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Na^+ POOL AND Na^+ CONCENTRATION IN EPIDERMIS OF FROG SKIN

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SUMMARY

1. Estimations of intracellular $[Na^+]_c$ in epithelial cells of non-short-circuited skins of *Rana pipiens* in sulfate Ringer's were made at varying $[Na^+]_0$ by two indirect methods. (a) Intracellular Na^+ content (μequiv/cm^2) was determined by the "Na^+-pool method". From this and estimations of total cellular and extracellular volume an average value for $\overline{[Na^+]_c}$ was calculated. (b) $\overline{[Na^+]_c}$ was determined by using a modified Nernst equation, assuming Na^+ permselectivity for the "outer border" of skin. Both methods yielded identical values for $\overline{[Na^+]_c}$ which varied in the same manner with $[Na^+]_0$.

2. From variations of $[Na^+]_s$ ($[Na^+]$ in the subcorneal space) and $\overline{[Na^+]_c}$ with changing $[Na^+]_0$, E_{ob} (potential difference across outer border) was calculated and the results compared to the experimentally found change of 35 mV for a 10-fold change in $[Na^+]_0$. The calculations based on $[Na^+]_s$ and $\overline{[Na^+]_c}$ gave the correct slope, but the absolute potential differences (PD) were too low when compared to known measurements of the intracellular PD values.

3. From this discrepancy it is suggested that $[Na^+]_c$ in the cells of the "first reacting cell layer" is lower, and $[Na^+]_c$ in the remaining cells is higher than $\overline{[Na^+]_c}$. This is illustrated by a sample calculation. Values for $[Na^+]_c$, first reacting cell layer, are 3–13 mM for $[Na^+]_0 = 7$–110 mM. This supports the hypothesis of passive entry of Na^+ into the epidermis. By computer simulation it is shown that a model can be constructed that maintains in a steady state such $[Na^+]$ gradients and yields flux characteristics in agreement with typical laboratory observations.

INTRODUCTION

In the present work on isolated, open skins of *Rana pipiens* in sulfate–Ringer's, two indirect methods for estimating $[Na^+]$ in epithelial cells of frog skin ($[Na^+]_c$) are employed: (1) estimation of the Na^+-pool size by the kinetic method[1–8], and (2) estimation of $[Na^+]_c$ by the potentiometric method, assuming with Koefoed-Johnsen and Ussing[9] that the "outer border" of skins in sulfate–Ringer's behaves as an Na^+-permselective membrane. This hypothesis seems to hold if modified[10]

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to account for apparent Na^+ diffusion delay in the cornified layer of the skin, and for changes in $[Na^+]_c$ with changing $[Na^+]_0$. When these modifications are made it is found that both the Na^+-pool size and the electrochemically effective $[Na^+]_c$ vary in the same way with $[Na^+]_0$. The potentiometric method affords the possibility of judging the results on $[Na^+]_c$ on the basis of intraepidermal potential profiles[11–14]. Thus, an additional criterion can be applied to evaluate the meaning of the average $[Na^+]_c$ obtained by either method. The present studies suggest that $[Na^+]_c$ in the outermost "first reacting cell layer" of epidermal cells of open skins (I. RCL of Voûte and Ussing[15]) is lower than $[Na^+]_c$ of the inner epithelial cells.

SKIN MODEL AND ASSUMPTIONS

A schematic picture of essential structural features of the epidermis, as revealed by light and electron microscopy[15–18], is shown in Fig. 1. Na^+ enters the epithelial cells *via* two possible pathways: (1) *via* epidermal cell junctions, (2) *via* the subcorneal space [S] crossing the "outer cell membrane" (ob) of the first reacting cell layer. Some degree of Na^+ leakage from the extracellular space into the "remaining epithelial cells" is assumed to occur, *e.g. via* leaking Na^+ pumps. S is a separate compartment within the epidermis by virtue of the existence of tight cell junctions (zonula occludens), two of which are shown in Fig. 1. The cornified layer constitutes a barrier of low ion (Na^+; SO_4^{2-}) permeability. This is in agreement with the studies of Nielsen[19] and Larsen[20,21]. Based on the findings of Koefoed-Johnsen and Ussing[9] it is assumed that ob is Na^+ permselective, but because it is a thin membrane (approx. 100 Å), its permeability coefficient for Na^+ is considered large, relative to that of the cornified layer. When "open" (not short-circuited) skin actively transports Na^+ in the inward direction (SO_4^{2-} not following across the skin) the arriving Na^+ is electrically balanced by, *e.g.* HCO^-_3 generated by metabolism, H^+ being partly eliminated by a buffer system. Skins in sulfate–Ringer's consume O_2 (or generate CO_2)[22] at the level of 0.5 μequiv· $cm^{-2}\cdot h^{-1}$. Net Na^+ transport for skins in sulfate Ringer's ($[Na^+]$ = 110 mM) occurs at the rate of 0.153 μequiv· $cm^{-2}\cdot h^{-1}$ (Table I). Movement *via* the tight cell junctions of K^+ and H^+ ions from

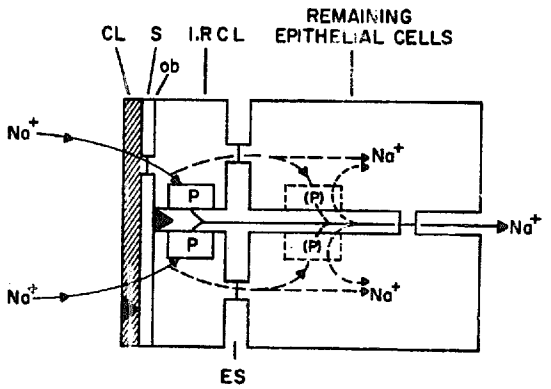

Fig. 1. Model of the frog skin epidermis showing: CL = cornified layer. S = subcorneal space. ob = outer border of the first reacting cell layer (1. RCL of Voûte and Ussing[15]) assumed to be Na^+ permselective. ES = extracellular space. P = "strong" Na^+ pump with no leak. (P) = "weak" Na^+ pump with leak. ▶ = tight cell junctions (zonulae occludens). Two possible pathways for entry of Na^+ into the first reacting cell layer (1. RCL) are shown with connections to remaining epithelial cells *via* existing cell junctions.

the extracellular space into S, from there into the outside bath, could contribute to maintenance of electroneutrality during active inward Na^+ transport[23–27].

