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ASPECTS OF IONIC TRANSPORT MECHANISMS IN CRAYFISH *ASTACUS LEPTODACTYLUS*

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SUMMARY

1. During the uptake of chloride from an external choline chloride solution, electroneutrality appears to be preserved by excretion of base, but base excretion independent of chloride absorption also occurs.

2. Sodium ion uptake from sodium sulphate solutions is compensated by excretion of hydrogen ions, but this has been established only in animals adapted to distilled water.

3. In animals preadapted to running water, the sodium ion influx can be abolished almost totally by amiloride without diminishing ammonium excretion.

4. The net fluxes of sodium and chloride are inhibited by acetazolamide and cyanide.

5. These results are interpreted as indicating that Na^+ and Cl^- are absorbed chiefly in exchange for H^+ and HCO_3^-, and are discussed in relation to an exchange diffusion mechanism.

INTRODUCTION

Crayfish can absorb Na^+ and Cl^- from dilute solutions, principally through the gills (Krogh, 1939; Koch, Evans & Schicks, 1954; Bryan, 1960*b*; Shaw, 1960*a*, *b*; Bielawski, 1964). Krogh found that the amounts of sodium and chloride absorbed were not necessarily equal. In experiments where the accompanying ion was impermeant it was possible to deduce that an endogenous ion of the same charge as that absorbed must be released in order to preserve electroneutrality.

Krogh suggested that the ion exchanged for Na^+ might be NH_4^+ in ammonotelic animals such as teleosts and crustacea. This proposal has found some experimental support (Shaw, 1959, 1960*a*, *b*; Maetz & Garcia-Romeu, 1964). However, ammonium excretion appears to be independent of the Na^+ concentration of the medium in crayfish, carp and trout (Shaw, 1960*b*; De Vooys, 1968; Kerstetter, Kirschner & Rafuse, 1970) and acetazolamide (Diamox) reduces the Na^+ flux without inhibiting the release of NH_4^+ (Kerstetter *et al.* 1970).

The possibility therefore arises that H^+ rather than NH_4^+ is exchanged for Na^+ in ammonotelic animals, as in the ureotelic frogs studied by Garcia-Romeu, Salibian & Pezzami-Hernandez (1969) and by Garcia-Romeu & Ehrenfeld (1972). Maetz, however, has found that in goldfish sodium absorption is correlated with the sum of the equivalents of H^+ and of NH_4^+ excreted (1973).

The work cited above on frogs showed that in these animals there is an exact stoichiometry between the chloride absorbed and the excreted base, which probably consists of bicarbonate.

The present work concerns the nature of the excretion of endogenous ions in exchange for Na^+ and Cl^- in the crayfish *Astacus leptodactylus leptodactylus*.

MATERIAL AND METHODS

The crayfish (*Astacus leptodactylus leptodactylus*), average weight 70 g were obtained from Turkey. They were kept unfed at a constant temperature (15 ± 1 °C) in tanks of running water. Before certain experiments they were preadapted for 10 days in distilled water which was renewed each day.

On the day of an experiment the animals were weighed and their nephropores blocked with dentists' cement in order to avoid contamination of the external solution by the urine. They were then placed in individual aquaria with 230 ml of the experimental solution. The temperature was maintained at 15 ± 1 °C. Ten ml samples were taken at the beginning and during the experiment which generally lasted 3 h. To all the experimental solutions 2·5 mM/l of Tris was added and the pH was adjusted to 7·0 with maleic acid. The pH was also controlled during the experiments.

Flux measurements

The netfluxes* of sodium, chloride, ammonium, hydrogen and base were calculated from the slopes of the lines representing the variation of external ionic concentration as a function of time. The sodium concentration of the sample was measured by flame photometry with an Eppendorf photometer and the chloride concentration with an Aminco Cotlove electrometric chloride titrator. Total ammonium concentrations ($NH_3 + NH_4^+$) in the external bath samples were determined with a Technicon auto-analyser (Logsdon, 1960; Assous, Dreux & Girard, 1960).

The sodium and chloride influxes (F_{in}) were calculated from the slopes of the curves representing the disappearance of ^{24}Na and ^{36}Cl from the external medium as a function of time, taking into account the specific activities of the radioisotopes.

$$F_{in} = \frac{\Delta Q/\Delta t}{Q/E},$$

where $\Delta Q/\Delta t$ is the variation of the quantity of radioactive isotope during the desired time unit and Q/E the specific radio-activity (RAS) of the ion in question (i.e. ratio of radioactive to total no. of atoms). The ^{24}Na (CEN Saclay) was provided in the form of $^{24}NaHCO_3$ and neutralized with 0·1 N-HCl. It was added to the external bath to give a final concentration of 0·2 mCi/l. The ^{36}Cl (Radiochemical Centre, Amersham) was obtained from a $H^{36}Cl$ solution neutralized with NaOH. It was added to the external medium to a concentration of 4 μCi/l. ^{24}Na activity was counted in 2 ml of sample in a Mecaserto well-counter type MO 13/100. The ^{36}Cl was counted in a Nuclear Chicago Scintillation Counter (Mark I, model 6860): 1 ml of the sample was

* The netflux (F_n) of an ion is considered positive when there is a net absorption of this ion by the animal, and negative when there is a loss of the ion into the external medium.

ded to 10 ml of the scintillation liquid (Bray, 1960). The count of each sample was corrected for quenching by the use of a standard quenching correction curve.

