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Influx and Efflux of Sodium at the Outer Surface of Frog Skin*

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Summary. The unidirectional Na influx, j_{12}, and Na efflux, j_{21}, at the epithelial surface of the frog skin were determined under various experimental conditions. The j_{21} was taken as the difference between j_{12} and the simultaneously measured short-circuit current (SCC). Errors in j_{12} determination originating from various transport rates within the skin were kept to a minimum using a normalization procedure. Under control conditions, j_{12} (1.20 μEquiv/cm² · hr) was found to be only slightly larger than the SCC (1.10 μEquiv/cm² · hr). After inhibition of the transepithelial Na transport by amiloride, ouabain, low temperature and low Na concentration, the reduction of j_{12} and SCC was almost identical, indicating that the entrance of Na into the epithelium is rate limiting for the transepithelial transport. Compared to the control, j_{21} remained unchanged after amiloride and ouabain, but was insignificantly reduced at low temperature and significantly reduced at low Na concentration. These data are consistent with the assumption that the Na efflux follows mainly an extracellular pathway.

The model of transepithelial Na transport proposed by Koefoed-Johnsen and Ussing [22] is based on the assumption that Na on its pathway through the epithelium has to cross a transport compartment limited by an outer and inner barrier, which have different transport properties. It was suggested by these authors that Na enters the transport compartment across the outer barrier by a passive transport step and is then actively extruded from the compartment across the inner barrier. In frog skin, the outer and inner barrier seem to be represented by the outer and inner facing cell membranes of the stratum granulosum [11, 31]. Whereas the involvement of an active transport step at the inner barrier is still generally accepted, the view that the entrance of Na into the epithelium proceeds by simple diffusion along an electrochemical gradient, has been called into question recently by several authors [4, 8, 26].

In order to obtain more detailed information about the transport properties of the outer barrier, different approaches have been used to determine the Na influx into the epithelium, j_{12} (Na fluxes in a three-

* Parts of the results were reported at the British-German Meeting on Pharmacology, Berlin 1973.

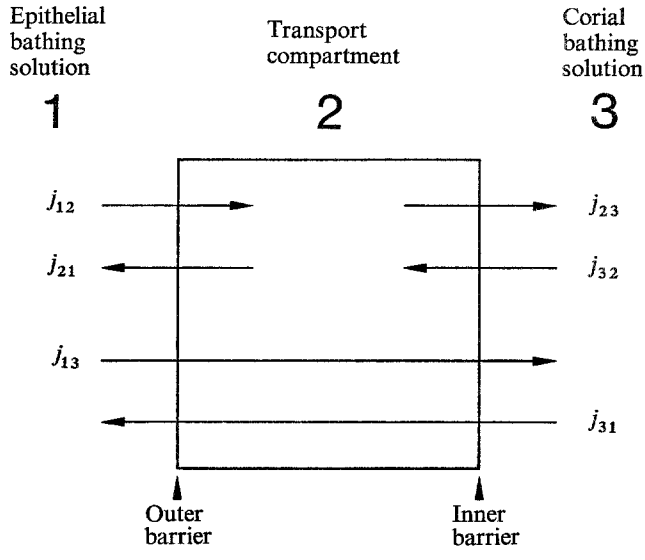

Fig. 1. Three-compartment model of Na transport in frog skin. Na fluxes across the outer barrier (j_{12}, j_{21}), across the inner barrier (j_{23}, j_{32}) and the transepithelial Na fluxes (j_{13}, j_{31}) are given

compartment system are depicted in Fig. 1). By analysis of the unidirectional transepithelial Na influx, j_{13}, on the basis of a three-compartment model, it has been concluded that for the outer barrier the backflux of Na, j_{21}, is relatively large compared to the net flux [10]. Results of direct j_{12} measurements, obtained with different methods by Biber and Curran [4] and Rotunno *et al.* [27], seemed to be compatible with this conclusion since they found that the rate of Na uptake into the epithelium, j_{12}, greatly exceeded the short-circuit current (SCC), or the unidirectional Na influx, j_{13}. However, more recent data from several authors, obtained using modifications of these initial methods, show smaller values of j_{12} under open-circuit and short-circuit conditions [3, 5, 17, 24].

