The Penetration of Sodium into the Epithelium of the Frog Skin

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ABSTRACT The aim of this paper is twofold. First, to describe a method for the measurement of the unidirectional flux of Na from the outer bathing solution into epithelium (J_{OT}), and second, to describe the use of this method under a variety of experimental conditions in order to obtain some insight into the nature of this flux. The method developed is based on the exposure of a frog skin to a Ringer solution containing ^{22}Na. The exposure is made so that neighboring points along the surface remain in contact with the ^{22}Na solution for gradually longer periods, ranging from 0 to 46 sec. Some 8 to 10 samples of the exposed part are used to obtain the time course of the uptake of ^{22}Na and this time course is used, in turn, to evaluate J_{OT}. This flux is then studied in skins mounted between two identical Ringer solutions with 115 mM Na (11.25 $\pm$ 0.10 [18] μmole$\cdot$hr^{-1} cm^{-2}), and in skins mounted with Ringer with 1 mM Na on the outside and 115 mM Na on the inside (0.43 $\pm$ 0.05 [18] μmole$\cdot$hr$^{-1}\cdot$cm^{-2}). From the observations that the flux is much larger than the net Na flux across the whole skin, that it is inhibited by K^+, and is unaffected by ouabain, it is concluded that the penetration of Na^+ into the epithelium does not occur by simple diffusion and is not directly dependent on an ouabain-sensitive mechanism. In the course of these experiments it was observed that when the skin was crushed between two chambers the uptake of Na in the neighboring exposed areas was decreased.

Only a small fraction of the total sodium contained in the epithelium participates in the process of active Na transport *across* the frog skin (6–9). This fraction of sodium—hereafter referred to as the sodium transporting compartment—is not confined to a particular cell layer but is distributed over the whole epithelium (7, 34). The mechanism used by Na to penetrate from the outer bathing solution into this transporting compartment must be a very efficient one since frog skins are known to take up a net amount of NaCl from solutions as diluted as 10^{-5} M (25); i.e., several orders of magnitude below the concentration in the plasma and in the epithelium (6, 34). On the basis of kinetic analysis of ^{24}Na movement Cereijido et al. (5) have demon-

strated that the mechanism involved in this uptake of Na^+ is not simple diffusion. Since the Na^+ pumping depends on the hydrolysis of ATP, the absence of ATPase at the outer solution–epithelium boundary (16, 34) also permits the conclusion that the mechanism used by Na to penetrate into the transporting compartment is not a pump either. In order to obtain more information on the mechanism used by sodium to penetrate into the epithelium it would be necessary to have a reliable method for the *direct* measurement of the unidirectional flux J_{OT} (see Fig. 1), and to study this flux under a variety of experimental conditions. However, this measurement has serious experimental difficulties. The known minimum value of J_{OT} and the small size of the transporting compartment indicate that this compartment would equilibrate with sodium tracer added to the outer solution in a very short time. This makes it necessary that any method for the evaluation of J_{OT}, based on the uptake of radioactive sodium, be fast enough to take a suitable amount of samples in the very early part of the exposure to tracer; *i.e.*, in a fraction of a

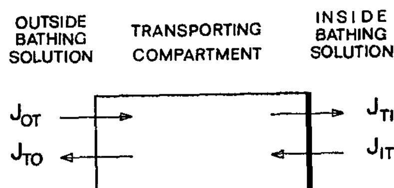

FIGURE 1. Compartment model for analysis of unidirectional fluxes across the outer and the inner facing membrane of the frog skin.

minute. A new method was devised, based on the exposure of a skin to ^{22}Na for progressive longer periods, to determine the time course of this rapid uptake. Although this method allows more than 15 samples to be taken in a few seconds, it was found that 7 to 8 samples over a period of 50 sec were sufficient to permit an accurate evaluation of J_{OT}. We then measured J_{OT} under a variety of experimental conditions.

EXPERIMENTAL METHOD

Principles

The abdominal frog skin is mounted as a flat sheet between two Lucite chambers (Fig. 2 *a*). The exposed part of the skin has a rectangular cross-section. A dual simultaneous infusion pump injects Ringer solution containing ^{22}Na into the chamber on the left, nonradioactive Ringer into the chamber on the right (Fig. 2 *b*). The radioactive solution is in contact with the outer side of the skin. The solutions are injected steadily into the bottom of the chambers, and the level rises homogeneously. The chambers can be filled in almost any desired time, but this time must be known accurately. Fig. 2 *b* illustrates a case in which the infusion was made in 48 sec. When the solution reaches the upper border and the exposure is interrupted, the lowest part

