Studies on the Effects of Applied Voltage and Duration on Human Epidermal Membrane Alteration/Recovery and the Resultant Effects upon Iontophoresis

Hirohiko Inada,[1] Abdel-Halim Ghanem,[1] and William I. Higuchi[1,2]

Received July 7, 1993; accepted December 27, 1993

The effects of applied voltage and the duration of application upon human epidermal membrane (HEM) alterations and recovery were investigated. All experiments were conducted using a two-chamber diffusion cell with constant DC voltage (250–4000 mV) applied over a predetermined period, and HEM changes were monitored by measuring the electrical resistance before and after voltage termination. The key findings were that the rate of decrease in resistance was strongly dependent upon the applied voltage, the reversible recovery times were dependent upon both the magnitude and the duration of the applied field (frequently were several orders of magnitude greater than times for attaining significant resistance reduction), and reversible recovery times were much longer when lower voltages were applied for longer times to attain the same decrease in electrical resistance than for higher voltages at short times. These findings closely parallel those obtained on electrical breakdown/recovery of bilayer membranes (electroporation). The second part of this work examined the hypothesis that decreases in HEM electrical resistance induced by the applied voltage are accompanied by proportional increases in HEM permeability. A study was designed to test this hypothesis involving a four-stage protocol with HEM: passive transport, 250-mV iontophoresis, 2000-mV iontophoresis for 10 min, then back to 250-mV iontophoresis. The data obtained strongly support the view that the HEM alterations induced by the electric field result in pore formation and in the expected changes in HEM permeability.

KEY WORDS: iontophoresis; skin alteration and recovery; human skin; electroporation; permeability.

INTRODUCTION

In previous studies, human epidermal membrane (HEM) alterations caused by electric fields were investigated using a limited number of voltage settings with a four-electrode potentiostat system (1–4). It was found that HEM may undergo continuous membrane alterations at a high applied voltage (1000 mV) but not at 250 mV or lower. These changes appeared to be completely reversible over time once the field was removed. As these voltage-induced effects appeared to be decreases in electrical resistance accompanied by proportional flux enhancement of both ionic permeants (iontophoresis) and polar, nonionic solutes (electroosmosis), the results suggested that these effects were primarily electric field induced pore formation (2,3). Clearly, these findings point to the need for more quantitative studies, as this phenomenon may be important for the iontophoretic delivery of both charged and uncharged drug molecules.

It is now generally accepted that reversible electrical breakdown of cell and model lipid membranes is based on the formation of pores (electroporation), including pore modification (i.e., growth/expansion of a preexisting pore). Several investigators have presented the results of theoretical and experimental studies of reversible electrical breakdown of planar lipid bilayers, cell membranes, and erythrocytes (5–19). Of particular interest, Glaser *et al.* and Chernomordik *et al.* (5–7) have obtained data with model bilayers supporting the view that (a) the rate of the pore creation (membrane conductivity) depends on the applied voltage and (b) an increase in the duration of applied voltage leads to an increase in the number of pores and increases in pore radii.

In the present study, the effects of applied voltage and its duration on the alteration and recovery of HEM were further investigated with the thought that there may be a parallel between the results of Glaser *et al.* and Chernomordik *et al.* (5–7) and those with HEM. Membrane resistance measurements were used to monitor continuously membrane alteration induced by the electric field and during its recovery after voltage termination. Also, as the second portion of this study, iontophoresis and electroosmosis experiments were designed and carried out to examine quantitatively the hypothesis that the field-induced alterations in HEM are consistent with the induction of pores, resulting in the proportional enhancement of mass transport of ions and neutral permeants.

MATERIALS AND METHODS

Materials

Phosphate-buffered saline (PBS), 0.1 *M* ionic strength, pH 7.5, was used in all experiments. Sodium azide (0.02%) was added to the buffer as a bacteriostatic agent. Chemicals were reagent grade and used as received. Human skin was obtained from Ohio Valley Tissue and Skin Bank (Cincinnati, OH). The HEM was obtained as described in the previous study (2). The range of electrical resistances of the HEM was 12–120 $k\Omega \cdot cm^2$.

Transport studies were conducted with the following permeants: [1-^{3}H]mannitol (sp act, 25.0 Ci/mmol; radiochemical purity, 99%) and [1-^{14}C]tetraethylammonium bromide (TEAB; sp act, 3.0 mCi/mmol; radiochemical purity, 99%) obtained from New England Nuclear Corporation, Boston, MA.

Methods

All experiments were conducted with the four-electrode potentiostat system (JAS Instrumental Systems, Inc., Salt Lake City, UT), which has been described previously in detail (1–4). This system is able to maintain a constant known voltage drop across a membrane positioned between the donor and the acceptor chambers of a two-chamber diffusion cell.

[1] Department of Pharmaceutics, College of Pharmacy, The University of Utah, Salt Lake City, Utah 84112.
[2] To whom correspondence should be addressed.

0724-8741/94/0500-0687$07.00/0

Membrane Alteration/Recovery Studies. First, to stabilize the HEM mounted in the cell, HEM was equilibrated for 3 days in the PBS buffer at 37°C. A constant HEM resistance was thus established before starting an experiment. All experiments were conducted at 37°C and consisted of two stages. In Stage I, a fixed DC voltage (250–4000 mV) was applied for a predetermined duration (10 sec to 60 min). During this period, the current was continuously monitored using a recorder (Houston Instrument, Austin, TX) and an oscilloscope with storage function (Tektronix Holland, NV, Heerenveen, The Netherlands). At the end of Stage I, the voltage was turned off and each chamber was flushed and refilled with fresh PBS. In Stage II (a period of 480 min directly after the termination of voltage drop), the current was measured at predetermined time intervals by applying a voltage drop of 250 mV and the membrane resistance was calculated according to Ohm's law.

Transport/Iontophoresis Studies. Experiments were designed to examine the hypothesis that the electric field-induced alterations in HEM are interpretable as reversible pore formation: that changes in electrical resistance are accompanied by proportional changes in the HEM permeability. Dual permeants (^{14}C-TEAB and ^{3}H-mannitol) were employed in a four-stage protocol to minimize the effects of skin-to-skin variability. Quadruplicate runs, each with a different and preselected HEM, were carried out through all stages. In the experimental setup, the anode and the cathode were in the donor and receiver chamber, respectively, with 5 mL PBS buffer in both chambers. To begin an experiment, appropriate levels of radiolabeled mannitol and TEAB (10,000–15,000 dpm/10 μL) were pipetted from their respective solutions into the donor chamber. All experiments with radiolabeled compounds were conducted with trace amounts of the permeants in the donor chamber, so that the ionic strengths of the donor and receiver were practically identical. Stage I was a passive transport experiment carried out by taking a 1-mL sample from the receiver chamber at predetermined time intervals and replacing it with 1 mL of fresh PBS. This basic sampling procedure was also followed at predetermined time intervals in Stages II–IV. In Stage II, a voltage drop of 250 mV was applied across the membrane over a 2-hr period. The current was continuously monitored, while samples were taken from the receiver chamber. At the end of Stage II, the voltage was turned off and the receiver chamber was flushed and refilled with fresh buffer solution. In Stage III (immediately after Stage II), a voltage drop of 2000 mV was applied for 10 min, the current monitored, and the sample taking resumed. At the end of Stage III, the voltage was reduced to 250 mV and maintained for 12 hr (Stage IV), while again monitoring current and sampling from the receiver chamber.

