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Permeability Parameters of the Toad Isolated *Stratum Corneum*

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Summary. A technique for isolating the *stratum corneum* from the subjacent layers of the epithelium was developed which permits studying the *stratum corneum* as an isolated membrane mounted between half-chambers. The method basically consists of an osmotic shock induced by immersing a piece of skin in distilled water at 50 °C for 2 min. When the membrane is bathed on each surface by NaCl-Ringer's solution, its electrical resistance is $14.1 \pm 1.3\ \Omega\ cm^2$ ($n = 10$). This value is about 1/100 of the whole skin resistance in the presence of the same solution. The hydraulic filtration coefficient (L_p) measured by a hydrostatic pressure method, with identical solutions on each side of the membrane, is $8.8 \times 10^{-5} \pm 1.5 \times 10^{-5}$ cm sec^{-1} atm^{-1} ($n = 10$) in distilled water and $9.2 \times 10^{-5} \pm 1.4 \times 10^{-5}$ cm sec^{-1} atm^{-1} ($n = 10$) in NaCl-Ringer's solution. These values are not statistically different and are within the range of 1/80 to 1/120 of the whole skin L_p. The *stratum corneum* shows an amphoteric character when studied by KCl diffusion potentials at different pH's. The membrane presents an isoelectric pH of 4.6 ± 0.3 ($n = 10$). Above the isoelectric pH the potassium transport number is higher than the chloride transport number; below it, the reverse situation is valid. Divalent cations (Ca^{++} or Cu^{++}) reduce membrane ionic discrimination when the membrane is negatively charged and are ineffective when the membrane fixed charges are protonated at low pH.

In the literature there are several reports dealing with the role of the *stratum corneum* in the transport of water and ions by amphibian skin and with the importance of this structure in electrophysiological observations.

During the moulting cycle, the amphibian skin undergoes profound morphological changes [3] which are accompanied by complex physiological alterations, with increase in water [4, 5, 10] and ionic [14, 16, 17, 23] permeabilities, and changes in the rate of active sodium transport [4, 15, 17, 23, 24, 33] and in the electrical resistance [13]. In all these reports the role played by the *stratum corneum* was not isolated from other alterations that probably occur during moulting and shedding of the *stratum corneum.*

Nielsen [23] has shown that once the cornified layer is spontaneously ruptured or mechanically removed, the short-circuit current rapidly rises to values above control levels. The cornified cell layer is a barrier located between the outside bathing solution and the outer membrane of the first reacting cell layer and, therefore, as suggested by Biber and Sanders [2] may shift the "effective" sodium concentration at the outside surface of this cell layer to a value which is different from the activity of Na^+ in the bulk solution. The magnitude of this shift could be a function of the permeability of the *stratum corneum* and of the rate of Na^+ transport. The results of Martinez-Palomo *et al.* [21] show that two diffusion barriers can be considered to be present in the external surface of frog skin: the first is formed by the cells of the *stratum corneum* and their *zonulae occludentes*, which are impermeable to ruthenium red and colloidal lanthanum, but seem to be permeable to La^{3+} (ionic lanthanum). The second barrier, formed by the outer membranes and *zonulae occludentes* of the cells of the *stratum granulosum*, is probably the most important selective external barrier of the skin. The role of the *stratum corneum* in electrophysiological experiments has been discussed by several authors. Thus, Whittembury [34], Rawlins *et al.* [26] and Nunes with Lacaz Vieira [25] suggested that the resistance of the *stratum corneum* is negligible compared to the total electrical resistance of the epithelium. On the other hand, discrepancies were observed between resistances of the *stratum corneum* measured with single- and with double-barreled glass microelectrodes [25]; the values measured with double-barreled electrodes were much higher than those obtained with single-barreled electrodes. Lindemann and Thorns [18], however, emphasize that in most epithelia investigated by them the main resistive barrier appeared to be at the outermost surface of the epithelium. To account for this observation, they raised the possibility that the cornified layer could be missing in their preparations. The presence of fixed charges in the matrix of the *stratum corneum*, as suggested in a previous report [25], may also contribute through a Donnan exclusion [12] to alter the Na^+ activity at the external surface of the cells of the *stratum granulosum*, especially when the outside bathing solution is of low ionic concentration.