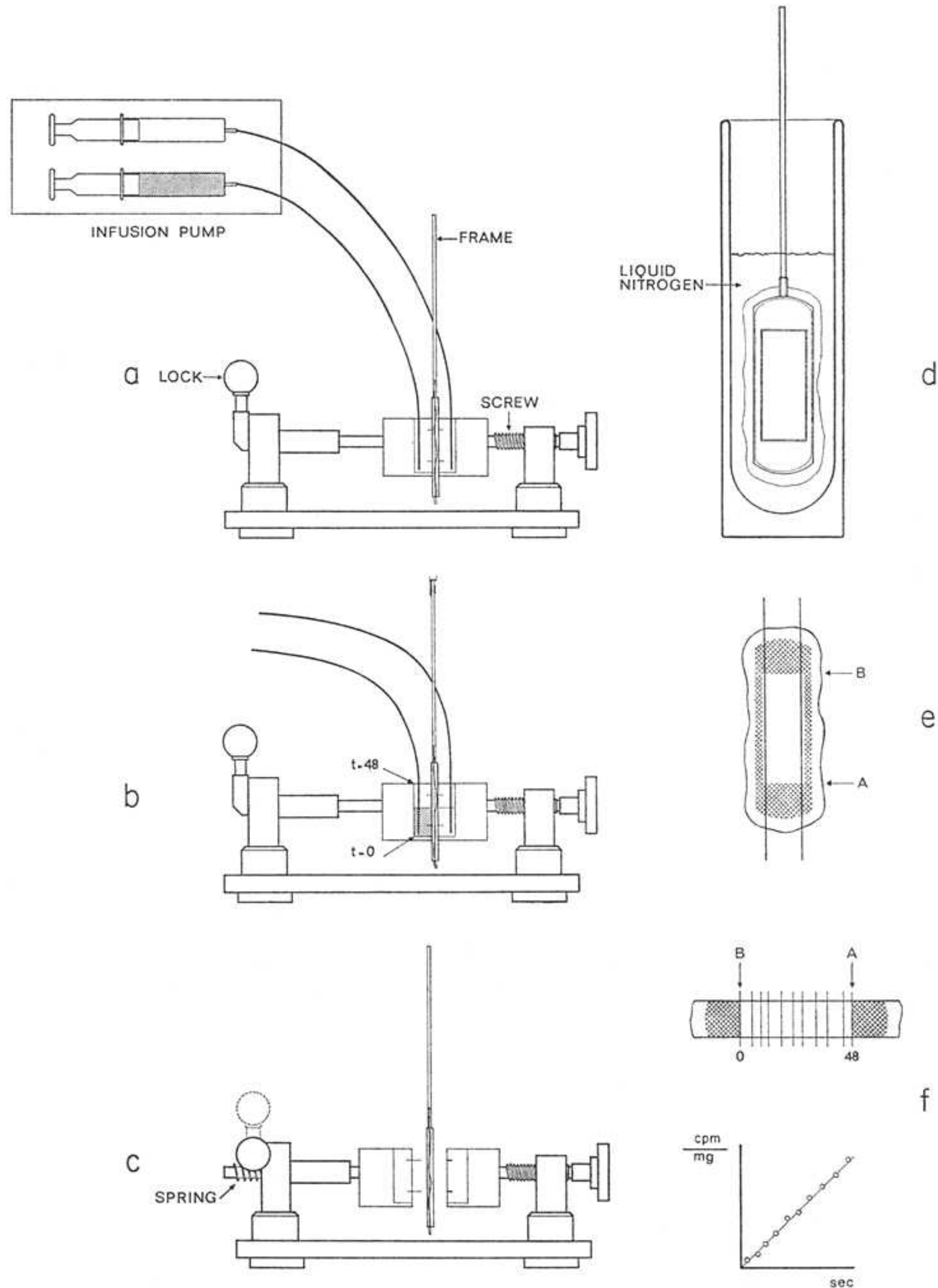

FIGURE 2. Schematic diagram of apparatus and experimental procedure. (*a*) The window of the frame allows the skin to be mounted as a flat sheet between two rectangular Lucite chambers. (*b*) The dual infusion pump injects Ringer with ^{22}Na into the chamber in contact with the outer face of the skin (left) and nonradioactive Ringer on the other. In this particular case the chambers are filled in 48 sec. (*c*) The lock releases

of the membrane is exposed for the whole time of the injection (48 sec). All other parts are exposed for a fraction of 48 sec. This fraction is proportional to the distance to the floor of the chamber. Once the solution reaches the upper border, the lock is released and a strong spring pulls the mobile shaft with the left chamber towards the "open" position (Fig. 2 *c*). The released frame with the skin is then immersed in chilled sucrose solution for 1 sec, and then put into a thermos flask containing liquid nitrogen (−196°C) (Fig. 2 *d*). The central part of the skin (Fig. 2 *e*) is cut into 10–15 transverse sections, each representing a certain uptake time of ^{22}Na (Fig. 2 *f*). The samples are analyzed for their concentration of ^{22}Na, and a kinetic curve, showing the rise in concentration of ^{22}Na as a function of time, is plotted. The slope of the curve, together with the specific activity of ^{22}Na in the bathing solution, can be used to calculate the unidirectional sodium flux from the outer solution to the epithelium (J_{OT}).

Material

The abdominal skin of specimens of either sex of the South American frog *Leptodactylus ocellatus* was used throughout. Animals were kept in a moist sink, and periods in captivity never exceeded 2 months. The water used in this sink had previously been gassed with moistened compressed air for 2 days. Most experiments were carried out on frogs from the same shipment. The different experiments reported were performed during all seasons over a period of 1 yr.

SOLUTIONS

Two Ringer solutions were used, one containing (mM): NaCl 115, $KHCO_3$ 2.4, $CaCl_2$ 1.0, glucose 2 g. In the other Ringer solution, 114 mM of NaCl were replaced with 228 mM of sucrose. All reagents were of analytical grade.

DRY WEIGHT

Small pieces of skin were collected in a tared small dish made out of Teflon, 0.9 cm in diameter and 1 mm thick at the border, or on a small tared piece of aluminum paper. They were then dried to constant weight at 90°C.

SODIUM CONTENT

The dried sample *and* the Teflon dish were placed in a polystyrene test tube with 2.0 ml of 0.1 N HNO_3, and stoppered with Parafilm (Marathon) paper (American Can Co., Neenah, Wisconsin). This was left overnight to be extracted in a shaker. Aliquots were then diluted and Na was measured by flame photometry (Hitachi-Perkin-Elmer spectrophotometer with photomultiplier, with flame photometry at-

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the spring which pulls the chambers apart. (*d*) The frame containing the skin is deep in a thermos flask with liquid nitrogen. (*e*) The skin is removed from the frame. The hatched area represents the marks left by the frame. The skin is cut along the vertical lines. (*f*) The central strip is cut into small pieces. Arrows *A* and *B* denote the bottom and the upper edges which were exposed for 48 and 0 sec, respectively. The counts per minute per milligram in each piece are represented as a function of time. The slope of the curve is a function of the flux J_{OT}.

tachment for C_2H_4 and O_2). Samples collected on aluminum paper were never used for Na measurements since traces of aluminum interfere with determination of alkali metals.