The samples taken during all four stages were mixed with 10 mL of scintillation cocktail (Opti-Fluor, Packard Instrument Co.) and were assayed using a Beckman liquid scintillation counter, Model LS-7500. The data were plotted as Q, the cumulative disintegrations per minute transported into the receiver compartment per cross-sectional area (0.678 cm^2) for diffusion, as a function of time, t. The drug permeation flux, J, for mannitol and that for the tetraethylammonium ion were calculated from

$$J = \frac{dQ}{dt} \tag{1}$$

where dQ/dt is the slope of the t–Q plot. In the case of nonlinear t–Q plots, we calculated the J values from the slope of successive data points.

RESULTS AND DISCUSSION

Electric Field-Induced HEM Alteration and Recovery Studies

HEM changes occurring during electric field application (Stage I) and recovery afterward (Stage II) monitored by resistance measurements were determined over a range of applied voltages (250 to 4000 mV) for three applied voltage durations: 60 min, 10 min, and 10 sec. The results are presented in Figs. 1–3. Recovery was followed in each case to 480 min after voltage termination. Each Stage I/Stage II pair run was with a different HEM. The ordinate in all cases refers to the percent of the zero-time resistance value.

The key findings of the data in Figs. 1–3 are as follows: membrane alteration (i.e., reduction in resistance) during Stage I was found to be strongly dependent on the applied voltage. There appeared to be a threshold voltage (~250 mV) below which there was little or no decrease in resistance with time. Above this threshold voltage, the resistance decreases were dependent on both the magnitude and the duration of the applied voltage. The recovery behavior after the electric field was turned off (i.e., Stage II) was found to be

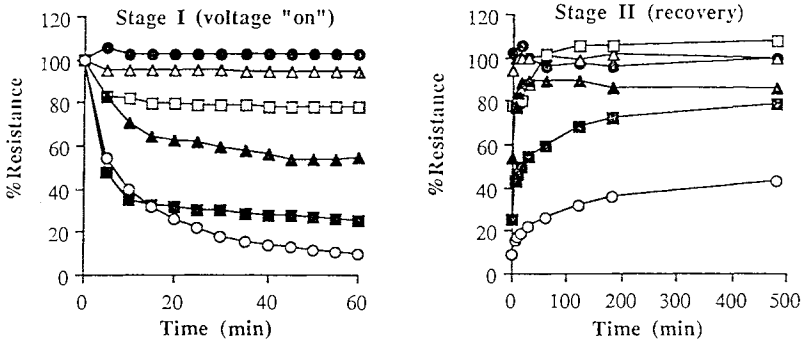

Fig. 1. Resistance profiles for HEM during application of voltage and during recovery. Voltage application period of 60 min: (●) 250 mV; (△) 500 mV; (□) 750 mV; (▲) 1000 mV; (■) 1500 mV; (○) 2000 mV.

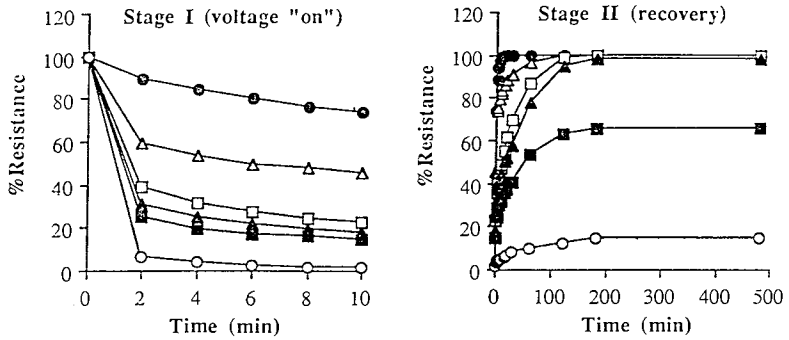

Fig. 2. Resistance profiles for HEM during application of voltage and during recovery. Voltage application period of 10 min: (●) 500 mV; (△) 1000 mV; (□) 1500 mV; (▲) 2000 mV; (■) 2500 mV; (○) 3000 mV.

dependent upon both the magnitude and the duration of the applied voltage. When the duration of the applied voltage (Stage I) was 60 min, at low voltages (<750 mV), there was rapid and complete recovery. For higher voltages, however, the recovery was only partial (see Stage II in Fig. 1). As the duration of applied voltage was shortened, the recovery was found to be both more rapid and more complete. It is shown in Fig. 2 that recovery was rapid and complete for 2000 mV applied for 10 min, but for 2000 mV applied for 60 min, the recovery was only about 40% at 480 min (Fig. 1). When the duration of Stage I was only 10 sec, the recovery was even more rapid and more complete (Fig. 3): even at the highest applied voltage of 4000 mV, there was full recovery in 100 min, while when the Stage I duration was 10 min, at 2500 and 3000 mV, the recoveries were not complete at 480 min.

As all of the data (i.e., the alteration/recovery runs) in Figs. 1–3 were obtained with different HEMs, it was decided to conduct an experiment involving successive alteration/recovery runs for a single HEM with the duration of an applied voltage of 1000 mV in the successive runs increasing as 10 sec, 1 min, 10 min, and 60 min. The results of this experiment are presented in Fig. 4, and the data show that there were no apparent "memory" effects and the consecutive runs demonstrated a relatively high degree of reproducibility.

It may be gleaned from Figs. 1–3 that the recovery behavior (Stage II) was not a single-valued function of the percentage resistance at the instant of termination of the applied field. For example, recovery for 1500 mV applied for 60 min (filled squares in Fig. 1) did not show full recovery (at 480 min), while 4000 mV applied for 10 sec showed rapid recovery (open circles in Fig. 3); in both cases, the percentage resistance values at the beginning of Stage II were about the same (i.e., ~30%). The same may be said when 2500 mV for 10 min (filled squares in Fig. 2) is compared to 4000 mV at 10 sec (open circles in Fig. 3). These results suggest that when a lower voltage applied for a longer time gave the same percentage resistance reduction as a higher voltage applied for a shorter time, the latter recovered more quickly and more completely.

To examine this point, the experiments presented in Fig. 5 were carried out. These data clearly show that, when a high voltage (3000 mV) applied for a shorter time (30 sec) attained the same resistance reduction (to about 40%) as a lower voltage (1000 mV) applied for a longer time (40–50 min), the recovery patterns for the two cases were quite different. In the former, the rate of recovery was found to be much faster than in the latter.

