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Effect of Ouabain, Amiloride, and Antidiuretic Hormone on the Sodium-Transport Pool in Isolated Epithelia from Frog Skin (*Rana temporaria*)

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Summary. When tracer Na^+ is added to the solution bathing the apical side of isolated epithelia the observed transepithelial tracer influx increases with time until a steady state is reached. The build-up of the tracer flux follows a single exponential course. The halftime for this build-up under control conditions was 0.92 $\pm$0.06 min, and in the presence of ouabain 4.51 $\pm$0.7 min. It is shown that the calculated Na^+-transport pool is located in the cells. The Na^+-transport pool under control conditions was 35.6 $\pm$3.4 nmol/cm^2, which corresponds to an intracellular Na^+ concentration of 7.9 mM. Activation of the active Na^+ transport by addition of antidiuretic hormone resulted in a highly significant increase in the Na^+ transport pool, and inhibition of the transcellular Na^+ transport with amiloride resulted in a decrease in the Na^+-transport pool.

Furthermore, the active Na^+ transport increased along an *S*-shaped curve with increasing intracellular Na^+ concentration (Na^+-transport pool). The Na^+ pump was found to be half saturated at an intracellular Na^+ concentration of 12.5 mM.

Key words transepithelial sodium transport · pool size · ouabain · antidiuretic hormone · amiloride

Introduction

The Na^+-transport pool in epithelia can be defined as the fraction of Na^+, located in the cells between the apical and the basolateral membrane, that participates in transepithelial transport. There has been much controversy about the size of such a pool and even about its existence (for reference, *see* Macknight & Leaf, 1978).

The Na^+ pool has been measured in whole frog skin by washout analysis of tracer Na^+ (Zerahn, 1969; Nagel, Dörge, Moshagen & Rick, 1974). From these experiments it was concluded that the major part of the Na^+ in the pool was already transported and located in an extracellular compartment, possibly the intracellular space between the epithelial cells. Nagel and Moshagen (1978) have repeated the pool determinations in frog skin. They found that ouabain (an inhibitor of the Na–K pump) had no effect on the halftime for the washout of tracer Na^+ and that there was a linear relationship between the active Na^+ transport and the pool size. This is the sort of results one would expect to get if the pool was a transported pool (e.g., a pool located in the extracellular space) or if a step different from the cellular events was rate determining for the washout of tracer Na^+.

The isolated frog skin consists of the epidermis (40–70 μm thick) and the underlying dermis (250–350 μm thick). The active transepithelial Na^+ transport takes place in the epidermis, therefore in a washout experiment tracer Na^+ located in the epithelium have to diffuse through the dermis.

For the mathematical treatment of this diffusion problem, the dermis may be treated as an infinite plane sheet. The solution to this problem can be found in Crank (1956) and in Dainty and House (1966). The halftime ($t_{\frac{1}{2}}$) for the diffusion process is given by

$$t_{\frac{1}{2}} = 0.38 \frac{l^2}{D_{Na}} \tag{1}$$

where l is the thickness of the dermis and D_{Na} is the diffusion coefficient for Na^+ in the corium. Hoshiko, Lindley and Edwards (1964) have reported that D_{Na} (for the corium of bullfrog skin) is about 4.2×10^{-6} cm^2 sec^{-1}. From Eq. (1) it can then be calculated that the halftime for diffusion of Na^+ through the corium of the thickness given above is, respectively, about 1 and 2 min. Therefore in the experiments presented in this paper, the Na^+ pool was determined on isolated epithelia.

With a method in which cellular tracer uptake was estimated, it was found that the active Na^+ transport increased along an *S*-shaped curve with increasing Na^+ pool; furthermore, inhibition of the $Na^+ - K^+$ pump with ouabain resulted in a highly significant increase in the halftime for the washout of tracer Na^+. Therefore it is concluded that the

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Na^+ pool measured in isolated epithelia is located mainly in the cells.

Materials and Methods

The experiments were performed on isolated epithelia from male and female frogs (*Rana temporaria*). The epithelia were dissected from collagenase-treated skins and mounted in perspex chambers (area, 1.5 cm^2; volume, 2.5 ml) and bathed in stirred Ringer's solution ((in mM) Na^+, 115; K^+, 2.5; Ca^{++}, 1; Mg^{++}, 1; Cl^-, 117; HCO_3^-, 2.5; PO_4^{3-}, 1; glucose, 5; at pH 7.8).

