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Barriers to Sodium Movement across Frog Skin

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Summary. The aim of this paper is to obtain information on the number, nature and location of the barriers to Na movement across the frog skin, and on the size and location of the Na-pool that might be contained between these barriers. On the basis that Na penetrates passively across an outer barrier, and is actively extruded across an inner barrier which is impermeable to passive movements of Na, we expected to detect at least the Na-pool of a single cell layer containing some 10^{-8} moles per cm^2 of epithelium (i.e., in a cell layer 5 μ thick and with 21 mM Na). Yet no Na-pool with these characteristics was found. The method employed could have detected a Na-pool at least an order of magnitude smaller than the one expected. It is concluded that either a Na-pool does not exist (except for the Na bound to the mechanisms operating the translocation), or else that the Na-pool is contained between barriers with different characteristics than the ones assumed above. In the first case, Na transport *across* the epithelium would consist of a translocation across a single asymmetrical functional "barrier". In the second case, the experimental results would require that ouabain either directly (by inhibiting an active step) or indirectly (through a mediated decrease of the Na permeability of the outer barrier) prevents Na penetration at the outer border.

The asymmetry of epithelial membranes like the frog skin is generally understood on the basis of two barriers of different ionic selectivity: an *inner barrier* which is permeable to K^+ but impermeable to passive movements of Na^+, and an *outer barrier* that is permeable to Na^+ but impermeable to K^+. With respect to what is in the middle of the two barriers, a variety of suggestions has been made: *a*) the cells of the *stratum germinativum* (Koefoed-Johnsen & Ussing, 1958); *b*) all cells in the epithelium (Ussing & Windhager, 1964; Biber, Chez & Curran, 1966; Farquhar & Palade, 1966); *c*) the outermost cell layer which is not yet cornified (Vôute & Ussing, 1970); and *d*) the polar groups of the plasma membrane of the epithelial cells (Cereijido & Rotunno, 1968).

Studies carried out in this laboratory have indicated first that at least 63% of the Na contained in the skin does not participate in the process of Na transport across (Cereijido & Rotunno, 1967), and then that the fraction that does participate is less than 10% (Cereijido, Reisin & Rotunno, 1968). Zerahn (1969) has subsequently shown that the fraction is in fact so small that it could escape detection by the methods so far employed. Yet this line of evidence appears to be contradicted by two sources of information: 1) Results obtained by washing out the ^{22}Na of epithelial membranes preloaded during at least 45 min, indicate that part of this ^{22}Na comes from a cellular compartment which might constitute a "transporting pool" (Schwartz & Snell, 1968; Nagel & Dörge, 1970; Vanatta & Bryant, 1970; Finn & Rockoff, 1971). 2) The electrical potential profile of the frog skin, as studied with microelectrodes, suggests that Na is transported from one cell layer to the inner neighboring one across most of the epithelium (Cereijido & Curran, 1965; Biber *et al.*, 1966; Rawlins, Mateu, Fragachan & Whittembury, 1970). If the view that Na is transported across the cytoplasm of epithelial cells were correct, this electrical profile would require that the Na transporting pool would consist of Na in the cytoplasm of all the cells in all the layers.

Therefore, the present status of the problem shows two main uncertainties: 1) Is the symmetry of epithelial membranes due to the presence of *two* anatomically and functionally different barriers, or to the asymmetry of a single step? 2) Should there be two barriers: what is the size and location of the Na pool contained in the middle? The aim of the present work is to obtain information that could help to answer these questions.

Materials and Methods

The abdominal skin of the local frog *Leptodactylus ocellatus* was used throughout. Animals of both sexes, at all seasons were studied at 20 to 22 °C. Unless otherwise stated (e.g., in some wash-out experiments), the inner bathing solution had (mM): 115 NaCl, 2.4 $KHCO_3$, 1.0 $CaCl_2$, and 2.0 glucose. The different outer solutions used in each case are specified below. The osmolarity of the different Ringer's was kept constant by adding sucrose. When gassed with air, the solutions had a pH of 8.2.

Influx of Na (J_{13})

The skin was mounted as a flat sheet between two Lucite chambers (5.0 ml each) with a circular exposed area of 3.14 cm^2. The electrical potential difference was measured by connecting the chambers through agar-Ringer's bridges to calomel half-cells and these cells to a Keithley 200B d–c electrometer. Short-circuit current was measured periodically. Fluxes were measured under open circuit. After a period of equilibration,

2.0 μC of ^{22}Na was added to the outside chamber. Duplicate samples of 250 μliters were withdrawn periodically from the inside bathing solution and the volume replaced with fresh Ringer's. At the end of the experiment, samples were taken from the outside bathing solution and analyzed for Na and ^{22}Na.

