Gene Transfer by Electroporation

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Abstract

Electroporation of cells in the presence of DNA is widely used for the introduction of transgenes either stably or transiently into bacterial, fungal, animal, and plant cells. A review of the literature shows that electroporation parameters are often reported in an incomplete or incorrect manner, forcing researchers to rely too much on a purely empirical trial and error approach. The goal of this article is to provide the reader with an understanding of electrical circuits used in electroporation experiments as well as physical and biological aspects of the electroporation process itself. Further, a simple paradigm is provided which unites all electroporation parameters. This article should be particularly useful to those new to the technique.

Index Entries: Animal cells; electroporation; microeukaryotes; plant cells; plant protoplasts; prokaryotes; transfection; transformation.

1. Introduction

No gene transfer technique other than electroporation can be applied equally successfully to prokaryotic and eukaryotic cells without major modifications and adaptation to cell type and origin. Therefore, the versatility of the electroporation process accounts for its popularity in transfection/transformation experiments. As the name indicates, electroporation consists of subjecting cells to an electric field, on which pores are formed in the lipid bilayer and other components of the cell membrane, allowing compounds to enter and leave the cytoplasm. Pore formation is reversible and cell survival ensues, provided some electrical parameters are not exceeded.

The first evidence that gene transfer can be achieved by electroporation was published 15 yr ago in the case of mouse lyoma cells *(1)*. Plant protoplasts were electrotransformed in 1985 *(2)*, while the first evidence for bacterial transformation was published in 1987 *(3)*. To date, many animal, plant, and bacterial cell types have been genetically engineered with this technique.

It is not the purpose of this review to give an exhaustive list of cell types and lines that have been successfully electrotransformed; this would achieve little. Rather, the goal here is to provide a solid theoretical introduction to electrical and biological parameters influencing the electroporation process. Commercial electroporation units being now available, and thus freeing one from building and understanding them, it is quite possible that researchers have stopped thinking about the process itself and simply "zap" cells, often without result. Negative results are frequently the result of a misunderstanding of the technique, and a considerable amount of time can be wasted trying to reproduce published electroporation conditions that may have been misrepresented in the first place. For example, one blatant omission is often that of the electrical resistance of the whole system (including the sample), which is seldom, if ever, given. Thus, the first section of this article will focus on the theory of electrical circuits in a user-friendly manner for molecular biologists.

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Most electroporation units in use today are based on the discharge of a capacitor in a sample containing cells. However, electrotransformation has also been achieved using square-wave pulses, and both approaches are discussed. Mathematical derivations in this section do not go beyond simple calculus and are required for a full understanding of electroporation parameters. Otherwise, electroporation becomes another "cookbook recipe" for gene transfer.

The second section of the article discusses some of the theory of membrane breakdown under the influence of an electric field with emphasis on concepts relevant to molecular biologists. Here again the purpose is not to get into arcane discussions on the latest model built to explain pore formation. Rather, this section attempts to make sense out of the theory as it may apply in the laboratory. The third section will cover examples of DNA transfer in prokaryotes and eukaryotes with emphasis on biological parameters. Finally, a good coverage of the topic cannot be achieved without resorting to the older literature, published as the electroporation process was being developed.

2. A Brief Theory of the Electric Field and Circuits

The basic law describing electric circuits can be written:

$$V = \oint E \, ds = I R \qquad (1)$$

where V is the electromotive force (or electric potential) expressed in volts (V), E is the electric field expressed in V/cm, s is the distance in cm, I is the current in amperes (A), and R is the resistance measured in ohms (Ω).

The current can also be described as $I = dQ/dt$ where Q is the charge (in coulombs) moving through a conductor and t is time in seconds.

The resistance R of a circuit is a quantity which depends on the length (l in cm) of the conductor, its cross-section (s in cm^2), and its resistivity (ρ in Ω/cm). Resistivity values vary enormously according to the chemical and physical nature of the conductor and its temperature.

Thus,

$$R = \rho \, l/s \qquad (2)$$

Therefore, the strength of the electric field E applied to a sample depends on a large number of interrelated parameters, all of which will influence the results of electroporation experiments. Fortunately, these parameters can be carefully controlled or measured.

2.1. Capacitor Discharge in a Circuit

As stated, most investigators currently perform electroporation of cells by means of a capacitor discharge. The critical parameters influencing the discharge are discussed in the following.

2.1.1. Interelectrode Distance

Electrode distance and electric potential (and hence electric field strength) are inversely related as discussed and illustrated in **Fig. 1A**. Let us assume two flat, parallel electrodes a and b separated by a distance d. Electrode a has an electric potential, V_a, whereas b has a different electric potential, V_b. If V_a is positively charged and V_b negatively charged, the electric field E will by definition be a vector quantity directed from a to b. Let us then assume that x_a and x_b are the coordinates of the electrodes on the x axis. Equation 1 can then be rewritten as:

$$V_{ab} = \int_a^b E \cos \theta \; ds \qquad (3)$$

where V_{ab} is the potential difference between a and b, E is the electric field, ds is the distance between the electrodes, and θ the angle they form. In electroporation experiments, V_{ab} is determined by setting the power supply to a given voltage value. In the case of **Fig. 1**, since the electrodes are parallel, $\cos \theta = \cos 0° = 1$, whereas $ds = dx$. Thus, solving **Eq. 3** one obtains:

$$V_{ab} = E \int_{x_a}^{x_b} dx = E(x_b - x_a) = E \, d \qquad (4)$$

or,

$$E = V_{ab}/d \qquad (5)$$

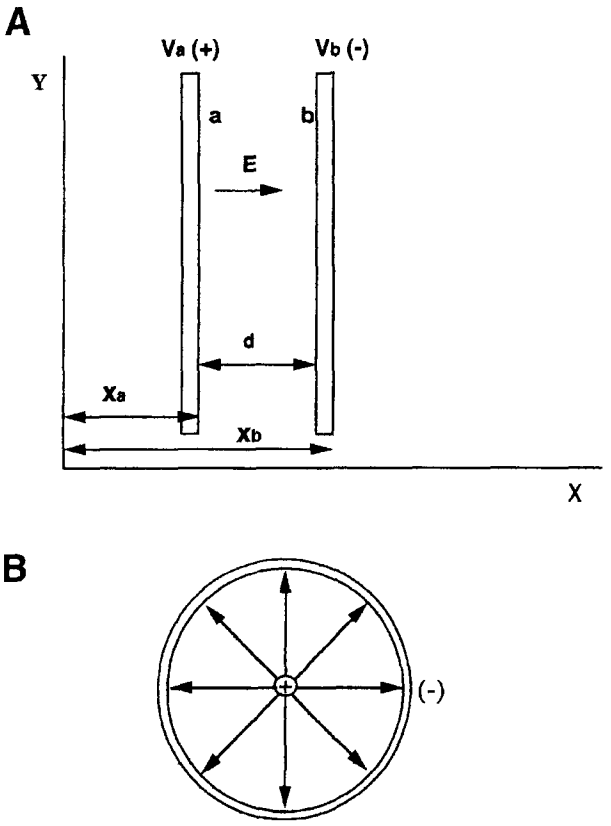

Fig. 1. **(A)** Relationship between electrode potential, gap, and electric field strength in the case of flat parallel electrodes. **(B)** Gaussian surface for a charged cylinder (cross-section) representing concentric circular electrodes. The central electrode is positively charged and the field is directed radially outward. For definition of symbols *see* **Subheading 2.1.1.**

where V_{ab}/d is the potential gradient measured in V/cm. Hence, the shorter the interelectrode distance, the higher the electric field strength at constant V_{ab}. For example, the value of E at a setting of 1000 V will be 1000 V/cm if the electrode gap is 1 cm but E will equal 2000 V/cm if this distance is reduced to 0.5 cm.

It should be noted that **Eq. 5** is valid only when parallel, flat electrodes are used. Indeed, in this case, the field will be homogeneous and normal to the plates. If however, circular electrodes are used, the field will be inhomogeneous and **Eq. 5**, which is the simplest consequence of Gauss' law describing the field at the surface of a conductor, will not apply. Circular electrodes generate electric fields as depicted in **Fig. 1B**.

2.1.2. RC Circuits

A simple *RC* circuit used in electroporation experiments is one consisting of a power supply providing an adjustable voltage plus a capacitance and a resistance in series. Such a circuit is diagrammed in **Fig. 2**.

There, C is the capacitor that can be charged to a maximum value Q (in coulombs). The capacitance of a capacitor is defined as $C = Q/V_{ab}$ where V_{ab} is the potential difference established across the capacitor. C is measured in farads (F).

When the switch S in **Fig. 2** is closed, the charge of the capacitor does not increase instantaneously to its final value Q. Let q represent the charge at a time t, and i the current in the circuit at that instant. The instantaneous potential difference across the capacitor is then $V_{ax} = q/C$ and the potential difference across the resistor is $V_{xb} = iR = R\,dq/dt$ (Ohm's law) since $i = dq/dt$. But, the total electric potential of the circuit is

$$V = V_{ax} + V_{xb} \tag{6}$$

Thus,

$$V = q/C + R\,dq/dt \tag{7}$$

or,

$$CV = (RC)\,dq/dt + q \tag{8}$$

and,

$$dq/(CV - q) = (1/RC)\,dt \tag{9}$$

Since $q = 0$ at $t = 0$, we integrate between limits as follows:

$$\int_0^Q \frac{dq}{(CV - q)} = \frac{1}{RC}\int_0^t dt \tag{10}$$

and get

$$-\ln (CV - q/CV) = t/RC \tag{11}$$

Hence,

$$CV - q = CV\,e^{-t/RC} \tag{12}$$

Since the asymptotic value at large t of CV is Q, we obtain:

$$q = Q\,(1 - e^{-t/RC}) \tag{13}$$

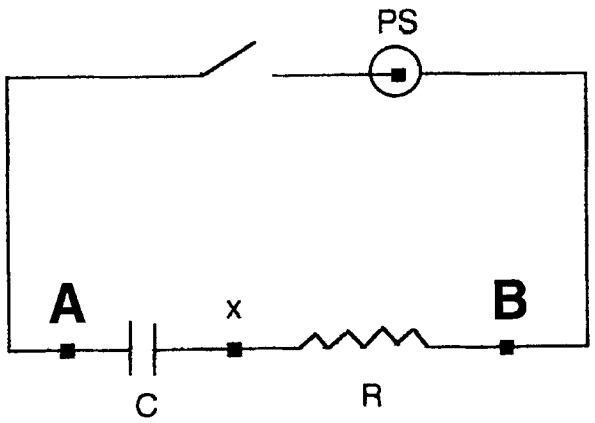

Fig. 2. Simple *RC* circuit. PS, adjustable power supply; *C*, capacitor; and *R*, resistor. Other symbols are defined in **Subheading 2.1.2.**

which is graphed in **Fig. 3A** and represents the rate of charge of a capacitor. If the capacitor is originally charged and then discharged through a resistance *R*, the process is reversed and the charge *q* decreases with time according to:

$$q = Q\,e^{-t/RC} \qquad (14)$$

which is graphed in **Fig. 3B**.

It can be seen that at $t = RC$, $q = Q/e$ or $q = (1/2.718) \times Q = 0.369\,Q$. Thus, at $t = \tau =$ the *RC* (or time) constant (in s) of the circuit, approx 63% of the initial charge will have been dissipated. At a time equal to approx 5 τ, the discharge reaches 99%. Also, since $q/C = V = iR$, and given that *C* and *R* are constant, both the voltage *v* and the current *i* across the resistance decay at exactly the same rate as *q*. Thus, **Eq. 14** can be rewritten:

$$vC = V\,Ce^{-t/RC} \qquad (15)$$

where *v* is the instantaneous voltage across *R* and *V* the voltage at $t = 0$. The initial current *I* at $t = 0$ is $I = V/R$. Therefore, if the charging voltage was 300 V and the resistance of the circuit is 300 Ω, *I* equals 1 A. Thus, a high voltage combined with a low resistance (circuit plus sample) can lead to very high current values, potentially produce arcing across the sample, and damage to the equipment, the researcher, and the cells in the sample. Damage to cells will be owing to high rates of electrolysis and temperature increase.

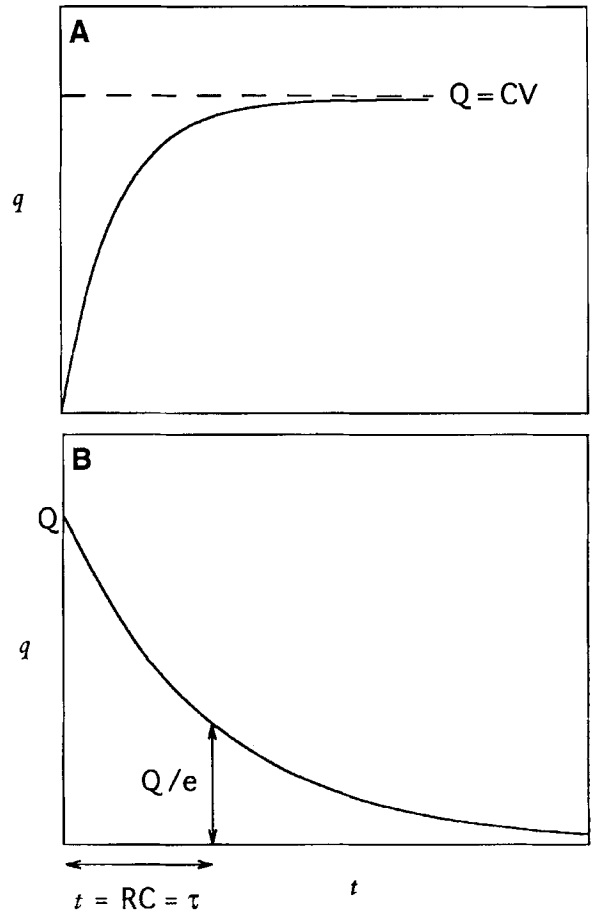

Fig. 3. **(A)** Charge of a capacitor as a function of time. *Q* is the maximum charge in coulombs and *q* is the instantaneous charge. **(B)** Discharge of a fully charged capacitor. Time *t* at $q = Q/e$ is by definition the time (or $RC = \tau$) constant of the *RC* circuit.

