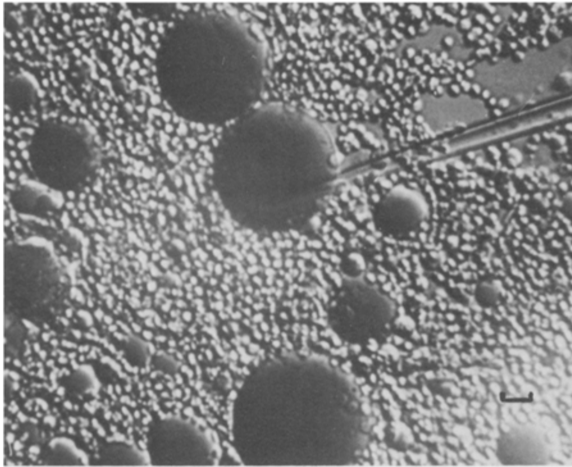

Fig. 5. Giant human erythrocytes obtained by electrically induced fusion of thousands of cells arranged in many pearl chains attaching each other (frequency of the a-c field 2 MHz, 1 kV/cm field strength). Fusion was induced by an electric field pulse of 6 kV/cm and 3 μsec duration. The size of the giant cells is obviously large enough to insert a microelectrode. The giant cells are surrounded by erythrocytes, which were not exposed to the electric field. (bar: 40 μm)

treated human erythrocytes are subjected to the field pulse technique. However, human red blood cells treated with pronase or neuraminidase can be fused with yields of up to 80% [57, 87]. High yields of giant human red blood cells can be obtained if high cell suspension densities are used, so that many pearl chains are formed in parallel to each other. Under these conditions, close membrane contact is established not only between cells in a given pearl chain but also between cells in adjacent chains. If the field strength is chosen sufficiently high (6 kV/cm) to also induce breakdown in larger membrane areas (*see* Laplace equation) fusion will obviously take place not only in cells aligned in parallel to the field lines, but also lateral between cells of adjacent pearl chains. Under these conditions giant cells with a diameter of up to 1 mm may be formed, which means that more than a thousand cells have to fuse together. On average the fusion process requires 3 min. Initially only a few cells (say 20) fuse and form a small sphere which continues to fuse with adjacent cells and grows until it reaches a given size. As indicated in Fig. 5 the cells are so large that it is possible to insert microelectrodes to measure the membrane potential and the membrane resistance.

The treatment of the red blood cells with enzyme before fusion can be interpreted in terms of a removal of the glycocalix in order to establish very close membrane contact between adjacent cells. However, recently it was shown that the formation of giant red blood cells is also possible without removal of the glycocalix [56].

Cultured Cells

As far as cultured cells of permanent cell lines are concerned, electric field-induced fusion has been studied in myeloma cells and mouse erythroleukemia cells (MEL, so-called Friend cells) [42, 84]. Hemoglobin synthesis in the latter cell line can be induced by DMSO-stimulation [21]. Evidence for hemoglobin synthesis is provided by the benzidine reaction within 2 to 3 days of DMSO treatment. This relatively easy and speedy way of testing whether a certain biochemical reaction pattern is still present after fusion makes Friend cells ideal tools for fusion experiments. If the cells are taken from the logarithmic phase and incubated for only a very short period of time in nonelectrolyte solution, fusion can be initiated by the application of a field pulse of 2 kV/cm strength and 20-μsec duration to the dielectrophoretically aligned cells. However, the yield is very low and the average number of cells to fuse is in the order of only 5 to 10, even in the presence of many pearl chains arranged in parallel. The yield can be considerably increased and the formation of giant cells can be induced if pronase (Serva GmbH, 1 mg/ml) or dispase (Serva GmbH, FRG, 10 μg/ml) are added at least 30 sec before the application of the alternating field to the cells between the two electrodes [42, 84]. Under these conditions up to 80% of the cells exposed to the field pulse will undergo fusion and form giant cells if the cell suspension density is sufficiently high (Fig. 6). The presence of pronase or dispase leads to a "stabilization" of the cells against high field strengths and long exposures. While individual cells suspended in a solution are mechanically destroyed if a field pulse of 2 kV/cm strength and 20 μsec duration is injected into the suspension [42, 84], cells in solutions containing these enzymes can be subjected to very high field strengths and longer pulse durations without any detectable deterioration of the cellular functions or the membrane integrity. It should also be noted that pronase and dispase lead to a mechanical stabilization of the cells, so that they are much easier to handle during the preparatory procedures without danger of lysis [42, 84]. If pronase is washed out the cells again become very sensitive to the electric field. The effect of pronase is useful in the timing of the fusion process and in controlling the yield and size of the fused cells. If field pulses of 5 kV/cm and a pulse length of 40 μsec are injected, for example, fusion both of MEL cells and of mouse myeloma cells occurs within a couple of seconds. On the other hand, if a field pulse of 4 kV/cm and a pulse length

