J. Membrane Biol. 39, 369–385 (1978)

Transients in Toad Skin: Short Circuit Current and Ionic Fluxes Related to Inner Sodium Substitution by Monovalent Cations

W.A. Varanda and F. Lacaz Vieira

Department of Physiology and Pharmacology, Institute of Biomedical Science, University of São Paulo, São Paulo, Brazil

Received 31 May 1977; revised 25 August 1977; revised again 18 October 1977

Summary. When the Na electrochemical potential difference across the skin ($\Delta\mu_{Na}$) is altered by perturbing the transmembrane electrical potential difference or the external Na concentration, effects on transport and associated oxygen consumption can be described by the formalism of linear nonequilibrium thermodynamics (Vieira, Caplan & Essig, 1972, *J. Gen. Physiol.* **59**:77; Danisi & Lacaz-Vieira, 1974, *J. Gen. Physiol.* **64**:372; Procópio and Lacaz-Vieira, 1977, *J. Membrane Biol.* **35**:219). We now show that with modifications of $\Delta\mu_{Na}$ by substitution of Li or choline for Na in the inner bathing solution, this formalism is no longer applicable. Inner Na by K substitution ($(Na \times K)_i$) causes profound alterations in short-circuit current (SCC), J_{Na}^{in}, K efflux (J_K^{eff}) and PD. SCC drops transiently after $(Na \times K)_i$ in Cl and in SO_4 media, increasing subsequently. In Cl medium, following the initial transient, there is a late decline in SCC toward a steady state. The rate of SCC decline in Cl medium is more pronounced than that observed in SO_4 medium. $(Na \times K)_i$ causes a transient increase in J_{Na}^{in} with a peak synchronous to the minimum in SCC, both in Cl and in SO_4 media. This was interpreted as due to depolarization of the inner membrane. In SO_4 medium, following the peak observed after $(Na \times K)_i$, J_{Na}^{in} drops, to increase again toward a steady state in which SCC and J_{Na}^{in} are not statistically different, resembling the control condition before $(Na \times K)_i$. In Cl medium, however, the J_{Na}^{in} steady state is approximately 100% higher than SCC. This difference is due to an important K efflux (J_K^{eff}), which builds up progressively after the substitution. The apparent K permeability $[J_K^{eff}/(K_i)]$ is of comparable magnitude in Cl and in SO_4 media before $(Na \times K)_i$ and also in SO_4 medium after $(Na \times K)_i$. However, in Cl medium, after $(Na \times K)_i$, the apparent K permeability increases one order of magnitude as compared to the control condition before the ionic substitution. In Cl medium, the high levels of J_{Na}^{in} and of J_K^{eff} observed in the steady state after $(Na \times K)_i$ were interpreted as being a consequence of cell swelling. SCC and PD follow very different temporal patterns after $(Na \times K)_i$ which are characterized by transients in SCC and a simple fall in PD. Reasons for these differences are discussed.

The isolated skin of several amphibian species in different experimental conditions actively transports Na against an electrochemical potential difference. Different aspects of this transport mechanism have been studied under the framework of irreversible thermodynamics (Essig &

0022-2631/78/0039-0369 $3.40

Caplan, 1968). Thus, a linear dependence was observed between the rate of active Na transport and the Na electrochemical potential difference across the skin ($\Delta\mu_{Na}$) in short-circuited (Danisi & Lacaz-Vieira, 1974) and in open-circuited skins (Procópio & Lacaz-Vieira, 1977) of *Bufo marinus ictericus*. Also, a linear dependence was observed between the rate of oxygen consumption associated with Na transport and $\Delta\mu_{Na}$, when varying skin PD by voltage clamping in *Rana pipiens* (Vieira *et al.*, 1972) or varying the external Na concentration in short-circuited skins of *Bufo marinus ictericus* (Danisi & Lacaz-Vieira, 1974). Phenomenological coefficients which characterize the transport system and its coupling to a metabolic driving reaction were evaluated for the skin of *Bufo marinus ictericus* (Danisi & Lacaz-Vieira, 1974).

The primary aim of the present work was to test further the adequacy of the irreversible thermodynamic formalism (Essig & Caplan, 1968) to fully describe the Na transport system of the skin of *Bufo marinus ictericus*. If the formalism of linear nonequilibrium thermodynamics is applicable, an increase in the Na electrochemical potential difference (independently of its electrical or chemical terms) would increase net Na transport rate if Na ions were pumped from higher to lower electrochemical potential level. Therefore, under short-circuited condition a reduction of inner Na concentration would be expected to increase the rate of net Na transport. Effects of changes in the inner Na concentration of epithelial membranes are still controversial. MacRobbie and Ussing (1961), Ussing (1965) and Rabito, Rodriguez-Boulan & Cereijido (1973) observed that in frog skin reduction of inner Na concentration at constant osmolarity is accompanied by decline in SCC. On the other hand, Finn and Reuss (1975) have shown that the increase in SCC observed by Lipton (1972) in the toad bladder was due to osmotic cell swelling rather than lower inner Na concentration. The present results show that in any circumstance increase in the rate of net Na transport could be ascribed as being directly related to Na removal from the inner solution. On the other hand, inner Na substitution was followed by different skin electrophysiological behavior according to the ion used to replace Na. The effects of Na by Li, choline, or K substitutions were studied in relation to short-circuit current and Na and K movements during the initial phase following the ionic substitution and until an apparent stationary state was attained. The present results show that when K is used as an inner Na substitute, long term transients develop and a final apparent stationary state is reached which is dependent on the anion present in major proportions if chloride or sulfate.

