Direct Measurement of Uptake of Sodium at the Outer Surface of the Frog Skin

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ABSTRACT A combination of the methods described by Schultz et al. (6) and by Ussing and Zerahn (9) was used to measure directly the unidirectional uptake of sodium from the outside solution into the frog skin, under short-circuit conditions. The sodium uptake was determined at six sodium concentrations ranging from 3.4 to 114 mM. NaCl was replaced by choline chloride in the solutions bathing both sides of the skin. Sodium uptake is not a linear function of sodium concentration but appears to be composed of two components, a saturating one and one that varies linearly with concentration. The sodium uptake is inhibited by the addition of lithium to the outside solution. The effect appears to be primarily on the saturating component and has the characteristics of competitive inhibition. In addition, lithium uptake by the skin is inhibited by sodium. The effects of lithium cannot be ascribed to changes in electrical potential difference. Measurements with microelectrodes indicate that under short-circuit condition there is no change in the intracellular potential when lithium chloride is added to the outside solution.

The functional model for Na transport across frog skin proposed by Koefoed-Johnsen and Ussing (1) has frequently served as a basis for consideration of transport across other epithelial tissues. One of the important features of this model was that entry of Na into the skin from the outside solution is due to simple diffusion. This concept was based primarily on the observation that in open-circuit conditions the outer surface of the skin behaved like a Na electrode. However, some recent observations are rather difficult to explain in terms of simple diffusion of Na across the outer surface. For example, Cereijido et al. (2) concluded from indirect estimates of rate coefficients for Na movement that the apparent Na permeability of the outer barrier decreased with increasing Na concentration. On the basis of studies of Na transport by skins bathed on the outside with dilute solution containing 1 mM NaCl, Biber et al. (3) suggested that if all epithelial Na were in a single

compartment, an active entry step might be necessary to allow net inward movement of Na. Rotunno et al. (4) measured more directly the Na concentration in the epithelium of skins bathed on the outside with low concentrations of Na; they found that the epithelial concentration was greater than the value expected for electrochemical equilibrium with the outside solution. However, in later studies Cereijido and Rotunno (5) found that only a part of the epithelial Na was exchangeable with sodium in the outside bathing solution. Thus, the nature of the entry step for Na is not entirely clear at present.

Recently, observations have been made which suggest that the Na uptake or influx in another epithelial tissue, the mammalian intestine, may not proceed by simple diffusion; Schultz et al. (6) estimated unidirectional Na fluxes across the mucosal border of the rabbit ileum and observed a flux ratio which cannot be attributed to simple diffusion. Even more significant in this context is the demonstration of coupling of glucose (7) and of amino acid uptake (8) with Na uptake, suggesting that part of the Na influx across the brush border into the intestinal epithelial cells involves an interaction with the membrane or a membrane component.

The present experiments were carried out in an effort to obtain more precise information on Na entry into the frog skin epithelium. Na influx was measured directly using a modification of the method developed by Schultz et al. (6) and of the short-circuit technique described by Ussing and Zerahn (9). The influx was studied as a function of Na concentration, and the effect of lithium was examined. Experiments were carried out under short-circuited conditions because under these conditions the electrical potential in the epithelium is not affected by changes in the Na concentration of the outside solution (10).

METHODS

Measurement of Influx

The chamber used is shown schematically in Fig. 1. It is similar in principle to the more usual chambers used for epithelial tissues, but it is designed to permit rapid changes of the outside bathing solution and rapid removal of the skin from the solutions. A circular piece of abdominal skin of *Rana pipiens* was mounted at the lower end of the upper chamber (part 2 in Fig. 1) with the outside surface facing downwards and was held in place by a cap with a circular hole exposing an area of 0.42 cm^2 of the epithelium. The skin separated the inside chamber (part 2 in Fig. 1) containing 1–2 ml solution from the outside chamber (part 1 in Fig. 1) containing 1.8–3.5 ml bathing solution. Both solutions were stirred vigorously by a stream of small air bubbles. The potential difference (PD) across the skin was measured by calomel half-cells connected to the solutions by agar bridges. A second pair of bridges connected to Ag-AgCl electrodes was used for passing current through the skin. The PD and short-circuit current (SCC) were determined by using an automatic clamping

device that was adjustable for changes in fluid resistance between the PD bridges; the PD or SCC was fed directly to a recorder (Texas Instruments Inc., Dallas, Tex.). The time required to clamp the tissue to zero PD following a step change in PD was less than 100 msec. This is considerably less than the time required for a change in the total skin PD after a rapid change in Na concentration of the outside solution (11).

A number of small discs were cut from the skin of each frog and were placed in beakers containing the solution used for equilibration in the chambers. Air was bubbled continuously through the solutions. After a disc was mounted in the chamber, identical solutions were placed on each side, and the tissue was allowed to equilibrate until a relatively steady SCC was obtained (less than 10% change in 10 min). The

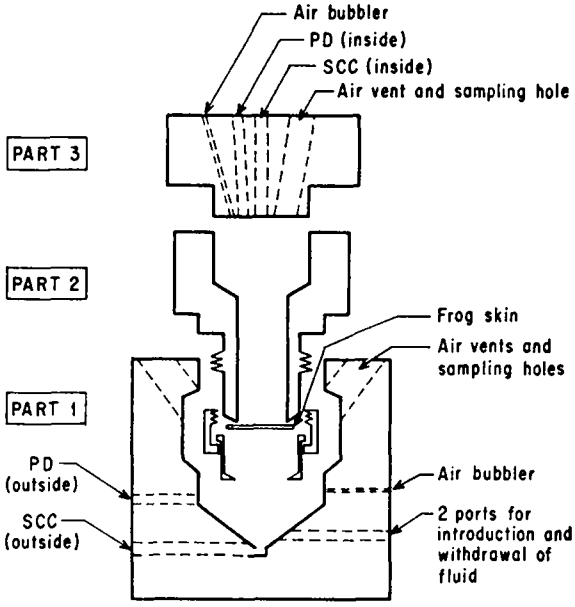

FIGURE 1. Chamber for measuring fluxes and uptake of tracers.