The present work was carried out with the object of developing a technique for isolating the *stratum corneum* from the subjacent layers and measuring its hydraulic and electric conductivities. It was also our aim to test directly the existence of fixed charges in the matrix of the *stratum corneum* suggested by the results of earlier experiments [25]. These fixed charges were postulated to account for observations made in the super-

ficial layer of the epithelium during alterations of the external bathing solution.

Materials and Methods

The experiments were carried out in the isolated *stratum corneum* obtained from the abdominal skin of the toad *Bufo marinus ictericus* in the temperature range of 22 to 25 °C.

Technique for Isolating the Stratum Corneum

A sheet of skin of about 2 cm × 5 cm is immersed in distilled water at 50 °C for 2 min and then placed on the surface of a glass plate with epithelium facing upwards. Holding the sheet of skin by one of its sides, the *stratum corneum* can be easily removed with the thumb by applying pressure on the skin and sliding the finger against the glass plate. Sheets of *stratum corneum* of several square centimeters thus obtained can be unfolded and easily mounted between half-chambers by performing the necessary operations with the membrane and the half-chambers totally immersed in distilled water. The membrane of *stratum corneum* (subsequently called "membrane") is sufficiently strong to resist the operations of mounting, changing of solutions, and the vigorous stirring of the solutions by means of an air turbine-driven propeller. The chambers were similar to those described by Ussing and Zerahn [31].

Electrical Measurements

Electrical Potential Difference. To measure the electrical potential difference across the membrane, an electrometer (Keithley mod. 615 – Keithley Instruments Inc., USA) coupled to a recorder (Varian mod. G-2500 – Varian Aerograph, Division of Varian Associates, USA) was used. Ag – AgCl electrodes were used as sensing devices. The transmembrane electrical potential difference ($\Delta\Psi$) was obtained by subtracting from the measured electrical potential difference (ΔE) the difference in electrode potential (ΔE_j):

$$\Delta\Psi = \Delta E - \Delta E_j \tag{1}$$

$$\Delta E_j = -(RT/F)\ln(a_2^{Cl^-}/a_1^{Cl^-}) \tag{2}$$

$$a^{Cl^-} = a_{\pm}^{KCl} \text{(according to MacInnes assumption [19])} \tag{3}$$

$$a_{\pm}^{KCl} = \gamma_{\pm m} \tag{4}$$

where: a^{Cl^-} = chloride activity; $a_{\pm}^{KCl}$ = KCl mean ionic activity; $\gamma_{\pm}$ = KCl mean ionic activity coefficient (from Glasstone [6]); m = KCl molality; R = gas constant and F = Faraday constant. The influence of HCl or KOH, used to adjust the pH of the solutions, on KCl activities was considered negligible in the range of concentrations used in the experiments, according to the thermodynamics of mixed electrolytes [8, 9, 27].

Ag – AgCl electrodes were made of a single silver wire of 2 mm diameter. Pieces of this wire were covered with epoxy resin (Araldite-Ciba-Geigy) leaving 5 mm uncoated at the tip, which was cleaned by abrasion and thoroughly washed in distilled water before being chloridized in 0.1 M HCl solution, against a Pt electrode using current density of approximately 10 $\mu A\,cm^{-2}$ for 30 sec. Pairs of electrodes were kept short-circuited in 0.1 M KCl until the moment of use. The potential measurements were considered successful when assymetry between electrodes was less than 1 mV (measured in 0.1 M KCl solution).