As indicated, $\tau = RC$ is measured in s. For example, if $R = 2000\ \Omega$ and $C = 400 \times 10^{-6}$ F (400 μF), the *RC* constant of the circuit will be 800,000 $\times 10^{-6}$ s $= (8 \times 10^5) \times 10^{-6} = 0.8$ s $= 800$ ms. If *R* is only 50Ω and $C = 2 \times 10^{-6}$ F (2 μF), then $RC = 10^{-4}$ s or 0.1 ms. As is obvious, *both* the capacitance *and* the resistance of the circuit determine the value of τ. It is critical to remember this, obvious as it is, because inasmuch as the value of *C* can be predetermined by installing capacitors of known capacitance, the value of *R* cannot be readily calculated. Indeed, **Fig. 4** demonstrates that the electroporation sample is very much part of the *RC* circuit, either in series (**Fig. 4A** or in parallel

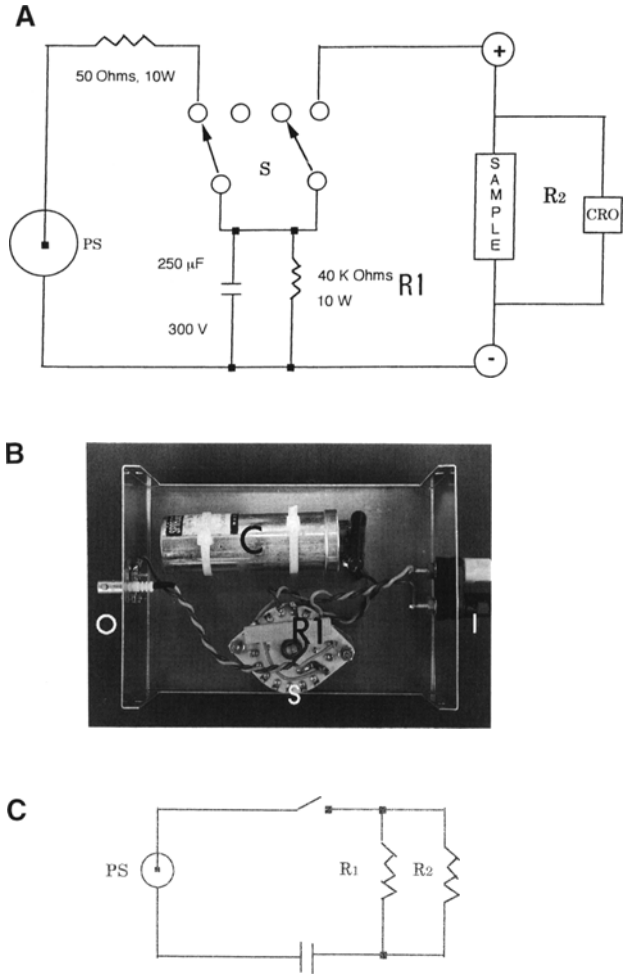

Fig. 4. (**A**) Simple electroporation unit built at Washington State University shown in its charge mode. S is the charge/discharge switch. PS is the power supply used to charge a 250-μF electrolytic capacitor to a maximum voltage of 300 V. The capacitor is installed in parallel with a 40-kΩ resistor that is part of the charge circuit. In the discharge mode, the capacitor releases its charge through R_1, a 40-kΩ resistor, and R_2, the electroporation sample. Since R_1 and R_2 are in series, the total resistance is $R_1 + R_2 +$ circuit resistance. A cathode ray oscilloscope (CRO) is used to monitor voltage decay and the RC constant. (**B**) Photograph of the unit in 6.A. C is the capacitor. Power supply input (I) is on the right while the output jack connecting to the electrodes (O) is on the left. S is the switch. (**C**) Schematic electroporation unit where a built-in resistor R_1 is in parallel with the sample R_2. In general, circuit resistance (other than R_1 and R_2) can be ignored.

(**Fig. 4C**) with a built-in resistor. The next section shows that not all electrical parameters influence the time constant.

2.1.3. Influence of R, C, and V on τ

As seen earlier, the resistance of the sample is critical in determining the value of the time constant. This resistance is determined by **Eq. 2**, $R = \rho\, l/s$, where in the case of the sample, ρ is its own resistivity, l is the electrode gap, and s is the surface area of the electrode in contact with the sample. Homemade electrodes are often built to fit in a disposable spectrophotometer cuvet and have a surface area approx equal to $2.8 \times 0.4 = 1.12$ cm^2. The electrode gap is usually 0.9 cm, a good fit, in order to maximize the number of cells present between the electrodes (in the case of prokaryotes, the gap is usually 0.2 cm in order to provide greater field strength).

The resistivity of the sample is not so simple to calculate. Indeed, ρ depends on the ionic strength of the medium and its temperature. In turn, the ionic strength depends on the salt composition, that is, salt concentration and degree of ionization. For example, **Table 1** gives the variation of ρ for KCl (a highly ionizable salt) in water as a function of concentration and temperature. As can be seen, ρ varies enormously as a function of concentration and very significantly with temperature. The equivalent of 1*M* KCl is of course well above what is found in physiological solutions. Most electroporation media will have an ionic strength somewhere between that of 0.01 and 0.1*M* KCl or lower (or sometimes higher, as in the case of physiological saline), meaning a range of resistivities spanning at least one order of magnitude. Thus, variations in the salt composition of electroporation media will have a drastic effect on the RC constant. For example, with $C = 500$ μF, $l = 0.9$ cm, $s = 1.12$ cm^2, and ρ = 600 Ω/cm, R will be equal to 482 Ω and RC = 241 ms. At ρ = 60 Ω/cm, RC will be equal to 24.1 ms.

To calculate an accurate value of R for the system, one must of course add to the resistance of the sample that of the circuit (R_1 in **Fig. 4A**). Such calculations are often impractical and direct measurement of R_2

Table 1
Resistivity of Aqueous KCl Solutions in Ω/cm as a Function of Concentration (moles/L) and Temperature[a]

	T (°C)		
KCl solution	0°	10°	25°
1*M*	15.3	12.0	8.9
0.1*M*	139.8	107.2	77.6
0.01*M*	1288.6	980.4	707.7

[a]Source: Handbook of Chemistry and Physics, Chemical Rubber Publishing Co.

is better done with an ohmmeter (alternatively, *RC* can be measured directly with an oscilloscope). Finally, since $\tau = RC$, it is obvious that the same time constant can be achieved either by changing *R* while keeping *C* constant, or the other way around. However, it would be false to think that an *RC* constant achieved by a high value for *R* should give the same results as one obtained at high *C* (and thus lower *R*) (*see* **Subheading 2.1.4.**).

It should be noted that commercial electroporation units usually have built-in resistors installed in parallel (rather than in series) with the sample. In a parallel circuit, the total resistance is calculated using the reciprocals of the individual resistance values. The built-in resistors are usually 200, 400, 600 and 800 Ω. Let us then assume for example, that the circuit resistance is set at 200 Ω and that the sample is poorly conductive and has a resistance of 2000 Ω. The total value of the resistance will be $1/R = 1/200 + 1/2000$ meaning that $R = 182$ Ω (as opposed to 2200 Ω in a series circuit). If the sample is moderately conductive and has a resistance of 200 Ω, then $1/R = 1/200 + 1/200$, making $R = 100$ Ω (instead of 400 Ω in series). If the resistance of the sample is ignored, *R* will be mistakenly thought to be 200 Ω, of course. The situation will get worse if the resistance is set, for example, at 800 Ω. Indeed, calculations show that with a resistance value of 2000 Ω for the sample, the total resistance will be 571 Ω, and if the sample has a resistance of 200 Ω, the total resistance will be only 160 Ω. Obviously, relying solely on the value of the set resistance will not allow one to compute a correct *RC* constant.

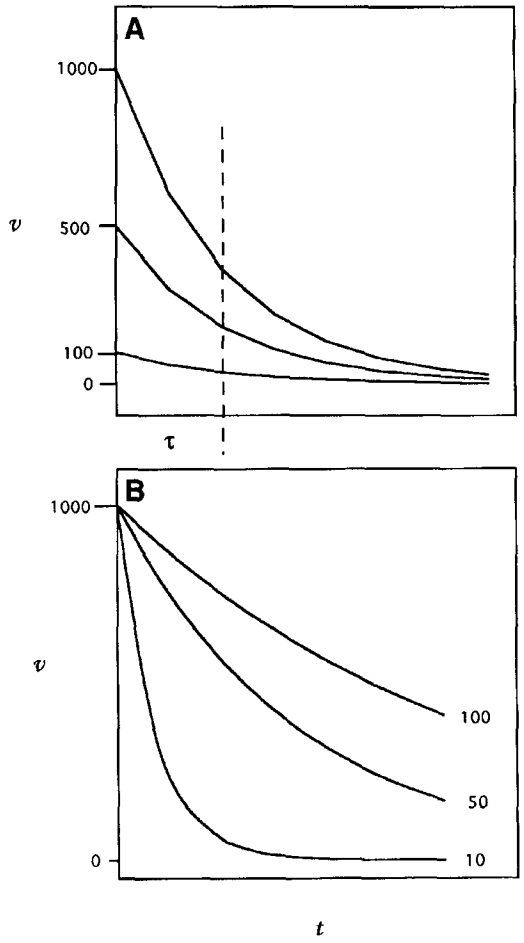

Fig. 5. Voltage decay in an *RC* circuit as a function of various parameters. **(A)** Variable voltage at *R* and *C* constant. The vertical broken line is drawn at $t = RC$ for all three curves. **(B)** Same initial voltage decay with variable *C* and *R* constant. The values of *RC* were set at 10, 50, and 100 ms. Same results as in (B) can be obtained by modifying *R* while keeping *V* and *C* constant. However, this will change the energy parameters of the discharge (*see text*).

Figure 5 shows the voltage decay in an *RC* circuit as a function of various parameters. Importantly, **Fig. 5A** shows that the *RC* constant is independent of the applied voltage. However, as we see later, it would be erroneous to conclude that voltage is an irrelevant parameter in electroporation experiments. Further, achieving different values for τ by keeping *C* constant and varying *R* has different implications regarding the amount of energy released into the system.

2.1.4. Energy Stored in a Capacitor

We have seen that $V_{ab} = q/C$ where V_{ab} is the potential difference across the capacitor, C is the capacitance, and q is the instantaneous charge of the capacitor during the process of charging. Thus, the work done to transfer the next charge dq is $dW = V_{ab}\, dq = (1/C)\, q dq$. Hence, the total work needed to reach Q (the maximum charge) is:

$$W = \int dW = \frac{1}{C}\int_0^Q q\, dq = \frac{1}{2}\left(\frac{Q^2}{C}\right) \quad (16)$$

Since $V_{ab} = Q/C$, **Eq. 16** can be rewritten:

$$W = 1/2\ (CV^2) \quad (17)$$

where W is expressed in joules (J).

Therefore, a discharging capacitor releases W amount of energy into the circuit, of which the sample is a part. Further, W varies with the square of the applied voltage and with the capacitance, but not with R. For example, a 500-µF capacitor charged to 200 V will release $1/2\ (500 \times 10^{-6}) \times 40{,}000 = 10$ J into the system.

The rate at which energy is released, or power, is $P = dW/dt$, expressed in J/s or watts. If the resistance of the circuit (including that of the sample) is 300 Ω, the RC constant equals 150 ms. Since capacitor discharge is 99% complete after $5 \times \tau$, 9.9 J will be dissipated after 750 ms meaning that the power will be 9.9/0.75 = 13.2 J/s. Of course, given the form of **Eq. 14**, 13.2 J/s is the *average* value of the power. At $t = \tau$, 63% of the energy is dissipated; hence, the power at that time is 6.3 J/0.15 s = 42 J/sec. At $t = 1/2\ \tau$, when approx 34% of the energy is released, the power is 45.3 J/s.

2.1.5. Maximum Voltage Applicable to a Capacitor

A capacitor consists of two conductors, known as plates or electrodes separated by an insulator, the dielectric. The capacitance of the capacitor depends on the area a of the plates and the thickness d of the dielectric. In addition, the permittivity of the dielectric ε, determines C in that $C = \varepsilon\, a/d$. Electrolytic capacitors can achieve a high capacitance (>1000 µF) in spite of a relatively low value for ε because d can be as little as 0.1 µm as the dielectric consists of a very thin layer of, for example, aluminum oxide. In addition, their large size (up to several cm in diameter) provides a large plate area. However, because of the low permittivity of the dielectric, excessive electric intensities (voltages) will lead to a breakdown of the insulating properties of the dielectric and the creation of a large current surge. Ceramic dielectric capacitors of the high-ε type can be operated safely at higher voltages but display a lower capacitance thanks to their smaller size. In brief, voltage ratings for capacitors should never be exceeded.

