Pore Disappearance in a Cell after Electroporation: Theoretical Simulation and Comparison with Experiments

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ABSTRACT The process of pore disappearance after cell electroporation is analyzed theoretically. On the basis of the kinetic model, in which the formation and annihilation of a metastable hydrophilic pore are considered as random one-step processes, a distribution function of cell resealing times, $F_r(t)$, is derived. Two cases are studied: 1) the rate of pore resealing, k_r, is significantly greater than the rate of pore formation, k_f; and 2) the rate of pore formation, k_f, is comparable with k_r. It is determined that the shape of the distribution function depends on the initial number of pores in a cell, n_i. If in the absence of an external electric field the rate of pore formation, k_f, is significantly less than the rate of pore resealing, k_r (case 1), pores disappear completely, whereas when $k_f \approx k_r$ (case 2), the cell achieves a steady state in which the number of pores is equal to k_f/k_r. In case 1, when $n_i = 1$, the distribution function $F_r(t)$ is exponential. The developed theory is compared with experimental data available in the literature. Increasing the time of incubation at elevated temperature increases the fraction of resealed cells. This indicates that the time necessary for the resealing varies from cell to cell. Although the shape of experimental relationships depends on the electroporation conditions they can be described by theoretical curves quite well. Thus it can be concluded that the disappearance of pores in the cell membrane after electroporation is a random process. It is shown that from the comparison of presented theory with experiments, the following parameters can be estimated: the average number of pores, $\bar{n}_i$, that appeared in a cell during an electric pulse; the rate of pore disappearance, k_r; the ratio k_f/k_r; and the energy barrier to pore disappearance $\Delta W_r(0)$. Estimated numerical values of the parameters show that increasing the amplitude of an electric pulse increases either the apparent number of pores created during the pulse (the rate of pore resealing remains the same) or the rate of pore resealing (the average number of pores remains the same).

INTRODUCTION

Electroporation of biological membranes has numerous applications in molecular biology, biotechnology, and medicine (Chang et al., 1992; Neumann et al., 1989; Orlowski and Mir, 1993; Weaver, 1993). However, experimental procedures have not yet been optimized, as the mechanisms of pore formation under the influence of an electric field and their subsequent resealing are not fully understood.

For a few decades investigators have been studying the phenomenon of electroporation, but their attention is mainly focused on studying the kinetics and mechanism of pore formation. There are a lot of theoretical studies on the formation of pores in biological membranes under the action of electric fields (Abidor et al., 1979; Crowley, 1973; Barnett and Weaver, 1991; Dimitrov, 1984; Glaser et al., 1988; Neumann, 1989; Neumann et al., 1992; Pastushenko et al., 1979a,b; Pastushenko and Chizmadzhev, 1982; Powell and Weaver, 1986; Sugar, 1989; Sugar and Neumann, 1984), and as a consequence the theory of this process is quite well developed (Barnett and Weaver, 1991; Freeman et al., 1994; Glaser et al., 1988; Weaver and Barnett, 1992).

Despite the fact that the process of the disappearance of pores after an electric pulse is particularly important for practical applications, relatively little is known about it (Weaver, 1994). Although there are a significant number of experimental studies in which some data on the kinetics of the resealing process are presented (Chernomordik et al., 1987; Deuticke and Schwister, 1989; Escande-Geraud et al., 1988; Glaser et al., 1988; Hibino et al., 1993; Kinosita and Tsong, 1977, 1979; Muraji et al., 1993; Rols et al., 1990; Serpersu et al., 1985; Zimmermann et al., 1980), no systematic study has yet been done. In addition, theorists have not paid proper attention to this process so far. In particular, very few such characteristics of this process that could be measured experimentally are described theoretically.

In this study the process of pore disappearance in a cell is investigated theoretically. The distribution function of cell resealing times, $F_r(t)$, an important characteristic that shows the dependence of the probability that a cell is resealed on the time passed after the pulse, is derived.

Theoretical simulations of the kinetics of pore resealing in the cell after electroporation are carried out on the basis of the kinetic model of the cell electroporation process, in which the formation and resealing of a metastable hydrophilic pore are considered as random one-step processes (Saulis and Venslauskas, 1993a). The distribution function of the cell resealing times $F_r(t)$ is derived for two cases:

1. the rate of pore resealing k_r is significantly greater than the rate of pore formation k_f and therefore the latter is neglected, and

2. the rate of pore formation k_f is taken into consideration.

In addition to theoretical simulations, the developed theory is compared with experimental data available in the

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literature, and the useful information that can be obtained from this comparison is dicussed.

THEORY

Kinetic scheme

Theoretical analysis and experimental data show that the resealing of the pore consists of several stages (Glaser et al., 1988; Hibino et al., 1993; Kinosita and Tsong, 1979; Saulis et al., 1991; Serpersu et al., 1985; Tsong, 1983), that is, the stages of the fast reduction of pore size until the small value $r \approx 0.5$ nm and the stage of the slower, complete pore closure (overcoming the energy barrier to pore resealing). However, the time constant of the last stage is significantly greater than that of the first ones (Saulis et al., 1991), and therefore in the first approximation the first stages can be neglected. Then the disappearance of a metastable pore can be considered a one-step process. The rate of this process can be expressed as (Saulis et al., 1991)

$$k_r = \Lambda \exp(-\Delta W_r(\Delta\Phi_m)/kT) \qquad (1)$$

where Λ is the preexponential factor with the dimension of velocity, $\Delta W_r(\Delta\Phi_m)$ is the energy barrier to pore resealing at the transmembrane potential $\Delta\Phi_m$ (Fig. 1), k is Boltzmann's constant, and T is the absolute temperature.

Let us consider the following.

1. The creation and disappearance of metastable hydrophilic pores are random one-step processess, i.e., the pores need only overcome one energy barrier (Fig. 1) (Glaser et al., 1988; Freeman et al., 1994).

