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Ionic Fluxes in Isolated Epithelial Cells of the Abdominal Skin of the Frog *Leptodactylus ocellatus*

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Summary. Unidirectional ion fluxes are measured in cells isolated by a trypsination-dissection method from the epithelium of the frog *Leptodactylus ocellatus*. Potassium seems to be contained in a single cellular compartment. The influx of potassium is 0.0068 μmole min^{-1} mg^{-1} of dry weight and is carried by a ouabain-sensitive pump. Sodium seems to be contained in two cellular compartments, one of which does not exchange its Na within the experimental period. The possibility that these compartments reflect the existence of different types of cells is not discarded. 49% of the rate constant for the Na efflux is ouabain-sensitive and 23% is ethacrynic-sensitive. Under control conditions the permeability to potassium (P_K), sodium (P_{Na}) and chloride (P_{Cl}) are 7.6×10^{-5}, 2.6×10^{-5} and 2.8×10^{-5} liters/min mg, respectively. The value of P_{Na} is much higher than predicted by current electrical models of the epithelium. The discrepancy might offer some insight into the nature of the "inner facing barrier" of the skin.

Epithelial cells carry out two important functions related to ion movement and distribution: 1) they maintain their own ionic balance as any other cell in the organism, and 2) they translocate a net amount of ions across the whole epithelium. There is little information on whether the same set of membrane mechanisms performs both functions, or whether there are two completely independent sets, including also several intermediate possibilities of partial coupling. This is particularly important in epithelia like the frog skin where, while the ionic balance of the cell must be a continuous function, ionic transport across the epithelium is limited to the periods when the frog is exposed to an aqueous environment. A necessary step in the understanding of the relationship between the two functions is the study of the mechanisms used by the epithelial cells to regulate their ion and water content. The present work is part of a series of studies aimed at obtaining information on the membrane of these cells. This series started with the design of a trypsination-dissection method to obtain a population of cells isolated from the epithelium of the frog skin, that maintains its steady state for several hours. It gave information on the balance of K^+, Na^+, Cl^- and H_2O in these cells and in the isolated

epithelium (Rotunno, Zylber & Cereijido, 1973; Zylber, Rotunno & Cereijido, 1973).

In the present study we examined ionic fluxes to characterize the mechanisms involved in these balances. The study shows that, while the value of K fluxes agree with those found in isolated epithelia and whole skin (Curran & Cereijido, 1965; Biber, Aceves & Mandel, 1972; Candia & Zadunaisky, 1972), those of Na are much higher than expected on the basis of electrical models.

Materials and Methods

Isolated epithelial cells were obtained from abdominal skin of South American frog *Leptodactylus ocellatus* (L) by a trypsination-dissection method described in detail in a previous paper (Zylber *et al.*, 1973). Essentially it consists of an incubation of the isolated abdominal skin in a Ringer's solution containing 2% trypsin, followed by dissection of the epithelium and a second period of incubation of the isolated epithelium with 0.2% trypsin. As estimated by nigrosin staining, only 6–7 per cent of the cells are damaged. The cells were incubated in Erlenmeyer flasks continuously stirred with magnetic bars. The Ringer's solution used contains (mM): 115 NaCl, 2.4 KCO_3H, 1.0 $CaCl_2$, 2.0 glucose, 600 mg per 100 ml bovine albumin. Other solutions used are described in Results. Once gassed with O_2 plus CO_2 the Ringer's had a pH of 7.4. All experiments were performed at room temperature (22 °C). The procedures to measure ion and water content were also those described by Zylber *et al.* (1973).

Potassium and Sodium Uptake

The cell suspension was allowed to equilibrate for a period of 30–60 min. Then 1 μC/ml of ^{42}K (or ^{24}Na) plus 0.3 μC/ml of ^{131}I-albumin was added. Duplicate samples of 5.0 ml were taken at several times and spun 4 min at 1500 × g in a special tube (Zylber *et al.*, 1973) which allows the collection of the cell pellet with a minimun of bathing fluid. The pellet was weighed, and dried at 80 °C to constant weight. The pellet and samples of the bathing fluids were counted for ^{42}K (or ^{24}Na) and, once the activity of these tracers became negligible, they were counted for ^{131}I. Both measurements were made in a well-type scintillation counter (Nuclear Chicago Autogamma) set as spectrometer on the corresponding peak. The dried samples were then extracted 24 hr with 1.0 ml of 0.1 N HNO_3 and samples were then taken and diluted for analytical determinations.

Sodium Washout

The cell suspension was incubated for 1 hr with ^{24}Na, 2 μC per ml. Duplicate samples of 5.0 ml were collected in a Teflon test tube containing 0.5 ml of the same Ringer's used during incubation to which ^{131}I-albumin was added. The two liquids were mixed by sucking and blowing with the same pipette and a sample of 5.0 ml was finally withdrawn and spun in a tube of the type mentioned above. Samples of this pellet and of the incubation medium were used to calculate the percent of equilibration with the tracer. This was taken as the zero time level. The whole cell suspension was then centrifuged, the supernatant was discarded, and the cells were resuspended in Ringer's without ^{24}Na but containing ^{131}I-albumin. Duplicate samples were periodically taken and processed as described for potassium uptake.

Chloride Washout

The cell suspension was incubated for 1 hr with ^{36}Cl (0.08 μC per ml) and processed as described for sodium washout except that no ^{131}I-albumin was added. Samples were counted in Bray's liquid scintillation solution in an ISOCAP 300 Nuclear Chicago counter.