In the present investigation, j_{12} was measured under short-circuit conditions using a modification of the method described by Moreno *et al.* [24]. j_{21} was calculated as the difference between j_{12} and SCC. The behavior of these fluxes under different experimental conditions was investigated to provide further information about the nature of the outer barrier.

Materials and Methods

Materials and Apparatus

The experiments were performed on the isolated abdominal frog skin. The frogs, *Rana temporaria* and *Rana esculenta*, were kept under running tap water at room temperature prior to the experiments. The arrangement used for measuring the uptake of

Na from the epithelial side was similar to that described by Rotunno *et al.* [27] and Cereijido *et al.* [9]. The isolated skin was mounted on a rectangular frame, exposing an area of 7.5 cm^2 (5 cm high and 1.5 cm wide) which was then inserted into a lucite Ussing-type chamber. The volume of each half-chamber was 9 ml. A dual infusion pump allowed the half-chambers to be filled separately but simultaneously at a constant rate. The bathing solutions could be rapidly sucked out of the half-chambers using a vacuum pump.

For measuring the potential difference (p.d.) and the short-circuit current (SCC), the chamber was equipped with calomel and Ag-AgCl electrodes. The calomel electrodes were connected to the half-chambers by Ringer's bridges. The ends of the Ringer's bridges were placed 2 mm above the bottom of the chambers and 0.5 mm away from the skin surface. The Ag-AgCl electrodes formed the wall of each half-chamber, opposite to the skin. With this arrangement of the electrodes it was possible to keep the skin short-circuited even while the chambers were being filled. The short-circuiting was performed by means of an automatic device [19].

The bathing solution used was frog Ringer's solution, containing (in mM): 110 NaCl, 2.5 $KHCO_3$ and 1 $CaCl_2$. In solutions containing only 10 mM Na, 100 mM NaCl of the Ringer's solution were replaced by Choline-Cl. The radioactive solution contained 50–500 μC/ml ^{24}Na[1]. Amiloride and ouabain were dissolved in Ringer's solution to give a final concentration of 10^{-4} M. All solutions were bubbled with air at all times except during the uptake measurements and had a pH of 8.0–8.3.

Experimental Procedure

At first the frog skin was preincubated on both sides with Ringer's solution until the SCC had reached a steady-state value (control SCC). The measurement of Na uptake was then commenced either immediately (control experiments) or after the transepithelial Na transport had been inhibited by one of the following experimental conditions:

a) open circuit conditions; short-circuiting was interrupted for 1 or 15 min;

b) low temperature; 2-hr equilibration at 0 °C;

c) low Na concentration; incubation of both sides of the skin for 20–40 min with Ringer's solution containing 10 mEquiv/liter Na;

d) amiloride; incubation of the epithelial side of the skin for 20–30 min with amiloride;

e) ouabain; incubation of the corial side of the skin for 60–90 min with ouabain.

For the measurement of j_{12} the bathing solutions were removed quickly, within 1 sec, and the half-chambers were each refilled from the bottom at a constant rate with the same experimental solutions except that the epithelial solution contained ^{24}Na. After the solutions had reached the top of the half-chambers, they were sucked out and the frame with the skin was removed. The epithelial surface of the skin was then immediately rinsed for 2 sec with isotonic sucrose and the central part of the exposed area of the skin was cut into 9 or 18 pieces of the same size (0.5 or 0.25 cm high and 1 cm wide). Cutting was completed within 5 sec after rinsing. In contrast to the method of Moreno *et al.* [24], the skin was not frozen in liquid N_2 after rinsing, since by using symmetrical halves of the same skin it could be shown that the uptake curve of the frozen half was identical to that of the unfrozen half.