Unidirectional Flux Outside → Epithelium (J_{12})

This technique was designed and described in detail by Rotunno, Vilallonga, Fernández, and Cereijido (1970) and modified by Cereijido, Moreno, Rodríguez Boulan, and Rotunno (1972). The following is a summarized description. The abdominal frog skin is mounted as a flat sheet between two Lucite chambers (Fig. 1). The exposed part of the skin has a rectangular cross-section. After a preincubation period with nonradioactive Ringer's, the chambers are emptied and a dual simultaneous infusion pump injects Ringer's solution containing tracer into the outer chamber and nonradioactive Ringer's into the inner chamber. The solutions are injected steadily into the bottom of the chambers and the level rises at a constant rate for 48 sec. When the solution reaches the upper border and the exposure is interrupted, the lowest part of the membrane has been exposed for the whole time of the injection (48 sec). All other parts have been exposed for a fraction of 48 sec. This fraction is proportional to the distance to the floor of the chamber. Once the exposure is interrupted, the outer face of the skin is washed with a jet of isotonic sucrose solution at 0 °C delivered with a squeeze bottle during 2 to 3 sec (15 to 20 ml). This procedure constitutes a modification of the method originally described by Rotunno *et al.* (1970) since in their case the rinsing was reduced to plunging the skin deep into a beaker with cold sucrose solution for 1 sec. The skin is then immersed into a thermos flask containing liquid nitrogen (−196 °C). The central part of the skin is cut into several transverse sections, each representing a certain uptake time of tracer. In previous papers (Rotunno *et al.*, 1970; Cereijido & Rotunno, 1971)

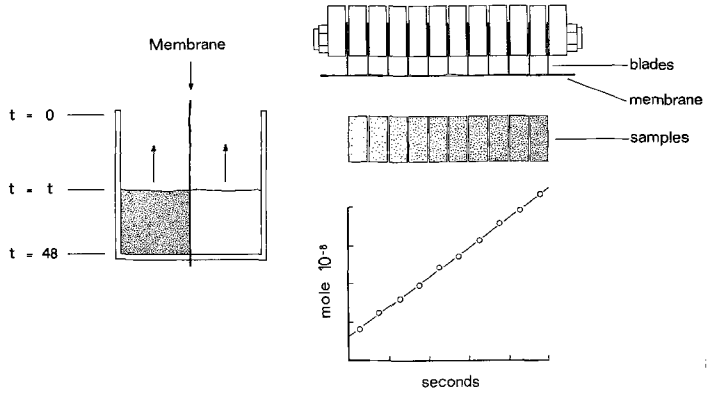

Fig. 1. Diagrammatic representation of the method used to measure J_{12}. After a period of preincubation in the desired Ringer's solution, the chambers are emptied. A simultaneous infusion pump injects Ringer's with ^{22}Na on the outside and nonradioactive Ringer's on the inside. The level rises steadily in 48 sec, so that when the level reaches the top, and the injection is interrupted, the lowest part was exposed for 48 sec and the rest was exposed for a progressively shorter period. A central strip (10 mm wide) of the exposed part is cut with a series of blades at 5 mm from each other. The amount of ^{22}Na in the samples is plotted *vs.* the exposure time (i.e., the distance to the top of the chamber)

the exposure time of a given sample was calculated as follows: the dry weight of the sample was added to the weight of all previous samples (i.e., from the coldest [top] to the given sample). This value was divided by the weight of all the samples. The fraction obtained multiplied by the total infusion time gave the time that the sample was exposed to the radioactive solution. This procedure was time consuming and relied on the constancy of the area/dry weight ratio. In the experiments reported in the present paper, the central strip of skin (1.0 cm wide) is cut with the device depicted in Fig. 1. It consists of a set of stainless steel blades held 5 mm apart from each other by small Lucite blocks; each sample, therefore, has 0.5 cm^2. This device was kept in a thermos flask with dry ice until it was needed to cut the central strip of membrane. The samples are analyzed for their content of tracer and a kinetic curve, showing the uptake of ^{22}Na as a function of time, is plotted. The slope of the curve is used to calculate the unidirectional flux from the outer solution to the epithelium (J_{12}).

Counting was done with a Nuclear Chicago Auto Gamma. Sodium was measured in an EEL flame photometer.

Results are expressed as mean $\pm$ standard error (number of observations).