2.1.6. Summing Up the Characteristics of Capacitor Discharge

Several general rules can be derived from the aforementioned theoretical considerations. They are:

1. Electric intensity at constant voltage is inversely proportional to electrode distance.
2. Flat parallel electrodes provide a uniform electric field. Circular electrodes do not.
3. The RC constant is independent of voltage but cannot be evaluated correctly if the resistance of the sample is unknown. Ignoring the value of the resistance can lead to severe under- or overestimation of RC by as much as one order of magnitude.
4. The resistance of the sample critically depends on the salt concentration and the temperature. Lowering the temperature increases R (and hence increases RC), while increasing the salt concentration decreases both R and RC.
5. At low R values (combination of high salt and room temperature), the current I can reach dangerous values. At 500 V, if R = 10 Ω (a highly conductive sample), then I = 50 A at t = 0. Highly conductive samples exist when plant protoplasts are suspended in high salt solutions used as osmoticum (osmotic protectant). It is clear that mannitol is a more preferable osmoticum in this case. It should also be remembered that for resistors in series, the total resistance of the circuit will be $R = R_1 + R_2 + \ldots R_n$, whereas for resistors in parallel, the reciprocal of the equivalent resistance equals the sum of the reciprocals of the individual resis-

tors, that is, $1/R = 1/R_1 + 1/R_2 + \ldots 1/R_n$. This relationship is reversed for capacitors.

6. The power (energy per unit time) released by capacitor discharge is very high at first and then tapers off as t increases. As voltage decays, the rate of energy release diminishes accordingly.
7. Voltage ratings for capacitors and power ratings for resistors should not be exceeded.

2.2. The Square-Wave Pulse

Square-wave pulses do not rely on capacitor discharge into the circuit.

2.2.1. Principle

Square-wave pulse generators time a voltage pulse through the use of fast switches. Basically, a power supply set to a given voltage value is connected to a square-wave pulse generator whose role is to close the circuit at $t = 0$ and open it microseconds or milliseconds later. Thus, the theoretical shape of the wave is as shown in **Fig. 6A**.

In the case of a capacitor discharge, the power supply is disconnected from the capacitor once the latter is charged and the discharge mode has been initiated (*see* **Fig. 4A**). On the contrary, with a square-wave pulse, the power supply remains part of the closed circuit at all times. Thus, the voltage applied to the sample should seemingly be limited only by the maximum voltage attainable by the power supply (400 V for ordinary and inexpensive electrophoresis power packs). However, Ohm's law always applies. Thus, at 400 V, if the resistance of the sample is 400 Ω, the current will be 1 A. However, if R is decreased to 40 Ω, the current value will be 10 A, an intensity that cannot be delivered by ordinary power supplies. In this case, the height of the pulse will be limited by the maximum amperage output of the power supply. Unfortunately, this reasoning is complicated by the fact that power supplies themselves contain capacitors. Thus, the latter may very well be able to provide (temporarily) the voltage set, but as the charge of these capacitors decays, the voltage determined by Ohm's law will prevail. Thus, a square-wave pulse may look not so square after all (**Fig. 6B**).

Unfortunately, that is not all. Other factors such as the impedance (very roughly equivalent to resistance in alternating current circuits) of the power supply, as well as the rate of charge of its capacitors as they discharge to meet set voltage demands, will influence the shape and height of the pulse. Therefore, the resistance of the sample plays a critical role and should be high for higher voltage values. In all cases, it is the maximum current output of the power supply which will eventually determine the height and shape of the pulse. Again, measuring the resistance of the sample or using an oscilloscope to track the pulse is a much better practice than simple guessing. Obviously,

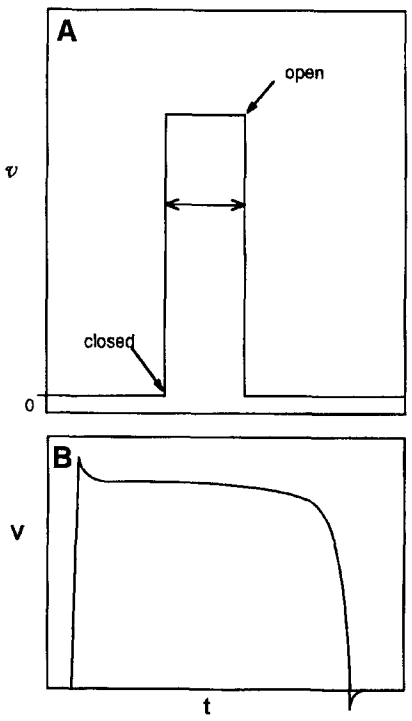

Fig. 6. **(A)** Idealized square-wave pulse in which switching is extremely fast compared to the length of the pulse. Switch positions (closed and open) are indicated by short arrows. The horizontal arrow gives pulse time or width. **(B)** Actual square-wave pulse often obtained in actual discharges. The (a) arrow indicates initial voltage decay owing to power supply capacitor discharge; the (b) arrow shows the square part of the wave at the voltage plateau determined by $V = RI$. The length and magnitude of the voltage spike (a) relative to the length and magnitude of the plateau (b) depend on the time constant(s) of the power supply capacitor(s) and its current limit. **(B)** Reproduced by permision from **ref. *64***.

as in the case of capacitor discharge, field strength is dependent on electrode gap.

2.2.2. Energy Released by a Square-Wave Pulse

The energy dissipated in a simple direct current circuit which contains no capacitance is

$$dW = Vdq \quad (18)$$

and the power

$$P = dW/dt = V\,dq/dt = VI = I^2R = V^2/R \quad (19)$$

where t is pulse time in s and q is the charge (in coulombs) crossing the circuit. For example, assuming that the voltage is 200 V (that is, 200 J/C), the amperage 1 A (that is, 1 C/s), then, the power $P = VI$ = 200 V x 1 A = 200 J/C x 1 C/s = 200 J/s. The energy, $W = Pdt$, for a 100-ms pulse will be 200 J/s x 0.1 s = 20 J. Thus, it can be seen (*see* **Subheading 2.1.4.**) that the energy here is of the same order as that released by a 500-μF capacitor charged to 200 V (W = 10 J). However, for a perfect square wave, the power (rate of energy dissipation) will be constant and P = 200 J/s. This is significantly higher than the *average* power provided by the capacitor (13.2 J/s) and higher than the power at $t = \tau$ (42 J/s). Indeed, whereas energy release in an *RC* circuit is proportional to 1/2 C and V^2, the phenomenon depends on V^2/R (or VI) and time in the case of a square-wave pulse. Since time can easily be adjusted in the latter case, energy release is in principle easier to control there.

2.3. Hybrid Systems

Some commercial electroporation units combine both capacitor discharge and fast switching to provide pseudo square-wave pulses. The principle here is to shut off capacitor discharge well before the *RC* constant is reached. **Figure 7** shows the approximate wave shape in such a system. It can be seen that in fact, the pulse corresponds to a partial capacitor discharge. The advantage of this principle is unclear since switching off the discharge at longer times will mimic full capacitor discharge more and more closely. However, this system does allow high-voltage (depending on the capacitor rating), short quasi-square-wave pulses not attainable with regular power supplies used with a square-wave generator (*see* **Subheading 2.2.1.**).

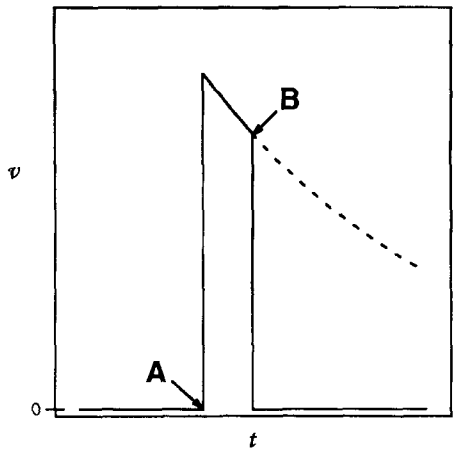

Fig. 7. Discharge shape in a hybrid system. Arrow (A) is the time at which capacitor discharge is initiated and arrow (B) shows the time at which the switch opens the circuit. Dotted line indicates the natural voltage decay if the discharge is not interrupted.

2.4. Heat Production in Electroporation Cells

Electrical energy is transformed into heat as an electric current passes through a resistance. The first law of thermodynamics states that energy in thermodynamic systems must be constant. Hence, the variation in energy of a system during a transformation is equal to the amount of energy that the system receives from its environment. Thus, electrical energy released into a system either as a capacitor discharge or a square-wave pulse will increase the energy level of the sample. Further, since electrical energy dissipation in a resistance produces heat, electroporation samples will have a temperature increase. To that effect, 4.186 J are equivalent to 1 cal, the amount of energy to raise the temperature of 1 g of water (1 mL for practical purposes) by 1°C. Thus, in the aforementioned examples, 13–20 J are released into the circuit, this amount of energy being enough to raise the temperature of 1 mL of water by 3.1–4.8°C. Obviously, long pulse times in the case of a square wave, high capacitance with a capacitor discharge, and high voltage with both systems will

raise the temperature even further. In theory, a release of 314 J will bring 1 mL of water initially at room temperature and 760 mmHg of pressure to the boiling point.

In practice, temperature increases are less drastic since the whole circuit (and not just the sample) is the unit in which energy is dissipated, and the electroporation cuvet is not a thermodynamically isolated system. Nevertheless, caution is de rigueur, in particular when multiple pulses are administered. Cooling the sample to 0°C can be done, bearing in mind that the resistance of the sample will be significantly lower (*see* **Subheading 2.1.3.**). As a rule, however, temperature increases of the sample will not reach critical values and will not alter membrane properties (fluidity), and hence should not be considered instrumental in the changes observed in membrane permeability.

3. Electric Field-Induced Pore Formation

The theory of the electroporation process is incomplete. But, there exists a vast amount of theoretical and empirical evidence demonstrating increased cell permeability as a result of exposure to electric fields. Much of this pathbreaking work was done earlier by the group of Ulrich Zimmermann at the Nuclear Research Center in Jülich, Germany.

3.1. Electrical Properties of Membranes in Electric Fields

Biological membranes are polarized and it is estimated that electric fields existing across them are as high as 10^5–10^8 V/cm. Thus, in some ways, the phospholipid bilayer of a cell membrane can be compared to a capacitor where the distance between plates is extremely thin. As seen in **Subheading 2.1.5.**, excessive voltages can lead to the breakdown of a dielectric by overcoming its permittivity. Thus, the reasoning underlying the electroporation process is that at a critical electric field value, the permittivity of the membrane will be exceeded and its conductivity dramatically increased. It is assumed that the increase in conductivity will be accompanied by local disruptions of the bilayer structure, hence the notion of pore formation and much enhanced permeability to macromolecules in particular.

Pore formation is reversible under the proper conditions and resealing of the membrane occurs within seconds or minutes, or even hours, depending on the temperature. This phenomenon was observed experimentally. The capacitance of membranes can be measured, and it has indeed been shown (reviewed in **ref. *4***) that capacitor breakdown (membrane poration) and regeneration (membrane resealing) do occur as one would expect in the case of a macroscopic capacitor. Thus, a brief electric (high-voltage) pulse is followed by membrane voltage relaxation, indicating increasing conductivity of the cell owing to breakdown of the dielectric (the lipid bilayer). Similarly, the resealing process was followed by measuring the decrease of current, hence the decrease of conductivity and voltage increase across the membrane. In other words, the dielectric, or the cell membrane, is perforated at a given voltage and allows current to pass, a process followed by restoration of membrane integrity after the pulse and reestablishment of its dielectric properties (higher voltage and low current).

Much empirical and theoretical work, well beyond the scope of this article, has been devoted to understanding the process of membrane breakdown in electrical fields. It was observed that electrical breakdown of artificial and natural membranes occurred in the range of 0.5–2 V ***(4)***. Further, irreversible breakdown was noted when the applied external electric field exceeded 4–6 times the threshold value or when the duration of the electric pulse was longer than 20–100 µs. However, this lethal effect of pulse duration was found to vary quite significantly according to cell type, size, and osmoticum. Further, breakdown voltage was found to be dependent on pulse duration, that is, the longer the pulse, the lower the breakdown voltage. For example, with the giant alga *Halicystis parvula* (diameter 0.5 cm, chosen for its large size giving the ability to make direct voltage measurements with microelectrodes), breakdown is observed at approximately 2 V for a pulse duration of up to 5 µs but is achieved at approx 0.5 V if pulse duration is 100–200 µs ***(4)***.

It is interesting to note that in practice, pulse durations of 100- to 1000-fold these values have been used successfully (*see* **Subheading 4.**). Given the negative correlation between breakdown voltage and pulse duration, it is not surprising that short pulses require higher field intensities than longer ones. In addition, breakdown voltage varies logarithmically with pulse duration *(4)*. The dramatic effect of membrane breakdown on cell conductivity (poration) is shown in **Fig. 8** (from **ref. 5**); it can be seen that membrane conductance surges vertically at a membrane potential of 0.85 V.

Since membrane breakdown is known empirically to occur at 0.5–2 V, it is possible to determine the magnitude of the external electric field necessary to achieve pore formation. This can be calculated using the equation:

$$V = 1.5\, r\, E \cos\theta \qquad (20)$$

where V is the breakdown voltage, r is the radius of the cell in centimeters (assuming a spherical cell), E is the applied electric field in V/cm and θ is the angle between the direction of the field and the normal to the membrane surface (**Fig. 9**). This equation shows that pore formation will occur first at the poles of the cell when E reaches the breakdown value (and where θ is 0°) and elsewhere as E increases.

