# The Nature of the In Vivo Sodium and Chloride Uptake Mechanisms through the Epithelium of the Chilean Frog *Calyptocephalella gayi* (Dum. et Bibr., 1841)

## *Exchanges of hydrogen against sodium and of bicarbonate against chloride*

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ABSTRACT The Chilean frog, *Calyptocephalella gayi*, placed in dilute NaCl solutions may pump $Na^+$ and $Cl^-$ at very different rates depending on the kind of bath solutions in which it was preadapted. Furthermore, $Na^+$ and $Cl^-$ may be absorbed from solutions in which the accompanying coion, such as sulfate and choline, respectively, is impermeant. In all these cases it is obligatory to postulate the existence of two ionic exchange mechanisms, $Cl^-$ and $Na^+$, being exchanged against endogenous anions and cations, respectively. It has been determined that $Na^+$ is exchanged against endogenous $H^+$ and that $Cl^-$ is exchanged against $HCO_3^-$. In animals pumping $Na^+$ and $Cl^-$ from dilute NaCl solutions $Na^+$ or $Cl^-$ uptake may be selectively inhibited, while the flux of the accompanying ion remains unchanged. This is considered to be an additional proof that both $Na^+$ and $Cl^-$ fluxes are always independent. The role of the ionic exchange mechanisms in the direct regulation of the $Na^+$ and $Cl^-$ levels in the internal medium is discussed as well as their relationship in the regulation of the acid-base equilibrium; other physioecological considerations have been treated.

## INTRODUCTION

Freshwater organisms face the problem of maintaining a high ionic concentration in the internal medium against the passive diffusive forces in action at the permeable limiting surfaces. Energy is continually expended in this process and ionic active pumps placed in these surfaces have the function of

coupling metabolic energy to the transport of ions "up-hill," opposing the electrochemical gradient. There is evidence that in freshwater animals there are at least two such active pumps, the transport of both chloride and sodium from the external to the internal medium being active (Jorgensen, Levi, and Zerahn, 1954; Shaw, 1960 *a*, *b*, *c*; Zadunaisky, Candia, and Chiarandini, 1963; House, 1963; García Romeu and Maetz, 1964; Dietz, Kirschner, and Porter, 1967; Stobbart, 1965, 1967). In addition, investigations in vivo of chloride and sodium fluxes have shown that the uptake of chloride from the external solution may be quantitatively very different from the uptake of sodium. Consequently, an ionic exchange of $Na^+$ and $Cl^-$ for endogenous cations and anions, respectively, must be postulated (Krogh, 1937 *a*, *b*; Jorgensen et al., 1954; Shaw, 1960 *a*, *b*, *c*; García Romeu and Maetz, 1964; Maetz and García Romeu, 1964; García Romeu and Motais, 1966; Stobbart, 1967; Dietz et al., 1967; Salibián, Pezzani-Hernández, and García Romeu, 1968). Indeed, ionic pumps seem to be only part of a more complex mechanism in which both $Cl^-$ and $Na^+$ pumps are linked to an anionic and a cationic exchanger, respectively.

A continuous comparison of the results obtained in vitro with those obtained in vivo may prove to be enlightening for the understanding of the actual physiological mechanisms involved in the transport of ions across the epithelia of aquatic organisms. In agreement with these considerations, our aim is to understand the relationships among ionic pumps, ionic exchangers, and the processes of control of these mechanisms in the living animal in accordance with their particular mode of life and evolutionary history. However, before this stage can be reached, it seems to be necessary to clarify the functioning of the ionic exchangers, answering the following questions.

(*a*) When the skin is pumping $Cl^-$ and $Na^+$ is it the total uptake of each ion exchanged against an endogenous ion, or is it only the difference between the incorporated $Na^+$ and $Cl^-$ which is exchanged against an endogenous ion? (*b*) Which are the endogenous ionic species involved in this exchange?

We initiated this work on the aquatic Leptodactylidae, *Calyptocephalella gayi*, with the aim of resolving these problems.

## MATERIAL AND METHODS

*Preparation of Animals* Frogs (180–200 g body weight) were captured in local streams. They were stored in outdoor aquaria with running tap water at uncontrolled temperature and fed with liver or the little frog *Pleurodema*. Before the experiments, and for the purpose of enhancing one or both $Cl^-$ and $Na^+$ fluxes, the animals were kept for 15 days in distilled water or in the following salt solutions: $Na_2SO_4$, choline-Cl, and NaCl (1.74 or 3.5 mEq/liter). The solutions were renewed once a day. The animals were maintained at room temperature.

*Measurements of Net Fluxes* After the preadaptation period the cloaca of the frogs was cannulated by the insertion of a plastic cannula, tightly fastened by a con-

centric subepithelial ligature. By this means urine was drained out of the external bath in order to measure only the epithelial electrolyte exchanges. The cannulated frogs were individually placed in flasks containing the same solution in which they had been preadapted (Fig. 1). The animals remained overnight in this condition, and the next day the experiments were undertaken changing each preadaptation solution, by siphoning, for an experimental one. The volume of this solution was about two times the weight of the animals. Aliquots (10 ml) of the external bath were taken immediately after immersing the animal in the experimental solution and every 30–60 min during the experiment (1.5–7 hr).

The concentration of the ionic species to be considered was plotted against time and the net fluxes were calculated from the slopes of the regression lines (Maetz,

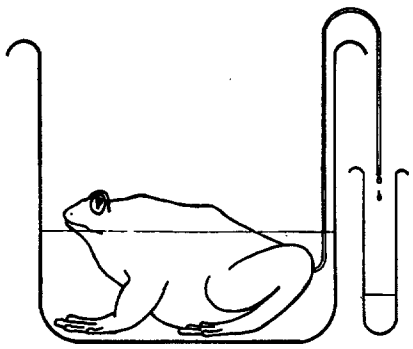

FIGURE 1. Arrangement used for measuring the ionic net fluxes through the frog skin.

1956*a*; García Romeu and Maetz, 1964) and expressed as microequivalents per hour × 100 g of animal weight. For this calculation a mean volume was considered. The experiments reported here were carried out over a period of 1½ yr, irrespective of season.

