J. Membrane Biol. 39, 313–331 (1978)

Electron Microprobe Analysis of Frog Skin Epithelium: Evidence for a Syncytial Sodium Transport Compartment

Roger Rick, Adolf Dörge, Elborg von Arnim and Klaus Thurau

Department of Physiology, University of Munich, Pettenkoferstr. 12, D-8000 Munich, W. Germany

Received 22 August 1977

Summary. For elucidation of the functional organization of frog skin epithelium with regard to transepithelial Na transport, electrolyte concentrations in individual epithelial cells were determined by electron microprobe analysis. The measurements were performed on 1-μm thick freeze-dried cryosections by an energy-dispersive X-ray detecting system. Quantification of the electrolyte concentrations was achieved by comparing the X-ray intensities obtained in the cells with those of an internal albumin standard.

The granular, spiny, and germinal cells, which constitute the various layers of the epithelium, showed an identical behavior of their Na and K concentrations under all experimental conditions. In the control, both sides of the skin bathed in frog Ringer's solution, the mean cellular concentrations (in mmole/kg wet wt) were 9 for Na and 118 for K. Almost no change in the cellular Na occurred when the inside bathing solution was replaced by a Na-free isotonic Ringer's solution, whereas replacing the outside solution by distilled water resulted in a decrease of Na to almost zero in all layers. Inhibition of the transepithelial Na transport by ouabain (10^{-4} M) produced an increase in Na to 109 and a decrease in K to 16. The effect of ouabain on the cellular Na and K concentrations was completely cancelled when the Na influx from the outside was prevented, either by removing Na or adding amiloride (10^{-4} M). When, after the action of ouabain, Na was removed from the outside bathing solution, the Na and K concentration in all layers returned to control values. The latter effect could be abolished by amiloride.

The other cell types of the epithelium showed under some experimental conditions a different behavior. In the cornified cells and the light cells, which occurred occasionally in the stratum granulosum, the electrolyte concentrations approximated those of the outer bathing medium under all experimental conditions. In the mitochondria-rich cells, the Na influx after ouabain could not be prevented by adding amiloride. In the gland cells, only a small change in the Na and K concentrations could be detected after ouabain.

The results of the present study are consistent with a two-barrier concept of transepithelial Na transport. The Na transport compartment comprises all living epithelial layers. Therefore, with the exception of some epithelial cell types, the frog skin epithelium can be regarded as a functional syncytium for Na.

The epithelium of frog skin has been extensively studied to gain some insight into the process of active Na transport across epithelial structures. Investigations on frog skin led to the first model of transepithelial

0022-2631/78/0039-0313 $3.80

Na transport, proposed by Koefoed-Johnsen and Ussing [26], in which there are two barriers to Na movement: Na passively enters the cellular transport compartment across the outer-facing membrane and is actively extruded across the inner-facing membrane. Originally, it was suggested by these authors that only the innermost epithelial layer, the stratum germinativum, constitutes the Na transport compartment. Subsequently, Ussing and Windhager [42] assumed that all epithelial layers, except the outer cornified layer, form a common transport compartment, so that Na enters the outermost living layer, the stratum granulosum, diffuses through intercellular communications into all cells, from where it is actively extruded into the interstitial space. More recently, Voûte and Ussing [45] concluded that only the outermost living layer, the stratum granulosum, is involved in transepithelial Na transport. Thus, the localization of the transport compartment within the multilayered frog skin remains unsettled.

During recent years experimental evidence has been obtained either implicating the outermost living cell layer, the stratum granulosum, as the site of the Na transport compartment or suggesting that all epithelial cells form a functional syncytium for Na. Electron microscopic examination of the distribution of an extracellular marker indicated that the tight junctions between the cells of the stratum granulosum represent a barrier to extracellular Na movement [18], thus the passage of Na, at least through these cells, must be assumed. An increase in cell volume [44, 45] and the formation of scalloped sacs [44] in the stratum granulosum, in proportion to the rate of active Na transport, strongly supports the participation of this layer in transepithelial transport. Furthermore, radiochemical analysis of whole skins [6, 7, 12, 36, 37] and isolated epithelium [1] showed only a small portion of total cellular Na to be exchangeable from the outer bathing medium, favoring the view that only a fraction of the cells constitute the Na transport compartment.

Alternatively, some observations lend support to the view that all epithelial layers share equally in the transportation of Na. Studies on the structural organization of the frog skin epithelium [19, 20] revealed many intercellular junctions between the cells, providing a possible morphological basis for intercellular Na exchange. The fact that small molecules can pass membrane junctions between adjacent epithelial cells has been demonstrated for the salivary gland [30]. Histochemical investigations on the distribution of the ATPase activity [21] and autoradiographic localization of ^{3}H-ouabain binding sites [31] within the frog skin gave evidence that all cell membranes lining the intercellular

spaces are potential sites of transepithelial Na transport. Transient current responses to changes of external Na concentrations supplied evidence for two distinct, communicating cellular compartments, the one rapidly accessible from the outer bathing medium, possibly comprising the stratum granulosum, and the other less rapidly accessible, perhaps representing the deeper layers of the epithelium [28, 32, 33].

The present experiments were undertaken to clarify whether a specific cell layer or all cell layers are involved in the transepithelial transport of Na in the frog skin. Measurements of intracellular electrolyte concentrations were made in individual cells of each of the cell layers and cell types of the frog skin at various functional states of Na transport using the technique of electron microprobe analysis [13, 14].

Materials and Methods

The experiments were performed on frogs of the species *Rana temporaria* and *Rana esculenta*, which were kept in running tap water prior to the experiments. Pieces of the isolated abdominal skin were mounted on lucite rings and inserted into Ussing-type chambers (exposed area, 1.6 cm^2). For incubation of the skin, the half chambers were continuously perfused. Except for short intervals (ca. 10 sec), occurring every 20 min, in which transepithelial potential difference was measured, the skins were kept under short circuited conditions using an automatic clamping device. During a preincubation period the skins were bathed on both sides in Ringer's solution for about 60 min, until the short-circuit current had reached a steady-state value. The incubation was then continued further for 90 min under various experimental conditions, according to the following protocol:

a) Control: both sides Ringer's solution

b) O.s. distilled water: outer side distilled water, inner side Ringer's solution

c) I.s. Na-free: outer side Ringer's solution, inner side Na-free Ringer's solution

d) Ouabain: both sides Ringer's solution, inner solution containing 10^{-4} M ouabain

e) O.s. Na-free, ouabain: outer side Na-free Ringer's solution, inner side Ringer's solution containing 10^{-4} M ouabain

f) Amiloride, ouabain: outer side Ringer's solution containing 10^{-4} M amiloride, inner side Ringer's solution containing 10^{-4} M ouabain.