Electrical Resistance. Resistance was always evaluated by applying current pulses and recording changes in $\Delta\Psi$ with the membrane bathed by identical solutions on each surface. One pair of Ag – AgCl electrodes (located close to the membrane) recorded $\Delta\Psi$; another

pair (far from the membrane) was used to inject current pulses. These pulses of variable duration (1 to 10 sec) and intensity (up to $\pm 100\ \mu A\ cm^{-2}$) were tapped off potentiometrically from a 10 V battery and measured by an electrometer. To correct for the voltage drop across the solution resistance between the voltage electrodes and the membrane, measurements were carried out before each membrane resistance determination using the same solution but without the membrane in the chamber. The membrane resistance was calculated by the difference in resistance obtained with and without the membrane. In each case the resistance was calculated as the slope of $\Delta\Psi$ as a function of the electrical current.

Volume Flow

Flow was measured as the rate of displacement of a liquid meniscus in a calibrated capillary tube connected to the lower pressure half-chamber. The pressure necessary to drive fluid through the membrane was measured by a mercury manometer. In all experiments in which pressure was used, the membrane was supported by a stainless steel disc with cylindrical holes of 140 μm diameter in order to prevent its rupture. The ratio between open area and total disc area was 0.192. The volume flow was calculated per unit area of the open area of the steel disc supporting the membrane. Preliminary experiments have shown that the filtration coefficient values obtained with discs of different open areas agree only when computed per unit area of the disc open area. In all experiments using pressure, identical solutions always bathed each side of the membrane and no stirring of the solutions was used.

Toads were captured in the vicinity of São Paulo, Brazil, and were kept with free access to running tap water. Straight lines were fitted by the least squares method. Results are presented as mean ± 1 SE.

Results

1. Morphological Aspect of the Isolated Stratum Corneum

Fig. 1 is a light microscopy picture showing that the isolated *stratum corneum* is a continuous membrane with a few islands of cell debris attached to its inner surface. Fig. 2 shows an electronmicrographic comparison between the transition between the *stratum corneum* and *stratum granulosum* in a normal skin and the delamination zone located between these two layers, after the osmotic shock.

2. KCl Diffusion Potential

These experiments have been made to test directly the previously formulated hypothesis [25] suggesting the existence of fixed charges in the matrix of the *stratum corneum*, and to study the parameters controlling their density and polarity.

KCl diffusion potentials have been measured with the membrane bathed on each surface by KCl solutions of different concentrations. KCl was used because K^+ and Cl^- have similar mobilities and hydrated radii

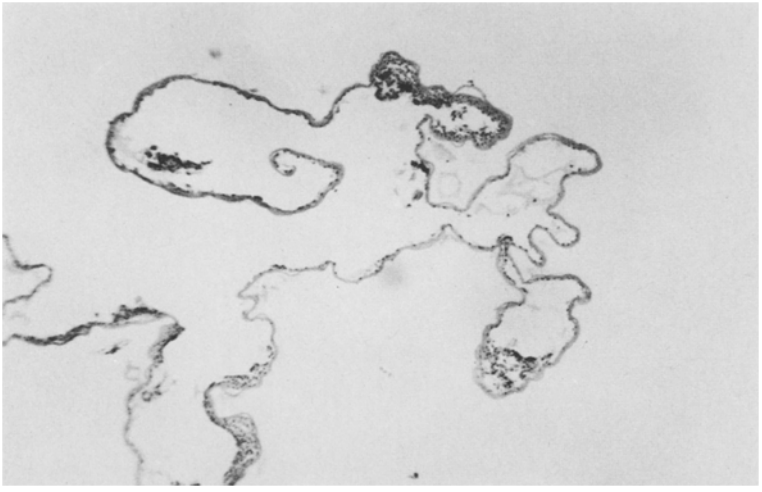

Fig. 1. Typical light micrograph of the isolated *stratum corneum* obtained by means of osmotic shock induced by immersing the skin in distilled water at 50 °C, for 2 min. (Stained with hematoxylineosin.) The isolated *stratum corneum* forms a continuous layer with few cell debris attached to its inner surface