The sodium and chloride effluxes (F_{out}) were taken as the differences between the respective netfluxes and influxes.

Since the gills of the crayfish are the site of respiratory CO_2 elimination, the samples had air bubbled through them for at least 30 min before any titration, in order to eliminate all volatile acidity. The hydrogen ion concentration was measured by titration with a 5 mM-NaOH solution and the base concentration with a 3 mM-HCl solution. The measurements were made on 2 ml of sample with a Radiometer TT2 automatic titrator linked to an automatic burette and a SB R 2C recorder.

All fluxes are expressed in μ-equiv./h/100 g.

Measurement of the anhydrase activity of the gill

Gills were isolated, perfused with a 9 % NaCl solution, dried and weighed. They were then ground in a teflon homogenizer, and the anhydrase activity measured by a manometric technique (Maetz, 1956; Istin & Girard, 1970) and expressed in international activity units per mg protein (Maren, 1967). The protein concentration of the homogenates was obtained by the so-called Folin technique.

Inhibitors utilized

Amiloride (Merck Sharp and Dohme Research Laboratory, West Point) was used at a concentration of 5×10^{-5} M in the external medium. Diamox (Acetazolamide, Theraplix Laboratory) was injected to give a final concentration of 2×10^{-4} M/1000 g animal. Potassium cyanide was added to the external medium to give a concentration of 5×10^{-4} M/l.

RESULTS

I. *Chloride/base exchanges from a choline chloride solution*

Table 1 summarizes the data on chloride influxes and effluxes, and netfluxes of chloride and base for a group of 13 animals placed in a 1800 μ-equiv./l choline chloride solution buffered at pH 7·0. The chloride influx is three times the netflux. There is thus a considerable chloride efflux. The netflux for base excretion is significantly higher than the netflux for Cl^- absorption. To demonstrate any correlation which may exist these two netfluxes the data for base excretion as a function of Cl^- absorption have been plotted in Fig. 1. The regression coefficient is $-1{\cdot}21 \pm 0{\cdot}15$, which is not significantly different from 1. The ordinate value when the abscissa is 0 is $16{\cdot}6 \pm 5{\cdot}1$ μ-equiv./h/100 g, representing a measurable base excretion which is independent of the Cl^- absorption. The excess of base excretion above this value bears a 1:1 relation to the chloride absorption.

II. *Sodium/hydrogen exchanges from a Na_2SO_4 solution*

To demonstrate the Na^+/endogenous ion exchange mechanism experimentally, the Na^+, H^+ and ammonium netfluxes were measured when the Na^+ was being absorbed from a Na_2SO_4 solution buffered at pH 7·0, the SO_4 ion being impermeant.

To check that the sulphate ion is truly impermeant the concentration of labelled

Table 1. *Chloride absorption and base excretion in* Astacus leptodactylus

(Crayfishes preadapted to running water. Fluxes measured in a buffered choline chloride solution (1800 μ-equiv./l) of pH 7·0. Fluxes expressed as μ-equiv./h/100 g (mean ± standard deviation of the mean).)

$F_{in}Cl^-$	$F_{out}Cl^-$	F_nCl^-	F_n base
147·8 ± 4·8 (n = 13)	94·7 ± 4·5 (n = 13)	+53·1 ± 2·8 (n = 13)	−80·8 ± 4·9 (n = 13)

$F_{in}Cl^-$, F_{out} Cl^-, F_nCl^- = influx, efflux and netflux of chloride.
F_n base = netflux of base.
Number of animals in parentheses.

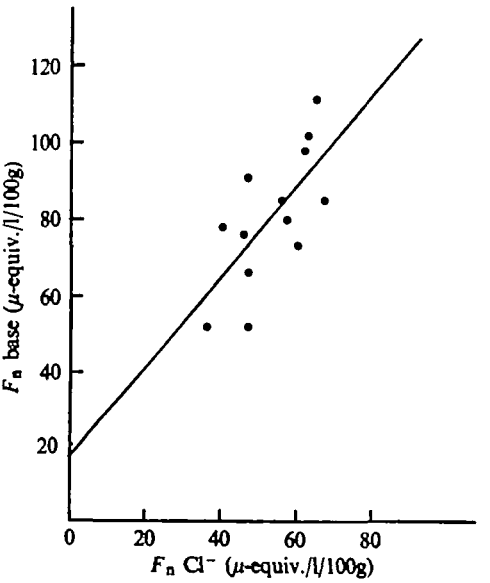

Fig. 1. Transepithelial netflux of chloride as a function of base excretion in *Astacus lactodactylus in vivo*. Animals were pre-adapted in running water. Fluxes are measured in a choline chloride solution (1800 μ-equiv./l) buffered to pH 7·0. F_n base = 1·21, F_n Cl^- + 16·60.

sulphate in the external medium was controlled throughout an experiment (4 h) and found to remain unchanged.