In two further experimental conditions the skins were first treated identical to *d*), but the incubation was continued afterwards for 60 min as described under g) and *h*).

g) Ouabain, o.s. Na-free: outer side Na-free Ringer's solution, inner side Ringer's solution containing 10^{-4} M ouabain

h) Ouabain, o.s. Na-free + amiloride: outer side Na-free Ringer's solution containing 10^{-4} M amiloride, inner side Ringer's solution containing 10^{-4} M ouabain.

The experiments were performed simultaneously on 3 or 4 pieces of the same skin. The following sets of experimental conditions were applied: $a+b+c$ (2 skins), $a+d+e+f$ (3 skins) and $a+d+g+h$ (2 skins). The Ringer's solution contained (in mM): 110, NaCl; 2.5, $KHCO_3$; and 1, $CaCl_2$. In Na-free Ringer's solution, NaCl was replaced by equimolar quantities of choline chloride. All solutions were bubbled with air and had a pH of about 8.3.

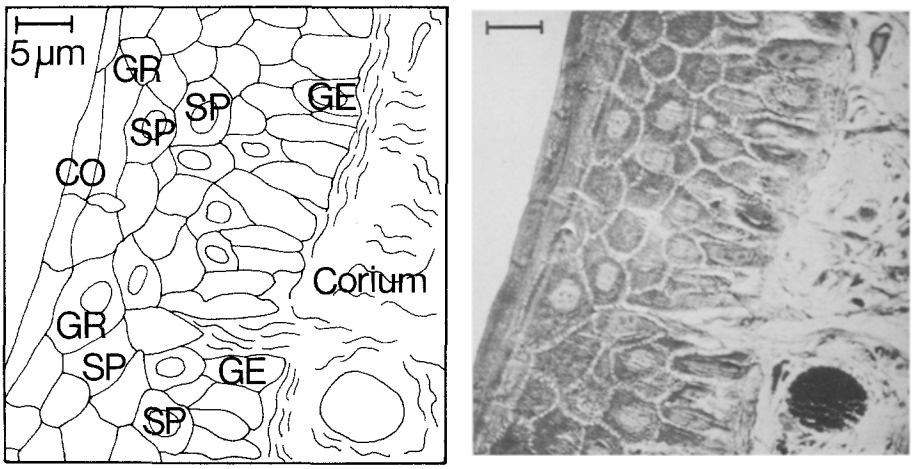

Fig. 1. Scanning transmission electron micrograph of a freeze-dried cryosection of frog skin epithelium (about 1-µm thick), together with a sketch of the various epithelial strata. *CO* = corneum; *GR* = granulosum; *SP* = superficial and deeper spinosum; *GE* = germinativum; *Corium* = corial soft connective tissue

At the end of incubation, the rings were quickly removed from the chambers. The epithelial surface of the skin was gently blotted with filter paper and, except for experiment *b*, covered with a thin layer of an albumin standard solution. The rings were then plunged into propane (−180 °C) to shock-freeze the skin. Between removal of the skins from the chambers and freezing, an interval of approximately 15 sec elapsed.

From the frozen material, 1-µm thick cryosections were cut at −80 °C (Reichert OMU 2), which were subsequently freeze-dried at −80 °C and 10^{-6} torr. Electron microprobe analysis of the sections was performed in a scanning electron microscope (Cambridge S4), to which an energy dispersive X-ray detector had been adapted (EDAX). The acceleration voltage used was 17 kV, and the probe current selected was 0.5 nA. Areas of 1–2 µm^2 were scanned for 200 sec, and the emitted X-rays were analyzed in the energy range between 0.6 and 4 keV, which includes the K-lines of the light elements Na to Ca. The discrimination between characteristic and uncharacteristic radiations (Bremsstrahlung) was performed with a computer program [3].

Quantification of elements was achieved by comparing the characteristic radiations of the cells with those of the adherent albumin standard layer. The standard solution was prepared by dissolving 20 g/100 g bovine albumin in the respective epithelial bathing solution. Since albumin per se contains some Na, the standard prepared with Na-free Ringer's solution (experiments $e+g+h$) had a Na concentration of approximately 30 mM. Although this Na is mainly protein bound, to exclude any Na uptake from the standard, amiloride (10^{-4} M) was added. Quantification of experiment *b* was based on the assumption that the sum of intracellular Na and K concentration was identical to the control. Experimental support for this assumption, as well as a more detailed description of this quantification method, will be given subsequently. The cellular dry weight content was calculated from a comparison of the Bremsstrahlung intensities of cell and standard. Details of the preparation of freeze-dried cryosections for X-ray microanalysis and of the method of quantification have been described earlier [13, 14].

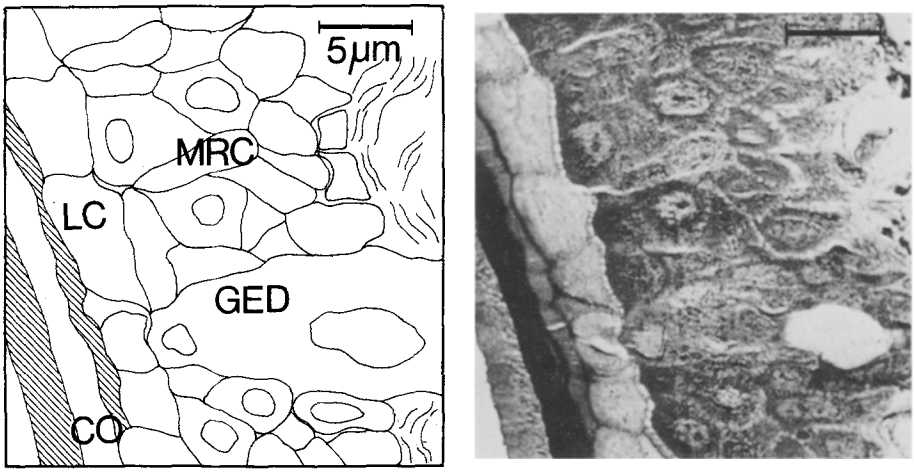

Fig. 2. Scanning transmission electron micrograph of a freeze-dried cryosection of frog skin epithelium (about 1-µm thick), together with a sketch of the epithelium. *MRC* = mitochondria-rich cell; *LC* = light cell; *GED* = gland excretion duct. The hatched areas correspond to the albumin standard layer

In contrast to precise histological morphology of frog skin [4, 19, 20], in this investigation only the shape of the cells and their localization within the epithelium could serve to discriminate the various epithelial cell types. A transmission image of a freeze-dried section of the skin, demonstrating the various epithelial layers, is shown in Fig. 1. Each of the strata, corneum, granulosum, spinosum, and germinativum, are easily distinguishable, as is the underlying connective tissue. In some sections other epithelial cells could also be observed. Fig. 2 shows a section which happens to contain most of these epithelial structures: a gland excretion duct, a layer of light cells underneath the just shedding corneum, and a mitochondria-rich cell, identified by its characteristic pear shape [15].