CALCULATIONS

Although the skin is a very complex multicompartmental system, it will be assumed that it can be treated as a simple three compartment model[3]. For steady state one may write:

$$J_n = P([Na^+]_0 - [Na^+]_s \cdot \gamma_s)$$

$$[Na^+]_s \cdot \gamma_s = [Na^+]_0 - J_n/P \tag{1}$$

Where P is the apparent permeability coefficient of the cornified layer for Na^+; γ_s is the activity coefficient for Na^+ in the fluid in S; γ_0 is taken as equal to 1 which is only approximately correct. The subscripts o and s refer to outer bath and subcorneal space, respectively. If outer border is strictly Na^+ permselective one has

$$E_{ob} = 58 \log \frac{[Na^+]_s \cdot \gamma_s}{[Na^+]_c \cdot \gamma_c} \tag{2}$$

where $[Na^+]_c$ and γ_c are in the intracellular $[Na^+]$, and the activity coefficient for Na^+ in the cell fluid. We are concerned, here, only with the exchangeable cellular Na^+, which does not exceed 50–60% of the total cell Na^+ (refs 6, 28). Combining Eqns 1 and 2 gives:

$$E_{ob} = 58 \log \frac{[Na^+]_0 - (J_n/P)}{[Na^+]_c \cdot \gamma_c} \tag{3}$$

This is a theoretical prediction, under the assumptions made. When $[Na^+]_0$ is changed from $[Na^+]_0^{I}$ to $[Na^+]_0^{II}$ one obtains from Eqn 3:

$$\Delta E_{ob} = 58 \log \frac{\{[Na^+]_0^{I} - (J_n/P)^{I}\}[Na^+]_c^{II} \cdot \gamma_c^{II}}{\{[Na^+]_0^{II} - (J_n/P)^{II}\}[Na^+]_c^{I} \cdot \gamma_c^{I}} \tag{4}$$

Experimentally it is found (see under Results) for skins of the species *R. pipiens* in sulfate–Ringer's, that the total skin potential changes by (mostly) 35 mV per decade change in $[Na^+]_0$, leaving the solution at the inside of the skin unchanged. Since the cornified layer is taken as a non-ion-specific diffusion barrier, and since an attempted calculation of the diffusion potential across this border showed only changes of the order of 1 mV, the experimental observation can be expressed by:

$$\Delta E_{ob} = 35 \log \frac{[Na^+]_0^{I}}{[Na^+]_0^{II}} \tag{5}$$

When the electrical response of the epidermis to changes in $[Na^+]_0$ as predicted by Eqn 4 is equated with the experimentally found response (Eqn 5) one obtains:

$$\frac{[Na^+]_0^{I} - (J_n/P)^{I}}{[Na^+]_0^{II} - (J_n/P)^{II}} = \frac{[Na^+]_c^{I} \cdot \gamma_c^{I}[Na^+]_0^{\beta I}}{[Na^+]_c^{II} \cdot \gamma_c^{II}[Na^+]_0^{\beta II}} \tag{6}$$

or:

$$\frac{[Na^+]_0^I - (J_n/P)^I}{[Na^+]_c^I \cdot \gamma_c^I \cdot [Na^+]_0^{\beta I}} = \frac{[Na^+]_0^{II} - (J_n/P)^{II}}{[Na^+]_c^{II} \cdot \gamma_c^{II} [Na^+]_0^{\beta II}} = \text{constant} = c \quad (7)$$

for the system in steady state under Conditions I and II. $\beta = 35/58 = 0.602$ (see Results).

In general, therefore, the following should hold:

$$\frac{[Na^+]_0 - (J_n/P)}{[Na^+]_0^{\beta}} = c \cdot \gamma_c \cdot [Na^+]_c \quad (8)$$

By the kinetic method of estimating the Na^+ pool S_2 (ref. 3), an average value for $[Na^+]_c$ (which will be designated as $\overline{[Na^+]_c}$) can be calculated, if one wishes tentatively to make the assumption (Fig. 1), that active Na^+ transport occurs transcellularly with all cells participating equally.

$$\overline{[Na^+]_c} = S_2/V_2 \quad (9)$$

An approximate value for V_2 is available (see Results). For calculation of S_2 (cellular Na^+ pool) one must know the amount of Na^+ in the extracellular space. Here, the assumption is often made that $[Na^+]$ in the extracellular (inulin) space is equal to $[Na^+]_0$. If this assumption is false (ref. 29), then one has:

$$\overline{[Na^+]}'_c = f(S_2/V_2) = f\overline{[Na^+]_c} \quad (10)$$

Where f is the fraction of the Na^+ pool (S_2) as measured by the method used and under the assumptions as stated above. It is this $\overline{[Na^+]}'_c$ that one might consider as the electrochemically effective cellular Na^+. Using $\overline{[Na^+]_c}'$ in Eqn 8 gives:

$$\frac{[Na^+]_0 - (J_n/P)}{[Na^+]_0^{\beta}} = c \cdot \gamma_c \cdot f \cdot \overline{[Na^+]_c} = \alpha \overline{[Na^+]_c} \quad (11)$$

Neither c, nor γ_c, nor f, the parameters that make up α, are known or readily available. However, an empirical, unique value for α can be obtained from the experimental data presented below which show that $\overline{[Na^+]_c} = S_2/V_2$, and $\overline{[Na^+]_c}$ as calculated from Eqn 11 varied in very nearly the same manner with varying $[Na^+]_0$ over the range from 7 to 110 mM. This was the case for $\alpha = 0.115$. This coincidence suggested to us to proceed with calculations of values for $[Na^+]_s \cdot \gamma_s$ from Eqn 1. From a discrepancy of the calculated values for E_{ob}, using Eqn 3 (γ_c is included in α), and data published in the literature on E_{ob} as obtained by microelectrode puncture of epithelial cells[11-14], certain inferences can be drawn as to the $[Na^+]$ profile in the epithelium.

