PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Anionic and Cationic Exchange Mechanisms in the Skin of Anurans, with Special Reference to Leptodactylidae in vivo

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Anionic and cationic exchange mechanisms in the skin of anurans, with special reference to Leptodactylidae *in vivo*

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In several species of anurans, the *in vivo* skin has been shown to absorb Na^+ and Cl^- independently from dilute external solutions. That the mechanism for sodium absorption is different from that of chloride absroption is born out by the following:

(1) Either of these ions is absorbed without an accompanying ion when this latter is impermeant.

(2) From NaCl solutions there can be an unequal absorption of sodium and chloride.

(3) A selective inhibition of the absorption of one of the ions can be produced experimentally, while the net flux of the other remains unchanged.

In all these situations, the absorbed ion has to be exchanged against an endogenous ion of the same charge. In *Calyptocephalella gayi*, H^+ and HCO_3^- are exchanged against sodium and chloride respectively.

A comparison of the relationships between H^+ excretion and Na^+ absorption in *in vivo* skins and short-circuited *in vitro* skins shows that in the latter no H^+ excretion occurs, only the Na^+ transport being maintained under these experimental conditions. From this, one must conclude that the active Na^+ transport is the motive factor of the transport mechanism. H^+ excretion by the *in vivo* skin plays the role of physiologically short-circuiting the Na^+ transport.

1. Introduction

Freshwater organisms are faced with the homeostatic problem of maintaining a high ionic concentration in the internal medium despite the passive diffusive forces acting at their permeable limiting epithelia. The process by which ions from dilute salt solutions are actively taken up by these animals is thus one of considerable importance in general and comparative physiology. Studies of ionic upake in crustaceans (Krogh 1938, 1939; Shaw 1960*a*, *b*, *c*, 1964), insect larvae (Stobbart 1967), teleosteans (Krogh 1937*a*, 1938; Garcia-Romeu & Maetz 1964; Maetz & Garcia-Romeu 1964; Garcia-Romeu & Motais 1966; Kerstetter, Kirschner & Rafuse 1970), adult and larval anurans (Krogh, 1937*b*, 1938; Jorgensen, Levi & Zerahn 1954; Salibian, Pezzani-Hernandez & Garcia-Romeu 1968; Garcia-Romeu, Salibian & Pezzani-Hernandez 1969; Alvarado & Moody 1970) and larval urodeles (Dietz, Kirschner & Porter 1967; Alvarado & Dietz 1970) demonstrate that, under a variety of circumstances, Na^+ and Cl^- can be absorbed by the animal at different rates from dilute solutions of NaCl. In fact, in some cases it has been shown that the absorption of one of these ions may occur unaccompanied by the ion of the opposite charge. The transporting epithelia in these diverse groups, therefore, must be capable of maintaining electroneutrality by exchanging the absorbed ions against counter ions of endogenous origin. It would seem that ion exchange is the basic way in which intact animals perform the uptake of ions from dilute salt solutions (see Keynes 1969). Such a mechanism, permitting the independent regulation of the simultaneous fluxes of different ions, allows for considerable flexibility. Furthermore, this regulatory system has the added advantage of being metabolically inexpensive because, as will be shown in this paper, at least in anurans, H^+ and HCO^- can be used as the endogenous counter ions for the exchange mechanism.

2. Evidence of the presence of independent chloride and sodium transports across anurans skin

Krogh (1937*b*) first showed the existence of independent Cl^- and Na^+ absorption mechanisms in the anuran *Rana esculenta*. He demonstrated that in a calcium chloride solution the external chloride was absorbed by exchange with endogenous HCO_3^-, the Ca^{2+} being impermeant. This stoichiometric relation was not found with sodium chloride solutions. His conclusions were that the Cl^- is absorbed with Na^+ from NaCl solutions, but from KCl, NH_4Cl and $CaCl_2$ it is not accompanied by cations; the Cl^- is in such cases replaced in the outside solution by HCO_3^- (Krogh 1938, 1939).

Table 1. (*a*): net fluxes of Na^+ from NaCl and Na_2SO_4 solutions of 0.43 mmol/l. (*b*): comparison between the Na^+ net fluxes in 0.43 mmol/l NaCl and 1.74 mmol/l Na_2SO_4 solutions.

Net fluxes mmol/h per 100 g (after Garcia-Romeu *et al.* 1969).

(*a*)			(*b*)		
NaCl 0.43 mmol/l	Na_2SO_4 0.43 mmol/l	*d*†	NaCl 0.43 mmol/l	Na_2SO_4 1.74 mmol/l	*d*†
$+7.2 \pm 1.6$ (6)	$+2.5 \pm 1.1$ (6)	-4.7 ± 1.1‡	$+6.2 \pm 2.2$ (4)	$+8.8 \pm 3.4$ (4)	$+2.7 \pm 1.1$

The number of animals is given in parentheses.
† Mean differences of paired data ± s.e.m.
‡ $P < 0.01$.