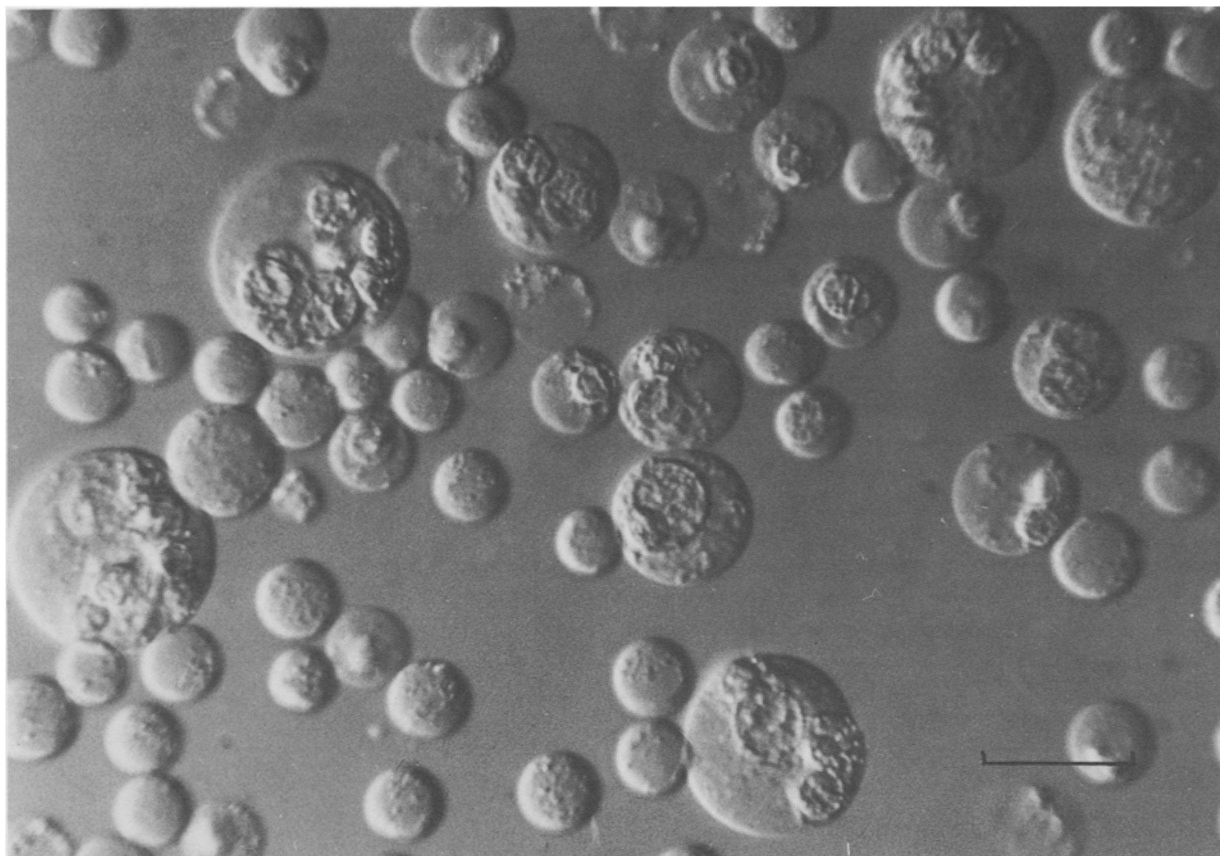

Fig. 6. Interference contrast micrograph of electrically fused MEL cells. Cells were suspended in 0.3 M mannitol solution to which 1 mg/ml pronase P was added before field application (field pulse 2 kV/cm and 40 µsec duration); rounding up of the giant cells was initiated by the addition of a solution containing 150 mM NaCl, 10 mM glucose and 1 mM $CaCl_2$ and is complete after 5 min. (bar: 25 µm)

of 40 µsec is applied, fusion takes at least 3 min. About 3 to 5 min appears to be the optimum time for fusion in order to obtain viable cells. If the fusion process is too fast, there seems to be a remaining disorder in the fused cells. The fused cells normally take on a spherical shape on transfer into an isotonic electrolyte solution. The viability of fused MEL cells could also be demonstrated by DMSO-stimulated hemoglobin synthesis (Fig. 7).

Recently, Pilwat et al. [42] reported that fusion of MEL cells and the formation of cells of different sizes, including giant cells, can also be achieved in the absence of these enzymes, if a train of field pulses of increasing intensity is gradually injected into the dielectrophoretically aligned cells. In general, a critical pulse of 2 kV/cm strength and 5-µsec duration is injected first, followed, after a couple of seconds, by two to three pulses of increasing field strength up to 5 kV/cm. Under these conditions the cells also become tolerant to field pulses of high intensity which they would otherwise succumb to if the first field pulse were as high as the last one. The fusion conditions described here for MEL cells also lead to the formation of homokaryon of mouse myeloma of different cell sizes (*unpublished data*).

Lymphocytes and Hybridoma Cells

There is also experimental evidence now available that mouse lymphocytes can also be subjected to electric field-induced fusion, provided that pronase or dispase are added during field application. Figure 8 shows fused aggregates of two and three mouse lymphocytes following a field pulse application (5 kV/cm field strength, 20 µsec pulse duration) and subsequent transfer to an electrolyte solution. The electric field-induced fusion technique seems to have particular potential in the production of hybridoma cells, i.e. cells producing monoclonal antibodies. Hybridoma cells are produced by fusing B-lymphocytes from the spleen with myeloma cells. The potential of clinical and commercial applications of this research is enor-

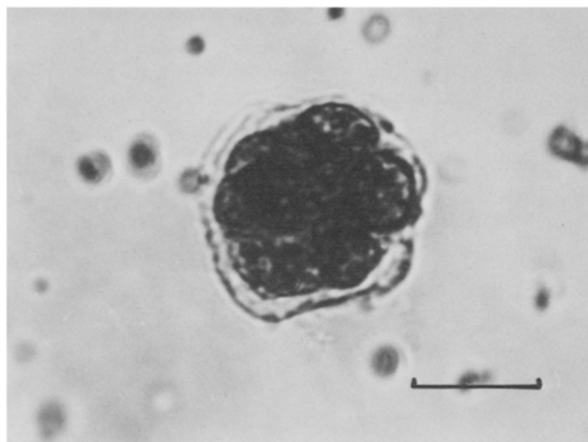

Fig. 7. Hemoglobin-synthesis in fused MEL cells can be induced by DMSO stimulation [21]. Evidence for hemoglobin-synthesis is provided by the benzidine reaction within 2 to 3 days of DMSO treatment, resulting in a blue (dark) staining of the fused cell [42]. (bar: 15 μm)

mous. It would be possible to prepare monoclonal antibodies to identify, quantify, classify and purify compounds of interest to investigators in almost every field of biology and medicine. The number of applications of monoclonal antibodies in clinical diagnosis is immense [32, 50, 72]. Monoclonal antibodies could also be produced against viruses or against the antigens on the outer membrane surface of cancer cells so that these are masked. This would totally alter the way in which these cancer cells affect the body's defense system.

