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SODIUM WASHOUT KINETICS ACROSS INNER AND OUTER BARRIERS OF THE ISOLATED FROG SKIN EPITHELIUM

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Summary

^{22}Na washout kinetics were measured from isolated frog skin epithelium that had been differentially loaded from the outer, inner, or both bathing solutions in a 7 cm^2 Ussing-type chamber. Regardless of the side from which loading occurred 96% of the exchangeable Na^+ washed out towards the inner solution. Under the three loading conditions the amount of Na^+ labelled was: (1) 0.33 μequiv./cm^2 from both solutions, (2) 0.16 μequiv./cm^2 from the outer solution, (3) 0.14 μequiv./cm^2 from the inner solution. Tissue Na^+ content determined by flame photometry was 0.33 μequiv./cm^2. Preincubation with ouabain before loading from the outside reduced the labelled Na^+ content to 0.05 μequiv./cm^2. However, when loading occurred from the inside solution, ouabain increased the labelled Na^+ to 0.92 μequiv./cm^2. The Na^+ transfer coefficient (epithelium-inside) was reduced by ouabain from 0.79 to 0.013 min^{-1}. These results show that: (1) the back leak from the epithelium across the outer barrier is negligible; thus the Na^+ uptake across this barrier is nearly equal to the net Na^+ transport; (2) the tissue Na^+ is almost completely exchangeable with the Na^+ in both bathing solutions; and (3) ouabain inhibits both Na^+ uptake across the outer barrier and the pump at the inner barrier, while increasing Na^+ flux from the inner solution into the epithelium.

Introduction

Transepithelial active Na^+ transport has been conceptualized in terms of a three-compartmental model [1—3]. The model consists of an outer and inner compartment separated by a middle compartment having opposing outer and inner barriers. The middle compartment represents all the epithelial cellular lay-

Abbreviation: I_{sc}, short-circuit current.

ers and the outer and inner compartments are the outer and inner bathing solutions, respectively. The cellular compartment may in reality be a multiple-compartment system including several cell layers. Active Na^+ transport includes then several distinct steps: entrance of Na^+ into an epithelial cell layer from the outer bathing solution across an "outer barrier", exit of Na^+ from this cell layer, and movement into deeper cell layers, intercellular spaces and the inner bathing solution. Across each barrier there exists a pair of influx and efflux vectors. In order to elucidate the steps involved in transepithelial Na^+ movement, individual vectors have been measured.

One approach was to determine the Na^+ influx across the outer barrier into the tissue, J_{12}. Some of these measurements gave values for J_{12} ranging between 7.77 and 11.25 μequiv./h per cm^2 [4,5]. These values are much larger than the net transepithelial Na^+ transport (I_{sc}) indicating that the outer barrier was not rate limiting, and suggesting an appreciable back leak, J_{21}. However, when appropriate corrections were made for the extracellular space, values more similar to the net transepithelial Na^+ influx were obtained [6]. The measurement of Na^+ uptake has also been done with a method not requiring correction for the presence of extracellular space. Such a technique has yielded values between 1.20 and 2.54 μequiv./h per cm^2 which are only slightly larger than the net transepithelial Na^+ influx [7,8].

We have taken another approach to the problem by measuring the washout kinetics of ^{22}Na from the isolated epithelium of bullfrog skin (fluxes J_{21} and J_{23}). To do these measurements, the basic requirement is that the cellular Na^+ pool be large enough, so that the washout of ^{22}Na into the respective bathing solutions can be readily measured. This requirement was met by using large sheets of isolated epithelium that contained about 3 μequiv. of Na^+.

We were able to measure ^{22}Na washout kinetics under a variety of loading conditions. Our results indicate that the outer barrier is rate limiting for transepithelial Na^+ transport and almost impermeable to Na^+ back leak. They also suggest that all the Na^+ in the epithelium is free to interchange and behaves as a single pool in communication with either bathing solution. The effects of ouabain on these parameters are described.

