Na^+/NH_4^+, Na^+/H^+ EXCHANGES AND NH_3 MOVEMENT ACROSS THE GILL OF *CARASSIUS AURATUS*

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Sodium uptake from the external medium in the absence of permeant co-ion occurs in most freshwater animals. Exchange of Na^+ for an endogenous cation is necessary to maintain the electroneutrality of the external and internal media.

For ammonotelic animals such as teleosts and crustaceans, in which the gill is the main site of ammonia excretion and sodium uptake, Krogh (1939) suggested that NH_4^+ is used as an exchange cation. Competition between NH_4^+ and Na^+ for sodium uptake from the external medium and the stimulation of Na^+ absorption by ammonia loading of the animal afford indirect evidence for the proposed Na^+/NH_4^+ exchange (Shaw, 1959, 1960*a*, *b*; Maetz & Garcia Romeu, 1964).

For ureotelic or uricotelic animals such as amphibians and insects, with part of their life-cycle in fresh water and actively absorbing sodium through specialized areas of the integument skin or anal papillae, H^+ ions rather than ammonia have been shown to be exchanged against Na^+ (Garcia Romeu, Salibian & Pezzani-Hernandez, 1969; Stobbart, 1971).

That the Na^+/NH_4^+ exchange is not obligatory in ammonotelic animals is suggested by various observations. In the absence of sodium uptake after transfer of the animals to a sodium-free medium, crayfish, carp and trout continue to excrete ammonia at similar if not higher rates than before (Shaw, 1960*b*; de Vooys, 1968; Kerstetter, Kirschner & Rafuse, 1970). Injection of acetazolamide, an inhibitor of carbonic anhydrase, is followed by a sharp decrease of sodium uptake, but ammonia excretion continues unimpaired (Kerstetter *et al.* 1970).

To explain the absence of stoichiometry between sodium uptake and ammonia excretion three hypotheses have been advanced. Shaw (1960*b*) suggested that ammonia is excreted in the ionized form but accompanied by HCO_3^- excretion when sodium uptake falls short of ammonia excretion. When sodium uptake exceeds ammonia excretion, H^+ ions are thought to supplement ammonia for the exchange. Kerstetter *et al.* (1970) suggested that ammonia is excreted in the free-base form; and that in fish, as in amphibians, H^+ is exchanged against Na^+. In trout, in the absence of a permeant anion, they observed a significant decrease of the pH of the external medium at the highest rates of Na^+ uptake. This pH shift suggests H^+ ion excretion by the gill of the trout. The authors were, however, unable to titrate the total hydrogen excreted by the fish. In recent reviews (Maetz, 1971, 1972*a*) the possibility that both forms of ammonia could cross the gill and that H^+ ions as well as NH_4^+ ions may be exchanged against Na^+ was considered.

In the present publication concerning *Carassius auratus*, the occurrence of H⁺ ion excretion by the gill is confirmed by recording the changes of the titrable acidity of the external medium in closed-circuit experiments. Permeability of the gill to both unionized and ionized forms of ammonia is demonstrated by ammonia-gradient experiments. The relative amounts of NH_3 and NH_4^+ varies according to the prevailing external pH and sodium concentration. When H^+ ion movement across the gill is taken into account, an excellent correlation between Na^+ uptake and NH_4^+ excretion is observed.

BIOLOGICAL MATERIAL AND TECHNIQUES

Carassius auratus obtained from a Paris dealer and weighing from 70 to 200 g were used.

Two main types of investigations were performed: ionic balance studies and ammonia-gradient experiments.

Ionic balance studies

These consisted of simultaneous measurements of Na^+ uptake, ammonia excretion and H^+ ion movement across the gill. For these investigations fish kept for at least 3 weeks in de-ionized water were used in order to obtain high rates of sodium uptake (Garcia Romeu & Maetz, 1964; Cuthbert & Maetz, 1972). Batches of 4–6 fish were maintained in 30 l tanks with de-ionized water renewed every day or two. The pH of the water usually rose from below 6 to about 7 between renewals as a result of NH_4HCO_3 accumulation. Temperature was controlled between 14 and 16 °C.

The fish were fitted with a urinary catheter and placed in running de-ionized water at 16 °C in the flux chambers at least 24 h before measurements. The ionic-balance studies were completed in sodium sulphate solutions at concentrations ranging from 10 to 1600 m-equiv l⁻¹. Sodium influx and efflux were measured simultaneously after closing the experimental circuit by adding ²⁴Na in the form of sulphate to the external bath. Sulphate has been shown to be an impermeant anion in the goldfish (Garcia Romeu & Maetz, 1964). The techniques have been described elsewhere (Maetz, 1956; Cuthbert & Maetz, 1972; Maetz, 1972b). For the calculation of the fluxes, the radioactive backflux from the fish was taken into account. All fluxes were measured at 16 °C.

The pH of the external medium was controlled in all the flux measurements. In most experiments imidazole at a concentration of 2 mM was used as a buffer. H_2SO_4 and KOH in molar solutions were added in variable amounts to change the pH. The pH range for the 'acid' periods was 5·5–6·9 and for the 'alkaline' periods was 7·0–7·6. In a few flux periods no imidazole was used and the pH was in the acid 5·8–6·9 range. As mentioned previously (Cuthbert & Maetz, 1972), imidazole does not seem to interfere with sodium uptake. In a few experiments goldfish were studied in de-ionized buffered water in order to measure the sodium loss in the absence of external sodium.

