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THE EFFECT OF SODIUM CONCENTRATION ON THE CONTENT AND DISTRIBUTION OF SODIUM IN THE FROG SKIN

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SUMMARY

1. The content and distribution of sodium in the epithelium of the frog skin (*Leptodactylus ocellatus* L.) have been studied.

2. The inulin space, the ^{22}Na exchange, and the amounts of water and sodium were measured in samples of connective tissue. The results indicate that the necessary assumptions generally made to calculate the sodium and water contents of the epithelial cells as the difference between the total content in the tissue and the amounts contained in the inulin space are not valid in the frog skin.

3. The mean concentration of sodium in the epithelium has been obtained from direct measurements of sodium and water in samples of epithelium. To measure the water content of the epithelium a new technique has been developed. When the skin is bathed with Ringer solution containing 115 mM-Na on both sides, the mean concentration of sodium in the epithelium is 79 mM. When the concentration of sodium in the Ringer is 1 mM the mean concentration in the epithelium is 25 mM. When the skin is bathed with Ringer with 1 mM-Na on the outside and 115 mM-Na on the inside—a situation which resembles the natural condition in the skin—the mean concentration of sodium in the epithelium is 52 mM.

4. The compartmentalization of Na was studied by comparing the sodium content and the degree of exchange with ^{22}Na in the bathing solutions. In these experiments the skins were exposed to Ringer solutions with different concentrations of sodium, and ^{22}Na on one or both sides.

5. The results indicate that the epithelium has a compartment of sodium which is not exchangeable in 40–80 min and whose size is not appreciably changed by a threefold change in the Na content in the epithelium and a hundredfold change in the concentration of the bathing solution.

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6. Sodium exchangeable in 40–80 min seems to be contained in two different compartments: (*a*) a large one that contains fixed sodium is mainly connected to the inside, and does not appear to participate directly in sodium transport across the frog skin; (*b*) a small one, that is bounded on the inside by a Na-impermeable barrier, and that seems to comprise the sodium involved in active transport. When the skin is bathed with Ringer solutions with 115 mM-Na on the inside and 1 mM-Na on the outside, the transporting compartment contains some 13 % of the total sodium in the epithelium.

7. The results are interpreted on the basis of a model recently proposed by Cereijido & Rotunno (1968). The major feature of this model is that the sodium transporting compartment is confined to the plasma membrane of the epithelial cells.

INTRODUCTION

The frog skin, as well as many other epithelia, transports a net amount of sodium chloride from the outer to the inner bathing medium. The fact that this property is observed even when the concentration of sodium chloride in the outside bathing solution is as low as 10^{-5} M (Krogh, 1937) poses the problem of explaining how the Na ions can enter an epithelium whose sodium concentration is much higher (Rotunno, Pouchan & Cereijido, 1966). A plausible explanation was afforded by the observation of Cereijido & Rotunno (1967) that when the skin is bathed in 5–10 mM-Na, only 37 % at the most of the epithelial Na is involved in active transport. This conclusion, based on a study of the distribution of ^{22}Na, coincided satisfactorily with an analysis of the nuclear spin resonance of the sodium in the frog skin which indicated that only 39 % of the total sodium is free as Na^{+} ion (Rotunno, Kowalewski & Cereijido, 1967). Despite the fact that the high concentration of Na in the epithelium and its compartmentalization were demonstrated in slices of tissue devoid of connective tissue, many conclusions relied on the assumption that Na and water contained in the connective tissue were essentially free and might be determined on the basis of the inulin space. Subsequent information, though, has indicated that the content of Na in the connective tissue and in the epithelium, has a complex relationship with the concentration of sodium in the bathing solutions. It was also shown that the connective tissue can fix Na (Imamura, Takeda & Sasaki, 1965). A series of studies was then performed to obtain more information on the content and distribution of sodium in the frog skin. The results of these studies, divided into three sections, are presented in this paper: in the first section it is shown that the concentration of Na in the epithelium cannot be calculated on the assumption that Na and water in the connective tissue are

free or, at least, bound in negligible amount, when Ringer with 1 mM-Na is used. In the second section the high concentration of Na in the epithelium, as well as its compartmentalization, are demonstrated from direct measurements of water, Na and ^{22}Na in the epithelium. In the third section the relationship between the different Na compartments and the inner and outer bathing solution is analysed.

METHODS

Material

The central part of the abdominal frog skin (*Leptodactylus ocellatus*, L.) was used throughout. Animals were kept in a moist sink. Each experiment was generally performed on frogs of the same shipment, but the different experiments reported in this paper were carried out over a period of 9 months. Experiments were carried out *in vitro* at room temperature (20–22° C).

Solutions

Two Ringer solutions were used, one containing (mM): NaCl 115, $KHCO_3$ 2·4, $CaCl_2$ 1·0; the other containing NaCl 1·0, $KHCO_3$ 2·4, $CaCl_2$ 1·0, choline chloride 114. All reagents were of Analar grade. After the solutions were gassed with air they gave a pH of 8·2.