^{22}Na FLUX

The solution injected into the chamber in contact with the outer side of the skin contained 1 μCi/ml of ^{22}Na Cl (Amersham or New England Nuclear). Once dried and weighed, the samples of skin were counted in a well-type scintillation counter (Nuclear Chicago Auto Gamma) set as spectrometer in the ^{22}Na peak. Samples of 500 μl of the outside bathing solution were diluted and counted in triplicate.

Experimental Procedure

The skin is mounted in a metallic frame with a central rectangular window measuring 5.2 × 2.1 cm as shown in Fig. 3. The central window allows the skin to be mounted in a rectangular Lucite chamber. The borders of this chamber make contact directly with the skin, just inside the metallic frame. The left chamber is mounted on a mobile shaft which, at the beginning of the experiment, is locked in the "closed" position (Fig. 2). The chamber on the right, which is mounted at the extreme end of a screw system, is then advanced from right to left until it contacts the skin. Four pins (Fig. 2 *c*) prevent sidewise displacements. The volume of each chamber is 16.9 ml. The exposed area is 8.9 cm^2.

Once mounted, the skin is left to equilibrate with the desired Ringer solutions. Electrical potential difference is measured by connecting the chambers through agar-Ringer bridges to calomel half-cells and these cells to a Keithley 200B DC electrometer (input impedance, 10^{14} Ω). The Ringer solutions were stirred by bubbling moistened air directly into the chambers. All experiments were carried out at room temperature (20–22°C).

After the equilibration period the solutions were quickly, but thoroughly, sucked out of the chambers. A Harvard (Model 941) simultaneous dual injection pump was used to deliver the radioactive (left chamber) and the fresh (right chamber) solutions. The rate and volume of the infusion were adjusted beforehand by selecting the speed of the pump and the volume of the syringes. Special care was taken to raise the level in both chambers at exactly the same rate and to avoid bulgings in the skin toward either side, because these not only modify the timing, but also they introduce profound modifications in the functional state of the membrane (31). A microswitch was built into the Harvard pump to stop the injection once the desired amount had been delivered. At this moment the chamber was opened by releasing the lock, and the skin was plunged deep into a 600 ml beaker with 238 mM sucrose at 0°C, and then transferred to a thermos flask containing liquid nitrogen at −196°C. The total operation from the interruption of the injection to the freezing in liquid nitrogen takes about 2 sec. When the skin is removed from the liquid nitrogen, it is impossible to cut it without breaking it into pieces, unless it is allowed to warm up for 10–20 sec. There is a period in which the skin is still frozen stiff but does not have a glass-like consistency. Cuts were made during this period. The skin is removed from the frame and a central strip is cut as shown in Fig. 2 *e*. The hot (bottom) side of this strip is caught with a

long hemostatic forceps. This forceps permits both the handling of the skin and the plunging of it again deep inside the nitrogen at any time that these become necessary. Some 10 cuts were made going from the "colder" to the "hotter" side so as to avoid contamination. Each sample was collected, dried, weighed, and counted as explained above.

The counts per minute (cpm) of ^{22}Na were divided by the milligrams of dry weight of the samples, and the values obtained were plotted vs. time. The time at which the

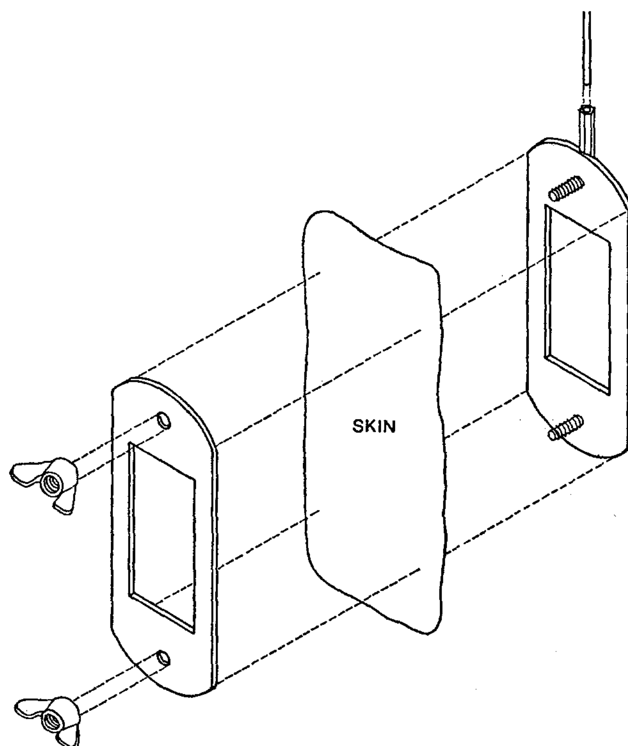

Figure 3. Exploded view of the mounting of the frog skin in a metal frame with a rectangular central window.

counts per minute per milligram of a given sample were plotted was obtained 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 gives the time that the sample was exposed to the radioactive solution. The slope of the curve is given in units of counts per minute per milligram per second. The measured specific activity of ^{22}Na in the loading solution was used to convert the counts per minute into micromoles, and this value, divided by the area per milligram (0.14 $cm^2 \cdot mg^{-1}$ of dry weight [34]) gives the flux of sodium in micromoles per square centimeter per second. Fluxes in this paper, though, are expressed in micromoles per square

centimeter per *hour*. Results are expressed as mean ± standard error (number of observations).

RESULTS

The Choice of a Total Injection Time

In order to evaluate the unidirectional sodium flux, J_{OT}, from the uptake of ^{22}Na it is necessary to take samples of the ^{22}Na content in the skin before the opposite flux (J_{TO}) becomes appreciable. This requires an estimate of how fast the specific activity of ^{22}Na in the transporting compartment (p_T^*) equilibrates with the specific activity of ^{22}Na in the outer solution.

