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Negative Potential Level in the Outer Layer of the Toad Skin

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Summary. The isolated skin of the toad *Bufo marinus ictericus* when impaled from the outer surface by glass microelectrodes filled with 3 M KCl shows a voltage profile which is a continuous function of the depth of impalement. The superficial intraepithelial potential difference measured with reference to the external solution (*PDi*) is negative with NaCl-Ringer's solution on both sides of the skin, displaying a minimum of -26.7 ± 3.6 mV at 6 ± 2 μm. Null value is obtained at 19 ± 3 μm, with positive values for deeper impalements. Indications of cell impalements (abrupt voltage and resistance jumps) were frequently observed at sites deeper than 25 μm from the outer surface. Measurements of the electrical resistance between the microelectrode and the external solution, made with single- and double-barreled microelectrodes, showed great discrepancies, which may be attributed to distinct pathways of different resistances in the *stratum corneum*. *PDi* measured at a depth of 5 μm was a logarithmic function of Na_2SO_4 or K_2SO_4 concentration in the external solution, increasing in negativity with a reduction in concentration. Substitution of Na by K in the external solution had only minor effects on *PDi*. Acidification of the external solution from pH 9 is accompanied by a reduction in the negative value of *PDi*. At pH 3 *PDi* was positive. *PDi* was interpreted as a diffusion potential at the tip of the microelectrode due to KCl diffusion from the electrode into the matrix of the *stratum corneum*. Differences in K and Cl mobilities, responsible for the origin of *PDi*, were attributed to fixed charges in the matrix of the *stratum corneum*, with density and polarity determined by their degree of protonation, controlled by the hydrogen ion concentration of the external solution. Skin potential, short-circuit current and their relationship to *PDi* were discussed.

There are several papers in literature dealing with the electrical profile of the isolated amphibian skin obtained with glass microelectrodes filled with 3 M KCl. A comparative analysis of these investigations shows many controversial points. Several unanswered questions still remain regarding the shape of the electrical profile and the number of potential jumps and their localization within the epithelium [7–9, 11, 23–25, 28, 29] as well as the dependence of the intraepithelial electrical potential on the ionic composition of the bathing solutions [3, 7–9, 28] and on the electrical condition (open-circuited or short-circuited state) [7–9, 28, 29]. A negative

potential (measured with reference to the external solution) in the range of a few to several millivolts, sometimes unstable, was observed in the superficial layer of the epithelium [3, 7, 24, 28, 29]. Some dependence on depth of impalement has also been reported [11]. Whittembury [29], using a technique for deposition of carmine spots from the electrode tip, has clearly shown that the superficial negative region lies within the *stratum corneum*. Several hypotheses have been put forward to explain the origin of this potential. Engbaek and Hoshiko [11] have suggested that it could be the membrane potential of epithelial cells of the *stratum corneum*. Whittembury [29] feels that this is rather unlikely, since the observed outer side resistance is practically zero. He also raised the possibility of this potential being a consequence of an ionic exchange character of the *stratum corneum*, or of artifacts due to distortion of the microelectrodes. On the other hand, Scheer and Mumback [25] and Chowdhury and Snell [8, 9] did not observe the existence of a negative site in the superficial layer of the epithelium.

The aim of the present investigation was to study in detail the nature of the electrical potential (observed with glass microelectrodes filled with 3 M KCl) in the region of the *stratum corneum*.

Materials and Methods

The experiments were performed on the skin of the toad *Bufo marinus ictericus* obtained from the medial surface of the leg, due to its smoothness and low density of large glands. The experiments were performed at room temperature (22–25 °C). The skin was mounted in a specially designed chamber, with the outer surface upwards, exposing an area of 1.13 cm^2, and held in place on the surface of a sintered glass disc by a reduced hydrostatic pressure (70 cm of water) in the lower hemi-chamber. In order to flatten the skin and to reduce the wrinkles on its external surface, the skin was gently stretched on the surface of the sintered glass disc, before assembling the two halves of the chamber, by means of a nylon ring fitted to a circular groove in the lower hemi-chamber. The chamber permitted rapid changes of the bathing solutions with the microelectrode in place in the epithelium, without disturbing the electrical measurements. Transmembrane potential difference (*PDt*) was recorded by an electrometer (Keithley, model 602 – Keithley Instruments, Inc., Cleveland, Ohio) coupled to one channel of a recorder (Varian model G-2500, two channels – Varian Aerograph, Division of Varian Associates, Walnut Creek, California) through 3 M KCl agar-bridges and saturated calomel half-cells. A second pair of 3 M KCl agar-bridges (adequately located to provide a uniform current density through the skin) connected to Cu-$CuSO_4$ half-cells was used in the voltage-clamp experiments as current bridges. An automatic voltage-clamp was used, having as the main component an operational amplifier (Burr-Brown model 3038/25 – Burr-Brown Research Corporation, Tucson, Arizona). In order to measure the intraepithelial potential difference (*PDi*) referred to the external solution (using an external agar-bridge and calomel half-cell pair as a reference electrode) glass microelectrodes (Kimax-

