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Ionic Exchanges in Isolated and Open-Circuited Toad Skin

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Summary. Net influxes of Na and Cl and effluxes of K and H (J_{Na}, J_{Cl}, J_K and J_H) and volume flow J_v across isolated open-circuited toad skins were measured using rotating chambers and a small volume of external solution, the ion fluxes being determined by chemical analysis of the external solution, in the range of 0.2 to 5.0 mM external Na concentration. In this concentration range, with skin potential varying with $(Na)_e$, J_{Na} is a linear function of the Na electrochemical potential difference across the skin, as expected on irreversible thermodynamic grounds. The L_{Na} coefficient calculated as $\Delta J_{Na}/\Delta \mu_{Na}$ is equal to 5.5×10^{-12} mole^2 joule^{-1} cm^{-2} min^{-1}, which is similar to values obtained for the same species in the short-circuited state and in higher ranges of $(Na)_e$. A positive correlation is observed between J_{Na} and J_K when J_{Na} varied with $(Na)_e$ and also when J_{Na} varies in randomly selected skins. Antidiuretic hormone stimulates J_{Na}, J_K and J_v in the range of 0.2 to 5.0 mM $(Na)_e$ and lowers the Na concentration in the equivalent solution absorbed by the skin (calculated as J_{Na}/J_v). Substitution of external Cl by SO_4 has no effect on J_{Na}, J_K and J_H and also in the skin potential in the range of $(Na)_e$ studied. Substitution of external Na by K abolishes J_{Cl} and reverses the skin polarity, the external solution now being positive to the internal one. Na removal from the external solution also reduces J_K almost to zero. J_H is significantly reduced in this condition; however, a basal secretion still persists with $(Na)_e$ equal to zero. The results of these experiments can be tentatively interpreted in terms of electrical coupling between ion fluxes, since only the procedures that result in alterations of skin potential are followed by changes in the rates of ion transport. The existence of other coupling mechanisms cannot be ruled out.

Ion transport from dilute external solutions by amphibian skin has been studied since the works of Krogh [27, 28] in the thirties.

Most of the experiments with dilute solutions on the outer side of the skin were carried out in intact animals and the ionic fluxes measured by chemical analysis [17, 22, 37] or with the use of radionuclides [24, 33, 38].

The movement of Na across isolated amphibian skins bathed externally by dilute Na solutions was studied predominantly with tracer methods [2–4, 36] and with the short-circuit technique [1, 3].

Studies on the mechanism of chloride transport across the in vivo amphibian skin have shown that this ion is absorbed independently of Na in

animals bathed externally by low (NaCl) solutions [15, 28] and that inhibition of Na transport has no effect on chloride absorption [17] under these conditions. On the other hand, investigations on the active or passive nature of chloride transport in the in vitro short-circuited skin have revealed two fundamental types of transport: an active transport when the external NaCl concentration is around 2 mM [11, 26, 32] and a passive one when the external Cl concentration is that of normal Ringer's solution. Recently, Garcia-Romeu and Ehrenfeld [16] have studied with tracer methods the movement of chloride through the isolated nonshort-circuited skin of the frog and have shown that the predominant component of the chloride transport is Na dependent with Ringer's external solution and Na independent with low external Na concentrations.

Potassium fluxes at the outer border of the frog skin were studied originally by Huf and Wills in 1953 [20]. These authors showed a correlation between rejection of K and transepithelial p.d. and demonstrated the existence of a relationship between sodium uptake and potassium rejection to the external medium. More recently, Nielsen [34] showed that the frog skin treated with Amphotericin B secrets potassium to the external medium, but that in normal conditions the external barrier is impermeant to potassium.

The ability of the isolated amphibian skin to establish and mantain an H ion gradient between the solutions bathing its sides is widely known and has long been reported in the literature [13, 14, 19]. More recent studies on this subject by Emilio and Menano [9] and Emilio *et al.* [8] have shown a marked dependence of the H flux on Na transport in the short-circuited toad skin suggesting that at least a fraction of the total H efflux is linked to the Na influx.

Despite the large number of works concerning the study of Na, Cl, K and H fluxes in toad and frog skins, there is no information relative to the simultaneous measurements of net Na, K, Cl and H fluxes in a same piece of skin bathed externally by dilute hypoosmotic NaCl solutions, the situation normally occurring in the habitat of the living frog or toad.

The present set of experiments were performed in the isolated and open-circuited skin of the toad *Bufo marinus ictericus* in an attempt to study some of the mutual relationships between the net Na and Cl uptakes and K and H outfluxes in conditions of low Na concentration, low ionic strength and low osmolarity in the external solution. The experiments attempted to simulate in vitro the in vivo conditions of the skin while at the same time avoiding the participation of control mechanisms operating in the intact animal.