Materials and Methods

The studies were carried out in modified Ussing-Zerahn chambers (Ussing & Zerahn, 1951), permitting measurement of transmembrane electrical potential difference (PD), short-circuit current (SCC) and unidirectional fluxes. Abdominal skins of the toad *Bufo marinus ictericus* were used by exposing an area of 7.1 cm^2. Fig. 1 is a chamber diagram. The hemichamber to which the isotope was added was connected to an air-lift pump driven by vacuum applied to its top. The volume of solution in his compartment was 6 ml. The other hemichamber (cold compartment) was open to the air and its solution stirred by an air turbine. The hydrostatic pressure across the skin was balanced to zero through the compensation of the liquid level difference between the compartments by reducing the pressure in the air-lift pump. This was accomplished by means of a regulator connected to the air-lift pump as shown in Fig. 1. With this setup the volume of solution in the cold compartment was reduced to a minimum. Every two minutes, all the cold compartment solution was drained into counting vials for ^{22}Na or ^{42}K assay. After being drained, the cold compartment was rinsed with approximately 50 ml of solution and refilled. At the end of each experiment, a sample of the hot solution (100 µl) was collected and diluted in order to determine its isotope specific activity. Experiments performed in the short-circuited condition had this condition interrupted for 5 to 10 sec (open-circuited state) every 2 min during drainage of the cold compartment. Unidirectional ionic fluxes were calculated as $J = \text{cpm}/(t \cdot a \cdot sa)$, where cpm is the counting rate of the whole volume of solution collected from the cold compartment, t is the duration of each period (110 to 115 sec), a is the area of membrane, and sa is the specific activity in the hot solution. Fluxes are expressed in their electrical equivalent, µA cm^{-2}. Approximately 50 µCi of ^{22}Na (New England Nuclear) was added to the external compartment to obtain Na influx (J_{Na}^{in}). Equilibration periods of at least 1 hr were observed before sampling started. ^{22}Na was counted in an automatic gamma-counter (Nuclear Chicago, mod. 4230). K efflux (J_K^{eff}) was measured by adding approximately 100 µCi of ^{42}K (Institute of Atomic

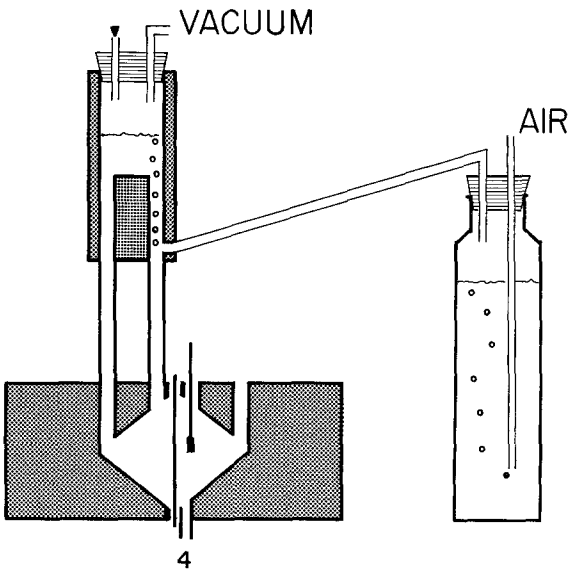

Fig. 1. Diagram of the experimental setup

Energy, São Paulo, Brazil) to the inner solution. Sampling of the cold side started immediately following addition of ^{42}K in order to obtain the kinetics of ^{42}K efflux. ^{42}K was counted in a liquid scintillation counter (Beckman, mod. LS 100) by the Cerenkov effect. Calibrations were carried out and linearity was observed between isotope concentration and counting rate in the range of concentrations used. Electrical measurements were carried out according to the method previously described (Danisi & Lacaz-Vieira, 1974). An electrometer (Keithley, mod. 615) was used to measure PD and a voltage clamp unit (Yale University Department of Physiology Electronic Laboratory) to set to zero the potential difference across the skin. PD and SCC were recorded in a two-channel recorder (Varian, mod. G-2500). One pair of 3-M KCl-agar bridges, connected to calomel half-cells, was used to record PD. A second pair, connected to Cu-$CuSO_4$ half-cells permitted passage of current. Membrane total electrical resistance was calculated as PD/SCC. The solutions used were (in mM): NaCl-Ringer's solution: NaCl, 115.0; $KHCO_3$, 2.5; and $CaCl_2$, 1.0: Na_2SO_4-Ringer's solution: Na_2SO_4, 57.5; $KHCO_3$, 2.5; and $CaSO_4$, 1.0. Solutions were gassed with air. Na by K, Li, or choline substitutions were carried out on equimolar bases, all Na being replaced by the other ions. Therefore, in the K substitution experiments, inner solution KCl was substituted for NaCl, and K_2SO_4 for Na_2SO_4. Results are presented as mean ±SEM.

Results

1. SCC, J_{Na}^{in}, *and the Effect of* Na *by Monovalent Cation Substitutions in the Inner Solution (*Cl^- *as the Major Anion)*

These experiments were performed to test the effects of inner Na removal on the rate of net transepithelial Na transport. Following a control period with short-circuited skins bathed by NaCl-Ringer's solution on both sides, Na in the inner solution was completely replaced by choline, Li, or K. Choline substitution (Fig. 2) does not significantly alter

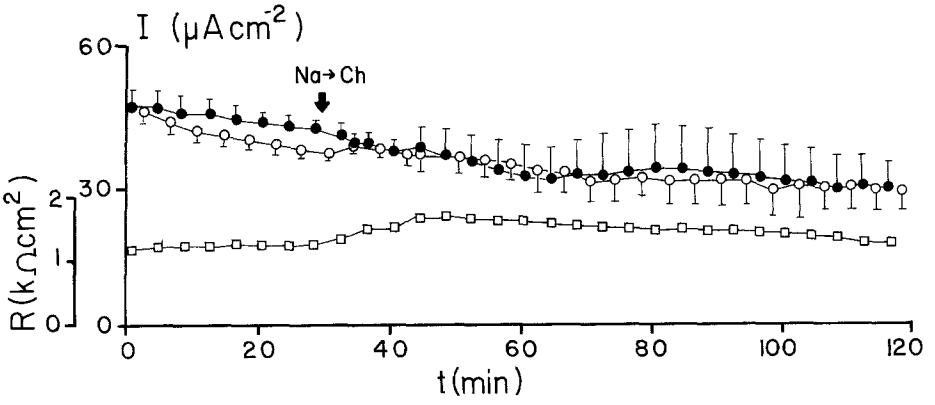

Fig. 2. Short-circuit current (●) and Na influx (○) in units of current density and skin electrical resistance (□) before and after inner Na by choline substitution. The ionic substitution is indicated by the arrow

the temporal patterns of SCC or of J_{Na}^{in}. In the control period, before choline substitution, mean values for the last 5 measurements were: SCC $=45.6 \pm 3.2$ μA cm^{-2} and $J_{Na}^{in} = 42.1 \pm 5.6$ μA cm^{-2}, which are not statistically different ($P > 0.6$, t test; $n = 4$). After choline substitution, SCC and J_{Na}^{in} displayed temporal evolutions similar to that of the control period. For the first 5 measurements after substitution, SCC $= 38.6 \pm 1.0$ μA cm^{-2} and $J_{Na}^{in} = 40.1 \pm 2.5$ μA cm^{-2} ($P > 0.6$, t test; $n = 4$). The skin