Measurements in individual epithelial cells were performed, and, where a clear distinction between nucleus and cytoplasm was present, measurements were performed in each. The values of intracellular electrolyte concentration were expressed as mmole/kg wet wt and given as mean $\pm$ SD, with the number of observations in parentheses. The Student's t test was applied to determine whether differences in the means attain statistical significance.

Results

Stratum Corneum

In the cornified cells, under control conditions, the Na concentration was 126.9 ± 12.4, the K concentration was 2.8 ± 0.5, and the Cl concentration was 91.1 ± 8.7 mmole/kg wet wt ($n = 52$). Neither ouabain, amiloride, nor corial Na-free Ringer's solution had any effect on these

values. When the outer bathing solution was Na-free choline-Ringer's solution (containing some 0.3–0.7 mM Na) the Na concentration in the cornified layer was reduced to 7.9 ± 4.3 mmole/kg wet wt ($n=47$). In contrast, both the K concentration at 3.1 ± 0.9 and the Cl concentration at 90.3 ± 9.1 mmole/kg wet wt were almost unchanged (n.s.). When the outer bathing medium was replaced with distilled water, the Na, K, and Cl concentrations in the cornified layer were reduced to 3.9 ± 2.6, 1.0 ± 0.8 and 2.6 ± 1.8 mmole/kg wet wt ($n=19$), respectively. Thus, under the various experimental conditions, as illustrated in Figs. 3 to 5, the Na and the K concentrations in this layer approximate those of the outer bathing solution but are always somewhat higher.

Stratum Granulosum, Spinosum, and Germinativum

In the control, as well as in all other experimental conditions, only slight, mostly insignificant differences of the cellular Na and K concentrations were observed between the various living cell layers. Therefore, mean values for all layers are generally given. In the control, the mean cellular Na concentration of all layers was 9.4 mmole/kg wet wt ($n=245$), ranging from 6.4 ± 2.1 to 14.4 ± 4.6 in individual skins, and the mean cellular K concentration was 118.4 mmole/kg wet wt, ranging from 110.6 ± 10.1 to 125.6 ± 9.9 in individual skins.

The effect upon cellular Na and K concentrations of incubating pieces of the same skin on both sides in normal frog Ringer's solution (control) or when Na was removed either from the outer or inner bathing solution, is shown for one experiment in Fig. 3. In the control, the Na concentration of the individual epithelial layers ranged from 16.3 ± 2.8 mmole/kg wet wt ($n=11$) for the stratum granulosum to 10.6 ± 4.2 ($n=9$) for the stratum germinativum. After replacing the outer bathing solution with distilled water, the Na concentration in all layers was reduced to almost zero; while after replacing the inner bathing solution with Na-free Ringer's solution, the Na concentration in the stratum granulosum, spinosum 1, and spinosum 2 was almost unchanged; only in the stratum germinativum was it significantly reduced to $2.8 + 2.7$ mmole/kg wet wt ($n=10$, $2P<0.01$). As evident from the lower part of Fig. 3, the K concentrations in all epithelial layers were very similar, and showed only small, albeit statistically significant, variations under the varying experimental conditions. After replacing the outer bathing solution with distilled water, the K values of all layers showed an increase, while after replacing the inner bathing medium with Na-free Ringer's solution, it

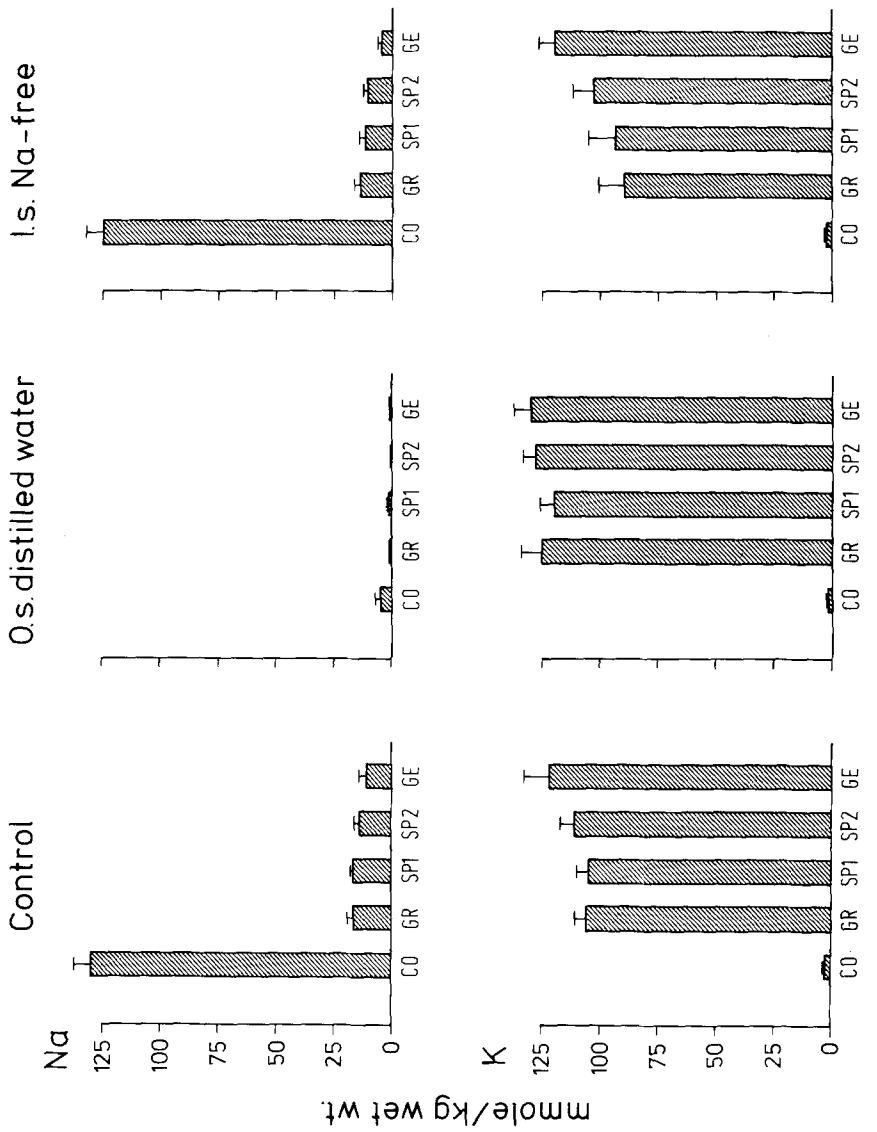

Fig. 3. Cellular Na and K concentrations in the different epithelial layers of frog skin in control and after incubating the outside with distilled water (O.s. *distilled water*) or the inside with Na-free Ringer's solution (I.s. Na-*free*). The different strata are: *CO* = corneum; *GR* = granulosum; *SP*1 and *SP*2 = superficial and deeper spinosum; *GE* = germinativum (each bar represents the mean of about 10 measurements in different cells, ± 2 SEM)