51—Kimble 46485 glass tubing) filled with 3 M KCl solution by boiling in vacuo, were used [18]. *PDi* was recorded by means of an electrometer (Keithley model 602) coupled to the second channel of the recorder. A micromanipulator (built from the body of an E. Leitz Wetzlar microscope) permitted advances of the microelectrode perpendicular to the external surface of the skin, in steps of approximately 1 μm, which were indicated by a mechanical micrometer (Kaefer—Germany) with the precision of 0.5 μm. In the voltage clamping experiments the level of transmembrane potential difference (*PDt*) was set and the clamping current recorded by one channel of the second recorder (Varian model G-2500). Measurements of the electrical resistance between the microelectrode tip and the external solution were carried out in two different ways: (a) With single-barreled microelectrodes (*Ri**). The resistance was calculated by changes in *PDi* associated with brief changes (less than 15 sec) in the clamping current, in voltage-clamping experiments. (b) With double-barreled microelectrodes (*Ri*). One barrel of the microelectrode was connected in a similar manner to that used for single-barreled microelectrodes. The other barrel was used to inject square pulses of current of variable duration (0.3 to 1.0 sec) and intensity (10 to 100 nA) and of both polarities. The current pulses were provided by a stimulator (Grass model S-4—Grass Instruments, Quincy, Mass.) through an isolating unit (Grass model SIU-4) connected to the microelectrode by a silver wire and the external solution through a 3 M KCl agar-bridge and Cu-$CuSO_4$ half-cell. The intensity of the current pulses was monitored by the voltage drop across a 10 MΩ resistor, measured by a differential amplifier (Grass model P-16 A) and recorded by the second channel of the second recorder. All the experiments were performed in a grounded Faraday cage. Always, before starting an impalement with a new microelectrode, its resistance was measured in the external solution. After that, the surface of the skin was lightly touched by the microelectrode and its resistance again evaluated in the external solution and the site of puncture changed. The initial procedure of touching the surface of the skin was always performed in order to discard mechanically inadequate microelectrodes which would present wide changes in resistance. Frequently, the microelectrodes had a small reduction in resistance after the initial procedure, and only those with resistance in the range of 2 to 10 MΩ were used. The problem of mechanically suitable microelectrodes has been previously considered by Cereijido and Curran [7]. These authors considered adequate electrodes in the range of 1 to 5 MΩ. Microelectrodes in the range of 1 to 30 MΩ have been used by others [7, 21, 24, 28, 29]. Measurements were considered successful when the tip potential varied less than ±2 mV and the resistance varied less than 1 MΩ after an impalement. Standard Ringer's solution used to bathe the internal surface of the skin consisted of: (a) Na_2SO_4-Ringer's solution: Na_2SO_4 57.5 mM, $KHCO_3$ 2.5 mM, and $CaSO_4$ 1.0 mM, with a pH of 8.2. (b) NaCl-Ringer's solution: NaCl 115 mM, $KHCO_3$ 2.5 mM and $CaCl_2$ 1.0 mM, with a pH of 8.2. The composition of the external solutions used in each experiment will be mentioned in the Results section. Toads were captured in the vicinity of São Paulo (Brazil) and kept with free access to running tap water, without food, for a period no longer than a month. Results are presented as mean ± standard error. Straight lines were fitted by the least-squares method.

Results

1. Voltage and Resistance Profiles in the Superficial Layer of the Epithelium

These experiments were performed as a survey in order to obtain information about the dependence of *PDi, Ri* and *Ri** (*see* Materials and

Methods for abbreviations) on depth of impalement. Skins were kept in the open-circuited state and bathed on both surfaces by NaCl-Ringer's solution. Electrical control was used to indicate the moment when the microelectrode touched the outer surface of the skin. At that moment, the micrometer reading was taken as the reference position for deeper impalements. This procedure was chosen since optical monitoring of the "touch" proved to be inadequate because of irregularities on the skin surface. We have assumed that the contact of the microelectrode with the skin surface was indicated by the first deflection observed in *PDi*. This assumption is supported by the simultaneous deflections observed in both *PDi* and *Ri* with double-barreled microelectrodes suggesting that the electrical "touch" is simultaneously seen by the voltage and resistance measurements. Fig. 1 describes a representative experiment with double-barreled microelectrode, showing *PDi* and *Ri* as a function of depth of impalement. No apparent indications of localized voltage or resistance barriers were visualized in these records. *PDi* recorded with single-barreled microelectrodes is a continuous function of the depth of impalement, showing a negative value at the touch, with a minimum of -26.7 ± 3.6 mV at 6 ± 2 μm ($n=25$), a null value at 19 ± 3 μm ($n=25$), and positive polarity for deeper impalements. With double-barreled microelectrodes a similar pattern was observed with a minimum of -16.3 ± 3.6 mV at 5 ± 1 μm

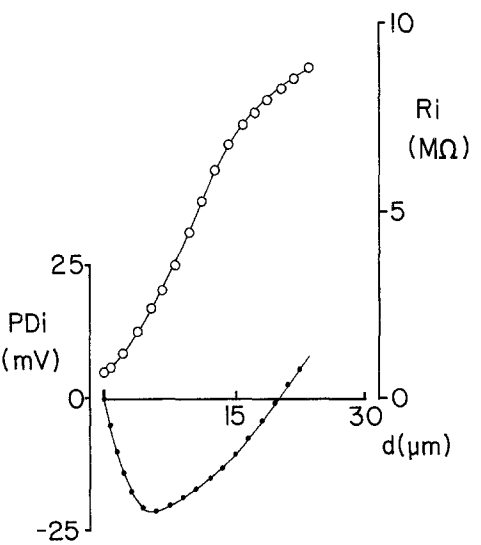

Fig. 1. Typical voltage and resistance profiles simultaneously recorded by double-barreled microelectrodes. Skin bathed on both sides by NaCl-Ringer's solution. *d* is the advance of the microelectrode

($n=21$). The lower absolute *PDi* mean value observed with double-barreled microelectrodes could be a consequence of thicker tips of these electrodes which could induce a higher degree of skin indentation. Contrary to the observations of Engbaek and Hoshiko [11] and Whittembury [29] this negative potential showed great stability for several minutes, even during renewal of the external solution.

It should be mentioned at this point that during impalements deeper than 25 μm, indications of cell impalements or crossing of barriers were frequently detected, being characterized by abrupt voltage and resistance jumps occurring at discrete positions in the course of microelectrode advancement. The pattern of these responses showed great variability, and their analysis is out of the scope of this paper. Variability in the shape and number of voltage jumps was also noticed in different degrees by Rawlins *et al.* [24], Whittembury [29], Cereijido and Curran [7], Ussing and Windhager [28] and Engbaek and Hoshiko [11].