Table I provides additional data and further insight regarding this behavior. Here experiments conducted at 3000 and 1000 mV for different voltage application periods are compared, and the $t_{50\%}$ (the time for 50% recovery) was estimated for each situation. Again, it is seen (as in Fig. 5) that the recoveries for high-voltage/short-time cases were much faster than those for the lower-voltage/longer-time cases. Additionally, it is interesting to note that, for the

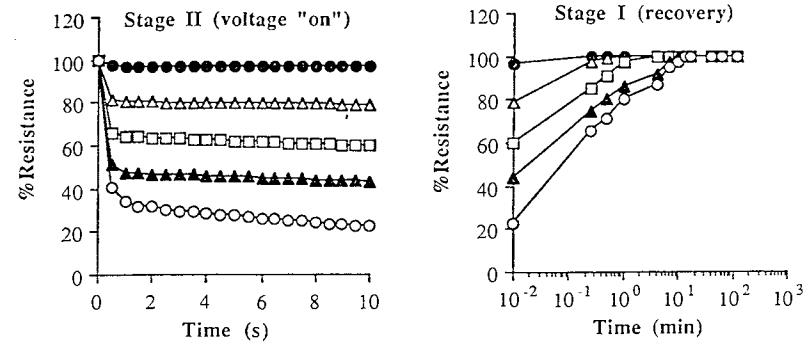

Fig. 3. Resistance profiles for HEM during application of voltage and during recovery. Voltage application period of 10 sec: (●) 500 mV; (△) 1000 mV; (□) 2000 mV; (▲) 3000 mV; (○) 4000 mV.

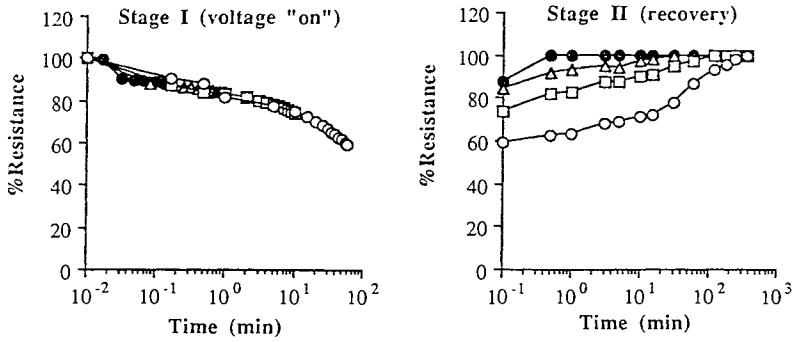

Fig. 4. Resistance profiles involving successive alteration and recovery experiments with a single HEM at various durations by an applied voltage of 1000 mV: (●) 10 sec; (△) 1 min; (□) 10 min; (○) 60 min. Each experiment was performed consecutively after 100% recovery.

shortest duration case (Experiment III), the $t_{50\%}$ values for Condition A and Condition B differed by a factor of 4 to 8, while for the longer duration case (Experiments I and II), the $t_{50\%}$ values differed by about 100-fold or more.

It should be instructive to compare these results (Figs. 1–5 and Table I) with those of Glaser *et al.* (5) and Chernomordik *et al.* (6,7). In their studies of the reversible electrical breakdown of lipid bilayer membranes, these investigators found electrical breakdown and recovery behavior qualitatively very similar to those reported here with HEM: first, the rate of increase in conductance was strongly dependent upon the applied voltage; second, the reversible recovery times were frequently many orders of magnitude greater than the times for attaining significant electrical breakdown; and third (and importantly), recovery times were much longer when lower voltages were applied for longer times to attain the same electrical conductance before recovery. These findings from the studies by Glaser *et al.* and Chernomordik *et al.* are remarkably similar to the present HEM results and therefore suggest that similar mechanisms are at play in the two situations, as discussed later.

It is worthwhile to mention here, while transdermal electroporation has been viewed by some authors (see, e.g., Refs. 19 and 25) as occurring only with "ultrashort" (a few

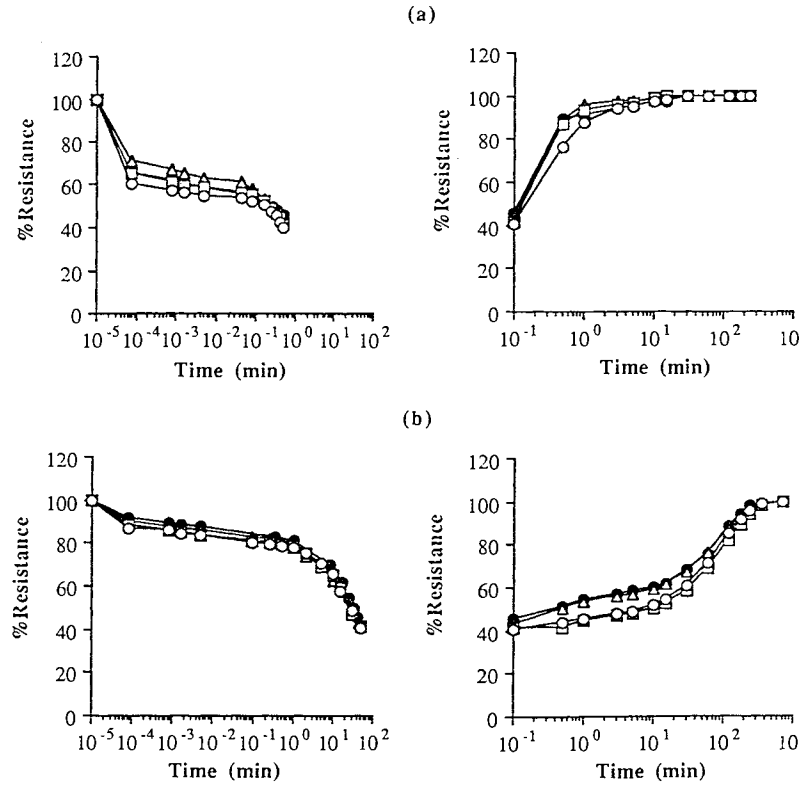

Fig. 5. Resistance profiles in alteration and recovery experiment: short duration at 3000 mV (a) and longer duration at 1000 mV (b). The percentage resistance at the end of Stage I is nearly the same in all cases. Each symbol presents a different HEM.

Table I. Time Required for 50% Recovery ($t_{50\%}$) After Voltage Termination in the Alteration/Recovery Experiments with the Short and Longer Voltage Durations[a]

Membrane (M)	Condition A: 3000 mV Duration (sec)	Condition A: 3000 mV $t_{50\%}$ (min)	Condition B: 1000 mV Duration (min)	Condition B: 1000 mV $t_{50\%}$ (min)	% Resistance at the end of Stage I
Expt I					
M-I	30	0.18	40	80	45
M-II	30	0.20	45	43	43
M-III	30	0.21	50	54	42
M-IV	30	0.37	50	56	40
Expt II					
M-V	10	0.29	34	95	35
M-VI	10	0.31	45	29	32
M-VII	10	0.30	39	63	34
M-VIII	10	0.24	36	30	35
Expt III					
M-IX	5	0.15	20	0.63	41
M-X	5	0.18	19	1.50	61
M-XI	5	0.17	24	0.96	59
M-XII	5	0.12	16	0.82	55

[a] It is clear that the reversible recovery times ($t_{50\%}$) are much longer when lower voltages are applied for longer durations (Condition B) to attain the same decrease in resistance than with higher voltages for shorter durations (Condition A).

milliseconds) pulses of high-intensity applied voltage across the skin, Chernomordik *et al.* (7) have pointed out that reversible pore induction with cell membranes and lipid bilayer membranes may take place over wide ranges of action times: e.g., with erythrocyte membranes, short times (microseconds to hundreds of microseconds) at high voltages (500–1000 mV) (see, e.g., Refs. 14 and 26) and long times (several seconds to minutes) at low voltages (150–200 mV) (see, e.g., Refs. 27 and 28). We have consequently taken the liberty to suggest that the term electroporation may be appropriate even for HEM pore induction at voltages of the order of a few volts applied over time periods of seconds to tens of minutes. It should be noted that even with the application of a few volts across the stratum corneum, the electric field intensity may be in the kilovolts per centimeter or tens of kilovolts per centimeter range because of the thinness of the stratum corneum layer (or the collective thinness of the total lipid bilayers of the stratum corneum).