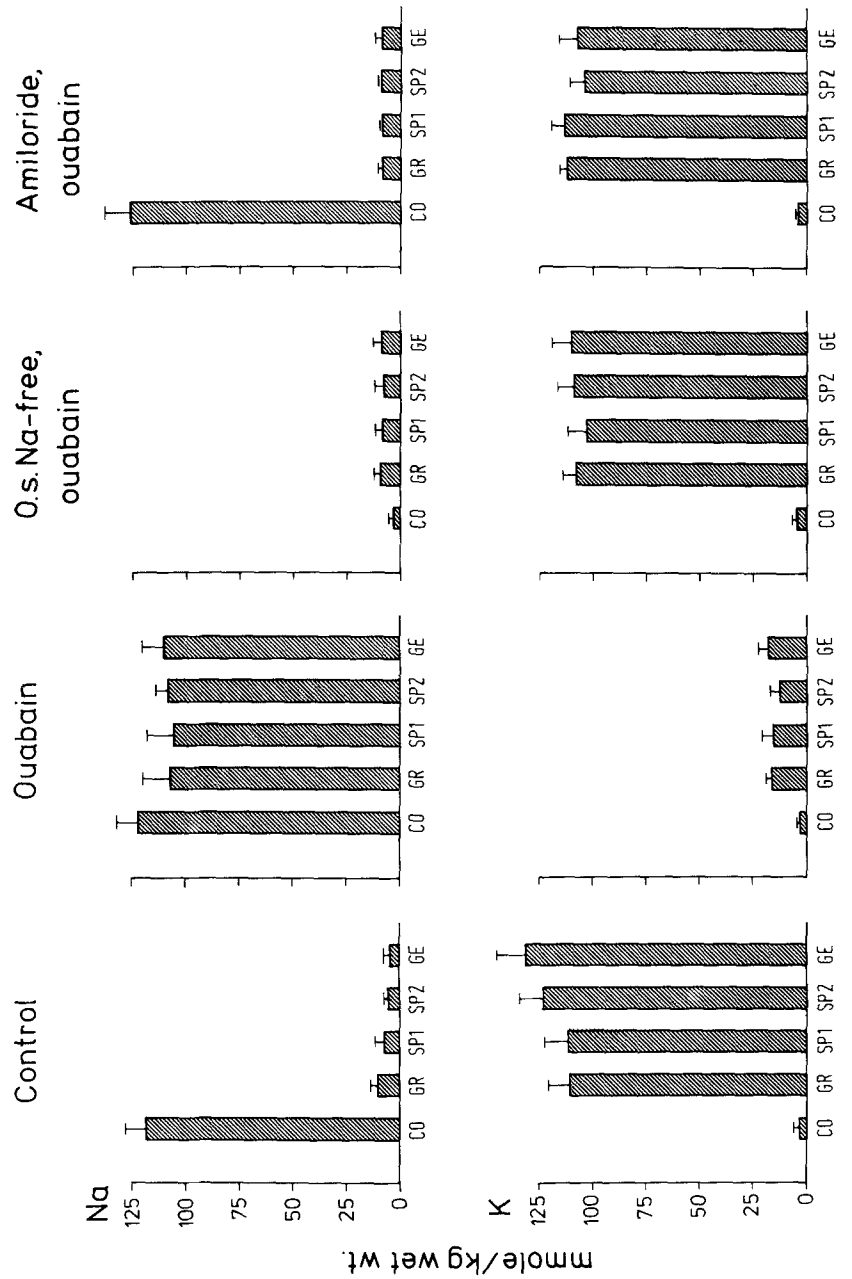

Fig. 4. Cellular Na and K concentrations in the different epithelial layers of frog skin in control, after ouabain and when, simultaneously with the application of ouabain, either the outside was incubated with Na-free Ringer's solution *(o.s. Na-free Ouabain)* or amiloride was applied. The abbreviations used for the different strata are the same as in Fig. 3 (mean $\pm$ 2 SEM)