For example, assuming a hypothetical breakdown voltage for a generic membrane of 1 V, the radius of a typical prokaryote equal to 1 μm and the radius of a typical eukaryotic cell equal to 25 μm, it can be calculated that pore formation at the poles will occur at $E = 6.6$ kV/cm for the prokaryote and $E = 260$ V/cm for the eukaryote. The calculated field intensity for the prokaryote is quite close to empirically successful values while that for the eukaryote tends to be on the low side, although plant protoplasts (nearly perfect spheres) have been electrotransformed at such low field values. The multiplier, 1.5, in **Eq. 20** is only valid for a sphere; obviously, not all living cells are spherical and thus the value of E will depend on cell shape as well. In fact, the value of the multiplier for a cylinder is 1.0. Likewise, not all biological membranes have a breakdown potential of 1 V. Nevertheless, **Eq. 20** gives a decent approxi-

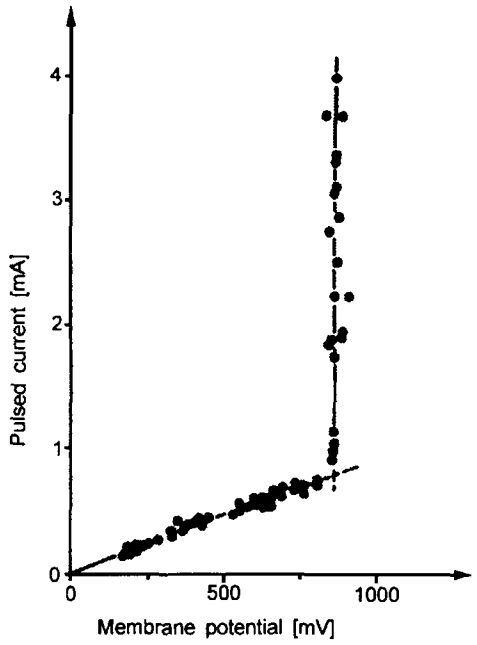

Fig. 8. Electropermeabilization of a *Valonia utricularis* cell as a function of membrane potential. Reversible breakdown was observed at 0.85 V. Reproduced with permission from **ref. 5**.

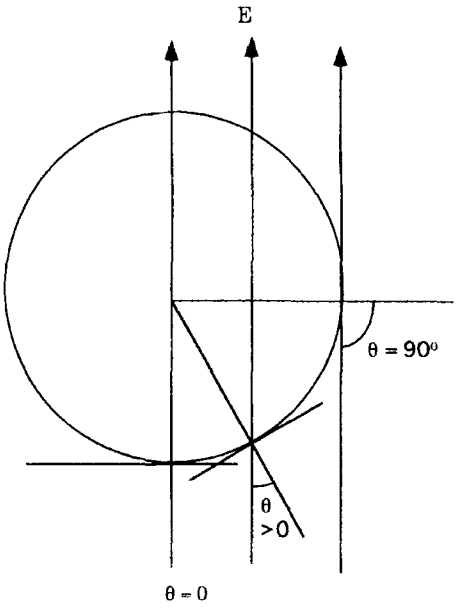

Fig. 9. Electroporation will occur first at the poles of a spherical cell where the angle between the normal to the cell diameter and the electric field *(E)* is 90° (θ = 0°) and will occur at higher field strength elsewhere with $\theta > 0°$.

mation as to what value of E should be chosen when initiating electrotransformation experiments with new types of cells. Clearly, a major difference in **Eq. 20** is brought about by cell radius (r) and shape, which determines the value of cos θ. As a rule, much higher electric fields are required for electroporation in small cells such as prokaryotes.

3.2. Dynamics of Pore Formation

An electric field imposed on a dielectric composed of polar molecules (such as membrane lipids and proteins) will strongly align the dipoles along the field. Thus, an electric field is expected to increase the dipole moment (the product of the electric charges and the relative displacement of the positive and negative electrical centers) across the membrane. This phenomenon certainly must have an effect on membrane properties with regard to permeability as dipole alignment will create stress by decreasing membrane fluidity. Neumann et al. ***(1)*** developed the classical thermodynamic aspects of membrane permeabilization by making simple assumptions. In this model, the membrane is supposed to exist in one of two states: the open state in which pores are formed and the closed state corresponding to the membrane at rest. When the field is applied, pore opening will occur at a rate determined by the kinetic constant, k_o and pore closure will occur at a rate determined by k_c. At rest, k_c is much larger than k_o since cells are not permeable to DNA. The law of mass action states that at equilibrium, the equilibrium constant $K = k_o/k_c$. Taking into consideration the influence of temperature, pressure, and electric field on K, one can write the standard equation describing the system as:

$$d \ln K = (\partial \ln K/\partial T)_{P,E}\, dT + (\partial \ln K/\partial P)_{T,E}\, dP + (\partial \ln K/\partial E)_{P,T}\, dE \quad (21)$$

where T is the absolute temperature, P the pressure, and E the electric field. This equation can be rewritten using the van't Hoff equivalents of the three terms and one gets

$$d \ln K = (\Delta H/RT^2)\, dT - (\Delta V/RT)\, dP + (\Delta M/RT)\, dE \quad (22)$$

where ΔH is the enthalpy change of the reaction, ΔV the volume change, ΔM the difference between the dipole moments before and after application of E, and R is the gas constant. As expected, this equation shows that K will be a function of T, P, and E. Since P is constant, the equilibrium will only depend on variations in T and E. Also, in many cases, the energy released into the sample will be low enough to leave T largely unaffected. Therefore, the predominant term controlling d ln K will be $(\Delta M/RT)\, dE$. Whether this equation truly represents the state of the system is questionable since the notion of equilibrium is ill-defined here. In fact, electroporation being a transient phenomenon, the system is by definition far from equilibrium except when the electric field value is steady. This is certainly not the case with a capacitor discharge, meaning that the terms $\partial \ln K/\partial E$ and $\Delta M/RT$ will vary over time since k_o and k_c will not be true constants.

In a less esoteric fashion, there is evidence that local membrane compression induced by the electric field contributes to pore formation ***(5)***. Indeed, ignoring the effects of internal osmotic pressure if cells are in an isotonic medium, the stress per unit area, P_a, caused by an electric field will be $P_a = - dW_a/ds$, where W_a is the energy in the field per unit area and s is membrane thickness. Keeping in mind **Eq. 17** and equating the membrane with a capacitance (**Subheading 2.1.5.**), $W_a = e\, V^2/2\, s$. Thus, $P_a = \varepsilon\, V^2/2\, s^2$, meaning that electromechanical stress, that is, thinning of the membrane, increases with the square of the membrane potential and the inverse square of the thickness. Here again, comparison with a capacitance has a heuristic value as it is well-known that the plates of a capacitor attract each other when charged.

Thus, the kinetic and thermodynamic aspects of pore formation are complex. Many articles (reviewed in **ref. *6***) have been devoted to theoretical and experimental studies of pore formation in natural and artificial membranes. Most of the results presented there, however, are of very little use to the molecular biologist as they focus almost exclusively on electrodynamic properties of membranes. From the results and theory discussed, a few simple concepts can be derived:

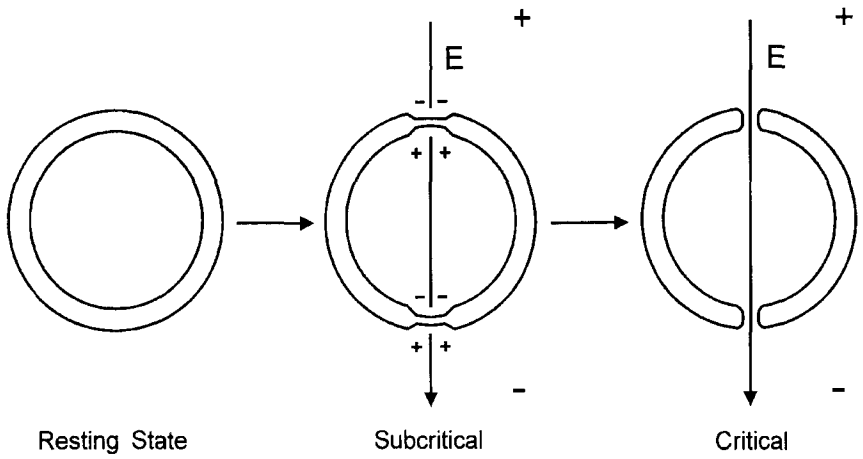

Fig. 10. Simple electromechanical model of cell poration. Electrocompression of the membrane occurs as a function of the imposed electric field and pore formation will be observed when the critical (breakdown) potential is reached. The stochastic model does not conform to this scheme (*see* **Subheading 5.**).

1. Membrane conductance increases dramatically at 0.5–2.0 V and is accompanied by leakage of cell constituents, indicating poration. This voltage is, of course, measured across the membrane and does not correspond to the macroscopic applied electric field. A normal membrane potential at rest is measured in mV.
2. Longer pulses cause membrane breakdown at lower field strength.
3. Breakdown voltage is directly proportional to cell radius. Field intensities needed are thus several kV/cm with prokaryotes but much lower with eukaryotes.
4. Breakdown depends on temperature and electric field strength and, in the latter case, can be accounted for by dipole alignment in the membrane and electrocompression. At least these factors contribute to membrane fragility and disruption in an electric field.

Figure 10 is a hypothetical representation of what may happen to a cell membrane as it is subjected to an electric field.

3.3. Electroporation Hardware

As electroporation of prokaryotes requires field strengths of several kV/cm, safety is a major concern and homemade instruments should be built with this in mind. Alternatively, electroporation units can be purchased from manufacturers at a high cost ($5000) if full flexibility regarding voltage, time constant, resistance, and above all, correct monitoring of the discharge are desired. As stated in **Subheading 2.**, it is imperative that discharge characteristics be measured at the level of the sample, that is, across the electroporation chamber. Most commercially available units do not do this.

Simple electroporation units suitable for the electrotransformation of animal and plant cells (and hence requiring voltage values lower by about one order of magnitude) can be built by trained personnel in any well-equipped electronics shop for a few hundred dollars, including the cost of the oscilloscope and the electrodes. Most of the time, high flexibility regarding electroporation parameters is not required, in particular when a single type of eukaryotic cell is under investigation. Further, work with most eukaryotic cells does not necessitate very high field strengths, meaning that units providing electric fields of several tens of kV/cm will never be used to their maximum capacity. Even when recombinant DNA work in *Escherichia coli* is necessary prior to electroporation of eukaryotic cells, other efficient and very inexpensive techniques are available to transform this host. Thus, several single-capacitor units can be built for a fraction of the cost of commercial units. However, building multiple electrodes for use in Petri plates and well plates

would probably not be cost effective. A comparative discussion of recent commercial electroporation hardware can be found in **ref. *6***.

4. DNA Transfer into Living Cells

Dozens of cell types have been successfully electrotransformed. The following discussion will analyze the conditions under which success was achieved in prokaryotic and eukaryotic systems. Emphasis will be put on electroporation conditions and cell pretreatment but not on the vectors, selectable or screenable markers, and gene constructs used, which, for obvious reasons, are as varied or more than the cell types investigated. A corollary of cell poration is of course leakage of constituents into the surrounding medium. As more pores are formed as a function of electrical parameters and more DNA molecules are transferred, leakage will also increase and lead to cell death. Electroporation conditions must thus reach an acceptable balance between rate of survival and frequency of DNA uptake.

4.1. Prokaryotes

A recently published compendium of electrotransformation protocols for microorganisms ***(6)*** provides a wealth of data regarding electroporation parameters used with *E. coli, Salmonella, Pseudomonas, Xanthomonas, Brucella, Francisella, Vibrio, Bacteroides, Agrobacterium, Helicobacter, Streptococcus, Lactococcus, Lactobacillus, Staphylococcus, Enterococcus, Clostridium, Mycobacterium, and Borrelia*, a group containing both Gram-positive and Gram-negative genera. Many of these genera cannot be transformed other than by electroporation, an indication of the value of this technique. Obviously, each genus (and species and strains within them) must be cultured in its appropriate growth medium prior to manipulation.

Remarkably, however, electroporation conditions and media are very uniform. For 12 of the 18 genera, field strength used was between 12.0 and 16.7 kV/cm. Six genera transformed well between 6.25 and 7.5 kV/cm. In all likelihood, voltage values were determined by the equipment used. In fact, it is known that *E. coli* can be transformed, albeit at lower frequency, at a field strength as low as 3.5–4.0 kV/cm ***(7)***. *Streptococcus* sp. could also be electrotransformed at such field strengths ***(8)***. This indicates that membrane breakdown starts to occur at those field values, increased transformation frequencies being observed at higher field strength when pore formation is made possible at multiple sites on the cell membrane. This is in excellent agreement with **Eq. 20**. Further, longer pulses at low field strength allow equally good electrotransformation of *E. coli* as short pulses at higher voltage ***(7)***. Here again, this observation is in excellent agreement with results discussed in **Subheading 3.1.**

Electroporation media most often consisted of sucrose or glycerol or both in double-distilled water and all discharges were done at 0–4°C. Those are wise conditions when working with high voltages; both the low temperature and the absence of added salt ensure high sample resistance (several kΩ/cm, depending on the purity of the water and the solutes) and thus limit current flow through the circuit. In fact, the presence of salt in DNA preparations was of concern to most workers. Some bacterial species require Mg ions for survival and $MgCl_2$ was sometimes present at a concentration of 1 m*M*. In a case where a comparatively low field strength of 4.0 kV/cm was tried, a 10 m*M* salt concentration was used, presumably safely.