*Analytical Techniques* Sodium concentration was measured by atomic absorption spectrophotometry with a Perkin Elmer model 290 and chloride was assayed by potentiometric titration (Sanderson, 1952). The concentration of ammonium was determined by a modified Conway microdiffusion technique (Balinsky and Baldwin, 1961).

The total acidity was estimated by titration with NaOH (1.87 mEq/liter) and the base with HCl (3.5 mEq/liter). These measurements were made on 2 ml samples with a Radiometer (Radiometer, Denmark) TTT 1 autotitrator in conjunction with the SBU 1 autoburet unit and SBR 2 Titrigraph recorder.

These determinations as well as pH measurements were made using separate glass and reference electrodes. Points of equivalence, pK of base, and buffering power of the samples were graphically determined.

*Anesthetic Solutions* In order to avoid alterations in the $Na^+$ and $Cl^-$ concentrations of the external bath when anesthetic was added in experiments on ionic uptake inhibition, the chloride and sodium of procaine and pentobarbital, salts, were substituted for sulfate and choline respectively.

Procaine sulfate was prepared by careful neutralization of the procaine base with sulfuric acid; the base was obtained by precipitation from procaine hydrochloride with KOH.

Choline pentobarbiturate was obtained by neutralization of pentobarbituric acid with choline base; the acid had been previously separated from its sodium salt by precipitation with $H_2SO_4$.

## RESULTS

### *Differential Rate of $Cl^-$ and $Na^+$ Uptake in Frogs Preadapted in Different Salt Solutions*

The rate of $Cl^-$ and $Na^+$ uptake may be very different, depending on the previous history of the adaptation of the frogs. In animals preadapted in NaCl

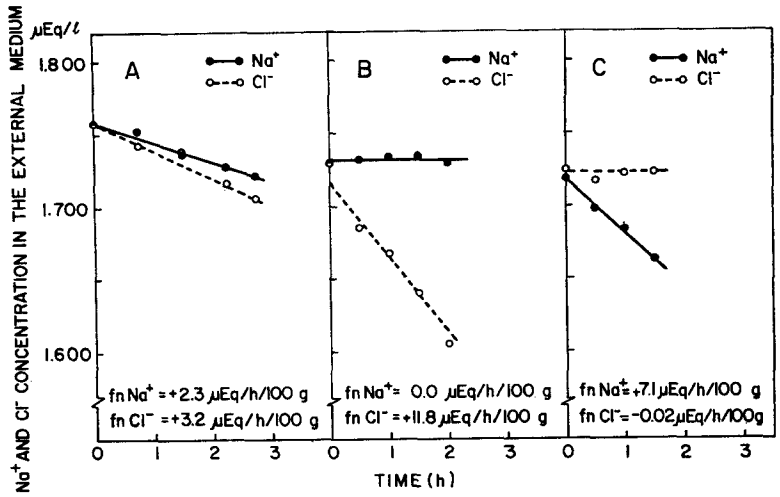


FIGURE 2. Net fluxes (fn) of $Na^+$ and $Cl^-$ from NaCl solutions (1.7 mEq/liter) from frogs preadapted in A, NaCl; B, $Na_2SO_4$; C, Choline-Cl solutions (3.5 mEq/liter). On the illustrations "h" is used instead of "hr" as an abbreviation for "hours." Also an italic "l" is used as an abbreviation for "liter."

solutions the net fluxes of both $Cl^-$ and $Na^+$ measured in NaCl are similar, but generally $Cl^-$ net flux is higher than $Na^+$ net flux. On the contrary, in frogs preadapted in $Na_2SO_4$, $Cl^-$ net flux exceeds $Na^+$ net flux, and the opposite occurs in frogs previously kept in choline-Cl solutions in which $Na^+$ uptake surpasses $Cl^-$ uptake (Fig. 2).

### *Relationship between $Cl^-$ and $Na^+$ Independent Uptake and Their External Concentrations. The Effect of Counterions*

In previous work on another Leptodactylidae (Salibián et al., 1968) we were not able to demonstrate a total independence between $Cl^-$ and $Na^+$ net

fluxes from 870 μEq/liter NaCl solutions. In order to see whether this failure was linked to the external ionic concentration, we compared the uptake of $Na^+$ from $Na_2SO_4$ and NaCl solutions at different concentrations, substituting these solutions one for the other in successive periods. Comparable experiments were done with the $Cl^-$ uptake mechanism in choline-Cl and NaCl solutions.

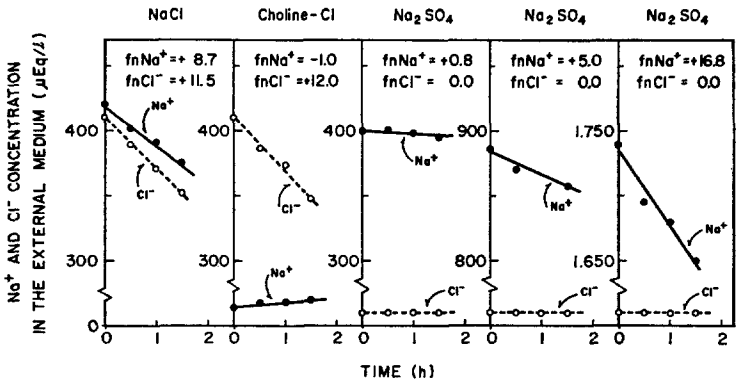


FIGURE 3. The effect of replacing external solutions of different concentrations on the net fluxes (fn) of $Na^+$ and $Cl^-$. All the measurements were carried out on the same frog. In this case the equality of $Na^+$ net fluxes in NaCl of 420 μEq/liter and $Na_2SO_4$ solutions was found at $Na_2SO_4$ concentrations between 0.87 and 1.74 mEq/liter.