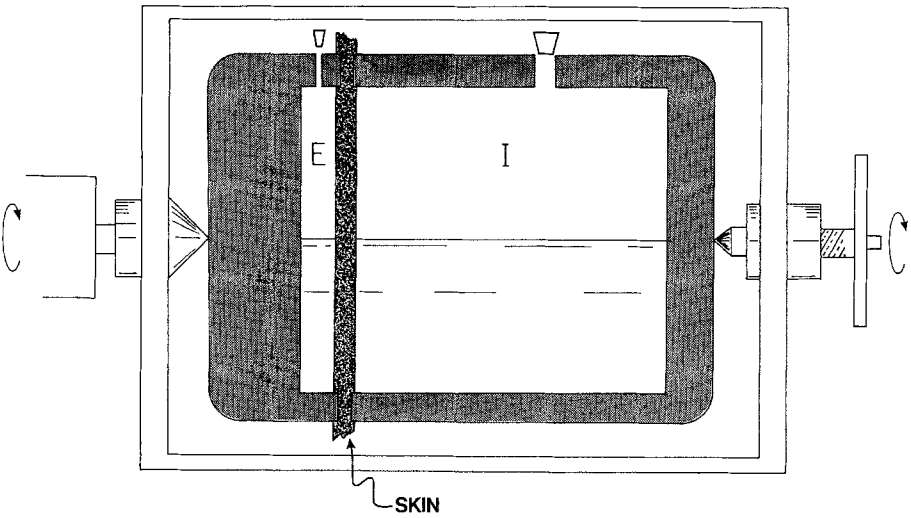

Fig. 1. Diagram of the rotating chamber. *E* and *I* refer to external and internal compartments

Materials and Methods

The experiments have been performed on the isolated abdominal skin of the toad *Bufo marinus ictericus* and carried out in the open-circuited state, at room temperature (20–25 °C). Water and ionic fluxes were measured with skin mounted in a specially designed rotating chamber (Fig. 1) exposing an area of 4.9 cm^2. After assembling together the two half-chambers, the system was coupled to a 100 rpm electric motor so that the chamber could rotate with its axis in the horizontal position. The volumes of the internal and external compartments were 13.2 and 3.9 ml, respectively. After mounting the skin, the external and internal compartments were filled with a volume of the desired solution equivalent to half of that of the compartment and the filling holes closed with plugs. With rotation of the chamber, a continuous flow of solution swept the surfaces of the membrane providing adequate stirring as well as the necessary aeration of the solutions. The air in each compartment was renewed approximately every 15 min during each experiment. This procedure can be shown to be adequate to keep the solutions well aerated if we compare the amount of oxygen trapped in the chamber (approximately 4×10^{-4} moles) with the estimated rate of oxygen consumption which should be in the range of 50 to 70 pmoles cm^{-2} sec^{-1}, according to previous data for the skin of *Bufo marinus ictericus* [6] and of *Rana pipiens* [41]. Experiments had a duration of 1.5 to 2.0 hr.

Flux Measurements

Volume flow. Volume flow was measured by evaluating the difference between the amount of solution initially added to the external compartment and that recovered at the end of the experiment. The average volume flow was evaluated according to:

$$J_v = (V_1 - V_2)/(A\,t)$$

Table 1. Test for the degree of recuperation of the amount of solution added to the external compartment[a]

V_1 (ml)	V_2 (ml)	$V_1 - V_2$ (μl)
2.327	2.315	12
2.299	2.298	1
2.292	2.289	3
2.291	2.286	5
2.270	2.270	0
2.269	2.268	1
2.269	2.261	8
2.257	2.255	2
2.260	2.255	5
2.258	2.258	0
		Mean ± SE 4 ± 1

[a] A given amount of solution (V_1) was added to the external compartment with skin mounted in the chamber. The chamber was rotated for a few seconds and the solution of the external compartment recollected (V_2). Volumes were measured by weighing a syringe before and after delivering or collecting the solution. Solution density was considered as 1.

where J_v is the volume flow, V_1 and V_2 are the initial and final volumes, A is the area of membrane and t is the duration of the experiment. The experiments were always performed in the following sequence after the skin had been mounted.

1. The internal and external compartments were half-filled with the desired solutions.

2. After an equilibration period of one hour, during which the chamber was kept in rotation, the internal solution was withdrawn and replaced by fresh Ringer's solution. The external solution was then slowly and carefully aspirated with a syringe, always at the same rate in order to obtain same equivalent residual film of solution on the skin surface. At that moment (time zero) a precisely known volume of solution (V_1) (calculated by weighing a syringe with precision of 1 mg before and after delivering the solution) was introduced into the external compartment which was sealed again.

3. At the end of the experimental period all the solution in the external compartment (except the remaining liquid film) was carefully withdrawn by means of a syringe. V_2 was calculated by weighing the syringe before and after collecting the solution. To transform weight to volume, the solution density was considered to be equal to 1. To test for the accuracy and for the reproducibility of the method preliminary experiments were performed in which a given volume of solution was introduced in the external compartment, the chamber rotated for a few seconds and the solution withdrawn. Table 1 summarizes the results. As can be seen, the error involved in the recollection is of a few microliters and, therefore, it can be neglected in the determination of volume flow.

Ionic fluxes. Average ionic fluxes were calculated as:

$$J_j = (V_1 C_{j1} - V_2 C_{j2})/(At)$$

where J_j is the ionic flux of species j, V is the volume of solution in the external compartment, C_j is the external ionic concentration of species j, t is the duration of the experiment, 1 and 2 refer to the initial and final measurements and A is the area of the membrane. Positive values for J_{Na} and J_{Cl} refer to net uptake from the external bathing solution; positive values for J_K

and J_H refer to net secretion to the external solution. A negative sign indicates reversion of those fluxes.