When the skin is mounted as a flat sheet between two Ringer solutions and sodium tracer is added to the outer chamber, the flux of tracer across the whole skin equilibrates in a short time, ranging from 2 to 15 min (1, 5, 7, 12, 20). This implies that the epithelial compartment from which the flux is coming (*i.e.* the transporting compartment) should equilibrate even faster than in 2 to 15 min (35). This suggests that, in order to evaluate the flux outer solution → epithelium, the samples should be taken in the first minute after adding the tracer on the outside.

One can make a better estimate of the half-equilibration time of the flux, J_{OT}, on the following basis: when ^{22}Na is added to the outside bathing solution, the variations in the amount of tracer in the transporting compartment are given by the following equation.

$$\frac{dP_T}{dt} = J_{OT}p_o^* - p_T^*(J_{TO} + J_{TI}) + J_{IT}p_I^* \qquad (1)$$

P_i = total activity of ^{22}Na in compartment i (counts per min);
J_{ij} = Na flux from compartment i to j (μmoles·hr^{-1}·cm^{-2});
p_i^* = ^{22}Na specific activity in compartment i (counts·min^{-1}·μmoles^{-1})

Since the bathing solutions are constant reservoirs, p_o^* remains constant and p_i^* may be taken as zero. Also, since the preparation is assumed to be in a steady state, the Na fluxes are constant and obey the following equation

$$J_{OT} + J_{IT} = J_{TO} + J_{TI} \qquad (2)$$

This allows us to modify equation 1 as follows:

$$\frac{dP_T}{dt} = J_{OT}(p_o^* - p_T^*) - p_T^* J_{IT} \qquad (3)$$

The inner membrane of the transporting cells has a very low permeability to Na (24, 30). It is crossed by the active flux, J_{TI}, in the inward direction. J_{IT} is negligible as compared with the other fluxes in equation 2 (6). Thus

equation 2 can be written

$$J_{OT} = J_{TO} + J_{TI} \tag{4}$$

and equation 3 becomes

$$\frac{dP_T}{dt} = J_{OT}(p_o^* - p_T^*) \tag{5}$$

The amount of tracer in the transporting compartment, P_T, is equal to

$$P_T = p_T^* S_T$$

Where S_T is the amount of Na in the transporting compartment. Introducing this value into equation 5 and rearranging

$$\frac{dp_T^*}{p_o^* - p_T^*} = \frac{J_{OT}}{S_T}\,dt \tag{6}$$

Integrating between $t = 0$ and $t = t$, and taking into account that at $t = 0$ the specific activity in the transporting compartment is zero, equation 6 yields

$$-\ln \frac{(p_o^* - p_t^*)}{p_o^*} = \frac{J_{OT}}{S_T}\,t \tag{7}$$

Therefore, the half-equilibration time ($t_{1/2}$) of compartment T is equal to

$$\frac{0.693\ S_T}{J_{OT}} = t_{1/2} \tag{8}$$

S_T, the Na-transporting compartment of the frog skin, has been evaluated in the skin of *Rana temporaria* and *Rana arvalis* as 0.063–0.070 (21) and 0.122 μmole cm^{-2} (1), and in the skin of *Leptodactylus ocellatus* as 0.032 μmole cm^{-2} (6). J_{TI}, which represents the active transport of sodium, has been found to be 2.32 μmoles. hr$^{-1}\cdot$cm^{-2} (39) in Ringer solutions with 115 mM Na. According to equation 4, J_{OT} can be *at least* as high as J_{TI}. Therefore, if one uses 2.32 as the value of J_{OT} and 0.070 μmole$\cdot$cm^{-2} as the value of S_T in calculating $t_{1/2}$ with equation 8, one obtains a maximum estimate of this parameter:

$$\frac{0.070 \times 0.693}{2.32} = 0.021\ \text{hr} = 75\ \text{sec}$$

Hence, if in this very short time the Na-transporting compartment reaches half the specific activity of the loading solution, in order to evaluate J_{OT} one

should sample the skin in an even shorter time. The time used was always between 46 and 48 sec.

Errors Due to Delay of [22]Na in Reaching the Outer Facing Membrane

The transporting compartment equilibrates so quickly with the outer loading solution that unstirred layers might introduce an error in the estimate of J_{OT} from samples taken in the first minute. Unstirred layers in the frog skin are of the order of 20–50 μ (22, 13). Na crosses these layers in some 0.2 sec, which is more than two orders of magnitude faster than the time used in this paper to evaluate J_{OT} (*i.e.* 46.5 sec), and should therefore cause a negligible error.

Errors Due to Diffusion of [22]Na along the Surface of the Skin

The method used to evaluate J_{OT} is based on the exposure of different parts of the skin for gradually varying periods of time; i.e., the farther from the bottom, the shorter the exposure time. Thus if ^{22}Na diffuses upward through the water which wets the outward face of the skin at a rate faster than the level of the loading solution is raised, the upper portions of the skin would have a longer exposure than the one measured by the rising rate of the solution in the chamber. It is possible to evaluate the magnitude of this error by considering that the injection is stopped at a given level. 90 sec later (i.e. twice the usual time of injection) the concentration of isotope (C) in the layer of the water wetting above the level of perfusing solution would be given by (10).

$$C = C_o \operatorname{erfc} \frac{x}{2\sqrt{D_{\mathrm{Na}}\, t}}$$

where C_o is the concentration of isotope in the liquid injected. The diffusion coefficient of Na (D_{Na}) is 1.5×10^{-5} cm^2, sec^{-1} (32). Since the width of a typical sample of skin is greater than 0.2 cm, the second sample above the level of the solution will be more than 0.2 cm ($x = 0.2$) from the loading solution. Introducing these values into the equation, one obtains

$$\frac{C}{C_o} = 1.3 \times 10^{-4}$$

Therefore, the concentration of isotope in the water wetting the surface of the skin at more than 2 mm above the level of the loading solution, would be almost four orders of magnitude lower than in the solution injected. This means that diffusion of ^{22}Na through the water wetting the surface would not constitute a source of error.