Transport/Iontophoresis Studies

This portion of the research was aimed at determining whether (or not) the decrease in resistance seen in Stage I in Figs. 1–3 reflects quantitatively proportional increase in the HEM permeability. Previously (2,3), it had been shown that HEM alterations induced at 1000 mV for 60- to 120-min durations correlated with proportional increases in both the iontophoretic flux and the passive permeation. These correlations were, however, somewhat crude and regarded as semiquantitative, the experiments being the first of their kind. In the present study, the case of 2000 mV with 10-min durations was selected for detailed transport/iontophoresis studies. A shorter (than 10-min)-duration experiment would not have been practical as a manual sampling (of the receiver chamber) procedure for flux determinations was employed. Also, a higher-voltage (higher than 2000-mV) run with a 10-min duration may have resulted in unacceptably long recovery times (see Fig. 2); a significantly lower voltage run would not have given the sufficiently large reduction in resistance needed for addressing the hypothesis.

Strategy. The design of the study was based upon the following equation (see Appendix for derivation and detailed discussion).

$$J_2 = \frac{\Delta\psi_2}{\Delta\psi_1}\frac{P_2}{P_1}\frac{CF_1}{CF_2}J_1 \tag{2}$$

Here, J_1 and J_2 are fluxes for the tetraethylammonium ion, TEA^+, at applied voltages of $\Delta\psi_1$ and $\Delta\psi_2$, respectively. P_1 and P_2 are the TEA^+ permeability coefficients for the two voltage situations, and CF_1 and CF_2 are "correction" factors to account for electroosmosis measured via the mannitol flux. For the present situation, where subscripts 1 and 2 in Eq. (2) correspond to 250 and 2000 mV, respectively, Eq. (2) may be written as

$$J_{2000} = 8 \cdot \left\{\frac{R_{250}}{R_{2000}(t)}\right\}\left\{\frac{CF_{250}}{CF_{2000}(t)}\right\}J_{250} \tag{3}$$

All of the terms on the right side of Eq. (3) have been defined (see the Appendix) and are known or obtainable from the experiments: R_{250} and R_{2000} are the electrical resistances at 250 and 2000 mV, respectively, and are given in Figs. 7 and 8.

Now, the principal question in this part of the research is, is the reduction in HEM electrical resistance during the application of the voltage reflected quantitatively in an increase in the HEM permeability?

Analysis of Experimental Results. First, let us examine the flux results at 250 mV (Stage II) and compare these to the passive flux data (Stage I). Typical results (run 1) of transport experiments with TEA^+ and mannitol during Stage I (passive) and Stage II (250 mV) are shown in Fig. 6 for Stage I and in Fig. 7 for Stage II. As shown, the data plots in each case are essentially linear and the fluxes were calculated for all experiments from the best-fit slope (dQ/dt) for both stages using Eq. (1). Membrane resistances monitored during Stage II remained essentially constant, as shown in Fig. 7. This constant resistance behavior is consistent with results in Fig.

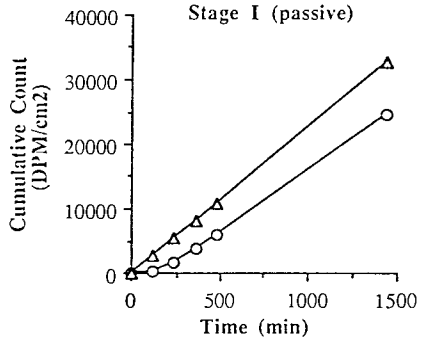

Fig. 6. Representative (run 1) results of [1-^{14}C]TEA^+ (○) and [1-^{3}H]mannitol (△) transport experiments for Stage I (passive).

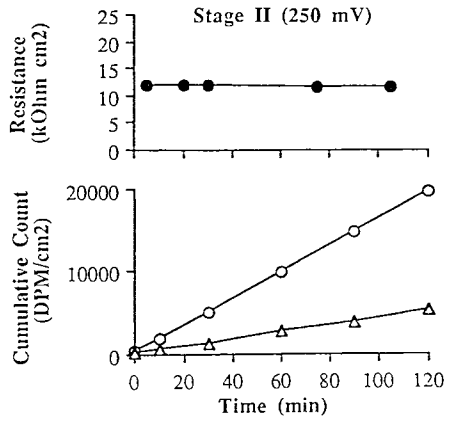

Fig. 7. Representative (run 1) results of [1-^{14}C]TEA$^+$ (○) and [1-^{3}H]mannitol (△) transport and resistance profile (●) for Stage II (250 mV).

1 and all previous work (2,3). Both transport and resistance data suggest that the HEM is not significantly altered by an applied field of 250 mV and this voltage is below the threshold voltage for any significant new pore formation. Thus, 250 mV was adopted throughout this study as the chosen reference in Eqs. (2) and (3).

Table II summarizes the Stage I and Stage II fluxes and membrane resistances for all four runs. The average mannitol enhancement factor based on the convective solvent flow, electroosmosis, (U_{250}) value of 2.3 and the average value for the TEA$^+$ enhancement factor due to both a direct field effect (Nernst–Planck) and a convective solvent flow effect (electroosmosis) ($E_+ = K_{250} + U_{250}$) of 11.9 are consistent with our previous work with HEM (2,3). Furthermore, 11.9 − 2.3 = 9.6 is in good agreement with the theoretically predicted value of 9.4 at 250 mV for a monovalent ion [based on the direct field effect, Nernst–Planck, without solvent flow using Eq. (A3) given in the Appendix]. This points out that the contribution of solvent flow to TEA$^+$ transport enhancement at 250 mV may be about 20% and is a secondary factor for TEA$^+$ iontophoresis with HEM.

The experimental results for Stage III (2000 mV, 10 min) and Stage IV (250 mV, 12 hr) are presented in Fig. 8 for the representative run 1. In contrast to the Stage II case (Fig. 7), the transport and the resistance data are nonlinear with time for both Stage III and Stage IV.

Figure 9 shows the comparisons between experiment and theory for TEA$^+$ transport during Stage III and Stage IV for run 1. The experimental TEA$^+$ fluxes were calculated from the slopes of two successive data points of the cumulative TEA$^+$ transport data presented in Fig. 8. The theoretical TEA$^+$ fluxes were calculated according to Eq. (3).

It is seen that the agreement between experiment and theory is quite satisfactory for run 1 in Fig. 9, strongly supporting the view that the resistance changes induced by applied electrical fields correlate directly with proportional changes in the permeability of HEM to ions and polar permeants. Figure 10 shows, furthermore, that the factor, $R_{250}/R_{2000}(t)$, is the primary determinant of the field-induced changes in J_{2000} and the factor, $CF_{250}/CF_{2000}(t)$, is relatively constant in both Stage III and Stage IV.