It is important to state that although advances of the microelectrode could be measured with the precision of 0.5 μm, it is impossible to place full confidence in these values as a measurement of depth of impalement, due to uncontrolled degree of skin deformation that certainly occurs mostly as a consequence of the rigidity of the *stratum corneum.*

The physical-chemical nature of *PDi* and its dependence on depth of impalement remain to be explained. Obviously, adequate understanding must depend on knowledge of the ionic nature of *PDi,* and is enormously impaired by our present ignorance of the degree of skin deformation in the course of microelectrode penetration.

2. Microelectrode Tip Potential as a Function of NaCl Concentration

On the assumption that the *stratum corneum* and the subjacent layers may function as a diffusion barrier for Na and its accompanying anion, it is conceivable that a concentration field could build up in these regions. This could be responsible for an apparent electrical profile observed during impalement by microelectrodes bearing salt-sensitive tip potentials. In literature there are several indications that glass microelectrodes filled with 3 M KCl may display such salt sensitivity [1, 5]. Table 1 presents mean values for tip potentials of single-barreled microelectrodes filled with 3 M KCl as a function of NaCl concentration in the solution bathing their tips. It shows two groups of microelectrodes selected according to

Table 1. Tip potential of glass microelectrodes filled with 3 M KCl as a function of NaCl concentration. Two groups of microelectrodes of different resistances

NaCl (mM)	Tip potential (mV)	
	R: 15 to 30 MΩ (n=15)	*R:* 2 to 10 MΩ (n=22)
1	−42.5±5.1	−1.2±1.0
5	−41.3±5.0	−0.9±0.7
10	−29.3±3.1	−0.8±0.5
100	− 8.6±2.2	−0.2±0.3

Mean±S.E.; n=number of observations.
Tip potentials were evaluated as the emf of the cell:

Sat. KCl calomel elect.	Micro-electrode	Test solution	Sat. KCl agar bridge	Sat. KCl calomel elect.

Electrical potential difference in the circuit was initially balanced to zero (by means of an external voltage source) with 3 M KCl as test solution. Tip potentials were then evaluated in the different NaCl solutions. Microelectrode resistance was measured in 3 M KCl solution.

their resistances evaluated in 100 mM NaCl. As seen, electrodes in the range of 2 to 10 MΩ (which is the range used througout this paper) present no salt sensitivity in contrast to microelectrodes with higher resistances. These results eliminate the possibility that the observed electrical profile (Results, Section 1) was a consequence of a concentration field within the superficial layers of the epithelium.

3. 5 μm *Impalements and Ionic Substitutions in the External Solution, with Na_2SO_4-Ringer's Solution Bathing the Internal Surface of the Skin*

In order to get insight into the ionic nature of *PDi,* it was imperative to reduce the number of variables, one of which is the depth of impalement. Consequently, all the following experiments were performed at a fixed 5 μm depth, where *PDi* is less sensitive to microelectrode position and presents its highest absolute value, as indicated in Fig. 1. In these experiments, following impalement, *PDi* was recorded until stable values were obtained, normally within a few seconds. A given impalement was considered successful when, after substitutions in the external solution, *PDi* returned to within ±2 mV of the initial value with restoration of the initial solution.

3.1. Substitution of Chloride by Sulfate in the External Solution

Literature shows that substitution of chloride by sulfate in the external solution induces an increase in the electrical potential difference across the skin which is interpreted as being due to lower sulfate permeability [19]. Therefore, this substitution was used as a tool to compare and correlate changes in *PDi* and *PDt*.

The skin was initially bathed on its external surface by 115 mM NaCl (pH 8.2 – Trizma base 2 mM and HCl). After impalement and stabilization of *PDt* and *PDi*, the external solution was changed to Na_2SO_4 57.5 mM (pH 8.2 – Trizma base 2 mM and H_2SO_4). After new equilibration values were reached for *PDt* and *PDi*, the external medium was changed back to the initial solution. Following each substitution the time for 100% voltage equilibration (within ±2 mV) was measured. After each study, microelectrode tip potentials were checked in the bathing solutions. Table 2 presents the results. It shows that the substitution of chloride by sulfate induces a significant increase in *PDt* and in the absolute value of *PDi*. Comparing the changes in *PDi* with those in tip potentials, and their equilibration times, we may infer that changes in *PDi* could possibly be a reflection of changes in tip potential. This inference is based on the assumption that changes in the external solution could induce similar

Table 2. Effect on transepithelial potential difference (*PDt*), intraepithelial potential difference (*PDi*) and tip potential (*TP*) of the substitution of chloride by sulfate in the external solution

		NaCl (115 mM)	Na_2SO_4 (57.5 mM)	NaCl (115 mM)
PDt	(mV)	28.1 ± 3.4	63,6 ± 1.5	29.2 ± 3.4
PDi	(mV)	−29.8 ± 1.6	−38.2 ± 1.9	−29.8 ± 1.6
TP	(mV)		−7.1 ± 0.2	0.5 ± 0.4
t_{PDt}	(sec)		131.0 ± 8.0	73.0 ± 6.5
t_{PDi}	(sec)		8.2 ± 0.5	8.2 ± 0.5
t_{TP}	(sec)		8.7 ± 0.5	8.6 ± 0.6

Voltages referred to the external solution.
PDt = transepithelial potential difference.
PDi = intraepithelial potential difference.
TP = variation in microelectrode tip potential from the value initially observed in NaCl solution.
t = 100% equilibration time (within ±2 mV).
Both external solutions had a pH of 8.2. Internal solution: Na_2SO_4-Ringer's solution.
n = 29 (*n* = number of observations).

alterations in the milieu around the microelectrode tip. Under the conditions of these experiments, a dissociation can be noticed between variations in *PDt* and *PDi*.