Flux periods were usually of 1·5–2 h duration separated by intermediate periods of 30 min during which the pH was adjusted and sodium sulphate and ²⁴Na were added to compensate for the rapid disappearance of sodium for the bath. This intermediate period is unsuitable for measuring sodium fluxes as for 10–15 min the
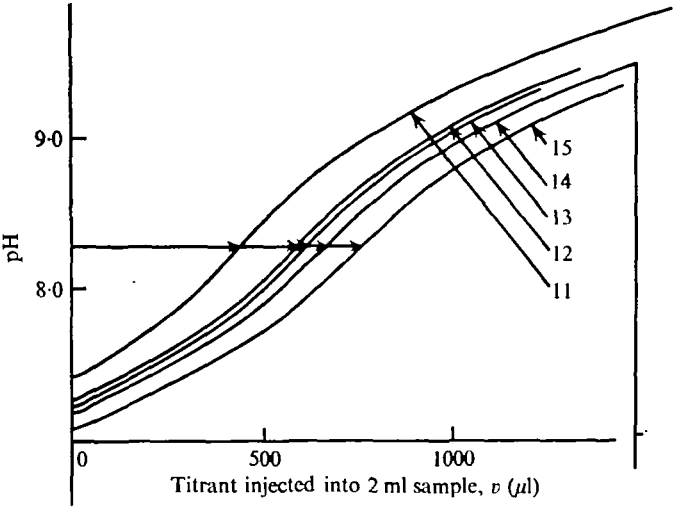
Fig. 1. Titration of acidity of aquarium water. Example of progressive acidification of the external medium. Photograph of recordings. 11, 12, 13, Successive sample numbers; the first sample is on the left. Note the equivalence point characterizing the imidazole buffer. Note the progressive increase of the buffering capacity of the external medium.
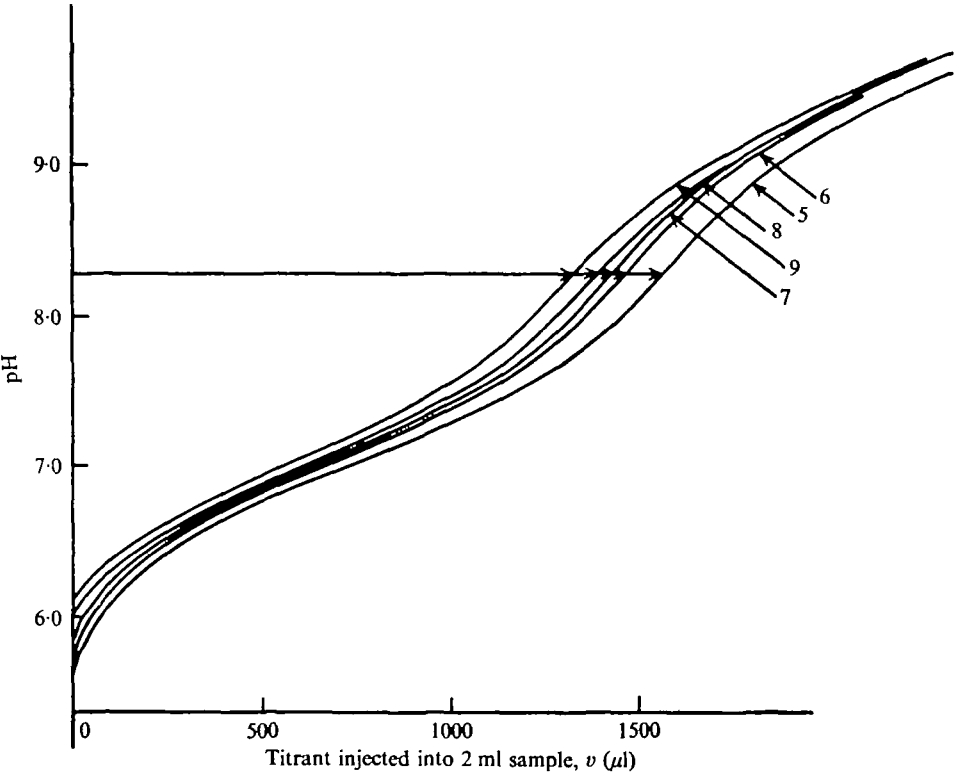

Fig. 2. Titration of acidity of aquarium water. Example of progressive alkalinization of the external medium. The first sample ($n = 5$) is on the right.

external sodium and [24]Na concentrations were not homogenous. This period may, however, be used for measuring ammonia excretion and for studying the immediate effects of pH on this parameter.

The withdrawal from the bath at regular intervals of 10 ml samples allowed for the determination of the Na$^+$ and K$^+$ concentrations by flame photometry, the chloride by amperometric titration and the concentration of total ammonia by the auto-analyser technique (Maetz, 1972b). The total acidity was measured by titration with NaOH (3·5 mM solution) on 2 ml samples with a Tacussel autotitrator, automatic burette and recorder. The NaOH concentration was re-checked every week against 2 mM HCl. To avoid interference with acidity originating from respiratory CO_2 from the fish, two precautions were taken: (1) vigorous air-bubbling of the external water was maintained throughout the flux measurements, (2) the samples were allowed to stand in contact with air for an additional 24 h before titration (see Garcia Romeu et al. 1969).

Figs 1 and 2 are photographs of serial recordings of H$^+$ titration from successive half-hourly samples. In Fig. 1, the fish was kept in an alkaline medium. The pH of the external medium decreased as a result of H$^+$ excretion by the fish and the quantity of titrant necessary to obtain the end-point increased. This quantity was, in fact, more important than would be expected from the actual pH decrease since the buffer-ing capacity of the external medium increased as can be seen from the initial slope of the titration curve. In Fig. 2 the fish was initially in an acid medium. The pH progressively increased and the quantity of titrant needed to obtain the end-point decreased. Again, the buffering capacity of the external solution increased.

Ammonia-gradient experiments

These were designed to correlate the net flux of ammonia across the gill with the difference of the partial pressures of free-base ammonia in the external and internal media. In order to test the capacity of the gill to excrete ammonia against a partial pressure gradient, exogenous ammonia was added in variable amounts to the external medium. The effects of external pH changes and the interference of external sodium with ammonia movements were also studied.

Ammonia exists in solutions as NH_4^+ and as NH_3 according to the relations des-cribed by the following equations:

$$NH_4^+ = NH_3 + H^+, \tag{1}$$

$$\frac{(H^+).(NH_3)}{(NH_4^+)} = K'_a, \tag{2}$$

$$pH = pK'_a + \log\frac{(NH_3)}{(NH_4^+)}, \tag{3}$$

The values in parentheses are concentrations expressed in moles litre^{-1}. Whether the free-base form or the ionic form or both are transferred depends on the character of the interposed membrane. Non-ionic diffusion is a passive process that conforms to physico-chemical laws. The distribution of ammonia as NH_3 should be dependent

on the partial pressure gradient of NH$_3$(pNH$_3$) across the membrane. Diffusion should occur from the side of greater tension to that of lesser tension.

pNH$_3$ is related to (NH$_3$) by the following equation:

$$(NH_3) = \frac{\alpha}{22 \cdot 1} p NH_3. \tag{4}$$

pNH$_3$ is expressed in mmHg; α, the solubility coefficient is given in litres SPTD per litre solution and 1 mmHg.

The α values for the external medium at 16 °C has been taken as 1, which is the mean of the values obtained by extrapolation using an Arrhenius plot from the data of Hodgman (1962) and Lange (1941). The α value for fish plasma at 16 °C was taken as 1·75 calculated by extrapolating the values given by Jacquez, Poppell & Jeltsch (1959) for human plasma.

The pK$'_a$ value for dilute saline solutions at 16 °C is taken as 9·55 from Bates & Pinching (1949) as against 8·89 at 37 °C. For plasma the ionic strength being equivalent to that of a 150 mM solution, the pK$'_a$ is higher by about 0·15 according to Bank & Schwartz (1960). For whole blood a correction factor of 0·25 should be used, according to Bromberg, Robin & Forkner (1960). In the present experiments, since the pH of the external and internal media was at least 2 units below the respective pK$'_a$, the total ammonia (Am) = (NH$_3$)+(NH$_4^+$) measured chemically corresponds within 1 % to (NH$_4^+$).

The experimental procedure for the ammonia-gradient experiments consisted of comparing the net flux of sodium and the movement of ammonia at low and high external ammonia concentrations. The duration of the periods was 1–2 h separated by an intermediate period of 20–40 min. At the end of the second period a blood sample was taken from the caudal artery by means of a heparinized syringe. Blood pH was measured immediately and compared with external ammonia within minutes of sampling the media. (NH$_3$), (NH$_4^+$) and pNH$_3$ were calculated using the α and pK$'_a$ values given above. Plasma concentrations rather than whole-blood concentrations of ammonia were used.

