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Gene transfer into mouse lyoma cells by electroporation in high electric fields

E.Neumann*, M.Schaefer-Ridder, Y.Wang, and P.H.Hofschneider

Max-Planck-Institut für Biochemie, D-8033 Martinsried/München, FRG

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Electric impulses (8 kV/cm, 5 μs) were found to increase greatly the uptake of DNA into cells. When linear or circular plasmid DNA containing the herpes simplex thymidine kinase (TK) gene is added to a suspension of mouse L cells deficient in the TK gene and the cells are then exposed to electric fields, stable transformants are formed that survive in the HAT selection medium. At 20°C after the application of three successive electric impulses followed by 10 min to allow DNA entry there result 95 (±3) transformants per 10^6 cells and per 1.2 μg DNA. Compared with biochemical techniques, the electric field method of gene transfer is very simple, easily applicable, and very efficient. Because the mechanism of DNA transport through cell membranes is not known, a simple physical model for the enhanced DNA penetration into cells in high electric fields is proposed. According to this 'electroporation model' the interaction of the external electric field with the lipid dipoles of a pore configuration induces and stabilizes the permeation sites and thus enhances cross membrane transport.

Key words: electric impulses/gene transfer/mouse L cells/pBR322 plasmid

Introduction

A variety of biochemical methods have been developed to transfer genes into eukaryotic cells including incubation of the recipient cells with co-precipitates of DNA and Ca-phosphate (Graham and van der Eb, 1973), direct injection of genes into the nucleus of the recipient cell (Diacumakos, 1973), use of viral vectors (Hamer and Leder, 1979; Mulligan *et al.*, 1979), and application of liposomes as vehicles for gene transfer (Fraley *et al.*, 1980; Wong *et al.*, 1980; Schäfer-Ridder *et al.*, 1982).

Here we report a physical method that leads to an enormous enhancement of DNA transport across cellular membranes. The new technique stems from the observation that biomembranes are made transiently more permeable by the action of short electric impulses above a certain field strength, without damaging the membrane structures (Neumann and Rosenheck, 1972, 1973; Zimmermann *et al.*, 1973; Kinosita and Tsong, 1977). The electrically induced increase in permeability leads to a transient exchange of matter across the perturbed membrane structures. The electric field effect on transport is clearly a membrane phenomenon (Sale and Hamilton, 1968; Rosenheck *et al.*, 1975; Lindner *et al.*, 1977). Electric impulses not only induce or enhance exchange of material across membranes but they also cause membrane-membrane fusion when two membranes are in close contact with each other (Neumann *et al.*, 1980; Scheurich and Zimmermann, 1980; Weber *et al.*, 1981).

When circular or linear DNA carrying the thymidine kinase (TK) gene is added to a culture of mutant mouse cells (LTK^- cells) deficient in this gene, which is then subjected to a sequence of electric field pulses at 20°C the LTK^- cells take up large amounts of the plasmid DNA within a period of ~10 min after pulsing. The newly acquired TK activity is demonstrated by the survival of the transformed cells in a selection medium (Pellicer *et al.*, 1978). The first electric field experiments with LTK^- cells and DNA containing the TK gene showed that there is enhanced colony formation after pulsing (Wong and Neumann, 1982). However, the number of stable transformants in these first experiments was rather low.

Here, data are presented which lead to the suggestion of some optimum conditions for electrically mediated gene transfer. They show that the electric impulse method is a simple, easily applicable and very efficient technique to transfer linear and circular DNA into recipient cells. The electric method to increase membrane permeability for artificial DNA transfer should be generally applicable, and particularly valuable with those cell lines with which biochemical methods have not been successful.

Results

The electrically mediated DNA transfer into LTK^- cells strongly depends on the initial field strength of the electric impulses (Materials and methods). As shown in Figure 1, there is a threshold of ~6–7 kV/cm and an optimum field strength range of 8 (±0.5) kV/cm for DNA transfer leading to colony formation in the HAT selection medium. At higher field strengths the cells are irreversibly damaged (Wong and Neumann, 1982; see also Sale and Hamilton, 1968).