Experimental arrangements

Two different techniques were used depending on whether it was desired to bathe the sides of the skin with the same or with different Ringer solutions.

(*a*) *Incubation in chambers.* The skin was rinsed in Ringer and mounted as a flat sheet between two lucite chambers with an exposed area of 3·14 cm^2. The volume of each chamber was 5·0 ml. The solutions were stirred by bubbling moistened air directly into the chambers. The electrical potential difference was 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).

(*b*) *Incubation in a tall graduated cylinder.* The skin was placed on a filter paper moistened with Ringer solution with the outer side in contact with the paper. Four to five pieces of 1·16 cm^2 were cut out with a cork borer and put into a tall graduated cylinder with about 3 ml. of Ringer solution per piece of skin. The Ringer solution was gassed and stirred by bubbling moist air vigorously, care being taken that the skins did not rest at the bottom of the flask.

Slicing

This technique was described in detail in a previous paper (Cereijido & Rotunno, 1967). In these experiments, slices were cut to obtain samples of epithelium or connective tissue and no study of the ion or water distribution as a function of depth was attempted. When the skins were incubated in a chamber they were removed, placed on a filter paper moistened with distilled water, cut with the cork borer and frozen on the stage of a freezing microtome (Jung Quick-Freeze Microtome). The time that elapsed between removal from the chamber and freezing on the microtome was never more than 60 sec. When the skins were incubated in the tall graduated cylinder they were blotted and frozen in about 20 sec since they were already cut. The skin was placed with the outside facing up or down depending on whether the samples required were of epithelium or connective tissue. Two or three samples, weighing together 1·2 mg at the most (15 % of the total tissue), were taken. This constituted about a half of the epithelium. In the case of samples of connective tissue this avoided including the glands.

Water content

Once the piece of skin was frozen on the stage of the microtome it was wiped with soft paper to remove any water which had condensed on the surface and a stop watch was started. A slice was made, lifted with a sharp stainless-steel needle and put into a miniature test-tube (length, 2 cm; outer diameter, 0·4 cm) and covered with a piece of Parafilm (Marathon) paper. The time between wiping the tissue and covering the tube was usually 20–30 sec. The miniature test tube and the piece of Parafilm paper were weighed beforehand, both together and separarely, in a Mettler Ultramicrobalance with an error of 2 μg. The tube stoppered with the slice inside was weighed and this weight minus the weight of the empty tube with the piece of paper was taken as wet weight. The tube was then opened, the Parafilm paper put aside and the tube dried to constant weight at 90° C. The weight of the tube plus the dried sample, minus the weight of the empty tube was taken as dry weight. The difference between wet and dry weight was taken as the amount of water of the sample. Figure 1 shows the water content in samples of epithelium of slices incubated in Ringer

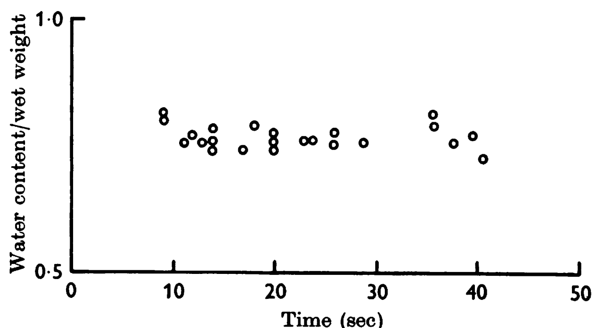

Fig. 1. Water content of slices of epithelium of frog skin incubated in tall cylinders with Ringer solution with 115 mM-Na as a function of time. The frozen piece of skin is gently rubbed with soft paper (zero time) and sliced. The slice is put into a miniature test tube and stoppered (time). Although with this technique a sample is obtained in 20–30 sec, in this particular experiment the time was varied to study whether desiccation or condensation of water in the slice would alter the water content.

solution with 115 mM-Na as a function of the time spent in collecting the sample. Although the time was usually 20–30 sec, in this particular experiment the time was varied to see whether it had any effect on the water content. It did not appear to have any. As an incidental observation it was noticed that if the skin was not deeply frozen, the slice melted before the tube was stoppered and gave erratic results.

Na content

One or more slices were put in a tared small dish made out of Teflon, 0·9 cm in diameter and 1 mm thick at the border. It was then dried to constant weight at 90° C, weighed and placed in a polystyrene test tube with 2·0 ml. of 0·1 N-HNO_3, and stoppered with Parafilm. It was left overnight to be extracted in a shaker. Aliquots were then diluted and Na was measured by flame photometry (Beckman DU spectrophotometer with photomultiplier, with flame photometry attachment for C_2H_4 and O_2).