Table 2 A (control period) summarizes results from a group of nine animals previously adapted to distilled water. It shows that the Na^+ netflux is slightly higher than the H^+ excretory netflux, and the excretory ammonium netflux is half that of the Na^+ netflux. No correlation between Na^+ absorption and ammonium excretion could be seen, but on the other hand, there was a good correlation between the Na^+ and H^+ netfluxes, as can be seen in Fig. 2. The slope of the regression line is −1·24 ± 0·13 which is not significantly different from 1. The ordinate value when the abscissa is 0 is −12·56 μ-equiv./h/100 g indicating that a measurable base excretion occurs even in the absence of a Na^+ netflux. Table 2 B summarizes the data from the group of 6 animals adapted in running water. A comparison of Table 2 A and B shows that preadaptation to distilled water causes a slight increase of the Na^+ influx, while the Na^+ efflux is higher in a running-water adapted animal than in a distilled-wat

Table 2. *Effect of Amiloride on the sodium absorption, acid excretion and ammonium excretion in* A. leptodactylus

A, Crayfishes preadapted to distilled water for a week.
B, Crayfishes preadapted to running water.
(Fluxes measured in a Na_2SO_4 (1800 μ-equiv./l) solution buffered at pH 7·0. Amiloride added to the external bath (final concentration 5×10^{-5} M). Fluxes expressed as μ-equiv./h/100 g (mean ± standard deviation of the mean).)

	$F_{in}Na^+$	$F_{out}Na^+$	F_nNa^+	F_nH^+	F_n ammonium
A (n = 9)					
Control period	43·3 ± 3·2	13·6 ± 2·2	+29·7 ± 3·4	−22·1 ± 4·7	−14·0 ± 2·7
Amiloride	0·7 ± 0·5	10·5 ± 1·1	−9·9 ± 1·0	+13·2 ± 3·1	−8·2 ± 1·8
Difference*	−42·6 ± 3·3	−3·1 ± 2·5	−39·6 ± 2·9	+35·3 ± 4·0	−5·8 ± 1·5
	$P < 0·001$		$P < 0·001$	$P < 0·001$	$P < 0·01$
B (n = 6)					
Control period	36·4 ± 5·2	21·1 ± 4·2	+15·3 ± 2·3	+20·1 ± 4·6	−6·7 ± 1·4
Amiloride	0·9 ± 0·6	22·1 ± 3·1	−21·2 ± 3·3	+32·3 ± 4·8	−5·5 ± 1·1
Difference*	−35·5 ± 4·9	+1·0 ± 3·3	−36·5 ± 3·4	+12·2 ± 6·2	−1·2 ± 1·7
	$P < 0·001$		$P < 0·001$	$P < 0·1$	

F_{in} Na^+, F_{out} Na^+, F_n Na^+ = influx, efflux and netflux of sodium.
F_n H^+, F_n ammonium = netflux of H^+ and ammonium.
Number of animals in parentheses.
* Mean of the difference between paired data ± S.E.M.

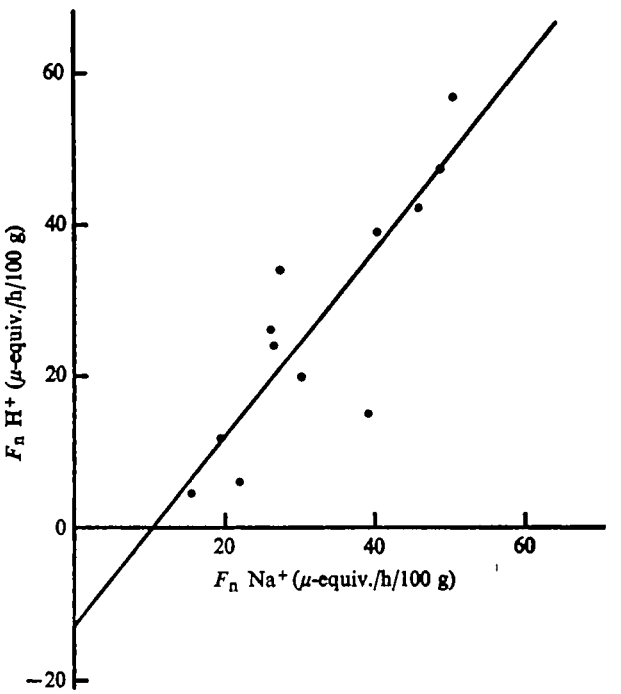

Fig. 2. Transepithelial netfluxes of Na^+ as a function of acid excretion in *Astacus leptodactylus in vivo*. Animals were preadapted in distilled water. Fluxes are measured in a Na_2SO_4 solution (1800 μ-equiv./l) buffered to pH 7·0. F_n H^+ = 1·24, F_n Na^+ − 12·56.

adapted one. The excretory ammonium flux was $-6{\cdot}7 \pm 1{\cdot}4$ μ-equiv./h/100 g, which is half that of the distilled water-adapted animal. The data in Table 2B obtained by titration of the external medium would appear to contradict the data of Table 2A. Thus for animals adapted in running water (Na^+ netflux relatively low) there is no increase of the H^+ concentration in the external medium but rather a reduction. Figs. 1 and 2 reveal the presence of a considerable base excretion into the external medium independent of the Na^+ or Cl^- netfluxes. It is to be expected therefore that this titrable base neutralizes the H^+ excreted in relation to Na^+ transport and thus masks the Na^+/H^+ exchange mechanism. The use of an inhibitor specific for Na^+, which also blocks the Na^+/H^+ exchange mechanism (Kirschner, 1973; Garcia-Romeu & Ehrenfeld, unpublished data) affords additional proof of the existence of this exchange mechanism in variously preadapted crayfish.