Although the presence of divalent ions like Mg^{2+} increases the amount of DNA bound to the cell surfaces (Wong and Neumann, 1982), Mg^{2+} actually reduces and finally prevents gene transfer. Figure 2 shows that increasing concentrations of $MgCl_2$ decrease the number of colonies; at 30 mM $MgCl_2$ the pulsed and unpulsed samples give the same number of colonies: 3 (±3) per 10^6 cells and per 1.2 μg DNA. When no

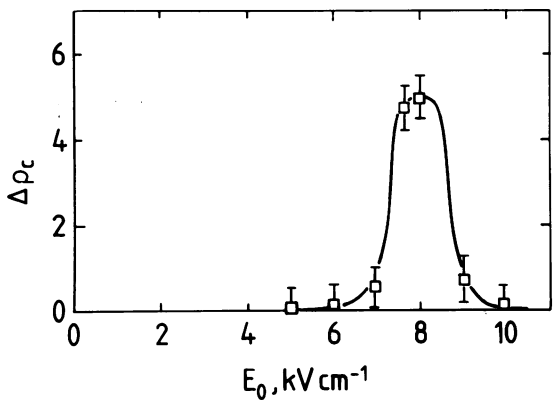

Fig. 1. The difference ($\Delta\varrho_c$) in the number of colonies (pulsed minus unpulsed sample) per 3.8 x 10^6 pulsed cells per 1 μg DNA at 20 mM $MgCl_2$ in HBS, as a function of the initial field intensity E_0 of exponentially decaying impulses with τ_E = 5 μs; see equation (13) of the text.

*To whom reprint requests should be sent.

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$MgCl_2$ is added to the mixture of cells and DNA the yield is 95 (±3) colonies per 10^6 cells and per 1.2 μg DNA.

To explore optimum conditions for this electrically induced gene transfer, non-optimum conditions with respect to $MgCl_2$ were chosen; eventual increases in the colony yield with increasing amounts of added DNA and higher cell densitities should be easily recognizable at 20 mM $MgCl_2$ (Figure 2). Figure 3 shows that the colony density increases with increasing amounts of DNA, both for linear and circular DNA. The numbers of colonies obtained with linear DNA are higher than those with circular DNA, for both the pulsed and the unpulsed samples.

At zero $MgCl_2$ the yield from a mixture of 20 μg circular DNA and 4 x 10^5 cells/0.4 ml is 30 (±3) colonies per 0.1 ml of the (pulsed) suspension. Since only 0.29 ml of the 0.4 ml sample volume are actually pulsed, this result means 500 (±20) transformants/10^6 cells/5 μg DNA. With the linear DNA the estimated yield is ~10^3 transformants/10^6 cells/5 μg DNA.

Figure 4 shows that the number of colonies also depends on the time interval between the electric pulses and the transfer of the pulsed cells to Dulbecco's minimal essential medium (DMEM; Materials and methods). At 20°C the saturation value is reached after ~10 min. This observation is in line with the fact that electrically induced perturbations of vesicle and cell membranes are long-lived compared with the duration of the impulse (Rosenheck *et al.*, 1975; Zimmermann *et al.*, 1976; Lindner *et al.*, 1977).

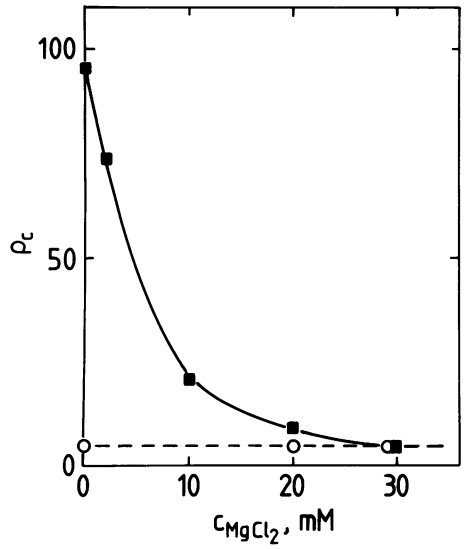

Fig. 2. Number of colonies (ϱ_c) per 10^6 pulsed cells and per 1.2 μg DNA in HBS, as a function of the $MgCl_2$ concentration; ■, pulsed; ○, unpulsed (control).