Inulin space

Pieces of skins were immersed in Ringer solutions containing 0·04 μc/ml. of [^{14}C]inulin (New England, Nuclear) during 40–80 min. They were then removed, blotted and frozen on the stage of the microtome. A slice or two of connective tissue of each piece were put in a miniature test-tube and stoppered. The wet weight of the sample was obtained as described above. [^{14}C]Inulin was then extracted in a way similar to that described under *Na content*. Duplicate samples of bathing solution and extraction fluid were placed in planchets. The planchets were counted in a windowless gas flow counter and the ^{14}C activity was compared with that in the bathing solution in order to estimate the inulin space.

^{22}Na uptake

After allowing 20–40 min for equilibration 2·0 μc of ^{22}Na was added to the tall graduated cylinder or one of the two chambers. When both sides of the skin were in contact with ^{22}Na special care was taken to use the same specific activity in both solutions, even if they had different concentrations of Na. This was achieved by preparing the different Ringer solutions with a stock solution of sodium labelled with ^{22}Na. After 40–80 min of equilibration with the tracer, one or two slices were obtained and were treated as described in *Na content*. The ^{22}Na distribution is expected to be in a steady state in less than 30 min (Cereijido & Rotunno, 1967). Samples of bathing solutions and extracting fluid were withdrawn for ^{22}Na and total Na measurements. ^{22}Na was counted in a well scintillation counter (Nuclear).

RESULTS

(1) *Studies in connective tissue*

Experimental data in this section refer to pieces of skins of the same frogs, some pieces being incubated in 115 and some in 1 mM-Na. Total Na, ^{22}Na and dry-weight determinations were performed in Teflon dishes, determinations of water and dry weight in miniature tubes. The comparisons are made on the basis of the dry weight. In the case of the inulin space, though, to facilitate the extraction of the [^{14}C]inulin, the sample was not dried and the figures for volume in μl./mg of *wet* weight given in Table 1 have to be converted to μl./mg of *dry* weight using the wet/dry ratio before they can be compared with data obtained in Teflon dishes.

Sodium and water content in slices of connective tissue. Connective tissue of skins incubated in Ringer solutions with 115 mM-Na have 0·276 μmoles Na and 2·39 μl. water/mg dry wt. (Table 1). Therefore the mean concentration of sodium is 115 mM as in the bathing solution. If it were not for the results presented below one would believe that Na and water penetrate freely into the connective tissue. However, when skins are incubated in Ringer solution with 1 mM-Na, the slices of connective tissue have 0·094 μmole Na and 2·12 μl. water/mg dry wt. The mean concentration of Na is thus 44 mM, which indicates that Na in the connective tissue is not contained in an extracellular space openly connected with the bathing solution and in a state of free solution.

Inulin space in connective tissue. It was expected that the inulin space in

the connective tissue would be close to the value of the water content. However, in slices incubated with 115 and 1 mM-Na the inulin space was 0·287 and 0·129 μl./mg wet tissue, which is 41 and 19 % of the amount of water in the tissue (Table 1). No difference was noticed between samples at 40 and 80 min. This would indicate that the water compartment where the inulin did not enter is not accessible, or is only very slowly accessible, to the molecule of inulin. The amount of water in the connective tissue appears to be slightly smaller at 1 than at 115 mM-Na but the difference is not significant ($0·05 < P < 0·1$). A smaller fraction of it may be occupied by inulin (41 *vs.* 19 %).

TABLE 1. Connective tissue

	Concentration of Na in the Ringer	
	115 mM	1 mM
Total Na (μmole/mg dry wt.)	0·276 ± 0·006 (13)	0·094 ± 0·005 (6)
Water content (μl./mg wet wt.)	0·703 ± 0·008 (10)	0·676 ± 0·010 (10)
Water content (μl./mg dry wt.)	2·39 ± 0·09 (10)	2·12 ± 0·11 (10)
Ratio: wet wt./dry wt.	3·39 ± 0·09 (10)	3·12 ± 0·11 (10)
Inulin space (μl./mg wet wt.)	0·287 ± 0·023 (10)	0·129 ± 0·015 (10)
Ratio of the ^{22}Na specific activity connective/Ringer (%)	40·5 ± 1·7 (13)	6·1 ± 1·2 (8)

Na and water content, inulin space and ^{22}Na exchange in slices of connective tissue of frog skin. They are expressed as the mean value ± the standard error of the mean, the number of experiments being given in parentheses. The pieces of skins incubated 40–80 min in tall cylinders with Ringer solution with 115 or 1 mM-Na belong to the same frogs.