in free solution [27]. The experiments were performed at pH 3 and pH 9, with solutions always of the same pH bathing each side of the membrane. The pH's were adjusted using no buffers, with HCl or KOH. The dilute solution (*sol.* 1) was vigorously stirred with a propeller driven by an air turbine. The stirring rate was considered adequate when an increase in propeller rotation induced no further increase in the recorded electrical potential difference. The rate of stirring of the dilute solution is extremely important in the stabilization value of the electrical potential difference, being critical at higher KCl concentration difference across the membrane. Steady-state electrical potential difference values, normally attained within a few seconds following solution changes, were used in the calculations. The concentrate solution (*sol.* 2) was not systematically stirred since preliminary experiments had shown the independence of ΔE on the rate of stirring of this solution. Fig. 3 presents the results. As may be seen, at pH 3 the concentrate solution is positive, indicating a higher Cl^- mobility. At pH 9, the reverse situation obtains. For a given pH the membrane potential ($\Delta\Psi$) is a linear function of the logarithm of KCl concentration in the dilute solution. The slope of this relationship is decreased with an increase in KCl concentration of the concentrate solution.

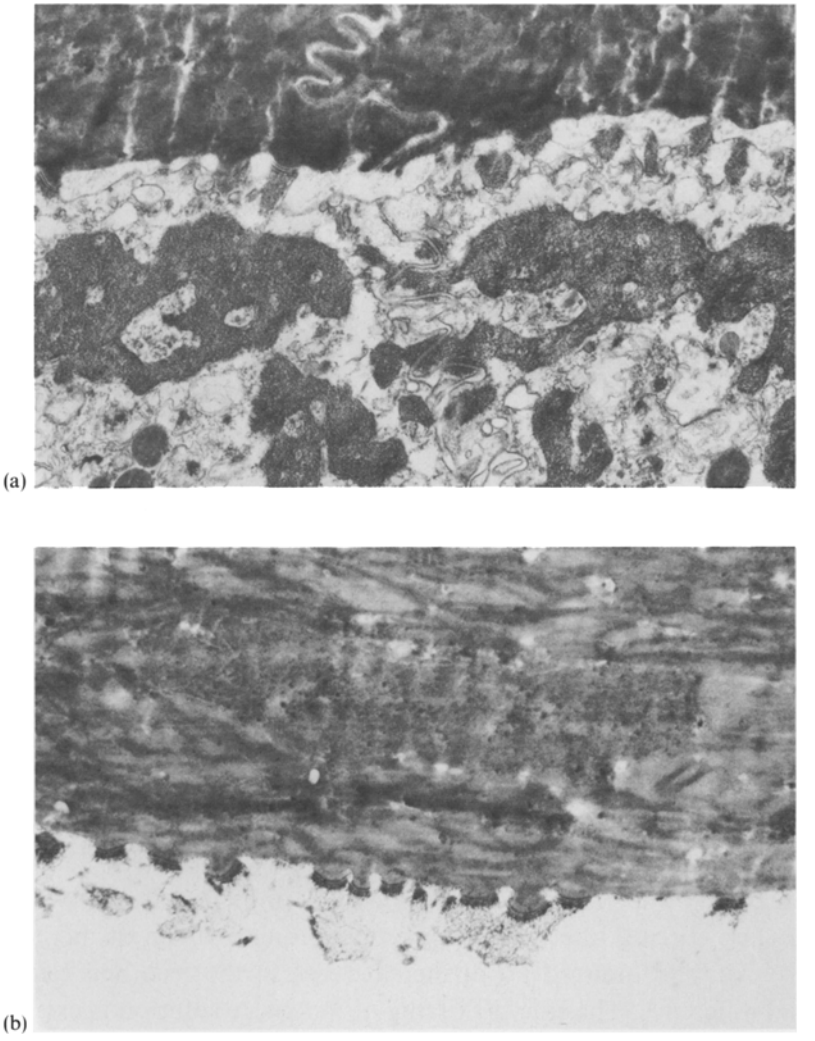

Fig. 2. Typical electronmicrograph of the *stratum corneum:* (a) Normal aspect of the transition between the *stratum corneum* and *stratum granulosum.* (b) Delamination zone after the osmotic shock. The inner surface of the *stratum corneum* is formed by the membranes of the cornified cells with disrupted desmosomes. (Fixed with glutaraldehyde-osmic acid and stained with lead citrate)