Results

Part I

The Transepithelial Influx of Sodium (J_{13})

Fig. 2 (full circles) shows the value of J_{13} as a function of the concentration of Na on the outside bathing solution. The concentration of Na in the inner bathing solution was always 115 mM. The hyperbola of Fig. 2 has a half saturation value Km of 22.1 mM and J_{13}^{max} of 2.7 μmoles per hr

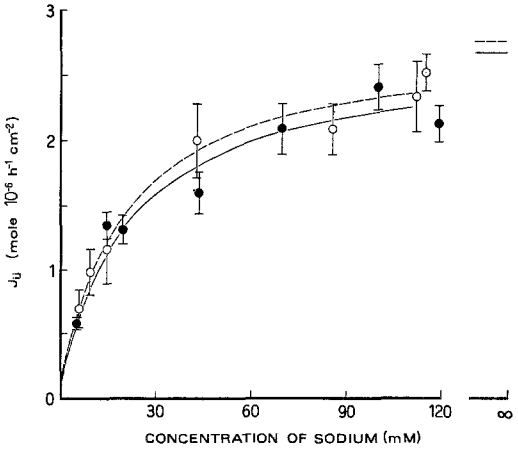

Fig. 2. Fluxes of Na across the outer border (J_{12}, open circles, dashed line) and across the whole skin (J_{13}, full circles, full line) as functions of the concentration of Na in the outer bathing solution. Each point is an average of 4 to 10 experiments. The values of Km and J_{ij}^{max} (asymptotes at $[Na]_0 = \infty$) calculated by the Lineweaver-Burke method for J_{13} were 19.1 mM and 2.79 μmoles per hr cm^2, and for J_{12} were 22.1 mM and 2.70 μmoles per hr cm^2

cm^2. This saturating behavior of the influx or the short-circuit current of the frog skin was observed previously by other workers (Kirschner, 1955; Morel, 1958; Cereijido, Herrera, Flanigan & Curran, 1964). The experimental value of J_{13} at 119 mM is 2.14 μmoles per hr cm^2 which compares with the value found by Curran, Herrera, and Flanigan (1963) in *Rana pipiens* (1.4 μmole per hr cm^2), by Zadunaisky, Candia, and Chiarandini (1963) in *Leptodactylus ocellatus* (3 μmoles per hr cm^2) and by Cereijido and Rotunno (1971) in a previous study (4.19 μmoles per hr cm^2).

The Flux of Sodium Across the Outer Border of the Epithelium (J_{12})

These experiments were made simultaneously and in the same shipments of frogs as the ones used for the measurement of J_{13}. Different shipments of frogs show considerable variations in the values of J_{12} and J_{13}. Fig. 3 illustrates the uptake curves obtained at several concentrations of sodium on the outside.

As pointed out by Martínez-Palomo, Erlij, and Bracho (1971), the existance of an extracellular space ECS on the outside might introduce serious errors in the evaluation of J_{12}. This stems from the fact that by dividing the amount of Na taken up by the skin over a short period of time, by the time of uptake one includes in the resulting flux the amount of Na trapped in the ECS, thus obtaining an overestimated value of J_{12}. Since Na does not reach directly the "outer barrier" of the epithelium, but has to travel first across the ECS, the delay in reaching the outer barrier might constitute

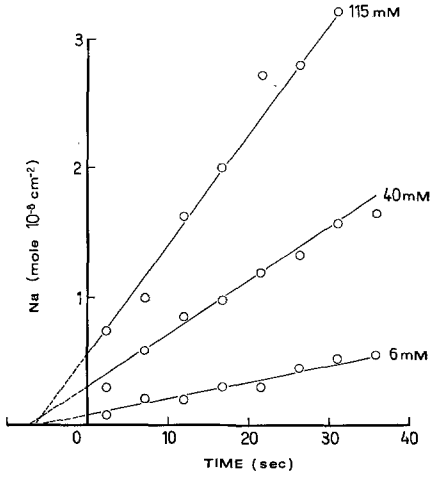

Fig. 3. The uptake of Na as a function of time at several concentrations of Na on the outside. Each line corresponds to a single experiment. Notice that they intercept the time axis at about −6 sec

another source of error. Also, in a skin standing vertically, when the loading solution is sucked out of the chamber at the end of the exposure to ^{22}Na, the solution wetting the outer side drains downwards. Its contribution to the measured uptake becomes proportionally larger as the samples approach the floor of the chamber. If the rinsing were not sufficient, this would constitute a serious source of error, as it would modify not only the intercept but the *slope* of the curve as well. As discussed by Rotunno *et al.* (1970) the delay of ^{22}Na in achieving the same concentration at the level of the Na^+-sensitive barrier that it has in the outer bathing solution is less than a quarter of a second (Kidder, Cereijido & Curran, 1964; Dainty & Gouse, 1966; Lindemann & Thorns, 1967). Since the period covered by even the first sample taken with our technique (4.8 sec), is much longer than the transient of equilibration, the error that this transient might introduce may be disregarded.