Na and K concentrations were measured by flame photometry (Baird Atomic Flame Photometer, model KY-4) with compensation for cross interference between Na and K by use of Li in the standard and test solutions. Cl concentration was evaluated potentiometrically by means of an Ag-AgCl electrode against a reference electrode (saturated KCl calomel half-cell and a 4 M $NaNO_3$ agar-bridge) with the electrical potential difference between the two half-cells measured by an electrometer (Keithley, model 615). The Ag-AgCl electrode was calibrated before and after each chloride determination, and the Cl concentration in the sample was interpolated between the two closest standards. Ag-AgCl electrodes were made of a silver wire coated with glass except at the tip which was cleaned by abrasion and thoroughly washed in distilled water before being chloridized in 0.1 M HCl. For pH determinations the solutions added to or removed from the external compartment were equilibrated with air until a stable pH was obtained. The remaining "fixed acid" was measured by one of the following procedures:

(*a*) the solution collected from the external compartment was titrated with 0.094 M NaOH to a pH equal to that of the initial solution (Beckman Expandomatic pH meter);

(*b*) the pH of the solution collected from the external compartment at the end of the experiment was measured with a glass microelectrode (Blood Micro System BMS 3 Mk 2 coupled to a Digital Acid Base Analyser PHM 72 Mk 2, Radiometer) and the amount of fixed acid present calculated by interpolation using a graph of pH versus amount of H added to an aliquot of external solution. This second method was used in some experiments in order to save solution for other measurements. No corrections were made for alterations in the buffer capacity of the external solution in the course of the experiments.

Transmembrane Electrical Potential Difference

For these measurements a piece of skin was mounted in Ussing and Zerahn chambers [39] and the electrical potential difference $(V_i - V_e)$ (i and e refer to the internal and external compartments) was measured by a digital electrometer with saturated KCl calomel half-cells and saturated KCl agar-bridges. In a few experiments $(V_i - V_e)$ was measured directly in the flux chambers at the beginning and at the end of the experiments, and the mean of these two values was taken as the average $(V_i - V_e)$ value. In these cases, the agar-bridges had very small point diameters to prevent contamination of the solutions with K or Cl, and were kept in contact with the solutions only a few seconds, this being sufficient for a stable reading. Voltages were measured by a digital electrometer. When necessary, corrections were made for the asymmetry in liquid junction potential between agar-bridges and solutions, using the Henderson equation [30].

Solutions

The external solutions were always solutions of NaCl or Na_2SO_4 in the concentration range of 0.1 to 5.0 mM. Exact compositions are given in the Results section. The internal solutions were: NaCl-Ringer's solution (NaCl 115.0 mM, $KHCO_3$ 2.5 mM, $CaCl_2$ 1.0 mM, with a pH of 8.2 after aeration) or Na_2SO_4-Ringer's solution (Na_2SO_4 57.5 mM, $KHCO_3$ 2.5 mM, $CaSO_4$ 1.0 mM, with a pH of 8.2 after aeration).

Toads were captured in the vicinity of Sao Paulo, Brazil, and kept with free access to running water without food and in healthy conditions for no longer than a month. Straight lines were fitted by the least-squares method. Results are presented as mean ± 1 standard error.

Results

1. Initial Survey

Experiments were carried out in different experimental conditions in order to test for *a*) the adequacy of the method, *b*) the reproducibility of the results and *c*) to determine the ionic concentration range in the external solution compatible with flux measurements by chemical determination. The adequate concentration range was found to be within 0.1 to 5.0 mM. Below 0.1 mM the errors involved in the chemical analysis were critical; above 5.0 mM variations in ionic concentration were too small to be correctly evaluated.

2. Sodium Uptake, Potassium Secretion, Volume Flow and Skin Electrical Potential Difference as a Function of the External Na Concentration. Effect of Antidiuretic Hormone

These experiments were performed in four different groups of skins to study the dependence of J_{Na}, J_K and J_v on the external sodium, without constraints on the skin electrical potential difference. Paired half-skins were

Table 2. Effect of antidiuretic hormone (ADH) on the rates of Na transport (J_{Na}), K transport (J_K) and volume flow (J_v) simultaneously measured in the same piece of skin[a]

Group	$(Na)_e$ (mM)	J_{Na} (nmole $min^{-1} cm^{-2}$)		J_K		J_v ($\mu l\, min^{-1} cm^{-2}$)		(Na)* (mM)	
		Cont	ADH	Cont	ADH	Cont	ADH	Cont	ADH
A	0.2	1.60	2.03	1.00	1.40	0.40	1.06	4.00	1.92
	($n=7$)	± 0.26	± 0.33	± 0.30	± 0.30	± 0.04	± 0.13		
		$p<0.025$		$p<0.01$		$p<0.01$			
B	1	1.90							
	($n=9$)	± 0.22							
C	2	2.43	4.16	0.93	1.96	0.33	1.41	7.36	2.95
	($n=16$)	± 0.26	± 0.53	± 0.16	± 0.40	± 0.03	± 0.15		
		$p<0.001$		$p<0.003$		$p<0.001$			
D	5	4.70	6.70	2.16	2.93	0.45	1.22	10.44	.5.49
	($n=8$)	± 0.63	± 0.97	± 0.50	± 0.70	± 0.06	± 0.21		
		$p<0.01$		$p<0.02$		$p<0.005$			

[a] Experiments carried out in paired half-skins (control and ADH) at different external Na concentration. (Na)* is the Na concentration in the equivalent solution absorbed by the skin, calculated as the ratio J_{Na}/J_v. $(Na)_e$ is the external Na concentration.

used, one half as a control and the other half as a test for antidiuretic hormone (ADH) (Pitressin, Parke Davis) added to the internal solution to give a final concentration of 0.4 Units/ml. The external bathing solution was NaCl (group A: 0.2 mM; group B: 1.0 mM; group C: 2.0 mM; and group D: 5.0 mM) with pH 8 (Trizma base 3 mM). The internal bathing solution was NaCl-Ringer's solution. Table 2 summarizes the results. As can be seen, ADH significantly stimulates J_{Na}, J_K and J_v in groups A, C and D (group B was not tested for ADH), and lowered the Na concentration in the equivalent solution absorbed by the skin, (Na)*, calculated as J_{Na}/J_v. Table 2 also shows that J_{Na}, J_K (except for one control sub-group) and (Na)* increases as the external Na concentration is raised for control as well as for ADH-treated skins.