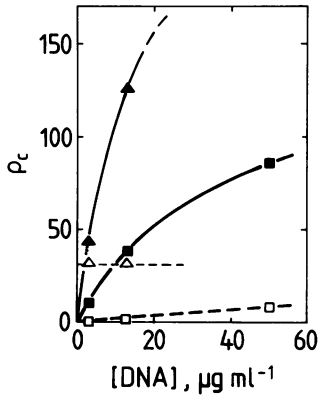

Fig. 3. Number of colonies (ϱ_c) as a function of the DNA concentration in the mixture of 5 x 10^7 cells/ml as a function of the DNA concentration, 20 mM $MgCl_2$, HBS. **(a)** circular DNA, ■; unpulsed control, □; **(b)** linear DNA, ▲; unpulsed control, △.

Discussion

Biological aspects

The results of this study on DNA transfer into LTK^- cells suggest some general guidelines for the determination of optimum conditions for electrically mediated gene transfer. In the case of LTK^- mouse cells, Mg^{2+} ions must be absent (see also Graham and van der Eb, 1973). At least three pulses of an initial field intensity of 8 (±5) kV/cm and a pulse decay time of ~5 μs should be applied at 20°C. The pulsed sample should be transferred into selection medium only 10 min after the pulsing. As expected, cell density and DNA concentration should be as high as possible; e.g. 5 x 10^7 cells/ml and 50 μg DNA/ml (cf. Figure 3).

Simple incubation of LTK^- cells with DNA occasionally leads to the formation of a few transformants (Figure 3). Therefore, DNA adsorbed on the cell surface is able to penetrate into the cell interior; the yield of colonies, however, is low compared with the colony yield after electric pulsing.

The detailed optimum conditions for the electric gene transfer into cells will depend on the cell type and the size of the cells (see below) and must be explored for each case along the lines described above. Even for non-optimum conditions the yield obtained with the field technique compares well with that of the present biochemical methods. The main advantage of the electric field procedure is, however, its ease of application and as a physical method it should be generally applicable.

Biophysical aspects

The mechanism of cross membrane transport of DNA is either an unspecific membrane process or it may be specifically mediated by permeases that are activated in high electric fields. A biological membrane is a co-operatively stabilized organization of lipids and proteins which contains dynamic, locally limited structural 'defects'. These local disorders are the candidates for the onset of further, electric-field induced perturbations, leading to permeation sites for enhanced material exchange. The additional electric potential difference

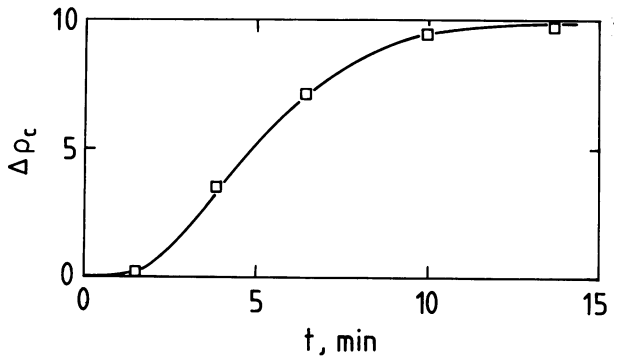

Fig. 4. The difference ($\Delta\varrho_c$) in the number of colonies (pulsed minus unpulsed) as a function of the incubation time after the pulsing. Experimental conditions: 5 x 10^6 cells/ml, 12 μg DNA/ml, 20 mM $MgCl_2$, HBS, 20°C.

ΔV which develops across the membrane is given by:

$$\Delta V = \Delta V_{(0)} \cdot \cos\theta \quad (1)$$

Therefore, the largest values of the field force vector exist at the 'pole caps' where $\theta = 0$; see Figure 5.

The maximum potential difference, ΔV_{max}, which a membrane of a cell or organelle experiences, depends on the cell size and is approximately given by:

$$\Delta V_{max} = \frac{3}{2} E_0 \, r \quad (2)$$

where E_0 is the initial field strength and r is the radius of the vesicle (Fricke 1953). When high electric fields are applied for only a short time (< 5 μs) the electric impulse lead to a reversible 'pore formation', probably in the lipid part of the membranes (Benz and Zimmermann, 1981; Teissie and Tsong, 1981) and various models have been proposed (Crowley, 1973; Zimmermann *et al.*, 1981; Abidor *et al.*, 1979).