The original version of the technique included the plunging of the frame with the skin in cold sucross solution for 1 sec. However, to avoid errors caused by the ^{22}Na that might be trapped in the ECS, we have sought a more rigorous way of rinsing, and the jet washing procedure described in Materials and Methods was adopted. As discussed elsewhere (Cereijido *et al.*, 1971), in this way all the ^{22}Na used to compute J_{12} represents ^{22}Na that has crossed a Na-sensitive barrier and which has the properties attributed to the "outer barrier". A similar conclusion will also be reached from the experiments shown below, in particular from those in Part II. In preliminary experiments we noticed that, provided it is ice cold, the washing with isotonic sucrose might be continued for at least 9 sec, without changing the slope of the uptake curve. This is expectable as it was observed that the flux of water (Grigera & Cereijido, 1971) and sodium (Rodríguez Boulán, Moreno, Rotunno & Cereijido, 1972, *unpublished*) (J_{12}^{THO} and J_{12}^{NA}) have a high activation energy.

The nonzero intercept of the Na curve was observed also in studies reported in previous papers but did not receive special attention. This was because of some uncertainty with respect to the level reached by the loading solution during the measurement of J_{12}. Although the mark left by the chamber in the lower part of the skin indicates precisely the limit of the first sample (i.e., the one exposed for the longest time) the upper limit reached by the rising loading solution leaves no marks. Since this fact does not impair the accurate estimate of the slope (hence no influence on the value of J_{12}) it remained obscure. In the present studies, the level reached was carefuly recorded. This confirmed the fact that the uptake curves have a nonzero intercept, and that this intercept increases with the value of

J_{12} (which is obtained from the *slope*). In trying to find an explanation for this nonzero intercept, we have measured the time elapsed from the interruption of the loading until plunging the skin into liquid nitrogen. It was always between 4 and 8 sec, typically 7 sec. Of this period, only the last 2 to 3 sec were devoted to the rinsing with cold isotonic sucrose. The previous 3 to 6 sec were spent in the suction of the tracer, the opening of the chamber, the removal of the frame from its position between the chamber, catching the squeeze-bottle, etc. During these 3 to 6 sec, the outerside is wet with loading solution and the skin keeps taking up Na. In support of this proposed origin of the intercept is the fact that, regardless of the value of the slope (and therefore of J_{12}), the curves always intercept the time axis at -5 to -8 sec.

Fig. 2 (open circles) shows J_{12} as a function of the concentration of Na on the outside. It can be seen that as the concentration of Na is raised, J_{12} tends to saturate. The saturation of the rate of entry of Na into the epithelium as the concentration increases was also shown by Cereijido *et al.* (1964) through an analysis of the kinetics of the transepithelial movement of ^{24}Na. The hyperbola fitting the experimental points has a Km of 19.1 mM and a J_{12}^{max} of 2.79. This value of J_{12} compares with the value of 2 μmoles per hr cm^2 calculated indirectly by Cereijido *et al.* (1964) for the flux of Na penetrating into a Na transporting pool. Also, the half-saturation value (19.1 mM) compares with 10 mM which is the value of Km found by Cereijido *et al.* (1964). As mentioned above, the method used by Biber and Curran (1970) and the original version of the technique employed in the present work overestimated J_{12} because it included the ^{22}Na trapped in the ECS. This flux appeared to be composed of two fractions: a saturating one with a Km of 14.3 mM and a J_{12}^{max} of 4.0 μmoles per hr cm^2 (Biber & Curran, 1970), and another fraction which increased linearly with the concentration of Na on the outside. Only the saturating fraction appeared to be related to the transport of Na across the skin. The linear fraction would represent Na penetrating in a compartment which is not directly related to Na transport, and roughly two-thirds of this amount would be constituted by Na in the outer ECS (Biber, Cruz & Curran, 1972). The curve of Fig. 2 does not have a linear component. It represents Na actually penetrating through a Na transporting mechanism. This stems from the fact that J_{12} should necessarily be as large as J_{13}. The kinetics parameters of J_{12} (Km and J_{12}^{max}) are similar to the ones of the saturating component of J_{12} found by Biber and Curran (1970).