When given, the capacitance used was 25 μF (14 times) or 0.4 μF (once). This shows that a unit equipped with a whole battery of capacitors is probably not necessary for the manipulation of prokaryotes. The value of the unit's adjustable resistance varied a little more. Fourteen values were reported, half of them being 200 Ω, three, 400 Ω, two, 1000 Ω (to reach an *RC* constant estimated to be 25 ms), and one each, 100 and 800 Ω. Interestingly, in the two cases where resistance was varied between 100 and 400 Ω (at constant *C*), no difference was found in transformation frequencies. What is one to conclude from this? Nothing. Indeed, the effect of changing *R* at constant *C* will of course modify the value of *RC*. However, without knowing the value of the resistance of the sample, it is impossible for one to esti-

mate the value of R and hence RC. Let us assume that the unit's variable resistance is in parallel with that of the sample (as is the case for commercial units) and let us call R_u the unit's resistance and R_s that of the sample. The total resistance will thus be $1/R = 1/R_u + 1/R_s$. Normally, R_s should be considerably lower than R_u to avoid large current surges and thus, the contribution of R_s will be much less in a parallel circuit than that of R_u. Let us further assume that R_s is 1000 Ω and R_u 400 Ω. Then, $1/R = 1/1000 + 1/400$ which gives $R = 285$ Ω and, with C = 25 μF, RC = 7.12 msec. If R_u is increased to 800 Ω and C kept constant, then RC = 11.10 ms, not 14.24 ms. If on the other hand, the sample has a ten times higher resistance, its effect on RC will be negligible. The problem here is that R_s is unknown and cannot be calculated since the contribution of the cells themselves as well as the conductivity of the water are not known. The problem gets even worse if a series circuit is used since in that case, resistance values are directly additive.

Hence, virtually all authors use the prudent phrasing that "at such and such settings the RC constant *should be* so much." Surveying the theoretical values reported for RC one finds that most values are 4–5 ms with 10 ms being the next most popular. The minimum reported was 2.5 ms and the maximum was 25 ms.

Transformation frequencies were of course extremely variable according to the genus, species, and strain. Frequencies thus ranged from a high of 10^{10} transformants/μg DNA in *E. coli* to a low of 20 transformants/μg DNA in one *Staphylococcus* strain. Most values were in the range of 10^4–10^8 transformants/μg DNA.

Based on this analysis, it would be tempting to derive universal parameters that should be applicable to all prokaryotes, within limits of survivability in high electric fields. First, capacitor discharge is by far the most common means used to apply an electric field to a suspension of prokaryotes. This may be the result of the unavailability of reasonably priced commercial units able to deliver a true square-wave pulse at very high voltage and the difficulty of building one locally (*see* **Subheading 2.2.**). Thus, a 25-μF capacitor charged to 2000 V and discharged in an electroporation cell whose electrode gap is 0.2 cm, placed in parallel with a 200-Ω resistor, will yield electroporation parameters (10 kV/cm, theoretical RC = 5 ms) that should lead to the transformation of any prokaryote surviving the treatment. Somewhat higher RC constants should also be acceptable. The sample resistance should be as high as possible (preferably several kΩ to avoid arcing and misrepresentation of the RC constant) and preferably measured. Finally, the sample should undergo electroporation at 0°C to prevent overheating. These electroporation conditions will release 50 J of energy into the system and the power at $t = \tau$ under the above conditions will be $1/2\ (CV^2)/t$ = 10,000 J/s.

Most interestingly, in none of the cases cited was it necessary to remove cell walls completely to achieve electrotransformation. Nevertheless, growth conditions in which the cell wall is more fragile, or gentle digestion with enzymes are known to enhance transformation efficiencies in Gram-positive bacteria. The vectors used were overwhelmingly covalently closed circular plasmids (including some of the integrating type) and sometimes phage DNA. Linear DNA was also used in cases where no circular replicons were available. Finally, plasmids of up to 30 kbp could be electrotransformed into cells, although transformation efficiencies tended to go down with increasing plasmid size.

4.2. Microscopic Eukaryotes

A range of very diverse microeukaryotes are now amenable to gene transfer by electroporation. In most cases, other transformation techniques are available that are usually much less expensive and possibly easier to conduct. Nevertheless, electroporation, owing to its great flexibility, equals or surpasses these techniques and is likely to succeed where all else has failed. However, the high consistency found in electroporation parameters used for prokaryotes is not present here. Further, it is necessary in several cases to partially or completely remove the cell wall to achieve high rates of transformation. All results presented below were obtained by capacitor discharge.

The genera *Saccharomyces*, *Schizosaccharomyces,* and *Candida* can be transformed as intact cells in sorbitol dissolved in double-distilled water (very low conductivity) or 30% glycerol in a significantly conductive buffer for *Schizosaccharomyces* at voltage values around 7.5 kV/cm, although a much lower voltage of 2.6 kV/cm was also reported for intact yeast cells ***(9–12)***. Here too, transformation frequencies were strain dependent. Capacitance values varied from 1–40 μF, resistances from 100–600 Ω and estimated (but apparently not measured) time constants from 0.5–32 ms. All discharges occurred in chilled samples. High transformation frequencies (10^6–10^7/μg DNA) were obtained with yeast spheroplasts treated with electroporation at 2.6 kV/cm ***(9)*** in a sorbitol-glycerol-low salt solution. The ability to store frozen yeast spheroplasts compensates for the added step to form them, and transformation frequencies are up to 1000-fold higher than with intact cells ***(10)***. Clearly, the presence of a cell wall hinders DNA transfer by electroporation. As with prokaryotes, vectors consisted of plasmid DNA of the independently replicating or integrative types.

Cell wall interference is also evident in the case of filamentous fungi such as *Neurospora* and *Aspergillus*. In both cases, techniques ***(13)*** were developed using germinating conidia (spores) treated with β-glucuronidase or other cell-wall degrading enzymes. However, complete spheroplast conversion was not necessary for successful electroporation, indicating that cell wall weakening was sufficient to allow the passage of DNA. Conidia and spores were transformed by electroporation in mannitol—1 m*M* HEPES at 12.5 kV/cm and 0°C at an *RC* constant said to be 5 ms. Since *R* was 400 Ω and *C* was 25 μF, the calculated *RC* constant is 10 ms. It is unclear whether the reported *RC* constant was actually measured. Stable transformation occurred here by homologous or illegitimate recombination with chromosomal DNA.

Next on the evolutionary scale one finds protists such as *Physarum*, *Dictyostelium,* and *Tetrahymena*. All three genera are now amenable to transformation by electroporation. In the case of *Dictyostelium*, cells were grown in regular medium and resuspended in a sucrose solution containing 10 m*M* sodium phosphate, a solution expected to have a resistance of the order of 200 Ω per 0.2 cm. Cells were pulsed twice at 5.5 kV/cm with *C* equal to 3 μF and a 5-Ω resistor in series. Since the reported RC constant was 0.65 ms (instead of the calculated 0.015 ms), it is quite possible that this was indeed the measured time constant, close to the predicted one taking into account the resistance of the electroporation medium. Both integrating and autonomously replicating vectors have been used to electrotransform *Dictyostelium* ***(14)***.

Physarum polycephalum has a complicated life cycle that includes a multinucleate syncytial stage and a haploid uninucleate amoeba stage. The latter was chosen for electrotransformation experiments. Optimal parameters were found to be 2.5 kV/cm at 800 Ω and 25 μF. Decreasing the resistance to 600 Ω or increasing it to 1000 Ω did not have an important effect on the transient expression of a *luc* reporter gene. However, electroporation at 1000 Ω (set resistance) was optimal at 2.1 kV/cm, meaning that, here again, a longer *RC* constant compensated for voltage. The electroporation buffer consisted of a sucrose—10 m*M* HEPES solution. Interestingly, diploid amoebae had a voltage optimum at 1.5 kV/cm, consistent again with **Eq. 20**, which relates membrane breakdown voltage to cell size. Transient transformation was achieved with circular and linear plasmids while stable transformation, which requires vector integration, was much more efficient with linearized vectors ***(15)***.

The life cycle of ciliated protozoans such as *Tetrahymena* is also complex, in particular at the level of the macro- and micronuclei which play different roles in somatic (vegetative) and conjugating cells. It was discovered ***(16)*** that electrotransformation efficiency strictly overlapped the period of macronuclear development in conjugating cells. Contrary to all other cases of protist electrotransformation, electroporation occurred at room temperature owing to the sensitivity of *Tetrahymena* to the cold. The electroporation buffer was 10 m*M* HEPES, pH 7.5, and parameters,

which remarkably were actually measured here, were 1.125 kV/cm at R = 13 Ω and C = 275 µF for a measured value of RC = 4 ms. The calculated RC constant is in this case 3.57 ms, indicating a significant effect of the electroporation buffer resistance. As seen before, a shorter RC constant of 1.6 ms obtained with a 50-µF capacitance and a 24-Ω resistance required a higher field strength of 1380 V/cm. Plasmid vectors used were of the replicative type (using an rDNA replication origin) or integrated by homologous recombination.

Lastly, a unicellular alga, *Chlamydomonas reinhardtii*, was also shown to be capable of electrotransformation with plasmid vectors through illegitimate recombination. This case is particularly interesting in that cell-wall deficient mutants (one of them being *cw-15*) are available, allowing useful comparisons with the wild type regarding the effect of the cell wall on DNA uptake ***(17)***. The wild type could be transformed at a field strength of 500 V/cm (2 pulses) in salt medium using a 25-µF capacitor and a calculated pulse length of 4.8 ms (resistance was not given). However, a pulse length of 3.8 ms at 1.0 kV/cm yielded more transformants. Increasing field strength to 2.25 kV/cm resulted in fewer transformants. Regarding the *cw-15* mutant, the highest frequency was obtained with a single pulse of 26.4 ms at 1.0 kV/cm. A single 5-ms pulse at 1.0 kV/cm in distilled water also yielded *cw-15* transformants with the addition of a 200-Ω resistor in parallel. RC constants were not actually measured in all cases and it is highly likely that calculated values are incorrect. Indeed, the salt medium had an ionic strength equivalent to that of physiological saline and was thus highly conductive, while distilled water is of course very poorly conductive. Fortunately, the wild type and the mutant were compared under similar conditions (high salt, 1.0 kV/cm, and calculated RC = 26.4 ms), and it was found that *cw-15* cells were transformed at a much higher rate (one pulse) than wild-type cells (two pulses).

In summary, electrotransformation experiments with fungi and protists lead to the following conclusions:

1. Intact fungal cells or fungi with weakened cell walls withstand electroporation at high field strengths with extremely variable reported RC constants. Yeast, however, could be transformed at 2.6 kV/cm either as intact cells or as spheroplasts. In the latter case, the cell wall definitely hindered transformation in terms of transformation efficiency. It is unclear whether it is the presence of a cell wall, the high threshold of membrane breakdown potential, or other factors which necessitate the use of higher voltages with other fungal genera. In terms of energetics, yeast could be transformed with the dissipation of only 0.8 J into the system. Other reports however used a 24-J discharge for yeast. *Candida* electroporation on the other hand, was done at the 45-J level, close to what is used with prokaryotes. However, owing to the different time constants used in different systems, the calculated power (based on information provided by the authors) at $t = RC$ was 1406 J/s for *Candida maltosa*, 5444 J/s for intact *Saccharomyces cerevisiaea,* and 15,600 J/s for *Neurospora crassa* conidia. This enormous range may be owing in part to the presence of the tough (even though weakened) cell wall present in *Neurospora* conidia. Also, since RC constants were computed without taking the resistance of the electroporation medium into account, it is likely that power values are somewhat overestimated.
2. Protists require significantly lower field strengths for electrotransformation. This is consistent with their larger size. Higher field strengths, as with prokaryotes, yield equal transformation rates at lower RC constants and vice-versa. Energy levels are considerably lower: *Chlamydomonas* underwent electroporation at 0.78 J, *Tetrahymena* at 8.6 J, *Dictyostelium* at 1.8 J, and the haploid state of *Physarum* at 8.0 J (the diploid state was transformed at 4.5 J). In terms of power, it can be calculated that *Tetrahymena* was transformed at 2150 J/s. This is an accurate value, since electroporation parameters were actually measured here. It can be calculated with a lesser degree of certainty that *Chlamydomonas* underwent poration at 195 J/s, *Physarum* at 225 J/s (diploid stage) and 400 J/s (haploid stage), and *Dictyostelium* at 2769

J/s. The meaning of these energy levels is discussed later.

4.3. Plant Cells

Given the existence of a thick wall surrounding plant cells, it is not surprising that early electroporation attempts were made with protoplasts, that is, cells rid of their wall by enzymatic digestion. Plant protoplasts must be maintained in the proper osmoticum where they assume a nearly perfect spherical shape. However, it is now clear that intact cells in suspension as well as plant parts and even embryos can pick up plasmid DNA molecules through electrically induced transfer. Transformation vectors used were almost invariably covalently closed circular plasmids containing a selectable (*neo* or a gene coding for herbicide resistance) or screenable marker such as *gus*. These markers were often chimeric and under the control of constitutive, inducible, temporally controlled, or organ-specific promoters. Viral RNA molecules have also been used in electroporation studies.