1 The irradiation of Na was kindly performed by Forschungsreaktor Garching, TU Munich.

For calculating the Na uptake the radioactivity of each piece of skin and that of a standard was determined in a well-type scintillation counter. Since the chamber is filled from the bottom to the top at a constant rate, the length of time that each level of the skin was in contact with the radioactive solution is proportional to the distance from the top. The exposure time of the individual pieces of skin can therefore be calculated as a fraction of the total infusion time plus the time which elapsed between emptying the chamber and rinsing the skin with isotonic sucrose (usually 2–3 sec). The time course of the Na uptake was obtained by plotting the uptake of the individual pieces against the corresponding exposure times.

The results, unless otherwise stated, are expressed as the mean $\pm$ standard deviation. The regression lines were calculated by the method of least squares.

Results

Evaluation of j_{12} and j_{21}

Preliminary experiments were performed to determine the time course of the Na uptake from the epithelial side of the skin within the first 5 min of exposure to tracer Na. It was found that ^{24}Na uptake was linear with time for the first 2 min and thereafter the slope of the uptake curve diminished gradually. This linear behavior during the first 2 min indicates that the tracer lost by effluxes out of the skin to the epithelial and corial side are negligible compared to the influx from the epithelial bathing solution. This view is supported by the observation that no tracer Na could be found in the corial bathing solution within the first 2 min of exposure. As a consequence, since in all other experiments the maximal exposure time did not exceed 1 min, the slope of the uptake curve was taken to be equal to the unidirectional influx of Na, j_{12}.

Under steady-state conditions the SCC equals the difference between the unidirectional transepithelial Na fluxes [30]. Since the Na net flux across the skin must also equal the net flux across the outer barrier, the difference between the SCC and the influx j_{12} equals the efflux, j_{21}. In those experiments in which j_{12} and SCC were determined simultaneously, the efflux j_{21} was calculated.

The results obtained from a typical experiment performed under control conditions are shown in Fig. 2. In *B* the Na uptake of individual pieces of skin is plotted against the time of exposure to tracer Na. Calculated from the slope of the regression line the j_{12} is 1.89 μEquiv/cm^2 · hr. The intercept of the regression line with the ordinate is 1.5 nEquiv/cm^2. In *A*, the time course of the SCC during the period of infusion is recorded. The mean SCC was 1.81 μEquiv/cm^2 · hr.

From network analysis, assuming a liquid film of about 100 μm at the skin surface, it can be calculated that those parts of the skin which are above the fluid level contribute to the SCC by only a negligible amount.

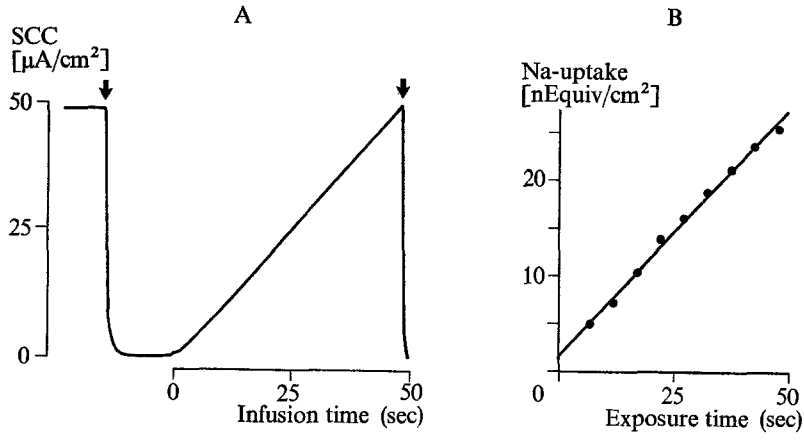

Fig. 2. The time courses of SCC (*A*) and Na uptake (*B*) obtained in a control experiment. The arrows in *A* mark the emptying of the half-chambers after the preincubation period and at the end of the infusion period