It has been shown by Carasso, Favard, Jard and Rajerison (1971) that epithelia isolated from collagenase-treated frog skins normally contains few mucous glands.

Results

Determination of the Na^+-Transport Pool

The Na^+-transport pool was determined on isolated short circuited epithelia. The epithelia was short circuited for at least 20 min or until the SCC was steady, so the experiments were conducted under conditions where the cellular Na^+ concentration was in a steady state. When the SCC was steady, $^{22}Na^+$ was added to the solution bathing the apical side of the epithelium and samples were withdrawn from the solution bathing the basolateral side after 30, 60, 90, 120, 150, and 180 sec and 4, 5, 6, 8, 10, 12, and 14 min incubation. The rates of appearance of $^{22}Na^+$ in the basolateral solution were calculated for each sampling period and plotted against time at the midpoints of each sampling period (Fig. 1). On the basis of such flux measurements and the simmultaneously measured SCC, the Na^+ pool was estimated by using the procedure described below.

The epithelium, which was mounted as a sheet between identical solutions in an Ussing-type chamber, can in these experiments be treated as a compartment (compartment 2) separating the solution bathing the apical membrane (compartment 1) from the solution bathing the basolateral membrane (compartment 3) (Fig. 2).

The calculation of the Na pool is based on the approximation that J_{12} (the flux from the apical solution into the cells) in the steady state is equal to $J_{23}^{(P)}$ (the flux from the cells via the $Na^+ - K^+$ pump to the basolateral solution); furthermore, it is assumed that the fluxes J_{13}, J_{31}, J_{21}, J_{23}, and J_{32} are so small compared with J_{12} and $J_{23}^{(P)}$ that they can be omitted from the calculations (*see* below).

In order to satisfy these approximations the epithelia were only used for the experiments if the steady-state transepithelial Na^+ efflux was less than 5% of the transepithelial Na^+ influx. J_{31} is equal to or less than the Na^+ efflux, depending on how big a

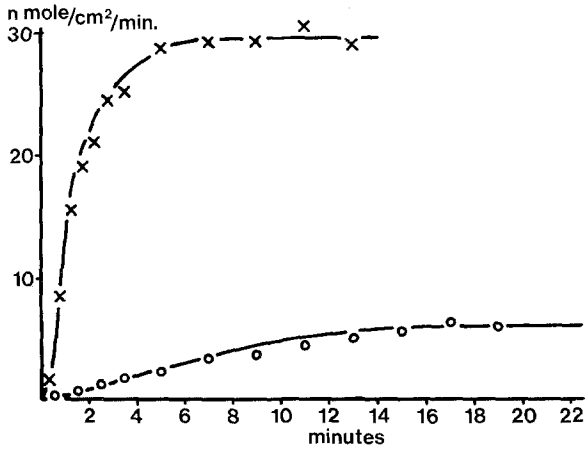

Fig. 1. Rate of appearance of $^{22}Na^+$ in the basolateral solution. At time zero $^{22}Na^+$ was added to the apical solution and samples were withdrawn from the basolateral solution 30, 60, 90, 120, 150, and 180 sec and 4, 5, 6, 8, 10, and 12 min after the addition of $^{22}Na^+$. The rates of appearance of $^{22}Na^+$ were calculated for each sampling period (the specific activity of $^{22}Na^+$ in the apical solution was taken as 1) and plotted against time at the midpoints of each sampling period. ×—×, tracer build-up under control conditions; ○—○, tracer build-up in an experiment where the epithelium had been treated with 2 μM ouabain

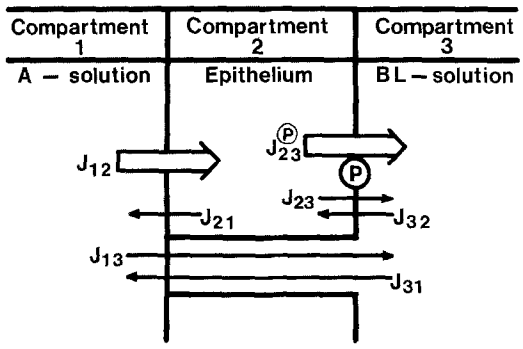

Fig. 2. Three-compartment model used in the description of the different fluxes across the isolated epithelium

fraction of the efflux passes via a cellular route (J_{32}, J_{21}). The experiments were performed under short circuited conditions with solutions of the same composition on each side of the epithelium; therefore J_{31} is equal to J_{13}.