^{22}Na exchange. ^{22}Na in the connective tissue of skins incubated with Ringer with 115 mM-Na reached 40·5 % of the specific activity in the bathing solution (Table 1). No difference was noticed between samples taken within the 40–80 min period. This suggests that the 40·5 % of Na exchanged belongs to a different compartment from the remaining 59·5 %. Since there is 0·276 μmole Na/mg dry wt., and 40·5 % of it is exchangeable in 40–80 min, there is 0·164 μmole of non-exchangeable Na per milligram of dry weight. From the extracellular space per milligram of wet wt. given in Table 1 (0·287) and the wet wt./dry wt. ratio (3·39) an extracellular space of 0·972 μl./mg dry wt. may be calculated. If the Na concentration in the solution which fills this space is 115 mM, as in the Ringer solution, then it contains 0·112 μmoles of Na per milligram of dry weight. It means that there are 0·164 (0·276 − 0·112) μmoles Na/mg dry wt. outside inulin space. The agreement between the quantity of Na outside the inulin space and the non-exchangeable Na (both 0·164 μmoles Na/mg dry wt.) would suggest that this Na is outside the compartment generally taken as the extracellular compartment. It should be noted that the concentration of Na in this compartment is also 115 mM. This fact makes it unlikely that the non-exchangeable

space is entirely composed of cells (in particular those of the *tela subcutanea*). From these results it seems clear that the assumption generally made in calculating the ion and water content of the epithelial cells—that the connective tissue is little more than an interstice full of free solution—may not be correct. Thus, to determine the concentration of Na in the epithelium it is necessary to measure directly the amount of Na and water in samples of epithelium free from connective tissue.

(2) *Concentration of Na and* ^{22}*Na exchange in the epithelium*

The extraction fluid of the miniature test-tubes used to obtain the water content gave high Na blanks. Thus, it was necessary to use the Teflon dish technique to measure the Na content in a different piece of

TABLE 2. Epithelium

Experiment	Concentration of Na in the Ringer (mM)	Na content μmole/mg dry wt.	Ratio of specific activity* (%)
A	115	$0{\cdot}264 \pm 0{\cdot}010$ (34)	$74{\cdot}8 \pm 5{\cdot}1$ (15)
B	1	$0{\cdot}084 \pm 0{\cdot}010$ (23)	$13{\cdot}8 \pm 1{\cdot}1$ (35)

* Specific activity of ^{22}Na in the epithelium/specific activity of ^{22}Na in the loading solution.

Na content and ^{22}Na exchange in slices of epithelium of frog skin incubated 40–80 min in Ringer solution with 115 and 1 mM-Na. All Ringer solutions listed in this and following tables were made isotonic with choline. The 35 values of ^{22}Na exchange at 1 mM-Na include twelve results of specific activities in which the Na content was not measured but assumed to be 0·084 μmole/mg dry wt.

TABLE 3. Epithelium

Na concentration in the Ringer (mM)	115	1
Amount of water (μl./mg wet wt.)	$0{\cdot}763 \pm 0{\cdot}005$ (23)	$0{\cdot}755 \pm 0{\cdot}005$ (8)
Amount of water (μl./mg dry wt.)	$3{\cdot}34 \pm 0{\cdot}09$ (23)	

Water content in slices of epithelium of skins incubated in tall cylinders as measured with the miniature tube technique. Since there is no significant difference between the amount of water at 115 and at 1 mM-Na ($P > 0{\cdot}2$) both groups of data were pooled to obtain the amount of water per milligram of dry weight to be used in other calculations.

tissue. However, the pieces of skin in which the water content was measured belonged to the same frogs as those used to obtain the Na content. Epithelium of skins incubated in 1 mM-Na made isotonic with choline had less than one third of the Na content of those incubated in regular Ringer (Table 2). Table 3 shows the water content in samples of epithelium as measured by the miniature tube technique. The amount of water seems to be independent of the Na concentration in the bathing solution. The value of $3{\cdot}34 \pm 0{\cdot}09$ μl./mg dry wt. given in Table 3 includes the data

obtained at 115 and the data obtained at 1 mM-Na, since no difference was observed between them. Using the data in Tables 2 and 3 the concentration of Na in the epithelium may be calculated. It is 79·0 mM in skin incubated in 115 mM-Na and 25·1 mM in those incubated in 1 mM-Na. This high concentration of Na in epithelium bathed with 1 mM Na confirms the conclusion of Rotunno, Pouchan & Cereijido (1966) that if Na is