solution in the outside (lower) chamber was then withdrawn, and a test solution containing inulin-^{3}H (6 μCi) and ^{24}Na (5–15 μCi) or ^{22}Na (8 μCi) was injected rapidly. After 30 sec, the top of the upper chamber (part 3 in Fig. 1) was removed, the upper chamber together with the mounted skin (part 2 in Fig. 1) was withdrawn from the lower chamber (part 1 in Fig. 1), and the mounted skin was blotted on several layers of No. 1 Whatman filter paper. Simultaneously, the fluid in the upper chamber was sucked into a test tube. The procedure, from removal of the skin from the loading solution to blotting the tissue, took less than 1 sec. The time of exposure of the outer surface to the test solution was taken as the time that elapsed between injection of the test solution into the lower chamber and blotting of the skin. After blotting, the exposed tissue was cut out of the chamber with a punch and extracted for at least 2 hr in 2 ml of 0.1 N HNO_3. Aliquots of the tissue extract and the test solution were assayed for ^{3}H and ^{22}Na or ^{24}Na using a liquid scintillation counter (Model Mark I; Nuclear-Chicago, Des Plaines, Ill.). The Na concentration in the

test solutions and in the solutions used for equilibration was determined by flame photometry. For evaluation of the Li uptake by the skin, the Li concentration of the extract and test solution was measured with an atomic absorption spectrometer (Perkin-Elmer).

The inulin in the test solution was used to estimate the volume of test solution adhering to the skin after blotting. Influxes were estimated from uptake of radioactive Na or of Li after correction for the inulin "space." This space usually varied between 0.1 and 0.16 μl for 0.42 cm^2 of skin and did not differ significantly under the various experimental conditions employed. In nearly all experiments, the solution bathing the inside of the skin was checked for appearance of isotopes. In less than 1 % of the flux measurements there was a significant amount of radioactive tracer in the inside solution at the end of a 30 sec exposure to tracers at the outside surface. These measurements were discarded, although in no case was radioactive Na present in excess of tritium when compared to the test solution. This means that there is no transepithelial movement of tracer Na in 30 sec and that the appearance of isotope was probably due to contamination of the inside bathing solution with test solution or to a hole in the skin preparation.

Measurement of Electrical Potential

Interpretation of the data on influxes requires information on changes in the electrical potential difference across the outer barrier of the skin. This PD was measured with microelectrodes and by a technique similar to the one described by Cereijido and Curran (10). The skin (6.6 cm^2) was mounted, outside surface facing upwards, between two Lucite chambers; it was supported by a wire mesh and held in place by a slight negative pressure in the lower (inside) chamber. Aerated solution was continuously circulated through the lower chamber (16 ml) at a rate of 5 ml/min by a gravity flow system. The upper chamber was stirred and aerated by a stream of small air bubbles. All the micropuncture studies were carried out under short-circuit conditions. The short-circuit was only released during the brief interval necessary for the reading of the open-circuit PD. Short-circuiting was achieved by the automatic clamping device used for the influx experiments. Microelectrodes were pulled with a micropipette puller (MI; Industrial Science Associates, Inc., Ridgewood, N.J.) and filled with distilled water by the method of Caldwell and Downing (12). Finally, a 3 M KCl solution was injected into the lumen of the microelectrode, and the air bubbles were dislodged with a fine glass capillary. At least 15 hr were allowed for diffusion of KCl into the tip. The microelectrodes used had a resistance between 13 and 22 Mohms and a tip potential of less than 8 mv. The PD between the reference electrode in the outside solution and the microelectrode was measured by a Keithley electrometer (Model 600A, input resistance 10^{14} ohms; Keithley Instruments Inc., Cleveland, Ohio). The four conditions for accepting a puncture as satisfactory were the following: (*a*) electrode resistance and tip potential measured after the puncture did not differ significantly from the values measured before the puncture; (*b*) the PD drift was less than 1 mv in 3 min; (*c*) the PD between either bathing solution and the microelectrode had to be at least 5 mv under open-circuit conditions; and (*d*) the PD between the bathing solutions and the microelectrode had to be more than

−5 mv under short-circuit conditions (10, 13). When conditions *b*, *c*, and *d* were satisfied with solution H (Table I) on both sides of the skin, the upper (outside) solution was removed and replaced by solution K. The microelectrode was left in place during this procedure.

All the solutions used in these experiments are listed on Table I. Fig. 2 shows the

TABLE I

COMPOSITION OF SOLUTIONS

Solution	NaCl	Choline Cl	LiCl
A	112–115	—	—
B	81	34	—
C	63	—	52
D	52	63	—
E	11 or 12	104	—
F	11	85	19
G	11	46	58
H	6	109	—
I	6	90	19
J	6	57	52
K	6	51	58
L	3	112	—
M	3	93	19
N	3	54	58

All solutions contained 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$.