In order to apply Eqn 11 experimentally, data on β, J_n, P and S_2 (needed to obtain the empirical constant α) at varying $[Na^+]_0$ were collected.

METHODS

(1) *Animals*

All experiments were conducted at room temperature (23 °C) on non-short-

circuited abdominal skins of *R. pipiens* which were apparently in good general condition.

(*2*) *Solutions*

Mixtures of Na_2SO_4 and K_2SO_4, buffered with 10 mM Tris (pH 8) were used throughout this work. The solutions were briefly oxygenated before use. $[Na^+]_0$ was varied between 3.4 and 110 mM. The inside bathing solution ($[Na^+]_1 = 110$; $[K^+]_1 = 10$; [Tris] = 10 mM) was kept constant in all experiments, but was replaced by fresh solution whenever the outside solution was changed.

(*3*) *Apparatus*

A typical double lucite chamber was used. Each half-chamber had a volume of 26 ml and a free skin area of 7.2 cm^2 (potentiometric studies) or 7.06 cm^2 (flux studies). Edge damage[30] could have resulted in underestimation of the PD values by no more than 2 mV. Continuous mixing of the solutions in the chambers was achieved by externally driven magnetic stirrers.

(*4*) *Potentiometric measurements*

PD responses of the epidermal side of the skins to varying $[Na^+]_0$ (7–115 mM, 4 steps) were determined by a method similar to that of Koefoed-Johnsen and Ussing[9]. All skins were kept mounted for 1 h prior to the first solution change. It was found that within 10–15 min after solution changes, reasonably stable PD values were obtained. PD was monitored with a Keithley electrometer, Model 600 A. Connections to the chamber fluids were made *via* chloride–Ringer agar bridges and calomel half-cells. Junction potentials were calculated from the Henderson equation. It was determined that the slope of the regression line (PD *vs* $[Na^+]_0$) could have been slightly over estimated by ignoring the junction potentials. The correction was so small, however, that it was neglected. Furthermore, in some experiments pencil-type calomel electrodes were momentarily dipped into the chamber fluids for PD measurement, and the results were the same as those obtained with bridges. Only those experiments were used in which at least 90% of the original PD was recovered upon return to the original solution (110 mM $[Na^+]_0$).

(*5*) *Steady state Na^+ flux measurements*

After equilibration of the skins for 20 min in non-radioactive sulfate–Ringer's, 2.7 μCi $^{22}Na_2SO_4$ (New England Nuclear) and 19.4 μCi $^{24}Na_2SO_4$ (Tracerlab) were added to the inside and outside chambers, respectively. Samples were taken from both compartments shortly after addition of tracer and again approx. 2 h later. Activities were measured using a γ-ray spectrometer (Picker) at two different settings of the spectrometer range for ^{24}Na counting. A final ^{22}Na count was made 4 weeks after the experiments. All ^{24}Na count rates were corrected for physical decay. The accuracy of the counting procedure was $\pm$ 2.1 and $\pm$ 1.6% for ^{24}Na and ^{22}Na, respectively. Na^+ fluxes (J, μequiv$\cdot cm^{-2} \cdot h^{-1}$) were calculated by dividing the increments in count rates in the inside (outside) fluid compartment by the specific activity of the outside (inside) solution used, the time and the free skin area.

(6) *Non-steady-state Na^+ influx measurements.*

The rate constants (k) for Na^+ movement across isolated frog skin were determined by the method of Curran *et al.*[3]. In the appendix of their paper it is shown that the presence of the corium compartment does not alter any of the calculations for obtaining the k parameters. The appropriate experiments were carried out on open skins in $Na_2SO_4 + K_2SO_4$ mixtures of varying $[Na^+]_0$ by adding $^{24}Na_2SO_4$ (0.4 mC; Isoserve) to the outside fluid compartment. Samples of 0.2 ml were taken from the inside compartment at 1-min intervals for 10 min, and 10-min intervals for an additional 40 min. Fluid samples and, at the end of the experiments, the briefly blotted skins (7.06 cm^2) were counted as mentioned under (5). The apparent permeability coefficients of the cornified layer (P) were calculated from $P = k_{12}V_0/A$. V_0 is the volume of the outside fluid compartment (26 ml), A is the skin area (7.06 cm^2) and k_{12} is the rate constant for entry of Na^+ into the skin in transcutaneous transport measurements.

(7) *Na^+ pool*

Na^+-pool measurement in the skins was determined by the method of Curran *et al.*[3] with correction for Na^+ in the corium as described by these authors[3,4]. To determine extracellular space available to Na^+ from the epidermal side, separate skins were incubated in sulfate–Ringer's in the outside fluid compartment to which 80 μCi [^{14}C]inulin was added. Inulin was extracted and measured by the method of Cereijido *et al.*[4]. Extracellular space in the epidermis was found to be 0.31 $\pm$ 0.05 $\mu l/cm^2$. This value is in excellent agreement with the extracellular space values reported for short-circuited skins in chloride–Ringer's[3] and for skins treated with amiloride[8]. Thus, the value was used as representative of skins bathed in all of the $[Na^+]_0$. For correction of the Na^+ pool (S_2) for Na^+ contained in this extracellular space it was tentatively assumed that the $[Na^+]$ in the epidermal extracellular space was the same as $[Na^+]_0$. Consideration for the case that this assumption is false is given in Calculations and Discussion. The total Na^+ pool (S_2 (μequiv/cm^2) was then calculated as $S_2 = P_{2\infty}/s_1$, where $P_{2\infty}$ is the total radioactivity in the skin and s_1 is the specific activity of the outside fluid compartment. It is assumed that at $t = \infty$, $s_2 = s_1$ in the readily exchangeable Na^+ fraction of the epidermal cells which takes part in transport.

RESULTS

(1) *Electrical potential response of the epidermal side of skins to varying $[Na^+]_0$.*

The mean PD of ten skins (*R. pipiens*) in sulfate solution (110 mM Na^+: 10 mM K^+) on both sides was 87 mV, inside positive. Upon serial changes of $[Na^+]_0$ in 4 steps (lowest $[Na^+]_0 = 7$ mM, at constant $[Na^+]_0 + [K^+]_0$) the skin PD decreased linearly ($R = 1.00$) with decreasing log $[Na^+]_0$. For a 10-fold change in $[Na^+]_0$ the PD changed by 34.6 mV. This agrees with the data in the literature[14,32]. Confirmation of this fact was of importance since it was our intention to combine this information with Na^+ flux data, applicable to non-short-circuited skins in sulfate–Ringer's, data for which have not been reported in the literature.