4.3.1. Protoplasts

Electroporation is now a well-established technique to transfer genes transiently or permanently into plant protoplasts. Stable transgene integration occurs through illegitimate recombination and is often accompanied by vector concatenation prior to integration. Production of transgenic plants by electrotransformation is limited only by our ability to regenerate whole plants from protoplasts. However, there have always been two approaches to protoplast electroporation: one school of thought advocates the use of high field strength, low *RC* constant (low capacitance), while the other favors low field strength, high *RC* constant (high capacitance). At present, most experiments are done at low electric field and high capacitance. In fact, both sets of parameters lead to good transformation frequencies. So, what is the difference? Possibly none as we will see in the following examples. Using **Eq. 20**, and assuming a breakdown potential of the membrane of 1 V, one calculates that for a perfect sphere, E = 266 V/cm for a radius of 25 µm, 333 V/cm for a radius of 20 µm and 666 V/cm if the radius is 10 µm. These diameter values are well within the range found in plant protoplasts. Tobacco mesophyll protoplasts often used for electroporation studies have a radius of 20–22 µm. Thus, low field intensities should be able to achieve electroporation of protoplasts, and they do. As in all other systems, electroporation media should be adjusted to the origin of the protoplasts to ensure maximum viability, independent of the electroporation process itself. Those conditions are much too varied to be discussed here.

In the first detailed study of plant protoplast electrotransformation, *Nicotiana tabacum* protoplasts underwent electroporation in the presence of plasmid DNA at 1–1.25 kV/cm (since the electrode gap was 1 cm, this represents the actual voltage) at a measured resistance of 1 kΩ using a 10-nF capacitor. This gave an *RC* constant of 10 µs ***(18)***. Under those conditions, the energy dissipated in the sample was very low, only 7.5 mJ. However, owing to the extremely short *RC* constant, the rate of dissipation, that is, the power was 750 J/s. Unfortunately, polyethylene glycol was present in the electroporation medium and this had an effect on electroporation parameters (*see* **Subheading 5.**). Others ***(19)*** used similar conditions to electrotransform the same type of protoplasts at 2000 V/cm (corresponding to a set voltage of 400 V), also using a 10-nF capacitor discharge. As is often the case, the resistance of the medium was not given, but can conservatively be estimated to have been around 3.5 kΩ since it consisted of mannitol dissolved in water. Under those conditions, an *RC* constant of 35 µs was probably achieved and, the energy being 20 mJ, the power was 570 J/s. In yet another study ***(20)***, DNA transfer into *Nicotiana plumbaginifolia* was achieved at 1500 V/cm (at an instrument setting of 300 V) using a 24-µF capacitor (thus having a 2400-fold higher capacitance than the one used in the previous studies) at a measured time constant of 5 ms. Therefore, the energy released was 1.08 J and the power 216 J/s. Thus, the use of a much bigger capacitor lengthened the *RC* constant and much increased the energy even though the voltage at the plates of the capacitor was much lower than in **ref. *18***. In all three studies, the field strength was well above the theoretical membrane

breakdown threshold while levels of power of the same order were achieved using completely different capacitance values. Thus, the factor which unifies the above parameters is power (energy), and not the capacitance value alone nor its corollary, the *RC* constant.

Much higher capacitance values yet have been used to electrotransform plant protoplasts at low field strength. Studies conducted with monocot and dicot protoplasts ***(21)*** demonstrated electrotransformation at voltages as low as 400 V/cm provided a big enough capacitor (1000 μF) was used for the discharge. Interestingly, no significant transgene activity was detected at 200 V/cm, regardless of the capacitance used (up to 2000 μF). This was probably due to the fact that the threshold for membrane breakdown was not reached. According to **Eq. 20** and assuming a 25-μm average radius for these protoplasts, this means that breakdown voltage was higher than 0.75 V. Breakdown was definitely achieved at 400 V/cm, corresponding to a 1.5-V membrane potential. Based on the same reasoning and type of experiment, it was estimated that membrane breakdown potential for yeast spheroplasts was 0.9–1.5 V ***(22)***. In a study where all electroporation parameters were measured and controlled ***(23)***, soybean (*Glycine max*) protoplasts were stably electrotransformed at 375 V/cm (375 V at the terminals) with a 490-μF capacitor, giving a measured time constant of 45 ms, that is, a system delivering 764 J/s. This value is definitely of the same order of magnitude as the power delivered in **refs. *19*** and ***20*** (570 and 216 J/s, respectively). At this point, and after analyzing several eukaryotic and prokaryotic systems, a trend regarding electrical parameters leading to successful electrotransformation begins to appear. First, the required electric field strength decreases dramatically as cell size increases. Second, electrotransformation efficiency increases sharply with voltage in all cases where optimization was attempted. These two observations are fully consistent with theoretical predictions about electrically induced membrane poration. Third, beyond the electric field strength required to achieve membrane breakdown, there is now the concept of *energy* ($W = 1/2\ CV^2$) and *power* delivered by the capacitor, that is, the *rate of energy delivery* rather than capacitance alone or *RC* constant alone. Power is defined as $dW/dt = (1/2\ CV^2) \times 1/t$, and thus relates capacitance, voltage, and time where *V* is the voltage applied to the capacitor, not the electric field strength. It can be seen from **Table 2** that energy and power requirements to electrotransform smaller cells are vastly higher than those needed to electrotransform large eukaryotic cells (effect of size and possibly other factors), and very similar energy and power values can be reached by modifying either the electric field strength or the capacitance. In this case, there will be a lower voltage limit below which no poration (no transformation) will occur in spite of capacitance increase; this limit is the membrane breakdown voltage. This was shown empirically ***(21)***. At constant *R*, it is the value of *C* that will determine the time constant, thus a low capacitance should be accompanied by a high electric field (within limits imposed by the current and cell viability), and vice-versa. This is because energy dissipation will be of the same order. It should be remembered, however, that energy and power vary with the square of the voltage but only linearly with the capacitance. Finally, modifying the value of the resistance (instead of the capacitance) to finely modulate *RC* can be done at constant *C*, keeping in mind that power decreases with increasing time and vice-versa since *t* in the equation describing power is in the denominator. Thus manipulating *RC* with either *C* or *R* does not have the same effect (increasing *C* will increase both the energy and the dissipation time, increasing *R* will increase only time). It is noteworthy that at relatively low power value ***(20)***, multiple pulses were administered to achieve maximum gene expression. In one particular case of very low power (and energy) use (C = 16 μF, measured RC = 40 ms, V = 330 V to give W = 0.72 J and dW/dt = 18 J/s **[24]**) up to 20 consecutive pulses had to be delivered for maximum electrotransformation. One study ***(25)*** compared the infection of tobacco protoplasts with tobacco mosaic virus RNA at different measured field strengths and measured *RC* constants. **Table 3** shows some of their results that demonstrate that

Table 2
Electroporation Parameters Used to Transform Selected Prokaryotic and Eukaryotic Cells[a]

Cell type	Ref.	*C* (μF)	*RC* (ms)	*E*/*d* (kV/cm)	*V* (kV)	*W* (J)	d*W*/d*t* (J/s)
E. coli	*6*	25	4.8	12.5	2.5	78.1	16,276
S. cerevisiae	*9*	25	4.5	7.0	1.4	25.5	5,444
Tetrahymena	*16*	275	4.0	1.125	0.25	8.6	2,150
N. tabacum	*18*	0.01	0.01	1.25	1.25	0.008	780
N. tabacum	*24*	100	6	0.75	0.30	4.5	750
N. tabacum	*25*	16	40	0.30	0.30	0.72	18
Glycine max	*23*	490	45	0.375	0.375	34.4	764
N. plumbaginifolia	*20*	24	5	1.5	0.30	1.08	216

[a]Electroporation with *Nicotiana tabacum*, *Glycine max*, and *Nicotiana plumbaginifolia* species was done with protoplasts. All results were obtained with flat, parallel electrodes except in **ref. *18*** in which circular electrodes were used. Polyethylene glycol was present in the electroporation medium in **ref. *18*** (*see* Discussion). Multiple pulses were used for optimized results in **refs. *20*** and ***25***. Single pulses were used elsewhere. Capacitor discharge was used in all cases. *E*/*d* is the field strength, *V* is the voltage at the capacitor terminals, *W* is the energy released by the capacitor, and the power $P = dW/dt$ is calculated at $t = RC$.

breakdown conditions were reached at 500 V/cm. However, higher field strength did not lead to protoplast infection when a 1-μF capacitor was used. Even doubling the field strength at this low capacitance did not result in any infection either, because the energy (power) was still below the necessary threshold. The use of a 790-μF capacitor did yield successful infection at 500 V/cm as the energy under those conditions was at least 50-fold higher. These observations are again in good agreement with the hypothesis that energy and power are the determining factors in electroporation.

4.3.2. Whole Cells

Conventional wisdom dictates that the presence of a cell wall should reduce electrotransformation efficiency or at least affect electroporation parameters. However, there is little empirical evidence to support this view, except perhaps in the case of yeast (*see* **Subheading 4.2.**). A major problem is that direct and exact comparisons between intact cells and fully isogenic spheroplasts or protoplasts regarding electrotransformation with identical vectors are not usually conducted, if at all. Nevertheless, given that cells with a cell wall (bacteria, yeast, *Chlamydomonas*, etc.) can indeed be electrotransformed leads to the inescapable conclusion that even if the cell wall may decrease transformation efficiency, it does not prevent DNA molecules from being internalized. Hence, the cell wall can be made permeable to macromolecules. The effects of electric fields on cell walls are not known and are bound to differ in different organisms. Recent experiments with intact plant cells support the view that plant cell walls do not present an insurmountable barrier to DNA uptake by electroporation. However, the mechanism through which DNA can cross plant cell walls is unknown.

Whole plant cell electrotransformation was first demonstrated ***(26)*** with maize embryogenic callus pieces treated at 375 V/ cm (225 V at the capacitor terminals) and 900 μF. Electroporation conditions were not better defined but, other work from the same laboratory ***(27)*** using very similar conditions reports a time constant of about 250 ms. Thus, under those conditions, the energy was 22.8 J and the power 91.2 J/s. Single pulses were delivered. The regeneration of transgenic plants from the treated cells demonstrated the value of low-power electroporation of callus cells.

To demonstrate this point further, transgenic sugarcane plants were obtained by electroporation of whole cells, actually cell clusters since plant cells usually do not grow as single cells in suspension cultures. Optimum electroporation con-

Table 3
Electroporation of Tobacco Mosaic Virus RNA into Tobacco Protoplasts as a Function of Capacitance and Field Strength[a]

C (μF)	RC (ms)	E/d (kV/cm)	V (kV)	W (J)	P (J/s)	Transfection
1	1	0.75	0.3	0.045	45	No
1	1	1.00	0.4	0.08	80	No
100	6	0.75	0.3	4.5	750	Yes
790	32	0.5	0.2	15.8	494	Yes
790	32	0.75	0.3	33.7	1053	Cell death

[a]Data calculated and tabulated from ref. *24*. Power P is calculated at $t = RC$.

ditions were found at 750 V/cm (300 V at the terminals) and 880 μF ***(28)***. Transgene activity was already detected at 660 V/cm. Unfortunately, the value of neither the resistance nor the time constant were provided in this article. It can be calculated in this case that energy delivered was 3.9 J. However, a precise calculation of the power cannot be made in the absence of values for R or RC. Nevertheless, the very large capacitor and moderately conductive electroporation buffer (around 1000-Ω resistance) used preclude short time constants. Thus, power release in this system must have been modest, possibly lower than the power used to electrotransform protoplasts. This again raises interesting questions regarding the mechanism of DNA transfer at low power and long RC constant. Indeed, it is intriguing that the plant cell wall does not make electrotransformation of intact cells more difficult (or require higher power) than that of protoplasts. Quite possibly, the mechanism of DNA transfer is different here.

4.3.3. Plant Parts and Embryos

Leaf bases isolated from cereal seedlings were used as recipients for a variety of chimeric transgenes introduced by electroporation ***(27)***. Again, low-voltage, long pulses were administered. Transgene expression was detected as low as 200 V/cm and 500 μF, at an energy of 3.6 J and a power estimated at 25 J/s. At high capacitance (900 μF), multiple pulses had no beneficial effect on transgene expression.

Later, transient expression of transgenes in embryos was reported by several groups ***(29–33)***. Cowpea embryos ***(29)*** were electrotransformed using a square wave pulse while maize ***(30)***, wheat ***(31)***, bean and other legumes ***(32)***, as well as rice embryos ***(33)*** were electrotransformed using a capacitor discharge. Single pulses at low voltage and high capacitance ***(30–32)*** proved quite effective; transgene expression was detected at 10.8 J and 42 J/s ***(30)***, 5.8 J and 27 J/s ***(31)***, and as low as 2.5 J and 9.7 J/s ***(32)***. In the latter case, since the lowest tested field strength leading to transgene expression was only 75 V/cm, it is doubtful that electroporation as classically understood was responsible for the observed effect.

Finally, transgenic grain legumes (pea, lentil, cowpea, and soybean) were obtained by microinjection of plasmid DNA into axillary buds and electroporation using several low-voltage square-wave pulses ***(34,35)***. In these cases also, classical electroporation may not have been the main mode of entry of DNA molecules. A possible mechanism for an alternative electrically induced DNA uptake process is discussed in **Subheading 4.3.4.**

4.3.4. Electroporation or Electrophoresis?

Electrotransformation results obtained with intact plant cells, tissues, and embryos at low voltage and high capacitance (also meaning low energy and low power) raise questions regarding the electropermeabilization of cells to DNA under those circumstances. As we have seen above, protoplast electroporation at modest energy and power levels requires several pulses. Presumably, pore formation there is due to the cumulative effect of the successive discharges while membrane breakdown potential is reached or exceeded. With intact cells, organs, or embryos, most authors

have used electric field values compatible with electrical breakdown, although graphs describing transgene expression as a function of field strength often show expression at field values lower than expected. Further, the sharp dependence of transgene expression vs field strength, observed in classical electroporation experiments, is no longer present (**Fig. 11**). Also, the presence of macroscopic objects, such as cell clumps, plant slices, and embryos, is bound to alter the shape and local strength of the electric field deeply, in completely unknown ways. Finally, the existence of the cell wall seems to be of little consequence. These results suggest that another mechanism of DNA transfer is at work here.