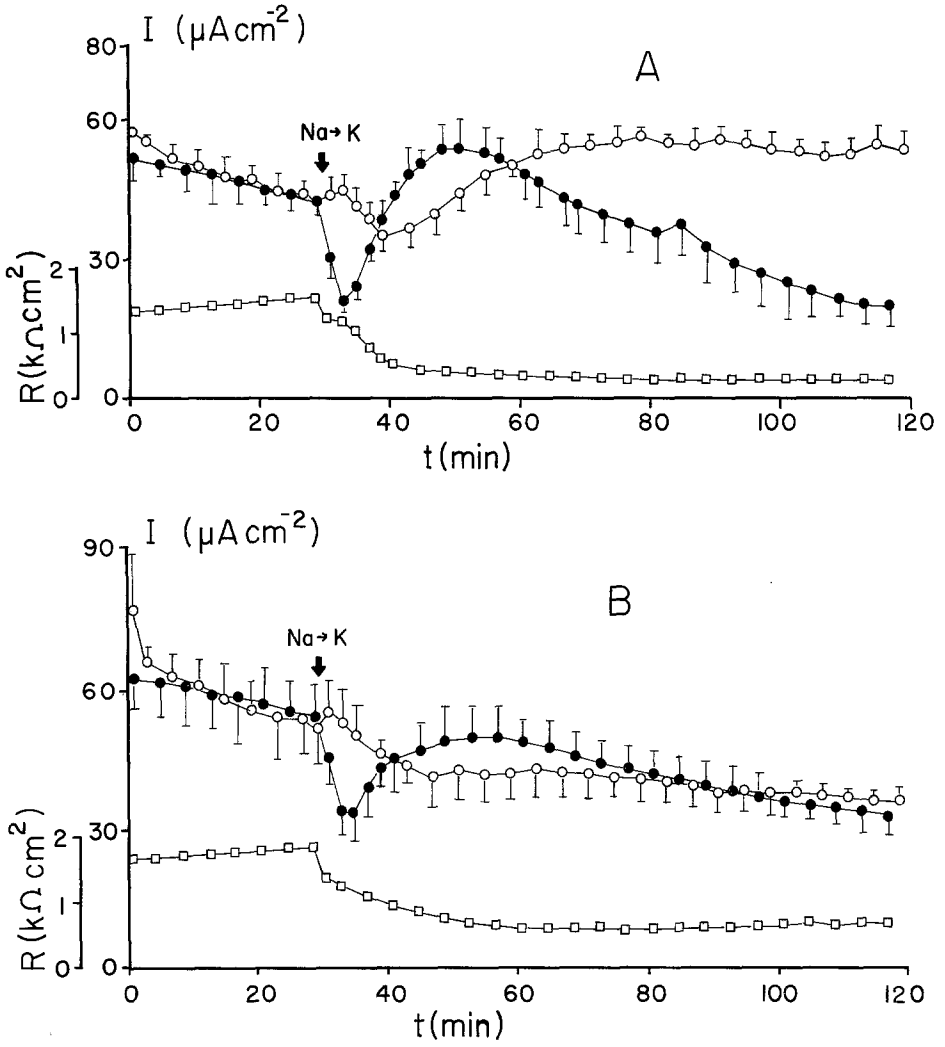

Fig. 3. Short-circuit current (●) and Na influx (○) in units of current density and skin electrical resistance (□) before and after inner Na by K substitution. The ionic substitution is indicated by the arrow. (*A*): Experiments performed with Cl as the major anion. (*B*): Experiments performed with SO_4 as the major anion

electrical resistance displayed a slight increase after Na by choline substitutuion, followed by a slow decline with time.

Na by Li substitution induced skin behavior very similar to that seen after choline substitution.

However, when Na was substituted by K in the inner solution, a contrasting behavior was observed as compared to choline or Li experiments. SCC and J_{Na}^{in} showed a very characteristic transient behavior before attaining an apparent steady state, normally 2 hr after the ionic substitution, as shown in Fig. 3*a*. In the control period, with NaCl-Ringer's solution on both sides of the skin, SCC and J_{Na}^{in} showed a behavior similar to that already described. For the last 5 measurements before substitution, SCC $=48.1 \pm 5.4$ μA cm^{-2} and $J_{Na}^{in} = 48.9 \pm 7.9$ μA cm^{-2} ($P > 0.9$, t test; $n=5$). Following inner Na by K substitution, SCC falls sharply to a minimum of 21.1 ± 1.9 μA cm^{-2} (approximately 50% of the pre-substitution values) 5 min after the substitution. Subsequently, SCC increases reaching values higher than those in the control period, to fall again, slowly, toward an apparent stationary state, 90 min after the ionic substitution. During the transient phase, SCC and J_{Na}^{in} display almost inverse relationships. Simultaneously to the initial decline in SCC, J_{Na}^{in} shows a peak of 44.6 ± 2.3 μA cm^{-2}, which is significantly higher than the mean of the last pre-substitution values (41.9 ± 3.2 μA cm^{-2}) ($P < 0.05$, paired t test; $n=5$). Subsequently, J_{Na}^{in} falls, to increase later toward a stationary state which is simultaneously attained by SCC. In the stationary state, $J_{Na}^{in} = 51.4 \pm 2.1$ μA cm^{-2} (for the last 5 post-substitution values), is approximately 100% higher than the corresponding SCC values (20.4 ± 6.7 μA cm^{-2}). Inner Na by K substitution is followed by a slow and progressive decline of the skin electrical resistance, which is 1.44 ± 0.02 kohm cm^2 in the control condition and 0.29 ± 0.01 kohm cm^2 for the last 5 post-substitution measurements (Fig. 3*a*).

2. SCC, J_{Na}^{in}, *and the Effect of* Na *by* K *Substitution in the Inner Solution* (SO_4^{--} *as the Major Anion)*

These experiments were performed to test the role played by changes in cell volume (induced by KCl movement from the inner solution to cell medium after Na by K substitution) on SCC and J_{Na}^{in} transients described in *Results*, section *1*. In order to prevent cell swelling, Cl was substituted in the bathing solutions by SO_4, a less permeant anion. Fig. 3*b* shows SCC and J_{Na}^{in} before and after Na by K substitution in the inner solution