TABLE I

A, net fluxes of $Na^+$ from NaCl and $Na_2SO_4$ solutions of 430 μEq/liter. B, comparison between the $Na^+$ net fluxes in 430 μEq/liter NaCl and 1.74 mEq/liter $Na_2SO_4$ solutions. Net fluxes in μEq/hr × 100 g.

| (A) | | | (B) | | |
|---|---|---|---|---|---|
| NaCl 430 μEq/liter | $Na_2SO_4$ 430 μEq/liter | $d$* | NaCl 430 μEq/liter | $Na_2SO_4$ 1740 μEq/liter | $d$* |
| +7.2±1.6 (6) | +2.5±1.1 (6) | − 4.7±1.1‡ | +6.2±2.2 (4) | +8.8±3.4 (4) | +2.7±1.1 |

In Tables I and II the number of animals is given in parentheses.
* Mean differences of paired data ± SEM.
‡ $P < 0.01$.

Fig. 3 illustrates an experiment in which the different solutions were assayed on the same frog.

Table I shows the effect of the accompanying anion and its concentration on the $Na^+$ net fluxes; each individual pair of values was obtained with the same animal by changing their external solution.

It is evident that a replacement of $Cl^-$ by $SO_4^=$ in the external solution drastically decreases the $Na^+$ net flux; this fall is statistically significant (Table I A). When the $Na_2SO_4$ concentration in the external bath is increased

four times (1.74 mEq/liter), the net fluxes of $Na^+$ are comparable to those in 430 $\mu$Eq/liter NaCl solution (Table I B).

Table II and the left side of Fig. 3 show that the rate of $Cl^-$ uptake is independent of the accompanying cation ($Na^+$ or choline) for the concentration used (430 $\mu$Eq/liter).

In summary, the uptake of $Na^+$ and $Cl^-$ may be accomplished without the uptake of the accompanying ion from very dilute external solutions, although the uptake of $Na^+$ is higher in NaCl than in $Na_2SO_4$ solutions of equal concentration.

TABLE II

Net fluxes of $Cl^-$ from NaCl and choline-Cl solutions of 430 $\mu$Eq/liter. Mean of net fluxes ± SEM in $\mu$Eq/hr × 100 g.

| NaCl 430 $\mu$Eq/liter | Choline-Cl 430 $\mu$Eq/liter | $d$* |
|---|---|---|
| +10.1±1.8 (6) | +9.9±2.3 (6) | −0.2±0.9 |

* Mean differences of paired data ± SEM.

*Selective Inhibition of $Na^+$ and $Cl^-$ Uptake*

In order to determine whether it is possible to inhibit selectively the $Na^+$ and $Cl^-$ mechanisms we carried out a series of experiments in NaCl solutions. As inhibitors for $Na^+$ we used procaine sulfate or sulfuric acid in the external bath; for $Cl^-$ we tried the addition of choline pentobarbiturate or choline base. The use of cationic procaine and anionic pentobarbiturate was suggested by the hypothesis of Blaustein and Goldman (1966) on the effects of ionic anesthetics as phospholipid charge-blocking agents.

INHIBITION OF $Na^+$ UPTAKE

Table III shows the effect of procaine on the $Na^+$ net flux and Fig. 4 A illustrates a typical experiment. The net flux of $Na^+$, positive during the control period, becomes suddenly negative after the addition of the inhibitor, while the $Cl^-$ net flux remains unchanged. Since procaine sulfate acidifies the bath to about pH 3.9, doubt arises as to whether the inhibition is due to acidification or not. In fact, experiments in which $H_2SO_4$ was added to give a pH of 3.9 demonstrate that acidification also produces a sudden inhibition of $Na^+$ uptake without affecting the $Cl^-$ uptake (Fig. 10). With the data here reported it seems to be impossible to decide whether procaine plays an active part in the inhibition of $Na^+$ uptake or whether only the pH is responsible for it. Notwithstanding, in our context the very important fact is that the $Na^+$ transport mechanism may be inhibited without affecting the $Cl^-$ uptake mechanism.

### INHIBITION OF $Cl^-$ NET UPTAKE

As procaine or $H^+$ inhibits $Na^+$ net uptake, pentobarbiturate dramatically inhibits the $Cl^-$ uptake; $Na^+$ uptake remains unchanged (Table III).

TABLE III

Comparison of the effects of procaine and pentobarbital on $Na^+$ and $Cl^-$ net fluxes. Mean of net fluxes ± SEM in $\mu$Eq/hr × 100 g.

| Inhibitor | No. of experiments | $Na^+$ net flux | | | $Cl^-$ net flux | | |
|---|---|---|---|---|---|---|---|
| | | Before | After | Difference* | Before | After | Difference* |
| Procaine | 4 | +4.4±1.4 | −5.5±1.3 | −9.9±1.3‡ | +9.5±1.9 | +8.4±0.6 | −1.1±0.9 |
| Pentobarbital | 8 | +8.0±0.7 | +8.9±0.9 | +0.9±1.0 | +9.2±1.0 | +1.4±0.5 | −7.8±1.3§ |

* Mean differences of paired data before and after inhibition ± SEM.
‡ $P < 0.01$.
§ $P < 0.001$.

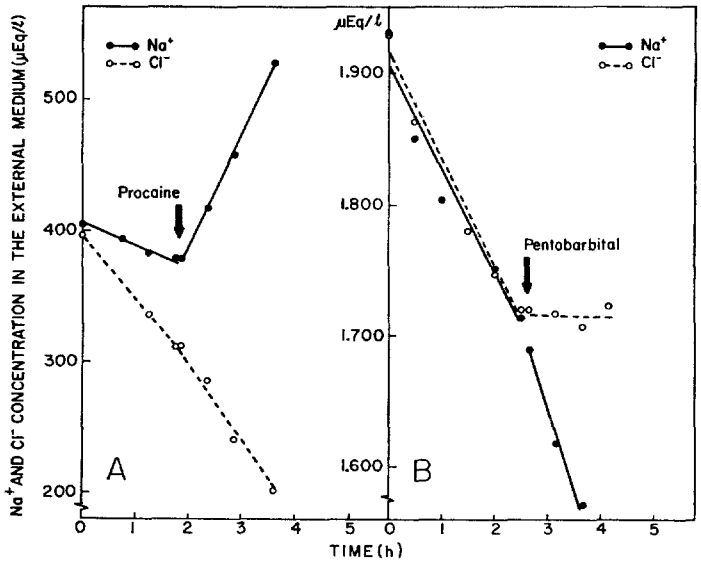


FIGURE 4. The effect of anesthetics (2 mM/liter) added to the external bath on the $Na^+$ and $Cl^-$ net fluxes measured from NaCl solutions. A, inhibition of $Na^+$ uptake by procaine sulfate; B, inhibition of $Cl^-$ uptake by choline pentobarbiturate.