In addition, blotting the skin surfaces with filter paper did not influence the shape of the SCC curve. Therefore, the linear increase in SCC reflects only the increasing area of skin below the fluid level. However, in many experiments the time course of the Na uptake and the SCC was not as linear as shown in Fig. 2. The records of Na uptake and SCC obtained from a skin showing extreme deviations from linearity are shown in Fig. 3 *A* and *B*. The varying slopes of the SCC record indicate that different parts of the skin have varying transporting rates. Therefore, it seems to be reasonable to correct the Na uptake for this inhomogeneity in transport rate. By differentiation of the SCC curve, the local SCC values were obtained (Fig. 3 *C*). The normalization of the Na uptake curve was performed by dividing the Na uptake of the individual pieces by the ratio of the corresponding local SCC to the mean SCC (Fig. 3 *D*). This procedure of "normalizing" the Na uptake of each piece of skin can be seen to greatly reduce the scatter of points and introduces linearity into the uptake curve.

j_{12} and j_{21} at Different Functional States of Na Transport

The measurement of Na uptake was performed under control conditions and after inhibition of the transepithelial transport by cold, open-circuit conditions, low Na concentration, after ouabain and amiloride. A typical uptake curve for each of these conditions is shown in Fig. 4. For better comparison, examples have been selected with almost identical control SCC's. Curve *A* represents the time course of Na uptake under control

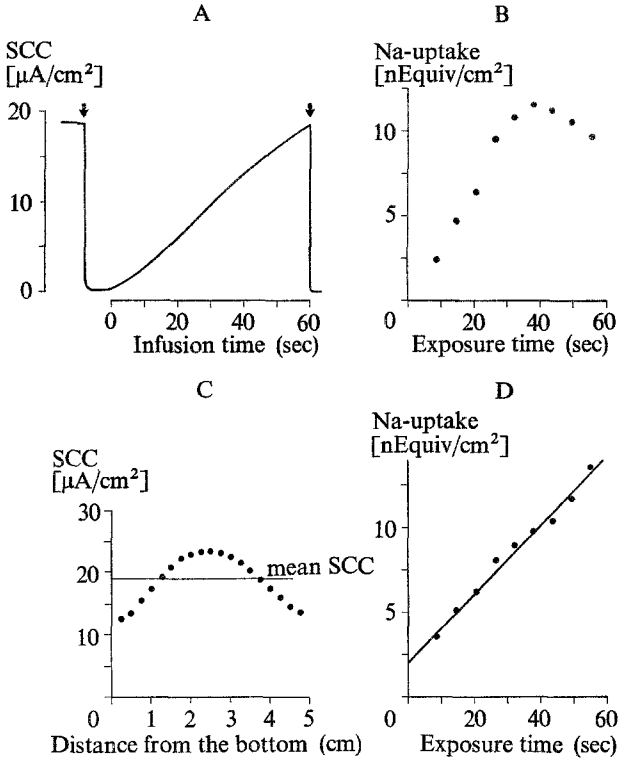

Fig. 3. Illustration of the normalizing procedure. (*A*, *B*) time courses of SCC and Na uptake in a control experiment. (*C*) the local transport rate (SCC's) at different levels of the exposed area of frog skin. (*D*) normalized Na uptake curve

conditions. Compared to the control, the slopes of the uptake curves under conditions *B* to *F* are reduced, indicating a smaller j_{12}. In the cold (*B*) the j_{12} is only 19% of the control value, under open-circuit conditions (*C*) 65%, with Na concentration of 10 mM (*D*) 56%, with ouabain (*E*) 12% and amiloride (*F*) 1%.

The *nonzero intercept* of the uptake curves seen in the examples shown in Fig. 4 was also observed in almost every experiment. On average, in the controls, it was 3.41 ± 3.43 nEquiv/cm^2. The small increase in intercept detected under open-circuit conditions and after inhibition by ouabain was not statistically significant. A significantly smaller intercept was found in experiments with low Na concentration (10 mM), 0.72 ± 0.76 ($p < 0.005$; *see* Fig. 4*D*).

The mean values of j_{12}, j_{21} and SCC obtained under the different experimental conditions are summarized in Fig. 5.