in SO_4 medium. In the control period, the behavior is very similar to that observed in Cl medium. For the last 5 measurements before the ionic substitution, SCC $=58.1 \pm 8.9$ μA cm^{-2} and $J_{Na}^{in} = 59.7 \pm 4.8$ μA cm^{-2} ($P > 0.9$, t test; $n = 5$). After the substitution, the transient phases of SCC and of J_{Na}^{in} are very similar to those displayed by the same variables in Cl medium. SCC goes through a minimum of 33.7 ± 5.7 μA cm^{-2} and J_{Na}^{in} through a maximum of 55.0 ± 7.3 μA cm^{-2} which is significantly higher than the mean of the last pre-substitution values (50.0 ± 7.1 μA cm^{-2}), ($P < 0.01$, paired t test; $n = 5$), both peaks occurring around 5 min after the substitution. However, a sharp difference can be noticed regarding SCC and J_{Na}^{in} stationary-state values, as compared to the behavior seen in Cl medium. In SO_4 medium, SCC and J_{Na}^{in} are no longer different 60 min after the substitution. For the last 5 post-substitution measurements, SCC $=33.1 \pm 5.4$ μA cm^{-2} and $J_{Na}^{in} = 36.7 \pm 6.7$ μA cm^{-2} ($P > 0.7$, t test; $n = 5$). This reflects the fact that in SO_4 medium there is no latter increase in J_{Na}^{in} as seen in Cl medium and, also, because the SCC does not decline as fact as it does in Cl medium. Inner Na by K substitution is followed by a slow progressive decline of the skin electrical resistance which is 1.74 ± 0.02 kohm cm^2 in the control condition and 0.72 ± 0.03 kohm cm^2 for the last 5 post-substitution measurements (Fig. 3*b*).

3. J_K^{eff} *and the Effect of* Na *by* K *Substitution in the Inner Solution*

In order to interpret the discrepancy observed between stationary state values of SCC and J_{Na}^{in} in Cl, but not in SO_4 medium, experiments were carried out to examine the role of an important K efflux after Na by K substitution in Cl medium experiments to reduce steady-state SCC to about 50% of J_{Na}^{in} (*Results*, section *1*). In these experiments a control period was carried out with Ringer's solution on both sides of the skin, and ^{42}K added to the inner solution. Sample collection in the outer compartment was started simultaneously in order to obtain the rate of ^{42}K appearance in the outer solution and to determine the steady-state J_K^{eff}. A steady-state J_K^{eff} is reached approximately 90 min from the moment of isotope addition. In Cl medium, steady-state $J_K^{eff} = (6.4 \pm 0.4) \times 10^{-2}$ μA cm^{-2} ($n = 5$), and in SO_4 medium, steady-state $J_K^{eff} = (1.6 \pm 0.1) \times 10^{-1}$ μA cm^{-2} ($n = 4$). These values are negligible as compared to SCC and to J_{Na}^{in} observed in similar conditions (*Results*, sections *1* and *2*). After J_K^{eff} had reached a steady state, Na by K substitution in the inner solution was carried out, with ^{42}K added to the new inner solution to give a final isotope concentration approximately equal to that pre-

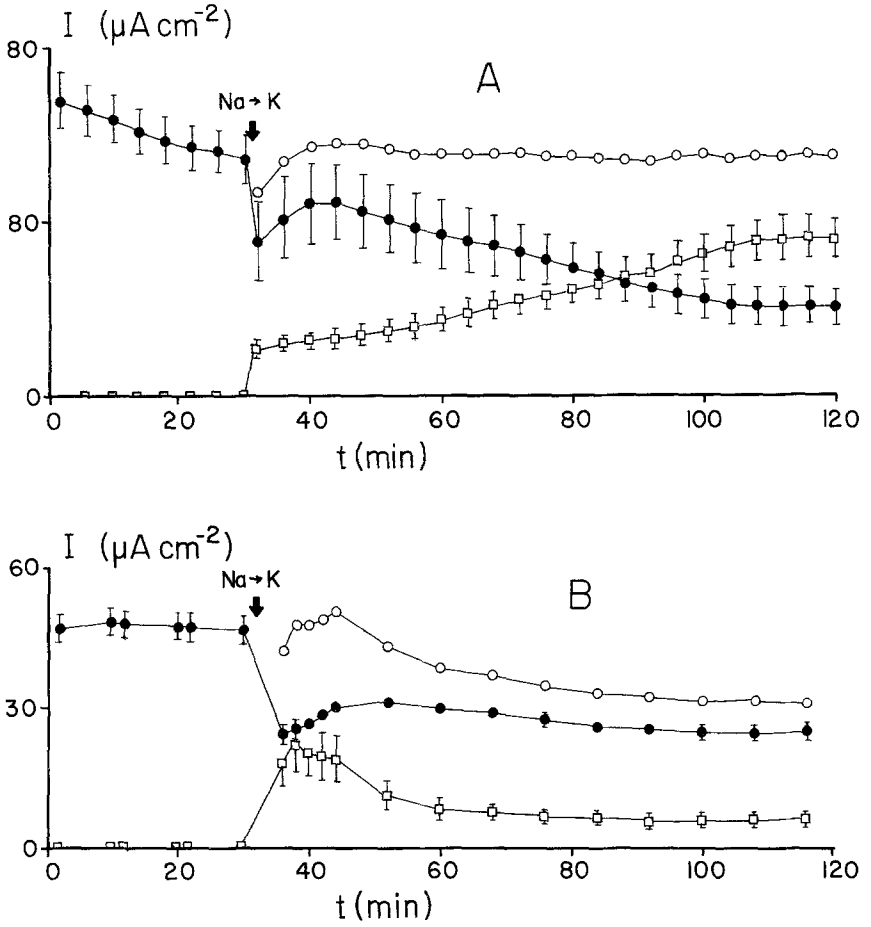

Fig. 4. Short-circuit current (●), K efflux (□), and the sum of SCC and J_K^{eff} absolute values (○) before and after inner Na by K substitution. The ionic substitution is indicated by the arrow. (*A*): Experiments performed with Cl as the major anion. (*B*): Experiments performed with SO_4 as the major anion

viously present. Sampling of the external solution followed as before. Figs. 4*a* and 4*b* show the rates of ^{42}K appearance in the external solution as a function of time for skins in Cl and in SO_4 media, respectively. In these figures, open circles correspond to the sum of SCC and J_K^{eff} absolute values. Table 1 summarizes the results. As can be seen, inner Na by K substitution increases steady-state J_K^{eff} in Cl and in SO_4 media. In the Cl group, this increase is much higher than that in SO_4, despite the fact that the pre-substitution values were of a comparable magnitude. The ratios $J_K^{eff}/(K_i)$ (where (K_i) is the K concentration in the inner

Table 1. Steady-state K efflux (J_K^{eff}) before and after Na by K substitution in the inner solution[a]

	Cl medium		SO_4 medium	
	Before	After	Before	After
J_K^{eff} ($\mu A\,cm^{-2}$)	0.10 ± 0.01	37.3 ± 0.50	0.16 ± 0.00	6.7 ± 0.16
P ($cm\,s^{-1}$)	4.0×10^{-7}	3.3×10^{-6}	6.5×10^{-7}	5.9×10^{-7}

[a] Experiments with Cl or SO_4 as the major anion in the bathing solutions.
Before: Na-Ringer's solution on both sides of skin.
After: Na-Ringer's solution on the outer side and K-Ringer's solution on the inner side.
P is the apparent K permeability calculated as $J_K^{eff}/(K_i)$, where (K_i) is the K concentration in the inner bathing solution, equal to 2.5 mM before and 117.5 after the ionic substitution

solution) are of a comparable magnitude in the Cl and in the SO_4 control groups and also in the SO_4 group after the ionic substitution. However, it is an order of magnitude higher in the Cl group after the ionic substitution.