Table III summarizes the results for all four runs. In all runs, the data indicated essentially full recovery by the end of Stage IV. It is evident from Table III that experiment and theory [Eq. (3)] generally agreed well for Stage III. Run 4 and, to same extent, run 2 showed some deviations at very early times of Stage IV, but the fluxes at later times were in relatively good agreement with predictions of Eq. (3).

Similarities Regarding Present HEM Results and Electroporation Behavior Involving Cell and Lipid Bilayer Membranes

The good agreement between predictions based on Eq. (3) and experiments (Fig. 9 and Table III) supports the view that the application of voltage in the range 250 to 2000 mV results in significant reversible increase in electrical conductance of HEM accompanied by a proportional increase in HEM permeability. This result suggests that HEM exhibits reversible electrical breakdown (with pore formation) similar to that found with cell membranes and lipid bilayer membranes (electroporation). In view of the potential practical importance, a comparison of the HEM results with the cell membrane and lipid bilayer membrane data should be worthwhile. In the following, several aspects of electroporation conditions/outcomes for two cases are examined and compared.

First, let us consider the possible electrical field strengths prevailing during electrical breakdown and pore formation with HEM. Two situations are examined: electrical breakdown and pore formation occurring transepidermally (Case A) and via appendageal routes (Case B). First, for Case A, one notes that the stratum corneum is principally responsible for the high electrical resistance (the resistance of the stratum corneum is three to four orders of magnitude greater than that of tape-stripped skin). Second, the thickness of human stratum corneum is 10–15 μM (see, e.g., Ref. 20) and it is typically composed of 15 to 20 cell layers. Also, the intercellular lipid regions represent one-tenth to one-twentieth of the thickness of the stratum corneum (see, e.g., Ref. 21); this intercellular lipid region (involving the lipid envelope surrounding each corneocyte, monolayers, and bilayer pairs) is about 20 to 40 nm thick, typically. With the above information we may do, for Case A, a "lower-limit" estimation and an "upper-limit" estimation of the electric field strength when 2 V is applied across the skin. For the upper-limit calculation, we may assume that the corneocyte interior is much more conductive than the intercellular lipid region, and the electric field strength, $d\psi/dx$, is

$$d\psi/dx = (2.0\ \text{V})/(0.05 \times 10^{-3}\ \text{cm}) = 40 \times 10^3\ \text{V/cm}$$

For the lower-limit estimation, we may assume a uniform field across the stratum corneum and

$$d\psi/dx = (2.0\ \text{V})/(1.0 \times 10^{-3}\ \text{cm}) = 2 \times 10^3\ \text{V/cm}$$

Thus, even the "worst case" (i.e., the lower-limit estimation) yields an electric field value in the kilovolt per centimeter range. It is suggested, however, that the corneocyte region likely would be much more conductive than the intercellular lipid regions (cell envelope and lipid bilayers), and therefore the upper-limit estimation is likely the better estimate of the electric field strength.

Further on this point, Keister and Kasting (23) suggest

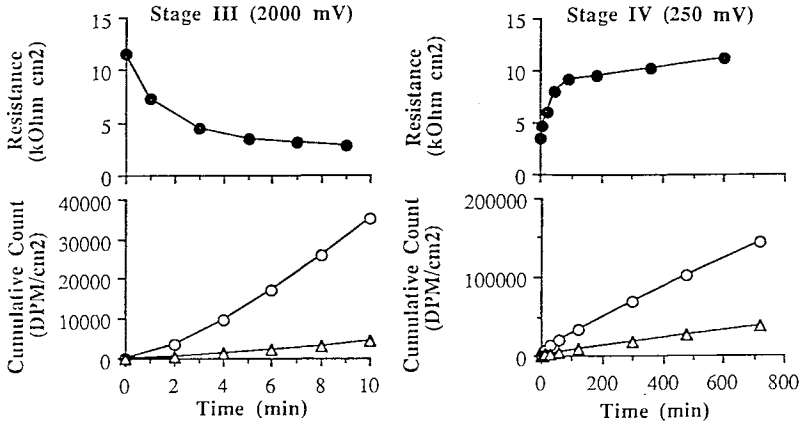

Fig. 8. Representative (run 1) results of [1-^{14}C]TEA$^+$ (○) and [1-^{3}H]mannitol (△) transport and resistance profile (●) for Stage III (2000 mV) and Stage IV (250 mV).

that, although there may be 15 to 20 cell layers, thinner portions of the stratum corneum may disproportionately contribute; therefore, the electric fields for Case A may be even greater than 40 kV/cm.

With regard to Case B, let us now consider a mechanism proposed by Kasting and Bowman (22). These investigators suggest that, if current flow occurs via skin appendages (sweat ducts and hair follicles), then it is likely that the number of cell layers and the number of transport-limiting lipid envelopes involved should be much smaller than those considered in the Case A calculations. Accordingly, the upper-limit field strength calculations could be 5 to 20 times greater than the 40 kV/cm estimated for Case A.

The reported electric field strengths in electroporation studies involving cells, cell membranes, and lipid bilayer membranes vary greatly: e.g., the onset of transport due to electroporation occurs at less than 1 kV/cm and increases with increasing field strength, then plateaus at higher field strengths (>2–5 kV/cm) when using erythrocyte ghosts (16); similarly, ATP leakage from bacteria increases with increasing field intensity from 2 to 6 kV/cm, then reaches a maximum plateau (12); and Teissie and Tsong (10) have reported that electric fields in the range of 20–40 kV/cm applied for short times (5 to 25 μsec) induced sucrose leakage from phospholipid bilayer vesicles. Chernomordik *et al.* (6,7) and Glaser *et al.* (5), using the voltage-clamp technique, showed that an applied voltage of 100 mV induced reversible electrical breakdown over time periods of seconds for cell membranes and lipid bilayer membranes. An applied voltage of 100 mV in these experiments may correspond to field strengths as great as 200 kV/cm (5). These investigators did not give in their reports the lowest applied voltages necessary for reversible electrical breakdown; relative to this point, Weaver notes, in a review article (24), that electroporation at low applied voltages (and at long times) "has not yet received much attention," but elsewhere (18) he suggests that "field strengths of 1.0 kV/cm to 10 kV/cm are typical for inducing reversible electrical breakdown accompanied by a tremendous increase in molecular transport across the membranes."

Let us next consider the matter of the duration of the applied voltages pertinent to reversible electrical breakdown (and pore formation). There has been an opinion expressed recently (see, e.g., Refs. 19 and 25) that transdermal electroporation differs from transdermal iontophoresis in that, for electroporation, one is involved with "ultrashort" pulses, lasting a few milliseconds, with an intensity of a few hundred volts, while conventional transdermal iontophoresis

Table II. Steady-State Fluxes of [1-^{14}C]TEA$^+$ and [1-^{3}H]Mannitol in Stage I (Zero Voltage) and Stage II (250 mV) and Their Enhancement Factors and Membrane Resistances

Run No.	TEA$^+$ flux (dpm/cm^2/min) Stage I	TEA$^+$ flux (dpm/cm^2/min) Stage II	E_+ factor[a]	Mannitol flux (dpm/cm^2/min) Stage I	Mannitol flux (dpm/cm^2/min) Stage II	U factor[b]	Membrane resistance (kΩ · cm^2)
1	16.1	161	10.0	22.7	42.3	1.9	11.6
2	5.8	72.1	12.4	5.7	15.6	2.7	17.1
3	3.7	53.7	14.5	3.0	7.2	2.4	28.5
4	0.6	6.5	10.8	1.7	4.0	2.3	59.5
Mean[c]			11.9			2.3	
+SD			1.9			0.3	

[a] The TEA$^+$ iontophoretic flux enhancement due to both a direct field effect, Nernst–Planck, and a convective solvent flow effect, electroosmosis.