An independent group of experiments was performed with solutions of a fixed KCl concentration difference across the membrane ($C_1 = 0.01$ molal; $C_2 = 0.1$ molal) and identical pH's which were varied by addition of HCl. The results show that the membrane presents an isoelectric pH of

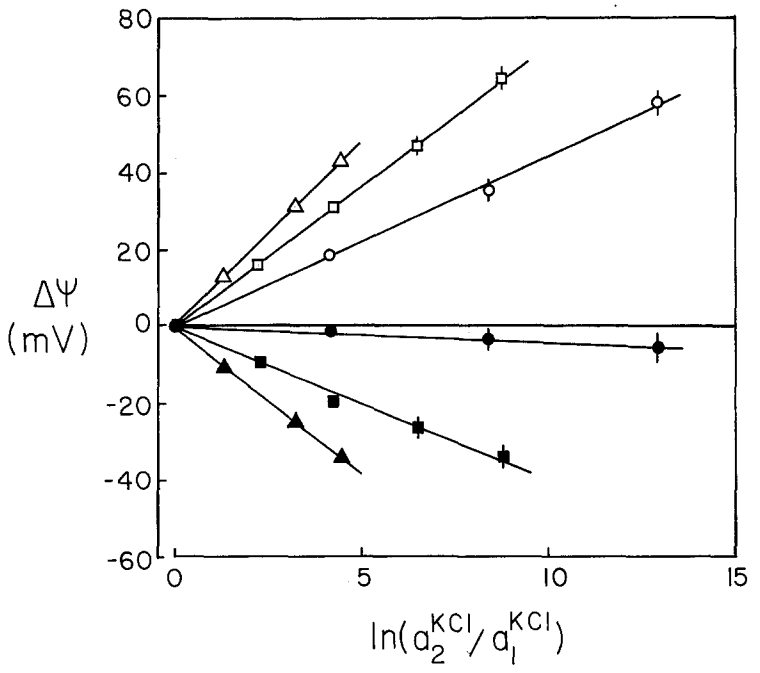

Fig. 3. Membrane electrical potential difference ($\Delta\Psi = \Psi_2 - \Psi_1$), due to KCl diffusion across the membrane, plotted according to Eq. (6). a^{KCl} is the KCl activity calculated as: $a = (m\gamma_{\pm})^2$, where m is the KCl molality and $\gamma_{\pm}$ is the KCl mean ionic activity coefficient. 1 and 2 refer to the dilute and concentrate solutions, respectively. Open symbols are experiments performed at pH 3; filled symbols, experiments at pH 9. pHs were always identical in the solutions bathing each side of the membrane, and were adjusted with HCl or KOH without buffer. The points (○ and ●) were measured with $m_2 = 1$ M and m_1 variable; (□ and ■) with $m_2 = 0.1$ M and m_1 variable; (△ and ▲) with $m_2 = 0.01$ and m_1 variable. The potassium transport numbers (t_K) were calculated according to Eq. (6) and were as follows: (○) $t_K = 0.42$; (□) $t_K = 0.36$; (△) $t_K = 0.29$; (●) $t_K = 0.51$; (■) $t_K = 0.59$; (▲) $t_K = 0.66$. Bars indicate 1 SEM. $n = 8$

4.6 ± 0.3, ($n = 10$). The isoelectric pH for each membrane was calculated by linear interpolation in a graph of $\Delta\Psi$ as a function of pH, using only the two values on either side of $\Delta\Psi = 0$.

For our present case the membrane potential can be expressed under the assumptions of irreversible thermodynamics as a diffusion potential without requiring any assumptions concerning membrane composition, structure, electric field within the membrane, or mobilities, as discussed by Stavermann [30], Scatchard [28], and tested for a cation exchange membrane by Gunn and Curran [7].