Source of Materials

^{42}K, ^{24}Na and ^{131}I-albumin were purchased from the Atomic Energy Comission of Argentina and ^{36}Cl from Amersham. Ouabain was from K & K Laboratories, Inc; dinitrophenol (DNP) was from Merck Laboratories; iodoacetic acid (IA) from BDH Laboratories and Ethylendiaminetetraacetic acid (EDTA) from Carlo Erba. For further details and reliability of the methods described see Zylber *et al.* (1973) and Rotunno *et al.* (1973). Results are given as mean ± standard error (number of observations).

Results

In agreement with previous work from this laboratory it was found that the cell preparation used maintains constant its concentrations of K, Na and Cl throughout the experimental period (3 hr maximal). The results will be divided into two parts. The first one is a general characterization of K, Na and Cl movement as studied by tracer kinetics. In Part II we study in more detail the mechanisms involved in Na movement, based on the results obtained in Part I.

Part I

Potassium

Curran and Cereijido (1965) found that 94% of the K in frog skin exchanges with ^{42}K in the bathing solution with a single time constant. Accordingly, the cell suspension was considered to be a closed two-compartment system described by

$$\frac{dP_c}{dt} = -J_{CB}p_C^* + J_{BC}p_B^* \tag{1}$$

where P_C is the amount of counts per minute of ^{42}K in the cells, the J_{ij} are unidirectional fluxes and the p_i^* the specific activities of ^{42}K. The subscripts C and B stand for *cell* and *bathing solution*, respectively. The rate constant k_{ij} is defined as

$$k_{ij} = \frac{J_{ij}}{S_i} \tag{2}$$

where S_i is the amount of potassium in compartment i. Since the preparation is in steady state $J_{CB} = J_{BC}$, and S_i is constant. Samples of the bathing

solution taken at the beginning and at the end of the experiment show that it behaves as a constant reservoir ($p_B^* = \text{constant}$).

Under these conditions integration of Eq. (1) yields

$$\frac{p_C^*}{p_B^*} = 1 - e^{-k_{CB}t}. \tag{3}$$

Fig. 1 shows the time course of penetration of ^{42}K into the epithelial cells. Except for a fast compartment containing 4% of the K in the cells, the experimental points can be fitted with Eq. (3). In agreement with results obtained by Curran and Cereijido (1965) we found that the whole K content is readily exchangeable. This seems to be different from the distribution of K in the bladder where only 45% is readily exchangeable (Finn & Nellans, 1972).

The fraction of K which is quickly labelled and which comprises 4% of the total K content might be constituted by K in damaged cells which, somehow, retain K (as mentioned above, by nigrosin staining this preparation has 6 to 7 per cent of damaged cells).

The content of potassium (S_C) was 0.47 ± 0.01 (70) μmole per milligram of dry weight. The content of water was 3.6 ± 0.1 μliters per milligram of dry weight. This gives a cellular concentration of K of 130 mM. Using the value of S_C and the value of k_{CB} shown in Fig. 1 (0.0145 min^{-1}), the efflux of K is found to be 0.0068 μmole min^{-1} mg^{-1} of dry weight. Since, under the conditions used, the cells maintain a steady state, this should also be the value of the influx.

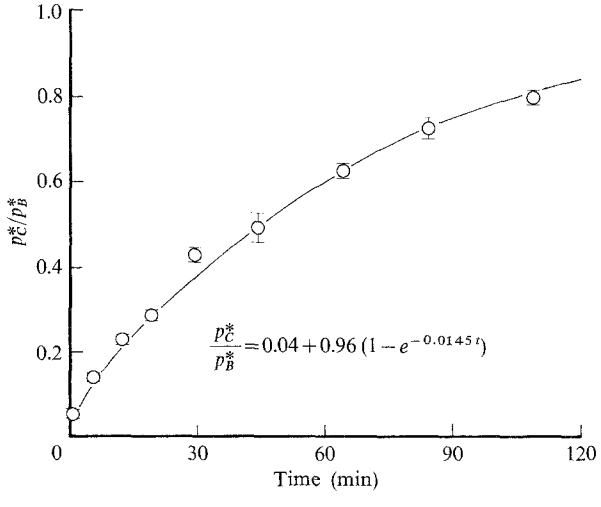

Fig. 1. Uptake of potassium in a population of isolated epithelial cells, expressed as a fraction of the specific activity of K in the cells (p_C^*) and in the bathing solution (p_B^*)

Effect of ouabain. Curran and Cereijido (1965) found that ouabain inhibits the uptake of K in the total frog skin, and Zylber *et al.* (1973) found that it decreases the K content of the isolated cells. As shown in Fig. 2, preincubation of the cells with ouabain 10^{-4} M for 1 hr before starting the experiment causes an almost total inhibition of the influx of potassium. This represents a much stronger inhibition than the one found by Biber *et al.* (1972) in the isolated epithelium of *R. pipiens* (42%).

Effect of low temperature (2 °C). After a period of equilibration at 21 °C the temperature was lowered. In 5 to 10 min it attained a steady temperature of 2 °C. ^{42}K was then added and this instant was taken as zero time. Fig. 3 shows that low temperature produces a strong inhibition of the uptake of potassium (94%), comparable to that produced by ouabain.