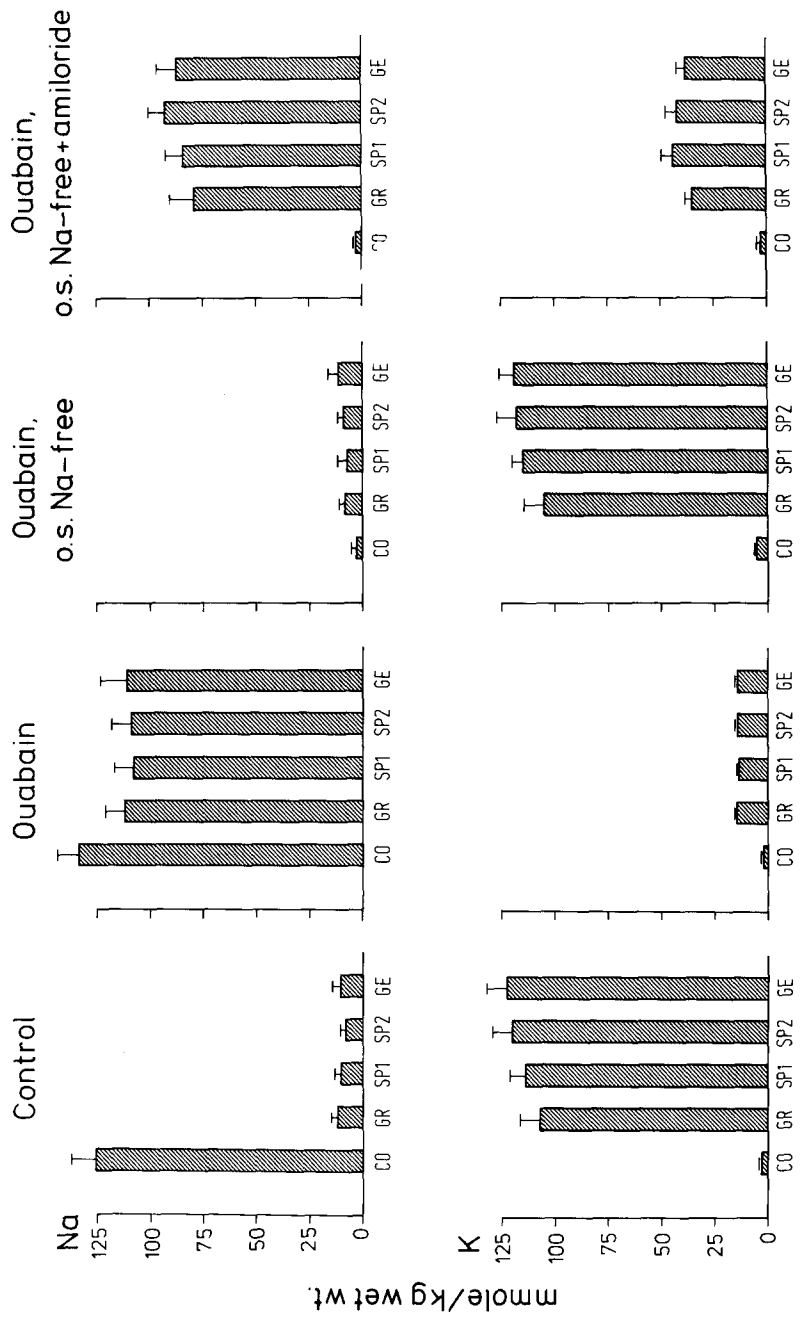

Fig. 5. Cellular Na and K concentrations in the different epithelial layers of frog skin in control after ouabain and when, after 90 min incubation with ouabain, the outer bathing medium was replaced either with Na-free Ringer's solution *(Ouabain, o.s. Na-free)* or with Na free Ringer's solution containing amiloride *(Ouabain, o.s. Na-free + amiloride)*. The abbreviations used for the different strata are the same as in Fig. 3 (mean + 2 SEM)

decreased. For the two experiments performed, when the epithelial side of the skin was bathed in distilled water, the mean cellular Na concentration dropped from 13.9 ± 5.2 mmole/kg wet wt ($n=84$) to 0.4 ± 1.1 ($n=109$), while the K concentration increased from 113.4 ± 11.2 to 124.0 ± 14.2. When the inner side of the skin was bathed in Na-free Ringer's solution, both the mean cellular Na concentration of 10.9 ± 5.1 mmole/kg wet wt ($n=79$) and the mean K concentration of 98.6 ± 16.0 were slightly, but significantly, lower than in the control ($2P<0.01$).

The effect of incubating different pieces of the same skin, either under control conditions or after addition of ouabain to the inner bathing solution, is shown for one experiment in Fig. 4. In addition to the effect of ouabain alone, the effect of also blocking the Na influx from the outer medium, either by using a Na-free Ringer's solution or by adding amiloride, is demonstrated. After ouabain, in all epithelial layers the Na concentration increased by about 100 mmole/kg wet wt, while the K concentration showed an almost equivalent drop. The effect of ouabain on the cellular Na and K values was completely cancelled in all layers when the outside solution was replaced with a Na-free Ringer's solution or Ringer's solution containing amiloride. In both experimental conditions the Na and K concentrations approximate those in the control. In three experiments, after ouabain, when the Na influx from the outside solution was possible, the mean Na concentration increased from 7.1 ± 3.1 mmole/kg wet wt ($n=91$) to 109.4 ± 11.5 ($n=103$), and the K concentration dropped from 119.5 ± 13.8 to 16.1 ± 4.1. When in addition to ouabain the Na influx was inhibited with Na-free Ringer's solution or amiloride containing Ringer's solution bathing the outer surface, the Na concentrations were 10.2 ± 5.0 ($n=73$) and 8.5 ± 3.2 ($n=81$), respectively, and the K concentration values 117.6 ± 14.1 and 116.8 ± 14.4, respectively, almost unchanged compared to control.

The effect of applying ouabain and afterwards replacing the outer bathing solution with Na-free Ringer's solution or Na-free Ringer's solution containing amiloride is shown for the experiment in Fig. 5. Using a Na-free Ringer's solution, in all epithelial layers the Na as well as the K concentrations returned to control values, whereas using Na-free Ringer's solution containing amiloride, only a small decrease in Na and also a small increase in K can be observed compared to ouabain. For the two experiments performed the Na concentration dropped from 108.4 ± 13.8 mmole/kg wet wt ($n=63$) to 6.9 ± 3.6 ($n=61$), and the K concentration increased from 16.8 ± 6.2 to 119.4 ± 16.1 when, after ouabain, the outer medium was replaced with a Na-free solution. When the

Na-free solution contained amiloride, the Na value decreased only to 82.6 ± 21.4 mmole/kg wet wt ($n=59$), and the K value increased only to 38.9 ± 11.9. The respective control values were 7.5 ± 3.5 mmole/kg wet wt ($n=70$) for Na and 122.9 ± 12.9 for K.

Mitochondria-Rich Cells

The frequency of this cell type in the individual skins showed large variations. Under control conditions the Na concentration of the mitochondria-rich cells was 6.5 ± 4.5 mmole/kg wet wt ($n=45$) and the K concentration was 127.2 ± 16.3. Although these values are very similar to those of the other epithelial cells, large variations could be observed within individual skins between these and other cell types. When the outer bathing medium was replaced with distilled water, the Na concentration decreased to 0.5 ± 1.5 mmole/kg wet wt ($n=11$, $2P<0.01$), but no significant change was observed when the inner bathing solution was replaced with Na-free Ringer's solution. After ouabain, an increase in the Na concentration was observed similar to that of other epithelial cells, but smaller in magnitude, and showing much more variation within the individual skins. After ouabain, the Na concentration was $42.1 + 16.2$ mmole/kg wet wt ($n=21$) and the K concentration 88.4 ± 20.1. The changes produced by ouabain were almost unaffected by the simultaneous application of amiloride, when the Na concentration was 38.3 ± 16.9 mmole/kg wet wt ($n=8$, n.s.) and the K concentration was 85.1 ± 22.1 (n.s.). When the outer bathing medium was Na-free Ringer's solution the effect of ouabain was found to be less pronounced.

Gland Cells

The data comprises measurements in cells of the excretion duct as well as in the acinus of the glands, except those in the apical part of mucous cells, which were characterized by higher S and lower K concentrations. In the control, the mean cellular Na concentration was 3.7 ± 3.1 mmole/kg wet wt ($n=31$), significantly lower than that of the other epithelial cells ($2P<0.01$). With distilled water on the outer side or Na-free Ringer's solutions on the inner side of the skin, the Na concentrations were found to be even less, at 1.5 ± 0.9 ($n=6$) and 2.0 ± 1.5 mmole/kg wet wt ($n=9$), respectively. In contrast to all other cells of the epithelium, the Na concentration after ouabain increased only slightly to

9.3 ± 8.5 mmole/kg wet wt ($n = 11$, $2P < 0.01$). This increase seemed to be smaller when the outer bathing solution contained amiloride or no Na. In the control, the cellular K concentration was 131.7 ± 10.9 mmole/kg wet wt. Under the various experimental conditions this value varied insignificantly and after ouabain was slightly reduced at 121.5 ± 15.1 mmole/kg wet wt ($2P < 0.05$).