Materials and Methods

All the experiments were done with the isolated epithelium obtained from the abdominal skin of *Rana catesbeiana*. The epithelium was obtained from large animals weighing about 600 g kept in a container with tap water at room temperature (21—23° C).

The procedure for the isolation of the epithelium was a method derived from that of Aceves and Erlij [9] and of Rajerison et al [10]. A detailed description of our method can be found in a paper by Siegel et al [11].

The isolated epithelial sheet was mounted into a plastic frame fashioned from X-ray film. The frame was made by first extending the tissue on a flat glass surface wetted with Ringer's solution so that the peripheral outline of the tissue could be traced onto a piece of film. An area somewhat smaller than the tissue's area was cut out so that the inside border of the cut-out film formed an overlap with the peripheral regions of the tissue. An identical cut-out was made

from a second piece of film with a handle. One member of the identical cut-out was layered with a thin film of Eastman 910 tissue adhesive and superimposed atop the extended tissue. The mounted tissue was then framed with the second cut-out also containing tissue adhesive so that the tissue formed a middle layer sandwiched between two pieces of film. The framed tissue was immediately immersed in Ringer's solution containing 103 mM NaCl, 2.5 mM KCl, 1.8 mM calcium gluconate and 2 mM Tris·HCl buffer. All solutions were bubbled with air at all times and had a pH of 7.4. The epithelia were loaded with ^{22}Na, (2 μCi/ml) which was added to the Ringer's solution. Two types of washout experiments were done.

Type I: Loading from both sides. Differential washout in an Ussing-type chamber

After loading the tissue for 1 h, the tissue was rinsed in a beaker containing 50 ml of Ringer's solution for 30 s prior to mounting in an Ussing-type chamber whose cross-sectional area (7 cm^2) was smaller than the framed tissue. Each half-chamber held 15 ml of Ringer's solution and had Parafilm rings around its borders. A piece of nylon hosiery was secured for tissue support across the open-face of one half-chamber. Under short-circuit conditions, the washout across each of the tissue's faces into the chamber compartments was studied. The voltage and current bridges were polyethylene tubing filled with 4% agar dissolved in NaCl Ringer's solution. Voltage bridges were connected to the recording equipment (Electrometer Model 200B, Keithley Instruments, Cleveland, Ohio; and recorder Model EU20, Heath Co., Benton Harbor, Mich.) by means of calomel cells. The short-circuit current (I_{sc}) was measured with an automatic voltage clamp system. Solution mixing was accomplished by vigorous bubbling and recirculation of the fluid in the chamber. During the washout period lasting 40 min, periodic samples of 3 ml were taken simultaneously from both chambers and immediately replaced with unlabeled Ringer's solution. Sampling was done in most cases 2, 4, 6, 10, 15 and 25 min after commencement of washout. The tissue was short-circuited during the washout, except for a few seconds every 5 min to record the potential differences (PD). The activity per unit volume in the chamber was always less than 3% of the activity in the tissue, so that there was negligible back leak of radioactivity into the tissue. After 40 min the last sample was taken and the tissue was removed from the chamber and washed for 30 s with 15 ml of unlabeled Ringer's solution from a wash bottle and then blotted gently prior to counting. All counting was done with a Packard autogamma spectrometer. Washout experiments were also done with tissues that had been loaded and unloaded in the presence of 5×10^{-4} M ouabain.

Type 2: Differential loading and differential washout in an Ussing-type chamber

In this type of experiment, loading was done for 1 h from one of two bathing solutions, while the preparation was short-circuited. During this period, the transepithelial Na^+ flux was also measured.

(a) Loading from the outside: In experiments where the loading solution was present in the outer bathing solution the washout kinetics of only those tissues whose forward flux were found to be not more than 10% larger than the I_{sc}

were studied. At the end of the loading period, one of two alternative procedures was followed to remove the isotope-containing solution before starting the washout. One procedure was to flush the chamber quickly five times so that the activity remaining in the chamber was near the background value. 15 ml of unlabeled Ringer's solution was added back to each side and the washout to both sides was determined as already described in the Type I experiments. The other procedure was to disassemble the chamber and rinse the tissue for 30 s in unlabeled Ringer's solution followed by remounting the framed epithelium in a new chamber. The washout measurement to both sides was determined as earlier described. The two procedures gave similar results. In addition to the control experiments, 5×10^{-4} M ouabain was added to the inside bathing solution either 3 h prior to or during the loading from the outside bathing solution.