The regression line for the $[Na^+]_0$–PD relationship using the data of Table I is: PD = 32.2 + 20.2 $[Na^+]_0$ ($R = 0.88$). In these studies, however, each individual

skin was not subjected to serial changes in $[Na^+]_0$. We consider the slope factor of 20.2 of questionable use. Nevertheless, in the Discussion the consequences of applying this extremely low value has been considered. The ideal, near 58-mV change in PD for a 10-fold change in $[Na^+]_0$ for skins of *R. temporaria*, suggesting perfect Na^+ permselectivity[9], was never observed in skins of *R. pipiens*. The skin of this species gives only a 24 mV PD change per decade change in $[Na^+]$ when SO_4^{2-} is replaced by the rather impermeant HCO_3^- (ref. 33). It has been shown[22] that in skins of *R. pipiens* the less than ideal response of the epidermis to changes in $[Na^+]_0$ can be observed in skins which retain their ideal, Nernst-type response to changes in $[K^+]_1$ on the corium side. This, as well as the better than 90 % recovery of the original PD, indicates that the skins used were in good condition. At $[Na^+]_0 < 1.0$ mM (*i.e.* mainly K_2SO_4 present) the PD response of skins of *R. temporaria* is less than 58 mV per decade change in $[Na^+]_0$, and the response is no longer linearly related to log $[Na^+]_0$ (ref. 9). Under these conditions electrical shunting of the skin PD by SO_4^{2-} appears to occur[13]. From the above results on skins of *R. pipiens* a value of $\beta = 35/58 = 0.602$ was calculated and used in Eqn 11.

(2) *Transcutaneous Na^+ fluxes (J_n).*

The results of these measurement, $J_n = J_{in} - J_{out}$, are given in Table I. When $1/J_n$ was plotted against $1/[Na^+]_0$ the results were describable by the empirical equation:

$$1/J_n = 6.55 + 31.3\ [Na^+]_0$$

J_{out} did not vary in a consistent manner with varying $[Na^+]_0$, but ranged from 0.02 to 0.07 μequiv·cm^{-2}·h^{-1}, indicating that the skins were not excessively leaky to Na^+. The flux ratio equation of Ussing[34] was applied to obtain calculated values of J_{in}/J_{out}. As expected, the found ratios were greater than the calculated ratios, suggesting active Na^+ transport across open skins in sulfate solutions. The flux ratios show no systematic variation with varying $[Na^+]_0$, and the low ratios are the result of low J_{in} rather than high J_{out} values.

TABLE I

DEPENDENCE OF NET Na^+ FLUX ON $[Na^+]_0$

Non-short-circuited ("open") skins in sulfate salt solutions. J_1/J_0 is the ratio of Na^+ influx/Na^+ outflux. $\Phi_1 - \Phi_4$ is the potential difference between the inside and outside. Standard errors of the mean values are given.

$[Na^+]_0$ (*mM*)	*No. of expts*	J_n (μ*equiv·cm^{-2}·h^{-1}*)	$\Phi_1 - \Phi_0$ (*mV*) *	J_1/J_0	
				Found	*Calculated*
3.40	6	0.065 ± 0.020	40.0 ± 3.7	5.50	0.02
6.90	5	0.092 ± 0.031	44.8 ± 6.1	2.30	0.03
13.8	7	0.109 ± 0.015	67.2 ± 4.0	6.44	0.04
27.5	6	0.129 ± 0.041	60.0 ± 6.3	3.38	0.10
55.0	6	0.143 ± 0.063	66.3 ± 9.9	4.43	0.19
110.0	6	0.153 ± 0.063	71.2 ± 5.9	3.48	0.33

* PD = 32.2 + 20.2 $[Na^+]_0$. $\beta = 20.2/58 = 0.348$ (see Results, Paragraph 1).

(3) *Rate constants (k) and apparent permeability coefficients (P)*

The results of these measurements are shown in Table II. Two points of interest are to be noted. First, P, referred here to the cornified layer, decreases sharply with increasing $[Na^+]_0$. From a plot of $1/P$ against $1/[Na^+]_0$ the empirical relationship

$$1/P = 40.4 + 2.02\ [Na^+]_0$$

applies over the range of $[Na^+]_0$ investigated. A decrease of the apparent permeability coefficient of a barrier at the entry side of skins is also seen in short-circuited

TABLE II

RATE CONSTANTS, k VALUES, FOR Na^+ MOVEMENT ACROSS FROG SKIN EPITHELIUM, AS CALCULATED BY APPLYING THE THREE COMPARTMENT MODEL OF CURRAN *et al.*[3]

Values for the apparent permeability coefficient, P, and of the Na^+ pool, S_2, were calculated from the equations given in Methods. Means ± S.E. are given.

$[Na^+]_0$ (*mM*)	*No. of expts*	$k^*_{12} \times 10^3$ (h^{-1})	k_{23} (h^{-1})	k_{21} (h^{-1})	$P \times 10^3$ (*cm/h*)	S_2 (*μequiv/cm²*)
6.90	6	10.3 ± 1.4	3.3 ± 0.6	3.9 ± 1.7	37.9 ± 5.2	0.04 ± 0.01
13.8	6	9.8 ± 1.6	1.9 ± 0.2	4.9 ± 0.6	36.1 ± 5.9	0.08 ± 0.01
27.5	6	6.8 ± 0.9	1.5 ± 0.5	4.5 ± 0.7	25.0 ± 3.3	0.11 ± 0.02
55.0	5	4.1 ± 0.7	1.2 ± 0.4	4.1 ± 0.8	15.1 ± 2.6	0.15 ± 0.02
110.0	6	2.4 ± 0.2	1.1 ± 0.2	4.0 ± 0.4	8.8 ± 0.7	0.18 ± 0.02

* Normalized values as described by Cereijido *et al.*[4].

skins (*R. pipiens*) in chloride–Ringer's when $[Na^+]_0$ is varied by replacement with choline ion[4]. Second, when the individual fluxes are calculated from the respective rate constants and other known parameters of the experimental system, a dependence of fluxes on $[Na^+]_0$ as shown in the right-hand section of Fig. 2 is obtained. For comparison a similar plot was made using the data of Cereijido *et al.*[4]. (see their Table I). Their J_{12}–J_{2i} was identical with the short-circuit current equivalent. We suspect that the large difference in J_{23} between the two experimental conditions with relatively smaller differences in J_{12} and J_{21} is a remote (osmotic?) effect on the active Na^+ transport mechanism. Anionic effects on Na^+ transport across frog skin have been reported by Ferreira [35] and Huf[36].