By measuring the unidirectional uptake of Na^+ from the apical solution into the isolated frog skin (J_{12}), Erlij and Smith (1973) have shown that J_{12} is equal to the SCC ($J_{23}^{(P)}$); thus (J_{21}) is negligible compared with J_{12}. The same has been shown by Biber, Cruz and Curran (1972) and by Rick, Dörge and Nagel (1975). Koefoed-Johnsen and Ussing (1958) showed that the basolateral membrane behaved like a nearly ideal K^+ electrode; this means that the permeability of the basolateral membrane to free

Na^+ must be small, thus J_{32} must be small. When the Na pump was blocked by ouabain, net uptake of Na^+ from the bathing solutions into the cells took place only from the apical solution (Rick, Dörge, von Armin & Thurau, 1978). This shows that J_{32} is much smaller than J_{12}. The passive flux J_{23} from the epithelial cells to the basolateral solution must be smaller than J_{32}, because the cells have a low Na^+ concentration and the cellular potential is negative with respect to the basolateral solution. Thus, when the Na^+ efflux is low as in the experiments presented here, the major part of the transepithelial Na^+ flux occur via the pathways described by J_{12} and $J_{23}^{(P)}$ (Fig. 2). Consequently, the change in concentration of $^{22}Na^+$ in compartment 2 (the epithelial cells) per unit area can be described by the following equation:

$$\frac{dc_2^*}{dt} = \frac{J_{12}}{V_2} \cdot \frac{c_1^*}{c_1} - \frac{J_{23}^{(P)}}{V_2} \cdot \frac{c_2^*}{c_2} \tag{2}$$

where V_2 is the volume of compartment 2, c_1 is the Na^+ concentration in compartment 1, and c_1^* is the $^{22}Na^+$ concentration in compartment 1. Since the volume of compartment 1 is very large (2500 µl) compared to the volume of compartment 2 (about 10 µl), the specific activity in compartment 1 remains constant and $c_1^*/c_1 = 1$ (apical Ringer's solution taken as 100% $^{22}Na^+$); since J_{12} is equal to $J_{23}^{(P)}$ (*see* above) Eq. (2) can be transformed to:

$$\frac{dc_2^*}{dt} = \frac{J_{12}}{V_2} - \frac{J_{12}}{V_2} \cdot \frac{c_2^*}{c_2}. \tag{3}$$

Equation (3) is the differential equation which describes tracer exchange between a reservoir with the labeled substance in a time-invariant concentration, communicating with a compartment, which at the beginning of the experiment contains no labeled substance. In Eq. (3) we can put:

$$\frac{J_{12}}{V_2} \cdot \frac{1}{c_2} = k. \tag{4}$$

After integration, Eq. (3) can be transformed to the following equation (*see*, e.g., Kotyk & Janacek, 1975):

$$2.3 \log \left(1 - \frac{c_2^*}{c_2}\right) = -kt. \tag{5}$$

The rates of appearance of $^{22}Na^+$ in the basolateral solution (m) were calculated for each sampling period and expressed in fractions of the final steady-state value (M). m/M is a measure of the specific Na^+ activity in the flux $J_{23}^{(P)}$ and is equal to c_2^*/c_2 (the specific activity in the compartment from where it appears). For each experiment the quantity $(1 - m/M)$ which is equal to $(1 - c_2^*/c_2)$ was plotted against time at the midpoints of each sampling period (Fig. 3). A straight line was fitted by inspection, and the halftime and rate constant were calculated for each experiment. Since the build-up of the $^{22}Na^+$ flux follows a single exponential course (at least in the first 3 min which account for the major part of the build-up period), the sodium pool (S) can be calculated from the following equation (Eq. (6)), which is derived from Eq. (4):