(b) Loading from the inside: In those experiments where loading was done from the inside bathing solution, only those tissues whose back fluxes were 10% or less of the I_{sc} were used for washout. Following loading, the chamber was either flushed or disassembled before commencing with the earlier described measurements. Also, the effect of 5×10^{-4} M ouabain added to the inner solution either prior to or during the loading period was studied.

Determination of Na^+ by flame photometry

The Na^+ content of epithelial tissue that had been loaded and then washed out under control conditions was determined by flame photometry in paired experiments. After the tissue had been unloaded, the tissue was removed from the chamber and each face of the tissue was quickly rinsed for 2—3 s with 10—15 ml of ice-cold isotonic sucrose solution [7]. The tissue was lightly blotted between filter paper and the activity of the area exposed to washout was punched out and counted. The tissue was then extracted overnight in 0.1 M HNO_3 for Na^+ determination by flame photometry. (Eppendorf Model 700, Brinkman Instruments, Westbury, N.Y.)

Measurement of epithelial thickness

A technique described by Mishima and Hedbys [12] to measure the thickness of the cornea (and other semitransparent membranes) with the slit lamp and pachometer was used to measure the thickness of the isolated epithelium. A piece of isolated epithelium was mounted over the 0.5 cm^2 cross-sectional area of a Ussing hemi-chamber and held in place with a Lucite ring. The chamber was secured by a clamp and positioned perpendicular to the light beam from the slit lamp with a micromanipulator. The refraction from the anterior and posterior surfaces of the epithelium was observed with a microscope at a 35° angle. The thickness of the epithelium could be measured as the displacement of the light beam by the pachometer with an error of less than 10%.

Results

Differential washout after loading from both sides

Washout curves towards each bathing solution were constructed from measurements of the activity appearing in each bathing solution plus the residual activity in the epithelium at the end of the washout. The cumulative activity in the

bathing solutions, plus the remaining activity in the tissue after 40 min (usually less than 10% of the total) was taken as the total amount of radioactivity present in the tissue at the beginning of the washout. Activity found in the bathing solutions as a function of time was subtracted from the total, and this difference (the activity remaining in the tissue as a function of time) was then plotted on a semilog scale. The amount of tissue Na^+ that equilibrated during loading was calculated by dividing the ordinate intercept of the washout curve by the specific activity of the loading solution. In seven experiments, the same area of skin epithelium exposed to washout was further analyzed for Na^+ content by flame photometry. In these experiments the mean value of exchangeable Na^+ as determined from the ^{22}Na activity was 0.37 ± 0.05 μequiv./cm^2 and the mean paired value of Na^+ content of the epithelium was 0.33 ± 0.04 μequiv./cm^2. This good agreement suggests that the tissue Na^+ was completely exchangeable and that the loading period was long enough for full equilibration. In sixteen other experiments the Na^+ pool available for isotopic exchange was 0.33 ± 0.02 μequiv./cm^2. Fig. 1 shows the activity in the epithelium as a function of time. The slopes of the curves are the transfer coefficients for efflux from the epithelium into the two bathing solutions. In Table I are shown values for the transfer coefficient, Na^+ pool and efflux towards both bathing solutions with control, ouabain incubated and "dead" (tissue with little or no spontaneous PD) epithelia. In Table I also are shown the values of the I_{sc} and electrical resistance. The high electrical resistance and small Na^+ efflux into the outer bathing solution indicate the absence of edge damage effects. The fraction of the Na^+ pool