The presence of an external electric field will favour charge and dipole configurations that leads to larger dipole moment components in the field direction. This in turn may lead to a thinning of membrane patches and finally to a hole, as proposed in Figure 5. The structural changes in a membrane domain forming a hole can be subjected to a general thermodynamic analysis. To a first approximation we may use a two-state model for the 'electroporation' process:

$$\{\text{closed structure}\} \underset{\bar{k}_{(c)}}{\overset{\bar{k}_{(o)}}{\rightleftharpoons}} \{\text{open structure}\} \quad (3)$$

The stability constant for this two-state model is given by the distribution of all membrane components B_j in the closed configuration, $B_j^{(c)}$, and in the open configuration, $B_j^{(o)}$, according to:

$$\bar{K} = \Pi\,[B_j^{(o)}]/\Pi[B_j^{(c)}] \quad (4)$$

For a stable, not permeable, membrane domain the inequality

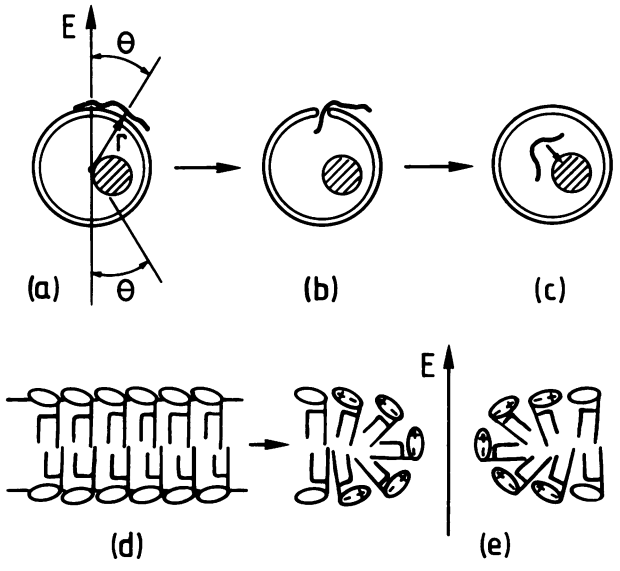

Fig. 5. Diagram of the electrically induced transfer of DNA into cells by the electroporation process. **(a)** Model of a spherical biological cell with nucleus and an external DNA on the cell membrane surface, relative to the direction of homogeneous electric field E; θ is the apical angle within which the membrane structure experiences an above-threshold field intensity and becomes permeable (cap). **(b)** Permeation of the DNA through a pathway induced by electroporation. **(c)** DNA association with the nucleus. **(d)** Unperturbed lipid domain in a membrane; **(e)** hole (pore), stabilized by a favourable lipid head group dipole interaction with the electric field.

$\bar{K} << 1$ holds, i.e., the concentration of hole areas is very small.

When we denote the stability constant of a single component interaction (of the B_j) by s_j and introduce an overall cooperativity parameter σ, then equation (4) may be rewritten in terms of these Zimm-Bragg parameters as:

$$\bar{K} = \sigma \Pi s_j \quad (5)$$

The net stability constant for the permeable structure is thus the product over all molecular contact sites j. The nucleation factor σ, with $\sigma << 1$ representing the low probability of nucleating a hole, assures a very steep structural transition curve, as may be derived from the data in Figure 1.

As an equilibrium constant, $\bar{K}$ will depend on the intensive state variables, temperatures (T), pressure (P), and a possibly applied electric field (E). The dependence of the domain stability on these state parameters may be generally written in a differential form as:

$$d \ln \bar{K} = \left(\frac{\partial \ln \bar{K}}{\partial T}\right)_{P,E} dT + \left(\frac{\partial \ln \bar{K}}{\partial P}\right)_{T,E} dP + \left(\frac{\partial \ln \bar{K}}{\partial E}\right)_{P,T} dE \quad (6)$$

Introducing the general van't Hoff relations, equation (6) is rewritten as:

$$d \ln \bar{K} = \frac{\Delta \bar{H}}{RT^2} dT - \frac{\Delta \bar{V}}{RT} dP + \frac{\Delta \bar{M}}{RT} dE \quad (7)$$

In this relationship the overall reaction enthalpy $\Delta \bar{H} = \Sigma_j \Delta \bar{H}_j$ is the sum of the component contributions $\Delta \bar{H}_j$ associated with s_j; $\Delta \bar{V} = \Sigma_j \Delta V_j$ is the overall volume change, and $\Delta \bar{M} = \Sigma_j \Delta M_j$ is the overall reaction dipole moment representing the difference between the dipole moments of the reaction products (hole configuration) and the components of the closed structure. From equation (7) it is clearly seen that a structural distribution, represented by $\bar{K}$, may be changed by a change of the temperature, or of the pressure, or by the application of an external electric field, provided that the overall reaction quantities $\Delta \bar{H}$, $\Delta \bar{V}$, and $\Delta \bar{M}$ have finite values, respectively.