2. Pores of only one type appear in the cell membrane.

3. The pores do not interact with each other.

4. The transmembrane potential is small and the same at all points on the cell surface.

On the basis of these assumptions, the cell electroporation process can be described by the following general kinetic scheme (Saulis and Venslauskas, 1993a):

$$\textcircled{0} \underset{k_{r1}}{\overset{k_{f0}}{\rightleftharpoons}} \textcircled{1} \underset{k_{r2}}{\overset{k_{f1}}{\rightleftharpoons}} \textcircled{2} \underset{k_{r3}}{\overset{k_{f2}}{\rightleftharpoons}} \cdots \underset{k_{rn}}{\overset{k_{f(n-1)}}{\rightleftharpoons}} \textcircled{n} \underset{k_{r(n+1)}}{\overset{k_{fn}}{\rightleftharpoons}} \cdots \qquad (2)$$

Here the numerals in circles refer to the number of pores in a cell, and k_{fn} and k_{rn} are the rates of pore formation and resealing, respectively, in a cell with n pores. In a general case, both rates are the functions of the transmembrane potential: $k_f = k_f(\Delta\Phi_m)$, $k_r = k_r(\Delta\Phi_m)$ (Saulis and Venslauskas, 1993a), but so as not to complicate equations, this will not be indicated. It should be mentioned that this kinetic scheme is a modification of the scheme used by Chizmadzhev's group to describe the electrical breakdown of bilayer lipid membranes (Pastushenko et al., 1979a).

In the analysis presented here, the process of pore formation is also taken into account, because the possibility of the spontaneous appearance of metastable hydrophilic pores cannot be excluded, even if the transmembrane potential is equal to zero (Taupin et al., 1975; Markin and Kozlov, 1985; Popescu and Rucareanu, 1991; Powell and Weaver, 1986). Some cells are permeable for membrane-impermeable substances without any exposure to an electric field (Joersbo et al., 1990; Marszalek et al., 1990), and the fraction of such cells can be as high as 18–22% (Joersbo et al., 1990). This fact is also in favor of the assumption that before electroporation some nonempty population of pores may be present in a cell.

Earlier the approach based on the analysis of such kinetic schemes was successfully used to study the kinetics of pore formation in both planar bilayer lipid (Pastushenko et al., 1979a) as well as cellular (Saulis and Venslauskas, 1993a) membranes. Here this approach will be used to analyze the opposite process, namely, the annihilation of pores in a cell membrane after electroporation. This process of pore disappearance can be characterized by the distribution function of cell resealing times, $F_r(t)$.

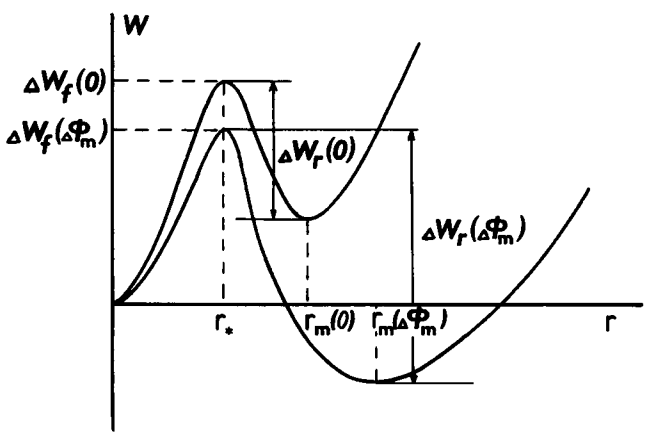

FIGURE 1 Pore energy W as a function of pore radius in the absence (*upper curve*) and presence (*lower curve*) of a transmembrane potential $\Delta\Phi_m$. Values of pore radius $r < r_*$ correspond to hydrophobic and $r > r_*$ to hydrophilic pores. Hydrophilic pores formed as a result of hydrophilization of hydrophobic pores are metastable, owing to the existence of an energy barrier $\Delta W_r(\Delta\Phi_m)$ that prevents their closing. At zero transmembrane potential, the energy of a membrane with one pore is greater than the energy of an intact membrane. Therefore it can be expected that the energy barrier to pore disappearance should be smaller than the barrier to pore formation as it is illustrated here. The transmembrane potential diminishes the energy barrier to pore formation, $\Delta W_f(\Delta\Phi_m)$, and raises the energy barrier to pore disappearance, $\Delta W_r(\Delta\Phi_m)$.

Distribution function of cell resealing times $F_r(t)$

The distribution function of cell resealing times, $F_r(t)$, shows the dependence on time of the probability that a cell is resealed. Obviously,

$$F_r(t) = 1 - F_p(t) \qquad (3)$$

where $F_p(t)$ is the time dependence of the probability that a cell is still porated after the end of the pulse, i.e., in the

absence of an external electric field. According to the definition of a porated cell given by us earlier (Saulis and Venslauskas, 1993a), $F_p(t)$ is

$$F_p(t) = \sum_{n=n_{cr}}^{\infty} P_n(t) \quad (4)$$

where $P_n(t)$ is the probability that there are n pores in the cell at an instant t and n_{cr} is the critical number of pores (i.e., the number of small metastable pores sufficient for the cell to be regarded as porated). Recall that a cell is porated, when, under the influence of the transmembrane potential or spontaneously, the cell membrane permeability to the smallest ions such as K^+, Na^+, and Rb^+ is so enhanced over the background level that this increase can be detected (Saulis and Venslauskas, 1993a). Therefore,

$$F_r(t) = \sum_{n=0}^{n_{cr}} P_n(t) \quad (5)$$

It has been shown that even one pore with a radius of 0.55 nm is sufficient for a human erythrocyte to be regarded as porated (Schwister and Deuticke, 1985). In addition, the shape of the experimental distribution functions of cell poration times, $F_p(t)$, shows that n_{cr} for human erythrocytes is close to unity (Saulis and Venslauskas, 1993b). Although the probability of a more complicated case cannot be excluded, for simplicity, the expressions for the distribution function $F_r(t)$ will be presented only for $n_{cr} = 1$. That is, it will be assumed that a cell is resealed if there are no pores in its membrane.

Thus the distribution function of cell resealing times, $F_r(t)$, shows the dependence of the probability that there are no pores in a cell on the post-pulse incubation time:

$$F_r(t) = P_0(t) \quad (6)$$

Kinetics of pore resealing

Under certain conditions, electric field-induced pores reseal after the electric pulse terminates. Usually, in considering the process of pore resealing after an electric pulse, it is assumed that additional pores do not appear in the membrane, that is, that the pore formation rate is equal to zero. Let us first analyze this simple case.

Case where $k_f \ll k_r$

If, in the absence of the transmembrane potential, the rate of pore resealing is significantly greater than the rate of pore formation ($k_r \gg k_f$), the latter can be neglected. When pores do not interact with each other, the disappearances of pores are independent. In such a case $k_{rn} = nk_r$ (Pastushenko et al., 1979a). Then, taking this into account, the kinetic Scheme 2 simplifies to

$$0 \xleftarrow{k_r} 1 \xleftarrow{2k_r} 2 \xleftarrow{3k_r} \cdots \xleftarrow{n_i k_r} n_i \quad (7)$$

where n_i is the number of pores in a cell just after the electric pulse (the initial number).