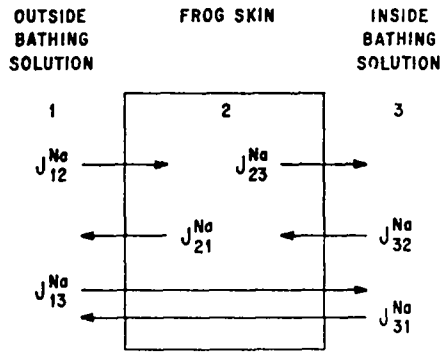

FIGURE 2. Three compartment model for movement of sodium across frog skin.

different fluxes across the frog skin. The three compartment system is, of course, an oversimplification, but it is nevertheless useful for the presentation of the data and for the discussion. All average values in this paper are given with the standard error of the mean (SEM).

RESULTS

In order to determine whether SCC measured in this chamber was representative of net Na transfer, the transepithelial Na influx (J^{Na}_{13}) was determined

in one preparation, first at 115 mM Na and then at 12 mM Na (solutions A and E, respectively). As shown in Table II, the average transepithelial influx is slightly greater than the SCC at both Na concentrations, in agreement with results obtained with more conventional chambers. Although Na outflux (J_{21}^{Na}) was not measured, these results suggest strongly that the SCC provides an adequate estimate of net Na transport under the conditions of the present experiments.

The uptake of ^{24}Na by the skin as a function of time of exposure to test solution is shown in Fig. 3. Each point represents the average of two flux determinations, and all tissues were obtained from a single frog. The tracer uptake is a linear function of time, and the line extrapolates to the origin. This observation indicates that over a 30 sec interval the specific activity of the skin compartment remains sufficiently low so that there is no significant return of tracer to the outside solution. Since, as discussed above, there is also no appearance of tracer in the inside bathing solution, the method should

TABLE II

COMPARISON OF TRANSEPITHELIAL INFLUX OF Na (J_{12}^{Na}) WITH SHORT-CIRCUIT CURRENT (SCC)

Condition	J_{12}^{Na}	SCC	Flux/SCC
	μeq/hr cm²	μeq/hr cm²	
112 mM Na	0.55	0.55	1.00
	0.57	0.56	1.03
	0.62	0.56	1.11
112 mM Na average	0.58	0.56	1.04
12 mM Na	0.16	0.13	1.23
	0.16	0.12	1.33
	0.20	0.21	0.95
12 mM Na average	0.17	0.15	1.17

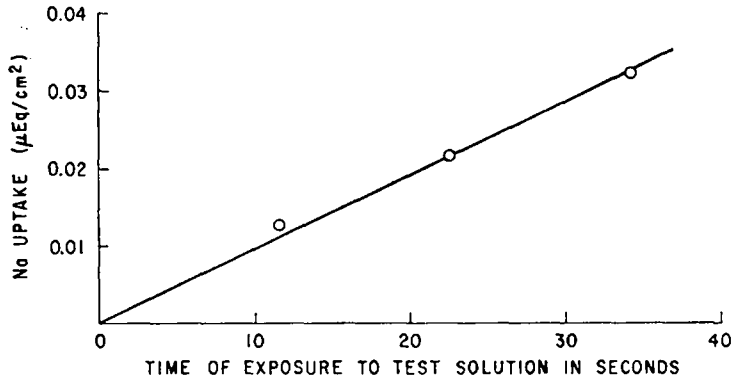

FIGURE 3. Na uptake measured by accumulation of ^{24}Na in the isolated frog skin after addition of the tracer to the solution bathing outside surface of the skin.

provide an accurate estimate of the unidirectional influx of Na from the outside solution into the skin (J_{12}^{Na}).

Na Influx (J_{12}^{Na}) at Different Na Concentrations

67 influx measurements were made on 10 frogs at sodium concentrations of 3.4, 6.1, and 11.1 mM (solutions L, H, and E, respectively). In each frog, except one, several influx measurements were made at all three concentrations. The sodium concentrations were always the same on both sides of the skin and were alternated for each determination. The first three lines on Table III give the results. In another set of experiments, 59 influx values were obtained from five frogs at Na concentrations of 52.1, 81.3, and 113.9 mM (solutions D, B, and A, respectively). Again, in each frog a similar number of observations was made in an alternating way at the different Na concentrations. The influx values are listed on the last three lines of Table III.

TABLE III
Na INFLUX (J_{12}^{Na}) AND SHORT-CIRCUIT CURRENT (SCC) AT DIFFERENT Na CONCENTRATIONS

Na concentration	Number of observations	$J_{12}^{Na} \pm$ SEM	SCC ± SEM
mM/liter		*μeq/hr cm²*	*μeq/hr cm²*
3.38	23	0.92±0.07	0.56±0.06
6.11	22	1.33±0.09	0.92±0.09
11.07	22	2.19±0.22	1.17±0.12
52.1	21	5.12±0.47	1.19±0.09
81.3	20	6.34±0.60	1.18±0.11
113.9	18	7.77±0.52	1.29±0.12

Both sides of the skin were bathed by identical solutions.