$$S = V_2 \cdot c_2 = \frac{J_{12}}{k} = \frac{\mathrm{SCC}}{k}. \tag{6}$$

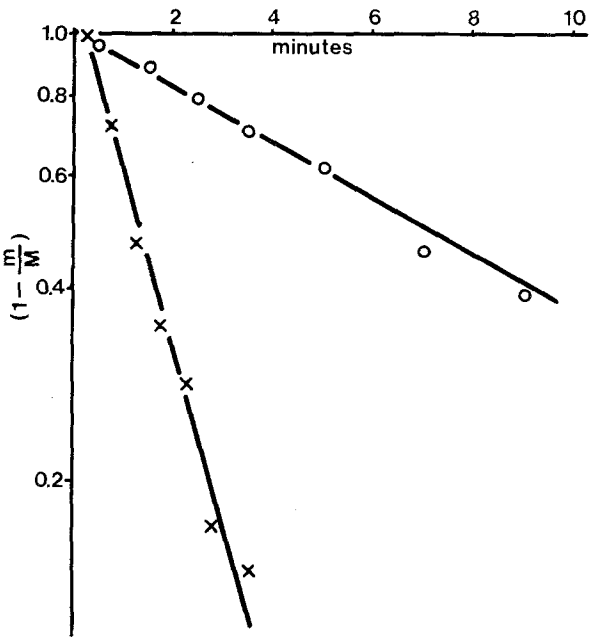

Fig. 3. The ^{22}Na-influx data from Fig. 1 presented as semilogarithmic plot of $\left(1 - \frac{m}{M}\right)$ where M is the steady-state influx and m is the influx at the time given on the figure. Zero time is the time of addition of $^{22}Na^+$ to the apical solution. ×—×, control epithelium; ○—○, epithelium treated with 2 µM ouabain

Half Time and Na^+ Pool

The build-up half time under control conditions was 0.92 ± 0.06 min ($n = 11$) and the calculated Na^+ pool was 35.6 ± 3.4 nmol/cm^2 epithelium (Table 1). If the Na^+ pool is located in the cells and uniformly distributed in all the cells, then the cellular Na^+ concentration can be calculated from the Na^+-transport pool by dividing it with the cellular water content. The isolated epithelia used in this experiments weighted 1.71 ± 0.06 mg dry wt/cm^2 and contained 2.63

Table 1. Halftime ($t_{0.5}$), Na transport pool from Na^+ influx build-up experiments on isolated epithelia, and short-circuit current (SCC)[a]

	n	$t_{0.5}$ (min)	Na pool (nmol/cm^2)	SCC (nmol/cm^2/min)
Control	11	0.92±0.06	35.6± 3.4	27.1±1.9
AVT	7	1.15±0.07	74.3±10.0	43.7±4.3
AVT+theo	4	1.19±0.13	100.6±15.8	58.1±5.1
Amiloride	3	1.02±0.12	11.0± 5.3	7.0±3.3
Ouabain	6	4.51±0.7	43.3± 8.5	6.4±0.6

[a] Mean values ±SE under control conditions, after arginine vasotocin (AVT, 40 ng/ml) and arginine vasotocin plus theophylline (AVT+theo, 40 ng/ml AVT and 1 mM theo), amiloride and 2 μM ouabain.

±0.04 mg cell water per mg dry wt (^{14}C-mannitol was used as extracellular marker); thus the epithelia contained 4.5 μl cell water/cm^2. From this data the cellular Na^+ concentration was found to be 7.9 mM.

The crosses on Fig. 4 represent the measured SCC in the individual control experiments plotted against the corresponding Na^+-transport pool. The triangles (Fig. 4) show the results obtained in a series of experiments where the SCC was partially or nearly completely inhibited by addition of the Na^+-channel blocker amiloride. The squares (Fig. 4) represent the results obtained in experiments where the SCC was activated by addition of the antidiuretic hormone (arginine vasotocin 40 ng/ml), and the open circles represent the results from experiments where the SCC was activated by both antidiuretic hormone and the phosphodiesterase inhibitor theophylline (arginine vasotocin 40 ng/ml and theophylline 1 mM).

The relationship between the Na^+-pool and the SCC could be described by Eq. (7), which is the equation used by Garay and Garrahan (1973) to describe the ouabain-sensitive Na^+ efflux in red cells.

$$SCC = SCC_{max}/(1+(K_{Na}/Na_c))^n. \quad (7)$$

n is the number of Na-binding sites per pump unit, K_{Na} is the apparent dissociation constant of the Na^+ sites of the Na^+ pump complex and SCC_{max} is the maximum Na^+ pump flux, and Na_c is the intracellular Na^+ concentration. A good fit to the data (Fig. 4, continuous line) was obtained for $n=3$, $K_{Na}=16$ nmol/cm^2/min, $SCC_{max}=90$ nmol/cm^2/min and half maximum saturation of the Na^+ pump at 56 nmol/cm^2, which corresponds to a cellular Na^+ concentration of 12.4 mM. The SCC increases with increasing Na^+ pool along an *S*-shaped curve rather than linearly (Fig. 4), this indicates that the Na^+ pool is located in the cells.