4. *Skin Electrical Potential Difference Before and After* Na *by* K *Substitution in the Inner Bathing Solution*

These experiments were carried out to test skin PD behavior after inner Na by K substitution in open- and in short-circuited skins.

(a) Skins kept in open-circuited state. Fig. 5*a* shows that in Cl and in SO_4 media PD falls sharply after the substitution and that a new steady-state is reached after about 2 min. In Cl medium, PD falls from 63.4 ± 7.9 mV to 5.5 ± 1.2 mV ($n=5$); in SO_4, from 107.5 ± 8.4 mV to 23.4 ± 2.3 mV ($n=5$). Fig. 5*a* shows that no transients resembling those of SCC (Figs. 3*a* and 3*b*) can be seen and that the behavior is similar in Cl and in SO_4 media, apart from the levels which are higher in SO_4 medium.

(b) Skins kept in short-circuited state. Fig. 5*b* shows the results for short-circuited skins (kept in the open-circuited state for only 5 to 10 sec every two min) bathed in Cl and in SO_4 medium, respectively. These results correspond to experiments of *Results*, sections *2* and *3*. As can be seen, no transients are observed, and PD falls sharply after the ionic substitution, following a slow decline with time. In Cl medium, PD falls from 63.9 ± 7.7 mV to 6.9 ± 1.6 mV ($n=5$); in SO_4, from 100.4 ± 12.1 mV to 24.6 ± 2.9 mV ($n=4$).

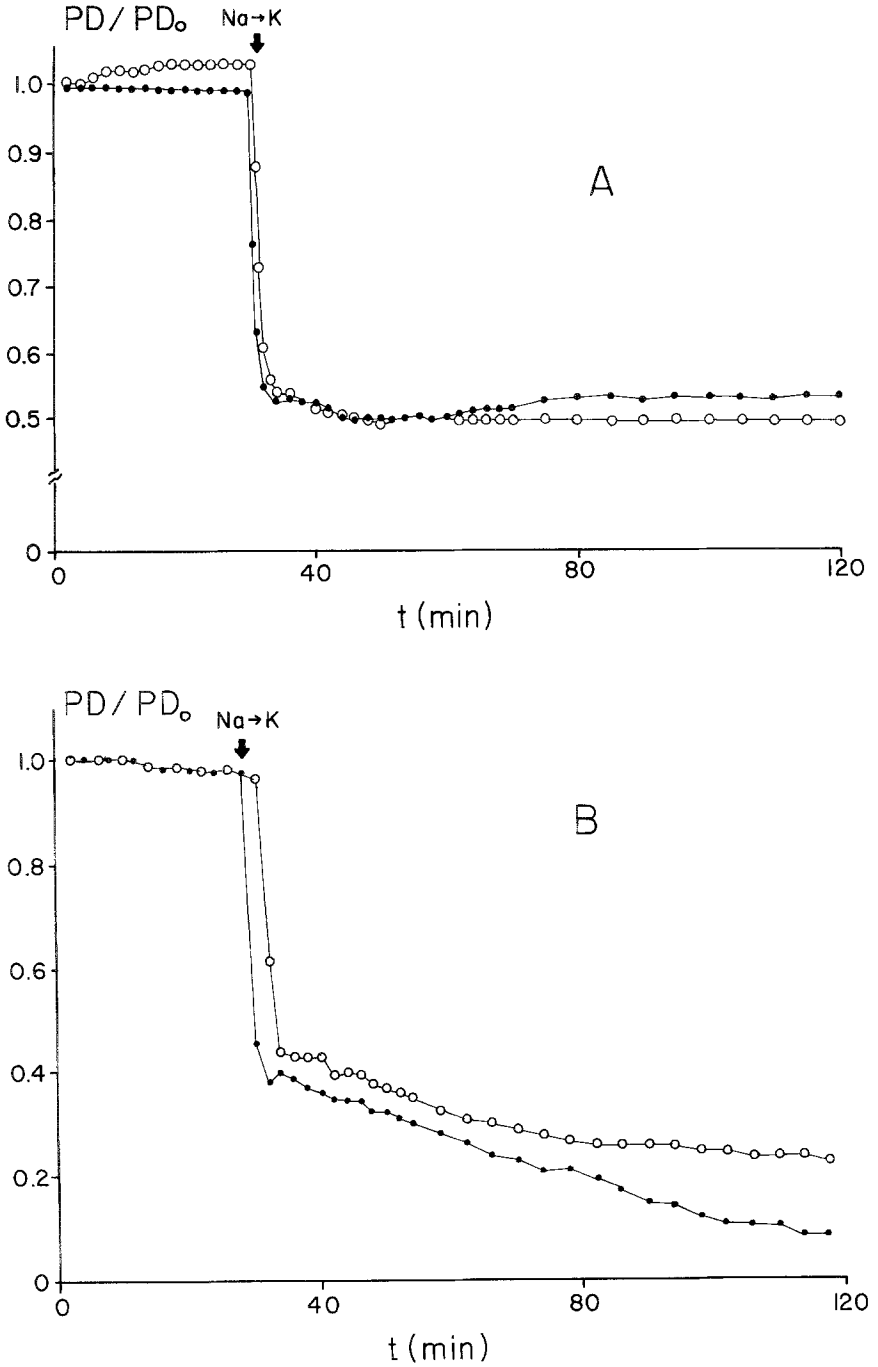

Fig. 5. Skin electrical potential difference normalized to the initial value (PD/PD_0), before and after inner Na by K substitution. (*A*): Skins kept in the open-circuited state. (*B*): Skins kept in the short-circuited state except for 5 to 10 sec in the open-circuited state for PD measurement. Experiments performed with Cl (●) or SO_4 (○) as the major anion