As the skin electrical potential difference is known to be a function of the external Na concentration [25] an independent group of experiments was carried out to measure $(V_i - V_e)$ as a function of $(Na)_e$, with solutions of similar composition to those used for the flux measurements. The skins were sequentially bathed by solutions of increasing NaCl concentration in the outer bathing medium and $(V_i - V_e)$ were:

$(Na)_e$ (mM)	$(V_i - V_e)$ (mV)	
0.1	-6.9 ± 2.9	
1	50.4 ± 3.2	
2	67.4 ± 3.2	$(n = 11)$.
5	86.9 ± 3.6	

In the range of 0.1 to 5.0 mM NaCl, $(V_i - V_e)$ was found to be a linear function of $\log (Na)_e$ according to:

$$(V_i - V_e) = 55.7 \log (Na)_e - 49.4. \tag{1}$$

Na electrochemical potential difference mean values $(\mu_i^{Na} - \mu_e^{Na})$ were estimated as a function of the Na concentration on each side of the skin and of the measured spontaneous skin electrical potential difference according to:

$$(\mu_i^{Na} - \mu_e^{Na}) = RT \ln [(Na)_i/(Na)_e] - F(V_i - V_e) \tag{2}$$

where R is the gas constant, T the absolute temperature, and F the Faraday constant. Although the term $F(V_i - V_e)$ increases with increase in $(Na)_e$, the rate of reduction of $RT \ln [(Na)_i/(Na)_e]$ with $(Na)_e$ is faster and, therefore, $(\mu_i^{Na} - \mu_e^{Na})$ falls with increase in $(Na)_e$. Fig. 2 shows a plot of mean J_{Na} values

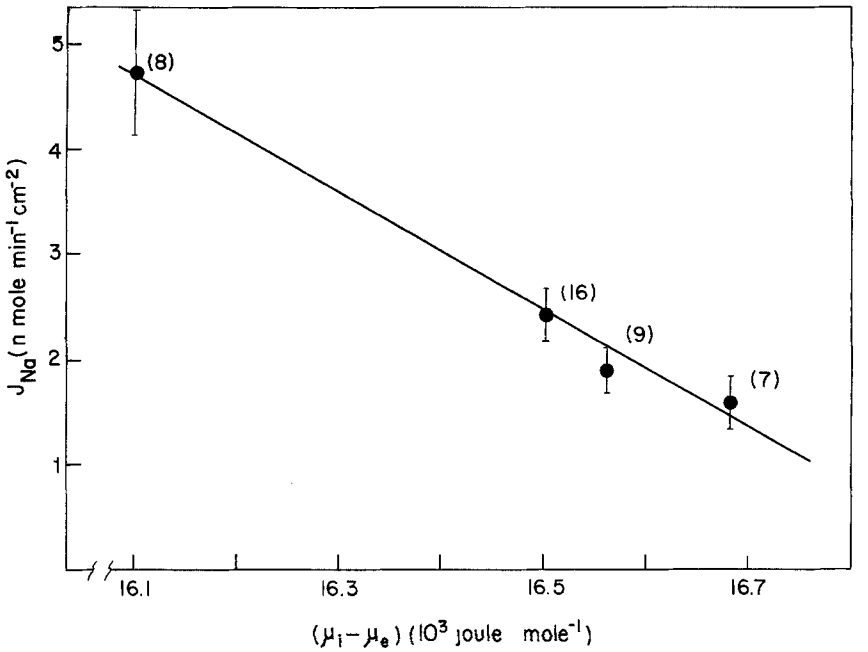

Fig. 2. Net Na transport (J_{Na}) as a function of the Na electrochemical potential difference across the skin, ($\mu_i - \mu_e$), calculated according to Eq. (2). Each point corresponds to a different group of skins and the numbers in the Figure indicate number of experiments. Internal solution was NaCl-Ringer's solution. External solution of NaCl (0.2 to 5.0 mM). Skins were kept in the open-circuited state

against the estimated Na electrochemical potential differences obtained according to Eq. (2).

3. Sodium Uptake, Potassium and Hydrogen Secretions, Skin Electrical Potential Difference and Substitution of Chloride by Sulfate in the External Bathing Solution

These experiments were carried out to evaluate the role of Cl ions, which are taken up actively by the skin (*see* Discussion) on net Na, K and H transports. Paired experiments with half-skins of the same toad were carried out. Na_2SO_4-Ringer's solution bathed the internal surface of the skin in the two groups in order to guarantee against Cl leakage to the external compartment. The external bathing solution was NaCl for one group and Na_2SO_4 for the other; both solutions on the external side had 2 mM Na concentration and pH 8 (Trizma base 2 mM). The fluxes for each experimental group are given below in units of nmole min^{-1} cm^{-2}:

Fluxes	External solution (mM)			
	NaCl	Na_2SO_4		
J_{Na}	3.63 ± 0.40	3.40 ± 0.40	$p > 0.20$	
J_K	2.40 ± 0.43	2.40 ± 0.40	$p > 0.50$	$(n = 10)$.
J_H	2.36 ± 0.16	2.36 ± 0.16	$p > 0.50$	

Paired t-tests showed that the differences between the two groups were not statistically significant.