However, the main barrier to the clinical application of monoclonal antibodies for treating human diseases is the murine origin of the antibodies. Recently Olsson and Kaplan [39] and Croce et al. [16] reported the production of human-human hybridoma cells which secreted monoclonal antibodies. However, in the meantime it has turned out, that the yield of human hybridoma cells is very low.

Pronase or dispase can be used to fuse cells of different sizes, a problem which arises, for example, when fusing myeloma cells with MEL cells or with lymphocytes. Mouse lymphocytes are smaller than murine myeloma cells. According to the integrated Laplace equation for spherical cells, the electric field strength required to reach the breakdown voltage is different for the two cell types because the field strength is a function of volume. However, if the myeloma cells are pretreated with a higher concentration of pronase for a longer period of time, the cells are stabilized against higher field strengths and longer exposure times. If these cells are then fused with lymphocytes which were either not exposed to pronase at all or only briefly exposed to low concentrations, high yields of viable heterokaryon products are obtained.

Electric field-induced fusion of mouse lymphocytes with mouse myeloma cells is shown in Fig. 9. A high yield of hybridoma cells which grow in HAT medium can be obtained with this technique (up to 50 to 80%). Since the fusion process can be followed under the microscope, the hybridoma cells can easily be identified. It may thus be possible to avoid the use of a selection medium (HAT medium) for the separation of the hybridoma cells from lymphocytes and myeloma cells.

Large-scale production of hybridoma cells against a great variety of antigens is possible, because the yield and the viability would be considerably increased and the time-consuming selection procedure for hybridoma cells in the HAT medium could be avoided.

Sea Urchin Eggs

Sea urchin eggs can also be fused with a high yield by the electric field pulse technique [52]. Prior to

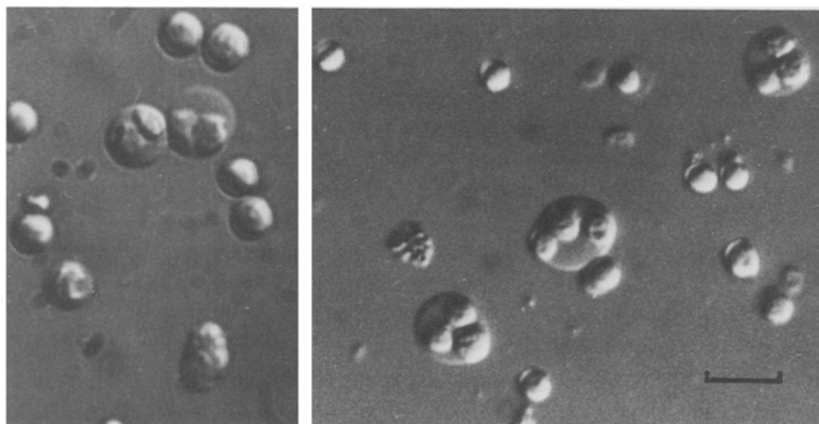

Fig. 8. Fusion of B-lymphocytes of mice consisting predominantly of two and three cells, respectively. Cells were collected dielectrophoretically (frequency of the a-c field 5 MHz, field strength 200 V/cm). Fusion was induced by an electric field pulse of 5 kV/cm and 20 μsec duration. Pronase P (1 mg/ml) was added prior to the experiment. (bar: 10 μm).

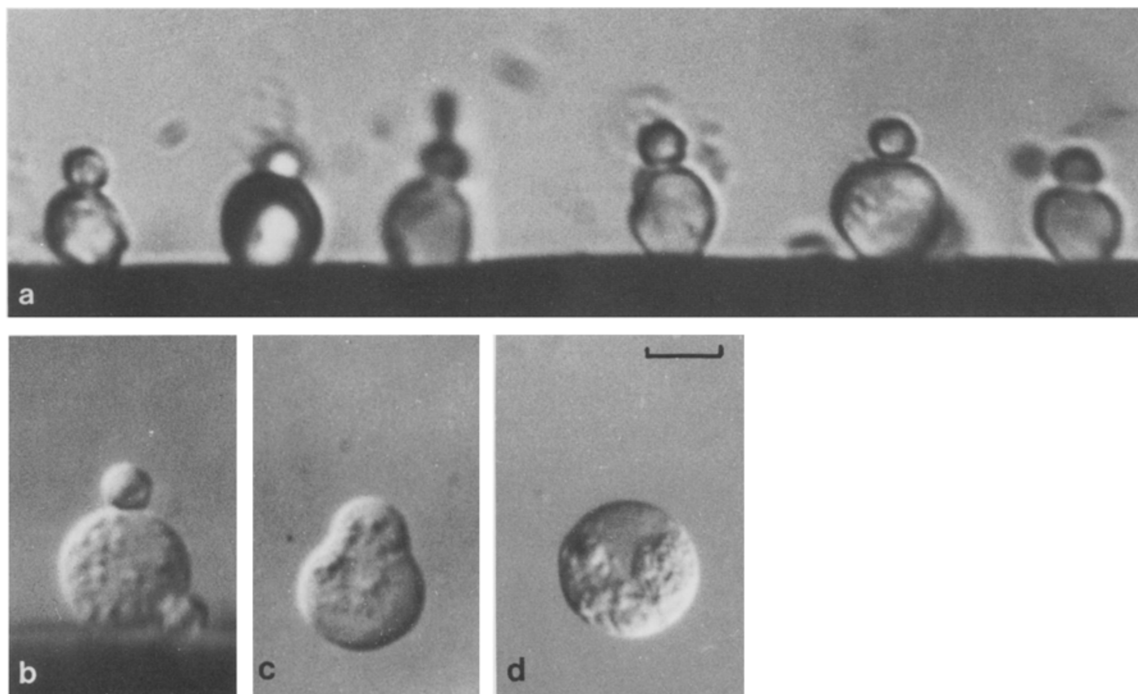

Fig. 9. Electrically induced fusion of mouse B-lymphocytes and mouse myeloma cells. The fusion of a lymphocyte and a myeloma cell results in the formation of a viable hybridoma cell. Using the fusion chamber, described in Fig. 2*e*, individual myeloma cells were attracted to the electrodes by dielectrophoresis. Lymphocytes were collected dielectrophoretically on top of the myeloma cells (*a*). Pronase P (1 mg/ml) was added prior to the experiment. After the field pulse (4 kV/cm, 20 μsec duration), fused cells were removed from the fusion chamber. For rounding up the cells were suspended in a solution containing 150 mM NaCl and 1 mM $CaCl_2$. Interference contrast micrographs (*b–d*) show the time course of fusion for a lymphocyte-myeloma union (*b*) 15 min (*c*) and 30 min (*d*) after field application. (bar: 10 μm)