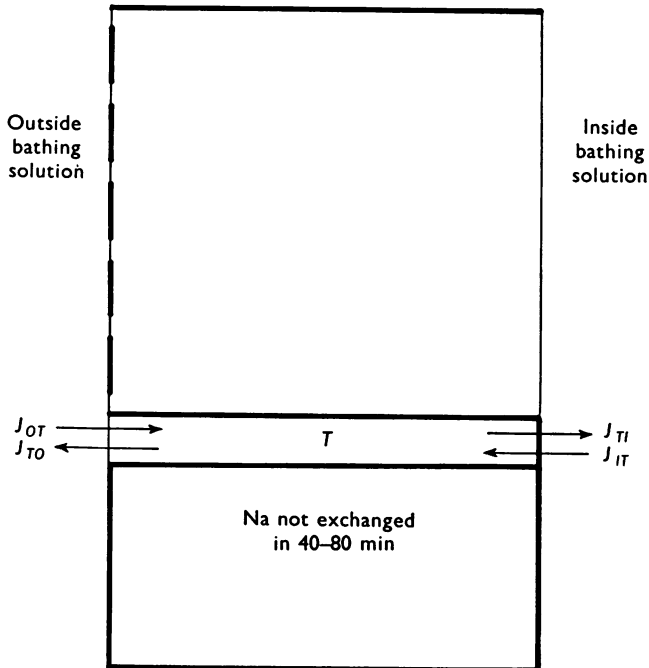

Fig. 2. Model used to analyse ^{22}Na movement. J_{OT}, etc., represents Na fluxes from compartment O to T. Thin lines represent boundaries which have a comparatively high Na permeability. T is the Na pool in the transporting compartment. When the skin is incubated in Ringer solution with 115 mM-Na on the inside, and 1 mM on the outside, about a third of the total Na contained in the epithelium is contained in the compartment which does not appreciably exchange in 40–80 min. The rest of the epithelial Na is contained in a compartment (ENTC) which does not appear to be directly involved in Na transport *across* the skin. The size of the compartments in the drawing is roughly proportional to the *amount* of Na they contain. However, the size does not represent the *volume* occupied by the compartment in the epithelium (see text).

free and contained in a unique cellular compartment, then the distribution of Na across the outer barrier of the epithelium cannot be understood on the basis of a passive distribution. The fact that a high sodium content is found even in epithelia bathed in low Na media with an ionic strength kept at physiological level with choline, rules out the possibility of non-

selective binding of Na by fixed anions as an explanation for the high sodium content.

The exchange of ^{22}Na *in the epithelium.* The column on the right of Table 2 shows the ratio of the ^{22}Na specific activity in the epithelium to the ^{22}Na specific activity in the Ringer solution. Experiment *B* in Table 2 includes data from slices collected in Teflon dishes in which both ^{22}Na and Na were determined, and also specific activities obtained from the counts/min measured in samples contained in miniature test-tubes divided by a corresponding amount of Na (0·084 μmole/mg dry wt.), i.e. in these particular samples the Na content was not measured but assumed. No difference was noticed in the amounts of ^{22}Na exchanged in skins incubated for periods between 40 and 80 min under a given condition of the several listed in Tables 2 and 4. This suggests that the sodium transport pool equilibrates in less than 40 min as was shown to occur with skins incubated in Ringer solution with 5–115 mM-Na on both sides (Curran, Herrera & Flanigan, 1963; Cereijido, Herrera, Flanigan & Curran, 1964; Cereijido & Rotunno, 1967). Na in the epithelium does not exchange completely with ^{22}Na in the bathing solutions. This observation confirms previous results obtained indirectly with 5–10 mM-Na when only 37 % of the total Na was exchanged (Cereijido & Rotunno, 1967). The amount of non-exchanged Na in 115 mM-Na is 0·067 μmoles/mg dry wt. (25·2 % of 0·264) and that in 1 mM-Na is 0·072 (86·2 % of 0·084). Thus, the non-exchanged amount seems to be little modified despite the 115-fold change in the Na concentration of the Ringer and the threefold change of Na content in the epithelium.

(3) *Characteristics of the Na compartments in the epithelium*

The purpose of the experiments in this section was to obtain some information on the size and connexions of the different Na compartments. In these experiments the skin was mounted as a flat sheet between two lucite chambers in order to permit the use of different Ringer solutions on both sides or to put tracer on one side only. From the data discussed above it is clear that the epithelium has some 0·070 μmole/mg dry wt. of Na which does not appear to be directly involved in Na transport. The fraction of Na in the epithelium which exchanges with ^{22}Na in the bathing solution includes the Na of the transporting compartment. To study the connexions of this compartment four fluxes should be considered: J_{OT}, the flux from the outside bathing solution (O) to the transporting compartment (T); J_{TO}, the opposite flux; J_{TI}, the flux from the transporting compartment toward the inside bathing solution (I) and J_{IT}, the opposite flux. When ^{22}Na is added to the bathing solution, care being taken to keep the ^{22}Na specific activity identical on both sides, the variations in the amount of

tracer in the transporting compartment P_T is given by the following equation:

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

where p_i^*, p_o^* and p_T^* are the specific activities of ^{22}Na in the inside, outside and transporting compartments respectively. Since the preparation is assumed to be in a steady state, the Na fluxes are constant. During the length of the experimental period (40–80 min) p_i^* remains constant. This makes p_T^* the only variable on the right-hand side of the equation. It achieves a constant value in less than 30 min (Cereijido & Rotunno, 1967). Therefore, after 30 min equation (1) may be rearranged as follows:

$$\frac{p_T^*}{p_i^*} = \frac{J_{IT}+J_{OT}}{J_{TI}+J_{TO}} \qquad (t > 30 \text{ min}). \tag{2}$$

Since the preparation is in steady state the following assumption should hold.

$$J_{IT}+J_{OT} = J_{TO}+J_{TI}. \tag{3}$$

With this condition equation (2) predicts that, after 30 min of adding the tracer, the specific activity of ^{22}Na in the transporting compartment should be equal to that in the outside bathing solution. These conditions are met in experiment *C* (Table 4). Only 16·0 % of the 0·140 μmole/mg dry wt. reaches the ^{22}Na specific activity of the loading solutions. It means that the transporting compartment has, at the most, 0·022 μmole/mg. Notice that this is a maximum estimate because the amount of Na in the transporting compartment (*T*) is less than or equal to the amount of Na exchanged.