Sodium

Fig. 4 shows the time course of ^{24}Na uptake in cells incubated in control Ringer's. There is a very fast compartment containing 0.055 µmole of sodium per milligram of dry weight which is not in the extracellular space measured by ^{131}I-albumin. Studies by Zylber *et al.* (1973) indicated that when the cells were switched from a control Ringer's to another in which Na was replaced by choline the concentration of Na in the cells dropped from 38.5 ± 0.9 mM to 15.8 ± 1.8 mM in the first few minutes, and that Na loss then continued at a much slower rate. As a possible source

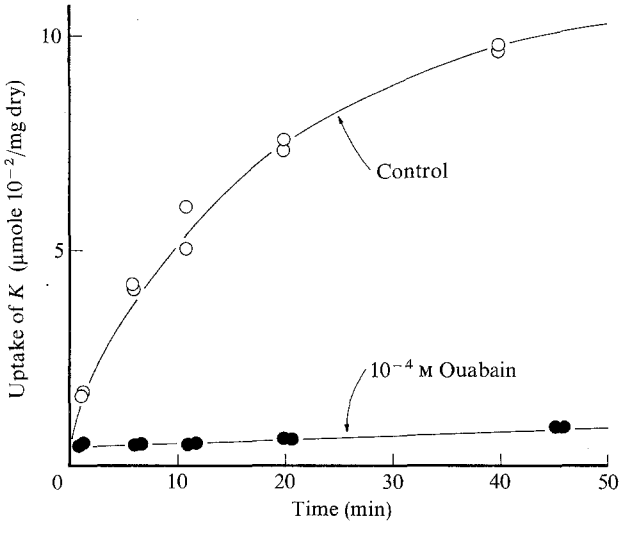

Fig. 2. Uptake of potassium under control conditions (open circles) and 10^{-4} M ouabain (full circles). The cells are in contact with ouabain since an hour before adding ^{42}K

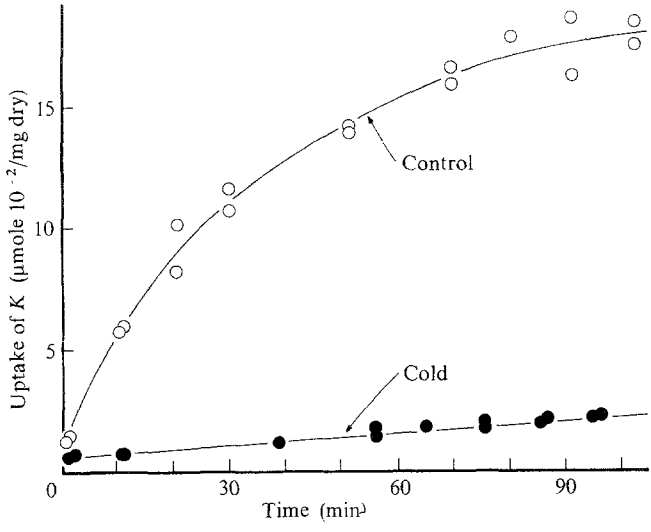

Fig. 3. Uptake of potassium at control (22 °C) and low (2 °C) temperature. The temperature was lowered 15 min before adding ^{42}K

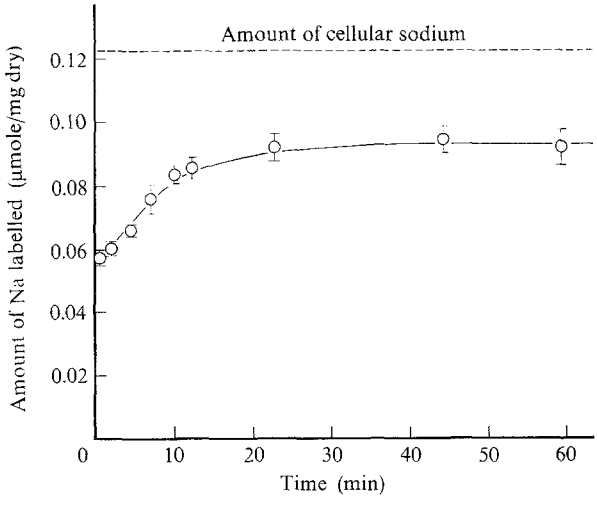

Fig. 4. Uptake of sodium in isolated epithelial cells expressed as amount of sodium labelled. Notice that there is a fraction of Na (0.0055 μmole/mg) which is probably not located into the cytoplasm and that is quickly labelled; another one (0.0026 μmole/mg) that does not exchange its sodium, and a third fraction containing 0.039 μmole/mg which labels its sodium in 20–30 min. The curve is described by Eq. (4)

of the fast sodium compartment they suggested (a) the fraction of damaged cells and (b) sodium bound to the intercellular substance with ion binding properties that fills the intercellular space in the intact epithelium, and part of which might remain attached to the surface of the isolated cells

(Rotunno *et al.*, 1973). A third possible source of the rapidly exchanging Na was suggested by the electron-microscopic appearance of the cells (Zylber *et al.*, *unpublished results*). Trypsin seems to detach the cells from the epithelium by cleaving desmosomes. The cleaved plaques, representing the halves of the desmosomes often appear to lie in vacuoles, as if they had been endocytized by the cells (*see also* Borysenko & Revel, 1973). If the connection of the vacuole with the bathing solution were not completely closed, at least in some vacuoles, the Na that they contain may, in principle, contribute to the fast compartment.

There is a cellular compartment containing 0.039 μmole of Na per milligram of dry weight which exchanges with a rate constant (k_{CB}) of 0.110 min^{-1}. According to Eq. (2) the Na flux from this compartment will be 0.039 μmole/mg weight × 0.110 min^{-1} = 0.0043 μmole min^{-1} mg^{-1}. The time course of Na exchange in Fig. 4 is therefore described by the equation:

$$\text{amount of labelled sodium} = 0.055 + 0.039\,(1 - e^{-0.119t}). \qquad (4)$$

The rest of the cellular sodium (0.029 μmole per milligram) does not exchange with ^{24}Na with a detectable time course in the 60 min of the experiment. Cereijido and Rotunno (1967) and Cereijido, Reisin and Rotunno (1968) have shown that the epithelium has a fraction of Na which does not exchange in 40 to 90 min.