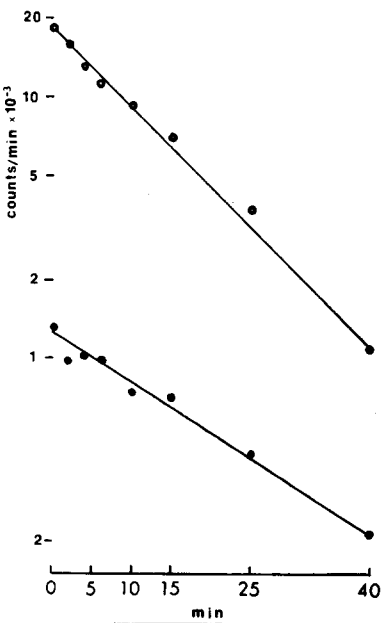

Fig. 1. Activity in the epithelium as a function of time. These curves are calculated from the activity in the inside bathing solution (top curve), or the activity in the outside bathing solution (bottom curve), plus the activity remaining in the tissue after washout. The slopes are the transfer coefficients. The Na^+ pool sizes are proportional to the values of their zero-time intercept.

TABLE I

TISSUE Na^+ POOL SIZES, TRANSFER COEFFICIENTS AND EFFLUXES CALCULATED FROM ^{22}Na WASHOUT TOWARDS THE INNER AND OUTER BATHING MEDIA IN CONTROL, OUABAIN-INCUBATED AND DEAD FROG SKIN EPITHELIUM

Results are means ± S.E. Number of experiments is indicated in parentheses. Ouabain concentration was $5 \cdot 10^{-4}$ M.

	Washout side	Na^+ pool size (μequiv./cm^2)	Transfer coefficient (min^{-1})	Efflux (μequiv./h per cm^2)	I_{sc} (μequiv./h per cm^2)	Resistance (kΩ/cm^2)
Control (16)	inner	0.320 ± 0.018	0.079 ± 0.005	1.530 ± 0.130	0.75 ± 0.14	3.2 ± 0.4
Control	outer	0.013 ± 0.003	0.049 ± 0.009	0.041 ± 0.004		
Ouabain (10)	inner	1.291 ± 0.211	0.028 ± 0.004	2.200 ± 0.638	0.02 ± 0.02	3.7 ± 0.5
Ouabain	outer	0.098 ± 0.028	0.021 ± 0.008	0.079 ± 0.018		
Dead (7)	inner	1.378 ± 0.237	0.031 ± 0.009	2.561 ± 0.759	0.15 ± 0.07	
Dead	outer	0.054 ± 0.029	0.047 ± 0.020	0.074 ± 0.025		

unloading into the inner bathing solution was much larger. 4% of the Na^+ pool unloaded into the outer bathing solution and the transfer coefficient of the outer component was 38% smaller than the transfer coefficient for unloading into the inner bathing solution. Under control conditions, the efflux across the outer barrier is less than 3% of the efflux across the inner barrier and 5% of the I_{sc}.

A second series of experiments was done with dead epithelia. These tissues were found to have significantly larger Na^+ pools and also smaller transfer coefficients than the controls for unloading towards the inner bathing solution. The small permeability of the outer barrier to Na^+ efflux was still observable. The larger Na^+ pool and the smaller transfer coefficient are consonant with an inhibition of the "pump" mechanism. The larger effluxes towards the two bathing solutions are due to the larger Na^+ pool.

A third series of experiments was done with epithelia loaded and washed out in the presence of 5×10^{-4} M ouabain. The similarity between the Na^+ pool of dead and ouabain incubated epithelia indicates that there is an inhibition of the pump mechanism which results in an increase in Na^+ accumulation accompanied by a smaller transfer coefficient for unloading towards the inner bathing solution. It should be noted that in all cases the washout curves consisted of only one component with a $t_{1/2}$ of at least 8.8 min. Since our procedure included flushing of the chamber or rinsing of the epithelium for 30 s after loading, components with a $t_{1/2}$ of less than 30 s may have been eliminated.