For such a kinetic scheme (see Appendix A),

$$F_r(t) = [1 - \exp(-k_r t)]^{n_i} \quad (8)$$

Theoretical distribution functions $F_r(t)$ for $n_i = 1$, 3, and 10, plotted according to Eq. 8, are shown in Fig. 2 (*solid lines*). Curves in Fig. 2 *B* are plotted in such a way that they would intersect at the point at which $F_r(t) = 1 - \exp(-1)$. From this figure it can be seen that the shape of the distribution function $F_r(t)$ depends strongly on the initial number of pores in a cell, n_i. The more pores that are created during the pulse, the longer the time required for the complete disappearance of pores in a cell.

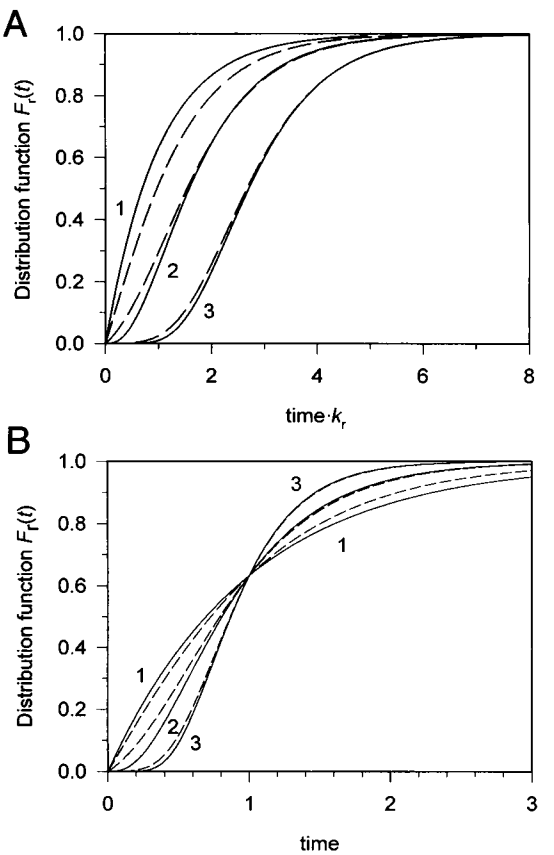

FIGURE 2 (*A*) Distribution functions of cell resealing times, $F_r(t)$, in the case where the pore formation rate is significantly less than the pore resealing rate, calculated from Eq. 8 for various values of the initial number of pores, $n_i = 1$ (*curve 1*), 3 (*curve 2*), and 10 (*curve 3*). When $n_i = 1$, the distribution function $F_r(t)$ is exponential. Dashed lines are distribution functions obtained assuming that n_i is a random value and is distributed according Poisson distribution (Eq. 10), calculated from Eq. 9 for the average number of pores $\bar{n}_i = 1$, 3, and 10. (*B*) The same dependences are plotted in such a way that they would intersect at the point at which $F_r(t) = 1 - \exp(-1)$.

We consider the process of the appearance of pores to be stochastic. Thus the number of pores that have appeared in the cell membrane during the pulse (the initial number of pores, n_i, for the resealing process) is a random value. Taking this into consideration, Eq. 8 must be rewritten as

$$F_r(t) = \sum_{n_i=1}^{\infty} P_{n_i}(0)[1 - \exp(-k_r t)]^{n_i} \quad (9)$$

where $P_{n_i}(0)$ is the probability that there are n_i pores in a cell just after the electric pulse.

We calculated $F_r(t)$ taking into account the fact that n_i is a random value. We assumed that the probabilities $P_{n_i}(0)$ that n_i pores have appeared in a cell during the pulse are given by the Poisson distribution,

$$P_{n_i}(0) = (\bar{n_i})^{n_i}\exp(-\bar{n_i})/n_i! \quad (10)$$

where $\bar{n}_i$ is the mean value of n_i and $n_i!$ is the factorial function $n_i! = 1 \cdot 2 \ldots n_i$.

Dashed lines in Fig. 2 are distribution functions $F_r(t)$ calculated according Eq. 9, in which the probabilities $P_{n_i}(0)$ are given by Eq. 10, for $\bar{n}_i = 1$, 3, and 10. From Fig. 2 it is seen that the deviations due to inhomogeneous distribution of the initial number of pores in the cells of a population are noticeable only for small n_i.

Here the pore formation process was neglected in what is a common approach. However, in a more general case the possibility of the spontaneous appearance of metastable hydrophilic pores cannot be excluded, even in the case of zero transmembrane potential (Taupin et al., 1975; Markin and Kozlov, 1985; Popescu and Rucareanu, 1991; Powell and Weaver, 1986). We now proceed to an analysis of the case of a nonzero pore formation rate.

Case where $k_f \approx k_r$

In his case the process of pore formation cannot be neglected. Note that we are analyzing the case where the transmembrane potential is small and the same at all points on the cell surface. Let us assume that the probability of an additional pore forming does not depend on how many pores already exist. This assumption should be valid as long as the fraction of membrane area occupied by pores is small (Barnett and Weaver, 1991), and factors that can noticeably change the rate of pore formation can be ignored. Furthermore, it has already been stated that there is no interaction between pores. Thus

$$k_{fn} = k_{fi} = k_f (i \neq n), \quad k_{rn} = nk_r \quad (11)$$

Then we have the following kinetic scheme:

$$0 \underset{k_r}{\overset{k_f}{\rightleftharpoons}} 1 \underset{2k_r}{\overset{k_f}{\rightleftharpoons}} 2 \underset{3k_r}{\overset{k_f}{\rightleftharpoons}} \cdots \underset{n_i k_r}{\overset{k_f}{\rightleftharpoons}} n_i \underset{(n_i+1)k_r}{\overset{k_f}{\rightleftharpoons}} \cdots \quad (12)$$

For such a kinetic scheme the distribution function $F_r(t)$ is (see Appendix B)

$$F_r(t) = \exp(-[k_f/k_r][1 - \exp(-k_r t)])[1 - \exp(-k_r t)]^{n_i} \quad (13)$$

If we take into account the assumption that the process of the appearance of pores is a stochastic one and therefore the initial number of pores, n_i, is a random value, Eq. 13 must be rewritten as

$$F_r(t) = \exp(-[k_f/k_r][1 - \exp(-k_r t)]) \times \sum_{n_i=1}^{\infty} P_{n_i}(0)[1 - \exp(-k_r t)]^{n_i} \quad (14)$$

It can be seen that Eq. 9 can be directly obtained from Eq. 14, upon substituting $k_f \ll k_r$.