Three series of experiments were performed mostly on 'de-ionized' fish. Two series concerned fish kept in 1 mM sodium sulphate solutions but the pH was in the alkaline range in one series and in the acid range in the other. The third series concerned fishes kept in alkaline, initially sodium-free media, i.e. choline chloride solutions. In all three series imidazole at 2 mM was used. In the first series sodium influx and efflux were determined using ^{24}Na. In the two other series only the net flux was measured. The pH ranges were similar to those reported for the ionic-balance studies. Acidification of the external medium in the second series was obtained by the addition of H$_2$SO$_4$ in appropriate amounts. In all three series ammonia was added in the form of a molar solution of ammonium sulphate. The final external concentrations varied from 1·5 to 12 mM. Since the ammonium solution is acid, simultaneous addition of KOH in appropriate amounts was used to keep the external solution in the alkaline range in the first and third series of experiments. Mean pH values, ammonia and sodium concentrations are given in Tables 4–6, which summarize the results of these experiments.

Titrable acidity changes were not recorded in the ammonia-gradient experiments.

Table 1. *Sodium exchange and ammonia excretion across the gill of sodium-depleted* Carassius *in relation to external sodium concentration and pH*

External Na concentration	Sodium				Ammonia excretion
	Influx	Efflux	Net flux	f_{in}/Na_{ext}	
I. At pH > 7 (7·25±0·03)					
69 (1)	28·0 (1)	19·2 (1)	+8·8 (1)	406 (1)	48·6 (1)
128±10 (5)	37·2±11·8 (5)	10·7±4·7 (5)	+26·5±7·4 (5)	274±66 (5)	26·5±5·5 (4)
241 (1)	54·5 (1)	7·5 (1)	+47·0 (1)	226 (1)	36·8 (1)
323±15 (4)	81·3±19·8 (4)	15·7±4·5 (4)	+65·6±23·4 (4)	245±46 (4)	44·5 (2)
479±11 (8)	95·7±9·0 (8)	15·6±6·5 (8)	+80·1±11·6 (8)	202±21 (8)	45·5±4·3 (3)
790±51 (4)	98·7±14·1 (4)	31·4±7·2 (4)	+67·3±14·8 (4)	126±17 (4)	31·2±14·1 (4)
1065±77 (4)	102·2±18·5 (4)	36·7±19·0 (4)	+65·7±14·0 (4)	100±21 (4)	40·9±11·9 (4)
II. At pH > 7 (6·19±0·11)					
421±31 (4)	27·3±5·7 (4)	15·2±4·9 (4)	+12·1±4·9 (4)	66±15 (4)	48·0±5·0 (4)
648±30 (4)	44·9±3·3 (4)	5·4±3·2 (4)	+39·5±2·9 (4)	69±3 (4)	54·6±16·1 (4)
832±47 (3)	42·8±66·4 (3)	6·7±3·3 (3)	+35·4±4·4 (3)	52±8 (3)	56·0±7·9 (3)
1326±147 (3)	30·3±15·8 (3)	12·3±2·7 (3)	+18·0±18·0 (3)	21±9 (3)	57·5±13·2 (3)

Concentrations in μ-equiv l^{-1}; fluxes in μ-equiv h^{-1} 100 g^{-1}; excretion rate in μM h^{-1} 100 g^{-1} f_{in}/Na_{ext} ($\times 10^{-3}$).

RESULTS

Ionic-balance studies

As these studies were made on 'de-ionized' fish with a high rate of sodium uptake, it will be convenient to describe first the pattern of branchial sodium exchange and ammonia excretion in these fish and compare it with that reported previously for control fish kept in tap water (Maetz, 1972b). The effects on this pattern of lowering the external pH will be considered next. Finally the correlation between Na^+ uptake and ammonia excretion will be discussed in relation to the simultaneous movement of H^+ ions across the gill.

Sodium exchanges and ammonia excretion as a function of external sodium

Table 1 summarizes the results concerning sodium influx, f_{in}, sodium efflux f_{out} and the net flux of sodium, f_{net}, the flux periods having been grouped according to arbitrarily chosen external sodium concentration (Na_{ext}) ranges. Fig. 3 compares the change of f_{in} as a function of Na_{ext} in sodium-depleted fish and in control fish as
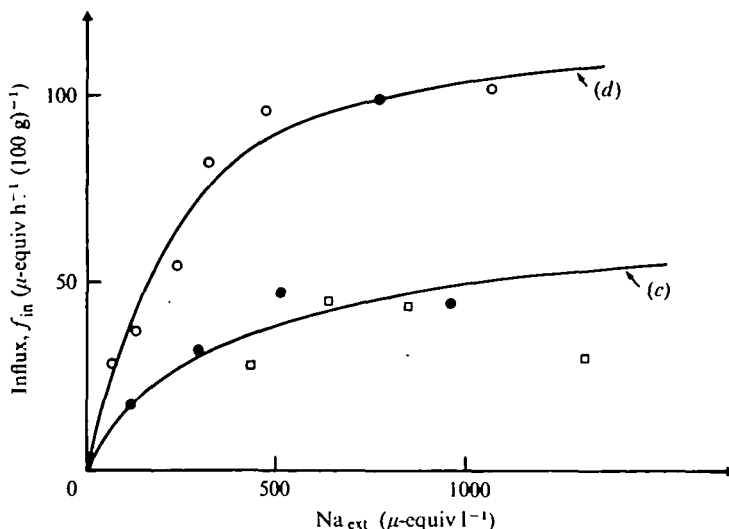
Fig. 3. Sodium influx as a function of external sodium concentration. Comparison between control and sodium-depleted goldfish. d, Sodium-depleted fish; c, control fish. □, influx in acid media (d).
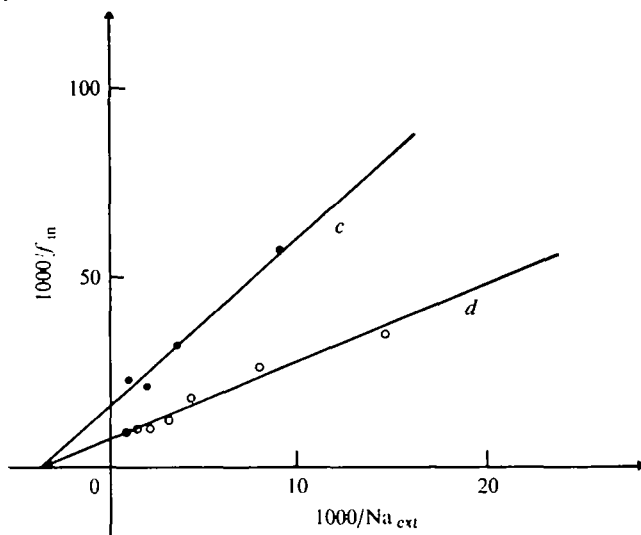

Fig. 4. Reciprocal plot of the data given in the preceding figure. Note identity of K_m and difference in f_{max}.

given by Maetz (1972b). It can be seen that the transport mechanism is saturated above 500 μ-equiv l⁻¹ in both types of fish. Fig. 4 gives the reciprocal plot of the data illustrated in Fig. 3 assuming Michaelis–Menten kinetics. The calculated maximal rate of uptake is 128·5 μ-equiv.h⁻¹.(100 g)⁻¹ for sodium-depleted fish, and 65 for control fish. The apparent affinity of the transport carrier is not significantly different, the K_m being 260 μ-equiv in sodium-depleted fish against 300 in controls.