(4) *Intracellular Na^+ pool (S_2) and $[Na^+]_c$*

When the data on S_2 (Table II) were plotted against $[Na^+]_0$ Fig. 3 was obtained. Na^+-pool sizes are to be read on the left side ordinate. A reasonably good value for the volume occupied by the epidermis of frog skin (*R. pipiens*) is 4.5 $\mu l/cm^2$, of which approx. 0.5 $\mu l/cm^2$ is epidermal extracellular space[3,4,7]. We attribute 1/8 of the cellular volume, *i.e.* 0.5 $\mu l/cm^2$, to the volume occupied by the first reacting cell layer (1. RCL[15]). The value of 4.5 $\mu l/cm^2$ is based on measurements[37] of the surface/weight relationship and the water content of the skins, and a value of 25 % of the total skin occupied by the epidermis[38]. No estimations were attempted for the volume of the subcorneal space, which must be quite small relative to the other spaces in the epidermis. Using these space figures, average $\overline{[Na^+]}_c$ values, applicable to all cells indiscrimantly, were calculated as $\overline{[Na^+]}_c = S_2/4.0 \cdot 10^{-3}$. The figures

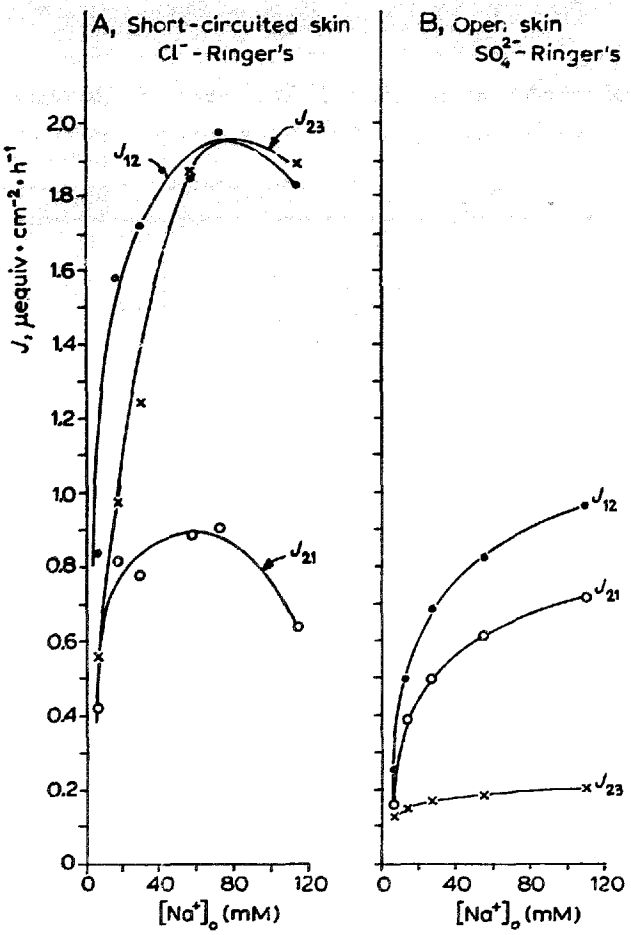

Fig. 2. A, Na^+ flux rates (J) across short-circuited frog skin (*R. pipiens*) in chloride–Ringer's at varying $[Na^+]_0$. Substitution for Na^+ was made with choline ion. The values were calculated from the data given by Cereijido *et al.*[4]. By comparison B shows similarly calculated flux rates obtained from the present studies on non-short-circuited (open) skins (*R. pipiens*) in sulfate–Ringer's at varying $[Na^+]_0$. Substitution for Na^+ was made with K^+.

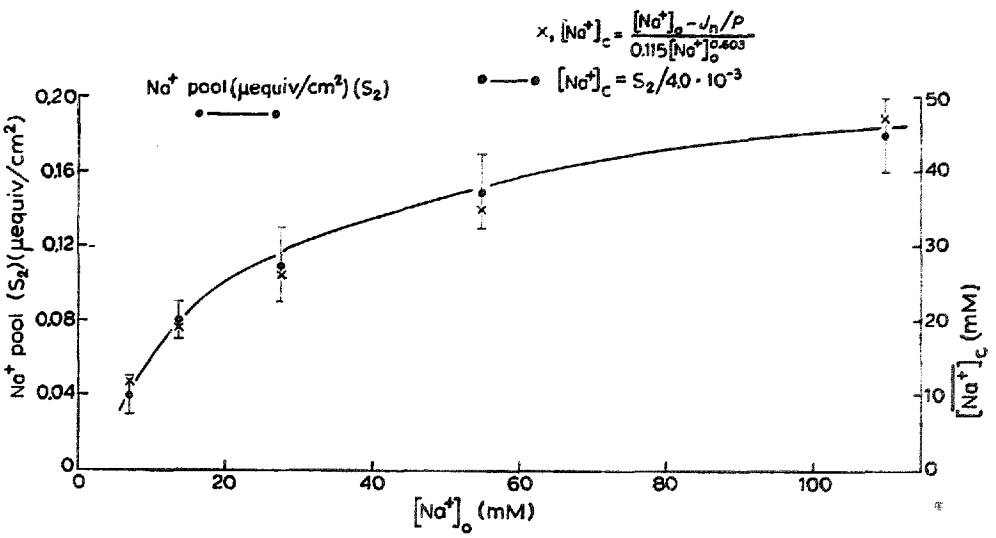

Fig. 3. Dependence of the intracellular Na^+ pool (S_2, μequiv/cm^2) in epidermis (left-hand ordinate), and average intracellular $[Na^+]$ ($\overline{[Na^+]}_c$, mM) (right-hand ordinate) on $[Na^+]_0$. The graphs are drawn by eye. Data were calculated as stated in the graph.