Distribution functions $F_r(t)$ calculated according to Eq. 13 for $n_i = 1$, 3, and 10 are presented in Fig. 3 (*solid lines*). As in the previous case, dashed lines represent distribution functions for the case where n_i is a random value and is distributed according to the Poisson distribution, with the mean value $\bar{n}_i$ equal to 1, 3, and 10 pores.

As can be seen from Figs. 2 and 3, when, in the absence of an external electric field the rate of pore formation k_f is significantly less than the rate of pore resealing k_r ($k_f \ll k_r$), pores disappear completely, whereas when the rate of pore formation is noticeable and thus cannot be neglected, the cell achieves the steady state with a nonzero number of pores. Substituting $t = \infty$ into Eq. 14 and taking into account Eq. 3, the fraction of cells that have pores in this state is

$$F_p(\infty) = 1 - \exp(-k_f/k_r) \quad (15)$$

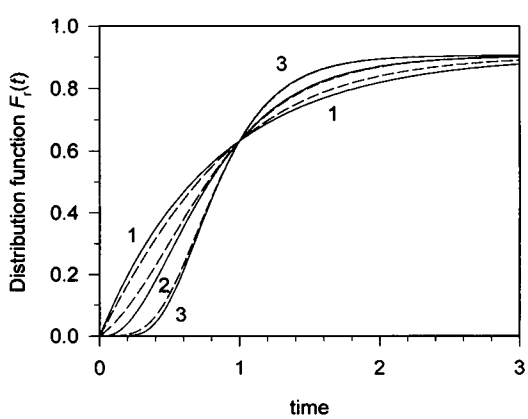

FIGURE 3 Distribution functions $F_r(t)$ in the case where the pore formation rate is taken into account, calculated from Eq. 13 for various values of initial number of pores, $n_i = 1$ (*curve 1*), 3 (*curve 2*), and 10 (*curve 3*), and assuming $k_f/k_r = 0.1$. Dashed lines are distribution functions $F_r(t)$, in the case where the initial number of pores is a random value and is distributed according Poisson distribution (Eq. 10) for the mean values of initial number of pores, $\bar{n}_i = 1$, 3, and 10.

For the kinetic scheme (Eq. 12), one can find that the average number of pores per cell, $\bar{n}$, depends on time as (see Appendix B)

$$\bar{n}(t) = (k_f/k_r)[1 - \exp(-k_r t)] + n_i \exp(-k_r t) \quad (16)$$

From this equation it follows that at the stationary state (at $t \rightarrow \infty$),

$$n(\infty) = k_f/k_r \quad (17)$$

The theoretical time dependences of the average number of pores calculated according Eq. 16 for $k_f/k_r = 0.05$ and $n_i = 0$, 1, 3, and 10 pores are presented in Fig. 4. It must be emphasized that the stationary state that a cell enters after a long enough time does not depend on initial conditions (see Fig. 4 and Eq. 17). Only the time necessary to achieve this state increases with an increasing initial number of pores in a cell. Therefore, if cell electroporation is fully reversible, the steady state should be the same for a resealed cell as well as the cell unexposed to an electric pulse.

However, cells have a nonzero resting potential that may vanish as a result of permeabilization of cell membrane. In such a case it is quite possible that the number of pores in the state when the resealing process is finished may be even lower than before electroporation. The lower number of pores should remain steady until the resting potential is restored.

Recently the expression for the initial rate of pore formation in a spherical cell was derived (Saulis and Venslauskas, 1991). In the case where the transmembrane potential is the same at any point of the cell surface, this expression gives us (see also Glaser et al., 1988)

$$k_f(\Delta\Phi_m) = \frac{4\pi\nu a^2}{a_l} \exp[-\Delta W_f(\Delta\Phi_m)/kT] \quad (18)$$

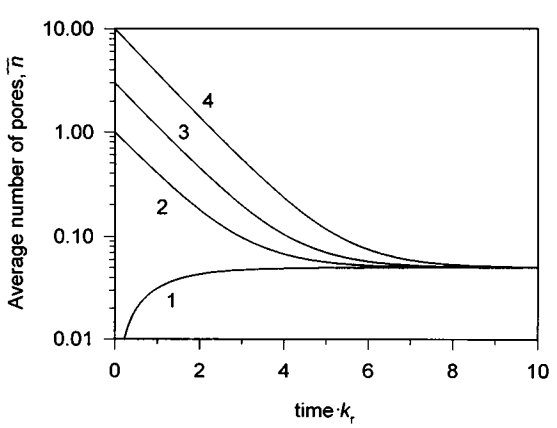

FIGURE 4 Theoretical dependence of the average number of pores in a cell, $\bar{n}$, on the time passed after the end of the electric pulse, calculated from Eq. 16 for the initial number of pores, $n_i = 0$ (*curve 1*), 1 (*curve 2*), 3 (*curve 3*), and 10 (*curve 4*) assuming that $k_f/k_r = 0.05$. Irrespective of the value of the initial number of pores, a cell achieves the same steady state. In this steady state the number of pores is equal to k_f/k_r.

where a is the cell radius, ν is the frequency of lateral fluctuations of lipid molecules, a_l is the area per lipid molecule, $\Delta W_f(\Delta\Phi_m)$ is the energy barrier for hydrophilic pore formation at the transmembrane potential $\Delta\Phi_m$, k is Boltzmann's constant, and T is the absolute temperature.

Equations 1 and 18 yield

$$k_f/k_r = \frac{4\pi\nu a^2}{a_l \Lambda} \exp\left[-\frac{\Delta W_f(\Delta\Phi_m) - \Delta W_r(\Delta\Phi_m)}{kT}\right] \quad (19)$$

This equation gives us the temperature dependence of the ratio k_f/k_r.

So far we have assumed that the process of cell electroporation is fully reversible. However, it is quite possible that some cells may undergo irreversible electroporation as a result of the appearance of irreversible pores or because of other reasons. In such a case, Eq. 14 should be modified in the following manner:

$$F_r(t) = (1 - F_{irr})\exp(-k_f/k_r[1 - \exp(-k_r t)]) \times \sum_{n_i=1}^{\infty} P_{n_i}(0)[1 - \exp(-k_r t)]^{n_i} - F_{irr} \quad (20)$$

where F_{irr} is the fraction of the cells that have been damaged irreversibly during electroporation.