A further case of absorption of Cl^- or of Na^+ without the accompanying ion has been described in the leptodactylid *Calyptocephalella gayi* '*in vivo*'; the impermeant ions choline and sulphate accompanied in the external solution the chloride and sodium respectively. The net flux of Cl^- from a 0.43 mmol/l solution of sodium chloride was the same as that from a choline chloride solution of equal concentration, namely 10.1 ± 1.8 and 9.9 ± 2.3 μmol/h per 100 g respectively (mean of 6 animals ± s.e.m.). While these data show that Cl^- absorption is independent of the nature of the accompanying cation, those given in table 1 demonstrate that Na^+ absorption is affected by the anion. In order to obtain similar Na^+ net fluxes in sulphate and chloride solution, the Na_2SO_4 solution must be four times more concentrated than that of NaCl (Garcia-Romeu, Salibian & Pezzani-Hernandez 1969). This effect of the sulphate ion on Na^+ transport has already been described in other amphibians, both '*in vivo*' and '*in vitro*' (Gil Ferreira 1968; Aceves, Erlij & Edwards 1968; Alvarado & Stiffler 1970).

Further evidence of the independent absorptions of Cl^- and Na^+ has recently been advanced by Alvarado & Moody (1970) for larvae of *Rana catesbeiana*.

Exchanges between absorbed and endogenous ions also occur when the simultaneous net fluxes of Cl^- and Na^+ are very different, or even in opposite directions. Such fluxes can be obtained by previous adaptation of the experimental animals in solutions lacking either Na^+ or Cl^-. Subsequently measured in NaCl, the absorption of the previously-lacking ion is always greater (figure 1). Table 2 resumes my data and those obtained by Jorgensen *et al.* (1954) for various anuran species. Although the experimental conditions were different, the results show that the relative importance of the Cl^- and Na^+ net fluxes depends on the preadaptation conditions. The role of the skin in the regulation of the ionic composition of the internal medium seems evident.

The independence of the Cl^- and Na^+ absorption mechanisms has also been shown in *C. gayi* by causing a selective inhibition of one of the fluxes without modifying the other (Garcia-Romeu *et al.* 1969). Table 3 shows the effect of procaine sulphate added to the external bath (final concentration 2 mmol/l) and figure 2*a* illustrates a typical experiment. The net flux of Na^+, positive during the control period, suddenly becomes negative after the addition of the inhibitor while the Cl^- net flux remains unchanged. Since procaine sulphate acidifies the bath to about

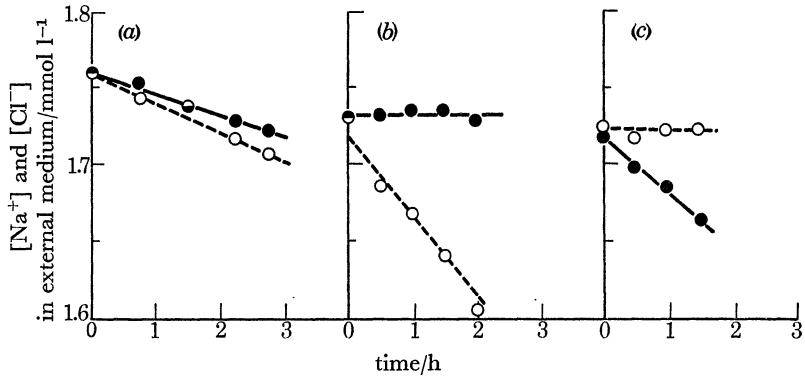

FIGURE 1. The effect of preadaptation on the Na^+ and Cl^- net fluxes of *C. gayi* measured in NaCl solutions of 1.7 mmol/l. ●——●, Na^+; ○– – –○, Cl^-. (*a*) Frog preadapted in NaCl solution. Net flux of Na^+ is +2.3 μmol/h per 100 g; net flux of Cl^- is +3.2 μmol/h per 100 g. (*b*) Frog preadapted in Na_2SO_4 solution. Net flux of Na^+ is 0 μmol/h per 100 g; net flux of Cl^- is +11.8 μmol/h per 100 g. (*c*) Frog preadapted in choline chloride solution. Net flux of Na^+ is 7.1 μmol/h per 100 g; net flux of Cl^- is −0.02 μmol/h per 100 g. (After Garcia-Romeu *et al.* 1969.)