Fig. 4 shows the sodium influx as a function of Na concentration in the outside solution. The relation is clearly nonlinear, and the fact that the points at the three highest concentrations fall on a straight line suggests that the curve may be a combination of a saturating and a linear component. Thus, the influx J_{12}^{Na} would be given by

$$J_{12}^{Na} = \frac{J^m [Na]_0}{K_{Na} + [Na]_0} + \alpha[Na]_0 \qquad (1)$$

in which J^m is maximal influx for a saturating component, K_{Na} is an "apparent Michaelis constant," α is a permeability coefficient, and $[Na]_0$ is sodium concentration in the outside solution. The solid line in Fig. 4 shows the best fit (least squares) of equation 1 to the experimental points. The values of the constants in equation 1 providing this fit were $J^m = 4.0$ μeq/hr cm², $K_{Na} = 14.3$ mM, and $\alpha = 0.037$ cm/hr. The fit to the points assuming

$\alpha = 0$ was much less satisfactory than that shown in Fig. 4. A further indication that the results cannot be described adequately by a single saturating component was obtained when the data were plotted as J_{12}^{Na} against $J_{12}^{Na}/[Na]$

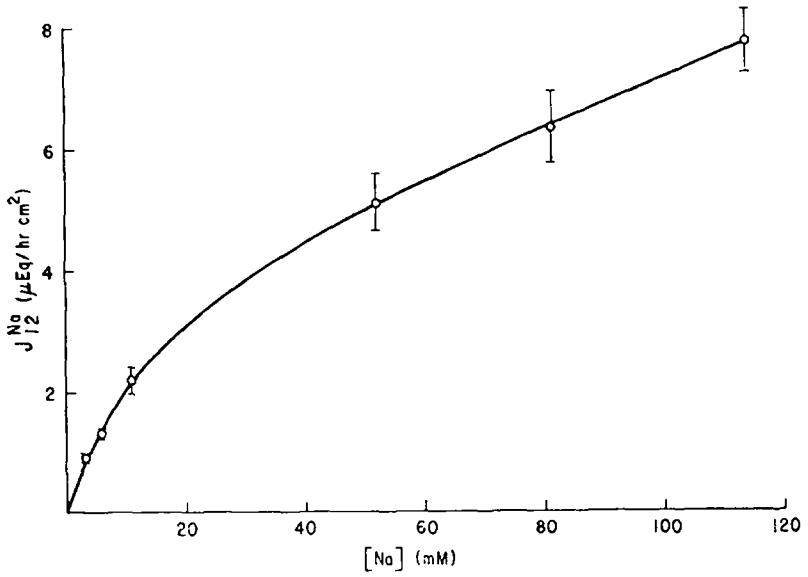

FIGURE 4. Na influx (J_{12}^{Na}) at different Na concentrations.

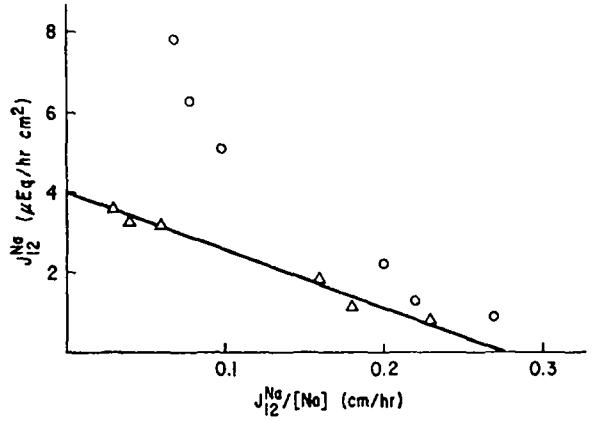

FIGURE 5. Na influx (J_{12}^{Na}) plotted against the Na influx divided by the sodium concentration ($J_{12}^{Na}/[Na]$) before (○) and after (△) subtraction of the linear component (0.037 [Na]).

(14–16) as shown in Fig. 5. The resulting relation was clearly nonlinear, but if a linear component given by 0.037 [Na] is subtracted from each point, the resulting points fall on a straight line. According to Dowd and Riggs (14), this method of plotting provides the most sensitive indicator of failure of data

to conform to simple Michaelis-Menten kinetics. Thus, the best explanation of Na influx involves a saturating component and a component linear with Na concentration. We cannot, of course, rule out the possibility that the linear portion of influx is actually a saturating component with a high apparent Michaelis constant, but there is no reason to postulate such an effect at present.

Inhibition of the Na *Influx* (J_{12}^{Na}) *by Lithium*

200 influx measurements were made using 10 frogs to examine the effect of Li on the Na influx. Two levels of Li concentration, 19.2 mM (solutions M, I, and F) and 57.5 mM (solutions N, K, and G), were tested. The results are summarized in Table IV. Na influx was decreased by the presence of Li, and at both Li concentrations the inhibition was relatively smaller the higher the

TABLE IV

INHIBITION OF Na INFLUX (J_{12}^{Na}) BY LiCl

Na concentration	J_{12}^{Na}		
	No LiCl	19.2 mM LiCl	57.5 mM LiCl
mM	*μeq/hr cm²*	*μeq/hr cm²*	*μeq/hr cm²*
3.38	0.92±0.07 (23)	0.65±0.07 (24)	0.43±0.03 (23)
6.11	1.33±0.09 (22)	1.08±0.09 (20)	0.74±0.05 (21)
11.07	2.19±0.22 (22)	1.66±0.17 (23)	1.25±0.14 (22)

Number of observations is given in parentheses. 10 frogs were used for these experiments. All nine solutions were tested at least in duplicate in all frogs except one in which only solutions with 3.38 mM Na were used.