III. *Action of Amiloride*

Table 2 shows that at a concentration of 5×10^{-5} M amiloride inhibits the Na^+ influx almost completely (99 %). The Na^+ netflux becomes strongly negative, its value approaching that of the efflux which remains unchanged. In distilled water adapted animals amiloride totally inhibited hydrogen excretion: the H^+ netflux which had been at $-22{\cdot}1$ μ-equiv./h/100 g became $+13{\cdot}2$ μ-equiv./h/100 g: a change of $+35{\cdot}3$ μ-equiv./h/100 g. This value is in good agreement with the Na^+ netflux in the control period ($+29{\cdot}7$ μ-equiv./h/100 g). It should be noted that when amiloride blocks all sodium transport the H^+ excretory netflux of $13{\cdot}2$ μ-equiv./h/100 g is equivalent to the value of titrable base in the external medium: this base excretion in the absence of sodium net absorption has already been discussed (Fig. 2). With running water adapted animals the Na^+ netfluxes were less important than those of distilled water adapted individuals and thus the Na^+/H^+ exchange would be more likely to be masked by the titrable base excretion. The difference between the netfluxes of base excretion during the control period and after addition of amiloride, however, provides a value for the quantity of H^+ excreted which can be compared with the quantity of Na^+ absorbed during the control period. Table 2B shows that this difference of $+12{\cdot}2$ μ-equiv./h/100 g agrees well with the $+15{\cdot}3$ μ-equiv./h/100 g value of the netflux for sodium absorption.

A fact which must be noted is that the inhibitor also causes a 40 % inhibition of the ammonium excretion in distilled water adapted animals.

IV. *Action of Diamox*

Diamox, well known as an inhibitor of carbonic anhydrase has an inhibitory action not only on Na^+ and Cl^- transports but also on the excretion of endogenous ions (Garcia-Romeu *et al.* 1972; Garcia-Romeu & Ehrenfeld, unpublished data). The action of this inhibitor was first tested in relation to the absorption of Na^+ and Cl^- from an NaCl solution buffered at pH 7·0. Table 3 shows that intraperitoneal injection of Diamox (2×10^{-4} M) strongly inhibits the netfluxes of Na^+ and Cl^- which become negative. The parallel inhibition of the two netfluxes suggested that the agent had the same action on the unidirectional fluxes of the two ions, but a study of Table 3 shows that this is not the case: the two transport mechanisms are independently affected. While the inhibition of the Na^+ netflux is due to a considerable reduction of the influx

Table 3. *Effect of injection of Diamox* (2×10^{-4} M) *on the sodium and chloride fluxes of* Astacus leptodactylus

(Crayfishes preadapted to running water. Fluxes measured in a NaCl solution (1800 μ-equiv./l) buffered at pH 7·0. Fluxes expressed as μ-equiv./h/100 g (mean ± standard deviation of the mean).)

	F_{in}	F_{out}	F_n
Na ($n = 7$)			
Control period	67·8 ± 6·5	21·7 ± 2·8	+46·1 ± 7·6
Diamox	19·6 ± 1·4	28·7 ± 3·1	−9·1 ± 3·0
Difference*	−48·2 ± 5·7	+7·0 ± 3·0	−55·2 ± 8·2
	$P < 0.001$		$P < 0.001$
Cl ($n = 7$)			
Control period	161·8 ± 6·9	103·0 ± 8·9	+58·9 ± 6·9
Diamox	202·1 ± 19·6	212·4 ± 19·4	−10·3 ± 3·6
Difference*	+40·3 ± 15·7	+109·4 ± 19·6	−69·2 ± 6·4
	$P < 0.05$	$P < 0.01$	$P < 0.001$

F_{in}, F_{out}, F_n = influx, efflux and netflux.
Number of animals in parentheses.
* Mean of the difference between paired data ± S.E.M.