Fig. 4 shows the results obtained with two skins at 115 and at 1.2 mM, respectively. In these experiments, the level of the loading solution was not

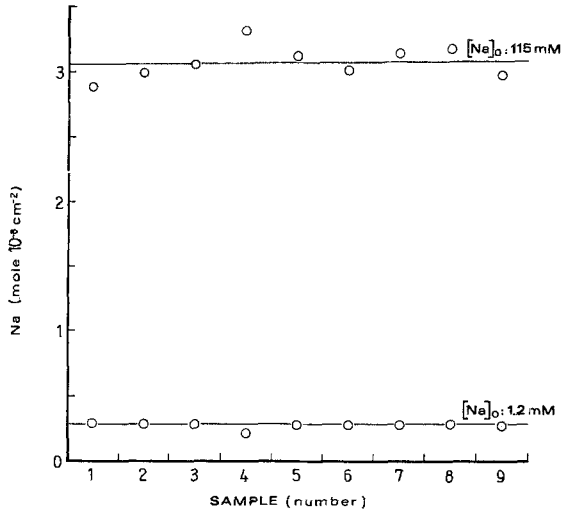

Fig. 4. Uptake of Na as measured with the technique depicted in Fig. 1, except that the whole area was exposed during the same length of time (44 sec). The concentration of Na was 115 mM (upper curve) and 1.2 mM (lower curve). Each point is an average of six experiments

progressively raised in 48 sec, but the whole area was exposed during 44 sec and then sampled as usual with the device described in Fig. 1. The purpose of these experiments was to check further whether the slope of the uptake curve used to compute J_{12} is a good estimate of this process. The uptake in 44 sec was 3.1×10^{-8} mole per cm^2 in 115 mM, and 0.28×10^{-8} mole per cm^2 in 1.2 mM Na. The corresponding fluxes are therefore 2.54 and 0.07 μmoles per hr cm^2; i. e., they agree with the values found at these concentrations using the slope of the uptake line which are shown in Fig. 2 (2.26 and 0.13, respectively). Another group of experiments to check this point was run in a somewhat different way: two groups of skins were exposed for 48 and 20 sec, respectively. They were not rinsed, but just blotted. Therefore, in both groups of skins the uptake of Na by the tissue was overestimated by the same amount of Na contained in the ECS. The *difference* in the amount of Na taken up by the two groups divided by the difference in the length of exposure to tracer gave the net influx. The uptake in 20 sec was $4.7 \pm 1.9 \times 10^{-8}$ and at 48 sec was 6.7 ± 2.2 mole per cm^2. Therefore, the corresponding influx is 2.57 μmoles per hr cm^2. This result is similar to those obtained with the procedure described in Fig. 1.

There is remarkable agreement between the curves relating J_{13} and J_{12} to the concentration of Na on the outside. In fact, the two curves of Fig. 2 fall within the experimental error of each other. J_{12} equal to J_{13} is a neces-

sary requirement to demonstrate the existance of a single barrier. However, models consisting of two membranes could also show $J_{12}=J_{13}$. It can be shown that $J_{12}=J_{13}$ may be observed with the two-barrier model when $J_{21}=0$ so long as $J_{23}-J_{32}=J_{12}$ in the steady state.

From these considerations one may conclude that the demonstration that $J_{12}=J_{13}$ fulfills a necessary but not sufficient requirement to support the existance of one barrier model.

Part II

The Effect of Amiloride and Ouabain

The aim of this section is to use the technique depicted in Fig. 1 and the action of amiloride and ouabain to try to detect the Na-pool that might be involved in transepithelial transport.

Estimation of the size of the Na-pool that would be involved. The concentration of Na in the epithelial cells has been evaluated through a variety of techniques: 1) *Isotopic methods in whole skin or slices of epithelium*: 21 mM (Cereijido *et al.*, 1968); 35 mM (Zerahn, 1969). 2) *Isolated epithelium*: 24.7 mM (Aceves & Erlij, 1971). 3) *Isolated epithelial cells*: 22 to 28 mM (Zylber, Rotunno & Cereijido, *unpublished observations*); 32 mM (toad bladder; Gatzy & Berndt, 1968). Taking the minimum estimate (21 mM), and considering that only the outermost cell layer (some 5 to 10 μ thick) participates in transepithelial transport (Vôute & Ussing, 1970), there would be some 10^{-8} mole of Na per cm^2 of skin in the Na transporting pool. Estimates made by other authors were (in 10^{-8} mole cm^2): 0.9 (Aceves & Erlij, 1971); 70 (Hoshiko & Ussing, 1960); 93 to 172 (Andersen & Zerahn, 1963); 150 (Curran *et al.*, 1963; Cereijido *et al.*, 1964). Most of these values are actually estimates of the amount of Na achieving the same specific activity as the Na tracer in the loading solution. Nevertheless, on the basis of these observations one must be prepared to detect at least 10^{-8} mole per cm^2. The procedure will be based on the comparison of the effects of amiloride and ouabain.