The addition of choline pentobarbiturate produces an upward shift of pH until 8.2–8.9 is reached. To determine whether the inhibition is due to the alkalinization of the external solution we tried the effects of a sudden shift in the pH by the addition of choline base to the external bath. In the pH range of 8.2-8.9 we were unable to reproduce the effect of pentobarbiturate.

In some experiments the addition of pentobarbiturate produced a sudden fall in $Na^+$ concentration of approximately 50 $\mu$Eq/liter in 1740 $\mu$Eq/liter (2.5 %). After this fall the slope of the $Na^+$ uptake remained identical with that of the control period. Fig. 4 B illustrates a typical experiment.

*Determination of the Endogenous Ionic Species Exchanged against External $Na^+$ and $Cl^-$*

The independence of cationic and anionic fluxes demonstrated above offers an experimental approach to study separately $Na^+$ and $Cl^-$ exchange mechanisms by maintaining alternatively one of the two mechanisms at rest. With this purpose we have measured (*a*) the $Na^+$ exchanged against endogenous $H^+$ or $NH_4^+$ in 1740 $\mu$Eq/liter $Na_2SO_4$, and (*b*) the $Cl^-$ exchanged against an endogenous base in 435 $\mu$Eq/liter choline-Cl solutions. Obviously, if both mechanisms functioned simultaneously, the exchanged endogenous ions would mutually react, and the equivalence between uptake and excretion could be masked.

ENDOGENOUS IONIC SPECIES INVOLVED IN THE $Na^+$ EXCHANGE MECHANISM

We looked for the possibility that the $NH_4^+$ excretion through the skin might be equivalent to the total $Na^+$ uptake. As in another Leptodactylidae (García Romeu and Salibián, 1968) we found that $NH_4^+$ is not the endogenous ion exchanged against $Na^+$. In two typical experiments we obtained a $Na^+$ net flux of +3.7 and +10.8 $\mu$Eq/hr × 100 g against an $NH_4^+$ net flux of +0.4 and −1.6 $\mu$Eq/hr × 100 g, respectively (Fig. 5 A).

In view of these results we have compared $Na^+$ uptake against $H^+$ excretion. As can be seen in Table IV and Figs. 5 B and 6 there is a stoichiometric correspondence between the $Na^+$ uptake and $H^+$ excretion.

The coefficient of correlation between the net fluxes of $Na^+$ and $H^+$ is very high ($r = 0.98$). A decrease of 0.30 ± 0.04 pH unit per hour in the external medium (mean ± SEM) was found. This $H^+$ decrease may seem very small, but the $H^+$ concentration as determined by the pH measurements never accounted for the actual $H^+$ concentration. This is due to a progressive buffering of the external medium which averages an increase in the buffering power of +15.97 $\mu$Eq of base $pH^{-1}$ $hr^{-1}$. There is no correlation between $Na^+$ uptake and increase in buffering power ($r = 0.40$; $P = 0.30$). It must be remarked that when the external solution is a NaCl solution and $Na^+$ and $Cl^-$ are incorporated in equivalent quantities there is no significant change in pH in the external medium.

In some experiments it was observed that the uptake of $Na^+$ progressively decreases and finally stops; the $H^+$ excretion follows a similar course (Fig. 7).

ENDOGENOUS IONIC SPECIES INVOLVED IN THE $Cl^-$ EXCHANGE MECHANISM

When the frogs pump $Cl^-$ from choline-Cl solutions there is a correlative base increase in the external bath (Fig. 8).

The coefficient of correlation between $Cl^-$ uptake and base excretion is very high and significant ($r = 0.96$). Nevertheless, in all cases the base excreted exceeds the $Cl^-$ incorporated (Table V, Fig. 9).

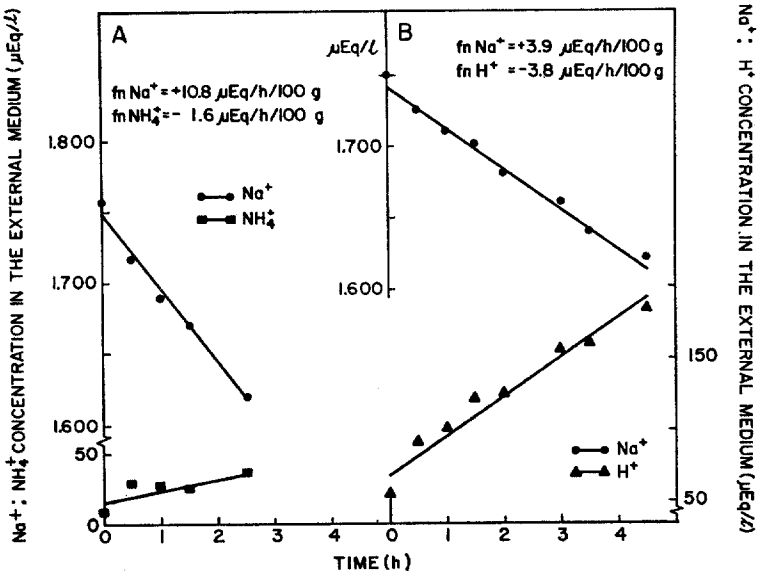


FIGURE 5. Comparison between the excretion of cations and the uptake of $Na^+$ in two different frogs submerged in $Na_2SO_4$ solutions. A, $NH_4^+$ excreted against $Na^+$ taken up; B, $H^+$ excreted against $Na^+$ taken up.