The Measurement of J_{OT}

Fig. 4 illustrates the time course in a skin of the ^{22}Na content obtained with Ringer solutions with 115 mM Na. The slope was calculated by the least squares method. The last two points on the right were not used to compute the

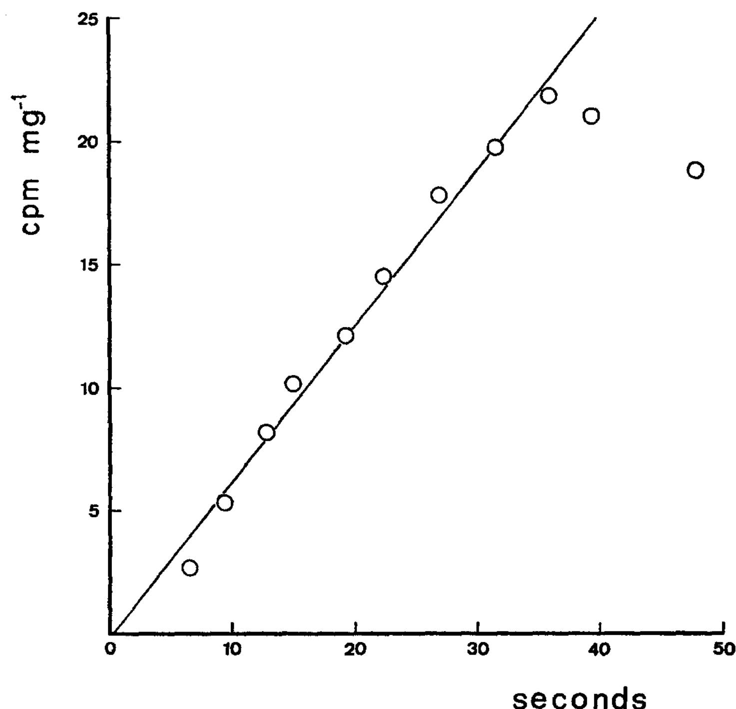

FIGURE 4. Uptake of ^{22}Na in a skin mounted in Ringer solutions with 115 mM Na. The last two points on the right were not taken into account in tracing the straight line. The slope is 0.67 cpm·mg^{-1}·sec^{-1}. The specific activity of ^{22}Na in the Ringer was 2110 cpm/μmole. The skin has 7.12 mg of dry weight per square centimeter. The corresponding J_{OT} is 8.13 μmoles·hr^{-1}·cm^{-2}.

slope. This decrease of the slope in the last points was observed in all the experiments. It would seem, at first, that they indicate a saturation of the transporting compartment. However, the observation that experiments carried out with faster infusion times (Fig. 5) also presented depression of the last points, and that this effect was also observed with Ringer solutions with only 1 mM Na (Fig. 6) cast doubts on the view that this represented a saturation effect. Moreover, saturation should only stop the rate of uptake at a constant level, not decrease it.

A possible clue to the origin of this effect was provided by the experiments

shown in Fig. 7. In this series of experiments the solution was not raised gradually, but the whole skin was exposed during the same period (30 sec). The skin was then removed, frozen, and sliced in vertical strips parallel to the lateral sides of the chamber. It can be observed that, as the samples approach the borders of the skin, the amount of tracer taken up is smaller. This effect suggested the convenience of taking only a central vertical strip of skin

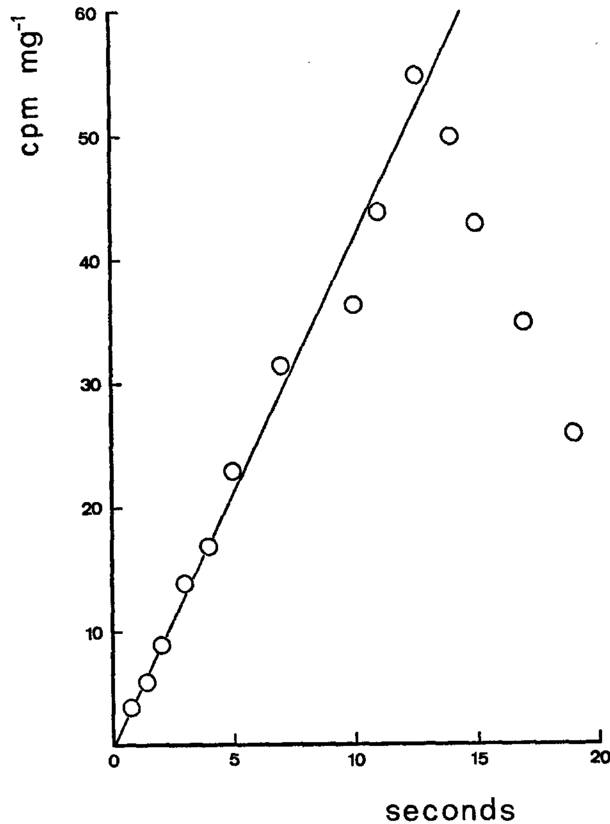

FIGURE 5. Measurement of J_{OT} with a total infusion time of 20 sec. The skin was mounted in Ringer solutions with 115 mM Na. Notice the depression of the last four points.