When the cells are loaded for 40 to 60 min with ^{24}Na and then re-suspended in Ringer's without tracer, they show first a steep drop of ^{24}Na

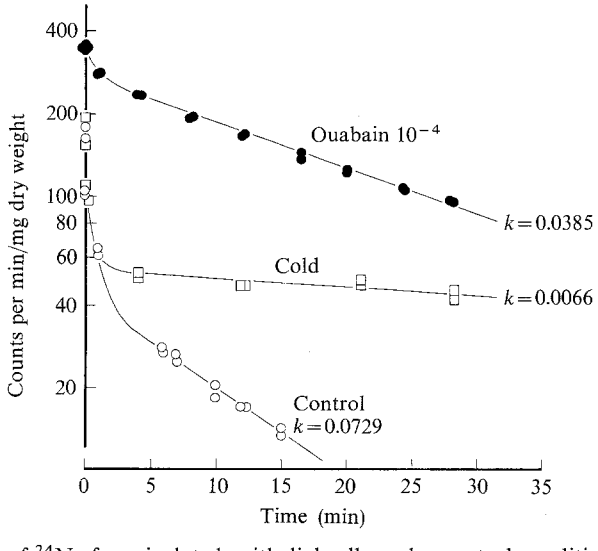

Fig. 5. Washout of ^{24}Na from isolated epithelial cells under control conditions (open circles), 10^{-4} M ouabain (full circles) and 2 °C degrees of temperature (squares)

activity, followed by a slower exchange (Fig. 5, open circles). The time course of this washout is the one expected on the basis of the experiment described above, except that in this case the rate constant (k_{CB}) is somewhat slower: 0.073 min^{-1}. It should be stressed though, that theoretically they should have identical value. Preparations obtained from frogs of the same shipment usually gave similar values, but since the present series of studies was performed during all seasons over a period of two years, the values of the constant show some variations. In the second part of this paper this is compensated by running a control experiment each time, and then normalizing to make comparisons.

Effect of ouabain. In this experiment the cells were preincubated in Ringer's with ^{24}Na containing 10^{-4} M ouabain for 1 hr. As shown in Fig. 5, ouabain reduces k_{CB} from 0.0729 (open circles) to 0.0385 min^{-1} (full circles) which represents an inhibition of 49%. For comparison, ouabain inhibits the Na rate constant efflux by 50–60% in oocytes (Dick & Lea, 1967), by 50–80% in frog muscle (Edwards & Harris, 1957; Horowicz & Gerber, 1965), by 42% in ascites tumor cells (Maizels, Remington & Truscoe, 1958) and by 80% in squid axon (Caldwell & Keynes, 1959).

Effect of low temperature (2 °C). The cell suspension was loaded with ^{24}Na for 50 min at room temperature and 10 min more at low temperature. The cells were then separated from the medium and resuspended in cold Ringer's without tracer. Cold produces a marked inhibition (90%) of the rate constant for the outflux of sodium (Fig. 5 squares). This inhibition is greater than that produced by ouabain (49%). This is different from the situation with K where the two inhibitions were similar. If we assume that at the concentration used, ouabain has elicited a near total inhibition of a K-Na pump (*see below*), then these results indicate that while the remaining flux of Na (51%) is cold-sensitive, the remaining flux of K (9%) is not. This is further explored in Part II.

Chloride

Zylber *et al.* (1973) have shown that when the cells are resuspended in sucrose Ringer's they lose Cl^- as if it were coming from two different compartments, the first one losing its Cl^- at a much faster rate than the second. The concentration of chloride also drops from 83.5 ± 4.7 (14) mM to a steady 57.2 ± 5.3 (11) mM. Fig. 6 shows that when the cells loaded with ^{36}Cl are transferred to the same NaCl Ringer's, but without isotope, they lose tracer with two very different rate constants. With respect to the

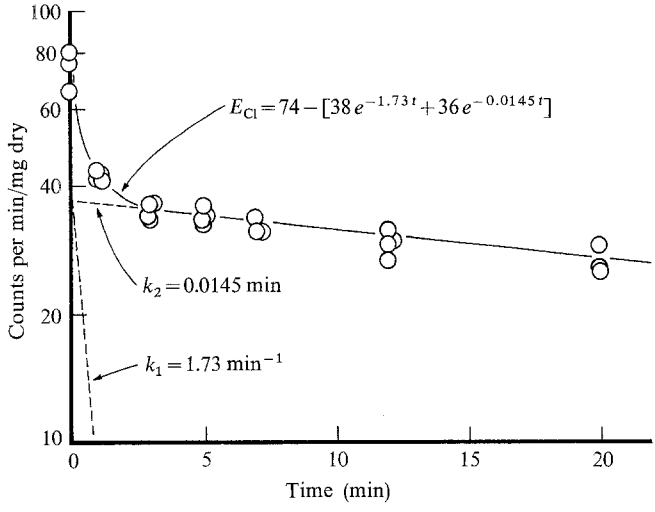

Fig. 6. Washout of ^{36}Cl from isolated epithelial cells. The experimental curve is composed of the two exponentials represented by the dotted lines

nature of the two compartments, the considerations made above for the washout of Na are also pertinent here. If the slow compartment alone is taken to represent Cl^- content in the cytoplasm of the cells, we may use the Cl^- content found by Zylber *et al.* (1973) in the cells washed with sucrose (0.15 ± 0.02 (12) μmole/mg dry weight) and Eq. (2) to compute the flux: 0.0022 μmole/mg dry weight min.