3.2. Changes in Na_2SO_4 Concentration in the External Solution

These experiments were performed to study the simultaneous effects on *PDt* and *PDi* when reducing ionic strength in the external solution (in the presence of Na, an actively transported cation). The skin was initially bathed on the external surface by a solution of 57.5 mM Na_2SO_4. After each impalement and stabilization of *PDt* and *PDi* values, the concentration of Na_2SO_4 in the external solution was changed in the following sequence: 5, 7.5, 12.5, 20, 35, and 57.5 mM. All the external solutions had a pH of 8.2 (Trizma base 2 mM and H_2SO_4), and were not corrected for osmolarity. Tip potentials were measured in all solutions following each impalement. In the range of 5 to 57.5 mM, *PDt* and *PDi* showed a logarithmic dependence on Na_2SO_4 concentration, as is seen in Fig. 2. Therefore, *PDt* and *PDi* can be expressed by: $PDt = a_t + b_t \log(Na_2SO_4)$, where $a_t = 65.6 \pm 1.2$ mV; $b_t = 19.1 \pm 1.3$ mV; $n = 18$ (of the 18 correlation

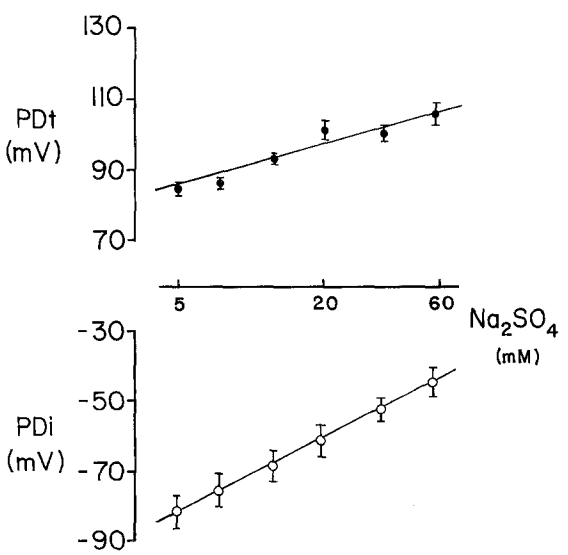

Fig. 2. Transepithelial potential difference (*PDt*) and intraepithelial potential difference (*PDi*) (5 μm impalements) as a function of external Na_2SO_4 concentration (pH 8.2 with Trizma base 2 mM and H_2SO_4). Internal solution was Na_2SO_4-Ringer's solution ($n = 10$)

coefficients, 16 had $p<0.01$ and 2 had $p<0.05$), and $PDi=a_i+b_i\log(Na_2SO_4)$, where $a_i=-117.2\pm7.3$ mV; $b_i=35.3\pm2.8$ mV; $n=18$ (of the 18 correlation coefficients, 17 had $p<0.01$ and 1 had $p<0.05$). Mean values for tip potential in the different solutions were: 5 mM (0.8 ± 2.4 mV, $n=18$); 7.5 mM (3.2 ± 1.9 mV, $n=14$); 12.5 mM (2.5 ± 0.7 mV, $n=14$); 20 mM (0.8 ± 1.0 mV, $n=17$); 35 mM (0.8 ± 0.6 mV, $n=14$); 57.5 mM (-0.6 ± 0.6 mV, $n=17$).

These results show that variations in tip potentials cannot account for the observed data. The slope of *PDt* as a function of Na_2SO_4 in the external solution ($b_t=19.1$ mV) is lower than the theoretical value expected considering the outer barrier as permeable only to Na [15]. On the other hand, there are in the literature discrepancies regarding the magnitude of the slope, which is significantly lower [3, 7] than 58 mV per 10-fold change in Na concentration [15]. Furthermore, substantial deviations from the behavior predicted by the model of Koefoed-Johnsen and Ussing [15] were observed under certain conditions [17].

The negative sign of *PDi* observed in all the results presented so far, as well as its behavior in the experiments of this section, argues against any possibility of *PDi* being measured across the postulated sodium-sensitive outermost barrier, which is considered to be a Na-selective barrier, through which Na would move by diffusion. If it is assumed that *PDi* recording sites are located more externally than the Na-sensitive barrier and that this potential difference is in series with the potential difference between the microelectrode tip and the internal solution (*PDr*), so that $PDt=PDi+PDr$, then *PDr* should increase with a reduction in the external sodium concentration. This behavior would then also be at variance with the expected electrical potential difference existing across the sodium-sensitive barrier, with the internal side being positive as referred to the external solution.

3.3. Changes in K_2SO_4 Concentration in the External Solution

In order to search further into the ionic nature of *PDi* and to evaluate the role of the active Na transport in the genesis of *PDi*, experiments were performed under conditions similar to those of Section 3.2, except for the substitution of Na by K. This eliminates the contribution of Na, the most important actively transported ion in this skin to the genesis of *PDi*. In the range of 5 to 57.5 mM K_2SO_4, *PDt* and *PDi* showed

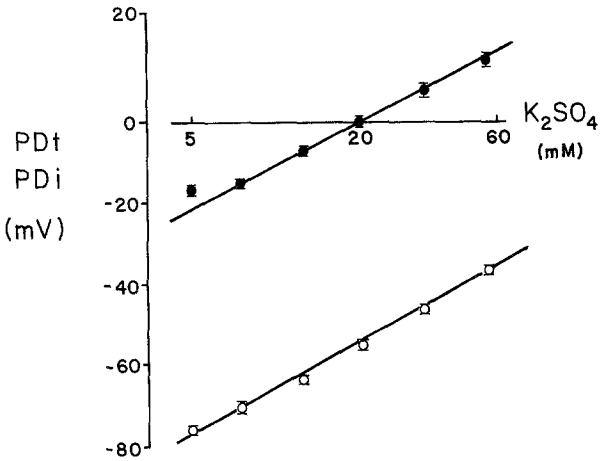

Fig. 3. Transepithelial potential difference (*PDt*) (closed circles) and intraepithelial potential difference (*PDi*) (5 μm impalements) as a function of external K_2SO_4 concentration (pH 8.2 with Trizma base 2 mM and H_2SO_4). Internal solution was Na_2SO_4-Ringer's solution ($n=24$)