the application of the alternating field and the field pulse, however, it is necessary to remove the vitellin layer with pronase. Like cultured cells, sea urchin eggs can also be subjected to high field intensities if pronase is added briefly to the cell suspension during the exposure to the field. Pronase also leads to a stabilization of the eggs against high field strengths and long exposure times to the field pulse [84]. At the time a mechanical stabilization of the eggs is observed, so that any danger of lysis of the eggs during the preparatory procedures can be minimized. The effect of pronase on the field intensity of the cells is also completely reversible. The fused 2 to 3 stage eggs can still be fertilized and subsequently divide [52].

Lipid Vesicles

Liposomes can be fused also to form giant vesicles using the electric field technique ([87], and Hub et al., *unpublished results*). It is interesting to note, that fusion also occurs between lipid vesicles made up of neutral lipids (e.g., egg-lecithin). Fusion of such lipid vesicles cannot be achieved by the currently used fusion techniques. Under certain conditions formation of large amounts of giant cylindrical lipid vesicles are observed which are quite stable when the field is switched off (dielectrophoretic frequency at 10 MHz and high electric field strengths of the alternating voltage which may exceed the breakdown voltage). Fusion of liposomes in which proteins are entrapped with planar lipid bilayers using the appropriate electrical arrangement should be possible in the future. This would be a very elegant way to incorporate proteins into artificial bilayer membranes.

Fusion Mechanism

On the basis of our knowledge of electrical breakdown we can propose the following model for the primary step in electric field-induced fusion (Fig. 10). As a result of electrical breakdown pores are generated in the two bilayers apposed to each other. In contrast to the situation in single suspended cells, the lipid molecules, which are randomly orientated in the pores, tend to aggregate between the two bilayers during the "resealing process" and form "bridges" such as the one illustrated in Fig. 10*c*. We

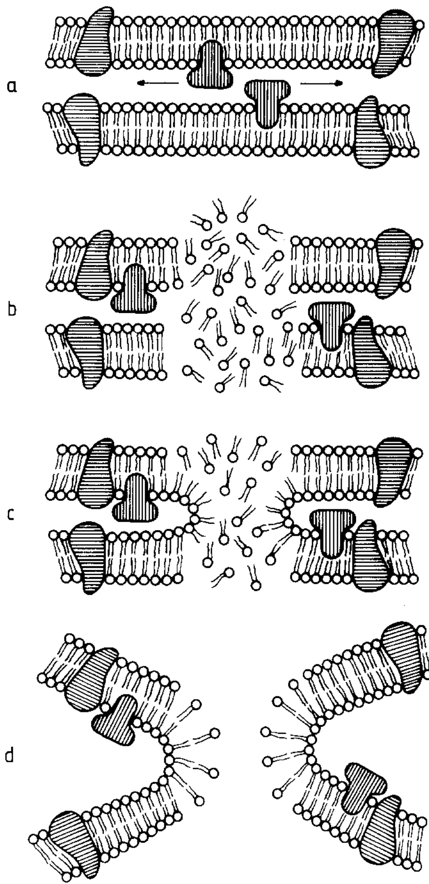

Fig. 10. Model for the molecular processes which may occur during electrically induced fusion. The following sequence of events may occur: (*a*) The lipid bilayers of the two membranes are brought in close contact due to dielectrophoresis, whereby it is assumed that protein-free areas are formed by lateral diffusion of proteins in response to the alternating field. (*b*) Electrical breakdown leads to a local disturbance of the membrane structure, so that (*c*) bridges may be formed between the bilayers of the two membranes during the subsequent resealing process. (*d*) Formation of spherical two-cell aggregates is energetically favored due to the small radius of curvature of the pores formed in this way

have to assume that the time constant of the bridging process is smaller than that of the resealing process of much of the individual bilayer membranes (say, less than 1 µsec). If the bridging process dominates an open channel is created. Its configuration is thermodynamically unstable because of the high surface curvature of the channels and the associated high tension in the membrane. The subsequent process of fusion resulting in the formation of a spherical cell is thus energetically favored and can proceed without any further input of energy. The formation of lipid bridges between the two apposed bilayers may be caused by the application of the field, or by mass transport through the pore, the latter being observed particularly often in the first phase after electrical breakdown when two protoplasts are being fused.

However, osmotic processes as proposed by Zimmerberg et al. [73] for the initiation of fusion are evidently not required in electric field-induced fusion. The higher field strength, which is found experimentally to trigger the fusion process, as compared to the field strength required for breakdown of the membrane area in the field direction, may suggest that either a few channels with a large diameter or many smaller channels have to be created by the field in a given membrane area. This can be explained on the basis of the Laplace equation. The formation of giant cells should also be considered from this point of view. The field intensities are so high that channels may be created as a result of an electrical breakdown in those membrane areas, which are oriented at an angle of about 70 to 80° to field direction, thus leading to lateral fusion between cells of different pearl chains.