TABLE IV

Comparison between the net fluxes of $H^+$ (fn $H^+$) and $Na^+$ (fn $Na^+$) in animals submerged in 1740 µEq/liter $Na_2SO_4$ solutions (mean of net fluxes ± SEM in µEq/hr × 100 g).

| No. of experiments | fn $Na^+$ | fn $H^+$ |
|---|---|---|
| 7 | +4.6±1.5 | −4.7±1.5 |

The pK of the base excreted, graphically determined, is 5.91 ± 0.03 (mean of five experiments ± SEM). By the same procedure we have determined the pK of choline bicarbonate in mixtures with choline-Cl at concentrations equivalent to those of the experimental solutions; the pK thus obtained was 5.90 ± 0.03.

When the exchange proceeds the increase in buffering power reaches −76.3 ± 16.3 µEq of acid $pH^{-1}$ $hr^{-1}$ (mean of six experiments ± SEM), that is, five times the increase in buffering power obtained when the frogs pump

$Na^+$ from $Na_2SO_4$ solutions. The coefficient of correlation between the rate of increase in buffering power and the $Cl^-$ and base net fluxes was 0.88 and 0.96, respectively; these values are statistically significant.

An increase of 0.15 ± 0.05 pH unit $hr^{-1}$ (mean of six experiments ± SEM occurs when frogs pump $Cl^-$ from choline-Cl solutions.

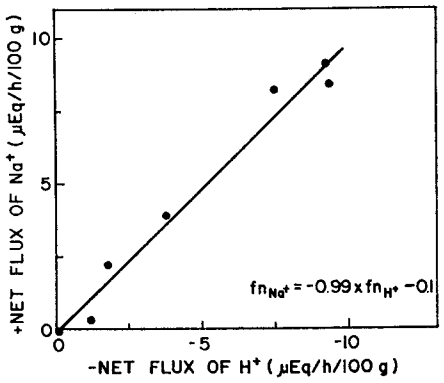


FIGURE 6. Regression line fitted by the method of least squares between $Na^+$ uptake and $H^+$ excreted in seven experiments.

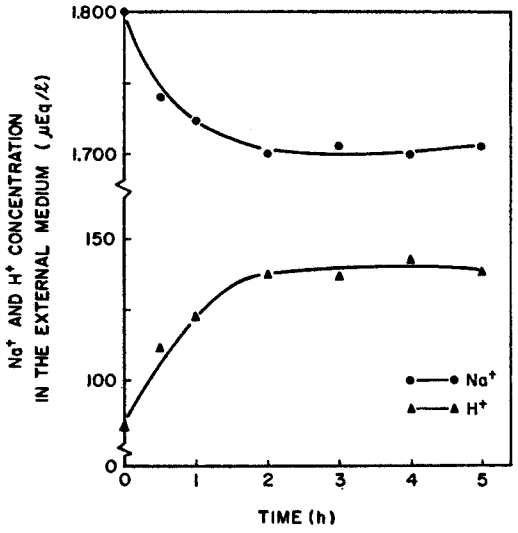


FIGURE 7. Comparison between progressive decrease of $Na^+$ uptake and a parallel decrease of $H^+$ excretion. The frog was submerged in $Na_2SO_4$ solution.

*Ionic Exchanges in Relation to Changes in the Buffering Power and pH of the External Solution*

As we have seen above, the pumping of $Na^+$ from $Na_2SO_4$ and $Cl^-$ from choline-Cl solutions promotes a pH shift to the acid or alkaline side, respectively. A dramatic and clear-cut demonstration of the role of $Cl^-$ uptake in the alkalinization of the solution was shown in experiments on the inhibition of

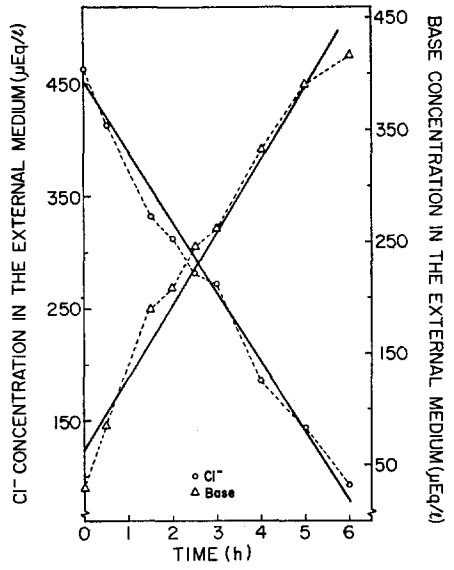


FIGURE 8. Comparison between the excretion of base and the uptake of $Cl^-$ in a frog submerged in choline-Cl solution.

TABLE V

Comparison between the net fluxes of base (fn $B^-$) and $Cl^-$ (fn $Cl^-$) in animals submerged in 435 μEq/liter choline-Cl solutions (mean of net fluxes ± SEM in μEq/hr × 100 g).

| No. of experiments | fn $Cl^-$ | fn $B^-$ |
|---|---|---|
| 6 | +8.6±1.8 | −11.8±2.5 |

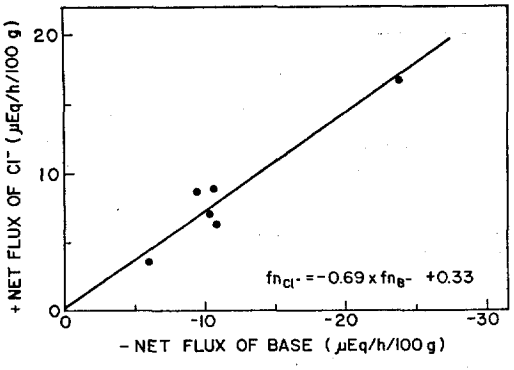


FIGURE 9. Regression line fitted by the same method as in Fig. 6 between base excreted and $Cl^-$ taken up in six experiments.

$Na^+$ uptake by acidification of an external NaCl solution. As may be seen in Fig. 10, while $Na^+$ and $Cl^-$ uptake proceeds there is no change in pH. When enough $H_2SO_4$ is added to produce a pH of 3.9, a sudden inhibition of the $Na^+$ uptake follows while the $Cl^-$ uptake remains unaffected. During the first hour the pH rises to a value of 5.7 at which the $Na^+$ uptake begins again.