Discussion

The initial aim of this work was to continue our studies in amphibia skin (Vieira *et al.*, 1972; Danisi & Lacaz-Vieira, 1974; Procópio & Lacaz-Vieira, 1977) in an attempt to test further in the isolated skin of the toad *Bufo marinus ictericus* the irreversible thermodynamic formalism of Essig and Caplan (1968) for the active Na transport. If the formalism were applicable, a reduction of the inner Na concentration, increasing the Na electrochemical potential difference across the skin ($\Delta\mu_{Na}$), would increase the rate of net Na transport across the skin. The effect of changing inner Na concentration on net Na transport is still a controversial matter in epithelial membranes (MacRobbie & Ussing, 1961; Bricker, Biber & Ussing, 1963; Ussing, 1965; Ussing, Biber & Bricker, 1965; Lipton, 1972; Rabito *et al.*, 1973; Robinson & Macknight, 1976*a–c*). In the present circumstance, a nominal zero Na concentration in the inner solution, carried out by Li or choline substitution (*Results*, section *1*) had no effect on the rate of net Na transport (measured by SCC) or on the rate of ^{22}Na appearance in the inner bathing solution (J_{Na}^{in}), despite the large thermodynamic force favoring active Na transport. This contrasts with the effects of changes in $\Delta\mu_{Na}$ caused by changes in the electrical potential difference across the skin or in the external Na concentration which alter not only the rate of net Na transport (Vieira *et al.*, 1972; Mandel & Curran, 1973) but also the rate of oxygen consumption associated with net Na transport (Vieira *et al.*, 1972; Saito, Essig & Caplan, 1973). The results indicate that the formalism of Essig and Caplan (1968), which successfully describes the transport system in other conditions (Vieira *et al.*, 1972; Danisi & Lacaz-Vieira, 1974; Procópio & Lacaz-Vieira, 1977), in the present circumstance does not fit the experimental results of changes in the inner Na concentration. Reasons for the nonlinear behavior between Na transport and changes in $\Delta\mu_{Na}$ are unknown. Asymmetry at the pump level could be responsible for the observed results; however, other possibilities may also contribute to the nonlinearity. The results of inner Na by Li or choline substitutions are consistent with observations of Finn and Reuss (1975) in the toad urinary bladder, showing that the increase in SCC induced by lowering inner NaCl concentration is due to an osmotic cell-swelling rather than to lower inner Na concentration. In the present case, effects of osmolarity changes were prevented by the ionic substitution, since the control and test solutions always had the same osmolarity.

Experiments with Li or choline substitution in the inner solution

indicate that these ions can replace Na even in long term experiments, at least regarding their ability to sustain SCC and J_{Na}^{in}. This behavior differs from observations of Mandel and Curran (1973) in *Rana pipiens*, showing that high inner choline concentration or low Na concentration may induce decline in SCC. It may, perhaps, reflect species difference.

Inner Na by K substitution, on the other hand, causes marked transient and stationary alterations in SCC, in J_{Na}^{in}, and in J_{K}^{eff}, which are clearly distinct from those observed with Li or choline substitutions. The stationary alterations are fundamentally dependent on the nature of the anion present in the solutions, if Cl or SO_4, as will be discussed later. Further, the electrical parameters, SCC and PD distinctly reflect what follows after inner Na by K substitution. Thus, a transient phase is clearly seen in SCC (Fig. 3*a* and *b*), but is completely absent in PD (Fig. 5*a* and *b*), both in open- and in short-circuited skins with brief open-circuited intervals for PD measurements. An abrupt fall in SCC follows inner Na by K substitution with a minimum, about 50% of the presubstitution values, 5 min after the ionic substitution. This is followed by a subsequent increase of SCC which may surpass presubstitution values. These SCC transients are very similar to those observed by Finn and Hutton (1974) and Robinson and Macknight (1976*a* and *b*) in the toad urinary bladder, following increase in the serosal K concentration. Fig. 3*a* and *b* show that the transients observed in SCC are independent of the major anion present in the bathing solutions, whether chloride or sulfate. The SCC fall that immediately follows Na by K substitution in the inner solution may be interpreted as being due to a transient K flow from inner solution to cell medium due to rupture of the K equilibrium across the inner cell membrane as a result of increase of inner K concentration. This interpretation is consonant with that given by Robinson and Macknight (1976*a* and *b*) to explain similar transients in the toad urinary bladder. The subsequent increase in SCC after the minimum could be a natural consequence of the transient nature of the K flow in a system drifting toward a new equilibrium state. Two variables should be considered in this evolution to equilibrium: changes in the cell K concentration and changes in the electrical potential difference across the inner cell membrane. It is reasonable to assume that increase in the inner K concentration could lead to some increase in cell K concentration. Results of Robinson and Macknight (1976*b*) indicate that this really occurs in the short-circuited toad urinary bladder following a rise in the serosal K concentration. Regarding the electrical potential difference, it is conceivable that the electrical potential well present in the

short-circuited condition (Whittembury, 1964; Cereijido & Curran, 1965; Rawlins *et al.*, 1970) could be reduced following a rise in the inner K concentration, according to the classical interpretation of the inner membrane potential as being a K diffusion potential (Koefoed-Johnsen & Ussing, 1958). This interpretation, however, has been challenged by more recent observations that indicate that the Na pump may be rheogenic, not only in amphibian skin but in other epithelial membranes (Frazier & Leaf, 1963; Snell & Chowdhury, 1965; Finn, 1974; Pour-Hassani & Finn, 1974; Nellans & Schultz, 1976; Varanda & Lacaz-Vieira[1]). Even in the case of a rheogenic pump, it is reasonable to assume that increase in the inner K concentration may lead to reduction in the inner membrane potential due to a shunting K current from inner to outer bathing solutions as shown to be present in Fig. 4*a* and *b*. Fall in the inner membrane potential after rise in the inner K concentration may lead to a new K steady state across the inner membrane with an increase in the cell K concentration smaller than what would be expected if the potential had not changed. Therefore, the puzzling fact that marked transients are observed in SCC after inner Na by K substitution while, on the other hand, only a fall, without any subsequent increase, is observed in PD after similar ionic substitution (either in open- or in short-circuited skins briefly unclamped for PD measurement, both in Cl or in SO_4 media (Fig. 5*a* and *b*)) may indicate that the new K steady state is reached due to a large depolarization of the inner membrane and a small increase in the cell K concentration.