TABLE III

ESTIMATIONS OF $[Na^+]_c$ AND E_{ob}

Data in Columns 1–6 are based on theoretical considerations given in the text and assuming uniform distribution of Na^+ in all epithelial cells ($[\overline{Na^+}]_c$). Data in Columns 7–9 are based on the assumption that the first reacting cell layer has a lower $[Na^+]_c$ than the rest of the epithelial cells.

1	2	3	4	5	6	7	8	9
$[Na^+]_0$	J_n/P*	$[Na^+]_s$**	$[\overline{Na^+}]_c$*** *All cells*	$[\overline{Na^+}]_c$§ *All cells*	E_{ob}§§	$[Na^+]_c$§§§ *All cells except first reacting cell layer*	$[Na^+]_c$† *first reacting cell layer*	E_{ob}††
(*mM*)	(*mM*)	(*mM*)	(*mM*)	(*mM*)	(*mV*)	(*mM*)	(*mM*)	(*mV*)
6.90	2.42	4.48	10.0	11.1	−22.8	12.2	3.1	+ 9.4
13.8	3.02	10.8	20.0	19.7	−15.1	21.7	5.5	+17.1
27.5	5.16	22.3	27.5	26.9	− 4.7	29.6	7.5	+27.4
55.0	9.47	45.6	37.5	36.5	+ 5.6	40.3	10.1	+38.0
110.0	17.4	92.6	45.0	46.2	+17.5	51.0	12.8	+50.0

* Data from Tables 1 and 2.

** $[Na^+]_s = [Na^+]_0 - J_n/P$.

*** $[\overline{Na^+}]_c = S_2/(4.0 \cdot 10^{-3})$ (μequiv per cm^2/cm^2 per cm^2).

§ Mean $[\overline{Na^+}]_c$ obtained by pool and potentiometric method.

§§ $E_{ob} = 58 \log [Na^+]_s/[\overline{Na^+}]_c$ (Line A, Fig. 4).

§§§ $[Na^+]_c = 1.103\ [\overline{Na^+}]_c$.

† $[Na^+]_c = 0.277\ [\overline{Na^+}]_c$.

†† $E_{ob} = 58 \log [Na^+]_s/[Na^+]_c$ first reacting cell layer, (Line B, Fig. 4).

thus obtained are given in Column 4, Table III and are to be read on the right side ordinate of Fig. 3. If one now calculates values for $[\overline{Na^+}]_c$ for varying $[Na^+]_0$ by Eqn 11, using the necessary variables listed in Table II and choosing $\alpha = 0.115$, one obtains results which are well within the statistical errors of the $[\overline{Na^+}]_c$ data obtained by the pool method (Fig. 3). In Column 5, Table III, the mean values for $[\overline{Na^+}]_c$ obtained by the two methods are listed.

DISCUSSION

(1) *Basic assumptions*

Both the Na^+-pool method and the potentiometric method of estimating $[\overline{Na^+}]_c$ are based on the assumption that the Na^+ flux across the skin can be analyzed by applying the three compartment model as presented by Curran *et al.*[3]. In view of the highly complex fine structure of the skin, this assumption must be considered as an oversimplification. We have adopted this model in the present studies, mainly because of the argument of several authors[1,2,7] that in Na^+ washout experiments the skin behaves as if it represented a single compartment.

In addition, two other critical assumptions were made. Applying the Na^+-pool method, it is assumed that the $[Na^+]$ in the extracellular space is equal to $[Na^+]_0$. Applying the potentiometric method, it is assumed that the outer border is Na^+ permselective. With regard to this point we have found it essential to modify

the Nernst equation for the intended purpose, recognizing that the outer border is not in direct contact with the outside bathing solution, but with the fluid of the subcorneal space. Its $[Na^+]_s$ is likely to be somewhat lower than $[Na^+]_s$ in steady state Na^+ flux across the skin. It seems safe to assume that both $[Na^+]_s$ and $[Na^+]_c$ rise with rising $[Na^+]_o$.

(2) *Intracellular* $[Na^+]_c$

Fig. 3 shows that $[\overline{Na^+}]_c$ obtained by the pool method and the potentiometric method give nearly identical values for $[\overline{Na^+}]_c$, varying in the same manner with $[Na^+]_o$ over the range from 7 to 110 mM. Since the pool method does not require knowledge of E_{ob}, and the potentiometric method does not require knowledge of the cellular volume, the agreement in the results tends to support the view that the assumptions made for calculations of $[\overline{Na^+}]_c$ by either method are reasonable. From the data given in Columns 3 and 5, Table III, E_{ob} was calculated by applying equation 3. γ_c is included in the empirical constant $\alpha = 0.115$ needed to obtain $[\overline{Na^+}]_c$. The results are presented in Column 6. A plot of E_{ob} against log $[Na^+]_o$ (Line A, Fig. 4) shows that the slope of the regression line agrees with the experimental results that a 10-fold change in $[Na^+]_o$ gives nearly $\Delta E_{ob} = 35$ mV. What is not in agreement with experimental facts reported in the literature is that for skins in 110 mM Na^+ sulfate solution, E_{ob} is only $+17$ to $+18$ mV. By neglecting the J_n/P terms in Eqn 3, one obtains slightly higher E_{ob} values, $+23$ mV for $[Na^+]_o =$ 110 mM. However, omission of these terms, although of minor significance relative to changes in $[Na^+]_c$ with changing $[Na^+]_o$, gives a less satisfactory fit to the $[\overline{Na^+}]_c/[Na^+]_o$ dependence relationship (Fig. 3). The $[\overline{Na^+}]_c$ values are too high at low $[Na^+]_o$. Microelectrode puncture studies by Engbaek and Hoshiko[11] (on epidermis of *R. temporaria*, *R. oxyrhinia* and *R. esculenta*), Whittembury[12] (on toad skin epidermis), Ussing and Windhager[13] (on epidermis of *R. temporaria*) and Cereijido and Curran[14] (on epidermis of *R. pipiens*) have demonstrated that the first potential step is much higher, $+40$ to $+65$ mV, outside bath negative. Since in our calculations allowance for $[Na^+]_s$ (subcorneal space) $< [Na^+]_o$ has been made,