In the simplest case, when $k_f \ll k_r$ and $n_i = 1$, Eq. 20 yields

$$F_r(t) = (1 - F_{irr})[1 - \exp(-k_r t)] + F_{irr} \quad (21)$$

and, taking into account Eq. 3, we have

$$F_p(t) = [1 - F_{irr}]\exp(-k_r t) + F_{irr} \quad (22)$$

Exactly the same equation, except for notations, was used by Muraji et al. (1993) and Neumann and Boldt (1990) for the description of their experimental data.

COMPARISON WITH EXPERIMENTAL DATA

Any theory should be compared with experiments. However, a comparison can be made only if the characteristics of the process, derived theoretically, can be obtained experimentally. In our case such a characteristic is the distribution function of cell resealing times, $F_r(t)$.

The kinetics of pore resealing after electroporation is mainly measured by studying the time course of the decrease in 1) membrane conductivity (Chen and Lee, 1994; Chernomordik et al., 1987; Hibino et al., 1993; Kinosita and Tsong, 1979; Kinosita et al., 1988; Pliquett and Wunderlich, 1983), 2) membrane permeability to small inorganic ions (Deuticke and Schwister, 1989; Glaser et al., 1988; Kinosita and Tsong, 1977; Serpersu et al., 1985) or other substances (Sowers, 1988; Sowers and Lieber, 1986; Yumura et al., 1995), and 3) the fraction of cells permeable to small inorganic ions (Saulis et al., 1991) or certain membrane-impermeant compounds (Escande-Geraud et al., 1988; Gabriel and Teissie, 1995; Muraji et al., 1993; Neumann and

Boldt, 1990; Rols and Teissie, 1989; Tatebe et al., 1995; Tsoneva et al., 1990; Zimmermann et al., 1980).

In the first two methods the parameters, the time dependences of which are recordered during resealing experiments, depend on both the size and the number of pores. Thus by measuring only the decrease in the conductivity or permeability after electroporation, it is impossible to determine whether this decrease is caused by the decrease in the size of pores or by the decrease in their number. In addition, these parameters are the sums of the values for individual pores. However, one large pore has the same conductivity or permeability as many small pores, and thus the shrinkage of one large pore is equivalent to the disappearance of many small pores. Because any pore should shrink at first before it can disappear completely, it is likely that the rapid decrease in conductivity or permeability that is observed just after an electric pulse is due mainly to the decrease in the size of pores (Barnett and Weaver, 1991; Saulis et al., 1991). This may also explain why the time course for the decrease in conductivity and permeability to inorganic ions after electroporation does not obey first-order kinetics, as was recently observed (Deuticke and Schwister, 1989; Glaser et al., 1988; Hibino et al., 1993). As the theory presented in this study describes the changes in the number of pores in a cell, the numerous experimental data obtained by using these two methods cannot be utilized, unfortunately, to test the validity of the theory.

In case 3 electroporation of any cell is considered an all-or-nothing event. That is, a cell is considered porated only if, upon termination of a certain period of time, a detectable number of test molecules have entered the cell. This can occur if a pore population, created during the electric pulse and consisting of a sufficient number of pores that are larger than the radius of a test molecule, is present in a cell. This means that in such a case only a change in the number of pores, which are so large that they can be detected by a particular method, is reflected.

The kinetic model presented in this study has been developed to describe the kinetics of the complete disappearance of pores in a cell. Unfortunately, there are very few experimental works in which such a kinetics was measured. The most popular experimental method for determining the kinetics of resealing of electropermeabilized plasma membranes is to measure the permeability of a particular molecular probe added to the medium at various times after the delivery of an electric pulse, e.g., trypan blue (Escande-Geraud et al., 1988), phloxine B (Muraji et al., 1993), or eosin (Zimmermann et al., 1980). However, the recovery of membrane barrier function to a particular probe molecule does not always mean that the pores have completely disappeared (Saulis et al., 1991).

Fortunately, the theoretical model developed here is also applicable to the description of the disappearance of pores that are larger than a certain chosen size, i.e., those that are large enough to allow test molecules (e.g., trypan blue) to pass through them. In such a case numerals in circles in kinetic schemes 2, 7, and 12 refer to the number of these pores. Thus the experimental data obtained in such a way can be used to ascertain whether the theory presented here is capable of describing the main features of the kinetics of pore disappearance after cell electroporation.

Now let us proceed to the comparison of the developed theory with experimental data. We will discuss the parameters that can be determined from such a comparison and how it can be made. Experimental data taken from several published papers (Muraji et al., 1993; Rols et al., 1990; Saulis et al., 1991) will be employed.

First of all, one should ascertain whether theoretical functions $F_r(t)$ obtained here can describe experimental dependences of the fraction of resealed cells on the time passed after the end of the pulse. It should be noted that in all experimental studies cited here, the time dependences of the opposite value, namely, the fraction of the cells that still have pores (are still porated), F_p, are presented. Thus the fraction of resealed cells was calculated from

$$F_r(t) = 1 - F_p(t)/F_p(0) \tag{23}$$

where $F_p(0)$ is the fraction of porated cells just after the electric pulse.

In Fig. 5 such dependences, taken from the papers published by Rols et al. (1990) (Fig. 5 *A*) and Muraji et al. (1993) (Fig. 5 *B*), are presented. Experimental points in Fig. 5 *A* show the dependences of the fraction of Chinese hamster ovary cells, the membrane of which has restored its impermeability to trypan blue, on the time elapsed after the pulse. Electroporation conditions were 10 square-wave pulses, $\tau_i = 100$ μs, $\nu = 1$ Hz, $E_0 = 1.5$ (*filled circles*), and 1.8 (*open triangles*) kV/cm, and resealing was monitored at $T = 21$°C.

Solid lines in Fig. 5 *A* are the theoretical distribution functions calculated from Eq. 20, assuming that the probabilities $P_{n_i}(0)$ that n_i pores have appeared in a cell during an electric treatment are given by the Poisson distribution (Eq. 10) and $F_{irr} = 0$. The rest of the unknown parameters in Eqs. 10 and 20, namely, the average number of pores, $\bar{n}_i$, that appeared in a cell during an electric pulse (Eq. 10); the rate of pore disappearance, k_r; and the ratio k_f/k_r were varied to obtain the best fit of the theoretical dependences $F_r(t)$ to experimental points.

It should be pointed out that an average number of pores, $\bar{n}_i$, that appeared in a cell during an electric pulse; the rate of pore resealing, k_r; and the ratio k_f/k_r between the rates of pore formation and resealing in the absence of an external electric field affect different features of the distribution function of cell resealing times, $F_r(t)$ (see Eq. 20 and Figs. 2 and 3). The average value of the initial number of pores, $\bar{n}_i$, determines the shape of the distribution function $F_r(t)$; k_r is the time constant of the process of pore disappearance; and the value of the distribution function $F_r(t)$ at $t = \infty$ depends on the ratio k_f/k_r. Therefore the estimations of these parameters are independent (in the case where a sufficient number of experimental points are available).