Concomitantly to the fall in SCC, a small transient increase in J_{Na}^{in} follows inner Na by K substitution, with a peak occurring simultaneously to the minimum in SCC (Fig. 3*a* and *b*). Different processes may contribute to this transient. The rise may be of an electrical nature, due to increase in the passive component of J_{Na}^{in} from cell to inner solution and/or to increase in the pumping rate of a rheogenic pump located in the inner membrane as a consequence of depolarization of the inner membrane. Certainly, it is not due to a reduction of the inner Na concentration, since it was absent in the experiments with Li or choline substitution (Fig. 2). In the toad urinary bladder, Robinson and Macknight (1976*a*) and Finn (1976) observed only a decline of Na influx after inner Na by K substitution, despite the fact that the first authors observed a temporal evolution for SCC very similar to what we have

---

1 W.A. Varanda and F. Lacaz-Vieira. 1977. Transient transepithelial K fluxes in toad skin. *(Manuscript in preparation)*

seen. In SO_4 medium, the decline seen in J_{Na}^{in} after the peak is progressive and 80 min after the ionic substitution J_{Na}^{in} and SCC are no longer different (Fig. 3*b*). This decline could possibly be a consequence of a reduction in the Na influx at the outer barrier of the skin, due to depolarization of this membrane as a consequence of depolarization of the inner barrier by high internal K concentration, by the condition of voltage clamp. This interpretation is consonant with results showing that the electrical potential difference across the outer barrier may control Na flux across this structure (Biber & Sanders, 1973). The small J_{Na}^{in} peak may be reflecting a small Na transport pool which is consistent with results of Finn and Rockoff (1971) and Macknight, Civan & Leaf (1975) in the toad bladder and of Aceves and Erlij (1971), Cereijido and Rotunno (1971), Moreno *et al.* (1973) and Cereijido *et al.* (1974) in amphibian skins. In SO_4 medium, steady-state SCC and J_{Na}^{in} are no longer different, resembling the control condition with Na-Ringer's solution on both sides of skin. This indicates that in the new stationary state after inner Na by K substitution the skin still pumps Na from the outer to the inner solution and that Na is the major ion responsible for the SCC. However, with Cl as the major anion in the bathing solutions, we have observed that a late decline in SCC normally occurs 50 min after inner Na by K replacement. This decline in SCC is not due to a drop in J_{Na}^{in}, since simultaneous measurements of SCC and of J_{Na}^{in} show that J_{Na}^{in} displays a late increase with a plateau starting 70 min after the ionic substitution, while SCC falls progressively. In the late steady state, J_{Na}^{in} is approximately twice the SCC value. These results are in agreement with those of Bricker *et al.* (1963) in Cl medium, which show the ability of the frog skin to maintain a short-circuit current when Na was completely replaced by equimolar concentration of K in the inner bathing solution. Reversible cell swelling, which takes place after inner Na by K substitution in Cl, but not in SO_4 medium (Ussing *et al.*, 1965), may explain the different behavior according to the anion present in major proportions in the bathing solutions. Epithelial cell swelling is associated with an increasing rate of Na transport in frog skin (Ussing, 1965) and in toad urinary bladder (Finn & Reuss, 1975). Hence, it is conceivable that the late increase in J_{Na}^{in} and in J_{K}^{eff} in Cl medium might be associated with cell swelling. Further, as previously noticed by Bricker *et al.* (1963) in Cl medium, the difference between J_{Na}^{in} and SCC values in Cl, but not in SO_4 medium, is due to an important K efflux, which builds up progressively with time after the ionic substitution. Epithelial cell swelling may lead to increase of the K permeability of the outer barrier

of the skin and, thus, to a significant K flow from cell to outer bathing solution along with the K electrochemical gradient. K efflux measurements in the control condition and after the ionic substitution show that in the former condition, with NaCl- or Na_2SO_4-Ringer's solution bathing both sides of skin, steady-state J_K^{eff} is insignificant as compared to SCC ($J_K^{eff}/SCC \cong 2 \times 10^{-3}$). Inner Na by K substitution increases J_K^{eff}, both in Cl and in SO_4 media. In sulfate medium, increase in the steady-state J_K^{eff} is proportional to the increase in the inner K concentration (K_i). The steady-state $J_K^{eff}/(K_i)$ ratio in control condition $(6.6+0.2) \times 10^{-7}$ cm s^{-1} is not significantly different from that after Na by K substitution $(5.9 \pm 0.1) \times 10^{-7}$ cm s^{-1} ($P > 0.7$, t test; $n = 5$). On the other hand, in Cl medium a steady-state J_K^{eff} is not completely attained. However, 1.5 hr after inner Na by K substitution the ratio $J_K^{eff}(K_i)$ $(3.3 \pm 0.04) \times 10^{-6}$ cm s^{-1} is approximately one order of magnitude higher than that in the control condition $(3.2 \pm 0.1) \times 10^{-7}$ cm s^{-1}. This indicates a facilitation of the K movement from inner to outer bathing solution, possibly as a consequence of an increase of the K permeability of the outer barrier of the skin, which is the main barrier to K movement across the epithelium (MacRobbie & Ussing, 1961) due to epithelial cell swelling, similar to what has been described for Na permeability in the toad urinary bladder (Finn & Reuss, 1975). A possibility that could also be raised to explain the transient declines observed in SCC and in J_{Na}^{in} after inner Na by K substitution would be that both are the result of a transient decline in the Na movement from the outer to the inner bathing solution, and that the temporal dissociation observed in Fig. 3*a* between the transients in SCC and in J_{Na}^{in} would be due to isotopic delay in the tissue. This would not affect the electrical measurement but would delay the appearance of ^{22}Na in the inner solution. We think, however, that this possibility is unlikely to occur for the following reasons: (i) what we first observe after inner Na by K substitution is a significant increase in Na influx which is concomitant to the fall in SCC either in Cl or in SO_4 media. A delay in isotopic flow could never give rise to this behavior; on the contrary, it could have masked the transient increase in J_{Na}^{in} if it were of an important magnitude. (ii) In Fig. 3*a* the area of the SCC transient calculated between the point where SCC started to decline and the point where it had returned to the initial value is 20% higher than that of the transient of J_{Na}^{in}, calculated in a similar way, which indicates that a transient reduction in J_{Na}^{in} could not, by itself, explain the transient reduction observed in SCC, suggesting the participation, as already mentioned, of a K flux from inner solution to cell medium. (iii) In SO_4 medium (Fig. 3*b*)

a transient, similar to that seen in Cl medium, is observed in SCC. However, only a decline without subsequent increase is observed in J_{Na}^{in}. This corroborates the hypothesis that the SCC transient is not due, at least entirely, to a transient decline in J_{Na}^{in} which would appear late in time due to isotopic delay in the tissue.