If instead of adding ^{22}Na to both sides, it is added only to the outside the variation in the amount of tracer in compartment *T* is given by

$$\frac{dP_T}{dt} = J_{IT}p_i^* - (J_{TI}+J_{TO})p_T^* + J_{OT}p_o^*. \tag{4}$$

If p_i^* is kept close to zero, after dP_T/dt becomes zero equation (4) may be rearranged to give

$$\frac{p_T^*}{p_o^*} = \frac{J_{OT}}{J_{TI}+J_{TO}}. \tag{5}$$

This condition is met in Expt. *D* (Table 4) where 17·4 % of the total Na reaches the same specific activity as the sodium in the outside bathing solution. This amount compares with 16·0 %, the fraction of Na labelled when the tracer is added to both side. The fact that adding ^{22}Na to the outside only (equation (5)) labels the epithelium to the same extent as

when it is added to both sides (equation (2)) implies that J_{IT} is negligible compared with J_{OT}. It may be concluded that the transporting compartment has some 0·020 μmoles Na/mg dry wt. and is bounded on the inside by a Na-impermeable barrier. A Na transporting compartment bounded on the inside by a Na-impermeable barrier has been suggested by Koefoed-Johnsen & Ussing (1958).

TABLE 4. Epithelium

Experiment	Concentration of Na in the Ringer (mM)		Na content in the epithelium	Tracer added to	Ratio of specific* activity (%)
	Outside	Inside	(μmole/mg dry wt.)		
C	115	1	0·140 ± 0·013 (11)	Both†	16·0 ± 1·1 (14)
D	115	5	0·110 ± 0·012 (8)	Outside	17·4 ± 2·5 (8)
E	115	115	0·234 ± 0·040 (8)	Outside	12·1 ± 1·7 (8)
F	1	115	0·175 ± 0·009 (26)	Both†	70·5 ± 2·7 (28)

* Specific activity of ^{22}Na in the skin/specific activity of ^{22}Na in the loading solution.
† Same specific activity on both sides.

Na content and ^{22}Na exchange in epithelium of frog skin incubated as a flat sheet between two lucite chambers of 3·14 cm^2 area. After a period of 20–40 min equilibration in the given condition, ^{22}Na was added to the outside or else the whole of the Ringer solutions in the chambers were replaced with Ringer solutions in which ^{22}Na had the same specific activity.

When the epithelium is bathed with Ringer solution with 115 mM on both sides it has some 0·249 μmole Na/mg (mean between 0·264 of Expt. *A*, Table 2, and 0·234 of Expt. *E*, Table 4). When the concentration of Na in the inside bathing solution is lowered to 1–5 mM, Na in the epithelium falls to 0·125 (mean between 0·140 and 0·110 of Expt. *C* and *D*, Table 4). Therefore there seems to be 0·134 μmole Na contained in a compartment connected to the inside. If the concentration of Na in the inner Ringer solution is kept at 115 mM and the concentration of Na in the outer Ringer is made 1 mM the Na content/mg of dry epithelium falls to 0·175 μmole (Expt. *F*, Table 4), i.e. it loses some 0·074 μmole with respect to the skins incubated in 115 mM-Na on both sides. If this is contained in the compartment accessible from the inside mentioned above, it means that the epithelium has a Na compartment connected mainly to the inside which contains about half of the total Na. It will be referred to as ENTC (exchangeable, non-transporting compartment). It may be inferred that this compartment is different from the transporting compartment from the fact that although J_{IT} was shown to be negligible when Ringer solution with 5 mM was used on the inside, the ENTC may be labelled from the inside (compare Expt. *A*, Table 2, with Expt. *E*, Table 4). The possibility remains that the ENTC represented just an increase in the sodium content of the transporting compartment. This would require, though, that J_{IT}, which with 1–5 mM-

Na on the inside was negligible compared with J_{OT}, became 8 times larger than this flux at 115 mM-Na. In summary, these results suggest the possibility that the epithelium contains at least three Na compartments: a non-exchangeable one; a transporting compartment; and another one whose Na exchanges more easily with Na in the inner bathing solution than with Na in the outer one.