[b] The mannitol iontophoretic flux enhancement due to convective solvent flow effect via electroosmosis.

[c] The mean values are consistent with our previous work with HEM (2,3). $(E_+ - U) = 9.6$ is in agreement with the theoretically predicted value of 9.4 at 250 mV for monovalent ion [based on the direct field effect without solvent flow using Eq. (A3) given in the Appendix].

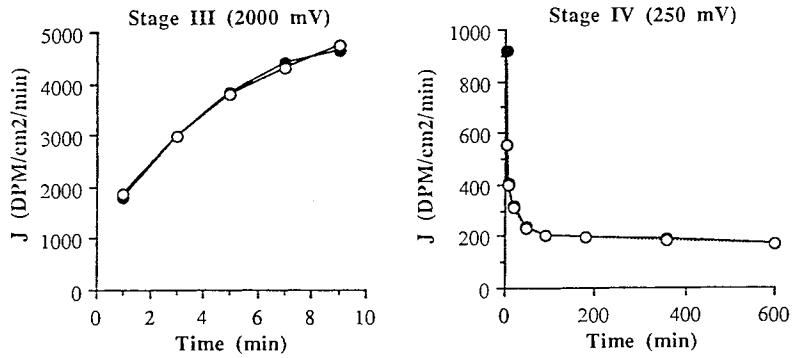

Fig. 9. Observed (●) and estimated flux profile (○) of [1-^{14}C]TEA$^+$ for Stage III (2000 mV) and Stage IV (250 mV). According to Eq. (3), estimation of flux profiles, $J_{2000}(t)$ in Stage III and $J_{250}(t)$ in Stage IV, were made using resistance profiles in Stage III, $R_{2000}(t)$, and in Stage IV, $R_{250}(t)$, mannitol enhancement profiles in Stage III, $U_{2000}(t)$, and in Stage IV, $U_{250}(t)$, membrane resistance measured in Stage II, R_{250}, and mannitol flux enhancement factor in Stage II, U_{250}.

may involve 3 to 12 V of electricity lasting from minutes to hours. This viewpoint suggests phenomenologic differences between the present HEM results (duration in the range of seconds to hours; see Figs. 1–5) and electroporation data for cell membranes and lipid bilayer membranes. The facts are clear, however, that electrical breakdown of erythrocyte membranes and appreciable increase in permeability were observed from tens of microseconds to several seconds to minutes when substantially different ranges of electrical actions were induced, i.e., at higher electric fields for short intervals (0.5–1 V, 1–500 μsec; see, e.g., Refs. 14 and 26) and, conversely, at lower voltages for long periods (0.1–0.2 V, seconds–minutes; see, e.g., Refs. 27 and 28). Chernomordik *et al.* (6,7) state, "The qualitative similarity of the general features and regularities of the breakdown, caused by the application to the membrane of voltage pulses of different amplitude and duration, indicate that electrical breakdowns, within *the wide range of voltage and times of their application,* are common in nature." All this supports the view that transdermal electroporation likely occurs during "conventional" iontophoresis (i.e., at low voltages applied for long times) as well as when short-duration (milliseconds), high-voltage pulses are involved. More work is needed to determine whether, for HEM, the site(s) or site distributions of transdermal electroporation may depend upon the amplitude and duration of the applied voltage.

It is worthwhile to mention at this point that transient field effects (capacitive current) in the stratum corneum are important on time scales of the order of tens of microseconds (29–31). Below 1000 Hz, the impedance is resistive, and a study in our laboratory (31) and those of Bagniefski and Burnette (30) show that, above a 1-msec pulse width, iontophoresis is essentially the same as continuous direct current (when corrected for duty cycle) because transient contributions are negligible. From this, it follows that even the earliest time points (see, e.g., Fig. 3) correspond to essentially DC conditions; and the HEM data suggest significant HEM electroporation having occurred in 2 sec with the application of 4.0 V.

In summary, the similarities are great between the present results and the electroporation data of other investigators. We have shown here that (a) the electric fields estimated for the HEM experiments are in the same range as those employed in electroporation studies of cell membranes and lipid bilayers and (b) the durations of the applied voltage in the present studies overlap with those reported in the studies by Glaser *et al.* and Chernomordik *et al.* (5–7). The most significant (and striking) similarity between the present

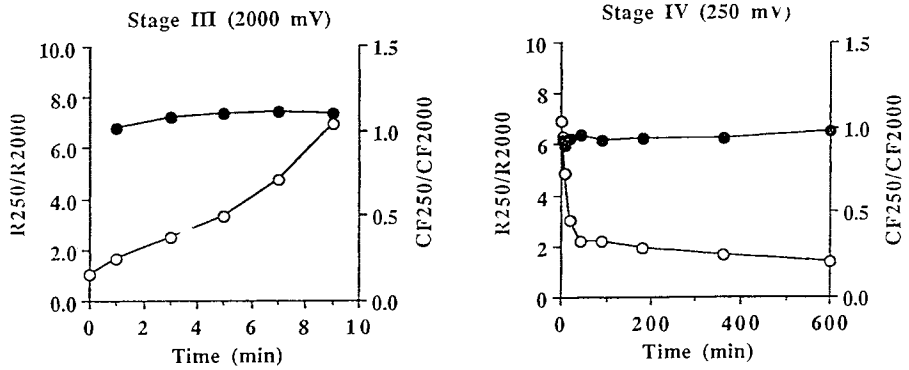

Fig. 10. Time profiles of R_{250}/R_{2000} (○) and CF_{250}/CF_{2000} (●) for Stages III and IV. These results support the view that resistance change (i.e., permeability change), caused by applied voltage is the main factor which determines the TEA$^+$ flux during and after iontophoresis.

Table III. Comparison Between Experimentally (Expt) and Theoretically Estimated (Est) Fluxes of [1-^{14}C]TEA$^+$ in Stage III at 2000 mV and Stage IV at 250 mV for the Four Runs[a]

Time (min)	Flux (dpm/cm^2/min)							
	Run 1		Run 2		Run 3		Run 4	
	Expt	Est	Expt	Est	Expt	Est	Expt	Est
Stage III								
1	1800	1850	338	507	443	484	90	94
3	2990	2970	664	723	710	695	165	156
5	3820	3780	897	929	858	870	211	208
7	4400	4300	1180	1070	929	932	334	304
9	4640	4730	1310	1310	992	993	443	441
Stage IV								
2.5	571	552	270	154	267	206	177	44
7.5	396	399	166	123	190	154	90	32
20	310	308	141	113	125	120	21	21
45	232	231	125	106	91	89	17	16
90	205	205	110	102	83	79	16	15
210	198	198	108	97	66	67	15	14
390	183	182	96	82	60	59	10	12
600	168	169	80	75	51	53	8	10

[a] It is seen that the agreement between experiment and theory is quite satisfactory and supports the view that the resistance changes induced by applied electrical field directly correlate with proportional changes in permeability of HEM for ionic permeant (TEA$^+$).