Amiloride when added to the outside is known to block the entry of Na from the outer solution into the transport pool (Eigler & Crabbé, 1968; Dörge & Nagel, 1970; Salako & Smith, 1970; Biber, 1971; Gebhardt, Fuchs & Lindemann, 1972). Ouabain, instead, inhibits Na translocation when it is added to the inside by acting on the Na-pump which is assumed to be located at the inner barrier (Koefoed-Johnsen, 1957; Zadunaisky *et al.*, 1963; Herrera, 1968).

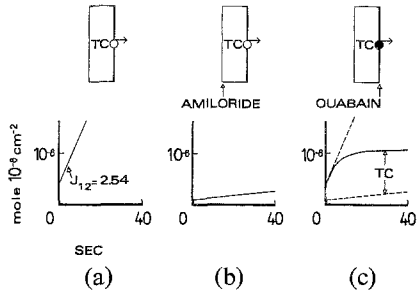

Fig. 5. Project of the experiment in Part II. *Top line:* kinetic model with an outer barrier permeable to Na and sensitive to amiloride, and an inner barrier impermeable to Na. The second barrier contains an ouabain-sensitive active step. *Bottom line:* schematic representation of the results expected from such kinetic model if it were applicable to the outermost cell layer containing 10^{-8} mole of Na per cm^2. The line in *a* corresponds to the intercept and flux of control J_{12} found experimentally in Fig. 6 (open circles)

The underlying idea of the procedure used in this section is as follows (Fig. 5): A control skin in 115 mM Na on the outside usually has an uptake with an intercept at 4 to 5×10^{-9} mole per cm^2 and a slope (J_{12}) of 2 to 3 μmoles per hr cm^2 (Fig. 5*a*). Amiloride is expected to block the Na entrance to the Na transporting pool. Ideally, if this blockage is complete, the curve would be horizontal (Fig. 5*b*). On the other hand, ouabain is thought to inhibit the pump at the inner barrier. The uptake is expected to proceed somewhat as in a control experiment until the ^{22}Na contained in the sodium in the pool achieves a steady specific activity (Fig. 5*c*). Notice that under the effect of ouabain the pool is expected to be at least 10^{-8} mole per cm^2. It might, in fact, increase making the difference with the uptake under the effect of amiloride even larger. In other words: since, according to current two-barrier models, amiloride and ouabain are supposed to act on the different ends of the transporting pool, the difference between the uptakes in the presence of ouabain minus the uptake in the presence of amiloride could be an estimate of the pool (TC).

Fig. 6 shows the experimental results. All experiments were made with Ringer's containing 115 mM Na^+. The values were obtained with the technique of Fig. 1. This time, though, instead of using the points in each experiment to calculate the slope and the flux J_{12}, a different manipulation of the data was done. All points at a given period were averaged and a straight line was fitted by the least-square method. The control curve (open circles) has a slope (J_{12}) of 2.54 μmoles per hr cm^2, and an intercept of 0.43×10^{-8} mole per cm^2. The curve obtained with 2×10^{-5} M amiloride outside (triangles) has a slope of 0.61 μmole per hr cm^2, and an intercept of 0.24×10^{-8} mole per cm^2. Thus, at the concentration used, amiloride inhibits the flux

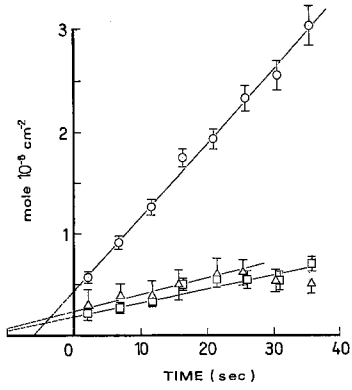

Fig. 6. Uptake of Na under control (open circles), amiloride (triangles), and ouabain (squares). The corresponding values of J_{12} are: 2.54, 0.61 and 0.49 μmoles per hr cm^2

J_{12} by 76%. This is a further indication that the ^{22}Na taken up by the skin, and which is used to compute the flux J_{12} represents Na which has actually crossed the outer barrier and is not contained in an ECS openly connected with the outer bathing solution. Ouabain, 10^{-5} M, added to the inner solution (squares) produces also a considerable inhibition of J_{12} (81%): the slope is 0.49 μmole per hr cm^2 and the intercept is 0.18×10^{-8} mole per cm^2. The difference between the intercept of the amiloride and the ouabain curves is 6×10^{-10} mole per cm^2 which is not only insignificant, but is oriented in sense opposite to that expected on the basis of the argument in Fig. 5. This amount of Na is roughly 1/17 of the minimum amount of Na that we expected to detect in a transporting compartment. Also, if the overall concentration of Na in the cells is, say, 25 mM, a square centimeter of epithelium 60 μ thick would contain 1.5×10^{-7} mole of Na. The comparison of this figure with the estimates of Fig. 6 suggests that, if the Na pool actually exists, it would contain a very small fraction of the epithelial Na.