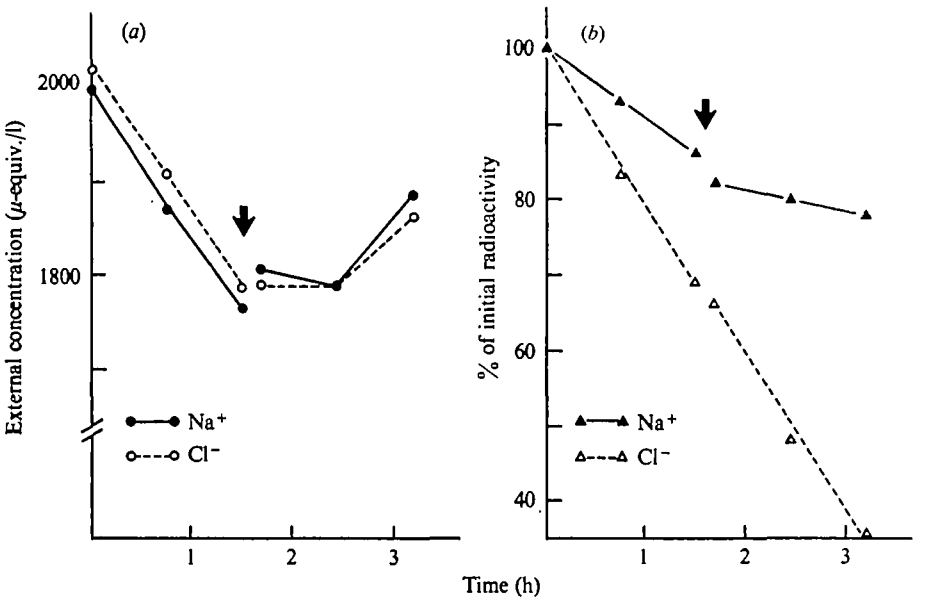

Fig. 3. Effect of injection of Diamox (2×10^{-4} M) on the Na^+ and Cl^- fluxes of *Astacus leptodactylus*: Fluxes are measured on the same animal in a NaCl solution (1800 μ-equiv./l) buffered to pH 7·0. The arrows indicate the injection of the inhibitor. (*a*) Netfluxes of sodium and chloride plotted against time. (*b*) Influxes of Na^+ and Cl^- plotted against time.

(71 % inhibition), that of the Cl^- netflux is caused by an increase of the Cl^- efflux (200 %), the influx increasing slightly. Fig. 3 illustrates an individual experiment.

When Na^+ uptake is from a sodium sulphate solution (1800 μ-equiv.; pH 7·0) Diamox simultaneously inhibited both Na^+ and H^+ netfluxes (see Table 4A). The Na^+ netflux became negative owing to a 82 % inhibition of the influx. Table 4 also shows that the NH_4^+ netfluxes were strongly inhibited by Diamox.

Table 4. *Effect of injection of Diamox* (2×10^{-4} M) *on the sodium and chloride fluxes and ammonium excretion of* Astacus leptodactylus

A, Animals preadapted in distilled water. Sodium fluxes measured in Na_2SO_4 solution (1800 μ-equiv./l) buffered to pH 7·0.
B, Animals preadapted in running water. Chloride fluxes measured in choline chloride solution (1800 μ-equiv./l) buffered to pH 7·0.
(Fluxes expressed as μ-equiv./h/100 g (mean ± standard deviation of the mean).

A (n = 5)	F_{in} Na^+	F_{out} Na^+	F_n Na^+	F_n H^+	F_n ammonium
Control period	73·4 ± 10·3	20·6 ± 2·7	+52·8 ± 8·5	−61·2 ± 6·6	−18·9 ± 1·4
Diamox	12·7 ± 1·3	37·4 ± 14·5	−24·7 ± 15·2	+5·9 ± 4·2	−4·5 ± 0·6
Difference*	−60·7 ± 9·5	+16·8 ± 15·4	−77·5 ± 18·2	−67·1 ± 7·3	−14·4 ± 1·6
	$P < 0·01$		$P < 0·02$	$P < 0·001$	$P < 0·001$
B (n = 5)	F_{in} Cl^-	F_{out} Cl^-	F_n Cl^-	F_n base	F_n ammonium
Control period	137·1 ± 6·7	87·0 ± 7·1	±50·1 ± 5·2	−79·6 ± 8·0	−4·1 ± 0·4
Diamox	205·6 ± 26·1	216·2 ± 26·6	−10·6 ± 2·5	−15·6 ± 2·4	−2·9 ± 0·5
Difference*	+68·5 ± 23·8	129·2 ± 31·2	−60·7 ± 6·6	−64·0 ± 7·4	−1·2 ± 0·5
	$P < 0·05$	$P < 0·02$	$P < 0·001$	$P < 0·001$	

F_{in}, F_{out}, F_n = influx, efflux and netflux.
Number of animals in parentheses.
* Mean of the difference between paired data ± S.E.M.

Table 5. *Effect of KCN* (5×10^{-4} M) *on* Na^+ *and* Cl^- *fluxes of* Astacus leptodactylus

(Animals preadapted in running water. Fluxes measured in a chloride sodium solution (1800 μ-equiv./l) buffered to pH 7·0. KCN added to the external bath (final concentration 5×10^{-4} M). Fluxes expressed as μ-equiv./h/100 g (mean ± standard deviation of the mean).)