Under *control conditions*, j_{12} was found to be only slightly larger than the simultaneously measured SCC. The mean value of j_{12} in 40 experiments

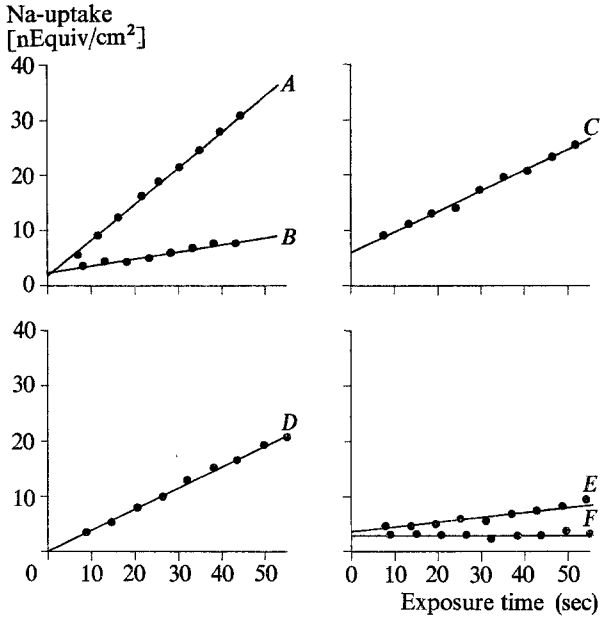

Fig. 4. Na uptake curves obtained under different experimental conditions. (*A*) control; (*B*) at 0 °C; (*C*) open circuit; (*D*) 10 mM Na concentration; (*E*) ouabain; (*F*) amiloride. Experiments *A*, *B* and *E*, *F* are performed on two halves of the same skin

Experimental Condition	j_{12}	j_{21}	SCC	Contr. SCC
	[μEquiv/cm² · hr]			
Control	1.20	0.10	1.10	1.10
Open circuit	0.98	–	–	1.38
0° C	0.30	0.06	0.24	1.80
[Na] = 10 mM	0.58	0.01	0.57	1.11
Amiloride 10^{-4} M	0.16	0.10	0.06	1.11
Ouabain 10^{-4} M	0.17	0.12	0.05	1.01

Fig. 5. Mean values of the Na influx (j_{12}), Na efflux (j_{21}), the SCC during the uptake measurement (SCC) and during preincubation period (control SCC)

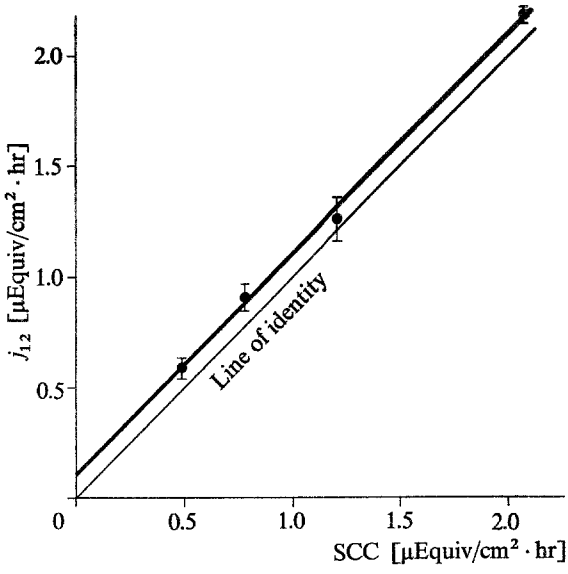

Fig. 6. Na influx, j_{12}, as a function of the corresponding SCC under control conditions (Mean values ± SEM)

was 1.20 ± 0.71 μEquiv/cm² · hr and that of the SCC was 1.10 ± 0.64 μEquiv/cm² · hr. Fig. 6 shows j_{12} as a function of the corresponding SCC. The four points were obtained by separating the control experiments into four equal classes with different SCC values. The relationship between j_{12} and SCC is linear. The j_{12} exceeds the corresponding SCC by only a small difference, 0.10 μEquiv/cm² · hr, which seems to be constant over a wide range of SCC values. No systematic deviation between the results obtained with or without the use of the normalizing procedure was detectable, but the standard deviation of the j_{21} determination was reduced from 0.32 to 0.14 μEquiv/cm² · hr.