a logarithmic dependence on the K_2SO_4 concentration, as is seen in Fig. 3. Therefore, *PDt* and *PDi* can here again be expressed as: $PDt=a_t+b_t\log(K_2SO_4)$, where $a_t=-52.4\pm1.2$ mV; $b_t=32.6\pm1.3$ mV; $n=24$ (for all correlation coefficients $p<0.01$), and $PDi=a_i+b_i\log(K_2SO_4)$, where $a_i=-114.4\pm2.9$ mV; $b_i=37.3\pm1.2$ mV; $n=24$ (for all correlation coefficients, $p<0.01$). Tip potential mean values in the different solutions were: 5 mM (-3.7 ± 1.9 mV); 7.5 mM (-4.8 ± 1.7 mV); 12.5 mM (-4.5 ± 1.4 mV); 20 mM (-3.7 ± 1.0 mV); 35 mM (-2.7 ± 0.5 mV); 57.5 mM (-1.5 ± 0.3 mV); ($n=24$).

These results show that reduction in K_2SO_4 concentration is followed by a decrease in *PDt* with reversal of its polarity in the vicinity of 20 mM, and by increasing negativity of *PDi*. The negative *PDt* value observed with low K_2SO_4 concentration in the external solution is in agreement with previous results in literature [21]. The dependence of *PDt* on K_2SO_4 concentration in the external solution could be interpreted as a diffusion potential mostly due to movement of Na and K ions in the shunt pathway. The positive *PDt* value observed above 20 mM and, above all, the positive value observed when Na and K concentrations are identical in the internal and external solutions, respectively, suggest a higher K selectivity in the shunt pathway as compared to that of Na. These results are in agreement with the observation of Mandel and Curran [20] of a higher K selectivity in the shunt pathway for *Rana pipiens*. Here also, as in the Na_2SO_4

study, variations in tip potential cannot account for the observed variations in *PDi*.

At this point it is worth comparing the slopes of *PDi* when varying Na_2SO_4 concentration ($b_i = 35.3 \pm 2.8$ mV, Section 3.2) or K_2SO_4 concentration ($b_i = 37.3 \pm 1.2$ mV). These slopes are not statistically different ($0.40 < p < 0.50$). Comparison of the intercepts with Na_2SO_4 ($a_i = -117.2 \pm 7.3$ mV) and with K_2SO_4 ($a_i = -114.4 \pm 2.9$ mV) shows that these values also are not statistically different ($0.60 < p < 0.70$). These observations indicate identical behavior of *PDi* as a function of external Na_2SO_4 or K_2SO_4 and, therefore, rule out any possibility of a direct contribution from active sodium transport. On the other hand, a complete dissociation of behaviors between *PDt* and *PDi* in the course of the experiments already shown, strongly supports the idea that *PDi* should not be considered as contributing to *PDt*, according to the relation: $PDt = PDi + PDr$, already presented.

3.4. Substitution of Na by K in the External Solution

The results of Section 3.2 and 3.3 indicate that the behavior of *PDi* is very similar, independent of whether the cation in the external solution is Na or K. In order to test this possibility directly, experiments were performed with skins alternately bathed by Na_2SO_4 and K_2SO_4 on the outer surface. After each impalement and stabilization of *PDi* and *PDt*, the external solution of 57.5 mM Na_2SO_4 was replaced by 57.5 mM K_2SO_4. (Both solutions had a pH of 8.2 adjusted with Trizma base 2 mM and H_2SO_4.) Subsequently, K_2SO_4 was replaced again by Na_2SO_4. Steady-state values of *PDt* and *PDi* were as follows:

Na 57.5 mM: $PDt = 128.0 \pm 0.8$ mV; $PDi = -26.6 \pm 3.0$* mV;

K 57.5 mM: $PDt = 40.1 \pm 3.4$ mV; $PDi = -24.4 \pm 3.0$* mV;

Na 57.5 mM: $PDt = 130.2 \pm 0.8$ mV; $PDi = -25.9 \pm 3.0$ mV; ($n = 14$).

The higher *PDt* values observed in these experiments with Na_2SO_4 and with K_2SO_4 in the external solution as compared to those in the same concentrations presented in Fig. 2 and Fig. 3, respectively, could possibly be a consequence of seasonal variations, suggested by the small standard errors within each group.

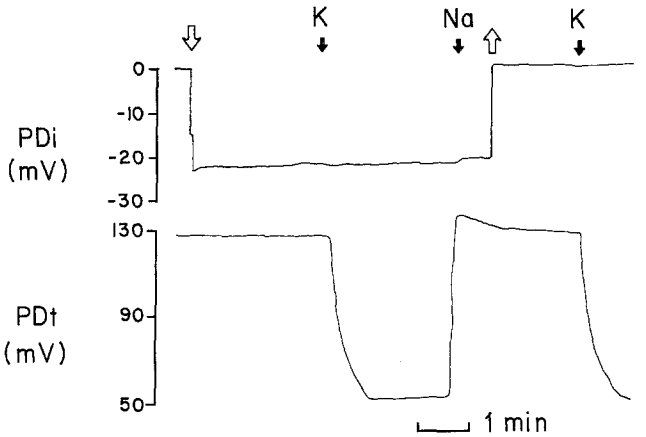

Fig. 4. Typical time course of intraepithelial potential difference (*PDi*) (5 μm impalement), transmembrane potential difference (*PDt*) and tip potential during alternation of external Na_2SO_4 and K_2SO_4 solutions (both 57.5 mM, pH 8.2 with Trizma base 2 mM and H_2SO_4). Internal solution was Na_2SO_4-Ringer's solution. Open arrows indicate impalement and return of the microelectrode to the external solution

These results show that the substitution of Na by K and vice-versa, induces large variations in *PDt*, with minor effects on *PDi*. The variations observed in *PDi*(*) were significant ($p < 0.01$, paired *t*-test). The effects of these substitutions on tip potential where negligible, as shown in Fig. 4.