An independent group of experiments was carried out to study the skin electrical potential difference in similar conditions to those of the previous experiments. Na_2SO_4-Ringer's solution bathed the internal surface of the skin. The external surface was bathed first by Na_2SO_4, then by NaCl and finally by Na_2SO_4. All these solutions contained 2 mM Na concentration and pH 8 (Trizma base 2 mM). Steady-state potentials were attained in a few seconds after substitution, as follows: Na_2SO_4: $(V_i - V_e) = 63.5 \pm 4.5$ mV; NaCl: $(V_i - V_e) = 62.6 \pm 4.2$ mV; and Na_2SO_4: $(V_i - V_e) = 63.8 \pm 5.2$ mV, $n = 11$. These values are not statistically different ($p > 0.20$, paired t-test with correction for the number of comparisons).

4. Sodium and Chloride Uptakes, Volume Flow and Skin Electrical Potential Difference and the Effect of Substitution of Sodium by Potassium in the External Bathing Solution

The aim of these experiments was to study the dependence of net chloride uptake on the presence of Na in the external bathing solution. Paired experiments with half-skins of the same toad were used. NaCl-Ringer's solution bathed the internal surface of the skin. The external solution for one group was 1 mM NaCl and for the other, 1 mM KCl. Both solutions had pH 8 (Trizma base 5 mM). For the NaCl group, $J_{Na} = 1.93 \pm 0.22$ nmole min^{-1} cm^{-2}, $J_{Cl} = 0.64 \pm 0.10$ nmole min^{-1} cm^{-2} and the volume flow, $J_v = 0.27 \pm 0.03$ µl min^{-1} cm^{-2}. For the KCl group, $J_{Cl} = -0.03 \pm 0.16$ nmole min^{-1} cm^{-2}, and $J_v = 0.25 \pm 0.02$ µl min^{-1} cm^{-2}, $n = 9$. In the KCl group, the Na concentration in the external bathing solution at the end of the experiments was found to be below the level of detection by flame photometry. Substitution of Na by K in the external medium lowered significantly J_{Cl} ($p < 0.001$, paired t-test). On the other hand, the volume flow was not statistically altered ($p > 0.20$, paired t-test).

An independent group of experiments studied the skin electrical potential difference in conditions similar to those of the previous experi-

ments. NaCl-Ringer's solution bathed the internal surface of the skin. The external surface was sequentially bathed, first by 1 mM NaCl and then by 1 mM KCl, both solutions with pH 8 (Trizma base 5 mM). Steady-state electrical potential differences, attained within a few seconds after the substitution, were as follows: NaCl: $(V_i - V_e) = 53.8 \pm 3.0$ mV; KCl: $(V_i - V_e) = -41.1 \pm 4.0$ mV. These results show that the substitution of Na by K in the external solution has a drastic effect on the skin electrical potential difference ($p < 0.001$, paired t-test) ($n = 9$), with reversion of skin polarity.

5. Sodium and Chloride Uptakes, Volume Flow and Skin Electrical Potential Difference in the Absence of an Osmotic Asymmetry Across the Skin

These experiments were carried out to measure J_{Na}, J_{Cl} and J_v in the absence of an osmotic gradient across the skin. The osmolarity of the external bathing solution was adjusted with sucrose to that of the internal solution. Skins were bathed on the internal surface by NaCl-Ringer's solution and on the external surface by 1 mM NaCl, pH 8 (Trizma base 0.1 mM). In these conditions the fluxes were: $J_{Na} = 2.29 \pm 0.55$ nmole min^{-1} cm^{-2}; $J_{Cl} = 0.69 \pm 0.24$ nmole min^{-1} cm^{-2} and $J_v = 0.01 \pm 0.002$ µl min^{-1} cm^{-2} ($n = 6$). The mean electrical potential difference measured directly in the flux chamber was approximately 56 mV.

6. Sodium Uptake, Hydrogen and Potassium Secretions and the Role of Sodium in the External Bathing Solution

These experiments were carried out to evaluate the role of Na ions in the external bathing solution on the simultaneously measured Na, K and H transports. Paired experiments with half-skins of the same toad were carried out. NaCl-Ringer's solution bathed the internal surface of the skin. The external solution was Na_2SO_4 with 3 mM Na and pH 8 (Trizma base 1 mM) for one group and only Trizma base 1 mM (pH 8) for the other group. For the group with external Na: $J_{Na} = 2.84 \pm 0.16$ nmole min^{-1} cm^{-2}, $J_K = 0.97 \pm 0.13$ nmole min^{-1} cm^{-2} and $J_H = 1.15 \pm 0.07$ nmole min^{-1} cm^{-2} and the mean pH value of the external solution at the end of the experiments, after equilibration with air, was 6.17 ± 0.18. For the group without Na: $J_{Na} = -0.05 \pm 0.05$ nmole min^{-1} cm^{-2}, $J_K = 0.03 \pm 0.03$ nmole min^{-1} cm^{-2}, and $J_H = 0.43 \pm 0.12$ nmole min^{-1} cm^{-2} and the final pH was 7.27 ± 0.06 ($n = 7$).

A second series of paired experiments was carried out with another batch of toads to test the effect of Na absence in the external bathing solution on net potassium secretion. Paired half-skins were bathed internally by NaCl-Ringer's solution. The external solution was also (with respect to the first series) 3 mM Na_2SO_4 plus 1 mM Trizma base for one group, and only 1 mM Trizma base for the other group. Both external solutions had pH 8. For the group with Na in the external solution, $J_{Na} = 4.70 \pm 0.32$ nmole min^{-1} cm^{-2} and $J_K = 1.62 \pm 0.20$ nmole min^{-1} cm^{-2}. For the group without external Na, $J_{Na} = -0.08 \pm 0.08$ nmole min^{-1} cm^{-2}, and $J_K = 0.05 \pm 0.05$ nmole min^{-1} cm^{-2} ($n=6$).