Under *open-circuit conditions* the mean j_{12} was found to be 0.98 ± 0.39 μEquiv/cm² · hr ($n = 12$). The mean control SCC in this series was 1.38 ± 0.82 μEquiv/cm² · hr. The j_{12} calculated as a percentage of the corresponding control SCC was on average 75 ± 10%. No significant difference could be detected between j_{12} values determined 1 min (78%, SEM 2%) and 15 min (72%, SEM 6%) after the release of the zero voltage clamp.

In experiments with *low Na concentration* ([Na] = 10 mM), j_{12} and SCC were almost identical. In 13 experiments the mean j_{12} was 0.58 ± 0.32 μEquiv/cm² · hr and the SCC was 0.57 ± 0.30 μEquiv/cm² · hr. The j_{21} was calculated to be 0.01 ± 0.07 μEquiv/cm² · hr and significantly different from the mean j_{21} in the controls ($p < 0.025$).

After inhibition of Na transport by *reducing the temperature* to 0 °C, j_{12} was found to be 0.30 ± 0.18 μEquiv/cm² · hr (n = 10). The SCC decreased from 1.80 ± 0.70 to 0.24 ± 0.05 μEquiv/cm² · hr. Compared to the control, the j_{21} (0.06 ± 0.14 μEquiv/cm² · hr) was reduced approximately by a factor of 2, but this reduction was not statistically significant. A mean Q_{10} was calculated to be 2.3 for the SCC and 2.1 for the j_{12}.

The inhibition of Na transport by *amiloride* produced a decrease in the SCC from 1.11 ± 0.41 to 0.06 ± 0.03 μEquiv/cm² · hr (n = 11). Under these conditions, j_{12} was 0.16 ± 0.13 μEquiv/cm² · hr. The j_{21} (0.10 ± 0.14 μEquiv/cm² · hr) was identical with the control value.

The same effect upon the SCC and j_{12} as under amiloride was observed under the action of *ouabain*. The SCC decreased from 1.01 ± 0.22 to 0.05 ± 0.04 μEquiv/cm² · hr (n = 10). The corresponding j_{12} was 0.17 ± 0.12 μEquiv/cm² · hr. The j_{21} (0.12 ± 0.13 μEquiv/cm² · hr) was not significantly different from the control nor from the value obtained under amiloride.

Discussion

The outer barrier to Na movement in the frog skin is thought to be situated at the apical cell membrane of the stratum granulosum [16, 21, 29, 31]. Consequently, when determining the Na influx, j_{12}, by measuring the Na uptake, the Na located in front of this barrier in adherent fluid, intercellular spaces and stratum corneum must be considered. The two methods available for direct determination of j_{12} [4, 27] differ with respect to the approach used to take this quantity of extracellular Na (Na_{ECS}) into account. In the method first described by Biber and Curran [4] the exact measurement of Na_{ECS} is essential since the Na which has been taken up is measured only once, after a definite exposure time. In contrast, in the method first described by Rotunno *et al.* [27], the time course of Na uptake is used to calculate the j_{12}.

In the present experiments, using this latter method, the Na influx j_{12} under control conditions was found to exceed the simultaneously measured SCC by only a small value (0.10 μEquiv/cm² · hr). This result is not in agreement with that reported by Biber *et al.* [3] who found a considerably larger difference between j_{12} and SCC under these conditions (1.94 μEquiv/cm² · hr). The larger values for j_{12} determined by these investigators may be due to an underestimation of Na_{ECS} using an ECS-determination. Although the Na concentration of the stratum corneum, as determined by microprobe analysis, is almost identical with that of the epithelial bathing solution [11], it can be expected that this space is not completely accessible to ECS

markers [7]. Therefore, the large difference between j_{12} and SCC reported [3, 4, 5] might be explained by an influx of Na into a space not exchangeable by ECS markers. Support for this hypothesis is provided by the fact that by using different ECS markers, different values of j_{12} were obtained [3, 17]. In addition, this view might provide an explanation for the much smaller difference between SCC and j_{12} found by Biber *et al.* [3, 4] at low Na concentration, since under these conditions the amount of Na in the space not marked by the ECS markers should be reduced.