Denoting by θ the degree of structural transition, then the overall equilibrium constant of the two-state model in equation (3) is generally given by

$$\bar{K} = \theta/(1 - \theta) \quad (8)$$

Note that $\theta = \Sigma[B_j^{(o)}]/(\Sigma[B_j^{(o)}] + \Sigma[B_j^{(c)}])$. It is now readily shown that the change in the degree of transformation is given by (Neumann, 1982):

$$d\theta = \theta\,(1 - \theta)\,\{\frac{\Delta \bar{H}}{RT^2} dT - \frac{\Delta \bar{V}}{RT} dP + \frac{\Delta \bar{M}}{RT} dE\} \quad (9)$$

This relationship demonstrates that the extent of a structural change depends, in addition, on the 'position' value θ of the distribution. Clearly, a maximum relative change is produced if $\theta << 1$, i.e., if the entire membrane initially is in a closed configuration.

Equation (9) rationalizes the structural domain transitions thermodynamically, but also the enormous difference in the kinetics of pore formation and annealing can be generally formulated. The overall rate constants for the forward and backward direction in equation (3) may be denoted by $\bar{k}_{(o)}$ and $\bar{k}_{(c)}$, respectively. We recall that the reaction dipole moment $\Delta M = (\partial M/\partial \xi)$ is the difference between the dipole contributions of the products and the reactions. For equation (3), we have:

$$\Delta \bar{M} = \left(\frac{\partial \bar{M}_{(o)}}{\partial \xi}\right)_E - \left(\frac{\partial \bar{M}_{(c)}}{\partial \xi}\right)_E \quad (10)$$

where $M_{(o)}$ is the macroscopic dipole moment of the open configuration, $M_{(c)}$ that of the closed structure, and ξ is DeDonder's general reaction variable describing the extent of structural transition. Since the open structure is favored in the field, the inequality:

$$\left(\frac{\partial \bar{M}_{(o)}}{\partial \xi}\right)_E > \left(\frac{\partial \bar{M}_{(c)}}{\partial \xi}\right)_E \quad (11)$$

must hold for the field strength range $E \geq E_{th}$, where E_{th} is the threshold value.

The formation of colonies of LTK^+ cells in the selection medium is certainly the result of DNA penetration through permeation sites induced by the electric field action on the cell membranes. The steep dependence of the colony density, ϱ_c, on the field strength, as shown in Figure 1, reflects a steep dependence of θ on E, and thus a large value of $\Delta\bar{M}$ which, most likely, is caused by a co-operative transition involving a larger number of membrane constituents in a (co-operative) domain.

The structural change leading to an open hole configuration is rapidly introduced by the field within a few microseconds. The annealing of the structural perturbation after the electric field has decayed to zero, is associated with time constants in the second and minute range. Obviously there is a great rate of enhancement for a transition of the type given in equation (3) in the presence of the field. Now, straightforward thermodynamics describes the field dependence of the opening rate constant according to (Neumann, 1982):

$$\bar{k}_{(o)}(E) = \bar{k}_{(o)}(0) \cdot \exp[(RT)^{-1} \int (\partial M_{(o)}/\partial \xi)_E dE] \quad (12)$$

Clearly at $E = 0$, the low background DNA penetration of the unpulsed sample must mean that $\bar{k}_{(o)}(0) << k_{(c)}(0)$ holds. The annealing process after the pulsing (where $E = 0$) is simply described by $k_{(c)}(0)$. As seen in equation (12), $\bar{k}_{(o)}$ is greatly enhanced in the field when $M_{(o)}$ increases during the structural transition.

The expressions (9)–(12) are fundamental relations for a thermodynamic and kinetic analysis of electric field-induced changes in the structure of membranes in general; they may be specifically applied to describe particle transfer through cellular membranes such as the electrically mediated DNA transfer into cells.