Na concentration. This behavior is suggestive of a competition between Li and Na and should involve the saturable component of Na influx. To test this possibility, the linear component of influx was evaluated using $\alpha = 0.037$ and subtracted from the total influx. The reciprocal of the residual influx was plotted against the reciprocal of the Na concentration as shown in Fig. 6. The results indicate that Li acts as a competitive inhibitor of the saturable component of Na influx since the three lines have identical intercepts but different slopes. The value of the inhibitor constant for Li, K_{Li}, was evaluated using a Dixon plot (17) ($1/J_{12}^{Na}$ vs. [Li] at various Na concentrations). K_{Li} was found to lie in the range 24–33 mM, about twice the value for K_{Na}. These calculations involve the assumption that Li does not alter the linear component of Na influx. Although there is no direct evidence to support this assumption, it seems clear that the major effect of Li must be on the saturating component, since at the low Na concentrations used in these experiments, the linear component accounts for only 15–20% of the total Na influx.

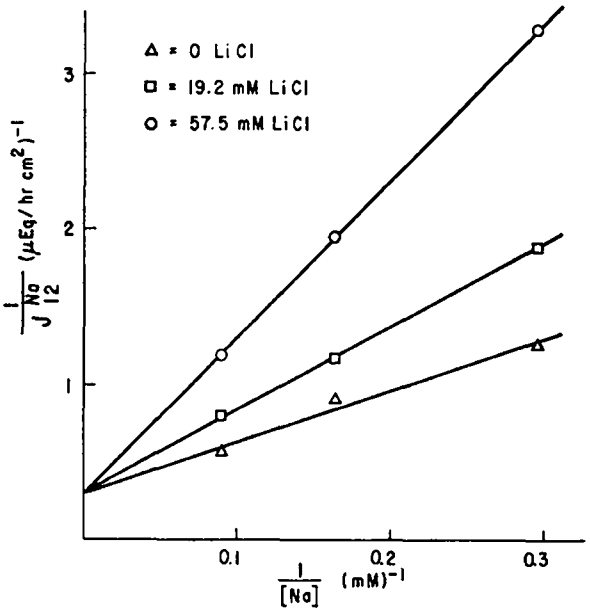

FIGURE 6. Double reciprocal plot for saturating component of Na influx (J_{12}^{Na}) at different Na and Li concentrations in outside bathing solutions.

Inhibition of the Li *Influx* (J_{12}^{Li}) *by Sodium*

If Li acts as a competitive inhibitor of Na influx, Li influx into the skin should also be inhibited by the presence of Na in the outside solution. To test this possibility 51 Li influx measurements were made in four frogs using solutions J and C (51.8 mM Li and either 6 or 63 mM Na). Due to a large variation in Li influx in different animals, the average influxes at 6 and 63 mM Na do not differ significantly. However, as shown in Table V, the ratio of Li influx in the presence of 63 mM Na to that with 6 mM was less than unity in each experiment, indicating that Na does inhibit Li influx significantly. We cannot at present show that this inhibition is competitive because experiments at a range of Li concentrations are difficult. At lower concentrations, Li influx is too small for accurate measurement, and at higher concentrations the effect will be obscured by the large linear component of influx.

Microelectrode Studies

The influxes of Na and Li reported above could have been influenced by changes in intracellular potential of the epithelial cells due to a change in Na and Li concentrations in the outside solution. However, Cereijido and Curran (10) found no significant change in intracellular potential under short-circuit conditions when the Na concentration in the outside solution was varied. In view of our Li inhibition experiments, we wanted to test whether the intraepithelial potential changes under short-circuit conditions during the first 30

sec after addition of LiCl to the outside solution. The frog skins were bathed on both sides in solution containing 6 mM Na (solution H) during the introduction of the microelectrode into the epithelium. A PD which was significantly different from the bathing media, i.e. at least 5 mv, and which changed

TABLE V

INHIBITION OF Li INFLUX (J_{12}^{Li}) BY INCREASED Na CONCENTRATION

Experiment	J_{12}^{Li} at 63 mM NaCl / J_{12}^{Li} at 6 mM NaCl
1	0.86
2	0.63
3	0.67
4	0.71
Average	0.72±0.05

In each experiment six flux measurements were made at a Na concentration of 63 mM, and four to nine determinations were carried out at a Na concentration of 6 mM.

TABLE VI

INTRACELLULAR POTENTIALS

	Microelectrode PD				Transepithelial PD or SCC 6 mM Na	
	6 mM Na		6 mM Na + 57.5 mM Li			
Puncture	Open	Short circuit	Open	Short circuit	Open PD	SCC
	mv		*mv*		*mv*	*μa/cm²*
1	+22.0	−13.2	—	−12.3	+40	38
2	+10.0	−13.9	+14.5	−12.0	+22	17
3	+18.5	−20.8	+27.0	−19.0	+36	44
4	+7.0	−20.1	+14.5	−19.0	+25	35
5	+13.5	−17.5	+13.0	−15.0	+28	29
6	+14.0	−11.5	+23.0	−19.0	+19.5	14
7	+9.0	−12.8	+41.0	−19.0	+20.0	21
8	+13.0	−14.0	+37.0	−12.5	+22.0	18
9	+10.5	−10.3	+19.5	−5.0	+17.0	15
10	+28.0	−21.0	+27.0	−20.2	+41.0	27
Average		−15.5±1.3		−15.3±1.5		

to a significant negative value (more negative than 5 mv) following short circuiting was judged to be sufficient evidence for intracellular location of the tip. Whittembury had located such potentials within the cells of the toad skin epithelium by iontophoresis of lithium carmine (13). Table VI lists the PD between microelectrode and the reference electrode in the outside bathing solution as well as the PD between the two bathing media. Under short-circuit conditions the PD obtained from the microelectrode shifted at most a few

millivolts when 57.5 mM LiCl (solution K) was added to the outside solution. The average change in 10 punctures was $+0.21 \pm 1.25$ mv.