The proposed mechanism presupposes that protein-free lipid domains are present in the membrane and that these domains are orientated in the field direction. The presence of proteins in the zone of breakdown would obviously disturb the bridging process between the two apposed bilayer membranes. Under these conditions one would expect the resealing process of the individual bilayer membranes to dominate because the presence of proteins should increase the time constant of the bridging process. The suggested involvement of virtually protein-free lipid domains in the primary step of fusion is along the same lines as the hypotheses introduced by Poste and Allison [46] for chemically and virus-induced fusion. In particular Lucy and colleagues [1, 2, 34] have pointed out, that the emergence of protein-free lipid domains has to precede fusion.

Freeze-fractures of the plasmalemma membranes of plant protoplasts (e.g. *Vicia faba, Avena sativa*) demonstrated that the inner monolayer of the plasmalemma is compartmentalized into areas with distinct, highly organized structures [60]. Membrane domains showing an extremely regular, planar hexagonal array of particles are interspersed between areas of intramembranous particles dispersed randomly at low concentrations on a relatively smooth fracture face. These intramembranous particles are believed to be of a lipo-protein nature (for details, *see* [68]). These characteristic properties of the membranes of plant protoplasts may be the reason why electrically induced fusion proceeds rapidly and reproducibly with high yields. In general, it seems to be well-established, at least for phospholipid bilayers, that as they approach each other, their structures are modified in a number of different ways. Following the arguments of Rand [51], as two spherical bilayer vesicles made up of egg PC and egg PE interact, PE is expected to accumulate

in the region of contact. The vesicles would then approach more closely. On the basis of the energy of interaction between PE and PC vesicles remixing of these lipids is unlikely. Having accumulated in the contact zone, the tendency of PE to form hexagonal structures could lead to the formation of inverted micelles, thence bilayer disruption leading to fusion.

We believe that such structural changes in a cell membrane associated with the emergence of particle-free lipid domains can also be caused, or at least facilitated, by the alternating electric field.

The equilibrium distribution of the mobile ions, the size and shape of the electrostatic potential of isolated surfaces and the resultant force between interacting charged surfaces can be described by the double layer theory (*cf.* [41]). According to this theory, the electrostatic pressure between two planar charged surfaces is approximately proportional to the product of the surface charge densities and falls off exponentially with surface separation. The rate of exponential decay is governed by the Debye constant κ which varies with the square root of the ionic strength of the intersurface medium. For physiological saline the Debye length $1/\kappa$ is about 1 nm, but increases to about 10 nm in nonelectrolyte solution [41]. Before fusion the short separation between the two membranes brings about a distortion of the counterion distribution which may, in turn, result in a decrease in the distribution of mobile counterions in the gap between the two membranes. The charge of the proteins is no longer neutralized, and the macromolecules repel each other and migrate towards the equatorial plane of the cell, thus creating macromolecule-free lipid domains in field direction. On the basis of this model, the effects of pronase and dispase on the fusion kinetics would be to alter the counterion distribution in the aqueous phase of the contact zone by adsorption of proteins onto the outer membrane surface. We can speculate that the repulsion hydration force is changed and the repulsive force on the proteins in the membrane is increased leading to the emergence of lipid domains. On the other hand several authors have pointed out, that an electric field parallel to the membrane should redistribute macromolecules bearing a net charge and being free to move laterally in the membrane. Externally applied electric fields do indeed redistribute lectin and acetylcholine receptors in cell membranes. McLaughlin and Poo [36] recently postulated that proteins protruding from the lipid bilayer redistribute due to an electro-osmotically induced movement of counterions in response to the tangential component of the field parallel to the membrane.

It is also conceivable that the proteolytic activity of the enzymes causes a partial degradation of some of the intramembranous particles [40], so that the mobility of the remaining proteins is increased. The mobility of the proteins could also be increased by the proteolytic activity of the enzymes on the cytoskeleton in the membrane contact zone following breakdown. On the other hand, one cannot rule out the possibility that the reaction of the enzymes in question may differ from that in normal electrolyte conditions because of the low ionic strength in the membrane contact zone. The water structure in the membrane contact zone may be quite different compared to that with electrolyte solution because of the low ionic strength and because of the close membrane contact [51].

If these assumptions are valid we would expect that the electric field-induced fusion process is altered in the presence of higher concentrations of electrolytes. This prediction could be tested experimentally by using flow chamber systems with an efficient cooling device (*see* Fig. 2*d, e*).

Alternatively, it is possible that macromolecule-free domains are created as a result of microaggregation of proteins within the membrane, which itself is caused by the tangential components of the alternating field operating in parallel to the membrane (Lindemann, Richter, Pilwat and Zimmermann, *unpublished data*). With the exception of membrane sites in field direction, tangential forces of the field arise throughout the membrane, with the tangential component increasing towards the equatorial plane of the cell. The tangential component of the field is thus largest on membrane sites oriented at an angle of 90° with respect to the field line. If we consider the first cycle of the alternating field, the macromolecules within the membrane accelerate in field direction (Fig. 11). It is important to bear in mind that the acceleration of the particles increases from the upper pole to the equator and decreases again from the equator to the lower pole. As a result, the membrane contact zone at the lower pole will initially tend to become depleted of macromolecules while the opposite membrane contact zone will become enriched with macromolecules. At the same time, the membrane sites in the equatorial plane of the cell will become more depleted of macromolecules because the tangential forces are strongest there. In the second cycle of the alternating field the field direction is reversed, and the macromolecules are accelerated in the opposite direction.