These results show that Na removal from the external bathing solution significantly lowers J_{Na} and J_K to near zero values and significantly lowers J_H ($p<0.01$, paired t-test). However, J_H is still different from zero in the absence of Na in the external bathing solution.

With a third batch of toads experiments were carried out in which half-skins of the same toad were used to measure J_{Na} and J_K in the same piece of skin, in order to eliminate skin variability. The external solution was Na_2SO_4 with a Na concentration of 1 mM for one group and 5 mM for the paired skin group. Both solutions had pH 8 (Trizma base 5 mM). Na_2SO_4-Ringer's solution bathed the internal surface of the skin. For the 1 mM group, $J_{Na} = 1.45 \pm 0.25$ nmole min^{-1} cm^{-2}, and $J_K = 0.33 \pm 0.12$ nmole min^{-1} cm^{-2}. For the 5 mM Na group, $J_{Na} = 2.30 \pm 0.23$ nmole min^{-1} cm^{-2}, and $J_K = 0.63 \pm 0.18$ nmole min^{-1} cm^{-2}. J_{Na} and J_K were significantly higher in the 5 mM Na group; for J_{Na}, $p<0.001$ and for J_K, $p<0.01$ (paired t-tests, $n=10$).

7. General Aspect of the Relationship Between Sodium Uptake and Potassium Secretion

Fig. 3 presents a general picture of the existence of a net potassium secretion in different experimental conditions (*see* Figure legend) and different groups of skins. It shows that the rate of net potassium secretion is always lower than the rate of net sodium uptake, both fluxes being measured in the same skin. This Figure also shows that there is a significant positive correlation between J_K and J_{Na} for randomly selected skins.

8. Potassium Secretion and the External Buffer Concentration

In the range of experimental conditions surveyed in this report, the skin is able to acidify the external bathing solution. The observed pH drop as

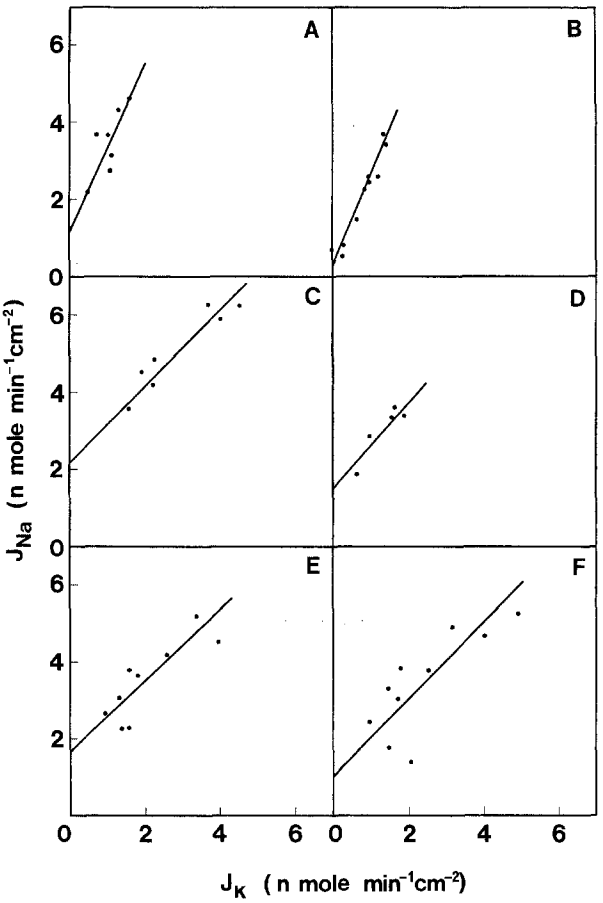

Fig. 3. Relationship between net Na uptake (J_{Na}) and net K secretion (J_K) in randomly selected skins for different experimental conditions. For each group the composition of the external and the internal solutions and significance of the linear correlation coefficients are given. *A*: Na_2SO_4 1.5 mM/NaCl-Ringer's, $p<0.05$; *B*: Na_2SO_4 1.5 mM/Na_2SO_4-Ringer's, $p<0.01$; *C*: Na_2SO_4 2.5 mM/NaCl-Ringer's, $p<0.01$; *D*: Na_2SO_4 2.5 mM/Na_2SO_4-Ringer's, $p<0.05$; *E*: NaCl 2.0 mM/Na_2SO_4-Ringer's, $p<0.01$; *F*: Na_2SO_4 1.0 mM/Na_2SO_4-Ringer's, $p<0.01$

well as J_H was found to depend on the rate of net sodium uptake. The rate of H secretion should, therefore, contribute with other ions to the maintenance of electroneutrality in the external bathing solution, hence J_{Na}, J_{Cl}, J_K and J_H should be mutually related. In the absence of Cl in the external bathing solution and for a reasonably constant J_{Na} value, J_K and J_H should, therefore, be inversely related. To test for this relationship, the following experiments were carried out in paired half-skins. Na_2SO_4-Ringer's solution bathed the internal surface of the skin. The external solution was

Na_2SO_4 with 3 mM Na concentration for both groups, one group with 5 mM Trizma base concentration and the other with no buffer. For the groups with and without external buffer J_{Na} was, respectively, 2.97 ± 0.53 and 2.58 ± 0.48 nmole min^{-1} cm^{-2}. These values were not statistically different ($p > 0.10$, paired t-test) ($n = 6$). The corresponding J_K values were for the same groups, 1.08 ± 0.25 and 1.75 ± 0.40 nmole min^{-1} cm^{-2}, respectively. These values are significantly different ($p < 0.025$, paired t-test). The pH of the external solution was not significantly altered in the course of the experiments using buffer; on the other hand, it dropped from 8 to 6.75 ± 0.09 pH units in the group without buffer in the external solution.