DISCUSSION

The method developed for the present studies appears to provide an adequate measurement of Na influx from the outside solution into the skin. The fact that the tracer uptake is linear with time (Fig. 3) indicates that the backflux of tracer from the skin is negligible over the time interval employed. Further, radioactive Na does not enter the inside solution during the time of measurement. Finally, with the chamber used, the influx can be measured under short-circuit conditions. Since, under these conditions, the electrical potential within the epithelium does not change appreciably with changes in Na concentration (10) or Li concentration (Table VI) in the outside solution, the influx can be determined without complications arising from changes in electrical potential difference.

There is one additional point that should be considered in light of some suggestions about Na movements in the frog skin. Andersen and Zerahn (18) have pointed out that the Na transport is extremely rapid, and most of the Na thought to be in a "transport pool" could, in fact, already have been transported and may be accumulated in intercellular spaces in the epithelium. Therefore, Na could be transported very rapidly out of the outermost cells into spaces or cells deeper in the epithelium. This possibility seems to be supported by more recent experiments of Ussing and coworkers (19–21), which indicate that a sizable part, if not most, of the transport in the frog skin is performed by the outermost lying living cell layer. Hence, we must consider whether, under these circumstances, the present method could actually be measuring, in part, a transfer of Na beyond the initial entry step. As shown in the appendix, this is not the case. Even if we assume rapid transfer of Na from an outer compartment to a deeper one in series, the present technique measures only the entry into the outer compartment provided the tracer uptake is linear with time. Thus, we feel that the technique provides a suitable estimate of influx, although we cannot, of course, reach any conclusion about the exact location of the tracer in the skin.

Three observations clearly suggest that the Na entry into the skin is not the result of simple free diffusion alone. First, the influx is not a linear function of the sodium concentration in the outside solution. Since the electrical potential in the epithelium is not altered appreciably with changes in outside Na concentration, a linear relation between flux and concentration would be expected provided the Na permeability of the barrier remained constant. The linear relation between flux and concentration above 50 mM Na suggests a constant permeability, but changes at lower concentrations cannot be ruled out a priori. Second, the results shown in Fig. 6 indicate a competitive inhibition

of Na influx by Li. Such an effect is not expected for a simple diffusion, and the simplest explanation involves an interaction of Na with a membrane component during passage across the "outer barrier" (note that the results in Table VI indicate that the effects of Li cannot be ascribed to changes in PD). Third, Na inhibits the influx of Li into the skin. This effect is again suggestive of interaction of both Na and Li with a membrane component during influx.

The exact nature of this interaction remains unknown, but at least three possibilities can be considered in terms of classical concepts of types of transport systems. The portion of influx exhibiting saturation kinetics and competitive effects could involve an active transport process, "carrier"-mediated facilitated diffusion, or "carrier"-mediated exchange diffusion.

The present observations do not permit a clear distinction among these possibilities. As discussed below, there are suggestions that the saturating component of influx may be the one involved in net transepithelial Na transport. If this is correct, the process cannot be entirely due to exchange diffusion because exchange diffusion cannot give rise to a net flux. Biber et al. (3) have suggested the possibility of an active Na entry into the skin on the basis of their studies on transport from low external Na concentrations, and Zerahn (22) has recently postulated that "the mechanism for active transport of Na is effective at the outer surface of the frog skin." The evidence for an active entry step is by no means compelling as yet, but some observations on Li may offer additional support for the concept.

Zerahn (23) demonstrated that Li can be actively transported across the isolated frog skin and that Li is accumulated in the skin during transport. Hvid Hansen and Zerahn (24) localized the accumulation of Li within the epithelial cells under short-circuit conditions. This finding could be explained on the basis of passive diffusion of ions across the outer surface of the skin by assuming a sufficiently negative potential in the epithelial cells. Since the maximum concentration of Li in the cells was 5–10 times greater than that in the outside solution, a negative potential of up to 60 mv is necessary for passive entry. On the other hand, our microelectrode experiments suggest that under short-circuit conditions the intracellular potential is not much more than 20 mv negative with respect to the outside solution in absence or presence of Li. Thus, an active entry process may be necessary to explain these observations. Since the present experiments indicate that Li and Na compete for the saturating influx process, these arguments could be taken as a suggestion that this process represents an active step. However, it is clear that additional studies of the entry step are necessary before any firm conclusion can be reached.

Our data provide a direct confirmation of the results obtained by Cereijido et al. (2) by indirect means. Assuming a single compartment for Na in the

skin, these investigators estimated rate coefficients for Na movement across the outer and inner barriers by observing the time course of approach of transepithelial tracer flux to a steady state. Their results suggested that the effective Na permeability of the outer barrier decreased with increasing Na concentration. Since the technique was indirect and involved assumptions about the properties of the skin, their results were open to some question. On the basis of experiments using a different but also indirect technique, Frazier et al. (25) concluded that Na entry into the toad bladder from the mucosal solution cannot proceed by free diffusion but must involve a considerable interaction of Na with some component of the mucosal cell membrane. The present results obtained by direct measurement indicate that the conclusions drawn from such indirect experiments were at least qualitatively correct; the data in Fig. 4 show clearly that the effective Na permeability of the outer barrier ($J_{12}^{Na}/[Na]o$) decreases with increasing Na concentration.