Macromolecules within the membrane which were accelerated from the equatorial planes towards the upper pole during the first cycle will not reach the equatorial plane again because the tangential forces acting on those particles at the new membrane site will be weaker. Those macromolecules still present

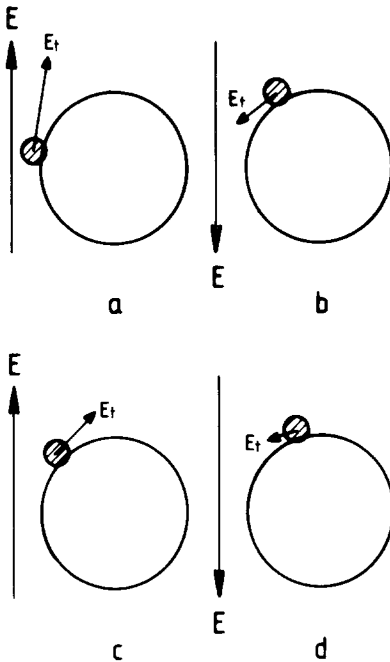

Fig. 11. Diagrammatic representation of the postulated process leading to the aggregation of intramembranous macromolecules in response to the external electric field. For reasons of clarity only one macromolecule (shaded sphere) and two cycles (*a*, *b* and *c*, *d*) of the a-c field are shown. The figure shows the movement of a macromolecule in response to the tangential forces of the electric field. Arrows indicate the direction of acceleration which depends on the position of the macromolecule and on the direction of the electric field. The length of the arrow corresponds qualitatively to the magnitude of the tangential electric field strength (E_t). For further details *see text*

in the equatorial planes will now be accelerated towards the lower pole. Furthermore, those macromolecules which migrated from the lower pole towards the equatorial plane during the first cycle will now be accelerated more strongly towards the lower pole because of the profile of the tangential forces. Thus after a few cycles of the alternating field the membrane sites at the equatorial plane may be partially free of macromolecules, whereas both membrane contact zones are enriched with intramembranous macromolecules in field direction.

At first glance the effect would appear to be opposite to that postulated for fusion in field direction, but these considerations apply only to a perfect sphere, i.e., to conditions where only point-to-point membrane contact is established at low field intensities for the alternating voltage. If the field strength is increased so that the spheres flatten in the membrane contact zone, the macromolecules will move only to the periphery of the flattened contact zone, because in the membrane contact zone itself the field lines are perpendicular to the membrane surface. The enzyme would facilitate this process of redistribution of macromolecules within the membrane by one of the mechanisms discussed above. If this latter mechanism works we would expect lateral fusion to be achieved without any problem, and this has indeed been verified experimentally by the formation of giant cells. At the same time we would expect the dielectrophoretically aligned cells to exhibit anisotropy with respect to the field stability. In theory such anisotropy can be verified experimentally by applying the breakdown pulse vertically to the pearl chains, although this still poses a few experimental problems which will, no doubt, be resolved in the near future.

In our opinion, the field stability of cells in the presence of enzymes is directly linked with the emergence of lipid domains and is an undoubtful proof for the presence of lipid domains. Any other explanation would be in contradiction with the available data and our current knowledge concerning electrical breakdown in individual cells and in artificial lipid bilayer membranes. The results can be explained, if the different resealing times of a bilayer membrane and of proteins are included in the considerations (*see above*). With less than 1 μsec the resealing times of lipid domains are so rapid ([10] and Benz, *personal communication*) that the structure of the bilayer at the two ends of the pearl chain is practically restored during the pulse itself or immediately afterwards, whereas breakdown at the lipid-protein junctions may lead to the flow of substantial currents through the cell for the periods of pulse application. This results in adverse side effects on the nucleus, cytoplasmic factors and organelles and eventually in the irreversible deterioration of the cells, if the field strength and duration exceed a certain supracritical level [87, 88]. It also seems conceivable that the osmotic processes observed after the event of breakdown result in irreversible changes at the cell and membrane levels, provided that the resealing time is longer than a couple of microseconds.

If we assume that in cells exposed to the field in the presence of pronase or dispase the protein-free lipid domains are orientated in field direction and therefore subjected to breakdown, it is evident that the rapid resealing of the lipid domains in the membranes is the primary reason for the field insensitivity of the cell under these conditions [42, 84].

The experimental finding described above, namely that a train of gradually stepped-up field pulses stabilizes cells against fields of high intensity, is consistent with the view that the emergence of lipid domains is a prerequisite for electric field-induced fusion. As a result of breakdown, a channel is created between the two membranes. Due to breakdown, proteins which are present at this site may be removed. The resealing of the pores will be effected predominantly

by diffusion of lipid molecules rather than by diffusion of proteins within the membrane, since the diffusion coefficients of proteins are at least two orders of magnitude lower than those of lipid molecules [71]. Thus, particle-free lipid domains might be generated in field directions, which in turn will lead to a higher field stability of the cells and a greater susceptibility to fusion.

Conclusion

Even though the electric field-induced fusion technique is in its infancy the presented results suggest that this new technique can be universally applied to fusion of all cells and artificial vesicle systems.

The technique is based on the dipole generation within material and on the breakdown of the cell membrane. Both effects are observed to all living and artificial systems. The universal application of the electric field-induced fusion suggests that changes in the intrinsic membrane electric field may be the primary step in cell-to-cell fusion.

It can readily be envisaged that changes in the local field distribution within the membrane can be generated by chemicals or by viruses resulting in intrinsic reversible breakdown events, and, in turn, in the formation of transient channels [41]. Using the electric fusion technique the mechanism of fusion may be elucidated in future by electron microscopy and optical measurements (e.g. fluorescence-spectroscopy). This is possible because of the synchronous process of electrically induced fusion. The gentle procedure and the high yield of hybrids obtained by electric field application suggest that this technique may have potential applications not only in membrane research but also in plant agriculture and medicine.

Abbreviations

a-c	alternating current
d-c	direct current
DMSO	dimethylsulfoxide
EDTA	ethylene-diamine-tetraacetate
MEL-cells	mouse erythroleukemia cells
PC	phosphatidylcholine
PE	phosphatidylethanolamine

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 160) and from the Bundesministerium für Forschung und Technologie (037266) to U.Z.

U.Z. would like to thank Dr. H. Pohl, University of Oklahoma, for very helpful discussions. A grant of the NATO (RG 273.80) to cover the travel expenses of U.Z. to the USA is greatly appreciated. We are greatly indebted to Dr. Pilwat for his experimental contribution and his help in preparation of this manuscript. We gratefully acknowledge the typewriting of the manuscript by Mrs. S. Alexowsky.