When the Na concentration is high, the permeability to Na of the outer barrier decreases (Cereijido *et al.*, 1964). If this permeability were also inversely related to the Na content in the cells, it might be expected that ouabain, by inhibiting a pump at the inner border, would increase the Na content in the cells and that this increase would, in turn, switch off the Na permeability of the outer barrier. Thus, while the site of ouabain inhibition would primarily be located at the inner border, its overall effect would be evidenced at the outer border. This would also be in keeping with the experimental results.

Discussion

The results obtained in the present study could be best explained by the existance of a single asymmetrical barrier which would be sensitive to amiloride added to the outer solution and to ouabain added to the inner solution. "Barrier" is taken here in a very broad sense, meaning a step or series of steps without the involvement of an appreciable amount of Na. Of course the results could also be understood on the basis of models with two or more barriers. However, the two barriers of the kinetic model needed might not have the properties specified in Fig. 5; i.e., a Na-permeable barrier on the outside, and a Na-impermeable one on the inside with an active step, in particular if the Na contained between the two barriers consists of the whole amount of Na in the epithelium. If the Na-pool were consituted only by Na in the first layer of cells (*stratum granulosum*), and if this amount can be calculated on the basis of the average concentration of Na in the cells (22 to 28 mM) and the thickness of this layer (5 to 10 μ), there will be some 1.1 to 2.8×10^{-8} moles of Na per cm^2 in this layer. The experiment described in Fig. 5 and summarized in Fig. 6 indicates that either the Na in the cytoplasm of the cells in this layer is not involved in transepithelial transport, or else that this Na is not contained between two barriers with the properties mentioned above.

Studies of the effect of ouabain on the flux of Na across the outer border (Biber & Curran, 1970; Nagel & Dörge, 1971), and on the accumulation of Li^+ (Leblanc & Lemonnier, 1971) suggest that the active step in Na transport might be located at the outer surface of the epithelium. If these observations can be taken as evidence of an active step in the outer barrier instead of on the inner one, then experiments in Part II of this paper would receive a plausible explanation. They would also agree with results obtained by Rotunno, Pouchan, and Cereijido (1966) indicating that Na can penetrate in a net amount from an outer solution with 1 to 10 mM Na into an epithelium with an overall Na concentration of 70 to 90 mM in the presence of a small electrical potential of 15 to 18 mV (cytoplasm negative). The interpretation made by these workers that an active step was needed at the outer border (Fig. 7, black pumps), met two major objections: 1) that epithelial Na was not contained in a homogeneous compartment but in three kinetically different ones (Cereijido & Rotunno, 1967; Cereijido *et al.*, 1968) and therefore one could not calculate how much of the epithelial Na^+ was actually contained inside the cells, and 2) that the binding of Na in the cytoplasm could lower considerably its chemical activity, thus decreasing the potential that Na should overcome to be able to penetrate from the outer bathing solution. This objection was based on the fact the amplitude

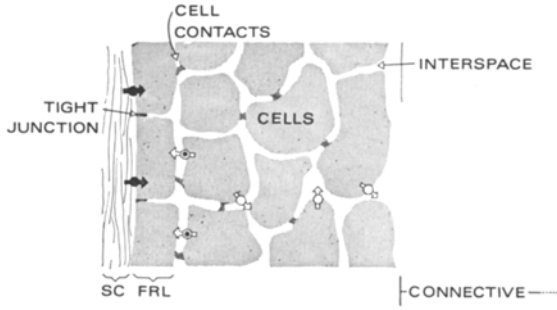

Fig. 7. Schematic representation of the epithelium of the frog skin showing pumps at the several hypothetical locations discussed in the text. SC: *stratum corneum*; FRL: first reactive layer of cells