When calculating j_{12} from the slope of the Na uptake curve, two possible sources of error occur if the different portions of the skin which are used to compile the uptake curve have either varying amounts of extracellular Na or have different transport rates. When the Na concentration in the bathing solution is relatively high, as under control conditions, the amount of Na located in front of the outer barrier (approximately 70 nEquiv/cm^2) is large compared to that which has crossed the barrier within the short exposure times. Therefore, inhomogenieties of Na_{ECS} will have a considerable influence upon the value of j_{12} obtained. The nonlinear uptake curves and the higher j_{12} values reported previously by Rotunno *et al.* [27] may be due, in part, to this effect. In the present experiments, in order to minimize this error, the epithelial surface of the skin was rinsed with isotonic sucrose solution after exposure to tracer Na as proposed by Moreno *et al.* [24]. This procedure reduces the Na concentration in the stratum corneum to almost zero [11] but leaves the concentration in the stratum granulosum unaffected (author's unpublished results from electron-microprobe analysis), thus demonstrating that cellular Na wash-out due to rinsing is negligible. This is supported by the observation that the rinsing procedure may be extended to 6 sec without affecting the Na uptake curves (authors' preliminary experiments and ref. [24]). A small amount of Na might, however, remain in front of the barrier even after rinsing. The nonzero intercept with the ordinate of the uptake curves may be attributable to this remaining extracellular Na. This explanation is strengthened by the observation that the size of the intercept seems to depend only upon the Na concentration of the bathing solution. The variation of the transport rate within one skin, that is indicated by the varying slopes of SCC record during filling of the chamber, was taken into account by the normalization procedure described. Since the scatter of the individual determinations can be substantially reduced in this way, relatively small numbers of experiments are sufficient to detect small changes of j_{12} or j_{21}.

Using these procedures to eliminate errors in j_{12} determination, even the small difference between j_{12} and SCC could be accurately determined.

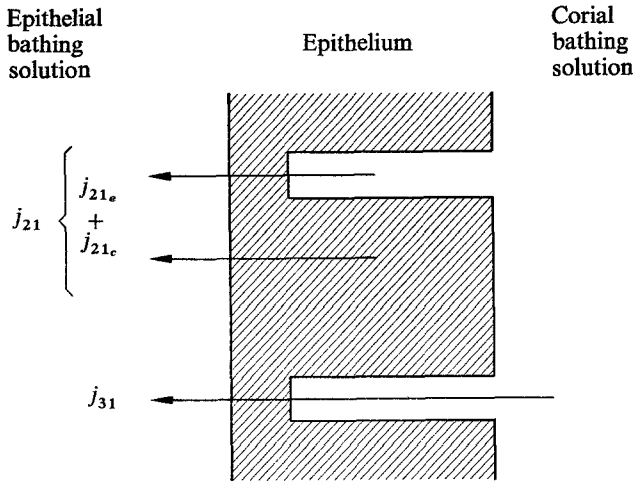

Fig. 7. Schematic diagram of possible routes of Na efflux. The subscripts *e* and *c* denote the extracellular and cellular component

The interpretation that this difference represents the unidirectional efflux from the skin to the epithelial side, j_{21}, is only valid if the net charge transfer across the skin, as measured by the SCC, results only from a net transfer of Na ions. However, a small net absorption of chloride [16, 23, 28, 32] and excretion of hydrogen ions [15] have been reported in some species of frog. If the transport of both these ions contributes to the net transfer of charge, the measured SCC would be somewhat smaller than the net Na transport, so that the actual Na efflux would be even smaller than calculated by the difference between j_{12} and SCC.