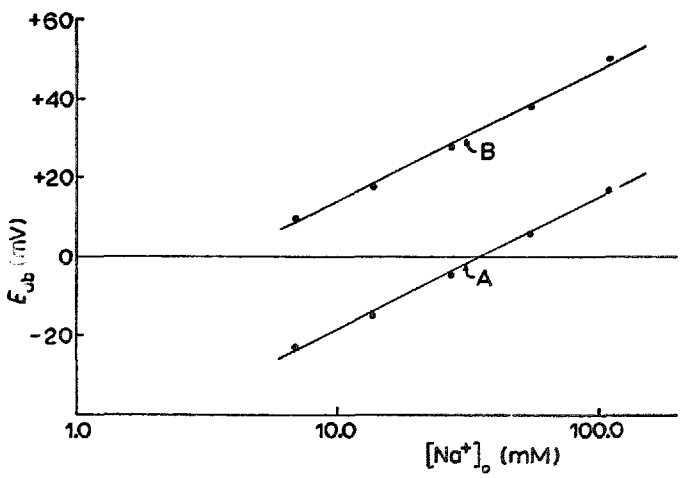

Fig. 4. Plots of dependence of the electrical potential difference across the outer barrier, E_{ob}, on $[Na^+]_o$, using Eqn 3 in the text. Values for the needed variables are given in Table III. Line A was calculated taking $[\overline{Na^+}]_c = [\overline{Na^+}]_c$, while Line B was calculated taking $[Na^+]_c$ (first reacting cell layer) $= 0.277\ [\overline{Na^+}]_c$.

the discrepancy between calculated and found E_{ob} suggests that $[Na^+]_c$ in the first reacting cell layer is lower than $\overline{[Na^+]}_c$. In Results, an estimated value of 0.5 μl/cm^2 is given for the volume of the first reacting cell layer. With this and a mean first PD step of, *e.g.* +50 mV, one can then calculate values for $[Na^+]_c$ in the first reaction cell layer. The data are given in Column 8, Table III. The calculated E_{ob} values, applying Eqn 3, are presented in Column 9, Table III. Line B, Fig. 4, shows that this result is in agreement both with respect to slope and observed E_{ob}. Since the condition for total Na^+-pool size also must be met, this analysis suggests that Na^+ is unequally distributed (Table III, Columns 7 and 8) in the epidermal cells. The possibility of increasing $[Na^+]_c$ with increasing depth from the external surface of the skin has also been considered by Biber *et al.*[39] and Erlij[40] has proposed that a very effective Na^+ pump may be located at the first reacting cell layer with little flow of Na^+ *via* the cell junctions into deeper cell layers. This interpretation would be in agreement with the observations of Voûte and Ussing[15] that morphological changes associated with current flow are most prominent in the first reacting cell layer. These cells swell upon shortcircuiting of the skin. The data shown in Column 8 Table III, support the hypothesis[9] of passive entry of Na^+ into the epidermis.

The case of $\beta = 0.348$ (instead of 0.602) has been considered. Calculations for $\overline{[Na^+]}_c$, then, deviate by no more than 18% from the values given in Table III, Column 5, with lower and higher values for $[Na^+]_0 = 7$ and 110 mM, respectively. Although the deviations are relatively small we question the usefulness of this very low β value for the reason stated under Results, Paragraph 1. A comparison of recently published data on Na^+ pool and $[Na^+]$, calculated for the first reacting cell layer and skins under differing experimental conditions is presented in Table IV. The differences seen may be explainable on the basis of the findings of Voûte and Ussing[15,18] that the first reacting cell layer appears swollen in short-circuited skins, and more dense and shrunk in short-circuited and non-shorted skins when using sulfate–Ringer's.

TABLE IV

CALCULATED VALUES FOR Na^+ POOL AND $[Na^+]$ IN EPITHELIAL CELLS OF THE FIRST REACTING CELL LAYER

(1 cm^2 × 5 μm = 0.5 μl).

Source of data	*Na^+ pool* ($\mu equiv \times 10^3$)	*$[Na^+]$* (*mM*)
Cereijido *et al.*[6]	2.7 *	5.4
Aceves and Erlij[28]	0.9 **	1.8
Present data $[Na^+]_0 = 6.9$ mM	1.6 **	3.2
Present data $[Na^+]_0 = 110$ mM	6.4 **	12.8

* Studies on whole, non-shorted skins (*Leptodactylus ocellatus*, L.) in chloride–Ringer's ($[Na^+]_0 = 115$ mM) without correction for Na^+ in the epidermal extracellular space. Maximum estimates.

** Studies on isolated, short-circuited epidermis (*R. pipiens*) in chloride–Ringer's ($[Na^+]_0 = 115$ mM) with correction for Na^+ in the epidermal extracellular space. Maximum estimates.

*** Studies on whole, non-shorted skins (*R. pipiens*) in sulfate–Ringer's with exclusion of Na^+ in the epidermal extracellular space.

(3) [Na+] in epidermal extracellular space

An alternate interpretation of our results is to suggest that the deeper cells, as well as the cells of the first reacting cell layer, have low $[Na^+]_c$ and that a considerable fraction of the Na^+-pool is, in fact, extracellular. This would be in agreement with the studies of Zerahn[7] on Na^* washout rates. If $[Na^+]$ in the extracellular space was indeed higher than $[Na^+]_o$, the intracellular Na^+-pool (S_2), as calculated by us as well as by others, would be too high. The possibility of an existing high $[Na^+]$ in the interstitial (extracellular) space would also explain earlier observations on water flow and water rectification associated with active Na^+ transport[38,41-43] which can be readily suppressed by cyanide poisoning. Current theory of fluid transport across epithelial tissues[29] supports the alternate hypothesis.