In sodium-depleted fish, as in control fish, the sodium efflux increases with Na_{ext} and the highest effluxes are in the same range when both types of fish are compared.

Table 2. *Effect of external pH changes upon sodium exchanges, ammonia excretion and handling of other monovalents ions by the gill of* Carassius

Medium	Alkaline	Acid	Paired difference	Statistics
pH	7.23 ± 0.05	6.13 ± 0.14	-1.10 ± 0.11	$P < 0.001$
Na_{ext}	650 ± 138	880 ± 147	$+230 \pm 80$	$P < 0.05$
$f_{in}Na^+$	74.1 ± 11.4	34.7 ± 6.0	-39.4 ± 8.8	$P < 0.01$
$f_{net}Na^+$	$+48.2 \pm 4.9$	$+24.2 \pm 7.3$	-24.0 ± 6.5	$P < 0.01$
$f_{out}Na^+$	25.9 ± 7.0	10.5 ± 2.3	-15.4 ± 7.0	N.S.
f_{in}/Na_{ext}	149 ± 27	47 ± 9	-102 ± 21	$P < 0.01$
Ammonia excr.	46.1 ± 5.1	54.3 ± 7.0	$+8.2 \pm 5.0$	N.S.
$f_{net}H^+$	-20.3 ± 9.5	$+24.2 \pm 6.0$	$+44.5 \pm 13.1$	$P < 0.02$
$f_{net}K^+$	-5.3 ± 1.0	-3.9 ± 0.9	$+1.4 \pm 1.5$	N.S.
$f_{net}Cl^-$	-10.3 ± 7.7	$+0.6 \pm 1.0$	-10.9 ± 7.3	N.S.

Number of animals: 8.

Na_{ext} in μ-equiv l^{-1}; fluxes in μ-equiv h^{-1} 100 g^{-1}; rate of ammonia excretion in μM h^{-1} 100 g^{-1}.

Table 3. *Ammonia excretion by the salt-depleted goldfish after abrupt acidification of the external medium* $(n = 7)$

Control period (1) (1·5 h; pH 7.23 ± 0.05)	Transition period (2) (0·5 h; pH < 6)	Acid period (3) (1·5 h; pH 6.23 ± 0.14)
51.8 ± 8.12	111.8 ± 16.51	58.9 ± 6.86

Paired differences: (1)−(2): $+60.0 \pm 14.26$ ($P < 0.01$)
(2)−(3): -55.9 ± 10.47 ($P < 0.01$)

Ammonia excretion rates in μmoles h^{-1} 100 g^{-1}.

The sodium balance is positive, even at the lowest range of Na_{ext} at which sodium exchanges were measured isotopically. In four fish placed in de-ionized water buffered with imidazole the net flux of sodium was negative, -4.1 ± 1.29, the mean Na_{ext} value being 22 ± 3.3 in these experiments. Equivalence between sodium influx and efflux across the gills is attained at external sodium concentrations between 35 and 40 μ-equiv l^{-1}, as shown in some experiments which will be discussed in the second part of this paper (see Table 6).

Ammonia excretion of sodium-depleted fish is twice as high as that of the controls, 37.3 ± 4.3 μM h^{-1} (100 g)$^{-1}$ against 19.8 ± 2.1, n being 20 and 32 respectively, and $P < 0.001$. Both groups of fish were unfed and from the same batch. A high rate of ammonia excretion is also found in the fish discussed in the second part of this paper (see Tables 4–6).

Effect of external pH changes on sodium exchange and ammonia excretion across the gill

The effects of external pH changes have been investigated comparatively in eight fish at high and low pH. Four fish were submitted to only one pH change; in three the 'acid' period followed the alkaline period and in one the reverse protocol was chosen. Four more fish were submitted to two pH changes, two to the sequence acid–alkaline–acid and two to the opposite. Figs 5 and 6 compare the changes of the main cation concentrations in the external bath in relation to pH changes. Table 2 summarizes the results. For the fish submitted to two pH changes, since the effects of
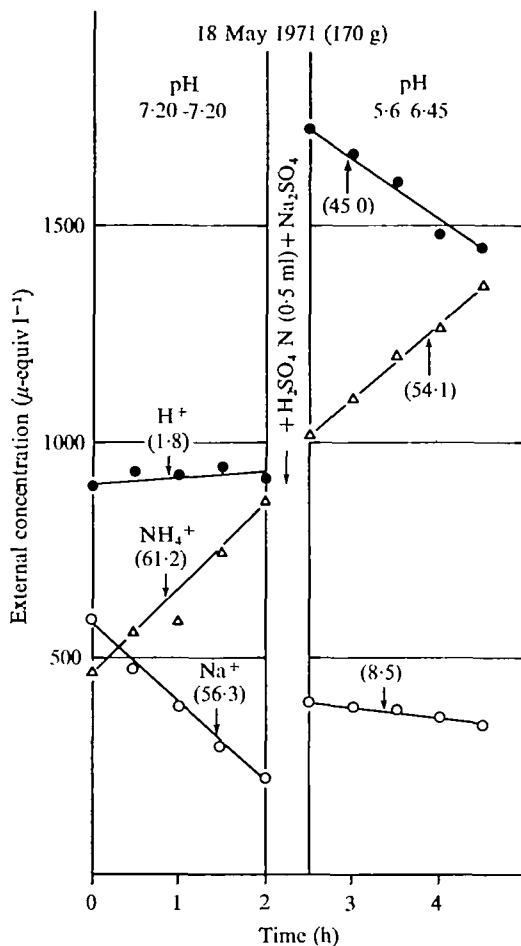
Fig. 5. Changes of the external sodium, total ammonia and titrable acidity as functions of time. Effect of acidification of the external medium. The slopes represent the net fluxes; values given in parentheses expressed in μ-equiv h^{-1} (100 g). Ordinate: ammonia given as being in the ionic form. The changes of the external pH are also indicated for each period.

pH upon the fluxes were found to be reversible, the mean values obtained for the first and the second periods were compared with those obtained for the second period.

Acidification of the external medium causes a 55 % reduction of the influx, even though the mean Na_{ext} is slightly higher in the 'acid' period. A decrease of the sodium efflux is also apparent, although not significant. The net absorption rate is twice as high in the alkaline period as in the acid period.