DISCUSSION

The inulin space and the extracellular space

Although the present study does not cover the whole connective tissue, the results presented are sufficient to show that the volume of distribution of inulin in 40–80 min cannot be taken as a measure of the amount of extracellular water in the frog skin. It might be possible that although the inulin and ^{22}Na are excluded from part of the water in the tissue, they will be absorbed above the concentration in the Ringer solution in some other regions so as to yield, in the total tissue, an apparent volume of distribution equal to the volume of water. Imamura *et al.* (1965), for instance, have shown autoradiographically that the connective tissue near the papillae binds Na. The coincidental fact that the Na concentration in the connective tissue of skins incubated with 115 mM-Na Ringer is also 115 mM could have led to the belief that Na in the chorion was in free solution. Even water does not seem to be in a free state. Once the inner side of the skin is blotted, no more water drips off, although in this condition the connective tissue consists of almost 80 % water. Persson (1953) has pointed out that in human skin there seems to be no interstitial water in liquid or flowing form and that that there are many macromolecules which are anionic polyelectrolytes with strong cation binding capacity capable of behaving as an ionic exchange resin and which associate themselves with a large volume of water. If this large volume of associated water were not available to dissolve the molecule used as tracer, the space measured would be smaller than the actual space occupied by water. This problem, however interesting, goes beyond the scope of the present paper. The only point that needs to be considered here is that the necessary assumptions generally made to calculate the Na and water content of epithelial cells from the difference between the total content in the tissue and the amount contained in the inulin space are not valid in the frog skin.

Localization of the sodium compartments in the epithelium

It should be emphasized that the fact that an important fraction of Na in the epithelium does not exchange with ^{22}Na in 40–80 min does not imply that it may not be exchanged in a much longer time. Because of the

observation that in the skins incubated in isotonic Ringer solutions with 5–10 mM-Na only 37 % of its Na was exchangeable (by ^{22}Na exchange) and 39 % the sodium was free (by nuclear magnetic resonance analysis) it was previously assumed that only the ionized sodium was readily exchangeable (Rotunno *et al.* 1967). However, in view of the results obtained in this paper it should be remembered that exchangeable does not mean ionized nor vice versa. Na may be fixed, that is non-ionized, but readily exchangeable; on the contrary, sodium may be in free solution but contained within a Na-impermeable barrier. When the skin is bathed with Ringer with 115 mM-Na on both sides the Na contained in the epithelium is 0·234 (chamber) and 0·264 (tall cylinder) μmoles/mg dry wt. and the corresponding water content is 3·34 μl./mg. This gives a concentration of sodium of 70–79 mM. Thus it is not surprising that the usual method of studying the Na transport in skins mounted as a flat sheet between two lucite chambers with solutions with 115 mM-Na has led to a neglect of active transport as a necessary mechanism for the entry of sodium across the outer facing membrane. However, it should be kept in mind that the technique of mounting epithelial membranes with identical Ringer on both sides and with the electrical potential difference abolished by the use of a short circuit current is an extremely useful method designed by Ussing & Zerahn (1951) mainly to cancel any difference in electrochemical potential gradient between the outside and the inside, thus putting in evidence active fluxes. The conditions used in Expt. *F* (Table 4) resemble more closely the natural conditions of the frog skin. Under these circumstances the concentration of sodium in the epithelium is 52 mM. However, even the situation in Expt. *F* is far from physiological since, owing to the isotonicity of the Ringer solutions on both sides, there is no osmotic inflow of water to help the entrance of sodium into the epithelium. Despite this fact, epithelial membranes exhibit a net influx of sodium when bathed on the outside with isotonic Ringer solutions with low concentration of sodium (Ussing, 1949; Kirschner, 1955; Frazier, Dempsey & Leaf, 1962; Cereijido *et al.* 1964; Rotunno *et al.* 1966). Rotunno *et al.* (1966) demonstrated that this entrance of sodium from the outer solution with low concentration into an epithelium with high sodium content cannot be ascribed to an active mechanism at the level of the outer facing membrane. On the other hand, a passive entrance requires that epithelial sodium be contained in different compartments, one of them being involved in the net transport of sodium. The results of this paper indicate that this requirement is fulfilled. The discussion which follows will attempt to gain some insight on the localization and characteristics of the transporting and the non-transporting compartment.

The exchangeable non-transporting compartment (*ENTC*). The results

presented here demonstrate that changes in the Na concentration in the inside solution drastically change the Na content in the epithelium. However, it was shown by MacRobbie & Ussing (1961) that changes in Na concentration on the inside fail to produce a variation in the osmotic volume of the epithelium. It is also known that those changes do not modify the electrical potential across the inner facing membrane (Cereijido & Curran, 1965; Snell & Chowdhubury, 1966). Therefore, two main possibilities are open to explain the increase in Na content which follows the increase in the concentration of Na on the inside: (*a*) Na penetrates the cells through the inner facing membrane but most of it is fixed in the cytoplasm, thus producing neither osmotic alteration nor modification of the electrical potential. The fact that the inner facing membrane is much more extensive than the outer one would explain why the ENTC exchanges more easily with the inside bathing solution than with the outer. (*b*) Na is fixed to the substance which fills the intercellular space (Farquhar & Palade, 1965). This space is opened toward the inside permitting a ready exchange with the inner solution, but closed on the outside by the *maculae occludens*. The large extent of complexing of Na in living tissues is a very well-known phenomenon in many other tissues (Shaw & Simon, 1955; Ling, 1962; Troshin, 1966; Cope, 1967). In fresh muscle, for instance, only 30 % of the Na is detectable by nuclear magnetic resonance analysis (Cope, 1967). Therefore, if the intercellular space were filled with an ion binding substance, it would not be surprising that it would show the properties assigned to the ENTC. The fact that, when bathed in regular Ringer solution, it binds sodium would be largely accounted for by the relatively high content of sodium with respect to other cations present in the Ringer.