References

1. Ahkong, Q.F., Blow, A.M.J., Botham, G.M., Launder, J.M., Quirk, S.J., Lucy, J.A. 1978. *FEBS Lett.* **95**:No. 1:147–152
2. Ahkong, Q.F., Botham, G.M., Woodward, A.W., Lucy, J.A. 1980. *Biochem. J.* **192**:829–836
3. Allen, N.S., Allen, R.D. 1978. *Annu. Rev. Biophys. Bioeneng.* **7**:497–526
4. Baker, P.F., Knight, D.E. 1979. *Trends Neurosci.* **9**:288–291
5. Benz, R., Beckers, F., Zimmermann, U. 1979. *J. Membrane Biol.* **48**:181–204
6. Benz, R., Conti, F. 1981. *Biochim. Biophys. Acta* **645**:115–123
7. Benz, R., Gisin, B.F. 1978. *J. Membrane Biol.* **40**:293–314
8. Benz, R., Zimmermann, U. 1980*a*. *Biochim. Biophys. Acta* **597**:637–642
9. Benz, R., Zimmermann, U. 1980*b*. *Bioelectrochem. Bioenerg.* **7**:723–739
10. Benz, R., Zimmermann, U. 1981*a*. *Biochim. Biophys. Acta* **640**:169–178
11. Benz, R., Zimmermann, U. 1981*b*. *Biophys. J.* (*submitted*)
12. Cole, K.S. 1968. Membranes, Ions and Impulses. University of California Press, Berkeley – Los Angeles
13. Coster, H.G.L., Steudle, E., Zimmermann, U. 1977. *Plant Physiol.* **58**:636–643
14. Coster, H.G.L., Zimmermann, U. 1975. *J. Membrane Biol.* **22**:73–90
15. Crane, J.S., Pohl, H.A. 1968. *J. Electrochem. Soc.* **115**:584–586
16. Croce, C.M., Linnenbach, A., Hall, W., Steplewski, Z., Koprowski, H. 1980. *Nature (London)* **288**:488
17. Edelson, P.J., Cohn, Z.A. 1978. *In:* Membrane Fusion. G. Poste and J.L. Nicholson, editors. pp. 369–385. Elsevier/North-Holland, Amsterdam
18. Engleman, E.G., Warnke, R., Fox, R.I., Dilley, J., Benike, C.J., Levy, R. 1981. *Proc. Natl. Acad. Sci. USA* **78**:1791–1795
19. Fricke, H. 1953. *J. Phys. Chem.* **57**:934–937
20. Friend, A.W., Jr., Finch, E.D., Schwan, H.P. 1975. *Science* **187**:357–358
21. Friend, C., Scher, W., Holland, J.G., Sato, T. 1971. *Proc. Natl. Acad. Sci. USA* **68**:378–382
22. Gauger, B., Bentrup, F.W. 1979. *J. Membrane Biol.* **48**:249–264
23. Glaser, R., Pescheck, Ch., Krause, G., Schmidt, K.P., Teuscher, L. 1979. *Z. Allg. Mikrobiol.* **19**:601–607
24. Hampp, R., Ziegler, H. 1980. *Planta* **147**:485–494
25. Hengartner, H., Du Pasquier, L. 1981. *Science* **212**:1034–1035
26. Holzapfel, Ch., Vienken, J., Zimmermann, U. 1982. *J. Membrane Biol.* **67**:13–26
27. Hui, S.W., Stewart, T.P., Boni, L.T., Yeagle, P.L. 1981. *Science* **212**:921–922
28. Jeltsch, E., Zimmermann, U. 1979. *Bioelectrochem. Bioenerg.* **6**:349–384
29. Kamiya, N. 1959. *Protoplasmatologia* **8**:3a
30. Kao, K.N., Michayluk, M.R. 1974. *Planta* **115**:355–367
31. Keller, W.A., Melchers, G. 1973. *Z. Naturforsch.* **28c**:737–741
32. Köhler, G., Milstein, C. 1975. *Nature* (*London*) **256**:495–497
33. Loyter, A., Zakai, N., Kulka, R. 1975. *J. Cell Biol.* **66**:292–304
34. Lucy, J.A. 1978. *In:* Membrane Fusion, G. Poste and G.L. Nicolson, editors. pp. 267–304. Elsevier/North-Holland, Amsterdam
35. Manegold, E. 1950. *Kolloid Z.* **118**:11–26
36. McLaughlin, S., Poo, M.M. 1981. *Biophys. J.* **34**:85–93
37. Muth, E. 1927. *Kolloid Z.* **41**:97–102
38. Neumann, E., Gerisch, G., Opatz, K. 1980. *Naturwissenschaften* **67**:414–415
39. Olsson, L., Kaplan, H.S. 1980. *Proc. Natl. Acad. Sci. USA* **77**:5429–5431
40. Passow, H. 1971. *J. Membrane Biol.* **6**:233–258