TABLE 2. THE EFFECT OF PREADAPTATION IN DIFFERENT SALT SOLUTIONS ON THE SODIUM AND CHLORIDE NET FLUXES OF DIFFERENT SPECIES OF FROGS

Fluxes measured in NaCl solutions.

species	*n*	pre-adaptation solution	net fluxes μmol/h per 100 g Na^+	net fluxes μmol/h per 100 g Cl^-	difference	reference
R. esculenta	6	Cl-free	+25.4	+50.7	−25.3	Jorgensen *et al.* (1954)
R. temporaria	4	Cl-free	−7.2	+20.3	−27.5	Jorgensen *et al.* (1954)
L. ocellatus	5	Cl-free	+2.9 ± 1.2†	+9.8 ± 1.1‡	−6.9 ± 1.1‡	Salibian *et al.* (1968)
C. gayi	6	Cl-free	+7.4 ± 0.8†	+20.6 ± 1.5†	−13.2 ± 1.5‡	Salibian & Garcia-Romeu (unpublished data)
R. esculenta	6	Na-free	+25.4	−7.7	+17.7	Jorgensen *et al.* (1954)
L. ocellatus	3	Na-free	+8.4 ± 2.2†	+6.2 ± 1.6†	+2.2 ± 0.9‡	Salibian *et al.* (1968)
C. gayi	6	Na-free	+7.8 ± 1.2†	+3.4 ± 1.0†	+4.4 ± 1.1‡	Salibian & Garcia-Romeu (unpublished data)
R. esculenta	4	distilled water	+15.0	+15.8	−0.8	Jorgensen *et al.* (1954)
L. ocellatus	4	NaCl	+3.9 ± 0.4†	+3.6 ± 0.9†	+0.3 ± 0.5‡	Salibian *et al.* (1968)
C. gayi	7	NaCl	+7.5 + 1.3†	+9.8 ± 1.8†	−2.3 ± 0.7‡	Salibian & Garcia-Romeu (unpublished data)

n: number of animals. †: mean ± S.E.M. ‡: Mean difference of paired data ± S.E.M.

pH 3.9, there was a possibility that the inhibition was due to acidification. Experiments in which H_2SO_4 was added to give a pH of 3.9 did in fact demonstrate that acidification does produce a sudden inhibition of Na^+ uptake without affecting the Cl^- uptake. Whether procaine inhibits sodium uptake directly or only in so far as it lowers the pH cannot be decided from these data, but the fact that the Na^+ transport mechanism can be inhibited without affecting the Cl^- uptake remains clear.

Choline pentobarbiturate (final concentration 2 mmol/l) strongly inhibits the Cl^- uptake while leaving the Na^+ uptake unchanged (table 3 and figure 2*b*). The addition of choline pentobarbiturate produces an alkalinization of the external solution; comparable alkalinization brought about by the addition of choline base did not, however, produce an analogous effect on the Cl^- net flux.

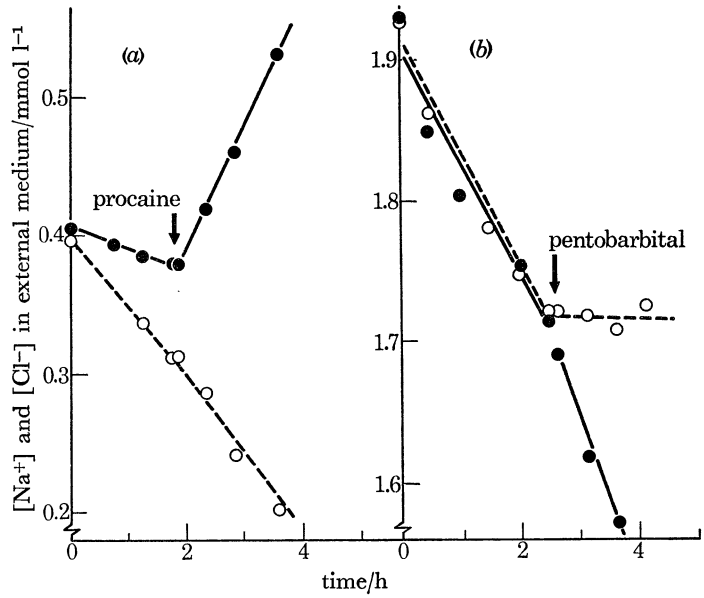

FIGURE 2. The effect of anaesthetics (2 mol/l) added to the external bath on the Na^+ and Cl^- net fluxes of *C. gayi* measured from NaCl solutions. ●——●, Na^+; ○– – –○, Cl^-. (*a*) Inhibition of Na^+ uptake by procaine sulphate. (*b*) Inhibition of Cl^- uptake by choline pentobarbiturate. (After Garcia-Romeu *et al.* 1969.)

TABLE 3. COMPARISON OF THE EFFECTS OF PROCAINE AND PENTOBARBITAL ON Na^+ AND Cl^- NET FLUXES

Mean of net fluxes ± S.E.M. in μmole/h per 100 g. (After Garcia-Romeu *et al.* 1969.)