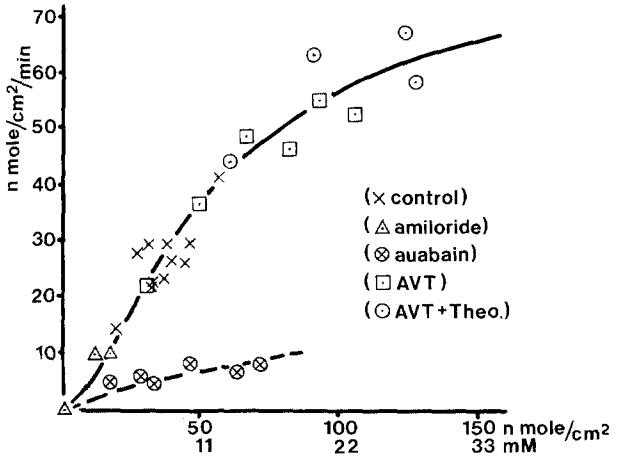

Fig. 4. SCC as a function of the Na^+ pool. ⊗----⊗, data from epithelia which had been treated with 2 μM ouabain. The fully drawn line has been calculated from Eq. (7). (×) Control experiments; (△) values after amiloride; (⊡) values after arginine vasotocin (AVT) 40 ng/ml; (⊙) values after arginine vasotocin 40 ng/ml plus theophylline (Theo) 1 mM. *Abscissa:* cellular Na^+ pool in nmol/cm^2, or in mmol/liter cell water (mM). Ordinate SCC in nmol/cm^2/min

Ouabain

Halftimes for the build-up were also measured in epithelia where the Na−K pump was partially inhibited by ouabain. In these experiments the epithelia were incubated for 60 min in the presence of 2 μM ouabain, whereafter the solution was changed to a solution without ouabain, which caused the ouabain-induced decrease in the SCC to become much slower. This ouabain treatment of the isolated epithelia resulted in 65–80% inhibition of the SCC (no recovery in the SCC was observed after the removal of ouabain). When the SCC was steady, $^{22}Na^+$ was added to the apical solution and the build-up of the tracer flux was measured (Fig. 3). In the presence of a low ouabain concentration the SCC is still equal to the net Na^+ flux across the epithelium (Koefoed-Johnsen, 1957). The halftime for the build-up of the tracer flux in the epithelium halves treated with ouabain were 4.5±0.7 min, and 1.11±0.09 min in the corresponding controls ($n=6$); thus, partial inhibition of the Na^+ pump by ouabain resulted in a highly significant increase in the halftime, but had only a small effect on the pool size (Table 1). In washout experiments on isolated epithelia Candia and Reinach (1977) also found that preincubation with ouabain increased the halftime for the wash-out of tracer Na^+.

Discussion

A clear distinction should be made between what is meant by the tissue-Na^+ pool (the amount of Na^+

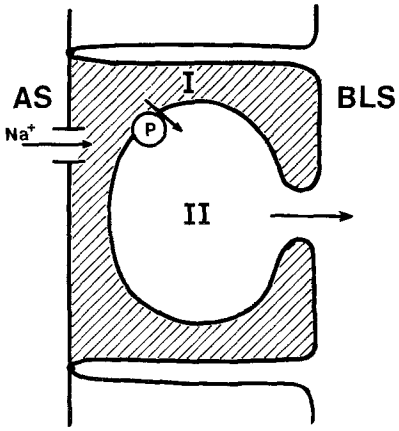

Fig. 5. Diagram of a two-compartment system in series. AS, apical solution; *BLS*, basolateral solution; *I*, compartment I (the cellular compartment); *II*, compartment II (the extracellular compartment); *P*, the $Na^+ - K^+$ pump

in the tissue) and the Na^+-transport pool. The Na^+-transport pool is usually defined as the fraction of the tissue-Na^+ pool that participates in transepithelial transport.

Na^+ involved in active transepithelial transport will successively be located in different compartments; it moves from the apical solution into the epithelial cells, continues into the extracellular space, and advances to the basolateral solution. Thus, Na^+ involved in active transepithelial transport moves through two compartments in series, namely, the cells and the extracellular space of the tissue. The movement of Na^+ between the extracellular space and the basolateral solution is a diffusion rather than a permeation process, but still it may be described approximately by a single exponential.