The Na which leaves the skin and moves into the epithelial bathing solution can be considered theoretically to arise from two different sources. This Na may originate from a cellular compartment and/or from the corial bathing solution, crossing the skin via the intercellular spaces and tight junctions (Fig. 7). Two experimental conditions which should influence mainly a cellular component of Na efflux exist during the application of amiloride or ouabain. Amiloride, which is thought to reduce the Na permeability of the outer barrier [12–14], should reduce a cellular efflux whereas ouabain, which is known to increase cellular Na concentration [20, 25], should increase a cellular efflux. Neither substance produced statistically different alterations in j_{21}, suggesting that the contribution of a cellular efflux component to the j_{21} is small. Two experimental conditions, which would be expected to reduce a paracellular component of Na efflux, would be lowering the Na concentration or the temperature of the bathing solution. In fact, j_{21} is reduced to 60% at 0 °C and to 10% at 10 mM Na

concentration compared to control conditions. Only the latter value was statistically different from the control, whereas the former was not statistically different owing to the variability of j_{21} and the relatively small effect of a temperature decrease to 0 °C. Nevertheless, it seems reasonable to conclude from these data that the major source of the Na which effluxes out of the skin to the epithelial side comes from between the cells.

If j_{21} does proceed almost entirely across a paracellular pathway, it must be identical with the transepithelial efflux, j_{31}. This appears to be the case, since the values of j_{21} obtained in these experiments under various experimental conditions are in good agreement with the values of j_{31} reported in the literature [12, 25, 29]. An important consequence of the identity of j_{31} and j_{21} is that no Na which has already passed across the outer barrier can recirculate to the epithelial bathing solution. Results obtained recently by Moreno *et al.* [24] confirm this idea. It was reported that under open-circuit conditions, j_{12} and the transepithelial influx j_{13} are nearly identical, indicating that none of the inwardly transported Na in j_{12} is available to contribute to the Na efflux j_{21}. Therefore, our observation that j_{12}, under open-circuit conditions, is smaller than the SCC of the skin, is to be expected, since j_{13} has been found to be smaller under open-circuit than under short-circuit conditions [30].

Reduction of the Na influx, j_{12}, under short-circuit conditions is observed when the transepithelial transport of Na is inhibited by amiloride, ouabain, low temperature and low Na concentration, a finding consistent with previous reports [2, 24]. The near identity and similar behavior of j_{12} and SCC under various experimental conditions suggests that it is the outer barrier which is the decisive step of transepithelial Na transport. However, this result does not provide direct evidence for the route Na takes when passing through the skin. Noncellular transport models, as proposed by Cereijido and Rotunno [8] and including the assumption already discussed by Moreno *et al.* [24] are compatible with the observed identity of Na fluxes across the outer barrier with the transepithelial fluxes. On the other hand, cellular transport models may explain the behavior of Na fluxes in two different ways: firstly, that the entrance of Na into the transport compartment involves an active step; secondly, that it occurs passively along a large electro-chemical gradient. Histochemical investigations of the ATPase localization in frog skin [18, 26] and energetic considerations do not lend support to the concept of an active transport step. For the passive entrance of Na across the outer barrier, low intracellular Na concentration and/or a negative intracellular potential would be necessary to explain the lack of detectable cellular Na efflux. The Na concentration, measured in isolated

epithelium [1], isolated cells [24] and by microprobe analysis [11], although relatively low, is not sufficiently low to explain why no cellular Na efflux could be detected. However, to make a passive influx of Na across the outer barrier possible in the almost complete absence of any cellular efflux, a large potential difference (p.d.) cell negative to the epithelial bathing solution, is necessary. A reduction of such a p.d. under experimental conditions, where the Na concentration in the bathing solution is the same as under control (open circuit, cold, ouabain), would then provide an explanation for the observed decrease of j_{12}. An increase of such a p.d. may also explain the observation that j_{12} was only 40% smaller after reducing the Na concentration of the epithelial bathing solution from 110 to 10 mM. Measurements presently available show an intracellular potential under control conditions of only -15 to -20 mV [6, 29], whose magnitude does not change at low Na concentrations [6]. Although these observations speak against a cellular transport pathway, the possibility of such a transport route cannot be ignored; the correlation between transcellular Na transport and the size [31] or the Na concentration [11] of the stratum granulosum indicates that this cellular layer is involved in transcellular Na transport.

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