The data of Cereijido et al. (see Table I in reference 2) can be used to calculate the Na influx as a function of the Na concentration. The resulting values are shown in Fig. 7, together with the influx via the saturating system obtained in the present experiments. Two points are of interest. First, Cereijido et al. obtained only a saturating component for the influx rather than a saturating component plus a linear one. Second, their results bear a marked similarity to the saturating component observed in the present experiments. They found a maximum flux of 2 μeq/hr cm^2 and a K_{Na} of approximately 10 mM compared to values of 4 μeq/hr cm^2 and 14 mM for the present study. This similarity raises an interesting possibility. Since the results of Cereijido et al. are based on the measurement of the transepithelial Na movement and yield only a saturating component for the influx at the outer barrier, it is tempting to suggest that only this component is involved to a significant extent in transepithelial Na transfer. Therefore, it seems possible that the linear com-

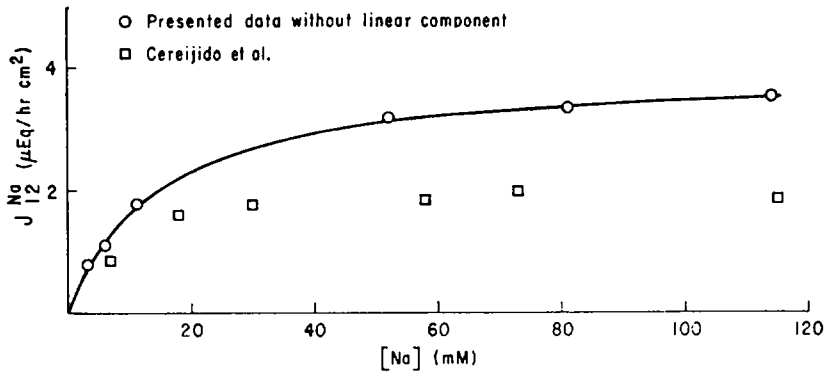

FIGURE 7. Saturating component of Na influx at different Na concentrations.

ponent of influx observed in the present experiments may represent a parallel pathway for Na entry into a pool of Na that is not related to the Na movement across the skin.[1] The observation of Cereijido et al. (2) that the short-circuit current saturates at a Na concentration of about 50–60 mM would be consistent with this view. These concepts may be of considerable interest with regard to the over-all mechanism of Na transport, but they must be regarded as speculative until further direct information is available on the nature of these two components of Na influx.

Rotunno et al.[2] have recently developed an entirely different method to measure the Na influx into the frog skin from the outside solution. At Na concentrations of 1 and 115 mM in the outside bathing solution, they obtained values for influxes which were about 50% higher than those expected from the data reported here. Additionally, they found that the Na influx is inhibited by K ions and by Li ions.[3] A quantitative comparison of the two sets of data is complicated by the fact that Rotunno et al.[2] used open-circuit conditions, while our studies were done under short circuit.

APPENDIX

We wish to consider the influence on the influx measurement of an additional Na compartment in series with compartment 2 of Fig. 2. We denote this compartment as compartment 4. When tracer Na is placed in compartment 1 (the outside solution), the rates of change of tracer in compartments 2 and 4 will be given by

$$\frac{dP_2}{dt} = k_{12}P_1 - (k_{21} + k_{24})P_2 + k_{42}P_4 \qquad (A\text{-}1)$$

$$\frac{dP_4}{dt} = k_{24}P_2 - (k_{42} + k_{43})P_4 + k_{34}P_3 \qquad (A\text{-}2)$$

in which P_i is total tracer in compartment i, and k_{ij} is the rate coefficient for transfer from compartment i to compartment j. We shall assume that the coefficient k_{42} is sufficiently small, that the term $k_{42}P_4$ in equations A-1 and A-2 can be neglected, and that $P_3 \approx 0$. As discussed in the text, we are interested particularly in the case in which $k_{24} \gg k_{21}$. Under these conditions tracer Na entering the outer skin compartment is transported onward very rapidly. With these assumptions, equation A-1 becomes

$$\frac{dP_2}{dt} + k_{24}P_2 = k_{12}P_1 \qquad (A\text{-}3)$$

[1] The linear component could involve entry into damaged tissue at the edge of the chamber, uptake into skin glands, and/or entry into a cellular or extracellular compartment in the epithelium. The present studies do not provide any information to distinguish among the various possibilities.

[2] Rotunno, C. A., F. A. Villalonga, M. Fernandez, and M. Cereijido. The penetration of sodium into the epithelium of the frog skin. Manuscript in preparation.

[3] Cereijido, M. Personal communication.

and equation A-2 becomes

$$\frac{dP_4}{dt} = k_{24}P_2 - k_{43}P_4 \qquad \text{(A-4)}$$

Since P_1 is constant, and $P_2 = 0$ at $t = 0$, integration of equation A-3 yields

$$P_2 = \frac{k_{12}P_1}{k_{24}}(1 - e^{-k_{24}t}) \qquad \text{(A-5)}$$

Introducing equation A-5 into equation A-2 and integrating (with the initial condition $P_4 = 0$ at $t = 0$) yields

$$P_4 = \frac{k_{12}P_1}{k_{43}} - \frac{k_{12}P_1}{k_{43} - k_{24}}e^{-k_{24}t} - \left[\frac{k_{12}P_1}{k_{43}} - \frac{k_{12}P_1}{k_{43} - k_{24}}\right]e^{-k_{43}t} \qquad \text{(A-6)}$$