Therefore, we can assume:

$$-F\Delta\Psi = \int_{\mu_1}^{\mu_2} (t_{K^+} - t_{Cl^-})\, d\mu^{KCl} \tag{5}$$

where the integral is from one solution to the other, μ is the chemical potential and t is the transport number. In using Eq. (5) it can be assumed that the water transport number is negligible, as is suggested in the results, paragraph 4. Eq. (5) can be easily integrated under the assumption of constant transport numbers. This can be considered a valid assumption, for a given pH value and for a given KCl concentration in the concentrate solution, as suggested by the results of Fig. 3, which shows a linear dependence of $\Delta\Psi$ on the logarithm of KCl activity in the dilute solution. This behavior could be a consequence of the concentration profile within the membrane being a strong function only of the KCl concentration in the concentrate solution. For each pair (pH and a_2^{KCl}) the integrated form of Eq. (5) is:

$$\Psi_2 - \Psi_1 = -(2t_{K^+} - 1)(RT/F) \ln (a_2^{KCl}/a_1^{KCl}) \tag{6}$$

where a_1^{KCl} and a_2^{KCl} are KCl activities in the solutions bathing each side of the membrane. KCl activities were calculated as $(m\gamma_\pm)^2$, where m = KCl molality and $\gamma_\pm$ = KCl mean ionic activity coefficient. R, T and F have their conventional meanings. An increase in a_2^{KCl} at pH 3, as well as at pH 9, induces a reduction in the absolute value of the slope of $\Delta\Psi$ as a function of $\ln (a_2^{KCl}/a_1^{KCl})$ (Fig. 3), this indicating that t_{K^+} approaches t_{Cl^-} and, therefore, that the membrane ionic discrimination is reduced. On the other hand, the pH of the solutions have a pronounced effect on the transport number. At pH 3, $t_{K^+} < t_{Cl^-}$, this indicating that the membrane is more permeable to Cl^- than to K^+ ions; at pH 9, the reverse occurs ($t_{K^+} > t_{Cl^-}$). (*See* Fig. 3.)

3. Effect of Divalent Cations on KCl Diffusion Potential

The presence of divalent cations (Ca^{++} or Cu^{++}) at equal concentrations on both sides of the membrane may have a pronounced effect on $\Delta\Psi$, according to the degree of protonation of the membrane. At pH 9, with the membrane negatively charged, divalent cations significantly reduce $\Delta\Psi$ due to KCl diffusion. At pH 3, the membrane being positively charged, they have no effect on $\Delta\Psi$. Fig. 4 shows the results of experiments with Ca^{++} at a 2 mM concentration on both sides of the membrane. The

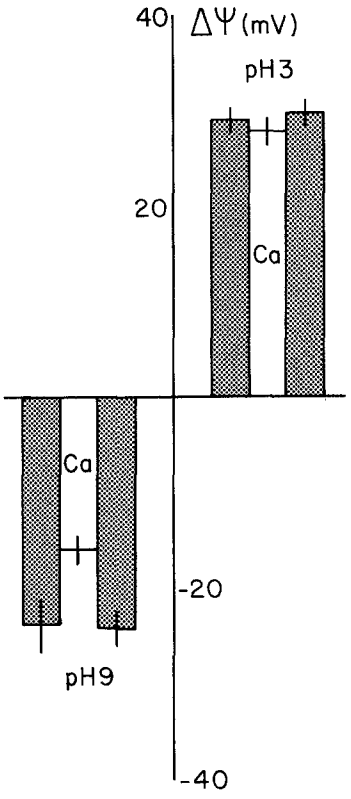

Fig. 4. Effect of Ca^{++} (2 mM on each side of the membrane) on membrane KCl diffusion potential ($\Delta\Psi = \Psi_2 - \Psi_1$), at pHs above and below the membrane isoelectric pH. KCl concentrations at each side of the membrane were: $C_1 = 0.01$ M; $C_2 = 0.1$ M. Shadded areas are control periods before and after an experimental period with Ca^{++}. 1 and 2 refer to each side of the membrane. Bars indicate 1 SEM. $n = 10$

effects of Ca^{++} or Cu^{++} are reversible upon washing; the reversion of Cu^{++} being slower than that of Ca^{++} suggests a strong Cu^{++} binding to the membrane.