Discussion

The results of the present work show that the isolated skin of the toad *Bufo marinus ictericus* in the open-circuited state and in the presence of low Na and hypoosmotic external solution absorbs Na and Cl from the external medium and secretes simultaneously H and K to this medium. These observations are not new; however, the relationships between the fluxes of these ions present interesting aspects under our experimental conditions. In all cases, J_{Na} was found to be higher than either J_{Cl}, J_K, or J_H; this may be related to our particular working conditions or to animal preadaptation [15] in running tap water.

The control and ADH experiments (Results section 2, Table 2) show an increase of J_{Na} with an increase in $(Na)_e$. The relationship between J_{Na} and $(Na)_e$ in the open-circuited state is certainly more complex than it is in the short-circuited state because not only the Na gradient across the skin is altered but the spontaneous electrical potential difference also changes with $(Na)_e$. In the control condition, $(V_i - V_e)$ increased logarithmically with increase in $(Na)_e$, with a slope of 55.7 mV. For a given Na concentration in the external bathing solution a negative correlation has been observed between skin potential and Na uptake [5, 20, 21, 31, 39]. This dependence has been interpreted according to different kinetic formalisms [29, 31]. According to the thermodynamics of irreversible processes [12, 23], the force directly related to J_{Na} in the open-circuited state is the Na electrochemical potential difference. Previous experiments of Vieira *et al.* [41] and Danisi and Lacaz Vieira [7] have shown the adequacy of the nonequilibrium thermodynamic formalism to describe Na transport in the amphibian skin, and also its coupling to the metabolic driving reaction. According to Essig and Caplan [12] the phenomenological coefficient L_{Na} can be evaluated as: $L_{Na} = (\Delta J_{Na}/\Delta X_{Na})_A$ where X_{Na} is the negative of the

Na electrochemical potential difference across the skin and A is the affinity of the metabolic driving reaction. For the results of Section 2, L_{Na} average values were estimated from the slope of J_{Na} as a function of X_{Na}. For the control experiments (Table 2 and Fig. 2), a value equal to 5.5×10^{-12} mole^2 joule^{-1} min^{-1} cm^{-2} is obtained. This value is within 6.6×10^{-12} and 4.0×10^{-12} mole^2 joule^{-1} min^{-1} cm^{-2} obtained in our laboratory by Danisi and Lacaz Vieira [7] for the isolated skin of toads of the same species in short-term and long-term experiments, respectively, both being performed in the short-circuited state with variations of $(Na)_e$ in the range of 5 to 115 mM. The close proximity between L_{Na} values estimated in the present work and those calculated previously [7] suggests an extended range of validity for the phenomenological coefficient L_{Na}. It can be argued that in the present case L_{Na} has been estimated less rigorously than it was previously since the formalism of Essig and Caplan [12] applies rigorously only to the transport of a single ion, and in the present circumstances other ions are simultaneously transported by the epithelium and an apparent coupling exists between J_{Na} and the fluxes of these ions. It seems improbable, however, that the close proximity between L_{Na} values obtained in different experimental conditions was due only to casual factors. If the relationship between J_{Na} and the other fluxes were mediated through an electrical coupling, the spontaneous electrical potential difference observed would reflect this coupling; and the L_{Na} calculated on the basis of transepithelial Na concentration and spontaneous electrical potential difference would still refer to the Na transport system itself.

Table 2 documents the existence of a net potassium secretion to the external bathing solution in the control and in ADH-treated skins; in these two groups there is a tendency for J_K to increase with increase in $(Na)_e$. This dependence is better characterized in the paired experiments (Results section 6), which show that the higher $(Na)_e$ group presents a significantly higher J_{Na} and an also higher J_K. The absence of Na in the external bathing solution reduces not only J_{Na} but also J_K to values not significantly different from zero. These experiments indicate a dependence of J_K on J_{Na} values as can also be inferred from the results of Section 7, where a positive correlation was observed between J_{Na} and J_K over different experimental conditions in randomly selected skins.

The K and Na fluxes obtained in the short-circuited condition with skins bathed bilaterally by Ringer's solution in the same species [40] show that there is a great difference in J_K and J_{Na} as compared to those of Table 2. In the former condition [40], J_K was of the order of 0.06 nmole min^{-1} cm^{-2} and J_{Na} of the order of 30 nmole min^{-1} cm^{-2}. Comparison of these values

with those of Table 2 shows that in spite of the higher Na fluxes observed with high external Na concentration and short-circuit condition, the K fluxes were of lower magnitude than those presented in Table 2 indicating that the apparent dependence of J_K on J_{Na} may be primarily a consequence of the J_{Na} dependent potential step at the outer border than of the Na fluxes per se.

In short-circuited skins a negative potential step has been described as one penetrates the epithelium [6, 42]. On the other hand, in open-circuited skins a positive potential step at the external membrane of the first cell layer has been described [6, 10, 42]. Therefore, the outflux of K is expected to be favored by the open-circuit condition as compared to the short-circuit condition.