The best agreement was obtained for $\bar{n}_i = 1$, $k_r = 3.9 \times 10^{-3}$ s^{-1}, and $k_f/k_r = 0.06$ in the case where the amplitude

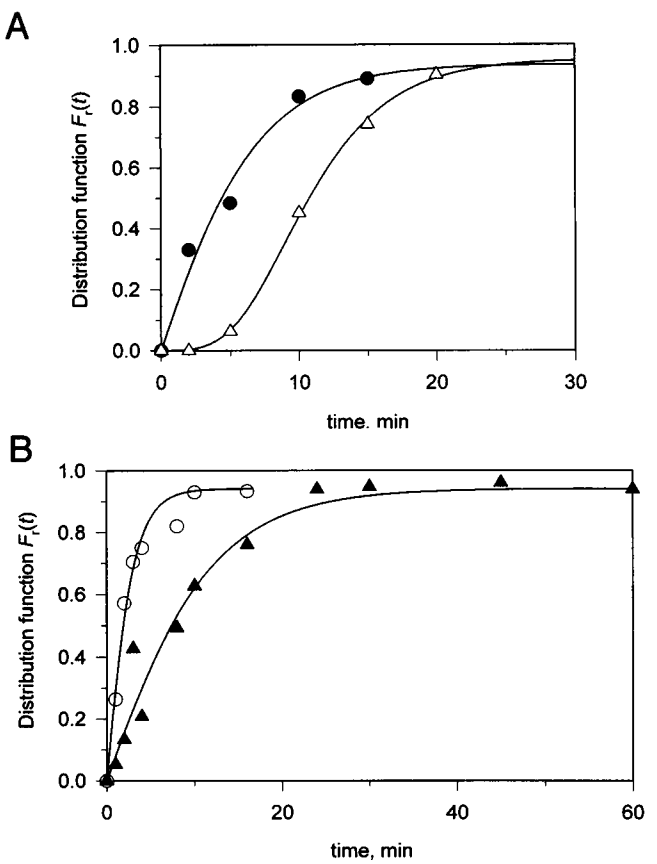

FIGURE 5 (*A*) Experimental points show the dependence of the fraction of Chinese hamster ovary cells, the membrane of which has restored its impermeability to trypan blue, on the time passed after the pulse. The data are taken from the paper published by Rols et al. (1990). Electroporation conditions were 10 square-wave pulses, τ_i = 100 μs, ν = 1 Hz, E_0 = 1.5 (●) and 1.8 (△) kV/cm, and resealing was monitored at T = 21°C. Solid curves are the theoretical distribution functions plotted according Eq. 20, assuming that the probabilities $P_{n_i}(0)$ that n_i pores have appeared in a cell during an electric treatment are given by the Poisson distribution (Eq. 10) and F_{irr} = 0. The parameters $\bar{n}_i$, k_r, and the ratio k_f/k_r were varied to obtain the best fit of the theoretical dependences $F_r(t)$ to experimental points. (*B*) The experimental dependence of the fraction of yeast cells, the membrane of which has restored its impermeability to phloxine B, on the time of incubation at 25°C temperature. The data are taken from the work of Muraji et al. (1993). Cell suspension was subjected to a single exponential electric pulse with a time constant of 70 μs. The amplitude of the pulse was 3.7 (○) and 4.6 (▲) kV/cm. Solid curves are the theoretical distribution functions calculated from Eq. 20, assuming that the probabilities $P_{n_i}(0)$ that n_i pores have appeared in a cell during an electric treatment are given by the Poisson distribution (Eq. 10) and F_{irr} = 0. The value of the ratio k_f/k_r = 0.06 was obtained from the tails of the experimental dependences, and then the parameters $\bar{n}_i$ and k_r were varied to obtain the best fit.

of electric pulses was 1.5 kV/cm and for $\bar{n}_i$ = 9, k_r = 4.1 × 10^{-3} s^{-1}, and k_f/k_r = 0.05 when E_0 = 1.8 kV/cm. Certainly these estimations are approximate, because too few experimental points were available. For the precise estimation of the unknown parameters, it is essential that more experimental points be used. In particular, to determine the ratio k_f/k_r, the fraction of resealed cells should be measured at longer times than was done in the work of Rols et al. (1990).

In Fig. 5 *B* the experimental dependences of the fraction of yeast cells permeable to phloxine B on the time of incubation at 25°C, taken from the work of Muraji et al. (1993), are shown. In this work cell suspension was subjected to a single exponential electric pulse with a time constant of 70 μs. The amplitude of the pulse was 3.7 (*open circles*) and 4.6 (*filled triangles*) kV/cm. Solid lines in this figure are the theoretical distribution functions calculated from Eq. 20, assuming that the probabilities $P_{n_i}(0)$ that n_i pores have appeared in a cell during an electric treatment are given by the Poisson distribution (Eq. 10) and F_{irr} = 0. In this case the value of the ratio k_f/k_r = 0.06 was obtained from the tails of the experimental dependences, and then the average number of pores, $\bar{n}_i$, and the time constant of the process of pore disappearance, k_r, were estimated by obtaining a best fit. The best agreement was obtained for $\bar{n}_i$ = 1, k_r = 9 × 10^{-3} s^{-1} for E_0 = 3.7 kV/cm and $\bar{n}_i$ = 1, k_r = 2.3 × 10^{-3} s^{-1} for E_0 = 4.6 kV/cm.

It can be seen from Fig. 5 that increasing the time of incubation at elevated temperature increases the fraction of resealed cells. This shows that the time necessary for the resealing varies from cell to cell, and thus the disappearance of pores from the cell membrane after electroporation is a random process. Although the shape of experimental relationships depends on the electroporation conditions, they can be described quite well by theoretical curves. Estimated numerical values of the parameters show that increasing the amplitude of an electric pulse increases either the apparent number of pores created during the pulse with an unchanged rate of pore resealing (experimental data from Rols et al., 1990), or the rate of pore resealing with a constant average number of pores (experimental data of Muraji et al., 1993).

As the number of pores, $\bar{n}_i$, created during the pulse can also be estimated from the kinetics of pore formation (Glaser et al., 1988; Saulis and Venslauskas, 1993a,b), one could compare the values for $\bar{n}_i$ obtained by two independent methods and find out whether these independent estimations are consistent with each other. Unfortunately, there are no experimental data available that allowed us to make such a comparison.