Light Cells

Occasionally in the first layer underneath the stratum corneum individual light cells could be observed. In one skin, in which the corneum was just starting to moult (*see* Fig. 2), the whole first cell layer consisted of such cells. Compared to the other epithelial cells they appeared to have swollen, as indicated by larger cell size and a markedly reduced Bremsstrahlung intensity and P concentration. In these cells, a typical extracellular electrolyte composition was observed; 121.3 ± 11.8 mmole/kg wet wt for Na, 3.3 ± 1.0 for K, and 109.0 ± 11.9 for Cl ($n = 12$). When the outside bathing medium was Na-free Ringer's solution, the Na concentration in the light cells was drastically reduced to 3.1 ± 2.5 mmole/kg wet wt ($n = 11$), whereas the Cl concentration was almost unchanged at 112.4 ± 14.3 (n.s).

Comparison of Element Concentration between Nucleus and Cytoplasm

The Na and K concentrations of nucleus and cytoplasm in all cell types were almost identical in both control and the other experimental conditions. In contrast, for P, Cl and Ca there was a systematic difference in concentration between nucleus and cytoplasm in both control and under the various experimental conditions. In the cytoplasm, the P concentration was lower than in the nucleus, whereas the concentration of Cl and Ca and the dry weight were higher. In Table 1 the concentration values for cytoplasm and nucleus under control conditions obtained mainly from granular and spiny cells are depicted.

Short-Circuit Current

At the end of the incubation the short-circuit current under the various experimental conditions was on an average 55.2 $\mu A/cm^2$ in the control, 0.5 with o.s. distilled water; 65.0 with i.s. Na-free; 3.2 with

Table 1. Cytoplasmic and nuclear concentrations of Na, K, P, Cl, Ca and dry weight

	Na	K	P	Cl	Ca	dry wt
	mmole/kg wet wt					g/100 g
Cytoplasm	7.4 ±4.5	111.8 ±12.1	98.8 ±19.2	36.5 ±5.0	1.1 ±1.2	25.4 ±2.3
Nucleus	5.1 ±3.9	115.0 ±11.4	144.4[a] ±19.1	32.9[a] ±3.7	0.3[a, b] ±0.6	21.8[a] ±1.9

Mean values ±SD, $n=19$.
[a] Significantly different from the cytoplasmic value, $2P<0.05$.
[b] Not significantly different from zero.

ouabain; −2.7 with o.s. Na-free, ouabain; 1.3 with amiloride, ouabain; −2.5 with ouabain, o.s. Na-free; and 1.0 with ouabain, o.s. Na-free +amiloride.

Discussion

The present investigation demonstrated almost identical concentrations for Na and K in the nucleus and cytoplasm under all experimental conditions. Thus it appears that the intracellular space of the epithelial cells essentially represents only one distributional space for these ions. This result agrees well with recent studies on salivary epithelial cells using ion sensitive microelectrodes [38], while it varies from chemical analysis of fractionated liver cells, demonstrating large differences between nuclear and cytoplasmic Na and K concentrations [24, 41].

According to the present data, the bulk of intracellular Na can be exchanged from the outside bathing solution. This is evident from the reduction of the Na concentration in all epithelial layers after replacing the outer bathing solution with distilled water, while almost no effect was detected after replacing the inside bathing medium with a Na-free solution. Also, when the active Na transport step is inhibited by ouabain, the increase in the cellular Na concentration results from a Na influx from the outside bathing solution, since it is prevented by using Na-free bathing solution or amiloride on the epithelial side. A Na outflux to the epithelial side can be induced in ouabain poisoned skins by removing Na from the outer bathing medium. This view is supported by the fact that the expected reduction of the cellular Na concentration could not be observed during simultaneous amiloride application. Using radio Na, a

Na efflux to the epithelial side under this experimental condition has already been reported by other investigators [28]. Since under short-circuited conditions the electrochemical gradients across the outer and inner barrier of the epithelium can be expected to be the same, the prevailing exchange of intracellular Na with the outer bathing medium indicates a considerably higher Na permeability of the outerfacing than the inner-facing membranes. This conclusion is consistent with the finding of the outer surface of the epithelium behaving like a Na electrode [25, 26, 29]. However, the present result varies from previous investigations demonstrating in whole skins [6, 7, 12, 36, 37] as well as in isolated epithelia [1] that the major quantity of cellular Na originates from the inner bathing medium. In addition, the present data do not provide evidence for any significant amount of nonexchangeable intracellular Na [6, 7].

During recent years conflicting results have been obtained regarding the localization of the Na transport compartment within the epithelium [8, 10, 26, 27, 34, 40, 42, 45, 46, 47]. Assuming a transcellular Na transport pathway, the cells comprising the Na transport compartment should be identified by characteristic behavior of their Na concentrations after inhibition of Na transport at the outer or inner barrier; when the active extrusion of Na towards the inner side is inhibited, Na should accumulate, whereas, when the passive Na influx from the epithelial side is blocked, cellular Na concentration should drop. According to the present results the cells of all epithelial layers, except the corneum, have shown this behavior. This finding supports the view of a syncytial Na transport compartment as suggested by Ussing and Windhager [42] and Farquhar and Palade [20]. Assuming intercellular junctions with high Na permeability between the various epithelial layers, the close similarity and parallel changes observed in the cellular Na concentrations under the different experimental conditions can easily be explained. Since in frog skin epithelium the extracellular shunt pathway is sealed up by tight junctions [18, 20], which are located between the cells of the outermost living epithelial layer, stratum granulosum, the deeper epithelial layers, stratum spinosum and germinativum, are not directly exposed to the outside bathing medium. Therefore, the finding that the intracellular Na of even the deeper layers originates mainly from the outer bathing solution provides direct evidence for a Na exchange via intercellular communications between deeper and superficial cells. However, the observation of an identical behavior of all epithelial layers with regard to their Na and K concentrations at different functional states of transep-

ithelial Na transport does not permit the conclusion that all epithelial cells share to exactly the same extent in the transepithelial Na transport rate. It is possible that most of the transepithelial transport is performed by a single epithelial cell type, to which the other epithelial cells are coupled. Since, however, recent studies on the distribution of ouabain binding sites [31] have demonstrated that the basolateral membranes of all epithelial layers are potential sites of transepithelial Na transport, it seems more probable that each epithelial layer shares significantly in the transepithelial Na transport activity. The present investigation provides no experimental support for the view that the transepithelial Na transport is accomplished by the sole activity of only the first reactive cell layer, the stratum granulosum [43, 45]. This layer showed identical behavior under the various experimental conditions, as the other epithelial layers, but occasionally it contained light, apparently swollen, cells. However, the extracellular-like electrolyte composition together with the fact that choline obviously can enter these cells indicates that they are dead cells, probably representing a first step of cornification. Since their Na concentration was not at all affected by amiloride, it seems most unlikely that the light cells share in the transepithelial Na transport pathway. In addition to these light cells the present electron microprobe study also revealed some further functional inhomogeneities of the frog skin epithelium. The outer cornified layer seems to represent an extracellular space, freely equilibrating with the outside medium [18]. Apparently, the mitochondria-rich cells as well as the gland cells of the epithelium are not coupled to those which constitute the functional syncytium for Na, since under some experimental conditions both cell types showed intracellular electrolyte concentrations largely different from those of neighboring granular, spiny, or germinal cells. Although the mitochondria-rich cells can be regarded as transepithelially transporting cells, since their Na concentration increased during inhibition of the active transport step and decreased when the Na influx from the outer medium was abolished, they obviously do not share in an amiloride-sensitive transport pathway. Therefore, since in the frog skin the net transepithelial Na transport [16, 12], as well as the unidirectional Na uptake from the outer bathing medium [34, 39], have been found to be extremely sensitive to amiloride, the contribution of this cell type to the rate of transport can be expected to be negligibly small. A more detailed analysis of the mitochondria-rich cells will be given in a subsequent paper.