Experimentally, the total activity (P_{tot}) in the skin is determined. According to the present considerations, this will be given by $P_2 + P_4$ so that

$$P_{tot} = \frac{k_{12}P_1}{k_{43}}(1 - e^{-k_{43}t}) - \frac{k_{12}P_1}{k_{43} - k_{24}}(e^{-k_{24}t} - e^{-k_{43}t}) + \frac{k_{12}P_1}{k_{24}}(1 - e^{-k_{24}t}) \qquad \text{(A-7)}$$

We are interested only in the portion of the time course in which P_{tot} is a linear function of time (Fig. 3), so that we may expand the exponential terms in equation A-7 and retain only the terms linear in t. Under these conditions, equations A-6 reduces simply to

$$P_{tot} = k_{12}P_1t \qquad \text{(A-8)}$$

Thus, the flux calculated from the uptake of activity by the skin will be the flux across the initial outermost barrier even if Na is then rapidly transported onward to deeper compartments in the tissue. Therefore, properties of the influx measured in this study are determined by the outer barrier. Note that this conclusion is unaltered if the term $k_{42}P_4$ is retained in equation A-4.

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REFERENCES

1. KOEFOED-JOHNSEN, V., and H. H. USSING. 1958. The nature of the frog skin potential. *Acta Physiol. Scand.* **42**:298.

2. CEREIJIDO, M., F. C. HERRERA, W. J. FLANIGAN, and P. F. CURRAN. 1964. The influence of Na concentration on Na transport across frog skin. *J. Gen. Physiol.* **47**:879.
3. BIBER, T. U. L., R. A. CHEZ, and P. F. CURRAN. 1966. Na transport across frog skin at low external Na concentrations. *J. Gen. Physiol.* **49**:1161.
4. ROTUNNO, C. A., M. I. POUCHAN, and M. CEREIJIDO. 1966. Location of the mechanism of active transport of sodium across the frog skin. *Nature (London).* **210**:597.
5. CEREIJIDO, M., and C. A. ROTUNNO. 1967. Transport and distribution of sodium across frog skin. *J. Physiol. (London).* **190**:481.
6. SCHULTZ, S. G., P. F. CURRAN, R. A. CHEZ, and R. F. FUISZ. 1967. Alanine and sodium fluxes across mucosal border of rabbit ileum. *J. Gen. Physiol.* **50**:1241.
7. GOLDNER, A. M., S. G. SCHULTZ, and P. F. CURRAN. 1969. Sodium and sugar fluxes across the mucosal border of rabbit ileum. *J. Gen. Physiol.* **53**:362.
8. CURRAN, P. F. 1968. Coupling between transport processes in the intestine. *Physiologist.* **11**:3.
9. USSING, H. H., AND K. ZERAHN. 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol. Scand.* **23**:110.
10. CEREIJIDO, M., and P. F. CURRAN. 1965. Intracellular electrical potentials in frog skin. *J. Gen. Physiol.* **48**:543.
11. KIDDER, G. W., III., M. CEREIJIDO, and P. F. CURRAN. 1964. Transient changes in electrical potential differences across frog skin. *Amer. J. Physiol.* **207**:935.
12. CALDWELL, P. C., AND A. C. DOWNING. 1955. The preparation of capillary microelectrodes. *J. Physiol. (London)* **128**:31P.
13. WHITTEMBURY, G. 1964. Electrical potential profile of the toad skin epithelium. *J. Gen. Physiol.* **47**:795.
14. DOWD, J. E., and D. S. RIGGS. 1965. A comparison of estimates of Michaelis-Menten constants from various linear transformations. *J. Biol. Chem.* **240**:863.
15. WOOLF, B. 1932. Cited in Allgemeine Chemie der Enzyme by J. B. S. Haldane and K. G. Stern. Steinkopff Verlag, Dresden und Leipzig. 119.
16. HOFSTEE, B. H. J. 1959. Non-inverted versus inverted plots in enzyme reactions. *Nature (London).* **184**:1296.
17. DIXON, M., and E. C. WEBB. 1964. Enzymes. Academic Press Inc., New York.
18. ANDERSEN, B., AND K. ZERAHN. 1963. Method for non-destructive determination of the sodium transport pool in frog skin with radiosodium. *Acta Physiol. Scand.* **59**:319.
19. USSING, H. H., AND E. E. WINDHAGER. 1964. Nature of shunt path and active sodium transport path through frog skin epithelium. *Acta Physiol. Scand.* **61**:484.
20. VOÛTE, C. L., and H. H. USSING. 1968. Some morphological aspects of active sodium transport. The epithelium of the frog skin. *J. Cell Biol.* **36**:625.
21. USSING, H. H. 1967. Active sodium transport across the frog skin epithelium and its relation to epithelial structure. *Ber. Bunsenges. Phys. Chem.* **71**:807.
22. ZERAHN, K. 1969. Nature and localization of the sodium pool during active transport in the isolated frog skin. *Acta Physiol. Scand.* **77**:272.
23. ZERAHN, K. 1955. Studies on the active transport of lithium in the isolated frog skin. *Acta Physiol. Scand.* **33**:347.
24. HVID HANSEN, H., AND K. ZERAHN. 1964. Concentration of lithium, sodium and potassium in epithelial cells of the isolated frog skin during active transport of Lithium. *Acta Physiol. Scand.* **60**:189.
25. FRAZIER, H. S., E. F. DEMPSEY, and A. LEAF. 1962. Movement of sodium across the mucosal surface of the isolated toad bladder and its modification by vasopressin. *J. Gen. Physiol.* **45**:529.