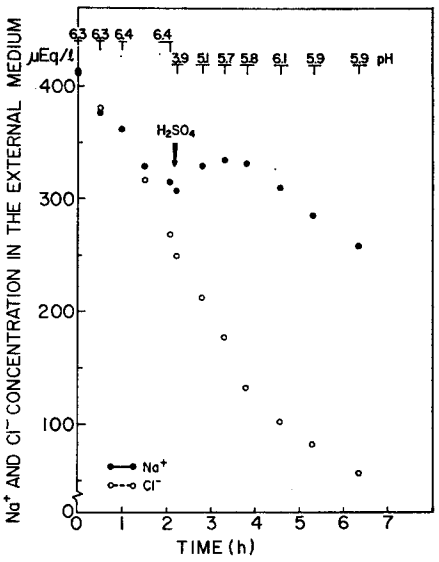


FIGURE 10. The transient effect of external acidification on the inhibition of $Na^+$ uptake and the pH evolution when the external bath is a NaCl solution. Frog preadapted in distilled water.

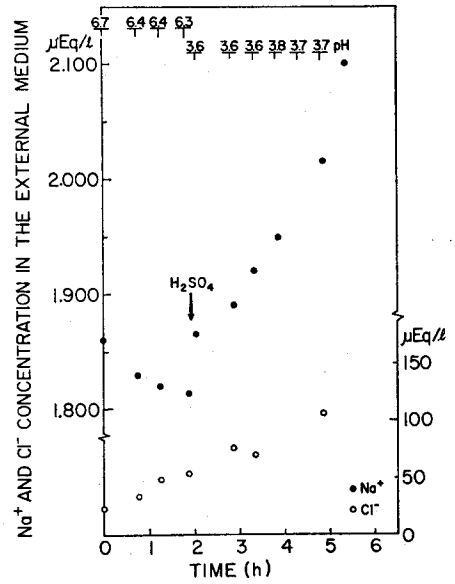


FIGURE 11. The permanent effect of external acidification on the inhibition of $Na^+$ uptake and the external pH evolution when the external bath is a $Na_2SO_4$ solution. Frog preadapted in distilled water.

Finally, the pH increase stops when the pumping of $Na^+$ is definitely reestablished. It must be considered that, if the base excreted is $HCO_3^-$ at the pH reached in the external bath (5.7), the system $HCO_3^-/H_2CO_3$ is in the region of maximal buffering effectiveness; at this point only minor pH changes are to be expected as a result of acid or base excretion.

If a similar experiment is barried out in $Na_2SO_4$ solutions in which there is not a $Cl^-$/base exchange, the external pH remains almost unchanged after

the addition of the acid and the inhibition of $Na^+$ uptake is not reversed (Fig. 11).

These facts tend to emphasize the dependence of the pH changes in the external medium on the ionic exchange mechanisms.

## DISCUSSION

### *Evidence for the Independence of $Cl^-$ and $Na^+$ Uptake Mechanisms and for the Existence of Two Ionic Exchangers in the Frog Skin*

The total independence of both $Na^+$ and $Cl^-$ uptake mechanisms is proved by the results of different groups of experiments, namely: (*a*) uptake of $Na^+$ and $Cl^-$ from solutions in which the accompanying ions are not permeant ($SO_4^=$ or choline; Kirschner, 1960; Ussing, 1960; García Romeu and Maetz, 1964), (*b*) differential rate of uptake of $Na^+$ and $Cl^-$ from NaCl solutions, depending on the preadaptive history of the frogs, and (*c*) selective inhibition of only one ionic flux while the other remains unchanged.

The fact that a sudden and single inhibition of ionic flux could be obtained in animals pumping both $Cl^-$ and $Na^+$ from NaCl solutions must be interpreted as an additional proof that the $Cl^-$ and $Na^+$ fluxes are always independent. If on the contrary, both fluxes were in some way linked, it would not be possible to affect selectively only one of them.

Once the independence of the fluxes is proved, the existence of two ionic exchangers is simultaneously demonstrated because it is evident that, in the case of differential or single ion uptake, the only way to maintain the obligatory electroneutrality of the external and internal solutions is by interdiffusion of endogenous counterions.

The fact that the concentration of $Na^+$ must be increased in $Na_2SO_4$ solutions in order to obtain the same rates of $Na^+$ uptake reached in NaCl solutions, shows that the accompanying anion affects the $Na^+$ permeability. A similar effect of accompanying ions has been reported in other animals (Stobbart, 1965; Shaw, 1960 *a*). It is possible that if the anion transport does not compensate the potential difference of (PD) established by the $Na^+$ transport mechanism, the $Na^+$ pump may not be able to overcome the enhanced PD. However, it must be remarked that in a system such as the one discussed here, the PD across the skin cannot any longer be considered as due only to the transport of $Na^+$. The relative mobilities of all the counterions exchanged should be considered.

### *The Endogenous Counterions Involved in the Ionic Exchange. Their Relationships with the pH Changes and the Buffering of the External Solution*

It has been known for a long time that the frog skin can establish a $H^+$ gradient between the solutions bathing their two surfaces, acidifying the external side

and alkalifying the internal solution (see Fleming, 1957; Friedman, LaPrade, Aiyawar, and Huf, 1967; Friedman, Aiyawar, Hughes, and Huf, 1967). Acidification according to our experimental data is only a consequence of the $Na^+/H^+$ exchange mechanism. This possibility was suggested 20 years ago by Ussing (1949).

The buffering capacity of the base excreted in exchange for $Cl^-$ excludes the possibility that it could be $OH^-$ and rather suggests that it may be $HCO_3^-$. The pK value obtained for the base excreted, similar to the pK of the choline bicarbonate, clearly point in this direction.