Part II

After the general characterization of K, Na and Cl fluxes carried out in Part I, we studied in more detail the mechanisms used by the cells to maintain their Na balance. The method employed was the same one used in the experiments described in Fig. 5 except that instead of taking samples at several times, we took them only at 3 and 10 min or at 5 and 12 min. This procedure was adopted because we wanted to start sampling once the fast compartment had vanished, but during a period in which the activity of Na was still high. Also during this short period the composition of the cells is not appreciably altered, so one may apply the derivations made above for steady-state conditions without introducing serious errors. As illustrated in Fig. 5 the rate constant can be measured with acceptable accuracy, so that samples taken from the same initial suspension have little scatter. Yet measurements performed in different months do show variations. To make comparisons easier, the values of the rate

constant obtained in all control experiments were averaged (0.0964 ± 0.0023 min^{-1} (36) experiments) and the values obtained in the different experimental conditions were normalized as follows:

$$\text{normalized } k = k \text{ experimental } \frac{k \text{ control}}{k \text{ average}}.$$

Effect of metabolic inhibitors. Fig. 7 summarizes the results obtained with metabolic inhibitors added 15 min before starting the washout. DNP 10^{-3} M reduces the rate constant for the outflux of Na by 27%. Iodoacetate 10^{-4} M produces an inhibition of 17%. This agrees with the evidence that both the glucolytic pathway and the Krebs cycle are in operation in the epithelium of the frog skin (Huf, Doss & Wills, 1957; Skjelkvale, Nieder & Huf, 1960; Curran & Cereijido, 1965). It also agrees with the observation that the inhibition of a single metabolic pathway does not supress completely the transepithelial transport of Na (Leaf & Renshaw, 1957). Fig. 7 also shows that the two inhibitors acting together reduce the rate constant by 72%, i.e. they produce a stronger inhibition than the sum of their individual effects.

Effects of ouabain. Fig. 8 (column 1) shows the effect of several agents on the rate constant for the outflux of Na. As mentioned before (Fig. 5),

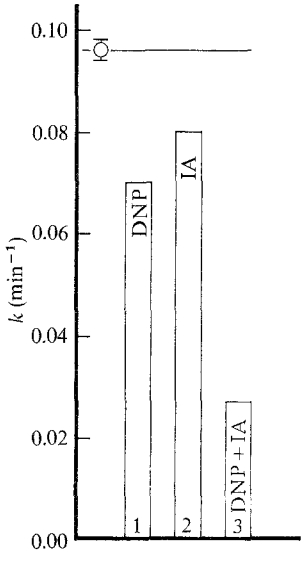

Fig. 7. Effect of metabolic inhibitors on the rate constant of sodium efflux from isolated epithelial cells. The open circle indicates the control value. (1) 10^{-3} M dinitrophenol; (2) 10^{-4} M iodoacetic acid; (3) dinitrophenol plus iodoacetic acid. Each bar represents the mean value of three measurements

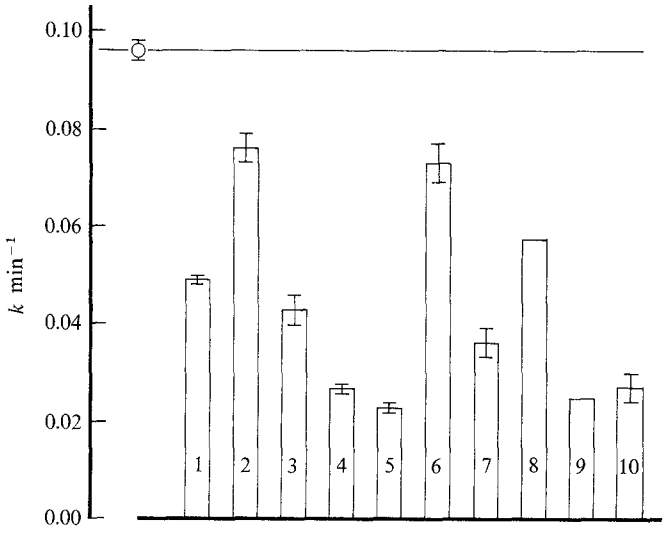

Fig. 8. Effect of several agents on the rate constant of sodium efflux from isolated epithelial cells. The open circle indicates the control value. (1) 10^{-4} M ouabain; (2) without Na^+ in the bathing Ringer's; (3) with ouabain and without Na^+; (4) without K^+ in the bathing solution; (5) with ouabain and without K^+; (6) with 2 mM ethacrynic acid; (7) with ouabain and ethacrynic acid; (8) with ethacrynic acid and without Na^+; (9) with ethacrynic acid and without K^+; (10) without Na^+ or K^+. The abscence of Na^+ or K^+ is replaced with choline. Each bar is the mean of 6 to 15 values, except columns 8 and 9 which are means of 3 values

ouabain reduces the outflux of Na. In the present case we checked that the rate constant is not different whether ouabain is added at zero time or 15 min before starting the washout, and that increasing the dose from the usual 10^{-4} M to 10^{-3} M does not elicit a stronger inhibition.

Removal of Na. Several authors have found that replacement of NaCl in the inner bathing solution for a less permeable solute produces a decrease of the short-circuit current (Mac Robbie & Ussing, 1961; Ussing, 1965; Rabito, Rodriguez Boulan & Cereijido, 1973) which is accompanied by a shrinkage of the cells. Fig. 8 (second column) shows that the removal of Na at zero time reduces k_{CB} by 21%. The amount of cellular water decreases from 3.32 to 2.97 μliters per milligram of dry weight (Table 1) indicating that the reduction of Na in the solution elicits a shrinkage. These results also show that, as mentioned above, the cells lose a significant amount of Na located in a fast compartment (Fig. 5).