(Fig. 2 *e*) in order to measure the flux. But even in this strip the total length is needed to obtain the timing (see Fig. 2 *f*), and the early and the last samples (i.e. those near the skin crushed at the top and the bottom) cannot be discarded. It should be emphasized though, that even when the crushing of neighboring areas decreases the rate of uptake, the uncertainty remains whether the decrease of the last points is also partially due to saturation. A third factor that might also play a role in the decrease of the last points is the fact that the lower and uppermost parts of the exposed skin correspond to the

lateral edges of the central abdominal skin of the frog. This skin might be somewhat thicker than the rest and, the corresponding amount of counts per minute per milligram might be lower. In view of the possibility that these factors may impair the evaluation of J_{OT}, samples near the edges are used

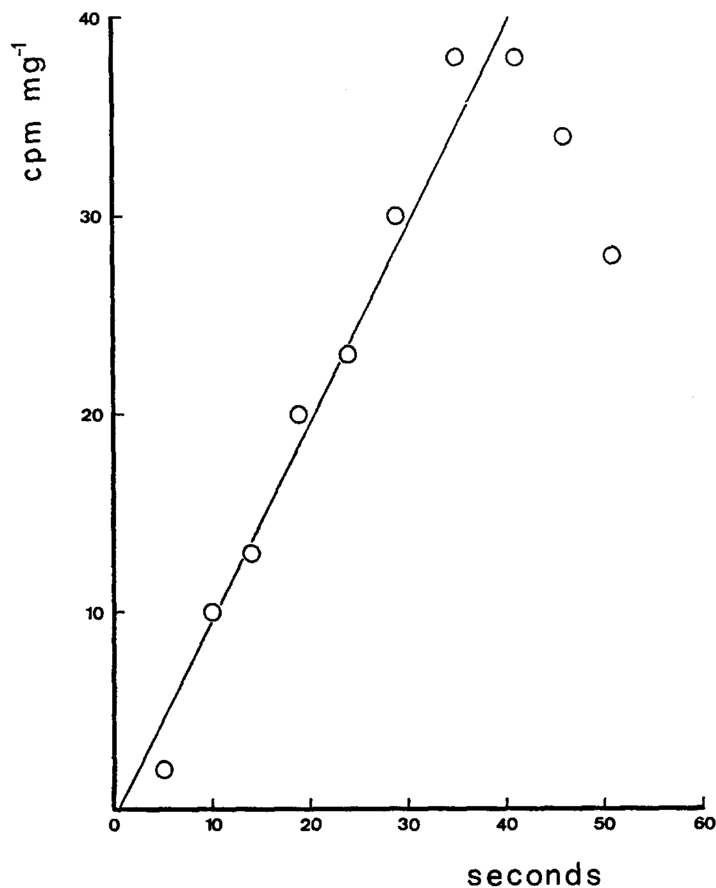

FIGURE 6. Measurement of J_{OT} in a skin mounted with regular Ringer on the inside, and with Ringer containing 1 mM Na on the outside. Notice the depression of the last two points.

only to calculate the timing of the samples, but disregarded when calculating the slope. As shown in Fig. 4, the rest of the samples fall in a straight line.

Table I summarizes the value of J_{OT} under two conditions:

(a) THE SKIN IS MOUNTED BETWEEN TWO IDENTICAL RINGER SOLUTIONS WITH A Na CONCENTRATION OF 115 mM The flux is 11.25 ± 0.10 (18) μmoles·hr^{-1}·cm^{-2}. This value is much higher than the value of the total net flux under short-circuited conditions (2.32 μmoles·hr^{-1}·cm^{-2}) (39). Al-

though in the present series of experiments the skin is not short-circuited, it should be noted that its spontaneous electrical potential is oriented in the direction that would decrease J_{OT}. This suggests that, under short-circuit conditions, the differences between J_{OT} and the net flux might be still larger.

As mentioned above, the half-equilibration time was estimated in order to set an adequate infusion rate. Equation 8 was used for this purpose and the value of the net flux (2.32 μmoles·hr^{-1}·cm^{-2}) was taken as a minimum estimate of J_{OT}. Now that the real value of J_{OT} is available, the real half-equilibration time can be computed

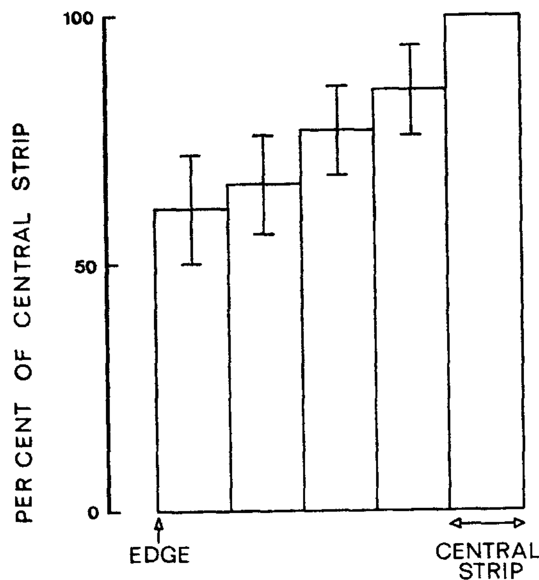

FIGURE 7. Uptake of ^{22}Na by frog skins in which the whole area was exposed for 30 sec. After the exposure the skins were cut vertically in pieces parallel to the lateral borders. The uptake in the central strip is taken as 100%. As the samples approach the edge of the chamber the uptake of ^{22}Na decreases.

$$\frac{0.693 \times 0.070}{11.25} = 0.0425 \text{ hr} = 15.3 \text{ sec}$$

Since neither the uptakes of Figs. 4–6 nor the other curves from which J_{OT} was computed appear to bend for at least 40 sec, a half-equilibration time of 15.3 sec results in a surprisingly short time. These results suggest that the current kinetic models used to evaluate the size of the transporting compartment (1, 6, 21), and which were used, in turn, to choose a suitable timing for the measurement of J_{OT}, need to be reevaluated.

(b) THE SKIN MOUNTED IN RINGER WITH 115 mM Na ON THE INSIDE AND 1 mM Na ON THE OUTSIDE The natural environment of frogs usually has a lower concentration of Na than their plasma. The exposure of the skin to 1 mM Na on the outside resembles more closely the natural condition in which the skin works than the condition tested above (i.e. with 115 mM Na on both

sides). Fig. 6 shows a typical experiment. With a low concentration of Na on the outside J_{OT} is 0.43 $\pm$ 0.05 μmole·hr^{-1}·cm^{-2}, which is of the same order as the net flux of 0.56 $\pm$ 0.07 μmole·hr^{-1}·cm^{-2} measured under the same conditions in *Rana pipiens* (3).