Differential washout after one side loading

Since the washout curves shown in Fig. 1 are linear, each component appeared to originate from a single compartment. The compartment unloading towards the inner bathing solutions contains about 96% of the Na^+ pool. We were interested in determining how much of this pool can be loaded from either bathing solution.

The sum of the two pools measured by differential loading should equal the pool measured after symmetrical loading. Previous work [13,14] has indicated

that whole skin can be loaded from the outside solution. This is to be expected since Na^+ transport occurs in the outside to inside direction across the cellular compartment. Thus, at least part of the tissue Na^+ should be labeled. There are no reports of loading from the inside solution, particularly with corium-free isolated skin epithelium.

(a) Loading from the outer solution. The PD and I_{sc} were 51.8 ± 5.3 mV and 0.59 ± 0.07 μequiv./h per cm^2, respectively. In Table II (row A) are shown the Na^+ pool, transfer coefficient, and efflux of the inner component washout. The pool was 50% of that measured when the epithelium was loaded symmetrically. The transfer coefficient was similar to the value measured after symmetrical loading. A negligible Na^+ efflux towards the outer bathing solution was observed, (not shown in Table II).

In another series of experiments the effect of 5×10^{-4} M ouabain in the inner bathing solution was tested. The I_{sc} decreased to 0.02 μequiv./h per cm^2 after 180 min. The tissue was then loaded from the outer bathing solution for 1 h. In Table II (row C), these experiments are referred to as ouabain-1. The Na^+ pool and transfer coefficient for washout towards the inner bathing solution were 0.05 μequiv./cm^2 and 0.027 min^{-1}, respectively. Less than 5% of the activity washing out towards the inner bathing solution appeared in the outer bathing solution. Only 12% of the initial ^{22}Na activity remained 40 min after beginning washout. Since the total Na^+ pool after outer and inner loading was 0.97 μequiv./cm^2 (cf. Table II: ouabain-1, rows C and D), these results indicate that only 5% of the Na^+ pool (i.e. 0.05/0.97) was labeled instead of 50% as in the control. This result is an indication that ouabain reduced Na^+ uptake across the outer barrier.

In another set of experiments, ouabain was added to the inner bathing solution 1 h after ^{22}Na was added to the outer bathing solution. In Table II (row E), these experiments are referred to as ouabain-2. In these experiments, the transepithelial Na^+ forward flux was measured before and after ouabain. As shown in Table II, the transfer coefficient was nearly the same as in the experiments in which ouabain was added prior to the labelling. On the other hand, the Na^+ pool

TABLE II

TISSUE Na^+ POOL SIZES, TRANSFER COEFFICIENTS AND EFFLUXES CALCULATED FROM ^{22}Na WASHOUT TOWARDS THE INNER BATHING MEDIUM AFTER DIFFERENTIAL LOADING IN CONTROL AND OUABAIN-INCUBATED FROG SKIN EPITHELIUM

Results are means ± S.E. Number of experiments is indicated in parentheses. Ouabain-1 type: epithelium was pre-incubated in $5 \cdot 10^{-4}$ M ouabain for 3 h before loading with ^{22}Na. Ouabain-2 type: epithelium was loaded with ^{22}Na either in the presence of $5 \cdot 10^{-4}$ M ouabain for 1 h or the drug was added 1 h after loading had begun.

Experiment type	Loading side	Na^+ pool size (μequiv./cm^2)	Transfer coefficient (min^{-1})	Efflux to inner solution (μequiv./h per cm^2)
A Control (9)	outer	0.16 ± 0.02	0.067 ± 0.009	0.66 ± 0.14
B Control (7)	inner	0.14 ± 0.02	0.061 ± 0.014	0.56 ± 0.11
C Ouabain-1 (5)	outer	0.05 ± 0.01	0.027 ± 0.005	0.08 ± 0.03
D Ouabain-1 (7)	inner	0.92 ± 0.13	0.013 ± 0.002	0.73 ± 0.13
E Ouabain-2 (9)	outer	0.14 ± 0.02	0.023 ± 0.002	0.18 ± 0.05
F Ouabain-2 (5)	inner	0.99 ± 0.09	0.040 ± 0.003	2.15 ± 0.44

was larger. This is probably due to the fact that the pool was labeled before ouabain reduced the J_{12} flux.