The third column in Fig. 8 shows the response to the removal of Na of cells whose pump was inhibited by ouabain. In this case the removal of Na adds little to the inhibition produced by ouabain (compare columns 1 and 3: 49 *vs.* 56%). This suggests that most of the inhibition produced by

Table 1. Water and ion content of epithelial cells incubated in Ringer's solution in which Na^+ was replaced by choline

Min of incubation	Water (μl)	Na (μmole)	K (μmole)	Cl (μmole)
0	3.32±0.01	0.123±0.004	0.443±0.007	0.260±0.007
20	2.97±0.01	0.032±0.004	0.418±0.007	0.214±0.007

Results are given per milligram of dry weight.

removal of Na is elicited by decreasing the active pumping and not by interrupting a Na/Na exchange. Mullins and De Weer (1973) found that trypsin injected into squid axon converts the Na pump into a mechanism of Na/Na exchange. The present observations that 49% of the outflux is sensitive to ouabain and that Na/Na exchange, if present, accounts for a very small fraction of ^{24}Na extrusion, would indicate that the trypsin added to the outside during the preparation of the cell suspension would have a different effect than when added on the cytoplasm side.

Removal of K. Fig. 8 (fourth column) shows that the removal of K decreases the rate constant for Na efflux by 72%. The removal of K from the bathing solution reduces the outflux of Na by 47% in L and LS cells (Lamb & MacKinnon, 1971) and by almost 100% in oocytes (Dick & Lea, 1967). The fifth column shows the response of cells whose pump was inhibited by ouabain. In this case the inhibition reaches 76%; i.e. ouabain adds very little to the inhibition produced by the lack of K. This shows that the removal of K has stopped the pump but, since the inhibition is greater than the one produced by ouabain alone (first column), it indicates that the removal of K is also impairing some other mechanism of Na outflux.

Effect of ethacrynic acid. Whittembury and Proverbio (1970) have shown that the renal cortex has two kinds of pumps: a Na/K one which is sensitive to ouabain and a second one which extrudes NaCl and can be inhibited by ethacrynic acid. Column 6 in Fig. 8 shows that 2 mM ethacrynic acid reduces the rate constant by 24%. The inhibition was no different in cells preincubated 15 min with ethacrynic acid than in cells that were put in contact with the drug et zero time.

The use of ouabain and ethacrynic acid in the same preparation produces an inhibition of 62% (Fig. 8, column 7). Although this is somewhat smaller than the sum of the inhibitions produced by the two

drugs acting individually (49 and 24%), it suggests that they act on two different populations of pumps.

When cells treated with ethacrynic acid are put in a Ringer's solution without Na (Fig. 8, column 8) the rate constant is reduced by 41%. This is close to the sum of the two individual inhibitions (columns 2 and 6: 45%) suggesting that the pump sensitive to ethacrynic acid does not depend on the presence of Na in the bathing solution.

Fig. 8 (column 9) shows that when Na efflux is inhibited by the removal of K from the bathing solution, the addition of ethacrynic acid does not increase appreciably the extent of the inhibition (compare with column 4), indicating that the ethacrynic-sensitive pump was already inhibited. Thus, the absence of K seems to stop both the ouabain-sensitive and the ethacrynic-sensitive pumps.

Removal of K and Na. As mentioned above, the removal of K lowers the value of K by 72% (Fig. 8, column 4). The replacement of Na by choline in the bathing solution does not produce further inhibition (column 10: 72%). In the present case Na removal seems to act on a mechanism that was already inhibited. This is in keeping with the conclusions reached above that the removal of K stops both pumps and the removal of Na affects only the ouabain-sensitive one.

Almost the whole uptake of potassium is carried by a ouabain-sensitive pump, whereas only 72% of the Na outflux is active: 49% carried by a ouabain-sensitive and 24% by an ethacrynic-sensitive pump. This is supported by the observation that combinations of several agents stop some 72% of the flux: DNP plus IA; ouabain plus ethacrynic acid; absence of K; absence of K plus ouabain or plus ethacrynic acid; or absence of K and Na in the bathing solution. The fact that neither ouabain nor ethacrynic acid increase the inhibition produced by the absence of K indicates that both types of pumps need K.

In control conditions the outflux of Na is 0.0043 μmole/min and per mg of dry weight. The ouabain-sensitive pump accounts for 49% of this flux: 0.0021 μmole/min mg. The influx of K (0.0068 μmole/min mg) is carried by a ouabain-sensitive pump. Therefore the Na/K ratio of this pump is 1:3. However, this ratio might not be the one existing when the cell is in the epithelium for at least two reasons: 1) because while all cells seem to handle K in a similar way, they could have great differences in Na permeabilities, i.e. the entire Na flux might be due to only one fraction of the population of cells; and 2) because, as discussed below, the isolated cells might have a higher permeability to Na than they had in the epithelium.

The effect of calcium and EDTA. Curran, Zadunaisky and Gill (1961) and Curran and Gill (1962) have shown that Ca inhibits the short-circuit current across the frog skin. This inhibition achieves and maintains its maximum within 3–4 min. Fig. 9 shows that the increase of Ca^{2+} concentration from control (1 mM) to 6.5 mM produces no decrease in Na extrusion from the cells (first column). This agrees with Curran's interpretation that the inhibition of transepithelial Na transport by Ca^{2+} is not elicited by modifications of the pumping mechanism itself, but by decreasing the penetration of Na across the outer barrier. Fig. 9 shows that the removal of Ca^{2+} plus the addition of 2 mM EDTA (third column) produce a small increase (22–23 %) of the rate constant for Na extrusion. This might be attributed to an increased leakiness of the cells, as Huf *et al.* (1957) have shown that skins incubated with EDTA lose K and gain Na. In the present case though, the cells have been exposed to EDTA for a period starting only 3 min before the measurements of unidirectional fluxes are made, and this time is too short to detect changes in the composition of the cell.