Materials and methods

Cells and DNA

Mouse LTK^- cells were grown in DMEM with 10% fetal calf serum. The cells were trypsinized, washed, and resuspended in HEPES buffered saline (HBS: 140 mM NaCl, 25 mM HEPES, 0.75 mM Na_2HPO_4, pH 7.1 at 20°C) to a final cell density of 5×10^7 cells/ml.

The recombinant plasmid, pAGO, carrying an active TK gene (2 kb of the *Pvu*II fragment of HSV I) inserted in the *Pvu*II site of pBR322 (Colbere-Garapin *et al.*, 1979) was used for transformation experiments, either in its supercoiled form or in the linear form by digestion with *Hind*III.

The solutions of the LTK^- cells were incubated for 10 min with various amounts of DNA, up to 20 μg DNA/0.4 ml, with and without $MgCl_2$, before transferring the solution to the electric measuring cell for pulsing. To maintain sterile conditions the sample cell was washed with 70% alcohol; gloves and mouth protection are necessary to avoid contamination.

About 10 min after the electric impulses, aliquots of 100 μl of the cell suspension were transferred into 10 ml DMEM and seeded into two 10-cm Petri dishes. To select for stable transformants the cell monolayers were incubated after 24 h with HAT medium (hypoxanthine, aminopterin, thymidine) under standard conditions (Pellicer *et al.*, 1978). After 2 weeks in HAT selection medium the TK-positive colonies were counted.

The electric impulse method

The measuring cell of an electric discharge circuit (Figure 6) is filled with 0.35 ml DNA cell suspension and then subjected to a series of impulses.

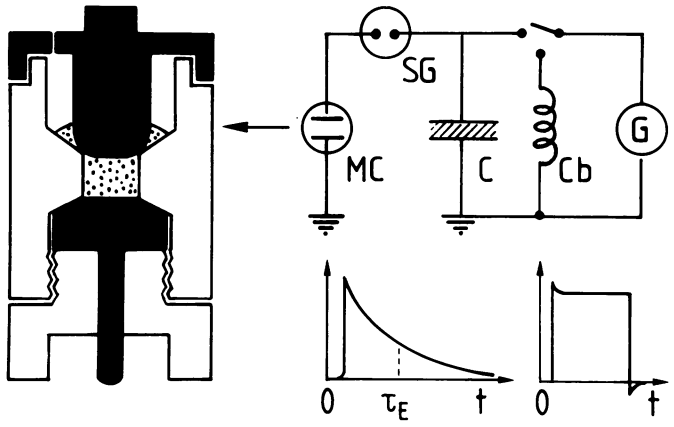

Fig. 6. Diagram of the measuring cell (MC), filling volume 0.35 ml, for the electric impulse experiments; discharge circuit for capacitor discharge (C), or alternatively, cable discharge (Cb); G, high voltage generator; SP, spark gap. Time courses of the electric field for capacitor discharge and for rectangular pulses from the cable discharge, respectively.

Usually three successive pulses are applied at an interval of 3 s. The special cell construction permits the required bubble-free closure of the measuring cell; 0.29 ml of the solution is exposed to the electric field.

A high voltage generator provides the electrical energy, which at first is stored in a high voltage capacitor or, alternatively, in a 1000 m coaxial cable. The discharge is triggered manually by a spark-gap (Figure 6). The temperature of the thermostated sample cell is 20°C.

In case of the capacitor discharge, the inductance of the circuit is so low that the voltage, U, across the conducting solution between the electrodes of the sample cell decays exponentially from an initial value U_0. Since the electric field strength is defined by $E = U/d$, where d is the distance between the electrodes, the exponential decay of the field force is given by:

$$E(t) = E_0 \exp(-t/\tau_E) \quad (13)$$

The field-decay time constant is $\tau_E = RC$; where C is the capacitance of the discharge capacitor (C = 12, 21, or 52 nF), $E_0 = U_0/d$ and R is the resistance of the discharge unit. The resistance of the sample cell (>200 Ω) usually determines the circuit resistance.

Because of the finite conductance of the solutions the electric energy of the impulse dissipates. The final temperature increase is given by:

$$\Delta T = 0.5\, C\, U_0^2/(\varrho c_p v) \quad (14)$$

where ϱ is the density of the solution, c_p is the heat capacity, and $v = 0.29\ cm^3$ is the effective volume that experiences the electric field. Here we may use the approximations $\varrho \approx 1$ g/cm, $cp \approx 4.12$ J/(gK) (see, e.g., Neumann and Rosenheck, 1972).