(b) Loading from the inner solution. The average PD and I_{sc} were 59.4 ± 9.0 mV and 0.87 ± 0.09 μequiv./h per cm^2, respectively. The control values for the tissue Na^+ pool, transfer coefficient, and efflux towards the inner solution are given in Table II (row B). Unloading occurred almost entirely towards the inner bathing solution. The Na^+ pool that equilibrated across the inner barrier was 44% of the control value (0.32 μequiv./cm^2, Table I). 4% of the initial activity remained in the tissue after 40 min of washout. The transfer coefficient was similar to the value found in epithelia loaded from both sides.

The effect of ouabain on the extent of loading occurring from the inner bathing solution was also studied. In one type of experiment designated ouabain-2, where 5×10^{-4} M ouabain and ^{22}Na were added simultaneously to the inner bathing solution, the epithelium was loaded for 1 h. The Na^+ transepithelial back flux was 0.04 μequiv./h per cm^2 and was not different from our control values of Na^+ back flux and from values reported in the literature [15]. The results are shown in Table II (row F). Little activity appeared in the outer bathing solution while the inner loading pool increased from a control value of 0.14 to 0.99 μequiv./cm^2 while the transfer coefficient decreased from 0.061 to 0.04 min^{-1}.

In another series of experiments, designated ouabain-1, incubation was done for 3 h followed by loading for 1, 2.5, 13.3, or 15.3 h from the inner bathing solution. The results are shown in Table II (row D). The activity washing out towards the outer bathing solution was 9% of the total amount in the epithelium. The Na^+ pool (0.92 μequiv./cm^2) that washed out towards the inner bathing solution was constant regardless of the time of incubation in ^{22}Na, which suggests that no further accumulation of ^{22}Na occurred after about 1 h. On the other hand, it took 2.5 h for the transfer coefficient to decrease to a minimum value. In seven experiments (row D) the mean transepithelial Na^+ back flux after 3 h of ouabain incubation was 0.05 ± 0.01 μequiv./h per cm^2.

Discussion

We have found that the isolated frog skin epithelium can be loaded with ^{22}Na from either or both bathing solutions. When loading was done simultaneously from both sides, the specific activity found in the epithelial cells was the same as in the bathing solutions, indicating that almost all the Na^+ in the epithelium was exchangeable. This result is in agreement with our previous finding, with corium-free epithelium, that practically all the Na^+ from the epithelium is lost after about 85 min when bathed in a Na^+-free solution [16]. The total tissue Na^+ pool calculated after symmetrical loading was 0.33 μequiv./cm^2 and the mean Na^+ content determined by flame photometry was 0.33 μequiv./cm^2. Na^+ washout from epithelia loaded from both sides occurred mainly towards the inner bathing solution: 96% of the exchangeable Na^+ appeared in this solution. Both the washout towards the inside and the small pool (4%) that washed out towards the outside solution could be described by a single exponential term. Nagel et al. [13] found with whole frog skin that only about 3% of the tissue Na^+ washes out towards the outer bathing solution. They obtained, however, a

three-component washout curve towards each bathing solution. Their more complex washout behaviour may, in part, result from the presence of fast components, since they did not prewash the tissue for 30 s. The retardant effect of the corium on Na^+ efflux may also play a role. Similarly, the computer simulation studies of Howell and Huf [17] indicated that most of the sodium in the epidermis is washed out towards the inner bathing solution. The fact that in our studies a single exponential term can account for about 96% of the tissue Na^+ suggests that this pool behaves as a rather uniform compartment, in spite of the several different cellular layers present in the skin epithelium.