4. *Hydraulic Filtration Coefficient* (L_p)

L_p was calculated by measuring the volume flow (J_v) as a function of the hydrostatic pressure (ΔP) across the membrane, in the range of 0 to 0.8 atm. In this pressure range, L_p is given by: $L_p = J_v/\Delta P$ [11]. With distilled water bathing each side of the membrane L_p is $8.8 \times 10^{-5} \pm 1.5 \times 10^{-5}$ cm sec^{-1} atm^{-1} ($n = 10$). With NaCl-Ringer's solution bathing each side of the membrane, $L_p = 9.2 \times 10^{-5} \pm 1.4 \times 10^{-5}$ cm sec^{-1} atm^{-1} ($n = 10$). These values are not statistically different ($P > 0.2$, t test).

5. *Electroosmosis*

No measurable water flows could be detected with current densities up to 200 $\mu A\ cm^{-2}$, with the membrane bathed on both sides either by KCl 0.1 or 0.01 molal, and either at pH 3 or at pH 9.

These results indicate that the water transport number in this membrane is very small and can be neglected in Eq. (5).

6. *Membrane Electrical Resistance*

Membrane electrical resistance (R_m) was measured with NaCl-Ringer's solution bathing each surface of the membrane in order to permit comparison with values obtained for the whole skin. $R_m = 14.1 \pm 1.3\ \Omega\ cm^2$ ($n = 10$). The resistance showed an ohmic behavior in the range studied ($\pm 100\ \mu A\ cm^{-2}$).

Discussion

A technique for isolating the *stratum corneum* was developed which obtains sheets of membrane suitable for *in vitro* studies with the membrane mounted between half-chambers. With this preparation it was possible to get direct information concerning permeability of the *stratum corneum.*

The electronmicroscopy of the *stratum corneum* (Fig. 2) shows that this membrane is almost a pure film of cornified cells where narrow intercellular spaces and desmosomes, as well as a highly dense intracellular content, can be clearly seen. The general appearance of the *stratum corneum* after the osmotic shock is identical to that of the *stratum corneum* of the control tissue not submitted to the osmotic treatment. Very few cell debris can be seen attached to the membrane inner surface. Normally, when fixation is performed after using the membrane in experiments with vigorous stirring of the solutions, it can be noticed that all cell debris are removed and the inner surface of the *stratum corneum* is formed simply by the membrane of the cornified cells, where disrupted desmosomes can be seen (Fig. 2). The osmotic shock induces rupture of all cells in the layers subjacent to the *stratum corneum.* The fact that the cornified cells are not disrupted by this treatment suggests that the membranes of these cells are freely permeable to water and solutes and, therefore, do not offer the conditions necessary for an osmotic water flow. This observation is in agreement with the results of Martinez-Palomo *et al.* [21] showing that

lanthanum (La^{3+}) penetrates readily through cells of the cornified layer, and with the experiments of MacRobbie and Ussing [20] showing that the thickness of the cornified layer is independent of the osmolarity of the external solution. These authors raised the possibility that their results could also be due to low water permeability of the cell membranes or to the cornified cells being mechanically resistant to volume changes. Whittembury [34] and Nunes and Lacaz Vieira [25] using single-barreled glass microelectrodes, varying the clamping current through the skin, have shown that the electrical resistance of the outermost layer of the epithelium is practically zero. On the other hand, these authors [25], using double-barreled glass microelectrodes (passing current pulses through one barrel and recording the resulting potential difference changes through the other), have shown that the impalement of the *stratum corneum* was followed by a significant increase in the resistance between the tip of the microelectrode and the outer solution. They interpreted the discrepancy between their results with single- and double-barreled microelectrodes as due to a lack of homogeneity in the resistivity of the *stratum corneum*, possibly as a consequence of the existence of low resistance pathways of relatively very small dimensions (such as the intercellular spaces) separating higher resistance regions (probably the cells themselves). The low resistance of the isolated *stratum corneum* could be mostly due to a shunting effect of the low resistance pathways postulated previously [25].