		Na^+ net flux			Cl^- net flux		
inhibitor	*n*	before	after	*d*†	before	after	*d*†
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§

n: number of experiments.
† Mean differences of paired data before and after inhibition ± S.E.M.
‡ $P < 0.01$.
§: $P < 0.001$.

3. THE ENDOGENOUS IONS EXCHANGED AGAINST SODIUM AND CHLORIDE

Krogh (1937*a*, 1938, 1939) suggested that the Na^+ was exchanged against the ammonium ion, but he did not produce any experimental support for this hypothesis. No direct evidence for this exchange has been advanced, although certain phenomena in some groups of animals lend weight to this hypothesis. Thus, the increase of Na^+ influx and net Na^+ absorption after intraperitoneal injection of an ammonium salt in the goldfish and eel would support a Na^+/NH_4^+ exchange hypothesis (Maetz & Garcia-Romeu 1964; Garcia-Romeu & Motais 1966), although other interpretations cannot be excluded and Kerstetter, Kirschner & Rafuse (1970) postulate a Na^+/H^+ exchange mechanism in the rainbow trout. In other cases, a relationship between the

average NH_4^+ excretion rate and the average Na^+ absorption rate have been shown to exist (Dietz *et al.* 1967; Alvarado & Dietz, 1970*b*).

An average ammonium excretion rate similar to the average sodium absorption rate may favour the Na^+/NH_4^+ exchange hypothesis, but it does not in itself prove that such an exchange occurs. To determine this, the two fluxes must be measured simultaneously on the same animals and their degree of correlation determined. These simultaneous measurements have been made for *Astacus pallipes* by Shaw (1960*b*). His data plotted graphically show that in reality there is no correlation between NH_4^+ excretion and Na^+ uptake (Maetz 1971).

No conclusions, therefore, can be drawn as to the role of the ammonium ion in cationic exchanges. As most freshwater animals are ammoniotelic, it would be very difficult as Maetz (1971) has pointed out, to distinguish between Na^+/NH_4^+ and Na^+/H^+ exchanges.

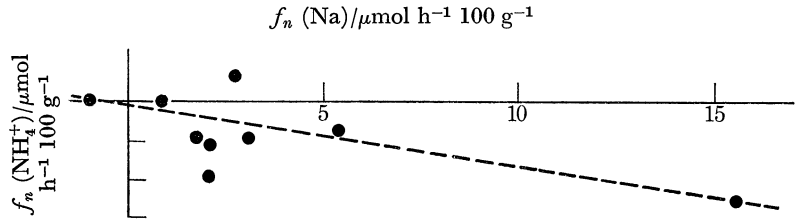

FIGURE 3. Correlation between Na^+ absorption and NH_4^+ excretion through the *in vivo* skin of *L. ocellatus*. (After Garcia-Romeu & Salibian 1968.)

Furthermore, if the NH_4^+ is to participate in cationic exchanges, it is essential that it should cross the epithelial membrane as the ionic NH_4^+. This, however, has never been proved in aquatic animals. This problem is worthy of study, but certain experiments (Garcia-Romeu & Salibian 1968) suggest that in the anuran *Leptodactylus ocellatus* it is the non-ionized NH_3 which crosses the membrane. There is a highly significant correlation between the Na^+ and NH_4^+ net fluxes in this species ($r = 0.78$; $0.001 < P < 0.01$) but the NH_4^+ excretion only accounts for 20% of Na^+ uptake (figure 3). At the time of publication this correlation was explained by a mechanism of regulation of acid-base equilibrium of the cells responsible for the transport of ions, We have since shown, however, that in another leptodactylid the ion exchanged against Na^+ is H^+ and not NH_4^+ (see below). So it would appear probable that the correlation discussed above was due to a pH gradient causing a passive NH_3 loss. It is in this way possible to observe a correlation between Na^+ absorption and NH_3 excretion even when this latter molecule is not participating in the exchange mechanism.

If the ammonium ion is not exchanged against sodium in the Leptodactylidae, one may assume, for reasons of metabolic economy, that H^+ should be the ion concerned. If at the same time Cl^- is exchanged against HCO_3^- the original stoichiometric relation becomes masked, the reaction between HCO_3^- and H^+ resulting in a temporary increase of the CO_2 pressure in the external medium. On the other hand, if the Cl^- and Na^+ are not absorbed simultaneously it should be possible to demonstrate the relation between absorbed and excreted ions. We therefore measured the net fluxes of Na^+, NH_4^+ (or NH_3) and H^+ in *C. gayi* in sodium sulphate solutions. Here again, in this species, it was not possible to discern any stoichiometric relations between Na^+ absorption and NH_4^+ (or NH_3) excretion (figure 4*a*), but on the other hand, an excellent relation between the net fluxes of Na^+ and H^+ was obtained (figure 4*b*; table 4); a 1:1 ratio for these ions and a very high correlation coefficient ($r = 0.98$) clearly show the linking

of H^+ and Na^+ in the exchange system, at any rate under our experimental conditions (figure 5) (Garcia-Romeu *et al.* 1969).