	F_{in} Na^+	F_{out} Na^+	F_n Na^+
Na^+ (n = 4)			
Control period	45·1 ± 12·2	20·4 ± 6·3	+24·7 ± 7·8
KCN (1st period), $t = 0$, $t = 45'$	12·4 ± 1·7	28·4 ± 7·1	−16·0 ± 7·8
KCN (2nd period), $t = 45'$, $t = 90'$	9·3 ± 0·5	21·2 ± 3·0	−12·0 ± 2·9
Difference* (control, 2nd period)	−35·8 ± 12·0 $P > 0·05$	+0·8 ± 3·9 NS	−36·7 ± 10·2 $P < 0·05$
	F_{in} Cl^-	F_{out} Cl^-	F_n Cl^-
Cl^- (n = 4)			
Control period	138·6 ± 4·8	77·7 ± 9·9	+60·8 ± 10·1
KCN (1st period), $t = 0$, $t = 45'$	86·3 ± 13·9	67·3 ± 16·8	+19·8 ± 7·0
KCN (2nd period), $t = 45'$, $t = 90'$	63·6 ± 2·3	71·9 ± 6·5	−8·3 ± 8·5
Difference* (control, 2nd period)	−75·0 ± 2·5 $P < 0·001$	−5·8 ± 14·6 NS	−69·1 ± 14·0 $P < 0·02$

F_{in}, F_{out}, F_n = influx, efflux and netflux.
Number of animals in parentheses.
* Mean of the difference between paired data ± SEM.

Table 6. *Excretion of total ammonium in different experimental conditions in* Astacus leptodactylus

A, Animals preadapted for a week in distilled water.
B, Animals preadapted in running water.
(Excretion of ammonium measured in a Na_2SO_4 solution (1800 μ-equiv./l) or a choline chloride solution (1800 μ-equiv./l) both buffered to pH 7·0. Fluxes as μ-equiv./h/100 g (mean ± standard deviation of the mean).)

Excretion measured in a Na_2SO_4 solution		Excretion measured in a choline chloride solution	
A	B	A	B
$-13{\cdot}8 \pm 2{\cdot}0$ ($n = 9$)	$-6{\cdot}3 \pm 1{\cdot}0$ ($n = 8$)	$-5{\cdot}0 \pm 1{\cdot}4$ ($n = 6$)	$-4{\cdot}8 \pm 0{\cdot}5$ ($n = 9$)

Number of animals in parentheses.

In animals absorbing Cl^- from a dilute choline chloride solution (1800 μ-equiv.; pH 7·0) injection of Diamox inhibits Cl^- absorption and base excretion, as can be seen in Table 4B. It should, however, be noted that although the base excretion is greatly reduced (by 80 %) a certain fraction of this endogenous ion excretion continues to alkalize the external medium independently of any net Cl^- absorption. This fact is in agreement with the results discussed above in relation to Fig. 1, in which the 16·6 μ-equiv./h/100 g at the origin indicates a base excretion independent of net Cl^- absorption.

As already mentioned, the inhibition of the Cl^- net flux is the result of an increase of the Cl^- efflux, not of a reduction of the influx, which on the contrary increases significantly.

V. *Effect of KCN on Na^+ and Cl^- transports*

The action of KCN on Na^+ and Cl^- uptake from a NaCl solution (1800 μ-equiv.; pH 7·0) was studied to throw light on the nature of the ionic transport mechanisms. Table 5 gives results obtained from a group of four animals preadapted in tap water.

The Na^+ and Cl^- netfluxes were strongly inhibited, becoming negative, after addition of KCN to the external medium. The effluxes of Na^+ and Cl^- were not affected by the inhibitor but the Na^+ influx was reduced by 79 % and the Cl^- influx by 37 %. The chloride influx and efflux values after KCN addition were not significantly different.

DISCUSSION

Independence of the Na^+ and Cl^- transport mechanisms. Exchange against endogenous ions

The results show that the Na^+ and Cl^- absorption mechanisms of *Astacus leptodactylus* can be completely independent (compare Tables 1 and 3). When the ion accompanying the Na^+ or Cl^- in the external solution is an impermeant one (i.e. $SO_4^=$ or choline), Na^+ and Cl^- must necessarily be exchanged against an endogenous ion of the same charge to maintain the electroneutrality of the solutions. Shaw (1960*b*) suggested that *Astacus pallipes* exchanged Na^+ against the ammonium ion or hydrogen according to its metabolic activity and the level of excretion of these ions.

Table 6 summarizes the data for ammonium excretion under different experimental

conditions. In all the cases except one it can be seen that ammonium excretion is independent of preadaptation or the presence of external Na^+. Thus, irrespective of preadaptation, the Na^+ netfluxes are much higher than the ammonium excretion values, and no correlation was found between Na^+ absorption and NH_4^+ excretion. Table 6 shows, however, that the ammonium excretion of animals adapted in distilled water and studied in sodium sulphate is twice that of animals in other experimental conditions. Part of the NH_4^+ excretion of the distilled water adapted animals is inhibited by amiloride and Diamox (Table 2A and 4A). In exceptional conditions, therefore, it would seem that part of the ammonium excretion may be in exchange for Na^+, but this Na^+/NH_4^+ exchange would only represent about 20% of the total net Na^+ absorption in these animals.