The Effect of Ouabain

The net movement of Na across the skin can be drastically reduced by ouabain (23, 39). This effect is assumed to be due to the inhibition of a pumping mechanism located at the "inner facing membrane" (flux J_{TI}). Flux J_{OT} is assumed to be passive and, therefore, should not be affected by ouabain. In order to test this assumption a concentration of 10^{-5} M was used on the inside. Concentrations of ouabain, one or two orders of magnitude lower, inhibit the active transport of Na^+ and of K^+ as well (11).

When the skin is bathed in Ringer with 115 mM Na on both sides the value of J_{OT} in control experiments is 14.01 $\pm$ 1.6 (8) μmole·hr^{-1}·cm^{-2} and in the

TABLE I

THE INFLUX OF SODIUM IN THE FROG SKIN

Concentration of Na in the outer* Ringer	Influx of sodium	n
mM	μmoles·hr^{-1}·cm^{-2}	
115	11.25 $\pm$ 0.10	18
1	0.43 $\pm$ 0.05	18

* Sodium concentration in the inside Ringer solution was 115 mM.

ouabain-treated skins is 13.90 $\pm$ 2.1 (8). In five experiments with normal Ringer on the inside, but 1 mM Na Ringer on the outside, the controls have 0.250, 0.255, 0.263, 0.330, and 0.433 μmole·hr^{-1}·cm^{-2} (mean, 0.306), and four ouabain-treated skins had 0.231, 0.307, 0.308, and 0.366 (mean, 0.303). This indicates that J_{OT} is not inhibited by ouabain. This absence of an effect of ouabain on J_{OT} agrees with the observation of Farquhar and Palade (16) and Rotunno et al. (34) that, at the outer anatomical border of the frog skin there is no ATPase to account for the movement of Na from the outside to the epithelium.

Effect of Potassium

The electrical potential and short-circuit current across epithelial membranes are sensitive to Na^+, but relatively insensitive to K^+ ions in the outer bathing solution (24, 20, 27, 4). Fig. 8 shows the effect of K^+ concentration on the unidirectional flux, J_{OT}, studied in skins with low (less than 2.5 mM) concentrations of Na^+ on the outside. The effect was studied at low concentrations of Na^+ in order to be able to increase the concentration of K^+ without making

the outer solution hyperosmotic, and also because the high Na over K selectivity requires a relatively low concentration of Na^+ in order to observe a clear inhibition by K^+. The curve shows that in agreement with this Na over K selectivity, it takes some 28 mM K^+ to reduce the sodium flux to half its control value. It also shows that, at the concentrations tested, K^+ does not inhibit completely the sodium flux.

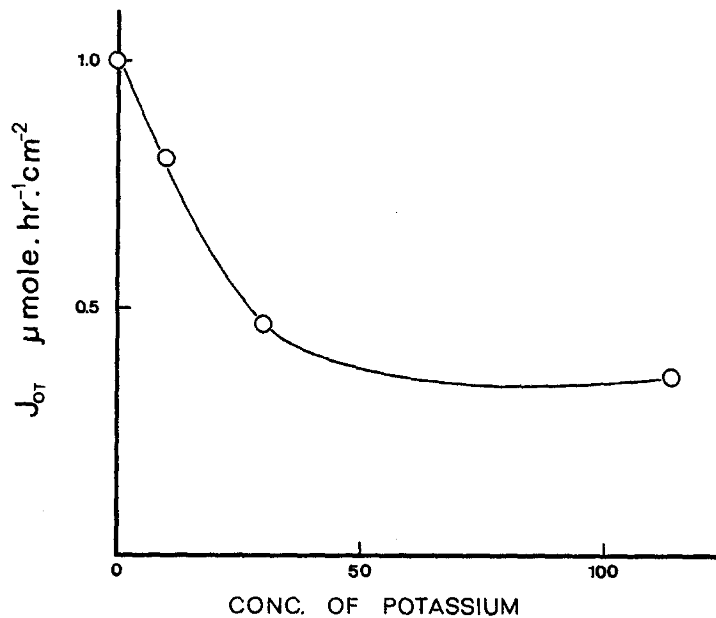

FIGURE 8. The influx of ^{22}Na as a function of the concentration of potassium in the outer bathing solution. The concentration of Na in the outer bathing solution is 1–2.5 mM, and in the inner solution 115 mM. Each point is the result of a duplicate experiment.

DISCUSSION

As stated in the Introduction, the aim of this paper is to describe a method for the measurement of the unidirectional flux medium → epithelium, and then to use it to obtain some insight into the nature of this flux. The value of J_{OT} from Ringer solution with 115 mM Na is 11.25 μmoles·hr^{-1}·cm^{-2}; i.e., almost five times higher than the flux from the epithelium to the inner side. The measurement of this flux was recently carried out by Curran and Biber[1] using a completely different experimental approach. These authors exposed the skin during a single period of some 30 sec, and then analyzed the whole exposed area for radiosodium. The values that they found, although somewhat lower than the ones reported here, also indicate that the Na flux from the

[1] Curran, P. F., and T. Biber. Private communication.

outside to the epithelium is much higher than the one from the epithelium to the inside.

In the trials necessary to design the method an incidental observation was made; i.e., the uptake of ^{22}Na is proportionally lower in the area of the skin close to the borders crushed by the chambers (Fig. 7). It is a very well-known characteristic of epithelial membranes that preparations mounted in bigger chambers (larger exposed areas) show larger fluxes per unit area than those mounted in smaller ones. The border: area ratio seems to play a role. Although some knowledge of cellular contacts and communication in epithelia is available (29), the mechanism by which a cell senses that thousands of microns away other cells are being crushed, and the mechanism which transforms this information into a reduction of the permeability to Na remain obscure. The fact that in this paper only the central (high flux) strip of exposed skin was used, might account for the somewhat larger fluxes reported here than in the work of Curran and Biber.