The hydraulic conductivity coefficient (L_p) of the isolated *stratum corneum* ($8.8 \times 10^{-5} \pm 1.5 \times 10^{-5}$ cm sec^{-1} atm^{-1} ($n = 10$) in distilled water and $9.2 \times 10^{-5} \pm 1.4 \times 10^{-5}$ cm sec^{-1} atm^{-1} ($n = 10$) in NaCl-Ringer's solution) can be compared to L_p values for the whole skin. For the *Bufo marinus ictericus* [32] with NaCl-Ringer's solution bathing the skin, $L_p = 1.1 \times 10^{-6} \pm 9.9 \times 10^{-8}$ cm sec^{-1} atm^{-1} ($n = 10$), and with Na_2SO_4-Ringer's solution bathing the skin, $L_p = 7.7 \times 10^{-7} \pm 1.4 \times 10^{-7}$ cm sec^{-1} atm^{-1} ($n = 10$). The comparison of these values shows that L_p for the *stratum corneum* is within 80 to 120 times the L_p for the whole skin. This indicates, therefore, that the *stratum corneum* cannot be considered an important barrier to the bulk flow of water across the whole skin. These results suggest that alterations in water permeability observed during the moulting cycle [4, 5, 10] are possibly associated with changes in structures more deeply situated in the epithelium, other than the *stratum corneum*. On the other hand, the fact that the *stratum corneum* does not offer an important resistance to filtration of water does not exclude that unstirred layers of water supported by this part of the epithelium may function as a barrier to diffusional flows of ions and nonelectrolytes.

The electrical resistance of the *stratum corneum* bathed by NaCl-Ringer's solution on both sides is $14.1 \pm 1.3\ \Omega\,cm^2$ ($n = 10$). This value is considerably lower than that for the whole skin resistance of *B. marinus ictericus* [32] bathed by NaCl-Ringer's solution ($R_{skin} = 1440 \pm 20\ \Omega\,cm^2$, $n = 5$) or by Na_2SO_4-Ringer's solution ($R_{skin} = 1740 \pm 20\ \Omega\,cm^2$, $n = 5$), indicating that the *stratum corneum* does not contribute significantly to the electrical resistance of the whole skin. At this point it is worth considering the observation of Nielsen [23] showing that once the cornified layer is spontaneously ruptured or mechanically removed, the short-circuit current rapidly rises to values above control. Probably, this increase is a consequence of removal of the unstirred layer supported by the matrix of the *stratum corneum* permitting, thereby, a higher Na^+ concentration to enter in contact with the transporting cells.

The KCl diffusion potential experiments at different pHs show that the *stratum corneum* bears fixed charges and that the density and polarity of these charges are controlled by the degree of protonation of ionizable sites within the membrane. The pH of the solutions, as well as the ionic concentration in the concentrate solution, have an important effect on the membrane ionic discrimination. Eq. (6) serves to describe correctly the behavior of KCl diffusion potential as a function of KCl concentration in the dilute solution (for a given pH and KCl concentration in the concentrate solution). The isoelectric pH of the membrane (4.6 ± 0.3, $n = 10$) is close to the isoelectric point (pH = 5.1) obtained for the whole frog skin in KCl diffusion potential experiments [1]. Isoelectric pHs have been calculated for different biological membranes from diffusion potentials and streaming potentials. They varied within the range of 2.7 to 5.3 [1, 22, 29, 35]. Above the isoelectric pH, for the *stratum corneum*, $t_K > t_{Cl}$; below the isoelectric pH, $t_K < t_{Cl}$.

Divalent cations (Ca^{++} or Cu^{++}) reduce membrane ionic discrimination at pH 9 and are ineffective at pH 3. This fact indicates the interaction of divalent cations with the membrane when it is negatively charged. These ions increase the ratio t_{Cl}/t_K when the membrane is negatively charged, suggesting that they titrate negative fixed charges in the membrane.

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