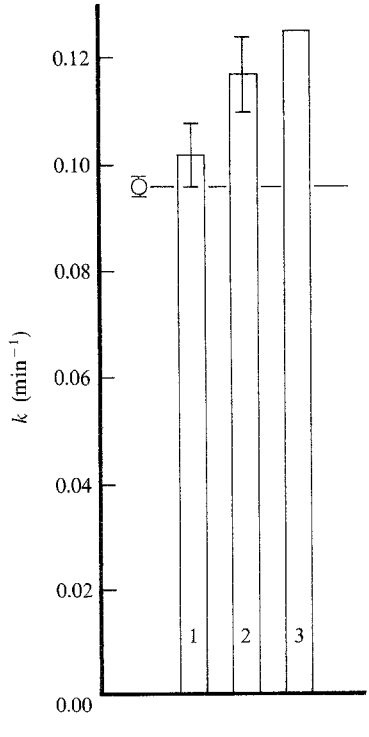

Fig. 9. Rate constant of the washout of sodium from isolated epithelial cells under control conditions: i.e. 1 mM $CaCl_2$ (open circle); 6.5 mM Ca^{2+} (column 1), abscence of Ca^{2+} (column 2) and abscence of Ca^{2+} plus 2 mM EDTA (column 3)

Discussion

The epithelium of the frog skin consists of several layers of cells attached to each other by numerous desmosomes, and to the basal lamina by hemi-desmosomes. Trypsin dissociates the desmosomes yielding unpaired attachment plaques which could be located along the outer membrane surfaces of the cells (Overton & Shoup, 1964; Sedar & Fortes, 1964). Such cleaved plaques often appear to lie in vacuoles, as if they had been endocytized by their cells of origin (Borysenko & Revel 1973; Zylber *et al., unpublished results*).

The procedure employed is an average method in the sense that it could not distinguish whether the preparation is constituted by cells with different transporting properties or if it consists of a homogeneous population. There may even be a third possibility: that all cells behave similarly with respect to a given ion, but would differ with respect to some other one. Thus K^+ seems to be exchanged with a single rate constant (Fig. 1) suggesting that from the kinetic point of view the cytoplasm of all cells constitute a single homogeneous compartment. On the contrary, Na^+ seems to be contained in three different compartments (Fig. 4). One of them is fast and imperturbable by the several conditions tested, and is probably located in a trapped space, not reachable by ^{131}I-albumin. It might be tentatively identified with the sites where the cleaved desmosomes are being endocytized. However, this compartment is even present hours after trypsin has been removed from the solution and its size does not vary during this time suggesting that, if it is related to endocytization, this process halts when trypsin is removed. The remaining two compartments seem to be truly intracellular as one is affected by the different agents tested, and the other does not label its Na within the experimental period.

Assuming that Cl^- is passively distributed and in equilibrium, Zylber *et al.* (1973) have calculated that the cells are 18 mV negative with respect to the bathing solution, a figure which agrees with 18.2 ± 1.7 mV, the value found by Cereijido & Curran (1965) with microelectrodes in short-circuited skins. It may be noticed that even if the isolated cells keep transporting a net amount of Na across themselves, the electrical potential *across* the cell that this might generate would be effectively short-circuited by the bathing solution. Therefore, the electrical potential inside the isolated cells would be equal to the p.d. recorded in cells of short-circuited skins. We may use this potential to compute β, the ratio between the permeability to sodium (P_{Na}) and to potassium (P_K) by using the following

equation (Hodgkin, 1957):

$$\Delta\psi = \frac{RT}{F} \ln \frac{[K]_C + \beta[Na]_C}{[K]_B + \beta[Na]_B} \tag{5}$$

$$\beta = P_{Na}/P_K \tag{6}$$

where $\Delta\psi$ is the electrical potential, R is the gas constant, T is the temperature and F is Faraday's constant. $[K]_B$ is 2.4 mM, $[Na]_B$ is 115 mM, $[K]_C$ is 131 ± 1.4 mM and $[Na]_C$ is 15.8 ± 1.8 mM. The ratio β is then 0.39.

Since the cells are in steady state, the influx and the outflux are equal. If the flux (J_{ij}) between compartments i and j occurs by diffusion and the electric field across the membrane is constant then

$$J_{ij} = P z [i] \left[\frac{-F\Delta\psi/RT}{1 - \exp(zF\Delta\psi/RT)} \right], \tag{7}$$

$[i]$ is the ion concentration in compartment i. Taking $\Delta\psi = 18$ mV and the value of potassium efflux equal to the potassium influx found in this paper (0.0068 μmole min^{-1} mg^{-1}) we may compute P_K as 7.6×10^{-5} liters/min mg. A similar calculation carried out for influx of Na^+ using the value of $J_{Na} = 0.0043$ μmole min^{-1} mg^{-1}, gives $P_{Na} = 2.7 \times 10^{-5}$ liters/min mg. These values of P_{Na} and P_K can be used to compute β with Eq. (6). It gives 0.34, which is in good agreement with the value calculated above on the basis of the electrical potential.