As shown above (Results), optimum conditions for successful gene transfer into LTK^- cells are $U_0 = 8$ kV, $C = 21$ nF, and $R = 250$ Ω. Thus, with equation (13), the field-decay constant is $\tau_E = 5.4$ μs. From equation (14) we obtain $\Delta T = 0.55$°C; thus the temperature increase per pulse is negligible.

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References

Abidor,J.Y., Arakelyan,V.B., Chernomordik,L.V., Chizmadzhev,Y.A., Pastushenko,V.F., and Tarasevich,U.R. (1979) *Bioelectrochem. Bioenerget.*, **6**, 37-52.

Benz,R., and Zimmermann,U. (1981) *Biochim. Biophys. Acta*, **640**, 169-178.

Colbere-Garapin,F., Chousterman,S., Horodniceanu,F., Kourilsky,P., and Garapin,A.C. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3755-3759.

Crowley,J.M. (1973) *Biophys. J.*, **13**, 711-724.

Diacumakos,E.G. (1973) in Prescott,D.M. (ed.), *Methods in Cell Biology*, Vol. 7, Academic Press, pp. 287-311.

Fraley,R., Subrami,S., Berg,P., and Papahadjopoulos,D. (1980) *J. Biol. Chem.*, **255**, 10431-10435.

Fricke,H. (1953) *J. Appl. Phys.*, **24**, 644-646.

Graham,F.L., and van der Eb,A.J. (1973) *Virology*, **52**, 456-467.

Hamer,D.H., and Leder,P. (1979) *Cell*, **18**, 1299-1302.

Kinosita,K.,Jr., and Tsong,T.Y. (1977) *Biochim. Biophys. Acta,* **471**, 227-242.
Lindner,P., Neumann,E., and Rosenheck,K. (1977) *J. Membr. Biol.,* **32**, 231-254.
Mulligan,R.C., Howard,B.H., and Berg,P. (1979) *Nature,* **277**, 108-111.
Neumann,E., and Rosenheck,K. (1972) *J. Membr. Biol.,* **10**, 279-290.
Neumann,E., and Rosenheck,K. (1973) *J. Membr. Biol.,* **14**, 194-196.
Neumann,E., Gerisch,G., and Opatz,K. (1980) *Naturwissenschaften,* **67**, 414-415.
Neumann,E. (1982) in Zimmerman,U. (ed.), *Electric Field Effects in Macromolecules and Membranes,* Springer Verlag, Berlin. pp. 95-193.
Pellicer,A., Wigler,M., Axel,R., and Silverstein,S. (1978) *Cell,* **14**, 133-141.
Rosenheck,K., Lindner,P., and Pecht,I. (1975) *J. Membr. Biol.,* **12**, 1-12.
Sale,A.J.H., and Hamilton,W.A. (1968) *Biochim. Biophys. Acta,* **163**, 37-43.
Schäfer-Ridder,M., Wang,Y., and Hofschneider,P.H. (1982) *Science (Wash.),* **215**, 166-168.
Scheurich,P., and Zimmermann,U. (1980) *Z. Naturforsch.,* **35c**, 1081-1085.
Scheurich,P., and Zimmermann,U. (1981) *Naturwissenschaften,* **68**, 45-46.
Teissie,J., and Tsong,T.Y. (1981) *Biochemistry (Wash.),* **20**, 1548-1554.
Weber,H., Förster,W., Jacob,H.E., and Berg,H. (1981) *Z. Allg. Mikrobiol.,* **21**, 555-562.
Weber,H., Förster,W., Berg,H., and Jacob,H.E. (1981) *Curr. Genet.,* **4**, 165-166.
Wong,T.-K., Nicolau,C., and Hofschneider,P.H. (1980) *Gene,* **10**, 87-94.
Wong,T.-K., and Neumann,E. (1982) *Biochem. Biophys. Res. Commun.,* **107**, 584-587.
Zimmermann,U., Schulz,J., and Pilwat,G. (1973) *Biophys. J.,* **13**, 1005-1013.
Zimmermann,U., Riemann,F., and Pilwat,G. (1976) *Biochim. Biophys. Acta,* **436**, 460-474.
Zimmermann,U., Scheurich,P., Pilwat,G., and Benz,R. (1981) *Angew. Chem.,* **93**, 332-351.