Fleming (1957) disclaims a $Na^+/H^+$ exchange in the isolated frog skin because the pH of the outside unbuffered solution does not follow the inward $Na^+$ transport. The hypothesis that the skin could not buffer the external solution and therefore the $H^+$ activity must be equal to the $H^+$ concentration is

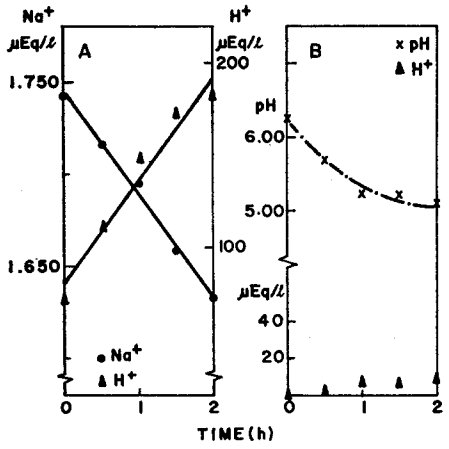


FIGURE 12. Comparison between $H^+$ excretion and $Na^+$ uptake. A, $H^+$ ion concentration measured by direct titration. B, pH evolution (upper) and $H^+$ concentration as calculated from pH data (below).

implicit in his conclusions. We have seen that this is not the case because the frog skin promotes a progressive buffering of the external solution. This buffering explains the sudden initial lowering of pH with the posterior progressive stopping that Fleming reports (compare Fig. 12 with Fleming's Fig. 2). Friedman, LaPrade, Aiyawar, and Huf (1967) also discarded the possibility of a $Na^+/H^+$ and $Cl^-/HCO_3^-$ exchange on what now seem to be inadequate bases. In a pair of frogs placed in distilled water they attempted to establish a relationship between the $Na^+$ and $Cl^-$ net fluxes and the acid or base excreted. As they kept constant the pH by adding HCl or NaOH, there was a progressive increase of either $Cl^-$ or $Na^+$ during the experiment while the other ion increased only by leaks from the animal. Furthermore, the animals were used immediately after handling. We know that a long-lasting shock effect, which upsets their mineral balance for several hours, follows any

disturbance of aquatic animals (Krogh, 1939; Jorgensen, Levi, and Ussing, 1946; Meyer, 1948, 1951; Maetz, 1956 *a*; Alvarado and Kirschner, 1963). It is clear that this is a very inadequate method for determining ionic fluxes and their relationships, since the system is not allowed to reach any state of equilibrium.

More recently, Friedman, Aiyawar, Hughes, and Huf (1967) have determined the net flux of $Na^+$ and the $H^+$ excretion in isolated skin bags submerged in 110 mM NaCl solutions. From these experiments they again concluded that no $Na^+/H^+$ exchange is produced. Since in this case the undetermined net flux of $Cl^-$ should be high, it is not possible to establish a relationship between $Na^+$ uptake and $H^+$ excretion, because the $H^+$ can be expected to be neutralized by the $HCO_3^-$ that should go into the bath in exchange for $Cl^-$. In this system, the final titratable acidity is determined by a complex relationship among all the ions involved, that is, $Na^+$, $Cl^-$, $H^+$, and $HCO_3^-$.

It has been reported that in the isolated frog skin the active transport of $Na^+$ is inhibited by the acidification of the internal facing solution (Schoffeniels, 1955; Snell and McIntyre, 1960). Funder, Ussing, and Wieth (1967) demonstrated that the $Na^+$ transport across the skin at low pH values is markedly dependent on the way in which the pH change is brought about, but they also demonstrated that the active $Na^+$ transport by isolated frog skin is affected by conditions lowering the intracellular pH of the transporting cells. In view of the unphysiological way in which the pH changes in the solutions bathing the isolated frog skin have been studied, and because it seems that with large pH variations the probably enhanced $Na^+$ transport promoted by a higher $H^+$ supply to the exchanger could be counteracted by an inhibition of the $Na^+$ pump, we believe that the effect of pH variations between the narrow "physiological" ranges of alkalosis and acidosis must be reinvestigated.

Our results do not exclude the possibility that acids or bases may be excreted without a parallel $Na^+$ or $Cl^-$ uptake, but these results clearly demonstrate that whenever $Cl^-$ or $Na^+$ is taken up a base or $H^+$ respectively is excreted at equivalent rates. Acidification when $Na^+$ is absorbed in $Na_2SO_4$, alkalinization when $Cl^-$ is taken in from choline-Cl, both point to the relationship between the $Cl^-$ and $Na^+$ fluxes and pH changes. Furthermore, the failure of the pH in the acidified $Na_2SO_4$ external solution to rise when there is no $Cl^-$ transport, and conversely, the rapid pH increase when the NaCl solution in which $Cl^-$ transport goes on is acidified (Figs. 10 and 11), are further proofs of the same phenomenon. As a consequence, a unified interpretation of the pH changes and the buffering produced by the frogs in their external solutions and the transport of $Cl^-$ and $Na^+$ is obligatory. They are only different aspects of the same process.

*The Origin of Endogenous Exchanged Ions*

In amphibians carbon dioxide is excreted mainly through the skin whereas the lungs are the main site for the intake of oxygen (Krogh, 1904; Dolk and Postma, 1927; Whitford and Hutchison, 1965; Hutchison, Whitford, and Kohl, 1968). This is a general rule, but the ratio of pulmonary to cutaneous oxygen absorption varies with the species and with the temperature and, in the case of *Bufo paracnemis*, the data do not indicate any predominance of the skin over the lungs as a site for $CO_2$ excretion (Johansen and Ferreira Ditadi, 1966).

If the skin has an important role in the excretion of $CO_2$, it is necessary to answer the question of whether in the frog skin, which lacks carbonic anhydrase (Maren, 1967), $CO_2$ is or is not given out as $HCO_3^-$ in exchange for $Cl^-$. Investigations on $CO_2$ and $HCO_3^-$ concentrations in the skin show that they are about the same as in the plasma, and it is reasonable to assume that the $HCO_3^-$ of the skin comes from the blood (Friedman, LaPrade, Aiyawar, and Huf, 1967). Furthermore, amphibians have carbon dioxide dissociation curves which show that their blood retains more carbon dioxide than does that of most vertebrates, a condition that can be explained by the retention in the blood of an unusually large concentration of bicarbonate (Redfield, 1933; Foxon, 1964; see Prosser and Brown, 1965). From all this it seems reasonable to postulate that the bicarbonate excreted by the skin in exchange for $Cl^-$ comes directly from the blood.