The experiments presented so far indicate that *PDi* is very insensitive to the substitution of Cl by SO_4 (section 3.1) and Na by K in the external solution, despite the fact that these substitutions drastically alter *PDt*. This behavior suggests that *PDi* could be related to the electrical conductivity of the external solution. This idea is reinforced by the observation that leakage of KCl from broken microelectrodes into the external solution induces a rapid disappearance of *PDi*, which is restored by repeated changes of the external solution. Therefore, based on the previous results, a working hypothesis, analogous to that formulated by Davis *et al.* [10] to explain a potential level in the stroma of frog cornea, was put forward to account for the genesis of *PDi*. It was assumed that *PDi* is a KCl diffusion potential generated by diffusion of this salt from the tip of the microelectrode into the matrix of the *stratum corneum*, where K ions would have a higher mobility than Cl ions (at the high pH used in the experiments), probably as a consequence of negative fixed charges in the matrix of the *stratum corneum*. According to Ives and Janz [13] a microelectrode filled with 3 M KCl when immersed in Ringer's solution establishes a steady diffusion from the tip of 6×10^{-14} mole sec^{-1}. This

is not sufficient to poison the cell interior but could be the origin of a diffusion potential in certain circumstances, as is assumed in the present case. The increase in ionic concentration of the external solution could induce its effects by increasing the ionic concentration in the matrix and, as a consequence, reducing the magnitude of *PDi* by short-circuiting the KCl diffusion potential. This effect would be analogous to the reduction in membrane potential observed in charged membranes as a consequence of an increase in salt concentration of the solutions, under the condition of a constant activity ratio across the membrane [26].

3.5. Changes in Hydrogen Ion Concentration in the External Solution

3.5.1. *Effects on PDt and PDi.* These experiments have been performed to test the hypothesis of *PDi* being a consequence of a diffusion potential at the level of the microelectrode tip, due to a diffusion of KCl from the electrode into the matrix of the *stratum corneum* bearing negative fixed charges at high pH in the external solution.

Skins were initially bathed by 57.5 mM Na_2SO_4 with pH 9.0 (Trizma base 2 mM and H_2SO_4) on the external surface. After each impalement, and stabilization of *PDt* and *PDi,* the pH of the external solution was changed in steps of 1 pH unit from 9 to 5, and back again to 9. Fig. 5 presents the behavior of *PDt* and *PDi,* showing that acidification of the external solution causes an increase in *PDt* (probably as a consequence of a diffusion of H ions from the outer to the inner solution) with a slope of 5.2 ± 0.3 mV, ($n=20$) per pH unit, and a reduction in the absolute value of *PDi,* in a nonlinear fashion. Microelectrode tip potentials were checked after each impalement and their pH dependence ruled out as a possible explanation for the observed *PDi* behavior, as indicated by the sequence of tip potentials as a function of pH: pH 5 (0.2 ± 0.3 mV); pH 6 (0.0 ± 0.3 mV); pH 7 (-0.1 ± 0.3 mV); pH 8 (-0.2 ± 0.3 mV) and pH 9 (-0.2 ± 0.3 mV), ($n=20$).

An independent group of experiments was performed in which the pH of the external solution of 57.5 mM Na_2SO_4 was changed directly from pH 9 to pH 3 and back again to pH 9. Fig. 6 is a representative experiment. Mean values for *PDt* and *PDi* in the above sequence are: for *PDt*: pH 9 (82.2 ± 5.7 mV); pH 3 (47.8 ± 3.5 mV) and pH 9 (82.9 ± 5.2 mV), ($n=12$); for *PDi*: pH 9 (-33.8 ± 1.9 mV); pH 3 ($+15.2 \pm 2.4$ mV) and pH 9 (-31.6 ± 2.8 mV), ($n=12$). The observed shift in tip potential from pH 9 to pH 3 was 0.2 ± 0.2 mV ($n=12$).

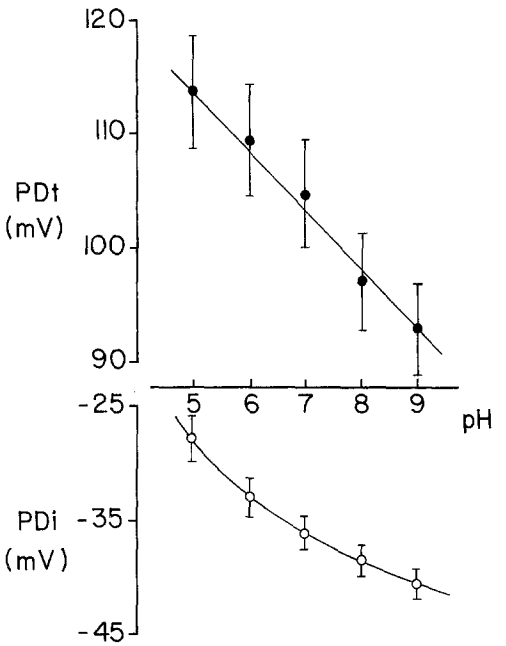

Fig. 5. Transepithelial potential difference (*PDt*) and intraepithelial potential difference (*PDi*) (5 μm impalements) as a function of the pH of the external solution (57.5 mM Na_2SO_4 and Trizma base 2 mM, pH adjusted with H_2SO_4). Internal solution was Na_2SO_4-Ringer's solution ($n=20$)

The conclusions of these results are that acidification of the external solution to a very low pH (pH 3) induces a shift in *PDi* polarity to positive values, and that this alteration is reversible when the external solution is returned to pH 9. This behavior could be predicted approximately by extrapolation of *PDi* values in Fig. 5 to lower pH values. On the other hand, the behavior of *PDt* could not be predicted in a similar fashion, since reduction of *PDt* was observed with acidification of the external solution to pH 3.