Ammonia excretion is slightly higher in the acid medium, but the difference is not significant. One important point about ammonia excretion must be emphasized however. The rate of ammonia excretion calculated for the intermediate period permitted the evaluation of the acute effects of acidification upon this parameter. Table 3 summarizes the results from seven fish which were submitted to the transition from alkaline to acid media. The rate of ammonia excretion increased twofold upon acidification, an increase which is highly significant. This augmentation was, however,
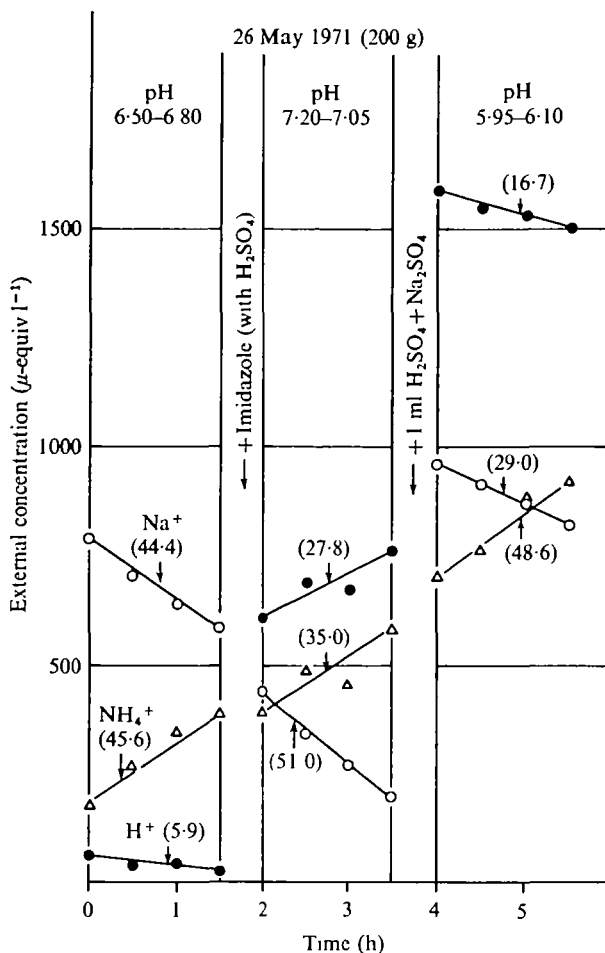
Fig. 6. Effect of successive alkalization and acidification of the external medium on net fluxes of H⁺, Na⁺ and ammonia. Co-ordinates as in preceding figure.

only temporary as the ammonia excretion rate fell again in the subsequent steady-state acid period. The augmented rate of ammonia excretion under these particular conditions suggests that the gill becomes permeable to the free-base form of ammonia, a point which will be discussed below.

In Table 2 the handling of the other monovalent cations by the gill is also summarized. K^+ is lost by all fishes, but the net loss is small. Cl^- is also lost, especially in alkaline media, but the chloride balance is not significantly different from zero in either type of medium. The most important results concern the behaviour of H^+ ions. In alkaline media the fish excretes H^+. The rate of excretion is, however, insufficient to account for the net sodium uptake. In acid media, on the other hand, the fish seems to absorb H^+ ions. The difference between the fluxes recorded in the two media is highly significant.

In Table 1 the fluxes measured in fish kept in acid media have also been grouped according to Na_{ext} in order to allow a comparison with the fluxes obtained in alkaline media.
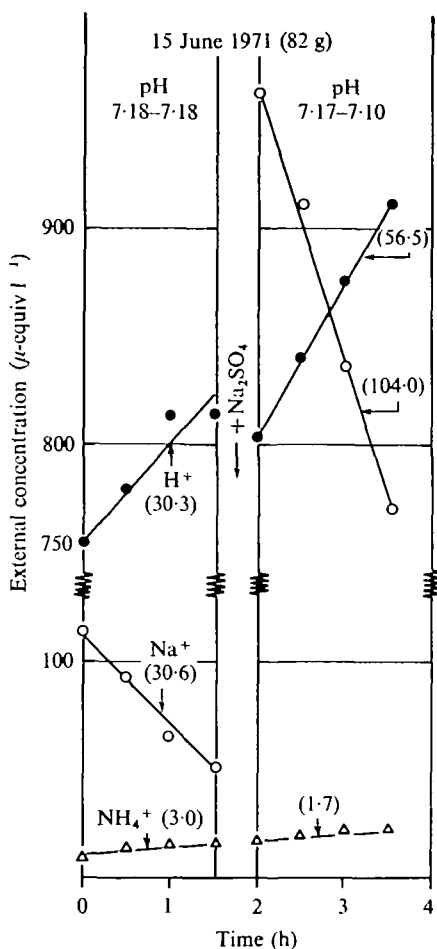
Fig. 7. Typical case of H^+/Na^+ exchange in goldfish with poor rate of ammonia excretion. Co-ordinates as in preceding figure.

Ionic-balance studies. Correlation between Na^+ uptake, NH_4^+ excretion and H^+ ion movements

In Figs 5 and 6 comparison of the slopes of the various straight lines representing the positive or negative net fluxes for the main cations strongly suggests that balance of the electric charges moving across the gill is achieved. For example, in Fig. 5, on the left-hand side, it may be seen that at pH 7·2 this particular fish gained Na^+ at approximately the same rate as it lost NH_4^+ ions, assuming that these ions cross the gill as such. Very little H^+ was excreted, the unchanged pH also indicating this. Upon acidification the sodium uptake was strongly inhibited while the rate of ammonia excretion, after a transient increase, resumed its original rate. H^+ ions rapidly disappeared from the external bath and the sum of the rates of disappearance of H^+ and Na^+ was equivalent to the rate of ammonia excretion. In Fig. 6, on the left-hand side, the external bath was not buffered during the first flux period. While H^+ movement across the gill was negligible, Na^+ absorption was matched by NH_4^+ excretion. In the
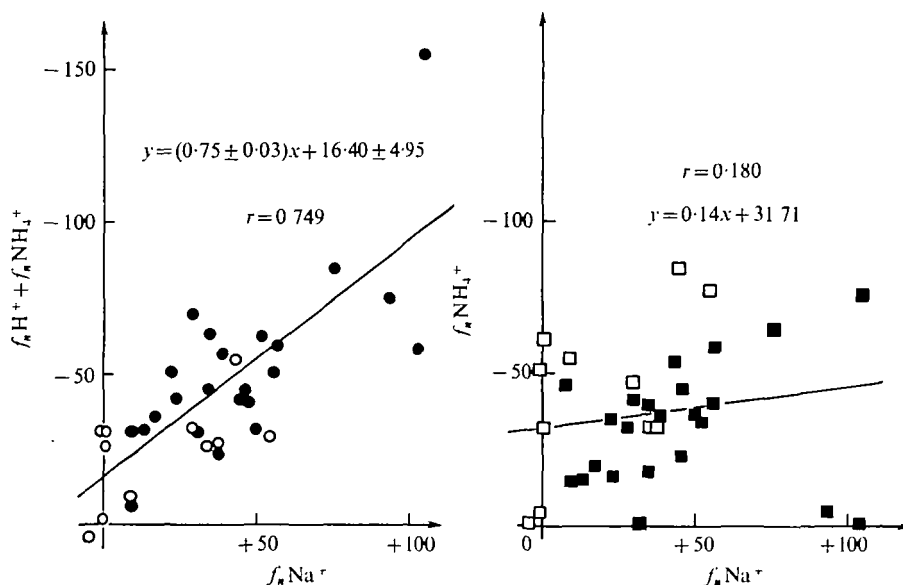
Fig. 8. Right: absence of correlation between ammonia excretion and sodium net uptake. Left: Correlation between ammonia excretion and sodium uptake when H⁺ ions movements across the gill are taken into account. Ordinate: net fluxes of ammonia (as NH_4^+) and H^+ in μ-equiv h^{-1} $(100\ g)^{-1}$. Abscissa: net sodium uptake in the same units. ●, ■, Experiments in alkaline media. ○ □, Experiments in acid media.

middle alkaline period the Na^+ gain more or less equalled the sum of the loss rates of NH_4^+ and H^+. On the right-hand side, after acidification, the sum of the absorptions of Na^+ and H^+ matched the NH_4^+ excretion.