41. Pethig, R. 1979. Dielectric and Electronic Properties of Biological Materials. Wiley, Chichester
42. Pilwat, G., Zimmermann, U., Richter, H.-P. 1981. *FEBS Lett.* **133**:169–174
43. Pilwat, G., Zimmermann, U., Schnabl, H. 1980. *In:* Plant Membrane Transport: Current Conceptual Issues. R.M. Spanswick, W.J. Lucas, and J. Dainty, editors. pp. 475–478. Elsevier, Amsterdam
44. Pohl, H.A. 1978. Dielectrophoresis. Cambridge University Press, Cambridge
45. Poste, G. 1972. *Int. Rev. Cytol.* **33**:157–252
46. Poste, G., Allison, A.C. 1973. *Biochim Biophys. Acta* **300**:421–465
47. Poste, G., Nicolson, G.L. 1978. Cell Surface Reviews, Membrane Fusion, Vol. 5. Elsevier/North-Holland, Amsterdam
48. Poste, G., Pasternak, C.A. 1978. *In:* Membrane Fusion. G. Poste and G.L. Nicolson, editors. pp. 305–367. Elsevier/North-Holland, Amsterdam
49. Power, J.B., Evans, P.K., Cocking, E.C. 1978. *In:* Membrane Fusion, G. Poste and G.L. Nicolson, editors. pp. 369–385. Elsevier/North-Holland, Amsterdam
50. Pressman, D., Bankert, R. 1980. *J. Surg. Oncol.* **15**:393
51. Rand, R.P. 1981. *Annu. Rev. Biophys. Bioeng.* **10**:277–314
52. Richter, H.-P., Scheurich, P., Zimmermann, U. 1981. *Dev. Growth Differ.* **23**:479–486
53. Ringertz, N.R., Savage, R.E. 1976. Cell Hybrids. Academic Press, New York
54. Saito, M., Schwan, H.P. 1961. *In:* Biological Effects of Microwave Radiation. M.F. Peyton, editor. pp. 85–97. Plenum Press, New York
55. Saito, M., Schwan, H.P., Schwarz, G. 1966. *Biophys. J.* **6**:313–327
56. Saleemuddin, M., Vienken, J., Zimmermann, U. 1982. *Biochim Biophys. Acta (in press)*
57. Scheurich, P., Zimmermann, U. 1981. *Naturwissenschaften* **68**:45–46
58. Scheurich, P., Zimmermann, U., Mischel, M., Lamprecht, I. 1980. *Z. Naturforsch.* **35c**:1081–1085
59. Scheurich, P., Zimmermann, U., Schnabl, H. 1981. *Plant Physiol.* **67**:849–853
60. Schnabl, H., Vienken, J., Zimmermann, U. 1980. *Planta* **148**:231–237
61. Schwan, P.H. 1957. *In:* Advances in Biological and Medical Physics. J.H. Laurence and C.A. Tobias, editors. Vol. 5, pp. 147–209. Academic Press, New York
62. Schwan, H.P., Sher, L.D. 1969. *J. Electrochem. Soc.* **116**:22–26
63. Schwarz, G., Saito, M., Schwan, H.P. 1965. *J. Chem. Phys.* **43**:3562–3569
64. Silva, P.P. da, Shimizu, K., Parkison, C. 1980. *J. Cell Sci.* **43**:419–432
65. Staehelin, T., Durrer, B., Schmidt, J., Takacs, B., Stocker, J., Miggiano, V., Stähli, C., Rubinstein, M., Levy, W.P., Hershberg, R., Pestka, S. 1981. *Proc. Natl. Acad. Sci. USA* **78**:1848–1852
66. Tazawa, M., Kikuyama, M., Shimmen, T. 1976. *Cell Struct. Funct.* **1**:165–176
67. Tazawa, M., Kishimoto, U. 1968. *Plant Cell Physiol.* (*Tokyo*) **9**:361–368
68. Verkleij, A.J., Ververgaert, P.H. 1978. *Biochim. Biophys. Acta* **515**:303–327
69. Vienken, J., Ganser, R., Hampp, R., Zimmermann, U. 1981. *Physiol. Plant.* **53**:64–70
70. Vienken, J., Jeltsch, E., Zimmermann, U. 1978. *Cytobiology* **17**:182–196
71. Webb, D. 1978. Electrical Phenomena at the Biological Membrane Level. E. Roux, editor. pp. 119–153. Elsevier, Amsterdam
72. Yelton, D.E., Scharff, M.D. 1980. *Am. Sci.* **68**:510–516
73. Zimmerberg, J., Cohen, F.S., Finkelstein, A. 1980. *Science* **210**:906–908
74. Zimmermann, U. 1978. *Annu. Rev. Plant Physiol.* **29**:121–148
75. Zimmermann, U. 1980. *In:* Animals and Environmental Fitness, R. Gilles, editor. pp. 441–459. Pergamon Press, Oxford
76. Zimmermann, U. 1981. *In:* Polymers in Biology and Medicine. E. Goldberg, L. Donaruma, and O. Vogl, editors. Vol. 2. Wiley, Chichester, New York (*in press*)
77. Zimmermann, U., Beckers, F., Coster, H.G.L. 1977. *Biochim. Biophys. Acta* **464**:399–416
78. Zimmermann, U., Benz, R. 1980. *J. Membrane Biol.* **53**:33–43
79. Zimmermann, U., Büchner, K.-H., Benz, R. 1982. *J. Membrane Biol.* (*in press*)
80. Zimmermann, U., Groves, M., Schnabl, H., Pilwat, G. 1980. *J. Membrane Biol.* **52**:37–50
81. Zimmermann, U., Pilwat, G. 1978. Electrical breakdown of cell membranes. Abstract IV-19-(H) p. 140, *Sixth In. Biophys. Congr.*, Kyoto, Japan
82. Zimmermann, U., Pilwat, G., Beckers, F., Riemann, F. 1976. *Bioelectrochem. Bioenerg.* **3**:58–83
83. Zimmermann, U., Pilwat, G., Péqueux, A., Gilles, R. 1980. *J. Membrane Biol.* **54**:103–113
84. Zimmermann, U., Pilwat, G., Richter, H.-P. 1981. *Naturwissenschaften* **68**:577–578
85. Zimmermann, U., Scheurich, P. 1981. *Planta* **151**:26–32
86. Zimmermann, U., Scheurich, P. 1981. *Biochim. Biophys Acta* **641**:160–165
87. Zimmermann, U., Scheurich, P., Pilwat, G., Benz, R. 1981. *Angew. Chem.* **93**:332–351; and *Angew. Chem. Int. Ed. Engl.* **20**:325–344
88. Zimmermann, U., Vienken, J., Pilwat, G. 1980*a*. *Bioelectrochem. Bioenerg.* **7**:553–574
89. Zimmermann, U., Vienken, J., Pilwat, G. 1980*b*. *Z. Naturforsch.* **36c**:173–177
90. Zimmermann, U., Vienken, J., Scheurich, P. 1980. *Biophys. Struct. Mechanism* **6**:86

Received 30 September 1981