of the peak of ionized Na^+ in the NMR spectrum of the frog skin was reduced to roughly one-third of the amplitude expected if it were completely free as Na^+ ion (Rotunno, Kowalewski & Cereijido, 1967). The first objection might no longer hold in view of the fact that even when the compartmentation of epithelial Na was later observed by other workers (*see*, for instance, Aceves & Erlij, 1971), the concentration of Na in the cells was found to be 22 to 28 mM (*see* Part II for references). This high concentration does not agree with a passive entrance from a 1 to 10 mM Na and an electrical difference of only 15 to 18 mV (cytoplasm negative), in particular if one takes into account that, while the net entrance is observed under shorted as well as opened circuit, this "helping" electrical gradient is only present under short-circuit conditions (Cereijido & Curran, 1965). As with respect to the second objection, recent studies by Shporer and Civan (1972) and by Edzes, Rupprecht, and Berendsen (1972) indicate that electric field gradients in the cells could cause a quadrupolar splitting of the ^{23}Na signal into three lines, the central of which (accounting for some 40% of the total intensity) being the only one observed. This means that the NMR spectra of ^{23}Na in the frog skin could not be taken as an unambiguous proof that its chemical activity is much lower than in free solution.

In conclusion, an active step at the outer border cannot be discarded and, if it exists, it will agree with a large amount of experimental results, including the ones in the present paper.

The difficulty arises when one tries to imagine where this active step delivers the pumped Na. If Na were delivered into the cytoplasm of the cells, one would be forced to assume another step to translocate this Na across the inner facing barrier (Fig. 7, white pumps). Because of the cell contacts of high Na conductance (Ussing & Windhager, 1964), the Na con-

tained in all cell layers would be labeled. This interpretation would disagree with the results obtained by Cereijido and Rotunno (1967), Cereijido *et al.*, (1968) and Aceves and Erlij (1971) indicating that most epithelial Na does not get labeled during transepithelial transport. However, if the amount of Na pumped by the first layer were much larger than the amount diffusing to inner layers, only the Na in the first layer will behave as participating in the Na transporting pool. This interpretation, though, would disagree with the observations made with microelectrodes in several laboratories, and mentioned in the introduction, which indicate that Na-pumping is not confined to a single cell layer, but is distributed over the whole epithelium. Also, the outermost layer is one of the final chronological steps in the evolution of epithelial cells. Cells in layers other than the outermost are assumed to have their pumps directed toward the interspace. Yet when these cells reach the outer layer a large fraction of their pumps (Fig. 7, star pumps) would have to work in reverse to become an inward pumping mechanism (Fig. 7, black pumps). Although this is not impossible, it would become a problem.

A completely different possibility was discussed by Cereijido and Rotunno (1968). These authors consider that epithelial cells have two different mechanisms to handle Na. The first mechanism, similar to the one in other cells in the organism, would be devoted to the maintenance of the Na balance of the cells themselves. The second one would translocate Na across the epithelium and might not use a transcellular route, but would proceed along the polar groups of the plasma membrane of the epithelial cells. Although these polar groups are distributed across the whole epithelium, the amount of Na that they could accommodate would be so small that the mechanism would be detected as a single translocating step (or "barrier") without a sizable Na-pool. Even when this model would agree with the present results as well as with micropuncture studies indicating that all cell layers participate in Na transport *across*, its main assumptions would require more experimental support.

The information collected in the present study and in the quoted papers, and the different working hypotheses mentioned above agree in, at least, one point: that Na is finally pumped into the interspace. This long, narrow and tortuous space is bordered by a large amount of cell membranes. The information available in frog skin (Aceves & Erlij, 1971) as well as in urinary bladder (Herrera, 1968) indicates that tracer sodium added to the *inside* can penetrate into the cells. Therefore, when tracer sodium is added to the *outside*, is pumped into the interspace, and diffuses inwards in contact with a large area of cell membrane, it may be expected to label some of the

Na contained in the cells. This view is supported by the observation made by Cereijido and Rotunno (1967) that, when the transepithelial flux of ^{22}Na reaches quasi steady state, the specific activity in the skin keeps increasing (although at a much slower rate than during the transient). Hence, the washout of an epithelium which was loaded from the outside during a period up to 10 times longer than the time it takes to equilibrate the flux across, is expected to show that a fraction of the washed Na comes from a cellular compartment (Schwartz & Snell, 1968; Nagel & Dörge, 1970; Vanatta & Bryant, 1970; Finn & Rockoff, 1971), even when this compartment might not have been primarily involved in transepithelial Na transport.

The present results also indicate that when one divides the amount of Na equilibrated with tracer sodium added to the outside, by the total amount of water in the cells, one does not necessarily obtain an estimate of the concentration of Na in the "transporting pool", nor in the cells, nor could one use the value thus obtained to decide where and how Na enters when it penetrates through the outer border of the epithelium.

In summary, the results obtained in the present study suggest that either there is no Na-pool (other than the Na attached to the molecules operating the translocation), or else it is contained between barriers with properties different to the ones generally assumed (e.g., those in Fig. 5).

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