It has been reported that DNA molecules can penetrate intact embryonic cells *(36,37)* by electrophoresis at low voltage (2–25 V) and low amperage (0.1–0.5 mA) for 60 or 15 min. In this technique, embryos were directly connected to the poles of the power supply and DNA was delivered to tissues through the cathode. Higher voltage and amperage values quickly led to cell death. Field strength values in those two reports were well below membrane breakdown threshold and the concept of electroporation does not apply any longer. In a sense, however, the conditions used in these experiments can be equated with very long pulses at low field strength. Thus, it is quite possible that electrophoresis, and not electroporation, could account for some of the above results obtained with embryos. How large molecules such as DNA can penetrate cell membranes (and walls) other than by electroporation is not known. Finally, transformation of plant tissues by electroporation or electrophoresis cannot yet be considered routine as more work is needed to confirm and extend published data. Nevertheless, embryo electrotransformation constitutes an attractive alternative approach to all other methods aimed at generating transgenic plants owing to its simplicity and independence from complex and lengthy plant regeneration protocols.

Results presented in **refs. *34*** and ***35*** and describing the production of transgenic plants by injection of terminal buds followed by electroinjection are probably not the result of classical

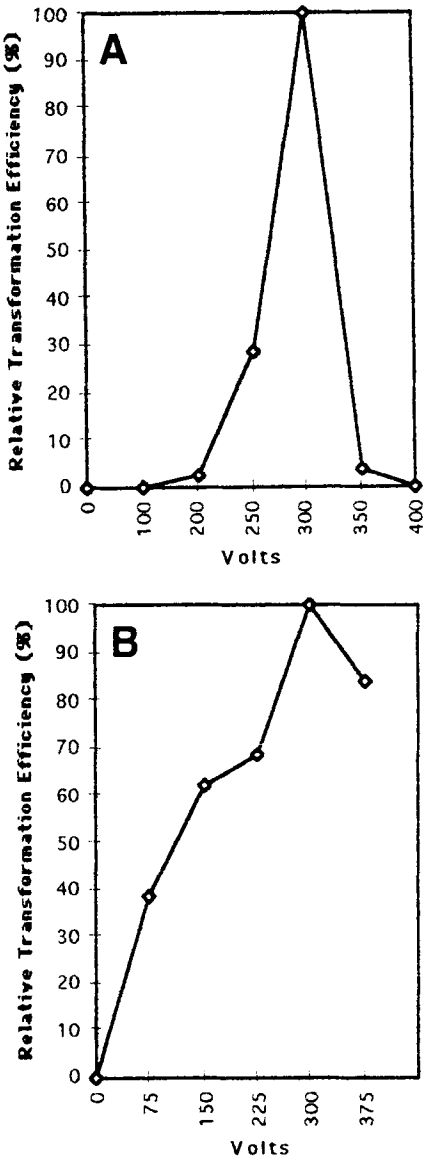

Fig. 11. Comparison of electroporation efficiency as a function of applied electric field in plant protoplasts **(A)** and plant cells present in an embryonic structure **(B)**. Data regraphed with permission from **refs. *24*** (A) and ***32*** (B).

electroporation since the electric field was only 166 V/cm, thus most likely below breakdown threshold. However, mechanical injection must have surrounded cells with DNA and damaged cell walls and membranes. It is thus likely that this step allowed internalization of DNA molecules. The electric shock may then have facilitated movement of DNA across the tissue, possibly through an elec-

trophoretic effect. Whether plasmodesmata were involved in this movement is unknown. It should nevertheless be remembered (*see* **Subheading 3.1.**) that breakdown threshold varies logarithmically with pulse duration. Thus, it is possible that long pulses may affect membrane fluidity and permeability in the absence of bona fide pore formation.

It is premature to try to generalize electrical conditions likely to lead to reproducible electrotransformation of plant cells, parts, or embryos. Nevertheless, electroporation parameters for protoplasts seem straightforward. Protoplasts isolated from mesophyll and tissue culture cells from most plant species should undergo poration at 400–500 V/cm, but no less than 300 V/cm in most cases. Single pulses from a 500 μF capacitor charged to 400–500 V, at 0°C and with $\tau = 50$ ms (and total $R = 100\ \Omega$) should lead to detectable transgene expression at energy and power levels of 40–62.5 J and 800–1250 J/s. At low-voltage values (compared to prokaryotes) the danger of arcing is much reduced and salt concentrations in the electroporation medium as high as the equivalent of 150 m*M* NaCl have been used. Of course, proper osmolality should be maintained (with mannitol, not high salt) to ensure protoplast integrity. These are by no means optimal conditions, obviously, and individual requirements for divalent cations in the medium, heat shock, pH, donor DNA concentration, and adjuvants such as polyethylene glycol should still be investigated. There is no agreement in the literature as to what such individual adjustments should be. However, full optimization of parameters may be of academic interest only if the goal is to generate transgenic lines at a reasonable frequency. It thus remains that very simple and inexpensive electroporation units (**Subheading 2.1.6.**) can be used to electrotransform plant protoplasts and isolate transgenic clones ***(38)***. Finally, plant protoplast electrotransformation frequencies in the 10^{-2} range have been reported although typical frequencies are more likely to be around 10^{-4}.

4.4. Animal Cells

As in the case of plant cells and protoplasts, transfection vectors can be linear or supercoiled DNA molecules, of the replicating or integrating type, usually containing a selectable or screenable marker. Biological parameters and electroporation media will depend on individual cases although phosphate-buffered saline or HEPES-buffered saline have been used successfully to achieve electroporation of various cell lines.

Mouse lyoma cells were the first to be electrotransfected by capacitor discharge ***(1,39)***. The electric field strength used in this pioneering work was extremely high (8 kV/cm), while the capacitance must have been very small (data not provided) given the very short *RC* constant (5 μs). Several years later, systematic studies ***(40,41)*** demonstrated that such high field strengths were unnecessary provided adequate capacitance values were used. Thus, as in the case of plant protoplasts, the debate between high voltage/low capacitance (or low *RC*) and low voltage/high capacitance (or high *RC*) was started. As before, there really is no debate at all since electroporation depends not on *RC* but on a combination of voltage and capacitance as determined by $W = 1/2\ (CV^2)$.

Human, monkey, and murine cell lines could be electrotransfected (frequency up to 1%) at an average field strength of 530 V/cm (200 V at the capacitor terminals) using a 1080-μF capacitor, giving a measured *RC* constant of 7 ms (thus more than 1000-fold higher than that observed or calculated in **ref.** ***1***) ***(40)***. Such a low *RC* constant obtained with a very large capacitance is explained by the 3.8 mm electrode gap, the small electrode surface area (0.017 cm^2), and the low resistance (calculated to be 6.48 Ω) of the electroporation medium (buffered physiological saline). Under the above conditions, the energy was 21.6 J and the power 3085 J/s. At 100 V, no transfection was observed, meaning that an energy of 5.4 J (power = 771 J/s) was not sufficient to achieve poration of the cells. Optimal field strength values at constant capacitance varied somewhat between cell lines, but energy values varied by a factor of less than two. The effectiveness of a lower capacitance was not investigated systematically.

Others ***(41)*** found that the human cell lines HEp-2 and 721 could be electrotransfected at

similar or somewhat higher field strengths at lower capacitance. HEp-2 cells were optimally transfected at 940 V/cm using a 25-μF capacitor (hence over 40 times smaller than in **ref. *40***) giving a measured *RC* constant of 10 ms (the larger *RC* constant obtained here in spite of the much lower capacitance was due to the larger distance between the electrodes and their much larger surface area, hence a much larger resistance even though buffered physiological saline was also used as electroporation medium). Calculations show that the energy released into the sample was 11 J (P = 1100 J/s) while W was 6.8 J (P = 338 J/s) in the case of 721 cells, which underwent electroporation at 520 V/cm with a 50-μF capacitor. Here again, seemingly tremendously different parameters yielded similar energy and power levels. Interestingly, no transfection of HEp-2 cells was observed at 500 V and 25 μF, meaning that an energy of 3.1 J was not high enough to achieve poration in these cells. First signs of successful electrotransfection appeared at an energy of 6.25 J. As can be seen, these energy values are quite consistent with those reported in **ref. *40***. Cell viability is an important consideration in selecting electroporation parameters. In this case, the viability of HEp-2 cells at 11 J was 80%, while that of 721 cells at the same energy was only 14%. Thus, better results for the latter were obtained at 6.8 J. Hence, high absolute electroporation efficiencies might be offset by cell death. In the above experiments, single pulses sufficed to achieve electrotransfection. When a very short time constant (5 μs, very low capacitance) was used, several pulses were required for optimal transfection frequencies ***(42)***, an observation also made with plant protoplasts.

Systematic studies as in **refs. *40*** and ***41*** were absolutely necessary since the literature at the time (even that published by prestigious laboratories) did not explicitly state electroporation parameters used and hence made duplication or extension of results impossible. Protocols for the electrotransfection of mammalian cells (and other cell types) are now available from manufacturers producing commercial electroporation units ***(43)***. In one such case, 112 protocols were counted, not all dealing with different cell types, however. Here again, it should be wondered whether so many recipes are necessary and how they differ. The examples given in **Table 4** were all obtained with the Bio-Rad Gene Pulser® system (Hercules, CA) which allows measurement of the *RC* constant. Electroporation medium was in all cases highly conductive and consisted of HEPES-buffered saline or phosphate-buffered saline. It can be seen that successful electrotransfection could be achieved, as expected, with the same type of cells (Chinese hamster ovary [CHO]), either at low capacitance and high voltage or the other way around, as long as the breakdown voltage and energy thresholds were exceeded. Other examples use low electric field strength and high capacitance and are representative of conditions that the vast majority of investigators use. Again, as in the case of bacterial cells and protoplasts, it does not seem that a wide flexibility regarding capacitors is necessary to achieve electrotransfection. A survey of dozens of reports (from **ref. *43***) shows that a typical mammalian cell able to sustain electroporation in a saline medium should be successfully electrotransfected at 500 V/cm (250 V at the capacitor terminals) using a 500-μF capacitance and a medium resistance of about 40 Ω to give an *RC* constant of about 20 ms, an energy of 15.6 J, and a power at $t = RC$ of 780 J/s. Again, these are by no means optimized conditions. However, low voltages are much safer at high ionic strength (saline solutions) as arcing is less likely to occur. It is remarkable, and perhaps not unexpected, that the above conditions are very similar to idealized parameters applicable to plant protoplasts (**Subheading 4.3.4.**).

Finally, cells in tissues can also be electrically permeabilized to drugs and DNA. Square-wave pulses at 400 V/cm lasting 100 μs were used to transfect neonatal mouse skin cells in vivo with a recombinant plasmid ***(44)***, while cells present in solid tumors could be electrically loaded with bleomycin ***(45)*** at 580 V/cm for 100 μs. Interestingly, in the latter case poration occurred at a threshold of 800 V/cm when the same cells were pulsed in suspensions. This led the authors to hypothesize that cells present in a tissue may

Table 4
Electrotransfection of Selected Mammalian Cell Lines[a]

Cell line	C (μF)	RC (ms)	E/d (kV/cm)	V (kV)	W (J)	P (J/s)	Protocol No.
CHO	25	0.5	1.88	0.75	7	14,000	088
CHO	960	35	0.625	0.25	30	857	091
HeLa	500	35	0.75	0.30	22.5	643	094
HeLa	960	30	0.425	0.17	13.8	462	097
Mouse ES	960	14.5	0.40	0.16	12.3	847	129
JEG-3	960	50	0.35	0.14	9.4	188	101

[a]Data obtained and calculated from protocols in **ref. *43***. Power P is calculated at $t = RC$. All results were obtained with the Bio-Rad Gene Pulser.

present lower breakdown voltage values resulting from their close contact with neighbors and the presence of intracellular junctions that would turn a tissue into an electrical continuum and affect the electric field. This report is reminiscent of what has been observed with plant cell clumps, organs, and embryos.

5. Discussion

This review has focused on electrical circuits and electrobiological parameters as they apply to gene transfer into cells. The theory of cell electroporation is incomplete, but there seems to be agreement that pore formation occurs through dipole orientation in the lipid bilayer and membrane compression as a consequence of an applied electric field, at least in one model. Pores may then appear at the level of lattice defects or protein channels ***(46)***. The current i induced in a defect will generate a certain amount of energy $\Delta W = i^2 r \Delta t$ (with r, the resistivity of the defect and Δt, the pulse duration) that will be dissipated locally as heat. This could also contribute to disordering of lipids and alteration of membrane protein and hence poration ***(46)***. One puzzling aspect of electroporation-mediated gene transfer is that pore size (1–10 nm) is too small to allow DNA entry. It has been suggested that only DNA molecules bound to the cell surface can be internalized through one of two mechanisms: (1) diffusion or endocytotic-like uptake of DNA, and (2) electrophoretic penetration of DNA enclosed in a vesicle formed by the disrupted lipid bilayer itself ***(46)***. In both cases, there is thus the intriguing possibility that electroporation might mimic liposome-mediated DNA uptake, which has been achieved in both prokaryotes and eukaryotes ***(47)***.

Another model holds that pore formation in a membrane is a stochastic, transient, and natural process in which microscopic pores occur randomly in a lipid bilayer as a result of thermal fluctuations. When an electric field is applied, pore size increases as a result of pore water polarization exerting pressure on pore walls ***(6,48)***. So far, neither model has been applied to the uptake of macromolecules.