HEM results and the studies by Glaser *et al.* and Chernomordik *et al.* is the pattern of (i) the strong dependence of the rate of increase in conductance upon the applied voltage, (ii) the reversible recovery times being frequently many orders of magnitude greater than the electrical breakdown times, and (iii) the reversible recovery times being much longer when lower voltages were applied for longer times to attain the same electrical conductance before recovery. While more work is needed along the lines of demonstrating permeability changes over a wider range of conditions, the present results cogently demonstrate reversible pore induction in HEM at modest applied voltages.

APPENDIX

Derivation of Eq. (3)

The Nernst–Planck equation has been modified to include a solvent flow velocity term (2–4). For this case, the flux, J, of a tracer-level permeant having a charge, Z, and an effective diffusion coefficient, D, through a porous membrane of thickness Δx, is given by

$$J = -D\left(\frac{dC}{dx} + \frac{ZFC}{RT}\frac{d\psi}{dx}\right) \pm vC \qquad \text{(A1)}$$

where C is the permeant concentration, ψ is the electric potential at any point x in the membrane, F is the Faraday constant, R is the gas constant, T is the absolute temperature, and v is the effective average solvent velocity. The term vC is a measure of the transport of permeant resulting from convective solvent flow. An enhancement factor, E, for a cationic permeant (such as TEA$^+$) may be defined as the ratio of iontophoretic flux ($J_{\Delta\psi}$) at an applied voltage $\Delta\psi$ across the membrane to passive flux (J_0) and can be obtained from Eq. (A1) (3,4) as

$$E_+ = \left[-K\left(1 - \frac{\text{Pe}}{K}\right)\right] \Big/ \left[1 - \exp K\left(1 - \frac{\text{Pe}}{K}\right)\right] \qquad \text{(A2)}$$

where

$$K = \frac{ZF\Delta\psi}{RT} \qquad \text{(A3)}$$

K is the ionic transport enhancement factor and involves the Nernst–Planck direct effect without solvent flow, and

$$\text{Pe} = \frac{v\Delta x}{D} \qquad \text{(A4)}$$

Pe is the Peclet number and characterizes the effect of convective solvent flow (via electroosmosis) on the flux of the permeant, and Δx is the membrane thickness.

Any enhancement in the flux of uncharged solutes can be assumed to be due to only convective solvent flow, and the enhancement factor (U) for a nonionic permeant (such as mannitol) can be obtained by taking the limit as K approaches 0 (i.e., $Z = 0$) in Eq. (A2) as described in the previous study (2):

$$U = \frac{\text{Pe}}{1 - \exp(-\text{Pe})} \qquad \text{(A5)}$$

In the case of $-K \gg \text{Pe}$ and $U \gg 1$, the following equation is obtained from Eqs. (A2) and (A5):

$$E_+ = -K + U \qquad \text{(A6)}$$

E_+ is the cationic iontophoretic flux enhancement due to both a direct field effect and a convective solvent flow effect.

The iontophoretic flux ($J_{\Delta\psi}$) for TEA$^+$ when applying a voltage $\Delta\psi$ may be described by

$$J_{\Delta\psi} = E_+ P_{\Delta\psi} C_D \qquad \text{(A7)}$$

where E_+ is defined by Eq. (A6), $P_{\Delta\psi}$ is the iontophoretic permeability coefficient, and C_D is the permeant concentration in the donor compartment. In the cases where $\Delta\psi = 250$ mV and $\Delta\psi = 2000$ mV, Eq. (A7) gives Eqs. (A8) and (A9), respectively.

$$J_{250} = (-K_{250} + U_{250})P_{250}C_D \qquad \text{(A8)}$$

$$J_{2000} = (-K_{2000} + U_{2000})P_{2000}C_D \qquad \text{(A9)}$$

Combining Eqs. (A8) and (A9) and noting that $K_{2000}/K_{250} = 8$, we obtain

$$J_{2000} = 8 \cdot \left\{\frac{P_{2000}(t)}{P_{250}}\right\}\left\{\frac{\text{CF}_{250}}{\text{CF}_{2000}(t)}\right\} J_{250} \qquad \text{(A10)}$$

where

$$\text{CF}_{250} = \left(\frac{K_{250}}{-K_{250} + U_{250}}\right) \qquad \text{(A11)}$$

and

$$CF_{2000}(t) = \left\{\frac{K_{2000}}{-K_{2000} + U_{2000}(t)}\right\} \quad \text{(A12)}$$

The K's are the respective TEA^+ transport enhancement factors based upon the Nernst–Planck equation without solvent flow and may be theoretically calculated by Eq. (A3) (3). The U's are enhancement factors based on electroosmosis (from mannitol flux experiments). P_{250} is assumed to be time (t) independent and the same as the passive TEA^+ permeability coefficient. U_{250} is defined by

$$U_{250} = \frac{J_{\text{mann, 250}}}{J_{\text{mann, passive}}} \quad \text{(A13)}$$

where $J_{\text{mann, 250}}$ is the mannitol flux at 250 mV and $J_{\text{mann, passive}}$ is the passive (zero-voltage) mannitol flux. $P_{2000}(t)$ is assumed to be time dependent. The "correction" factor, $CF_{2000}(t)$ may be time dependent because $U_{2000}(t)$ may be time dependent. $U_{2000}(t)$ is defined by

$$U_{2000}(t) = \left\{\frac{J_{\text{mann, 2000}}(t)}{J_{\text{mann, passive}}}\right\}\left\{\frac{P_{\text{mann, passive}}}{P_{\text{mann, 2000}}(t)}\right\} \quad \text{(A14)}$$

where $J_{\text{mann, 2000}}(t)$ is the mannitol flux at 2000 mV, and $P_{\text{mann, passive}}$ and $P_{\text{mann, 2000}}(t)$ are the mannitol permeability coefficients at zero voltage and 2000 mV, respectively.

Now the principal question is, is the reduction in HEM electrical resistance during the application of the voltage reflected quantitatively to the increase in HEM permeability? This key question is addressed by substituting the following expression into Eqs. (A10) and (A14):

$$\frac{R_{250}}{R_{2000}(t)} = \frac{P_{2000}(t)}{P_{250}} = \frac{P_{\text{mann, 2000}}(t)}{P_{\text{mann, passive}}} \quad \text{(A15)}$$

where R_{250} and $R_{2000}(t)$ are the electric resistances at 250 and 2000 mV, respectively.

Finally, Eq. (3) (given in the text and repeated here) can be obtained by combining Eqs. (A10)–(A15) to calculate the theoretical TEA^+ flux as follows:

$$J_{2000} = 8 \cdot \left\{\frac{R_{250}}{R_{2000}(t)}\right\}\left\{\frac{CF_{250}}{CF_{2000}(t)}\right\} J_{250} \quad (3)$$

where

$$CF_{250} = \frac{K_{250}}{-K_{250} + (J_{\text{mann, 250}}/J_{\text{mann, passive}})}$$

and

$$CF_{2000}(t) = \frac{K_{2000}}{-K_{2000} + \{J_{\text{mann, 2000}}(t)/J_{\text{mann, passive}}\}\{R_{2000}(t)/R_{250}\}}$$

All of the terms on the right side of Eq. (3) have been defined and are known or obtainable from the experiments: R_{250} and R_{2000} are from Figs. 7 and 8, and the K's are calculated from Eq. (A3). Equation (3) is the main equation for testing the hypothesis that the decrease in HEM resistance induced by applied voltage is proportional to the increase in HEM permeability as demonstrated in Fig. 9 and Table III.