In Fig. 7 an exceptional case of a sodium-depleted fish with a low ammonia excretion is represented. On the left-hand side, at low Na_{ext}, Na^+ absorption was equivalent to H^+ excretion. On the right-hand side, at high Na_{ext}, the Na^+ absorption was increased almost threefold. A concomitant increased H^+ ion excretion was observed, although not equivalent to the augmentation of the Na^+ uptake.

To verify whether the law of electroneutrality of external and internal media applies despite the high rates of ionic exchanges across the gill in sodium-depleted fish, the ionic balance of the moving ions, i.e. $\Sigma\ zf_{net}$ was calculated for 22 alkaline and 12 acid periods for which the f_{net} of the positive $(z = 1)$ and negative $(z = -1)$ monovalent ions were measured. For this calculation ammonia was considered to be excreted in the ionic form. The overall balances were found to be -6.8 ± 5.8 and -8.9 ± 5.1 for the alkaline and acid periods respectively, and are thus not significantly different from zero, even though the movement of the bivalent cations was neglected.

In Fig. 8 the 34 flux periods are presented once again to illustrate the main results of the investigations discussed here. On the right-hand side the rates of ammonia excretion, the ammonia being considered in the ionized form, are plotted against the concomitant rates of sodium uptake. No correlation is apparent, a point which is also seen in Table 1. Some fish, with very low rates of sodium uptake, exhibit rather high rates of ammonia excretion. This is evident in experiments with low external sodium and in acid media. Other fish, as already mentioned, had very low

Table 4. *Effects of the addition of ammonia to the external medium on the sodium exchanges and ammonia excretion across the gill in the presence of external sodium and at alkaline pH (n = 7)*

	Am_{ext}	Am_{int}	$f_n Am$	Na_{ext}	f_{in}	f_{out}	f_{net}
Before	222 ± 26	—	-61.9 ± 6.3	1049 ± 139	132.0 ± 7.9	33.9 ± 7.8	$+98.1 \pm 140$
After	6951 ± 1374	1120 ± 134	-62.1 ± 43.1	1217 ± 187	93.4 ± 10.6	50.8 ± 28.7	$+42.6 \pm 35.6$
Difference	—	—	N.S.	—	-38.6 ± 7.8 $P < 0.01$	N.S.	N.S.

Am: total ammonia concentration in μmoles l^{-1}; Am_{int}: the plasma concentration.
Na: concentration in μ-equiv l^{-1}.
Fluxes in μmoles or μ-equiv h^{-1} $(100 \text{ g})^{-1}$. N.S.: not significant.
At the end of the experiment the pH of the external medium was 7.14 ± 0.03 and that of blood, 7.71 ± 0.08.
The blood/plasma ratio was 1.93 ± 0.09 for ammonia concentration.

rates of ammonia excretion, yet absorbed Na^+ normally. On the left-hand side of the figure the sum of the net fluxes of NH_4^+ and H^+ have been plotted against Na^+ uptake for the same experiments. A very good correlation is obtained ($P < 0.001$). It may be seen from the equation of the regression line, however, that the stoichiometric relationship diverges from the expected 1-for-1 ratio and that the line does not pass through the origin, the intercept with the y axis being significantly different from zero.

Ammonia-gradient experiments

These experiments were designed to test the ammonia-excreting activity of the gill against a concentration gradient in various experimental conditions.

Experiments at alkaline pH with Na present in the external medium

Table 4 and Fig. 9 summarize the results of seven experiments of this kind. It may be seen that addition of ammonia to the external medium, the final $(Am)/(Na_{ext})$ concentration ratio being 5.6 ± 0.4, produces a significant reduction of the sodium influx, while the sodium efflux increases but not significantly. Ammonia output remains unchanged. The absence of any effect on ammonia excretion suggests that the reduction in sodium influx is due to competition between NH_4^+ and Na^+ for common sites of entry through the gill, a suggestion already made by Shaw (1960b) for the crayfish.

At the end of these experiments the blood was found to be more alkaline than the external medium and the external ammonia concentration to be about 6 times that of plasma. The ammonia concentration ratio of whole blood and plasma was nearly 2, however.

In Fig. 9 the ammonia movement across the gill observed during the second period was plotted against the calculated pNH_3 difference between external and internal media. A negative net flux – that is, ammonia excretion – is seen in six out of seven fish although the partial pressure of NH_3 was higher in the external medium. Movement of ammonia is uncorrelated with the pNH_3 gradient ($r = 0.159$). This experiment thus confirms the preceding observations obtained on control fish and on ammonia-loaded fish kept in alkaline fresh water with added NaCl (Maetz, 1972).
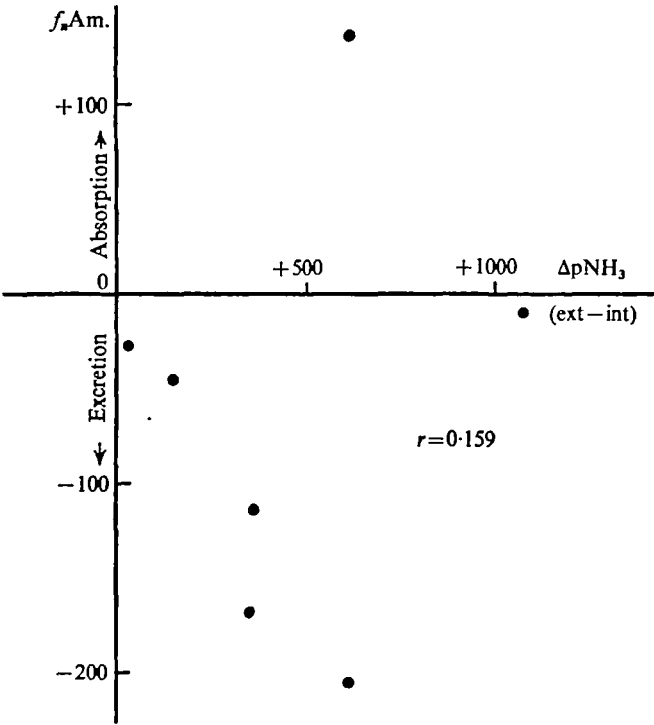
Fig. 9. Absence of correlation between net flux of ammonia and the gradient of partial pressure of ammonia across the gill. Experiments in the presence of external sodium and in alkaline pH. Ordinate: flux in μmole h^{-1} (100 g) $^{-1}$. Abscissa: difference between external and internal ammonia partial pressure is 10^{-4} mmHg.

Table 5. *Effects of the addition of ammonia to the external medium on the net flux of sodium and ammonia across the gill in the presence of external sodium and at acid external pH ($n = 7$)*

	Am_{ext}	Am_{int}	$f_{net}Am$	Na_{ext}	$f_{net}Na$
Before	211±29	—	−47·7±11·0	828±193	+39·7±23·0
After	6048±2112	584±149	−31·2±20·8	1022±203	−10·3±55·3
Difference	—	—	N.S.	—	N.S.

At the end of the experiment the external pH was 6·20±0·43 and the blood pH was 7·72±0·05. The blood/plasma ammonia ratio was 1·85±0·33.