When the epithelium was loaded from the outer bathing solution, 50% of the total epithelium Na^+ was labeled at equilibrium. Similarly, loading from the inner bathing solution labeled also about 50% of the total epithelial Na^+. This is to be expected, since labeling from either side should add up to the total amount that is labeled after symmetrical loading. The interesting finding is that regardless of the side from which the epithelial Na^+ was labeled, almost all of the Na^+ washed out towards the inner solution. Furthermore, the washout transfer coefficients were similar: 0.067 min^{-1} when labeled from the outside and 0.061 min^{-1} when labeled from the inside.

A clear distinction should be made between what is meant by the tissue Na^+ pool (0.33 μequiv./cm^2) and the Na^+ transport pool. The transport pool is usually defined as the fraction of the epithelial Na^+ pool that participates in transepithelial transport. Our measurements do not permit a precise determination of its size, but an estimation of a range of values can be made. Since 50% of the total Na^+ pool was labeled from the outer bathing solution, and 96% of this Na^+ washed out towards the inner bathing solution, at least 48% of the tissue Na^+ pool effectively participates in transepithelial transport, and this amount can be considered as a minimum value for the transport pool.

We will consider two extreme possibilities: (a) that the transport pool is 48% of the total pool and (b) that all the Na^+ in the skin epithelium participates in transport. We will show that either model is consistent, with certain restrictions, with our results. Possibilities representing intermediary values are also compatible with our results. The first possibility is represented schematically in Fig. 2. Two separate compartments are depicted; each containing half (0.16 μequiv./cm^2) of the total pool. Compartment A is only accessible from the outside; thus, when ^{22}Na is only in the inner solution, compartment A will not be labeled. When ^{22}Na is in the outer bathing solution, all the Na^+ in compartment A will be labeled and at equilibrium the specific activities in compartment A and the outer bathing solution will be equal, since in this model there is no recirculation of unlabeled Na^+ across the inner border of compartment A. Compartment B will not be labeled from the outside bathing solution, because the activity in the intercellular spaces surrounding it will be essentially similar to that in the inner solution, which was maintained below 1% of the activity in the outer solution. When ^{22}Na is in the inner solution the intercellular spaces will have the same activity and compartment B will be totally labeled at equilibrium. The arrows indicate the fluxes across the compartment barriers. Notice that fluxes 1 and 2 must be almost equal, except for the small back leak across the outer border. Fluxes 3 and 4 should also be equal. The value of flux 2 is determined from washout towards the inside after outside loading and was 0.66 μequiv./h

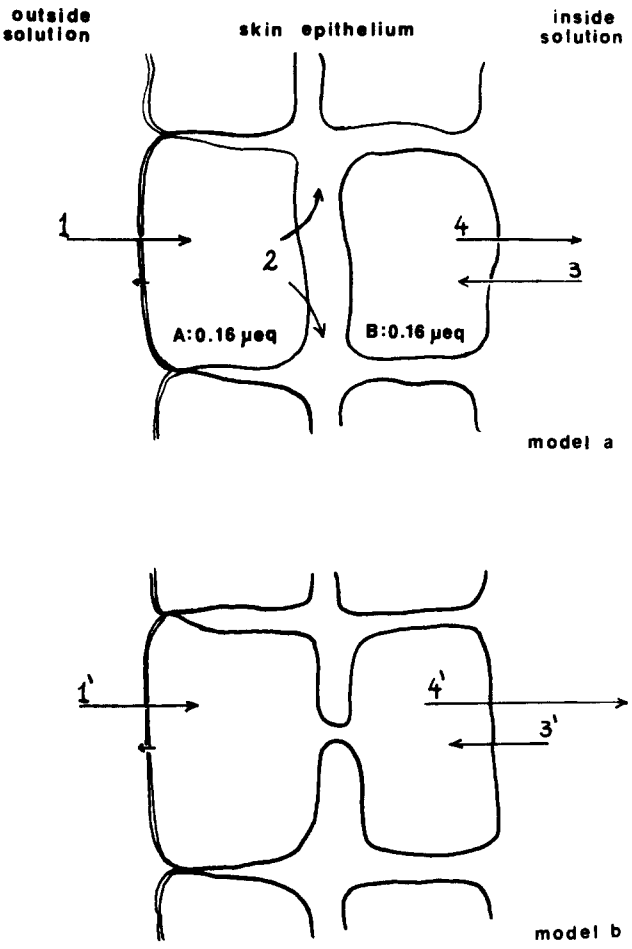

Fig. 2. Two alternative models for Na^+ movement across frog skin epithelium are shown and the directions of Na^+ fluxes are indicated by the arrows. In model a the compartments are A,B. Model b consists of a single compartment.