(4) Computer simulation of the model Fig. 1.

(E. G. Huf and J. R. Howell, personal communication.) The question arose whether it is possible to have the system in steady state such that: extracellular $[Na^+]$ > "remaining cell $[Na^+]$" > first reacting cell layer $[Na^+]$ with net Na^+ flux at a rate commonly observed in laboratory experiments. The following five compartment system was considered: Outside fluid (1) ⇄ first reacting cell layer (2) ⇄ remaining cells (3) ⇄ extracellular fluid (4) ⇄ inside fluid (5), with flows also between 2 ⇄ 4. Strong Na^+ pumps with little leakage are assumed to operate between (2) and (4), and weak, leaky pumps between (3) and (4). Five linear differential equations of the form as generally used in compartmental flow analysis (see *e.g.* Curran *et al.*[3]) were set up with a total of 10 rate constants. These equations were then written in matrix form for computer solutions as follows: ($\dot{S} = dS/dt$, in which S is the amount of material in a given compartment):

$$\begin{bmatrix} \dot{S}_1 \\ \dot{S}_2 \\ \dot{S}_3 \\ \dot{S}_4 \\ \dot{S}_5 \end{bmatrix} = \begin{bmatrix} -k_1 & k_{21} & 0 & 0 & 0 \\ k_{12} & -k_2 & k_{32} & k_{42} & 0 \\ 0 & k_{23} & -k_3 & k_{43} & 0 \\ 0 & k_{24} & k_{34} & -k_4 & k_{54} \\ 0 & 0 & 0 & k_{45} & -k_5 \end{bmatrix} \begin{bmatrix} S_1 \\ S_2 \\ S_3 \\ S_4 \\ S_5 \end{bmatrix}$$

The k values are the rate constants with $-k_1 = -k_{12}$; $-k_2 = -(k_{21} + k_{23} + k_{24})$; $-k_3 = -(k_{32} + k_{34})$; $-k_4 = -(k_{42} + k_{43} + k_{45})$; $-k_5 = -k_{54}$. Assumed numerical values for k values and other specifications of the system, resembling a laboratory experiment with frog skin, are given in the legend of Fig. 5. The k values between two compartments are chosen such that their ratios are inversely related to the respective volumes involved where passive diffusion is assumed to occur ($k = A \cdot P/V$, where A is the area and P is the permeability coefficient). This was not applied to flows between (2) and (4), and between (3) and (4) where it has been assumed that strong and weak pumps, respectively, are located, pumping Na^+ into the extracellular spaces. Initial conditions: Compartments (1) and (5) = $500 \cdot 10^n$ Na^+, zero Na^+ in all other compartments at zero time. Solutions were obtained with an IBM 1130 computer with application of the Continuous System Modeling Program (CSMP).

The result is shown in Fig. 5. [Na^+] in mM as the ordinate is plotted against time in min. The print-out numbers were converted into [Na^+] by letting $500 \cdot 10^n$ ions per 5000 μl be equal to 100 mM. The system reaches steady state

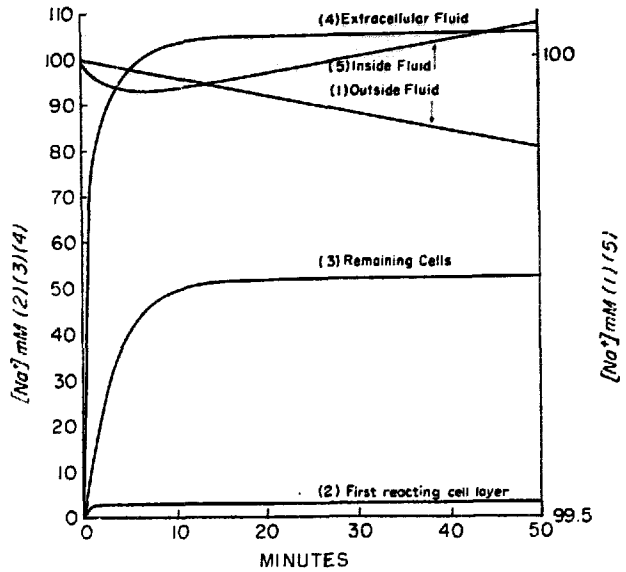

Fig. 5. Computerized flows of Na^+ in the skin model shown in Fig. 1. Assumed volumes: $V_1 = V_5 = 5$ ml; $V_2 = 0.5$ μl/cm^2; $V_3 = 4$ μl/cm^2; $V_4 = 0.5$ μl/cm^2. The following k values (min^{-1}) were used: $k_{12} = 0.00002$; $k_{21} = 0.2$; $k_{23} = 0.04$; $k_{32} = 0.005$; $k_{24} = 10$; $k_{42} = 0.1$; $k_{34} = 0.5$; $k_{43} = 2.0$; $k_{45} = 4.0$; $k_{54} = 0.0004$.

in all skin compartments in about 15–20 min. At that time [Na^+] in the first reacting cell layer is 3.18 mM; [Na^+] in the remaining cells is 51.95 mM, and [Na^+] in the extracellular fluid is 104.87 mM. As expected, [Na^+] in (1) and (5) decreases first (right hand side ordinate), but whereas [Na^+] in (1) continues to fall, [Na^+] in (5) rises again and after 15–20 min increases linearly with time. From the slope and the data given in the legend one calculates a net flux of 0.58 μequiv$\cdot$cm$^{-2}\cdot$h^{-1}. From separate influx and outflux studies it was found that outflux is 1.2% of the influx. It is not claimed that the model meets other requirements. For instance, it can be calculated that with the assumed k values about 10% of the net Na^+ passing the pump (2)$\rightarrow$(4) is Na^+ which recirculated in the skin compartments. 90% of the Na^+ passing this pump comes from outside compartment (1). This and other features of the model are presently under investigation. Unequal intradermal distribution of $[Na^+]_c$ as strongly suggested by the present study could explain earlier observations from this laboratory (Huf *et al.*[24]; Winn *et al.*[22]) that "maintenance electrolyte equilibrium" and "unidirectional active ion transport" are two aspects of the Na^+ and K^+ metabolism of epithelial cells of frog which show different drug sensitivities.

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