Ammonia was found to be excreted against the concentration gradient of the free-base form. In those fish, however, the internal pH was lower than that of the external medium.

Experiments at acid external pH and with Na present in the external medium

These experiments were designed to verify whether a decreased external pH favoured the transfer of the free-base form of ammonia across the gill. Table 5 and Fig. 10 summarize the results obtained on seven fish. In these experiments the mean external pH was 6·2. Blood pH was not found to be altered by the acidification of the
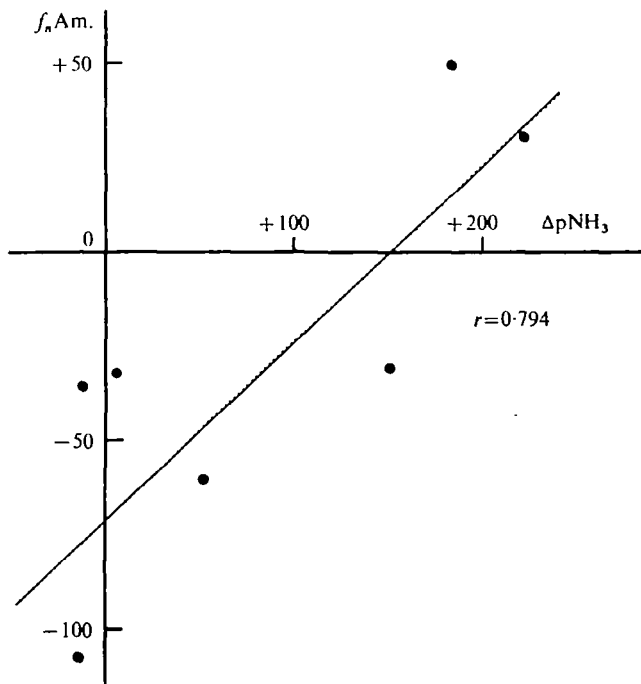
Fig. 10. Correlation between the net flux of ammonia and the gradient of partial pressure of ammonia across the gill. Experiments in the absence of external Na^+ and in alkaline pH. Co-ordinates as in preceding figure. Equation of the regression line:

$$y = (71 \cdot 89 \pm 26 \cdot 01) + (0 \cdot 476 \pm 0 \cdot 90)\, x.$$

external medium. Only the net sodium flux was measured. During the control period sodium absorption was found to be significantly lower ($P < 0 \cdot 05$) than that of fish kept in alkaline media (see preceding table). Thus the results presented in Table 2 are confirmed. Ammonia excretion was similar in acid and alkaline media.

Addition of ammonia, the Am/Na_{ext} ratio being nearly identical to that given in the preceding table, produced a slight reduction of the rate of ammonia excretion and a reversal of the net flux of sodium.

In Fig. 10 the net flux of ammonia across the gill appears to be correlated ($P < 0 \cdot 05$) to the pNH_3 gradient whereas in Fig. 9 it does not. Thus, in acid media movement of ammonia follows the pNH_3 gradient. It may be seen from the equation of the regression line, however, that the intercept of the regression line with the y axis is significantly different from zero. This indicates that at $\Delta pNH_3 = 0$, for example, ammonia is being excreted. In most fish ammonia is excreted against the pNH_3 gradient. Thus movement of ammonia is correlated with the ammonia gradient as it would be if ammonia moved passively; but in many cases, movement of ammonia takes place against the gradient. This apparent contradiction is resolved if one assumes that ammonia moves in both the free-base and the ionized form, only the former form conforming to the physico-chemical laws.

Table 6. *Effects of the addition of ammonia to the external medium on the net flux of* sodium and ammonia across the gill at low external Na and at alkaline pH (n = 5)

	Am_{ext}	Am_{int}	$f_{net}Am$	Na_{ext}	$f_{net}Na$
Before	398±67	—	−36·6±8·2	37·8±12·0	+2·3±0·9
After	4126±2512	1371±749	−3·0±17·2	53·6±14·5	−6·1±4·8
Difference	—	—	N.S.	—	N.S.

At the end of the experiment the external pH was 7·36±0·09, the blood pH was 7·59±0·06.
The blood/plasma ratio was 1·36±0·06.
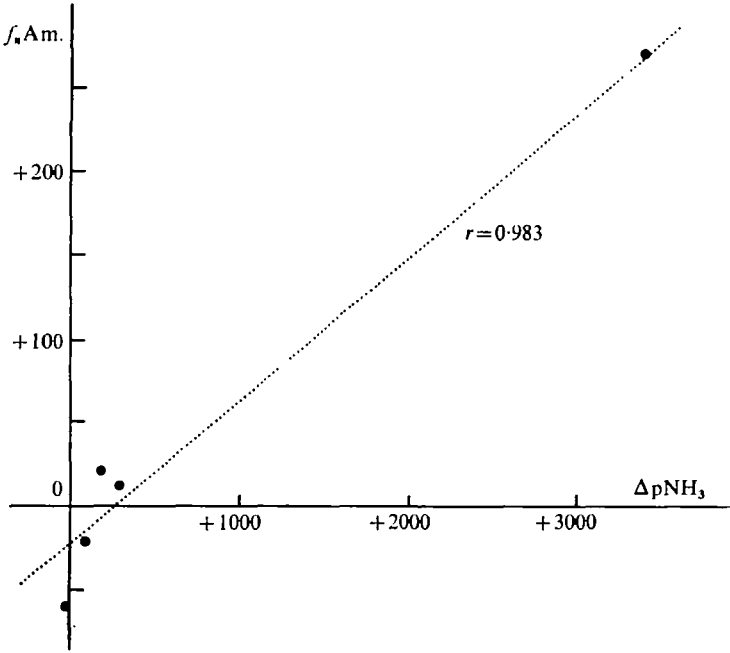For the experiments, fish were kept in mM choline chloride solution.

Fig. 11. Correlation between net flux of ammonia and the gradient of partial pressure of ammonia across the gill. Experiment in the presence of external Na⁺ and in acid pH. Co-ordinates as in preceding figure. Equation of the regression line:

$$y = (-22·78 \pm 62·22) + (0·087 \pm 0·023)x.$$

Experiments at very low external Na and at alkaline pH

Table 6 presents the results of five experiments of this kind carried out on fish maintained in choline chloride solutions initially sodium-free. The external sodium concentration recorded in the table represents sodium lost until influx balances efflux. After addition of ammonium sulphate to the external medium ammonia excretion is stopped and sodium is again lost by the fish, although in variable amounts.

When the movement of ammonia is plotted against the pNH_3 gradient (see Fig. 11) an excellent correlation between these two variables is observed, and the equation of the regression line shows that in this series of experiments the intercept of this line with the y axis is not different from zero. Thus in these experimental conditions

movement of ammonia follows the ammonia gradient and conforms to the laws of passive transfer. In the absence of external sodium, or at very low sodium, all ammonia thus moves in the free-base form.