The ratio of 1:21 between hydrogen excretion and Na^+ absorption indicates a Na^+/H^+ exchange mechanism with a 1 to 1 relationship. This exchange mechanism is best seen in distilled water adapted animals but more difficult to demonstrate in tap water adapted individuals where the net Na^+ absorption is lower. With the titration method used here, the excretion of a titrable base (HCO_3^-, OH^-, organic base) results in a reduction of external acidity and masks the Na^+/H^+ exchange. The fact that amiloride inhibits both Na^+ netflux and H^+ netflux to the same extent confirms the presence of a Na^+/H^+ exchange irrespective of preadaptation (Table 2). It is impossible to decide by titration of the external medium, however, whether the acidity resulting from Na^+ absorption is due to H^+ excretion or possibly to the absorption of OH^- ions along with the Na^+ (cf. Garcia-Romeu & Ehrenfeld, unpublished data). A Na^+/H^+ exchange has also been recorded in many ureotelic or ammonotelic animals (Stobbart, 1971; Garcia-Romeu *et al.* 1969; Garcia-Romeu & Ehrenfeld, 1972; Maetz, 1972). Kirschner, Greenwald & Kerstetter (1973) in a comparative study of three freshwater animals (frog, trout, crayfish) found evidence for Na^+/H^+ exchange mechanisms. In our experiments we found a correlation between Na^+ netflux and net H^+ excretion whereas the above authors found a good correlation between Na^+ influx and H^+ netflux. This discrepancy between my results and those of Kirschner *et al.* may be due to the use of different methods for measuring the external acidity. It still remains a possibility that under our conditions the exchange is a Na^+ influx/H^+ efflux one. In this case, if the Na^+ efflux were to be accompanied by an equivalent HCO_3^- efflux, part of the H^+ efflux exchanged against the Na^+ influx would be neutralised by the bicarbonate and the experimentally discernible process would be an apparent Na^+ netflux/H^+ netflux exchange. The fact that the Na^+ and base effluxes are equal after addition of amiloride to the external medium would argue in favour of such a possibility (Table 2).

Chloride transport

The absorption of Cl^- from a choline chloride solution implies that the chloride is exchanged against an endogenous ion of the same charge. Our data give a good correlation between net Cl^- absorption ($F_n Cl^-$) and endogenous base excretion (Fig. 1); the slope of the regression line is not significantly different from 1, indicating a 1 for 1 exchange mechanism. Such a Cl^-/base exchange mechanism has been proposed for *Astacus pallipes* (Shaw, 1960), several species of frog (Krogh, 1937; Garcia-Romeu *et al.* 1969; Garcia-Romeu & Ehrenfeld, 1972) and an insect larva (Stobbart, 1971). Although no direct evidence was given by these workers as to the nature of

endogenous base excreted it is probable that bicarbonate ions are involved. The present experimental technique, does not determine the nature of the excreted base. The alkalinisation of the external medium could also be the result of a simultaneous absorption of Cl^- and H^+ although this is unlikely (Garcia-Romeu & Ehrenfeld unpublished data). The presence of a Cl^-/HCO_3^- exchange would seem to be the most probable explanation: the action of Diamox on the Cl^- transport argues in favour of this hypothesis (see below). The base excretion is 34 % higher than the net Cl^- absorption (Fig. 1: ordinate -17 μ-equiv./h/100 g when abscissa is 0). A base excretion independent of net Cl^- absorption therefore exists; the nature of this titrable base remains to be determined as also that of the accompanying cation. The possibility of an excretion of $NaHCO_3$ has already been considered: other cations such as potassium may accompany this endogenous base excretion. It is also possible that ammonia is in part responsible for the alkalinization of the external medium. If ammonia, as non-ionized NH_3, is excreted into a medium of pH 7·0, because of its pK of 9·3 it will act as a hydrogen ion trap and cause an alkalinization of the external medium.

Action of Diamox

Our results show that Na and Cl ionic fluxes are inhibited by Diamox. These results obtained with *Astacus leptodactylus* agree with those from *R. esculenta* (Garcia-Romeu & Ehrenfeld, unpublished data). Diamox totally inhibits Na^+ and Cl^- netfluxes and the excretory netfluxes of H^+ and base linked with Cl^- transport. The inhibition, however, is not brought about in the same manner for the two ions. Thus the Na^+ netflux inhibition is the result of a strong drop in Na^+ influx, while the Cl^- netflux inhibition is due to a large increase in the Cl^- efflux, the influx not being inhibited (Tables 3 and 4B). This difference of reaction of the Na^+ and Cl^- transport mechanisms to Diamox reveals the independence of the two mechanisms and poses the problem of the site of action of the inhibitor.

Maetz & Garcia-Romeu (1964) proposed the presence in the freshwater fish *Carassius auratus* of Na^+/NH_4^+ and Cl^-/HCO_3^- exchange mechanisms dependent upon carbonic anhydrase. By inhibiting this enzyme Diamox would modify the cellular concentrations of hydrogen and bicarbonate and in this way block the ionic exchanges. The fact that Cl^- absorption in *C. auratus* is stimulated by the injection of bicarbonate argues in favour of this hypothesis. An important quantity of cellular carbonic anhydrase ($2{\cdot}17 \pm 0{\cdot}23$ U/mg, $n = 12$) was found in the gills of *Astacus leptodactylus*. It would be reasonable to suppose therefore that in this species Diamox inhibits the transmembranous ionic fluxes by acting directly on the cellular carbonic anhydrase. This hypothesis may be questioned however, since there are other possible explanations of the action of Diamox on ionic transports. Thus an action has been reported in tissues which do not contain carbonic anhydrase (Kitahara, Fox & Hogben, 1967; Fuhrman, 1952; Istin *et al.* unpublished data) or under conditions in which an alternative hypothesis of the action is feasible (Hogben, 1967). The problem of the site of action of Diamox in our experiments remains unresolved.