By measuring the kinetics of pore disappearance at various temperatures, one can obtain the dependence of the rate of pore disappearance on temperature. Such a dependence may be useful for a better understanding of the mechanism of pore resealing after electroporation and the factors regulating it. In addition, from Eq. 1 it follows that

$$\ln[k_r(T)] = \ln \Lambda - \Delta W_r(0)/kT \qquad (24)$$

where $\Delta W_r(0)$ is the energy barrier to pore resealing at zero transmembrane potential. Thus, by plotting the temperature dependence of the rate, $k_r(T)$, of the complete disappearance of pores in Arrhenius coordinates, the energy barrier to pore resealing, $\Delta W_r(0)$ (or, in the case where the time course of the decrease in the number of pores that are larger than a certain size is measured, an apparent activation energy for the decrease of pore size), and the preexponential factor Λ can be estimated. These values can give some information

about the process of pore disappearance (or the process of pore size decrease) and could be useful for the selection of their numerical values in theoretical models (see, for example, Freeman et al., 1994).

To illustrate the possible estimation of the energy barrier to pore resealing, $\Delta W_r(0)$, and the preexponential factor Λ, the experimental data taken from the work of Saulis et al. (1991) were used (Fig. 6). In this work the time course of the complete resealing of human erythrocytes exposed to a single exponential electric pulse (E_0 = 3.25 kV/cm, τ_i = 22 μs), was measured at two different temperatures, 32°C and 37°C. The values of the ratio k_f/k_r were estimated from the last few experimental points, and then the average number of pores, $\bar{n}_i$, and the time constant of the process of pore disappearance, k_r, were estimated by obtaining a best fit of Eq. 20 to the experimental functions $F_r(t)$, assuming that the initial number of pores is distributed according the Poisson distribution and $F_{irr} = 0$. The best coincidence was obtained for $\bar{n}_i = 1$. The estimated values of k_r were 9.6×10^{-4} s^{-1} at 32°C and 2.1×10^{-3} s^{-1} at 37°C. From Fig. 6 it can be seen that the theoretical curves do not ideally fit the experimental points.

The energy barrier $\Delta W_r(0)$ was calculated from

$$\Delta W_r(0) = [kT_1T_2/(T_2 - T_1)]\ln[k_r(T_2)/k_r(T_1)] \quad (25)$$

where k is the Boltzmann's constant, T_1 and T_2 are the temperatures, and $k_r(T_1)$ and $k_r(T_2)$ are the rates of pore disappearance at temperatures T_1 and T_2, respectively. The value $\Delta W_r(0) = 49\ kT$ was obtained. Such a value is on the same order of magnitude as the value of the energy barrier to pore formation $\Delta W_f(0) = 40-45\ kT$, estimated recently for bilayer lipid membranes (Glaser et al., 1988; Leikin et al., 1986) and human erythrocytes (Saulis and Venslauskas, 1993b) from the kinetics of pore formation at elevated transmembrane potentials. Almost exactly the same numerical value of the energy barrier to pore disappearance ($\Delta W_f(0) = 50\ kT$) was used by Weaver's group in their recent model (Freeman et al., 1994). Upon substituting an obtained value of $\Delta W_f(0)$ in Eq. 24, we determine that the preexponential factor Λ is $\sim 3 \times 10^{18}$ s^{-1}.

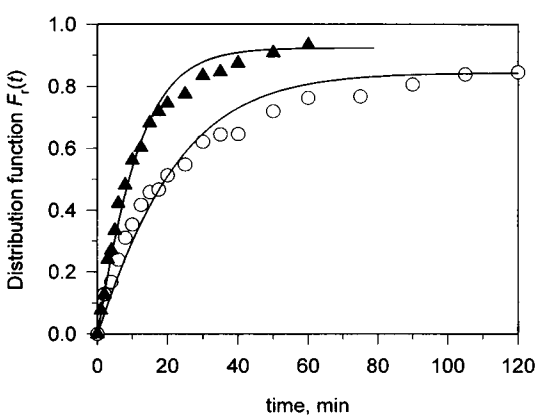

FIGURE 6 Distribution functions $F_r(t)$ of human erythrocyte resealing times at 32°C (○) and 37°C (▲) temperatures. Cells were subjected to a 3.25 kV/cm pulse (time constant τ_i = 22 μs) at 20°C, and immediately mixed with five volumes of incubation medium at 37°C or 32°C. An aliquot was taken at regular intervals and the fraction of cells which, after incubation at high temperature, have no pores was determined from the extent of hemolysis after prolonged incubation (20–24 h) in 0.9% NaCl solution at 4°C (Saulis et al., 1991). The solid curves are the theoretical curves plotted in accordance with Eq. 20, assuming that the initial number of pores is distributed according to the Poisson distribution and $F_{irr} = 0$. The values of the ratio k_f/k_r were estimated from the last few experimental points, and then the average number of pores, $\bar{n}_i$, and the time constant of the process of pore disappearance, k_r, were estimated by obtaining a best fit of Eq. 20 to the experimental points. The best coincidence was obtained for $\bar{n}_i = 1$. The estimated values of k_r were 9.6×10^{-4} s^{-1} at 32°C and 2.1×10^{-3} s^{-1} at 37°C.

Certainly these are rough estimations. For intelligent line extrapolation and a more accurate estimation of $\Delta W_r(0)$ and Λ, the detailed dependence $k_r(T)$ should be obtained. However, this is beyond the scope of this paper. The aim of this paper is to show what useful information can be obtained from the comparison of the presented theory with experimental data, and how such a comparison can be made.

From the fraction of porated cells before electroporation or the value of the distribution function $F_r(t)$ at $t = \infty$ (in the case where none of the cells are damaged irreversibly during electroporation), the ratio k_f/k_r can be estimated. This ratio allows us to evaluate the rate of pore formation at zero transmembrane potential (or at least its upper limit). For human erythrocytes the rate of pore resealing is $\sim 2 \times 10^{-3}$ s^{-1} at 37°C. Usually k_f/k_r is less than 0.1. Thus for human erythrocytes, $k_f(0)$ should be no more than 2×10^{-4} s^{-1}. This value could be used to test the estimations of the energy barrier to pore formation and the preexponential factor in Eq. 18, which were determined from the kinetics of pore formation.