When *C. gayi* pumps Cl^- from choline chloride solutions there is a related base increase in the external bath (figure 6). 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 5; figure 7).

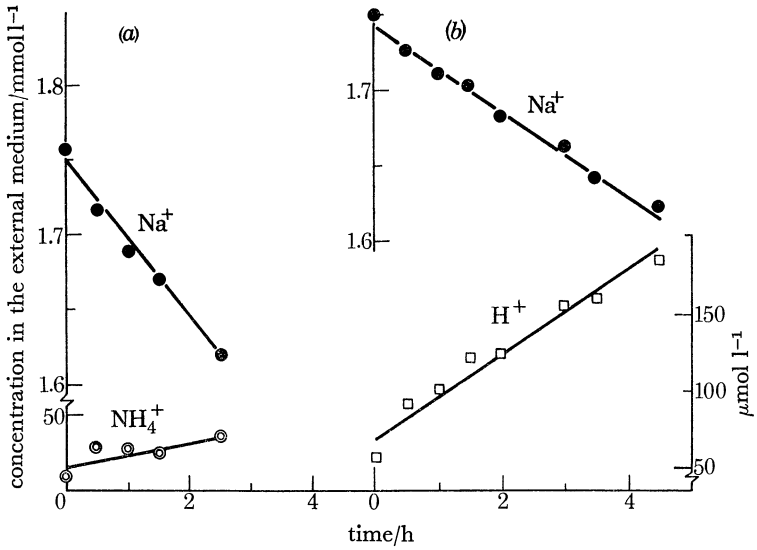

FIGURE 4. Comparison between the excretion of cations and the uptake of Na^+ in two different individual *C. gayi* submerged in Na_2SO_4 solutions. ●——●, Na^+; ◎——◎, NH_4^+; □——□, H^+. (*a*) NH_4^+ excreted against Na^+ taken up. (*b*) H^+ excreted against Na^+ taken up. (After Garcia-Romeu *et al.* 1969.)

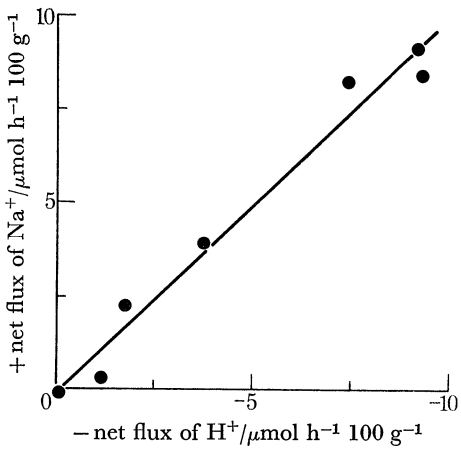

FIGURE 5. Regression line fitted by the method of least squares between Na^+ uptake and H^+ excreted in seven experiments on *C. gayi*. (After Garcia-Romeu *et al.* 1969.)

TABLE 4. COMPARISON BETWEEN THE NET FLUXES OF H^+ (f_n H^+) AND Na^+ (f_n Na^+) IN ANIMALS SUBMERGED IN 1.74 mmol/l Na_2SO_4 SOLUTIONS.

Mean of net fluxes ± S.E.M. in μmol/h per 100 g. (After Garcia-Romeu *et al.* 1969.)

n	f_n Na^+	f_n H^+
7	+4.6 ± 1.5	−4.7 ± 1.5

n: number of experiments.

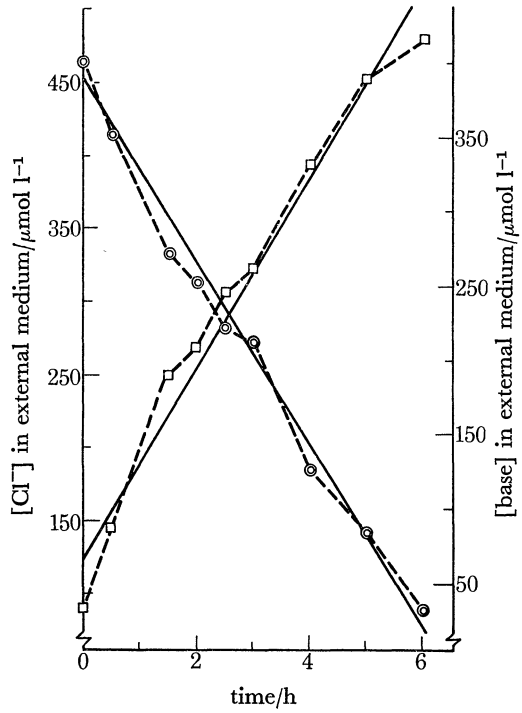

FIGURE 6. Comparison between the excretion of base and the uptake of Cl^- in a frog (*C. gayi*) submerged in choline chloride solution. ◎---◎, Cl^-; □---□, base. (After Garcia-Romeu *et al.* 1969.)