This work was supported by a grant from Fundação de Amparo à Pesquisa do Estado de São Paulo (04-biol. 73/1195).

W.A. Varanda wishes to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo for a fellowship which made possible the development of this research (04-biol. 72/687).

References

Aceves, J., Erlij, D. 1971. Sodium transport across the isolated epithelium of frog skin. *J. Physiol. (London)* **212**:195

Biber, T.U.L., Sanders, M.L. 1973. Influence of transepithelial potential difference on the sodium uptake at the outer surface of the isolated frog skin. *J. Gen. Physiol.* **61**:529

Bricker, N.S., Biber, T.U.L., Ussing, H.H. 1963. Exposure of the isolated frog skin to high potassium concentration at the internal surface. I. Bioelectric phenomena and sodium transport. *J. Clin. Invest.* **42**:88

Cereijido, M., Curran, P.F. 1965. Intracellular electrical potentials in frog skin. *J. Gen. Physiol.* **48**:543

Cereijido, M., Rabito, C.A., Rodriguez-Boulán, E., Rotunno, C.A. 1974. The sodium-transporting compartment of the epithelium of frog skin. *J. Physiol. (London)* **237**:555

Cereijido, M., Rotunno, C.A. 1971. The effect of antidiuretic hormone on Na movement across frog skin. *J. Physiol. (London)* **213**:119

Danisi, G., Lacaz-Vieira, F. 1974. Nonequilibrium thermodynamic analysis of the coupling between active sodium transport and oxygen consumption. *J. Gen. Physiol.* **64**:372

Essig, A., Caplan, S.R. 1968. Energetics of active sodium transport processes. *Biophys. J.* **8**:1434

Finn, A.L. 1974. Transepithelial potential in toad urinary bladder is not due to ionic diffusion. *Nature (London)* **250**:495

Finn, A.L. 1976. Changing concepts of transepithelial sodium transport. *Physiol. Rev.* **56**:453

Finn, A.L., Hutton, S.A. 1974. The kinetics of sodium transport in the toad urinary bladder. III. The role of potassium. *J. Membrane Biol.* **17**:253

Finn, A.L., Reuss, L. 1975. Effects of changes in the composition of the serosal solution on the electrical properties of the toad urinary bladder epithelium. *J. Physiol. (London)* **250**:541

Finn, A.L., Rockoff, M.L. 1971. The kinetics of sodium transport in the toad bladder. I. Determination of the transport pool. *J. Gen. Physiol.* **57**:326

Frazier, H.S., Leaf, A. 1963. The electrical characteristics of active sodium transport in the toad bladder. *J. Gen. Physiol.* **46**:491

Koefoed-Johnsen, V., Ussing, H.H. 1958. The nature of the frog skin potential. *Acta Physiol. Scand.* **42**:298

Lipton, P. 1972. Effect of changes in osmolarity on sodium transport across isolated toad bladder. *Am. J. Physiol.* **222**:821

Macknight, A.D.C., Civan, M.M., Leaf, A. 1975. The sodium transport pool in toad urinary bladder epithelial cells. *J. Membrane Biol.* **20**:365

MacRobbie, E.A.C., Ussing, H.H. 1961. Osmotic behaviour of the epithelial cells of frog skin. *Acta Physiol. Scand.* **53**:348

Mandel, L.J., Curran, P.F. 1973. Response of the frog skin to steady-state voltage clamping. II. The active pathway. *J. Gen. Physiol.* **62**:1

Moreno, J.H., Reisin, I.L., Rodriguez-Boulán, E., Rotunno, C.A., Cereijido, M. 1973. Barriers to sodium movement across frog skin. *J. Membrane Biol.* **11**:99

Nellans, H.N., Schultz, S.G. 1976. Relations among transepithelial sodium transport, potassium exchange, and cell volume in rabbit ileum. *J. Gen. Physiol.* **68**:441

Pour-Hassani, H., Finn, A.L. 1974. Effect of serosal K-Na substitution on Na and K kinetics in toad bladder. *Fed. Proc.* **33**:216

Procópio, J., Lacaz-Vieira, F. 1977. Ionic exchanges in isolated and open-circuited toad skin. *J. Membrane Biol.* **35**:219

Rabito, C.A., Rodriguez-Boulán, E., Cereijido, M. 1973. Effect of the composition of the inner bathing solution on transport properties of the frog skin. *Biochim. Biophys. Acta* **311**:630

Rawlins, F., Mateu, L., Fragachan, F., Whittembury, G. 1970. Isolated toad skin epithelium: Transport characteristics. *Pfluegers Arch.* **316**:64

Robinson, B.A., Macknight, A.D.C. 1976*a*. Relationships between serosal medium potassium concentration and sodium transport in toad urinary bladder. I. Effects of different medium potassium concentrations on electrical parameters. *J. Membrane Biol.* **26**:217

Robinson, B.A., Macknight, A.D.C. 1976*b*. Relationships between serosal medium potassium concentration and sodium transport in toad urinary bladder. II. Effects of different medium potassium concentrations on epithelial cell composition. *J. Membrane Biol.* **26**:239

Robinson, B.A., Macknight, A.D.C. 1976*c*. Relationships between serosal medium potassium concentration and sodium transport in toad urinary bladder. III. Exchangeability of epithelial cellular potassium. *J. Membrane Biol.* **26**:269

Saito, T., Essig, A., Caplan, S.R. 1973. The effect of aldosterone on the energetics of sodium transport in the frog skin. *Biochim. Biophys. Acta* **318**:371

Snell, F.M., Chowdhury, T.K. 1965. Contralateral effects of sodium and potassium on the electrical potential in frog skin and toad bladder. *Nature (London)* **207**:45

Ussing, H.H. 1965. Relationship between osmotic reactions and active sodium transport in the frog skin epithelium. *Acta Physiol. Scand.* **63**:141

Ussing, H.H., Biber, T.U.L., Bricker, N.S. 1965. Exposure of the isolated frog skin to high potassium concentrations at the internal surface. II. Changes in epithelial cell volume, resistance, and response to antidiuretic hormone. *J. Gen. Physiol.* **48**:425

Ussing, H.H., Zerahn, K. 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol. Scand.* **23**:110

Vieira, F.L., Caplan, S.R., Essig, A. 1972. Energetics of sodium transport in frog skin. II. The effects of electrical potential on oxygen consumption. *J. Gen. Physiol.* **59**:77

Whittembury, G. 1964. Electrical potential profile of the toad skin epithelium. *J. Gen. Physiol.* **47**:795