The dependence of *PDi* on the hydrogen ion concentration of the external solution strongly supports the hypothesis of the existence of fixed charges in the matrix of the *stratum corneum* with net charge density being controlled by the degree of protonation of ionizable sites. The behavior of *PDt* with acidification of the external solution to pH 3 sharply contrasts with its behavior in the pH range of 9 to 5, where acidification produced only a continuous increase in *PDt*. The biphasic behavior of *PDt* that follows the acidification of the external solution to pH 3, displaying an initial increase followed by a subsequent decrease to a new stable

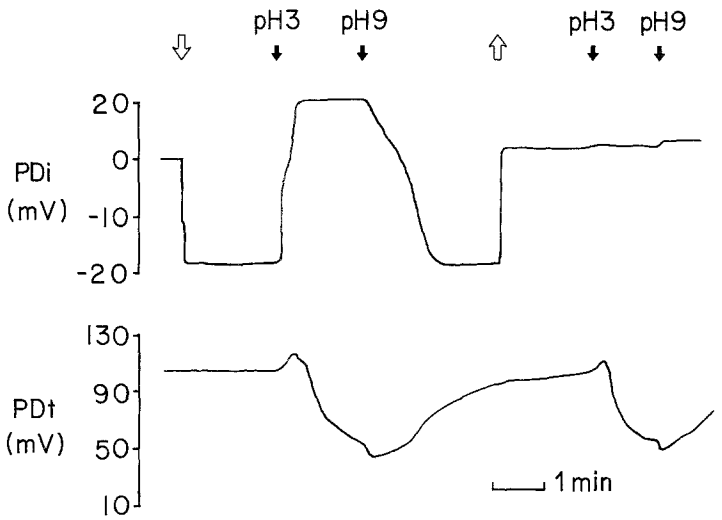

Fig. 6. Typical time course of intraepithelial potential difference (*PDi*) (5 μm impalement), transmembrane potential difference (*PDt*) and tip potential during pH changes in the external solution (57.5 mM Na_2SO_4, Trizma base 2 mM, pH adjusted with H_2SO_4). Internal solution was Na_2SO_4-Ringer's solution

value, which is lower than that observed at pH 9, is compatible with a double effect of this intense acidification. This was interpreted as due initially to a hydrogen diffusion potential, followed by a *PDt* fall, probably by a late inhibitory effect of low pH on the active sodium transport mechanism, more deeply situated in the epithelium.

3.5.2. *Effect on Short-Circuit Current.* These experiments were performed to adduce more information concerning the behavior of *PDt* during a decrease in the external pH. The experiments were performed in the short-circuited state with 57.5 mM Na_2SO_4 (pH 9 to 3 adjusted with Trizma base 2 mM and H_2SO_4) bathing the outer surface of the skin. Results are shown in Table 3. As seen, acidification in the pH range of 9 to 5 induces an increase in short-circuit current and in *PDt*, to new stable values, with minor effects on the electrical resistance across the skin. The observed increase in short-circuit current with acidification of the external solution in the pH range of 9 to 5 might be a consequence of a hydrogen ion diffusion current from the outer to the inner chamber, due to hydrogen ion migration along with its chemical potential difference, although a stimulatory effect on the active sodium transport mechanism cannot be ruled out. Acidification from pH 5 to pH 3 drastically reduces

Table 3. Effect of acidification of the external solution on short-circuit current (I), transepithelial potential difference (PDt) and transepithelial resistance (Rt)

pH	I ($\mu A\ cm^{-2}$)	R_t ($k\Omega\ cm^2$)	PDt (mV)
3	25.9 ± 1.0	2.7 ± 0.1	69.3
5	86.7 ± 4.0	1.1 ± 0.0	93.6
6	82.4 ± 4.6	1.1 ± 0.0	89.8
7	78.8 ± 4.9	1.1 ± 0.1	87.5
8	69.1 ± 5.1	1.2 ± 0.1	79.4
9	58.6 ± 3.7	1.1 ± 0.1	66.8

I=Short-circuit current.
R_t=Transmembrane resistance calculated by voltage pulses of ± 20 mV.
PDt=Transmembrane potential calculated by: $I \times R_t$.
$n=18$ (n=number of observations).
External solution: Na_2SO_4 57.5 mM (Trizma base 2 mM, pH adjusted with H_2SO_4).
Internal solution: Na_2SO_4-Ringer's solution.

short-circuit current and PDt, and significantly increases the electrical resistance. These results are in agreement with previous results of Ussing [27] showing that acidification of the external solution from pH 6.6 to 5.25 had little influence on Na-influx; when, however, the outside pH was lowered to 3.3, the Na-influx nearly stopped.

The temporal evolution of short-circuit current and PDt being very similar and biphasic following acidification of the external solution to pH 3, is consistent with a sequential double effect of the strong acidification of the outer solution.

4. *Electrical Resistance of the 5* μm *Superficial Epithelial Layer*

These experiments were performed in order to evaluate the electrical resistance of the outermost 5 μm layer of the epithelium using single-barreled microelectrodes and to compare these results with those obtained with double-barreled microelectrodes (Results, Section 1) as well as with those of literature [29]. The internal surface of the skin was bathed with Na_2SO_4-Ringer's solution and the external surface by 57.5 mM Na_2SO_4 with a pH of 8.2 (Trizma base 2 mM and H_2SO_4). By means of voltage clamping PDt was set at different levels (0, ± 20, ± 40, ± 60 and ± 80 mV) with the microelectrode in place in the epithelium (at a depth of 5 μm), and PDi was simultaneously monitored. The current-voltage relationship

displayed ohmic behavior for both *PDt* and *PDi* in the above range. *PDi* can be expressed as: $PDi = a_i + b_i I$, where I is the clamping current. After returning the microelectrode to the external solution at the end of each impalement, with its tip 2 μm off the external interface (position obtained by touching the skin and withdrawing the microelectrode 2 μm) an identical series of clamping voltages were again applied. The relationship between the current and the voltage recorded by the microelectrode again showed ohmic behavior, which can be expressed by: $PDs = a_s + b_s I$. The resistance Ri^* was estimated as $(b_i - b_s)$. It should be pointed out that the sensitivity of the measurements enables detection of variations in *PDs* due to 10 μm advances of the microelectrode in the external solution. Mean values for $b_i = 0.190 \pm 0.002$ kΩ cm^2 and for $b_s = 0.190 \pm 0.002$ kΩ cm^2 ($n = 12$) indicate that the electrical resistance of the 5 μm outermost layer is very small and within the error of measurements. These observations are compatible with the results of Whittembury [29] that the observed outer side resistance is practically equal to zero, and of Bracho *et al.* [4] showing that lanthanum penetrates readily through cells of the cornified layer.