Compared to most chemical or radiochemical analysis of electrolytes

in frog skin epithelium [1, 6, 7, 12, 36, 37], the present mean cellular value for Na is at 8 mmole/kg wet wt much lower than previously reported, while that for K is much higher at 120 mmole/kg wet wt. The difference in values obtained between electron microprobe and chemical analysis is especially large when the chemical analysis is performed on whole skins with the corial connective tissue present [6, 7, 12, 36, 37], whereas with epithelia isolated from the connective tissue [1, 27], there is a better agreement. Using isolated epithelial cells, which have been washed in Na-free solutions [48], the chemical analysis provides almost identical values as the present data. The fact that the discrepancy between both methods is smaller, when the extracellular space is reduced or the Na concentration in the extracellular space is lowered, lends support to the view that the chemical analysis is falsified by an underestimation of the extracellular space [11]. This view is also supported by the finding that the alterations of cellular Na and K concentrations after ouabain found in this study are much more pronounced then those found by chemical analysis [37]. The total amount of cellular Na calculated from the present data is even lower than the quantity of Na which exchanges with radio Na from the outer bathing medium in whole skins [2, 6, 7, 9, 12, 37], which has been called the Na transport pool [22]. Perhaps some of this epithelially exchangeable Na is located in the lateral intercellular spaces of the epithelium [47] or is trapped in the underlying corial connective tissue. There is a much better agreement between the size of the Na transport pool determined in isolated epithelia [1, 18] and the total cellular Na calculated from the present data, indicating that in isolated epithelia the Na transport pool represents truly intracellular Na.

An additional finding of this study is the fact that the sum of the cellular concentrations of Na and K was very stable despite large variations in the concentrations of these ions, suggesting a close inverse correlation between the cellular Na and K concentrations. After ouabain the K depletion of the cells could be completely prevented by inhibiting the Na influx from the outside medium by using either amiloride or Na-free solutions. The decrease in K concentration following ouabain could even be reversed by inducing a Na outflux towards the outer medium with Na-free solutions. Since these effects were observed in the presence of ouabain, it seems most probable that they are of a passive nature. A large negative intracellular potential with amiloride or Na-free solutions on the outside might explain the persistence of high cellular K values despite the application of ouabain. On the other hand, an intracellular

hyperpolarization, caused by a Na diffusion potential, could account for the reaccumulation of cellular K after exchanging the outside medium for Na-free solution.

As to the nature of transepithelial Na movement, all the findings of this study are compatible with the transcellular two barrier concept developed by Koefoed-Johnsen and Ussing [26]. So far, alternative transport models, such as a three barrier model [47] or an extracellular one [8], have not gained experimental support. Regarding the transport properties of the inner-facing barrier, the Na increase after ouabain fits with the localization of an active ouabain-sensitive transport step at this site. Regarding the outer-facing barrier, the low cellular Na concentration value found in this study implies that a large chemical concentration gradient exists when the skin is bathed in normal Ringer's solution. Since X-ray microanalysis detects all cellular Na, whether it is in solution or bound, the cellular Na activity might be even lower. Assuming a negative intracellular electrical potential, which has been thought under short-circuited conditions to be about -20 mV [5, 17], or according to more recent investigations to be -73 mV [35] or -90 mV [23], the low cellular Na value implies that a large electrochemical gradient must be present. Therefore, since the Na concentration will be reduced further when the Na concentration of the outer bathing medium is lowered, a net Na uptake by passive forces can be expected to occur even from bathing media containing less than 0.1 mM Na. Thus, the behavior of the Na uptake across the outer barrier [34, 39] could be explained without the need to assume an active transport step [10, 27, 40].

The authors wish to thank Dr. June Mason for her valuable comments and criticism during the preparation of this manuscript. This work was supported by the Deutsche Forschungsgemeinschaft.

References

1. Aceves, J., Erlij, D. 1971. Sodium transport across the isolated epithelium of the frog skin. *J. Physiol. (London)* **212**:195
2. Andersen, B., Zerahn, K. 1963. Method for non-destructive determination of the sodium transport pool in frog skin with radio sodium. *Acta Physiol. Scand.* **59**:319
3. Bauer, R., Rick, R. 1977. A computer program for the analysis of energy dispersive X-ray spectra of thin sections of biological soft tissue. *X-ray Spectrom. (in press)*
4. Carasso, N., Favard, P., Jard, S., Rajerison, R.M. 1971. The isolated frog skin epithelium. I. Preparation and general structure in different physiological states. *J. Microsc. (Paris)* **10**:315