When the cells are in the epithelium their membranes constitute the "inner facing barrier" (except for the outer face of the outer cell layer). Since the frog skin has several layers of cells, only a small fraction of the cell membrane of about a fifth of the population of cells is expected to have the properties of the "outer facing barrier". Therefore the membranes of the cells studied are expected to reflect mainly the characteristics of the inner facing barrier. One of these characteristics is a very low Na-permeability. It is therefore surprising that we found that P_{Na} is as high as 34–39 % of P_K. The opinion generally held, that P_{Na} should be negligible, is based on the demonstration that the electrical potential of epithelia like the frog skin and the urinary bladder is almost unsensitive to changes of the Na concentration of the inner bathing solution (Koefoed-Johnsen & Ussing, 1958). Yet recent observations of Chowdhury (1973) and Finn (1974) in frog skin and urinary bladder indicate that the electrical potential is mainly related to the work of the pump and is relatively independent of the ion gradients and specific permeabilities of the barriers. Another observation taken as a supporting evidence of the low Na permeability of the inner barrier is that isolated epithelia exposed for 15, 30 or 60 sec

to ^{24}Na on the inside take up a similar amount of tracer, and that this corresponds to the extracellular space (Biber *et al.*, 1972). It was expected that, in spite of delays and scattering introduced by the extracellular space, if sodium permeability was similar to potassium permeability the value taken along the interval of 45 sec would not fall on a horizontal line but would show a positive slope. This constitutes the main experimental support of the view that the inner barrier has a negligible Na permeability. However, subsequent work from the same laboratory (T. Biber, *personal communication*) have demonstrated that inulin is not a good marker of the ECS and, therefore, estimates of Na penetration based on ^{24}Na and inulin measurements may not be reliable. On the other hand, Rawlins *et al.* (1970) have shown that the electrical resistance of the inner strata of the epithelium rises from 420 ± 60 to $780 \pm 160 \Omega cm^2$ when K is substituted by Na in the bathing Ringer's, indicating that even when these strata are less permeable to Na they have an appreciable Na conductivity. Another indication that the "inner facing barrier" might not have a negligible P_{Na} comes from the observation of Herrera (1971) that when the epithelium of the urinary bladder increases its Na content upon ouabain treatment, this Na enters from the inner bathing solution. Also, Aceves and Erlij (1971) found that when Na is added to the inner bathing solution 11.1 ± 1.34 out of 24.7 ± 4.17 (8) mmoles of Na per liter of cellular water is labelled (45%). This figure agrees with our opinion that, of all the Na assumed to be intracellular (0.068 μmole/mg) only 57% (i.e. 0.039 μmole/mg) is exchangeable. We may conclude that the information available on the permeability to Na of the inner barrier of whole epithelia is still incomplete and that comparisons with the data of the isolated cells obtained in this work are not possible.

With respect to K^+ the situation is different and the data available permit some comparisons. The epithelium is some 70 μ thick. Assuming a density of 1.1, a square centimeter of fresh epithelium weighes 7.7 mg. The wet/dry weight ratio of the epithelium is 4.32 (Zylber *et al.*, 1973). Then a square centimeter of epithelium has 1.88 mg of dry weight. With this data the K influx found in the isolated cells (0.0068 μmole/mg min) can be converted to 0.75 μmole per hour and square centimeter of the epithelium. This figure can now be compared with the unidirectional K-flux from the inner bathing solution to the cell found in whole skin and in isolated epithelia by several authors. Working with whole skin Curran and Cereijido (1965) found 1.04 μmole hr^{-1} cm^{-2} and Candia and Zadunaisky (1972) found 0.3 μmole hr^{-1} cm^{-1}. This last figure might be underestimated as Candia and Zadunaisky introduced no corrections

for dampings due to the presence of connective tissue. In isolated epithelium, Biber *et al.* (1972) found 0.312 μmole hr^{-1} cm^{-2}. Therefore the values of J_K found in isolated cells are those expected from measurements in whole skin and in isolated epithelium. Trypsin does not seem to modify the permeability to potassium of the epithelial cells.

A similar calculation carried out for the Na flux indicates that a piece of epithelium 70 μ thick will have a J_{Na} of 0.47 μmole hr^{-1} cm^{-2}. Unfortunately, there are at present no values of J_{Na} (measured from the inner bathing solution to the cells) in whole epithelia available to make comparisons.

Trypsin makes the membrane of chick embryo hearts lose its sensitivity to tetrodotoxin (McDonald, Sachs & Dehaan, 1972) and when injected into the cytoplasm, it transforms the Na-pump into a ouabain-sensitive Na – Na exchange mechanism (Mullins & De Weer, 1973). The main structural changes produced by trypsin are the removal of glycoproteins (Winzler *et al.*, 1967) and a clustering of sites sensitive to concanavalin A (De Petris, Raff & Mallucci, 1973). This opens two main possibilities. The first one is that trypsin, besides being a valuable tool for obtaining free epithelial cells, would make them permeable to Na. This would not be due to an unspecific leaking of the membrane, as it is sensitive to agents like K^+, ouabain, ethacrynic acid, DPN and IA, and does not seem to increase the permeability to K^+. The fact that trypsin acts on glycoproteins together with the permeabilizition of the "inner barrier" to Na would suggest that the low Na-permeability attributed to this barrier would be related to the presence of glycoproteins. It is conceivable that when the cells mature and arrive at the outermost cell layer of the epithelium, the portion of their membrane in contact with the outer environment loses its glycoproteins thereby acquiring the characteristic high Na-permeability of the "outer facing barrier".

The second possibility is that trypsin would not alter the permeability to Na. The population of cells obtained by trypsinating the epithelium maintains its Na^+, K^+, Cl^- and water balance (Zylber *et al.*, 1973), and the ionic concentrations found are similar to those found in epithelia isolated with collagenase (Aceves & Erlij, 1971) or with trypsin (Rotunno *et al.*, 1973) indicating that trypsin, at the concentration and with the procedure used in this study, does not impair the membrane mechanisms which, as seen in this paper, remain sensitive to most agents known to modify transport phenomena. Therefore, the possibility exists that the value of P_{Na} that we find both, by measuring fluxes and through considera-

tions on the electrical potentials, would reflect the relationship between P_{Na} and P_K existing in whole epithelia.

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