In the experiments presented here it was found that the build-up halftime for the tracer Na^+ flux under control conditions was 0.92 ± 0.06 min and the calculated Na^+ transport pool was 35.6 ± 3.4 nmol/cm^2 (Table 1). Since the build-up of the tracer flux could be described by a single exponential curve (Fig. 3), the Na^+ transport pool measured must be localized mainly in a single compartment, namely, a cellular or an intracellular compartment.

Figure 5 shows the two possible locations of the pool, compartment *I* represents the cellular and compartment *II* represents the extracellular compartment. Both the size and the halftime for the build-up of the tracer concentration in an extracellular pool might depend on the rate of the active transepithelial Na^+ flux; but both the pool size and the halftime would be expected to be modified in the same way, independent of whether the steady-state active transepithelial Na^+ transport is inhibited by addition of $Na^+ - K^+$-pump inhibitor ouabain or by Na^+-channel inhibitor amiloride, because the steady-state active Na^+ flux from the cells to the extracellular compartment under both conditions will be the same, if the SCC is reduced to the same level by ouabain or amiloride. The size of the pool was nearly independent of the transport rate in the presence of ouabain, but not in its absence (Fig. 4). Consequently, the pool measured cannot be located in the extracellular space, but must be located in the cells (in a compartment placed before the $Na^+ - K^+$ pump). The same conclusion is reached when one compares the halftimes; the inhibition of the transepithelial Na^+ transport by amiloride had no effect on the halftime for the build-up of the tracer flux, whereas addition of ouabain resulted in a highly significant increase (Table 1).

Table 2. Comparison of the data from Fig. 4 with the data found by Larsen et al. (1978)

	K_{Na} (mM)	$J_{Na\ max}$ (nmol/cm^2/min)	"K_m" (mM)	n
Larsen et al., 1978	3.4 ± 0.3	117 ± 22	13.1 ± 1.1	3
Fig. 4	3.6	90	12.5	3

[a] "K_m", half-maximal saturation of the Na^+ pump.

If the Na^+ pool under control conditions (35.6 nmol/cm^2) is uniformly distributed in all the cells in the epithelium, then it corresponds (as shown in a previous section) to a cellular Na^+ concentration of 7.9 mM. Rick et al. (1978) found by microprobe analysis that the cellular Na^+ concentration was 9 mM in the presence of 110 mM Na^+ in the bathing solutions. Microelectrodes showed that the cellular Na^+ activity in frog skin was 14 mM (Nagel, Garcia-Diaz & Armstrong, 1980) and 7 mM in rabbit urinary bladder (Lewis & Wills, 1980). This shows that most intracellular Na^+ takes part in the active transepithelial Na^+ transport under control conditions.

From Fig. 4 and Table 1 it is seen that activation of the Na^+ transport by addition of antidiuretic hormone results in a highly significant increase in the Na^+ transport pool (the cellular Na^+ concentration), whereas inhibition of the Na^+ transport by addition of amiloride resulted in a decrease in the Na^+-transport pool (the cellular Na^+ concentration).

Furthermore, the active Na^+ transport increased along an *S*-shaped curve with increasing Na^+ concentration (Fig. 4). In experiments where the inward-facing membranes were depolarized with K_2SO_4 and the "Na^+-selective" apical membrane was used for estimating the intracellular Na^+ activity, Larsen, Fuchs and Lindemann (1979) also found an *S*-shaped relationship between the cellular Na^+ concentration and the active Na^+ transport in frog skins.

The results found by the method used in this paper and the method used by Larsen et al. (1979) are nearly identical (Table 2).

Cereijido et al. (1964) found that the Na^+ pool under control conditions in whole skins was 150 neq/cm^2, whereas *I* found that the Na^+ transport pool in isolated epithelia under the same conditions was 35.6 neq/cm^2.

The discrepancy between this data is probably due to the presence of Na^+ in the corium and the diffusion delay caused by the corium.

The present work indicates, as also shown by Parsons and Hoshiko (1971), that the halftime (2–4 min) typically seen under control condition in whole skin washout or build-up experiments (Hoshiko & Ussing, 1960; Cereijido et al., 1964; Nagel & Moshagen, 1978) is due to the corium.

Thus, the results in the present paper are in agreement with the data obtained by other methods (Rick et al., 1978; Larsen et al., 1979), but they are different from data obtained in experiments where tracer washout from whole skins were used.

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