If the skin fills a role in respiration, this role must be very different in air and in water. The uptake of oxygen by the skin in water must be very low with respect to its uptake in air, due to the low solubility of this gas in water. As a result, the efficiency of the skin as a respiratory organ is diminished in water with respect to its role in air, and the small surface of the frog skin becomes insufficient to compensate for the lower $O_2$ concentration. The higher uptake of oxygen in air than in water and the conversion of hemoglobin into oxyhemoglobin might tend to lower the pH and to dissociate the bicarbonate into carbon dioxide and water in a reaction catalyzed by the carbonic anhydrase of the red cells. Thus, while the physicochemical condition in air would cause the $CO_2$ to be the dominant chemical species in the respiratory excretion by the skin, in water the bicarbonate would substitute for the $CO_2$, thus enabling its exchange for exogenous chloride. Immersion in water must act as a switch that promotes a shift of $CO_2$ to $HCO_3^-$ excretion by the very different rate of oxygen uptake through the skin in both media.

With respect to the $H^+$ excreted through the skin in exchange for $Na^+$, one could postulate its origin from the blood. If $HCO_3^-$ is extruded by the skin, $H_2CO_3$ must be ionized to $H^+$ plus $HCO_3^-$ in order to maintain the $HCO_3^-/H_2CO_3$ equilibrium.

In summary, this view corresponds to a separation of $H^+$ and $HCO_3^-$ by means of membrane loci of differential anionic and cationic permeability.

*Concluding Remarks*

In fish $NH_4^+$ seems to be the counterion involved in the exchange for $Na^+$ (Krogh, 1937 *b*; Maetz and García Romeu, 1964; García Romeu and Motais, 1966). Similarly, for the aquatic larvae of salamanders, Dietz et al. (1967) gave figures for ammonium excretion equivalent to the $Na^+$ uptake. As we have seen, the ammonium excretion in *C. gayi* is not comparable to the $Na^+$ uptake; the same situation was found in another Leptodactylidae, *Leptodactylus ocellatus* (García Romeu and Salibián, 1968).

The $Na^+/H^+$ exchange here described may be a general case in the more terrestrial amphibian species in which epithelial ammonium excretion is maintained at a very low level, reflecting the shift from ammoniotelism to ureotelism and the assumption by the kidneys of the function of nitrogenous compound excretion. Then, replacement of $NH_4^+$ by $H^+$ in the ionic exchange mechanism appears to be linked to an ecological change. This replacement would have been imposed as a physiological adaptation developed during the transition to life on land.

The modern amphibians have preserved a very primitive form of respiration, the hyoid force-pump of lung ventilation, a system of very limited efficiency (Szarski, 1962; Cox, 1967). This can be explained on the assumption that their ancestors had not evolved the costal method of ventilating the lungs (Cox, 1967). Hence, the skin has been developed as an accessory respiratory area to compensate for the inefficient pulmonary gas exchange. But, as Cox emphasizes, cutaneous respiration involves a further restriction. In the short term, it is advantageous in permitting more active life on land. However, the permeability of the skin to gases means that it is also permeable to water and the resulting high rate of water loss has, in the long run, restricted the amphibians to wet or moist habitats. Therefore, modern amphibians do not represent a straightforward link between fishes and fully terrestrial tetrapods; the adult dependence on water is not a relic of the water-dependence of fishes, but a limitation imposed by the development of the cutaneous respiration.

The permeability to gases and water is inseparable from the permeability to ions, and the skin had to develop mechanisms to make good the losses of ions, as in the case with all other known respiratory surfaces in contact with water. In this context it seems that the loss of branchiae must have been a very critical moment in the evolution of the ancestors of modern amphibians, in which the skin had to take care of functions originally foreign to it and primitively accomplished by gills. The more complex function added to the skin was that of transporting ions from the external to the internal medium.

In fish it has also been shown that $Cl^-$ is exchanged for $HCO_3^-$ (Maetz and García Romeu, 1964). In these animals the over-all respiratory exchange takes place in the gills, where the oxygen uptake by the hemoglobin must liberate hydrogen ions and then the conversion of bicarbonate to carbon dioxide should proceed catalyzed by the carbonic anhydrase of the red cells. Hence, a reconversion of carbon dioxide into bicarbonate is needed for maintaining the exchange of bicarbonate for chloride. That this may in fact occur is supported by the demonstration that the branchial cells contain carbonic anhydrase (Leiner, 1938*b*; Maetz, 1956*b*) the inhibition of which inhibits the $Cl^-/HCO_3^-$ exchange (Maetz and García Romeu, 1964). On the other hand, the amphibian skin lacks carbonic anhydrase, a condition that seems to be imposed by the primitive function of this tissue that is not primarily endowed with ionic transport mechanisms as are the gills of fishes. The large concentration of bicarbonate in the blood of amphibians may be a security device to overcome the lack of carbonic anhydrase in the skin. This fact, together with the low uptake of oxygen by the skin in water, that assures a minimal conversion of bicarbonate in carbon dioxide, makes possible the $Cl^-/HCO_3^-$ exchange. In species in which the skin plays an important respiratory function in water (see Czopek, 1967) the high concentration of $HCO_3^-$ in the blood must be the most important factor that allows the maintenance of the exchange of $Cl^-$ for $HCO_3^-$. We see that a novel solution has been evolved through which an independent cationic and anionic exchange is obtained similar to that present in the gills of fishes. This coincidence in the same final mechanism suggests that the exchange of endogenous for exogenous ions is obligatory for ion transport.

The capacity of the frog skin for handling independently the anionic and cationic fluxes by means of ionic exchangers has very important physiological consequences. It is necessary to emphasize that the skin assumes by this mechanism a direct function in the maintenance and regulation of the $Cl^-$ and $Na^+$ levels of the internal medium. Furthermore, the exchange of $H^+$ for $Na^+$ and $HCO_3^-$ for $Cl^-$ is perforce linked with the regulation of the acid-base equilibrium. Another approach to the regulative power of the frog skin was followed by Steinbach (1967) who has demonstrated that the skin can secrete, towards the inside, a fluid balanced with respect to the ions that is reasonably comparable to a normal physiological saline.

In summary, the frog skin is a very complex organ, functionally similar to the kidney and playing a role complementary to it.

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