The sodium not exchanged in 40–80 *min.* As mentioned above, the inward-facing membrane has a very low permeability to sodium. If the epithelial cells depended on the inward facing membrane to maintain their balance of sodium, it would be expected that this sodium would constitute a very slow compartment. Therefore, the possibility exists that the 0·07 μmole/mg of sodium which does not exchange in 40–80 min constitutes the intracellular sodium. If this were so, and all the epithelial water (3·34 μl./mg) were contained in the cells, then the concentration of sodium in the cells would be 21 mM. However, the identification of the ENTC with the Na trapped in the intercellular space and the identification of the non-exchanged Na with the intracellular Na would require more information.

Transporting compartment. This compartment contains 0·02 μmole of Na/mg of dry tissue. Using a different approach that allows minimum estimates to be made, Hoshiko & Ussing (1960) demonstrated that the Na

transporting compartment of several European frogs contained 0·063–0·070 μmole of Na/cm^2 of skin. For the sake of comparison one can assume that the specific gravity of the epithelium is 1·10; then the 0·02 μmole/mg of dry weight found in the present paper can be converted to 0·0053 μmole/μl. of epithelium, which corresponds to a piece of 1 cm^2 in area and 10 μ in thickness. A skin with an epithelium 60 μ thick will thus contain a transporting compartment of 0·032 μmole/cm^2, a figure lower than the one found by Hoshiko & Ussing (1960) but in satisfactory agreement considering the difference in species and the completely different experimental approaches.

With respect to the properties and location of the transporting compartment, it is difficult to attempt an interpretation of the results of the present paper in terms of current models of epithelial membranes, because they generally assume that the epithelial sodium is homogeneously distributed in a unique cellular compartment. Therefore the discussion of the transporting compartment will be based on a model, recently proposed by Cereijido & Rotunno (1968), the main characteristics of which may be summarized as follows: sodium from the outer bathing solution reaches the outward facing membrane and migrates over the outer leaflet of the plasma membrane from one Na-selective fixed negative charge to another. It passes though the *zonulae occludens* and reaches the Na-selective polar groups of the inner facing membrane. It crosses to the Na-selective polar groups of the plasma membrane of deeper layers of cells by travelling over the fixed charged groups of the pillars which are composed of the extracellular material of the desmosomes. The model assumes that the barrier of very low permeability to sodium, of the inner facing membrane (Koefoed-Johnsen & Ussing, 1958; MacRobbie & Ussing, 1961), is located between the polar groups where sodium migrates, and the intercellular space. The pumps located all over the inner facing membrane translocate Na^+ from the Na-selective polar groups to the intercellular space across the Na-impermeable barrier. The site left by a pumped Na is refilled by sideways migration. The over-all effect is to move the empty site towards the outward-facing membrane where another Na can be adsorbed. Some of the properties of the transporting compartment of this model are relevant to the discussion of the results of the present paper. For instance, since the transporting compartment of the model is confined to the cell membranes alone, it is expected to contain a small fraction of the total Na in the epithelium. This view is in keeping with the evidence presented in this paper indicating that when the skin is bathed in normal Ringer solution on both sides only 8 % of the total sodium (0·020 μmole in 0·264 μmole) is involved in active transport. The model also assumes that the transporting compartment is bound on the inside by a barrier with very low perme-

ability to sodium, which agrees with the fact that J_{IT}, the sodium flux from the inside bathing solution to the transporting compartment, is negligible as compared with the other fluxes. Besides, in the model, the mechanism for sodium transport *across* the epithelium is different and relatively independent of the mechanism for regulating the balance of sodium in the cells. This agrees with the results showing that the sodium involved in the mechanism of transport across the skin is contained in a compartment separated from the rest of the epithelial sodium. It is conceivable that the possibility of stopping the net transport across the epithelium without disturbing the electrolyte balance, using either metabolic inhibitors (Huf, Doss & Wills, 1957) or xantines (Levinsky & Sawyer, 1953), arises from the separation of the Na involved in these functions observed in the present study. The studies of Curran & Cereijido (1965), demonstrating that the balance of potassium is independent of the concentration of sodium in the outer bathing solution and of the magnitude of the net transport of sodium, give further support to the view that the Na undergoing net transfer does not get directly involved in the ionic equilibration of the cell.

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