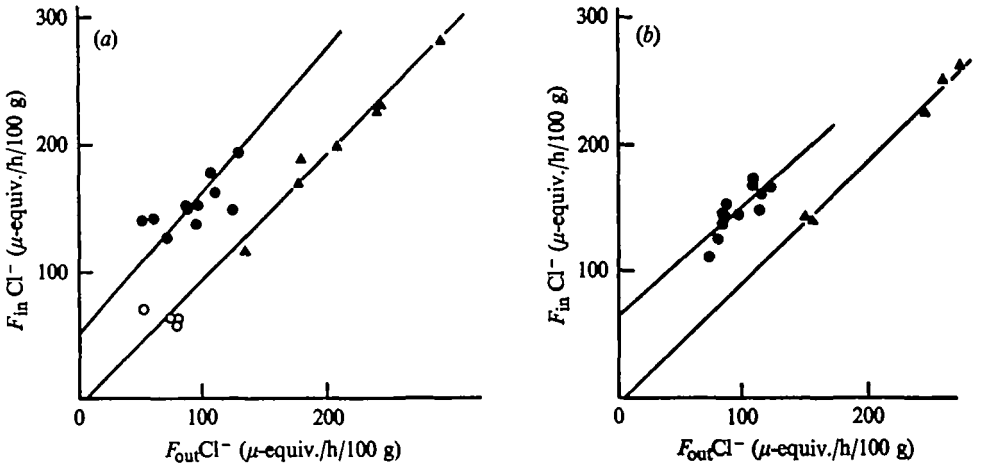

Fig. 4. Chloride efflux in relation to chloride influx in *Astacus leptodactylus*. Animals were pre-adapted in running water. (*a*) fluxes measured in a NaCl solution (1800 μ-equiv./l) buffered to pH 7·0. Each point represents an individual experiment of a control period (black dot) followed by either a 'Diamox period' (white dots) or a KCN period (cross). (*b*) fluxes are measured in a choline chloride solution (1800 μ-equiv./l) buffered to pH 7·0. Each point represents an individual experiment of a control period (black dot) followed by a 'diamox period' (white dot).

Possibility of the occurrence of an exchange diffusion for chloride. Action of cyanide

In the present experiments, with Cl^- transport from a 1800 μ-equiv./l NaCl solution the Cl^- efflux was 103 μ-equiv./h/100 g, and animals placed in distilled water had a chloride loss of a few μ-equiv./h/100 g. Similar results were found by Shaw (1960) who suggested that a Cl^-/Cl^- exchange occurs in *Astacus pallipes* accounting for 90% of the influx. Fig. 4 illustrates the correlation between the Cl^- influxes and effluxes. This correlation can be explained by an exchange diffusion mechanism, linked to the active transport pump, which accounts for two-thirds of the chloride efflux when the external Cl^- concentration is 1800 μ-equiv. Garcia-Romeu & Ehrenfeld (1972) have recently reported a correlation between the influxes and effluxes of both Cl^- and Na^+ but did not have sufficient data to advance an hypothesis concerning the nature of the linkage between influx and efflux.

Table 5 shows that the Na^+/H^+ and Cl^-/base exchange mechanisms are inhibited by KCN and are thus dependent on metabolic energy. Part of the Cl^- influx, on the other hand, is not inhibited. This part has a value comparable to that of the Cl^- efflux, which is also unmodified by addition of inhibitor (Table 5, Fig. 4), and affords evidence in favour of the presence of an exchange diffusion. Exchange diffusion as defined by Ussing (1947) is independent of metabolic energy and the present Cl^-/Cl^- exchange satisfies that criterion. It has been shown that the chloride transported across the crayfish gill against an electrochemical gradient is by an active process (Shaw, 1960; Bielawsky, 1964, 1971; Croghan, Curra & Lockwood, 1965). This active chloride transport is brought about by exchange against an endogenous ion of the same charge and is responsible for the Cl^- netflux; since a large proportion of the chloride influx seems involved in a Cl^-/Cl^- exchange reaction, the question arises as to whether one is dealing with two different mechanisms, or one and the same.

Shaw (1960) concluded that when crayfish are Cl^- deficient 'a chloride-system activation is brought about. This system activation does not bring about an increase in chloride influx but serves to switch the mechanism from a predominantly Cl^-/Cl^- exchange to a useful exchange of chloride for some other anion.'

As can be seen from Table 3 and Fig. 4, the main action of Diamox is to increase the Cl^- efflux, the value of which becomes roughly equal to that of the influx. Thus the netflux becomes zero and the bicarbonate excretion linked to this flux is reduced proportionately. This gives the impression that the Cl^-/HCO_3^- exchange has become a Cl/Cl^- exchange as an effect of Diamox. Further investigation of the exact mode of action of Diamox is necessary to explain this possible shift of exchange systems and more work has to be done before confirming the existence of an exchange diffusion mechanism.

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