A frequently raised question is that which concerns the relative merits of a capacitor discharge versus a square-wave pulse. Clearly, most researchers favor electroporation by capacitor discharge over square-wave pulses. It is legitimate to ask oneself the question as to why this is so. Certainly, one overarching reason is that units delivering true square-wave pulses are no longer on the market, at least in the United States. Yet, this is not truly answering the question. Rather, it may be that the answer lies in the history of the effects of electric fields on cells. Electrically induced cell fusion, based on the same principles as electroporation, was a reality before gene transfer by electroporation became popular ***(49)***. Units built to achieve cell fusion are much more complex than the ones required to effect electrotransformation; yet, given their existence at the time, they were the only ones available to test DNA transfer into cells. These units did not rely on capacitor discharge but used a square-wave voltage pulse and were extremely expensive. Hence,

the very first reports of cell electrotransformation either used such a unit *(39)* or were ambiguous as to whether a square-wave pulse or a capacitor discharge was used *(1)*. Also, electroporation/electrofusion chambers used early in this type of research were minuscule and better fit for microscopic observations of fusing cells than selection and propagation of transgenic lines. Further, electroporation parameters used with early instruments able to deliver square-wave pulses were incompletely described *(51–53)* and possibly not reproducible unless one used the very same equipment available to these authors. However, a subsequent study *(54)* was detailed enough to allow a direct (albeit theoretical) comparison between energy factors involved in the square-wave pulse and the capacitor discharge methods. In this report, carrot protoplasts underwent electroporation at 540 V/cm (above the membrane breakdown potential) with six square-wave pulses in a solution of ionic strength very close to that of physiological saline. Pulses lasted 10 ms and were repeated six times. Since the authors reported the electrode gap, electrode surface area, and temperature used, it is possible to calculate with a good degree of certainty that the resistance of the electroporation medium was 572 Ω (calculated resistivity at 0°C was approx 200 Ω/cm, gap was 0.75 cm, and electrode surface 0.2625 cm^2). Voltage at the terminals was 405 V. Under those conditions, the power $P = V^2/R$ was 286.7 J/s and the energy was $W = P$ x 0.01 s = 2.87 J. The current $I = V/R$ was thus theoretically 0.71 A. These parameters are well within limits reported in **Table 2**, which led to the successful electrotransformation of plant protoplasts with a capacitor discharge. Also, a current value of less than 1 A is acceptable to many power units. Not surprisingly, these authors were successful in observing transgene activity in their protoplasts. Thus, if breakdown voltage is low (and hence, cell size is large), it is expected that a square-wave pulse will be able to achieve electroporation of cells quite well, and this was indeed demonstrated. Similarly, mammalian cells were electrotransfected using square-wave pulses at 1-1.5 kV/cm, with a threshold of 0.5 kV/cm *(54)*. These authors compared transfection efficiencies obtained under these conditions with those observed after capacitor discharge. Twice the transfection efficiency was obtained at 6 kV/cm using a 50-nF capacitor, which explains why the field strength had to be so high. In this study, the time constant (unreported) must have been extremely short but unfortunately, since neither the resistance nor the current were given, it is impossible to draw an accurate comparison between the two systems.

Prokaryotes, however, would not be easily transformed with this kind of equipment. Assuming that the power required to achieve electroporation in a generic prokaryote is 10,000 J/s at $t = \tau$ for a capacitor discharge (**Subheading 4.1.**) and that the membrane breakdown voltage is 10 kV/cm (2 kV for a 2-mm electrode gap). Then, with a square-wave pulse, since $P = VI$ = 10,000 J/s = 2000 V x I, this gives a value for I of 5 A at $R = V/I$ = 400 Ω. This amperage cannot be sustained by simple power supplies. If the resistance of the system is increased to 2000 Ω, the current will be reduced to 1 A, and of course the power will then be only 2000 x 1 = 2000 J/s, which is below the level required to electrotransform bacteria efficiently, even though the membrane breakdown potential is reached. Thus, simple square-wave generators, even though they can electrotransform eukaryotic cells, do not usually have the flexibility and power to be used with prokaryotic cells. Nevertheless, specially built equipment was used to electrotransform *E. coli* and *S. typhimurium* with a square-wave pulse at 8 kV/cm *(55)*.

As we have seen in **Subheading 4.3.1.** and above, energy and possibly power are the factors that unite electroporation parameters. An interesting confirmation of this notion was presented indirectly in a study aimed at rationalizing electroporation conditions *(56)*. In this article, plant protoplasts underwent electroporation using a square-wave pulse at various voltages and pulse times in the presence of a dye and electroporation efficiency was evaluated according to the fraction of stained protoplasts. It should be remembered that plant protoplasts are excellent candidates for this kind of study owing to their spherical shape.

Computer calculations were made to fit the various curves obtained and a general empirical equation was derived which correlated E (field strength) and t (pulse time) as follows: $t \times E^q = K$ where q is a constant and K is a value that varies with the size of the protoplasts and their origin. The average value of q (calculated from the authors' data) is 1.89. It did not escape the authors' attention that electroporation results varied with the first power of time and roughly with the square of the electric field. I propose that Joersbo et al. ***(56)*** actually demonstrated empirically that electroporation results depend directly on the energy dissipated in the system and that their equation is the same as $W = t \times (V^2/R)$, which defines energy in a system containing no capacitor (such as that of **ref. *56***). Thus, a paradigm seems to exist that unifies electric parameters and explains why more than one set of conditions can yield nearly identical results. Therefore, it is not necessary, as has been suggested ***(57)***, to invoke the idea of two different mechanisms for electroporation, one at high field strength and another one at low field strength. Experiments with plant protoplasts, mammalian cells, and prokaryotes clearly show that in systems using a capacitor discharge, no transformation/transfection will occur even beyond voltage breakdown values if the capacitance is too small. This is in good agreement with the idea that an energy threshold ($W = 1/2\ CV^2$) must also be reached. But then, how does the power (dW/dt) factor in? This question is more difficult to resolve since by definition, small capacitors will yield small RC constants and hence comparatively high power values. On the other hand, large capacitors will give lower power (comparatively, not absolutely, since power also depends on V^2) values by definition since RC will be larger. Thus, it is difficult to determine what minimum power level is necessary to electrotransform since, intrinsically, C determines both power and time. This question could be resolved experimentally by keeping V *and* C constant while changing R (and hence RC), which will not modify the energy dissipated but will affect the power. To my knowledge, these experiments have not been done systematically. Modifying R can be achieved either by manipulating the internal resistance of the equipment, the interelectrode distance, or that of the electroporation medium. The latter however, is not recommended since physiological parameters would also be affected. Alternatively, the energy can be kept constant and the power made to vary by manipulating *both* V and C in such a way that $W = 1/2(CV^2)$ remains constant. **Figure 12** represents the principle graphically. For example, a 25-µF capacitor charged to 1549 V will discharge 30 J of energy with an RC constant of 12.5 ms and a power of 2400 J/s. Then, a 400-µF capacitor charged to 387 V will also release 30 J of energy, but with a time constant of 200 ms and thus a power of 150 J/s. It should be noted that power decreases rapidly with increasing capacitance initially, but as capacitance reaches 150 µF in this example, the rate of change tapers off (**Fig. 12**, inset). Such experiments should thus be conducted using appropriate capacitance values (here for example, between 25 and 100 µF) and of course, the same cell line.

An additional conundrum in electroporation experiments has to do with **Eq. 20**, which states that breakdown voltage is directly proportional to cell radius. Thus, since high-capacitance capacitors (of the electrolytic type) cannot accept voltage values in the kV range needed to achieve poration of prokaryotes, the RC constants obtained there, with mandated low capacitances, will be short and, de facto, power values will be high (given the high V^2). Therefore, it follows that prokaryotes will always be electrotransformed at high power while larger eukaryotic cells (plant protoplasts, protists, and mammalian cells) will be successfully transformed at power values one or two orders of magnitude less than with prokaryotes.

Factors affecting membrane integrity are rarely used in electroporation experiments. However, we have seen in **Table 2** that tobacco protoplasts could achieve electroporation at extremely low energy (although the power was high) in the presence of polyethylene glycol (PEG). It is well known that PEG considerably affects membrane properties and, in all likelihood, this is what happened in **ref. *18***. Indeed, a study on the effects of PEG on electropore formation in *Schizosacchar-*

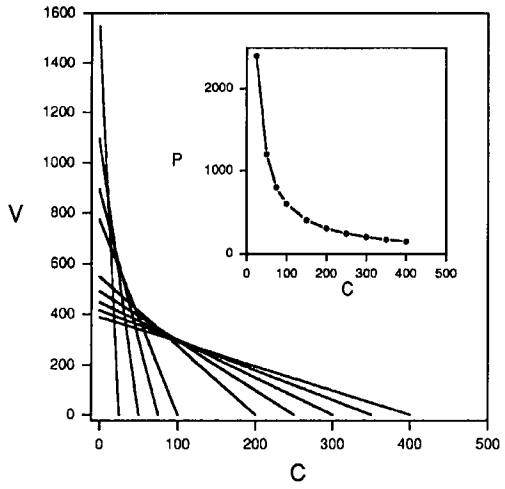

Fig. 12. Voltage and capacitance combinations, each yielding 30 J of energy, enough to electrotransform mammalian cells and plant protoplasts. At an arbitrarily set resistance of 500 Ω (to give a reasonable I_{max} value of 3.1 A at 1549 V, the highest voltage), the RC constants will range from 12.5 ms to 200 ms. Inset: variation of power d (1/2 CV^2)/dt as a function of capacitance. V is in volts, C in μF, and P at $t = RC$ in J/s.

omyces pombe clearly showed enhanced pore stability and size with PEG. Further, PEG may cause membrane compression and destabilization, possibly explaining why low energy values caused poration ***(58)***. Based on the same concept, it can be predicted that increasing the osmotic pressure inside cells through the use of a slightly hypotonic medium should also lead to greater electroporation efficiency (*see* **Subheading 3.2.**).

DNA concentrations used in electroporation experiments vary considerably according to the type of cell to be transformed or transfected. Highly competent *E. coli* cells only require a few pg/mL DNA while other bacterial genera may need several μg/mL. Typical values for yeast are around 2 μg/mL. Plant protoplasts and mammalian cell lines usually achieve electroporation in the presence of 10–50 μg/mL DNA or even higher. It is a general rule that transformation/transfection efficiencies increase linearly with DNA concentration until a plateau is reached. Since transformation vectors are usually amplified in *E. coli* or by PCR, DNA availability is generally not a problem, except in cases of cDNA cloning. In terms of size, it is not known at which point DNA molecules will cease to be internalized by electroporation. Very large viral and plasmid genomes have been transferred to mammalian cells (HSV-1, approx 150 kbp **ref. *41***) and plant protoplasts (pTi C58 from *Agrobacterium tumefaciens*, approx 220 kbp **ref. *50***), although in the latter case, it is not evident that intact plasmid molecules were taken up.

Another important parameter, cell viability after treatment, depends on poration parameters, temperature, and electroporation medium. No single recommendation exists that would fit all cell types, although, invariably, electroporation of cells under conditions that allow gene transfer will always produce significant mortality. It has been observed ***(59)*** that different mammalian cell types display different levels of electrosensitivity, regardless of cell size. Some cell lines showed very poor (10%) survival at 5 kV/cm while others had a 70% survival rate at the same field strength. The reasons for these variations are not known but may involve the ability of the plasma membrane to reseal after the shock, possibly owing to surface geometry and effect on the cytoskeleton. Nevertheless, significant cell death only occurred beyond the electropermeabilization threshold (observed at 1–2 kV/cm using a square-wave pulse).

No other transformation/transfection technique is as far-reaching as electroporation. The calcium chloride shock so successful in the transformation of *E. coli* does not work with most bacterial genera. Alkaline cation-mediated transformation that works well for yeast does not work for most other microscopic fungi, while PEG-mediated transformation of plant protoplasts is not applicable to animal cells. Further, the calcium phosphate-DNA coprecipitate technique is not applicable to many mammalian cell lines. The only true competitor of the electroporation process in the latter case seems to be the technique known as lipofection (transfection aided by small unilamellar cationic liposomes), which has been shown to work well with mammalian cells but is not used in other systems.

Finally, a last twist on the electroporation process is its adaptation to electrotransformation of microorganisms without DNA purification. The principle here is that if electrically induced pores can allow DNA uptake, they should also allow DNA escape from cells. Further, if a second population of microorganisms is concomitantly present in the electroporation medium or is added later and undergoes electroporation separately, these cells should be able to internalize the released DNA molecules. Such quick transformation technique was shown to allow plasmid transfer between different bacterial genera and between yeast and *E. coli* ***(60–62)***.

In conclusion, the electroporation process as a means to achieve gene transfer in an extremely wide variety of cells supersedes all other techniques. This is because this process is based on a physical principle, the release of electrical energy, at the level of the boundary between the intracellular and outside worlds, the membrane bilayer.

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Note Added in Proof

C. Guangyu, A. J. Conner, J. Wang, A. G. Fautrier, and R. J. Field (in a manuscript entitled "Energy dissipation as a key factor for electroporation of protoplasts," submitted) demonstrated empirically that the electroporation efficiency of asparagus protoplasts increases linearly with energy dissipation between 0 and 100 J. A 10-fold range in the value of the RC constant, at three energy levels, had very little effect on electroporation efficiency. Their empirical results are thus in excellent agreement with the theoretical considerations developed in this review.

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