ACKNOWLEDGMENT

This research was supported by NIH Grant GM 43181.

REFERENCES

1. T. Masada, W. I. Higuchi, V. Srinivasan, U. Rohr, J. Fox, C. R. Behl, and S. Pons. Examination of iontophoretic transport of ionic drugs across skin: Baseline studies with the four-electrode system. *Int. J. Pharm.* 49:57–62 (1989).
2. S. M. Sims, W. I. Higuchi, and V. Srinivasan. Skin alteration and convective solvent flow effects during iontophoresis. I. Neutral solute transport across human skin. *Int. J. Pharm.* 69:109–121 (1991).
3. S. M. Sims, W. I. Higuchi, and V. Srinivasan. Skin alteration and convective solvent flow effects during iontophoresis. II. Monovalent anion and cation transport across human skin. *Pharm. Res.* 9:1402–1409 (1992).
4. S. M. Sims, W. I. Higuchi, and V. Srinivasan. Interaction of electric field and electroosmotic effects in determining iontophoretic enhancement of anions and cations. *Int. J. Pharm.* 77:107–118 (1991).
5. R. W. Glaser, S. L. Leikin, L. V. Chernomordik, V. F. Pastushenko, and A. I. Sokirko. Reversible electrical breakdown of lipid bilayers: Formation and evolution of pores. *Biochim. Biophys. Acta* 940:275–287 (1988).
6. L. V. Chernomordik, S. I. Sukharev, I. G. Abidor, and Y. A. Chizmadzhev. Breakdown of lipid bilayer membranes in an electric field. *Biochim. Biophys. Acta* 736:203–213 (1983).
7. L. V. Chernomordik, S. I. Sukharev, S. V. Popov, V. F. Pastushenko, A. I. Sokirko, I. G. Abidor, and Y. A. Chizmadzhev. The electrical breakdown of cell and lipid membranes: The similarity of phenomenologies. *Biochim. Biophys. Acta* 902:360–373 (1987).
8. J. C. Weaver, G. I. Harrison, J. G. Bliss, J. R. Mourant, and K. T. Powell. Electroporation: High frequency of occurrence of a transient high-permeability state in erythrocytes and intact yeast. *FEBS Lett.* 229:30–34 (1988).
9. R. Benz and U. Zimmermann. The resealing process of lipid bilayers after reversible electrical breakdown. *Biochim. Biophys. Acta* 640:169–178 (1981).
10. J. Teissie and T. Y. Tsong. Electric field induced transient pores in phospholipid bilayer vesicle. *Biochemistry* 20:1548–1554 (1981).
11. I. P. Sugar. A theory of the electric field-induced phase transition of phospholipid bilayers. *Biochim. Biophys. Acta* 556:72–85 (1979).
12. S. Sixou, N. Eynard, J. M. Escoubas, E. Werner, and J. Teissie. Optimized conditions for electrotransformation of bacteria are related to the extent of electropermeabilization. *Biochim. Biophys. Acta* 1088:135–138 (1991).
13. U. Zimmermann and J. Vienken. Electric field-induced cell-to-cell fusion. *J. Membr. Biol.* 67:165–182 (1982).
14. K. Kinoshita and T. Y. Tsong. Voltage-induced pore formation and hemolysis of human erythrocytes. *Biochim. Biophys. Acta* 471:227–242 (1977).
15. E. H. Serpersu, K. Kinoshita, and T. Y. Tsong. Reversible and irreversible modification of erythrocyte membrane permeability by electric field. *Biochim. Biophys. Acta* 812:779–785 (1985).
16. M. R. Prausnitz, B. S. Lau, C. D. Milano, S. Conner, R. Langer, and J. C. Weaver. A quantitative study of electroporation showing a plateau in net molecular transport. *Biophys. J.* 65:414–422 (1993).
17. J. C. Weaver and A. Barnett. Progress toward a theoretical model for electroporation mechanism: Membrane electrical behavior and molecular transport. In D. C. Chang, B. M. Chassy, J. A. Saunders, and A. E. Sowers (eds.), *Guide to Electroporation and Electrofusion,* Academic Press, New York, 1992, pp. 91–117.
18. J. C. Weaver. Electroporation: A general phenomenon for manipulating cells and tissues. *J. Cell. Biochem.* 51:426–435 (1993).
19. M. R. Prausnitz, V. G. Bose, R. S. Langer, and J. C. Weaver. Transtissue molecular transport due to electroporation of skin.

In M. Blank (ed.), *Electricity and Magnetism in Biology and Medicine,* San Francisco Press, San Francisco, 1993, pp. 122–124.
20. K. A. Holbrook and G. F. Odland. Regional differences in thickness (cell layers) of the human stratum corneum: An ultrastructural analysis. *J. Invest. Derm.* **62**:415–422 (1974).
21. D. C. Swartzendruber. Studies of epidermal lipids using electron microscopy. *Semin. Dermatol.* **11**:157–161 (1992).
22. G. B. Kasting and L. A. Bowman. DC electrical properties of frozen, excised human skin. *Pharm. Res.* 7:134–143 (1990).
23. J. C. Keister and G. B. Kasting. The mechanism of iontophoresis. In *Transdermal Delivery of Drugs,* Proceedings of the workshop on current status and future directions, NIH Publication No. 91-3075, 1990, pp. 137–154.
24. J. C. Weaver. Electroporation. In *Interfacial Phenomena in Biological Systems,* Marcel Dekker, New York, 1991, pp. 253–277.
25. B. M. Wallace and J. S. Lasker. Stand and deliver: Getting peptide drugs into the body. *Science* **260**:912–913 (1993).
26. A. E. Sowers and M. R. Lieber. Electropore diameter, lifetimes, numbers and locations in individual erythrocyte ghosts. *FEBS Lett.* **205**:179–184 (1986).
27. J. A. Donlon and A. Rothstein. The cation permeability of erythrocytes in low ionic strength media of various tonicities. *J. Membr. Biol.* **1**:37–52 (1969).
28. A. V. Putvinsky, S. A. Popov, T. V. Puchkova, Y. A. Danilov, and Y. A. Vladimirov. Electrical breakdown of erythrocyte membranes produced by diffusion potential difference. *Biofizika* **28**:505–506 (1983).
29. K. Okabe, H. Yamaguchi, and Y. Kawai. New iontophoretic transdermal administration of the beta-blocker metoprolol. *J. Control. Release* **4**:79–85 (1986).
30. T. Bagniefski and R. R. Burnette. A comparison of pulsed and continuous current iontophoresis. *J. Control. Release* **11**:113–121 (1990).
31. A. H. Ghanem, J. A. Rogers, W. I. Higuchi, and V. Srinivasan. An examination of pulsed iontophoresis with Nuclepore^{R} and human epidermal membranes (submitted for publication).