J_H also shows a dependence on $(Na)_e$ since the withdrawal of Na from the external bathing solution (Results, section 6) significantly decreases J_H. In this condition, J_K is reduced to a value not significantly different from zero, while J_H is reduced but a basal secretion persists. These results are to some extent in agreement with those of Emilio *et al.* [8] for the isolated skin of the toad *Bufo bufo* which in the short-circuited state show that at least a fraction of the total H efflux is linked to Na influx. On the other hand, the same authors [9] observed that this exchange mechanism does not seem to be operative in the isolated skin of the frog *Rana ridibunda* under similar conditions, indicating species' different behavior.

The results of Section 2 show that with $(NaCl)_e = 1$ mM, J_{Cl} occurs against an electrochemical potential difference of the order of 6700 joule mole^{-1}, thus fulfilling the requirement of an active transport process [35]. Experiments with $J_v = 0$ obtained by eliminating the osmotic gradient across the skin (Results, section 5) show that J_{Cl} is of a magnitude comparable to J_{Cl} in the presence of an osmotic water flow. This suggests that the role of a solvent drag mechanism on J_{Cl} should be minimal under these conditions.

Substitution of Na by K in the external bathing solution reduces J_{Cl} to a value not statistically different from zero (Results, section 4). This substitution also drastically alters the spontaneous skin electrical potential difference $(V_i - V_e)$, which goes from 53.8 ± 3.0 mV with 1 mM NaCl in the external solution to -41.1 ± 4.0 mV with 1 mM KCl. The reversal of skin polarity observed when Na is replaced by K in the external solution increases the Cl electrochemical potential difference against Cl net flux from 6700 to 15,700 joule mole^{-1}. It is conceivable that such a large electrochemical step is of sufficient magnitude to completely block J_{Cl}. These results show, however, a sharp difference between J_{Na} and J_{Cl}

regarding the power of the pumping mechanism. In our experiments Na uptake occurs normally against an electrochemical potential difference of the order of 16,000 joule mole^{-1} while a comparable thermodynamic force apparently blocks J_{Cl}.

In the range of 0.1 to 5.0 mM $(NaCl)_e$, the slope of $(V_i - V_e)$ as a function of $\log(Na)_e$ is very close to the theoretical value calculated by the Nernst equation (Results, section 2), suggesting that in these experimental conditions the importance of chloride shunting must be minimal. On the other hand, substitution of chloride by sulfate in the external bathing solution has neither significant effect on J_{Na}, J_K or J_H nor on the skin electrical potential difference. This electrical behavior is quite distinct from that observed when solutions of high salt concentration bathe the external surface of the skin [25]. In that condition, substitution of Cl by SO_4 has a marked effect increasing the skin electrical potential difference and this is interpreted as being due to a lower sulfate permeability in relation to chloride. Possibly, in the case of the present work, the shunting effect of Cl ions is very small due to its lower concentration and, therefore, its substitution by a less permeant anion would induce an insignificant effect on the electrical potential difference.

The existence of an electrical coupling between the Na, K, H and Cl fluxes is suggested by the observations that only those procedures that result in alterations of $(V_i - V_e)$ are followed by changes in the rates of ion transport. Thus, substitution of Cl by SO_4, which does not significantly alter $(V_i - V_e)$, fails to alter significantly J_{Na}, J_K, or J_H (Results, section 3). On the other hand, changes in $(V_i - V_e)$ are always followed by changes in the transport rates of Cl, K and H.

The observation that J_{Na}, J_K and J_H were not altered by the substitution of Cl by SO_4 is compatible with a possible exchange of Cl by an intraepithelial anion such as proposed for the in vivo skin of anurans by Garcia-Romeu [15] and Garcia-Romeu *et al.* [17].

Antidiuretic hormone added to the inner bathing solution has a significant effect increasing J_{Na}, J_K and J_v in all experimental conditions tested in Table 2 (Results, section 2). In all cases, the Na concentration in the equivalent solution absorbed by the skin, $(Na)^* = J_{Na}/J_v$, is higher than the Na concentration in the external medium. Therefore, in the present experiments the effect of ADH was to lower (Na)*. This is a consequence of a proportionally higher effect of the hormone on J_v than on J_{Na}. This fact is physiologically compatible with the role of ADH being released in response to an increase in plasma osmolarity.

The effects of ADH reported in Table 2 can tentatively be interpreted on

the basis of the nature of Na penetration at the outer border of the epithelium. If we assume a passive Na entry at the outer border there must be a net thermodynamic force acting on Na ions, directed from the external solution to the epithelium and the increase in J_{Na} by ADH treatment could be interpreted on the basis of an ADH-induced increase in Na permeability of the outer barrier. However, the possibility of an active Na entry at the outer border cannot be discarded.

The increased K secretion observed in ADH-treated skins might have been the result of an increase by ADH of the K permeability of the external barrier of the epithelium, since the internal barrier is already greatly permeable to K and is probably not a limiting factor for the K movement across the epithelium. Under these experimental conditions, the K ion is certainly subjected to an electrochemical gradient favoring its outward net flux since the epithelial cell concentration is of the order of 130 mM [18] and the K concentration in the external medium is always lower than 1 mM and, also, the electrical potential difference across the outer barrier is certainly not sufficient to equilibrate the K ion across this barrier. However, it is also possible that the increase of J_K with ADH could be secondary to the ADH-mediated increase in J_{Na}, since a relationship between these fluxes was observed in different experimental conditions (Results, section 2).

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