5. Cereijido, M., Curran, P.F. 1965. Intracellular electric potentials in frog skin. *J. Gen. Physiol.* **48**:543
6. Cereijido, M., Reisin, I., Rotunno, C.A. 1968. The effect of sodium concentration on the content and distribution of sodium in the frog skin. *J. Physiol. (London)* **196**:237
7. Cereijido, M., Rotunno, C.A. 1967. Transport and distribution of sodium across the frog skin. *J. Physiol. (London)* **190**:481
8. Cereijido, M., Rotunno, C.A. 1968. Fluxes and distribution of sodium in frog skin. A new model. *J. Gen. Physiol.* **51**:280
9. Curran, P.F., Herrera, F.C., Flanigan, W.J. 1962. The effect of Ca and antidiuretic hormone on Na transport across frog skin. II. Sites and mechanisms of action. *J. Gen. Physiol.* **46**:1011
10. Cuthbert, A.W. 1972. A double (series) pump model for transporting epithelia. *J. Theor. Biol.* **36**:555
11. Dörge, A., Gehring, K., Nagel, W., Thurau, K. 1974. Intracellular $Na^+ - K^+$ concentration of frog skin at different states of Na-transport. *In:* Microprobe Analysis as Applied to Cells and Tissues. Th. Hall, P. Echlin and R. Kaufmann, editors. p. 337. Academic Press, London-New York
12. Dörge, A., Nagel, W. 1970. Effect of amiloride on sodium transport in the frog skin: II. Sodium transport pool and unidirectional fluxes. *Eur. J. Physiol.* **321**:91
13. Dörge, A., Rick, R., Gehring, K., Mason, J., Thurau, K. 1975. Preparation and applicability of freeze-dried sections in the microprobe analysis of biological soft tissue. *J. Microsc. Biol. Cell* **22**:205
14. Dörge, R., Rick, R., Gehring, K., Thurau, K. 1977. Preparation of freeze-dried cryosections for quantitative X-ray microanalysis of electrolytes in biological soft tissues. *Pfluegers Arch. (in press)*
15. Ehrenfeld, J., Masoni, A., Garcia-Romeu, F. 1976. Mitochondria-rich cells of frog skin in transport mechanisms: Morphological and kinetic studies on transepithelial excretion of methylene blue. *Am. J. Physiol.* **231**:120
16. Eigler, J., Kelter, J., Renner, E. 1967. Wirkungscharakteristika eines neuen Acylguanidins – Amiloride-HCL (MK 870) – an der isolierten Haut von Amphibien. *Klin Wschr.* **14**:737
17. Engbaek, L., Hoshiko, T. 1957. Electrical potential gradients through the frog skin. *Acta Physiol. Scand.* **39**:384
18. Erlij, D. 1971. Salt transport across isolated frog skin. *Phil. Trans. R. Soc. London B* **262**:153
19. Farquhar, M.G., Palade, G.E. 1964. Functional organization of amphibian skin. *Proc. Nat. Acad. Sci. USA* **51**:569
20. Farquhar, M.G., Palade, G.E. 1965. Cell junction in amphibian skin. *J. Cell. Biol.* **26**:263
21. Farquhar, M.G., Palade, G.E. 1966. Adenosine triphosphatase localization in amphibian epidermis. *J. Cell. Biol.* **30**:359
22. Frazier, H.S., Dempsey, E.F., Leaf, A. 1962. Movement of sodium across the mucosal surface of the isolated toad bladder and its modification by vasopressin. *J. Gen. Physiol.* **45**:529
23. Helman, S.I., Fisher, R.S. 1977. Microelectrode studies of the active Na transport pathway of frog skin. *J. Gen. Physiol.* **69**:571
24. Hooper, G., Dick, D.A.T. 1976. Non-uniform distribution of sodium in the rat hepatocyte. *J. Gen. Physiol.* **67**:469
25. Kidder, G.W., Cereijido, M., Curran, P.F. 1964. Transient changes in electrical potential differences across frog skin. *Am. J. Physiol.* **207**:935
26. Koefoed-Johnsen, V., Ussing, H.H. 1958. The nature of the frog skin potential. *Acta Physiol. Scand.* **42**:298

27. Leblanc, G. 1972. The mechanism of lithium accumulation in the isolated frog skin epithelium. *Pfluegers Arch.* **337**:1
28. Leblanc, G., Morel, F. 1975. Na and K movements across the membranes of frog skin epithelial associated with transient current changes. *Pfluegers Arch.* **358**:159
29. Lindley, B.D., Hoshiko, T. 1964. The effect of alkali cation and common anions on the frog skin potential. *J. Gen. Physiol.* **47**:749
30. Loewenstein, W.R. 1970. Intracellular communication. *Sci. Am.* **222**:78
31. Mills, J.W., Ernst, S.A., Dibona, D.R. 1977. Localization of Na^+-pump sites in frog skin. *J. Cell. Biol.* **73**:88
32. Morel, F., Leblanc, G. 1973. Kinetics of sodium and lithium accumulation in isolated frog skin epithelium. *In:* Alfred Benzon Symp. V. Transport Mechanisms in Epithelia. H.H. Ussing and Na.A. Thorn, editors. p. 28. Munksgaard, Copenhagen
33. Morel, F., Leblanc, G. 1975. Transient current changes and Na compartmentalization in frog skin epithelium. *Pfluegers Arch.* **358**:135
34. Moreno, J.H., Reisin, I.L., Rodriguez Boulán, E., Rotunno, C.A., Cereijido, M. 1973. Barriers to sodium movement across frog skin. *J. Membrane Biol.* **11**:99
35. Nagel, W. 1976. The intracellular electrical potential profile of the frog skin epithelium. *Pfluegers Arch.* **365**:135
36. Nagel, W., Dörge, A. 1970. Effect of amiloride on sodium transport of frog skin. I. Action on intracellular sodium content. *Pfluegers Arch.* **317**:84
37. Nagel, W., Dörge, A. 1971. A study of the different sodium compartments and the transepithelial sodium fluxes of the frog skin with the use of ouabain. *Pfluegers Arch.* **324**:267
38. Palmer, L.G., Civan, M.M. 1977. Distribution of Na^+, K^+ and Cl^- between nucleus and cytoplasm in *chironomus* salivary gland cells. *J. Membrane Biol.* **33**:41
39. Rick, R., Dörge, A., Nagel, W. 1975. Influx and efflux of sodium at the outer surface of frog skin. *J. Membrane Biol.* **22**:183
40. Rotunno, C.A., Pouchan, M.I., Cereijido, M. 1966. Location of the mechanism of active transport of sodium across the frog skin. *Nature (London)* **210**:597
41. Siebert, G., Langendorf, H. 1970. Ion balance in the cell nucleus. *Naturwissenschaften* **57**:119
42. Ussing, H.H., Windhager, E. 1964. Nature of shunt path and active sodium transport path through frog skin epithelium. *Acta Physiol. Scand.* **61**:484
43. Voûte, C.L., Hänni, S. 1973. Relation between structure and function in frog skin. *In:* Transport Mechanisms in Epithelia. H.H. Ussing and N.A. Thorn, editors. Alfred Benzon Symp. V, p. 38. Munksgaard, Copenhagen
44. Voûte, C.L., Mollgard, K., Ussing, H.H. 1975. Quantitative relationship between active sodium transport, expansion of endoplasmic reticulum and specialized vacuoles ("scalloped sacs") in the outermost living cell layer of the frog skin epithelium *(Rana temporaria). J. Membrane Biol.* **21**:273
45. Voûte, C.L., Ussing, H.H. 1968. Some morphological aspects of active sodium transport. *J. Cell Biol.* **36**:625
46. Zerahn, K. 1969. Nature and localization of the sodium pool during active transport in the isolated frog skin. *Acta Physiol. Scand.* **77**:272
47. Ziegler, T.W. 1976. A new model for regulation of sodium transport in high resistance epithelia. Med. Hypothesis **2**:85
48. Zylber, E.A., Rotunno, C.A., Cereijido, M. 1973. Ion and water balance in isolated epithelial cells of the abdominal skin of the frog *Leptodactylus ocellatus. J. Membrane Biol.* **13**:199