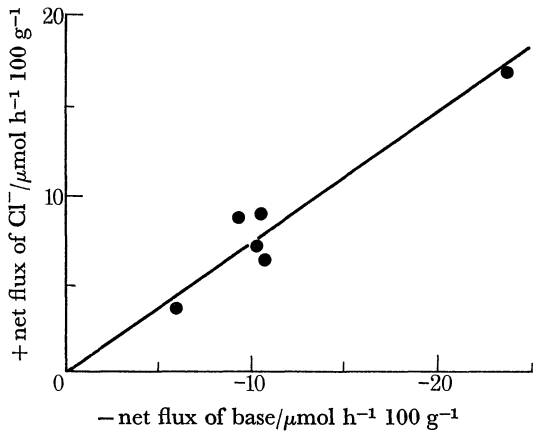

FIGURE 7. Regression line fitted by the same method as in figure 5 between base excreted and Cl^- taken up in six experiments on *C. gayi*. (After Garcia-Romeu *et al.* 1969.)

TABLE 5. COMPARISON BETWEEN THE NET FLUXES OF BASE (f_n B^-) AND Cl^- (f_n Cl^-) IN ANIMALS SUBMERGED IN 0.44 mmol/l CHOLINE CHLORIDE SOLUTIONS

Mean of net fluxes ± S.E.M. in μmol/h per 100 g. (After Garcia-Romeu *et al.* 1969.)

f_n Cl^-	f_n B^-
$+8.6 \pm 1.8$	-11.8 ± 2.5

n: number of experiments.

The pK of the base excreted, graphically determined, is 5.91 ± 0.03 (mean of five experiments $\pm$ S.E.M.). We have similarly determined the pK of choline bicarbonate in mixtures with choline chloride at concentrations equivalent to those of the experimental solutions; the pK thus obtained was 5.90 ± 0.03.

As the exchange proceeds the increase in buffering power reaches -76.3 ± 16.3 μmol of acid pH^{-1} h^{-1} (mean of six experiments + S.E.M.), that is, five times the increase in buffering power obtained when the frogs pump Na^+ from sodium sulphate 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.

Both the buffering capacity of the excreted base and its pK indicate that it is HCO_3^-.

4. Changes of external pH and ionic transport

The excretion of acid or base by the frog skin has been otherwise interpreted by Friedman, Laprade, Aiyawar & Huf (1967*b*) who suggest that the skin by such excretion counters pH

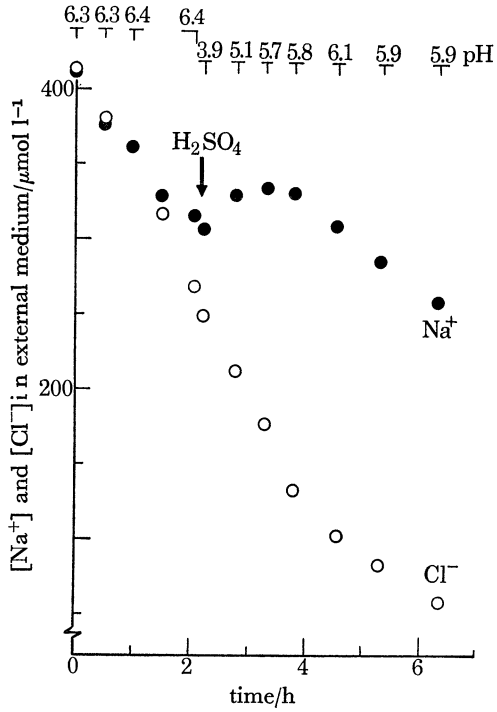

Figure 8. The transient effect of external acidification on the inhibition of Na^+ uptake and the pH evolution when the external bath is a NaCl solution. Frog (*C. gayi*) preadapted in distilled water. ●, Na^+; ○, Cl^-. (After Garcia-Romeu *et al.* 1969.)

variations of the external medium and maintains the pH near neutrality; it has not been possible to confirm their results (Garcia-Romeu *et al.* 1969; Emilio, Machado, & Menano 1970). Figure 8 shows the changes in pH and in the external concentrations of Cl^- and Na^+ before and after acidification to pH 3.9 with H_2SO_4. Before the addition of acid, Cl^- and Na^+ are absorbed simultaneously by the frog (*C. gayi*), the pH remaining constant. After acidification, the Na^+ net flux becomes negative while that of Cl^- is increased. The pH rises progressively and at about 5.7 sodium absorption restarts. Thus, one could conclude that the skin reacts to acidification by base excretion to readjust the pH, but figure 9 shows that in fact this is not a valid interpretation.