Equation 19 gives us the temperature dependence of the ratio k_f/k_r. If the preexponential factor in this equation is independent of temperature, the temperature dependence is governed only by the difference $\Delta W_f(\Delta\Phi_m) - \Delta W_r(\Delta\Phi_m)$ between the energy barriers to pore formation and resealing. At zero transmembrane potential, the energy of a membrane with one pore is greater than the energy of an intact membrane (Abidor et al., 1979; Barnett and Weaver, 1991; Pastushenko and Chizmadzhev, 1982). Therefore it can be expected that the energy barrier to pore disappearance should be smaller than that to pore formation, as illustrated in Fig. 1. In such a case, increasing the temperature should lead to an increase in the ratio k_f/k_r. However, the assumption that the energy barrier to pore creation should be larger than the energy barrier to pore destruction was also made (Freeman et al., 1994). Thus by measuring the temperature dependence of the ratio k_f/k_r, the sign of the difference $\Delta W_f(\Delta\Phi_m) - \Delta W_r(\Delta\Phi_m)$ can be evaluated (certainly if the ratio k_f/k_r is not an infinitesimal quantity and can be measured experimentally).

CONCLUSIONS

In this paper the analysis of the process of pore disappearance in a cell after electroporation was carried out on the

basis of a kinetic model in which the creation and disappearance of metastable pores are considered random one-step processes. The theoretical expressions for the distribution function of cell resealing times, $F_r(t)$, derived on the basis of this model, describe quite well the experimental dependence of the fraction of resealed cells, F_r, on post-pulse incubation time. This indicates that the process of pore annihilation after cell electroporation is fundamentally stochastic. Although the theory presented here is simplified, it can be used to estimate various parameters from the experimental data.

The author hopes that the theoretical analysis presented here can be helpful in the further development of a more general quantitative theory of the process of pore disappearance in a cell after electroporation. With this theory it should be taken into consideration that 1) pore resealing is a multistep process, 2) a few types of pores in a cell membrane may be present, and 3) the precise value of the critical number of the pores n_{cr} may differ from unity.

APPENDIX A

We analyze the kinetic scheme in Eq. 7. Let us denote the states of cells with a definite number of pores as B_0, B_1, B_2, etc. and the probability that a cell is in state B_n at instant t as $P_n(t)$.

We consider the transition between these states a stochastic process in continuous time. In such a case probabilities $P_n(t)$ satisfy the following set of differential equations (Feller, 1964):

$$dP_{n_i}(t)/dt = -n_i k_r P_{n_i}(t) \tag{A1}$$

$$dP_n(t)/dt = (n+1)k_r P_{n+1}(t) - n k_r P_n(t), \quad 1 \le n < n_i \tag{A2}$$

Because the states B_1, B_2, etc. of a cell are incompatible, we have

$$\sum_{n=0}^{n_i} P_n(t) = 1 \tag{A3}$$

The initial conditions for our system are

$$P_{n_i}(0) = 1, \quad P_n(0) = 0, \quad n \neq n_i \tag{A4}$$

where n_i is the initial number of pores.

Solving the differential equations A1–A3 with the initial conditions A4 for $n_i = 1$, we obtain, when $n_i = 1$,

$$P_0(t) = 1 - \exp(-k_r t) \tag{A5}$$

Then solving Eqs. A1–A3 with the initial conditions A4 for $n_i = 2$, 3, and so on, one can find that

$$P_0(t) = [1 - \exp(-k_r t)]^{n_i} \tag{A6}$$

APPENDIX B

We analyze the kinetic scheme in Eq. 12. In such a case the probabilities $P_n(t)$ satisfy the following infinite set of differential equations (Feller, 1964):

$$dP_0(t)/dt = k_r P_1(t) - k_f P_0(t) \tag{B1}$$

$$dP_n(t)/dt = k_f P_{n-1}(t) - (k_f + n k_r)P_n(t) + (n+1)k_r P_{n+1}(t), \quad n \ge 1 \tag{B2}$$

$$\sum_{n=0}^{\infty} P_n(t) = 1 \tag{B3}$$

with initial conditions

$$P_{n_i}(0) = 1, \quad P_n(0) = 0, \quad n \neq n_i \tag{B4}$$

The solution of this infinite set of differential equations can be obtained by deriving the partial differential equation for the generating function

$$P(s, t) = \sum_{n=0}^{\infty} s^n P_n(t) \tag{B5}$$

The derivatives of the generating function are

$$\partial P(s, t)/\partial s = \sum_{n=1}^{\infty} n s^{n-1} P_n(t) \tag{B6}$$

$$\partial P(s, t)/\partial t = \sum_{n=0}^{\infty} s^n dP_n(t)/dt \tag{B7}$$

Multiplying Eq. B1 by s^0, Eq. B2 for $dP_n(t)/dt$ by s^n, and then summing them up, we obtain

$$\sum_{n=0}^{\infty} s^n \frac{dP_n(t)}{dt} = k_f \sum_{n=1}^{\infty} s^n P_{n-1}(t) - \sum_{n=0}^{\infty}(k_f + n k_r)s^n P_n(t) + k_r \sum_{n=0}^{\infty}(n+1)s^n P_{n+1}(t) \tag{B8}$$

Combining Eqs. B5–B8, we get the result that our generating function $P(s, t)$ is valid for the following partial differential equation:

$$\partial P(s, t)/\partial t = (1 - s)[-k_f P(s, t) + k_r \partial P(s, t)/\partial s] \tag{B9}$$

From Eq. B4 we obtain in the same way the initial conditions for the equation

$$P(s, 0) = s^{n_i} \tag{B10}$$

The solution of Eq. B9 with initial conditions B10 is (Feller, 1964)

$$P(s, t) = \exp[-(k_f/k_r)(1 - s)(1 - \exp(-k_r t))] \times [1 - (1 - s)\exp(-k_r t)]^{n_i} \tag{B11}$$

Substituting $s = 0$ into Eq. B11 and taking into account Eq. B5, we readily obtain

$$P_0(t) = \exp(-[k_f/k_r][1 - \exp(-k_r t)])[1 - \exp(-k_r t)]^{n_i} \tag{B12}$$

One can also obtain the time dependence of the average number of pores per cell, $\bar{n}(t)$, which is equal to

$$\bar{n}(t) = \sum_{n=1}^{\infty} n P_n(t) \tag{B13}$$

From Eqs. B6 and B13 it follows that

$$\bar{n}(t) = \partial P(s, t)/\partial s|_{s=1} \qquad \text{(B14)}$$

Taking the partial derivative of Eq. 14 with respect to s and substituting $s = 1$, we obtain the required dependence of the average number of pores per cell, $\bar{n}(t)$, on time:

$$\bar{n}(t) = (k_f/k_r)[1 - \exp(-k_r t)] + n_i \exp(-k_r t) \qquad \text{(B15)}$$

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