Current models of Na transport envisage the sodium-transporting compartment as being connected only to the outer and inner bathing solutions, and bound on the inside by a Na-impermeable barrier. This model of a transporting compartment was used to derive an equation relating J_{OT} to the size of the Na pool (S_T) (equation 8). The use of this equation with values of S_T published by several authors (1, 6, 21) and the experimental value of J_{OT} measured in this paper predict a half-equilibration time of some 15 sec. The uptake curve of ^{22}Na, though, does not show a tendency to saturate during the whole time of the experiment (46 sec). This would indicate that the kinetic model mentioned above, which is currently used to evaluate the size of the transporting compartment, might not be correct.

Is the ^{22}Na Uptake Operated by a Single Mechanism?

The observation that potassium does not inhibit completely the flux (Fig. 8) and the observation that the slopes of the curves counts per minute per milligram vs. time do not saturate by the time the transporting compartment is assumed to be in equilibrium (Figs. 4 and 5), suggest that the unidirectional flux from the outer bathing solution to the epithelium might enter into some other compartment as well. Epithelial Na is not contained in a single compartment (6). The possibility exists that the different compartments might be independently connected with the outside bathing solution. Moreover, the Na flux to a given compartment is not necessarily carried by a single mechanism. This suggests that the unidirectional flux, J_{OT}, could be the result of a sum of several fluxes. This view is supported by the observation of Curran, Herrera, and Flanigan (12) that even though the effects of Ca^{++} and antidiuretic hormone on the flux of Na^{+} are due to the effects of these agents on the same "outer barrier," they act entirely independently. Bentley's (2) studies of

the effects of vasopressin, aldosterone, and amphotericin B on the urinary bladder point to the same multiplicity of J_{OT}.

One of the components of the influx must necessarily penetrate into the transporting compartment. The question arises of where the other (or others) component goes. The net influx comprises 94% (i.e. 2.32 out of 2.47 μmoles·hr^{-1}·cm^{-2} [39]) of the influx. Therefore, the compartment where this "extra flux" goes might not be connected with the inner bathing solution through an exchange diffusion mechanism, otherwise the unidirectional flux from the outer to the inner bathing solution would be much greater than the net influx.

The Nature of J_{OT}

The observation that ouabain fails to inhibit J_{OT} (Table I) in a preparation in which the active flux is known to depend on ATP hydrolysis and to be sensitive to ouabain (23) would suggest that J_{OT} is primarily a passive flux, i.e. it is not *directly* coupled to the hydrolysis of ATP. Whether J_{OT} could transfer Na^+ against its electrochemical potential gradient by virtue of a coupling to the movement of other molecular species, cannot be answered on the basis of the present results. The fact that the outer face of the skin has a high Na over K selectivity, together with the observation that the entrance of Na^+ is inhibited by K^+ (Fig. 8) suggests that Na^+ does not enter by free diffusion either, but it interacts with some sites in the epithelium. On the basis that a Na^+-transporting mechanism going through the epithelial cells would require Na^+ to penetrate the hydrophobic component of the membrane twice (once to get into the cell from the outer bathing solution, and another to get out at the opposite side of the cell), Cereijido and Rotunno (8) suggested the possibility that Na^+ migrated over the polar groups of the outer leaflet of the cell membrane of the epithelial cells. Vilallonga, Fernández, Rotunno, and Cereijido (36) have demonstrated that the lipid monolayer can even exhibit ion selectivity. Therefore, the possibility exists that one of the first things that Na^+ does when entering the epithelium is to interact with the polar groups of the outer leaflet of the cells. This view is supported by a study of the electrical excitability of the frog skin carried out by Lindeman (26) indicating that, in order to enter into the epithelium, Na interacts with a limited number of sites. Interaction of Na with sites in the skin must be a fairly common feature since Rotunno et al. (33) demonstrated that some 60% of the total Na in the skin is not free as Na^+ ion but complexed. Whether these sites are negative fixed charges (14, 28) or mobile carriers (15) cannot be answered on the basis of the present results.

The Rate-Limiting Step in Na^+ Transport across the Epithelium

The unidirectional flux, J_{OT}, is some five times greater than the total influx of Na^+. This would indicate that the Na pumping at the epithelium-corium

boundary is the rate-limiting step. The outer facing barrier however, offers a more significant restriction to the movement of water and ions than the inner facing barrier (4, 20, 30, 38). Studies by Curran et al. (12), Cereijido et al. (5), and Whittembury (37) have in fact indicated that the movement of Na^+ across the outer barrier is a rate-limiting step in the transport of sodium and that many agents elicit an effect on the over-all Na^+ transport by modifying the entrance of Na into the transporting compartment. The fact that the *net* entrance of Na into this compartment is rate limiting, while the *unidirectional* flux, J_{OT}, is much greater than the net flux, is a further indication that, in order to penetrate into the transporting compartment, Na^+ must interact with sites constituting a mechanism of obligatory exchange diffusion: large unidirectional fluxes of ions with low or even zero net fluxes are characteristics of such systems.

In discussing the rate-limiting step in the translocation of Na^+ it must be taken into account that under physiological (nonshort-circuited) conditions the skin does not transport just Na^+, but some salt of this ion. Fischbarg et al. (17) and Gil Ferreira (19) have shown that Na^+ transport across frog skin is indeed influenced by the nature of the anion. The translocation of Na^+ through a system which can exhibit facilitated diffusion can be sensitive to the nature of the anion and would offer also the possibility of exchanging external Na^+ for another cation from the inside. This exchange, that does not seem to play a significant role in in vitro preparations, seems to be of paramount importance in the living animal (18).

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