The discrepancy between these results (showing that the outer side resistance is practically equal to zero) and those obtained with double-barreled microelectrodes (Results, Section 1) showing a significant increase in the resistance between the electrode and the external solution, already noticed at the moment at which the electrode touched the surface of the skin, and increasing with depth of impalement, is worth consideration. This difference of resistance strongly suggests a lack of homogeneity in the resistivity of the *stratum corneum*. It is consistent with the existence of low resistance pathways of relatively very small dimensions (probably the intercellular space) separating higher resistance regions (probably the cornified cells themselves). The tip of the microelectrode would be located with greater probability within the higher resistance regions, accounting for the relatively high resistances measured by the double-barreled microelectrodes. In the case of single-barreled microelectrodes, also located in these high resistance regions, major potential shifts would not be detected, since most of the applied current would flow along the lower resistance interspace.

Discussion

The results of the present experiments show that the electrical potential difference (*PDi*) measured with reference to the external solution with

glass microelectrodes filled with 3 M KCl can be considered as a diffusion potential at the tip of the microelectrode due to KCl diffusion from the microelectrode into the matrix of the *stratum corneum*. Differences in K and Cl mobilities in this matrix, as compared to those in free solution [22] may be attributed to fixed charges with density and polarity determined by their degree of protonation, controlled by the hydrogen ion concentration in the external solution. In the pH range of 5 to 9, the matrix behaves as though negatively charged, and at pH 3 as positively charged. The effect of increasing external ionic concentration on *PDi* is consistent with the hypothesis of *PDi* being progressively short-circuited by increasing ionic concentration in the matrix. This effect would be analogous to the reduction of membrane diffusion potential, observed in charged membranes, as a consequence of an increase in salt concentration of the solutions bathing the membrane, under the condition of a constant activity ratio across the membrane, as discussed by Teorell [26]. In order to check the hypothesis formulated in this paper, we [16][1] have developed a method for isolating the *stratum corneum* as a flat sheet of membrane suitable for mounting between hemichambers and have shown that this membrane displays very high electrical and hydraulic conductivities and, when bathed by KCl solutions of different concentrations on both sides, gives rise to a diffusion potential with a polarity that is pH dependent. Above pH 4.6 (the isoelectric pH for this membrane), K ions present higher mobility than Cl ions; below the isoelectric pH, the reverse situation occurs. Increasing KCl concentration on both sides of the membrane, with a constant activity ratio (above as well as below the isoelectric pH), is followed by a reduction in the magnitude of membrane potential, similar to the behavior of charged membranes [26].

These results, besides adducing information to the electrophysiological aspects of the *stratum corneum*, raise important and more general points regarding the interpretation of electrical potentials obtained with glass microelectrodes filled with 3 M KCl. Thus, the negative potential level observed in the *stratum corneum*, which is an artifact due to a unique combination of electrode and puncture site characteristics, was interpreted by Engbaek and Hoshiko [11] as a membrane potential of epithelial cells. This possibility was challenged by the experiments of Whittembury [29] because of his observation of a very low outer side resistance at the site of puncture. Martin and Curran [21] impaling skins of *Rana pipiens*

1 *Also* F. Lacaz Vieira and M.A. Nunes. Electrophysiological parameters of the isolated *stratum corneum* (*In preparation*).

and *Rana esculenta* (bathed by diluted solutions of KCl on the outer surface) with glass microelectrodes filled with 3 M KCl, have shown that when the microelectrode was advanced through skins under conditions producing a reversed overall potential difference (inside solution negative) several steps in potential were usually observed. Their Fig. 1 shows a typical profile of four distinct steps with the electrode becoming progressively more negative relative to the outside reference solution. This figure shows clearly that each negative step is a consequence of a single advance of the electrode. This profile resembles very closely the one presented in our Fig. 1, which does not differ very much from others we have obtained with external solutions of Na_2SO_4 and KCl of different concentrations. Martin and Curran [21] have not given any explanation for the nature of the negative steps, nor precise information on their localization within the epithelium. However, their Fig. 1 suggests that these negative steps were located superficially in the epithelium, since the first deflection observed with the microelectrode is already a negative one. It is conceivable that the negative steps observed by Martin and Curran [21] could have an origin similar to that of our *PDi*. Under that assumption, these negative steps could not account for the reversed overall potential difference observed, and more deeply situated structures could certainly be responsible for the alterations of *PDt*. On the other hand, the positivation shown by Martin and Curran [21] of the negative level, when Na-Ringer's solution bathes the external surface of the skin, goes against our interpretation of their results.

Studies with glass microelectrodes filled with 3 M KCl have raised controversial points in the interpretation of data on electrical potentials in frog and rabbit corneas. Candia *et al.* [6] attemped to localize the site of the Cl-transport mechanism by studying the potential profile across the cornea with microelectrodes. They reported that the stroma was negative to the solution bathing both surfaces and suggested that the Cl-transport mechanism was located in the endothelium. Controversial opinions regarding the stromal negative level are mentioned in literature [2, 10, 12, 14]. Davis *et al.* [10] using glass microelectrodes filled with 3 M KCl or Ringer's solution suggested that the negative stromal potential could be a KCl junction potential, being the consequence of the presence of negative fixed charges in the stroma, possibly due to existence of acidic polysaccharides in that region.

Several aspects regarding the behavior of *PDi* and *PDt* as well as their mutual relationship in the course of alterations in the ionic composi-

tion of the external solution have been dealt with throughout the Results section.

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