When the frog is in a sodium sulphate solution there is no such adjustment of pH after acidification: obviously, when no Cl^- can be absorbed, no external alkalinization can occur. The variations of pH brought about by the skin must thus be interpreted as consequences of the Cl^-/HCO_3^- and Na^+/H^+ exchanges concerned in ionic transport.

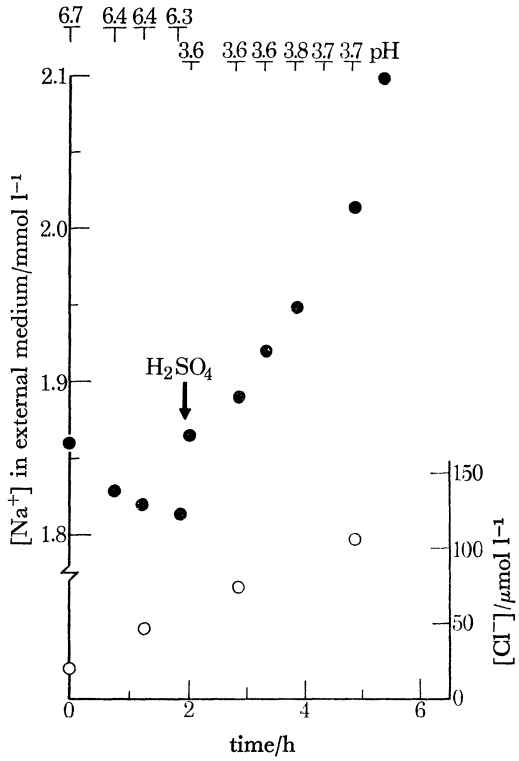

FIGURE 9. The lasting effect of external acidification on the inhibition of Na^+ uptake and the external pH evolution when the external bath is a Na_2SO_4 solution. Frog (*C. gayi*) preadapted in distilled water. Key as in figure 8. (After Garcia-Romeu *et al.* 1969.)

5. SODIUM ABSORPTION AND HYDROGEN EXCRETION OF THE '*IN VIVO*' AND '*IN VITRO*' SKIN

It has long been known that frog skin can establish an H^+ gradient between the solutions bathing its two surfaces, acidifying the external solution and alkalizing the internal one (see Fleming 1957; Friedmann *et al.* 1967*a*, *b*). This phenomenon has been recently studied by Emilio *et al.* (1970) in the isolated skin of *R. ridibunda*. A progressive acidification of the mucous side and an alkalinization of the serous surface were shown to occur over a period of 3 or 4 h resulting in a difference of 3 or even more pH units between the two skin surfaces. The acidification stops at an external pH of approximately 5 but may start again if the solutions are renewed. At a steady pH of 7.0 the H^+ excretion rate (0.07 μmol $cm^{-2}h^{-1}$) was maintained for a rather long period before showing any tendency to decrease. Finally, the absolute amounts of H^+ excreted by the skin were higher in conditions of constant pH than in the 'free-run' experiments.

Emilio *et al.* did not find any relation between the short-circuit current, measured every 30 min, and the H^+ excretion; even the inhibition of H^+ production by Diamox did not inhibit the short-circuit current. Gil Ferreira (1968) showed that in *R. ridibunda* the s.c.c. measures the Na^+ net flux as in other *Rana* species. Since such a relation is only possible if the short-circuited

skin is solely concerned with the net Na^+ transport, one is forced to conclude that during short circuiting the skin no longer transports hydrogen. Also, it is known that the sodium influx/outflux ratio increases considerably under short-circuiting (Ussing & Zerahn 1951; Linderholm 1952). As a consequence of these two phenomena, namely the absence of H^+ net flux and the increase of Na^+ net flux, it can be seen that no relation between the fluxes of these two ions can be found in the short-circuited skin.

The differences in the responses of the '*in vivo*' skin and the '*in vitro*' short-circuited skin are significant in the consideration of certain aspects of the Na^+ and H^+ transport mechanisms and of their interrelation. The work of Emilio *et al.* discussed above, together with all that is known of the responses of '*in vitro*' short-circuited skin thanks to the technique of Ussing & Zerahn (1951), show that there is a net Na^+ transport against an electrochemical gradient when the conditions are such that neither H^+ nor any other ion is being transported. It is evident, therefore, that H^+ transport towards the mucous face is not a necessary condition for net Na^+ transport. The Na^+ transport mechanism, in fact, must be the motive force of the cationic exchange system, and the H^+ excretion must serve to equilibrate the charges, in other words, to act as a physiological short circuit for the sodium transport. If the charges are equilibrated by an external circuit, as in the technique of Ussing & Zerahn, the linking of the two ionic fluxes disappears and only the Na^+ continues to be transported.