per cm^2. Similarly, the value of flux 4 is obtained from washout towards the inside after inside loading and was 0.56 μequiv./h per cm^2. Notice that the value of flux 2 is obtained by multiplying the transfer coefficient (0.067 min^{-1}) times a pool size. Since the value of flux 2 could not be smaller than the I_{sc} measured during the washout (0.59 μequiv./h per cm^2) the transport pool should be about 0.15 μequiv./cm^2 to obtain a flux that is comparable to the I_{sc}. The value of the pool we calculated from the Y-intercept of these washout experiments was 0.16 μequiv./cm^2. This provides an independent confirmation of the minimum size of the transport pool.

The second possibility shown diagrammatically in Fig. 2 is that all the epithelial Na^+ behaves as a single uniform compartment, due perhaps to the presence of intercellular communications. With this model all the Na^+ participates as a transport pool. The fact that only 50% of it is labeled from either side is readily explained considering that fluxes 1′ and 3′ are equal and flux 4′ is equal to 1′

plus 3′. When the label is in the outer solution, the specific activity in the cellular compartment only reaches a maximum value of 50% of that in the labeled solution, due to the recirculation of unlabeled Na^+ (flux 3′). Similar arguments explain the 50% labeling from the inside. Only when fluxes 3′ and 1′ carry ^{22}Na (namely during loading from both sides) can the specific acticity of the cell compartment be equal to that of the bathing solutions. It is probable that the true representation of the transport pool lies in between these two extremes: a partial anatomical or functional connection may exist between compartments A and B.

From an earlier study [16], we have additional presumptive evidence suggesting an appreciable Na^+ permeability between the tissue and the inner bathing solution. In the isolated frog skin epithelium under short-circuit conditions, the I_{sc} decreased more than 50%, 60 min after the inner bathing solution had been replaced with a Na^+-free Ringer's solution, and we found that the Na^+ content after 85 min in a Na^+-free Ringer's solution decreased to less than 1% of the control value.

It is of interest to compare our measurements of the Na^+ pool with those of other workers. Under control conditions our value of 0.33 μequiv./cm^2 can be expressed as 42 mM, since the epithelial thickness of *R. catesbeiana* is 70—80 μm. Values obtained by other workers in whole skin [18,19] isolated epithelium [9] and isolated skin epithelial cells [20] are 35, 21, 25, and 39 mM, respectively. Our observed increase in the Na^+ pool after ouabain incubation are in accord with Aceves and Erlij [9] in the isolated epithelium and Zylber et al [20] in isolated epithelial cells.

Preincubation with ouabain increased the exchangeable Na^+ pool from 0.33 to 1.29 μequiv./cm^2, and this affect agrees with the results of other workers [9,20]. The differential loading experiments with ouabain indicated that most of the Na^+ gain originated from the inner solution. In addition, Na^+ uptake from the outer solution was markedly reduced, as previously reported [6—8]. When ouabain was added during loading, partial labeling from the outer solution occurred (Table II). As with the control, the washout occurred mainly towards the inner solution.

The rate of exchange of Na^+ between the tissue and the inner bathing solution appeared to reach a steady state in less than 1 h with ouabain, since loading periods up to 15.3 h did not produce a significant increase in the Na^+ pool beyond that measured after loading for l h. It is noteworthy that the measurements of Zylber et al. [21] of the transfer coefficient under control and ouabain-incubated conditions were 0.0729 and 0.0385 min^{-1}, respectively, which are in close agreement with our measurements of 0.079 and 0.04 min^{-1} (cf. Tables I and II).

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