6. Ionic exchanges and fixed charges in the membrane

From the independence of Cl^- and Na^+ transport mechanisms in anuran '*in vivo*' skin it might be deduced that the site concerned with cation transport is separated from that concerned with anion transport in the membrane.

The presence of a membrane with fixed charges (see Teorell (1953) and Helfferich (1962) for a general review of the subject) could explain the following characteristics of ionic transport across the skin:

(*a*) Na^+/H^+ *and* Cl^-/HCO_3^- *exchanges*

If pores with a negative charge (cationic exchanger) and others with a positive charge (anionic exchanger) occur in the skin, cation and anion fluxes could be independent of each other. In the ionic exchanger membranes the transport of ions of opposite charge to those fixed on the membrane would be facilitated while that of co-ions would be retarded.

(*b*) *Effects of* pH *on* Na^+ *and* Cl^- *transport*

Ussing (1949) has shown that in the isolated non-short-circuited skin of *R. temporaria* raising the external pH increases the Na^+ influx and diminishes the Cl^- influx; conversely, acidification produces the opposite effect (see also Schoffeniels 1955; Funder, Ussing & Wieth 1967). These results can be explained by the differential effect of pH on the ionization of the positive and negative fixed charges of the membrane.

(*c*) *Development of* '*streaming currents*'

In a membrane performing ionic exchanges, pressure on one of the faces establishes an electrical potential (streaming potential) by shifting the liquid pore, the charge of which is determined by the counter ions. The streaming potential may be short circuited whereupon the current (streaming current) becomes a measure of the excess counter-ion transfer.

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Nutbourne (1968) showed that small hydrostatic pressures on the isolated skin of *R. temporaria* increased the short-circuit current when the pressure was higher on the outside of the skin. These results suggest the presence of negative fixed charges in *R. temporaria* skin. Vargas (1968), in studying the streaming potentials in the giant axon of the squid *Dosidicus gigas*, calculated the density of fixed charges, and Diamond & Harrison (1966) concluded that negatively charged canals exist in the gall bladder from the streaming potentials established upon application of osmotic pressure gradients.

(*d*) *Effects of* pH *on cationic selectivity*

In a cationic exchanger the selectivity sequence for the cations is largely determined by the strength of the negative fixed charge field. A change of pH, altering this field, modifies the selectivity sequence. Acidification, which can cause a complete inversion of the selectivity sequence, changes the preference of the ionic exchanger towards ions of the smallest apparent hydrated diameter (Eisenman 1961, 1962; Diamond & Wright 1969). Pesente (1969*a*, *b*) states that at pH 5.0 the permeability order for the alkaline cations of the isolated skin of *R. esculenta* shifts to one in which the ion with the smallest hydrated diameter is preferred.

All these results suggests that on the path of Na^+, towards the transport compartment, there are sites at which negative fixed charges play an important role. In this connexion it is interesting to note that Cereijido & Rotunno (1968) propose a model in which negative fixed charges direct the Na^+ towards the pumping sites (see also Cereijido, Reisin & Rotunno 1968).

As to the Cl^- transport mechanism, our understanding is severely limited by the fact that, except in certain rare cases, this ion is passively transported '*in vitro*' (Koefoed-Johnsen, Ussing & Zerahn 1952). It would seem that the Cl^- transport mechanism is irreversibly damaged by '*in vitro*' techniques.

7. Conclusions

The occurrence of Na^+/H^+ and Cl^-/HCO_3^- exchanges, enables the skin to play a role in the regulation of Na^+ and Cl^- ions in the internal medium by permitting it to transport different amounts of the two ions. The question remains whether all the sodium and chloride absorbed is exchanged against endogenous ions or whether the exchange mechanisms only serve to equilibrate any difference between amounts of Na^+ and Cl^- pumped.

Although we have stressed here the occurrence of mechanisms permitting the independent uptake of sodium and chloride, it should be pointed out that under normal physiological conditions sodium and chloride uptake are never completely isolated from one another. The question arises as to the level at which regulatory integration of the uptake occurs: to what extent and in what way it is brought about directly by the skin and to what extent superimposed integrations phenomena determine the characteristics of the system. More detailed comparisons of the responses of *in vivo* and *in vitro* skins will elucidate this problem. Before such comparisons can yield valid conclusions, however, the reasons why the Cl^-, actively transported *in vivo* (Jorgensen *et al.* 1954), behaves passively according to its electrochemical gradient *in vitro* (Koefoed-Johnsen *et al.* 1952) must be ascertained. In this connexion, the few studies reporting active *in vitro* Cl^- transport (Zadunaisky, Candia & Chiarandini 1963; Zadunaisky & De Fisch 1964; Martin 1964; Martin & Curran 1966) are particularly significant.

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