<div align="center">DISCUSSION</div>

Interpretation of ionic-balance studies in the light of results obtained in ammonia-gradient experiments

The ionic-balance studies were made with fish placed in high or low external sodium and in alkaline or acid media. Correlation between ammonia excretion and sodium uptake was observed only when H^+ ion movement was taken into account. In alkaline media, at high external sodium, the net uptake of sodium was higher than total ammonia excretion. The excretion not only of NH_4 ions but also of H^+ ions must be considered to equate the Na^+ uptake. At low Na_{ext}, when the Na^+ uptake is less than ammonia excretion, a variable fraction of the ammonia is excreted in the free-base form. Below the sodium concentration which corresponds to the equivalence between f_{in} and f_{out}, all ammonia is eliminated in the unionized form.

At high external sodium, but at acid pH, the net flux of sodium is reduced as a result of f_{in} inhibition. The net flux of sodium is less than the total rate of ammonia excretion. A variable fraction of the ammonia is excreted in the free-base form and the medium becomes alkaline, as shown by the progressive decrease of the titrable acidity of the aquarium water. *Disappearance of the H^+ ions merely reflects the progressive trapping of the protons by NH_3 eliminated by the gill rather than absorption of H^+ by the fish.* NH_4^+ thus formed, being a weak acid, escapes the titration technique. To be titrated, the trapped protons would have to be liberated by formaldehyde (Karlmark, 1971). Thus in Fig. 5, on the right-hand side, the H^+/NH_4^+ exchange suggested by the slopes of opposite signs of the linear functions representing the ammonia and H^+ concentration changes respectively, illustrates in reality NH_3 extrusion into the bath.

The transitory 100% increase of the rate of ammonia excretion observed within the 30 min of acidification of the external medium results from two factors: (1) The gill suddenly becomes preferentially permeable to the free-base form of ammonia and (2) after a one-unit decrease of the external pH the external NH_3 concentration decreases tenfold. In the well-buffered internal medium the pH and thus the $(NH_3)/(NH_4^+)$ ratio remain constant and internal pNH_3 largely exceeds external pNH_3. Ammonia is lost until the external and internal pNH_3 are the same. The loss observed during the intermediate period represents about one-half of the ammonia content of the fish. After equilibration the former rate of loss is resumed as presumably the limiting factor is the endogenous ammonia production which remains unchanged.

Interpretation of the pH effects on the sodium exchanges and on the form of ammonia excreted by the gill

The experiments reported in Table 2 show that a tenfold increase of the external proton concentration produces a 50–60% inhibition of sodium influx and efflux. In a preliminary set of experiments on the goldfish in collaboration with Garcia Romeu, we observed that at the pH range of 3·5–4·5 inhibition of sodium influx was complete (see Maetz, 1972a). Similarly in the trout, Packer and Dunson (1970) found the sodium influx to be totally inhibited at pH 4·0.

Similar results have been reported on other transporting epithelia. In the crayfish gill the pH has no effect in the range of pH 10–6 but below pH 6 the influx falls off sharply, and at pH 4·0 it is reduced to 20–30% of the normal value (Shaw, 1960b). The effect of pH is comparable to that found for the isolated frog skin (Ussing, 1949; Schoffeniels, 1955) and for sodium uptake by frogs in vivo (Garcia Romeu et al. 1969). Ussing (1949) made the additional significant observations on isolated non-short-circuited frog skin that rising external pH results in a diminution of Cl⁻ influx while acidification had the opposite effect. Furthermore, Pesente (1969a, b) noted that acidification at pH 5 produces a shift in the permeability order for the alkaline cations across isolated frog skin. In a recent review, Garcia Romeu (1971) interpreted these various observations on frog skin in terms of an apical epithelial membrane envisaged as a mosaic of negatively (cationic exchanger) or positively (anionic exchanger) charged channels. In a cationic exchanger the selectivity sequence for the cations is largely determined by the strength of the negative fixed-charge field (Diamond & Wright, 1969). A change of pH altering this field would modify the selectivity sequence to one in which the ion species with the smallest hydrated diameter is preferred.

It should be noted that inhibition of sodium influx is observed at an H^+ ion concentration of about 0·6 μ-equiv.l^{-1}, which is about 1000 times less than the Na^+ concentration. From the few influx values given in Table 1 and in Fig. 3 for acid media, it is apparent that the maximal rate of influx is reduced by about 60% irrespective of the external Na^+ concentration. Thus it may be suggested that H^+ and Na^+ ions do not compete for a common site or carrier but rather that pH changes result in a variation in the number of sites available for Na^+ transport by alteration of the negative fixed-charge field.

Alteration of the negative fixed-charge field after acidification of the external medium may also drastically alter the permeability of the gill to the ionized ammonium ion, which is characterized by a rather large hydrated diameter. The rather small highly lipid-soluble free-base form of the molecule may thus preferentially cross the gill epithelium.

We do not know why the presence or absence of external sodium results in modification of the gill permeability to either form of the ammonia molecule. It is suggested that the presence of Na^+ induces conformational changes in the apical carrier for Na^+ entry into the epithelial cell.

Effects of salt depletion on the sodium exchange and the rate of ammonia across the gill of the goldfish

Several interesting points arise when the handling by the gill of sodium ions and ammonia in 'de-ionized' and control goldfish are compared.

The increased sodium-pumping activity of the gill in sodium-depleted fish results from an apparent increase of the number of transport sites rather than from an increased efficiency of these sites. Ammonia excretion is also augmented in sodium-depleted fish; this confirms the results of de Vooys (1968) for the carp. It is of interest that both ammonia-loaded fish (see Maetz, 1972b) and salt-depleted fish exhibit an increased sodium absorption activity with very similar maximal rates and affinity constants. Sodium-depleted fish behave as ammonia-loaded fish. Indeed the level of ammonia measured in the blood of 5 de-ionized fish was found to be 1045 ± 123 μmoles

as compared with 227 ± 27 in seven control fish, both types of fish being kept in similar confinement conditions (about 2 l of water per 100 g fish per day).

The observed effects of sodium depletion upon sodium exchange across the gill result (at least in part however) from the removal of Ca^{2+} from the external medium as was demonstrated by Cuthbert & Maetz (1972). The relative importance of sodium depletion and of Ca^{2+} removal is now being investigated in this laboratory by G. de Renzis.

The origin of the increased ammonia excretion and ammonia plasma level in relation to salt depletion is unknown. It is suggested that sodium depletion induces increased endogenous ammonia production perhaps as a result of interrenal hyperactivity. Olivereau observed a mild interrenal hyperactivity in de-ionized eels (Olivereau, 1966, 1967). Pora & Precup (1971) recently found that injection of rather high doses of glucocorticoids augments nitrogenous excretion in *Gobius* and *Trachurus*, an effect ascribed to the gluconeogenetic effect of corticoids. The investigations of Butler (1968) on the eel suggest that, in fish as in mammals, the hypophysial–interrenal axis controls gluconeogenesis. Very recently Bergström (1971) reported in sodium-depleted salmon an increased blood glucose level. Finally, the interrenals have been implicated in the control of sodium uptake in the freshwater eel (Maetz, Motais & Mayer, 1968; Mayer, 1970; Chester Jones *et al.*, 1969). All this evidence suggests that the feedback mechanisms triggered off by salt depletion simultaneously affects the metabolism of salt, glucose and ammonia and is mediated by the hypophysial–interrenal system.

SUMMARY

1. Sodium exchange, ammonia excretion and H^+ ion movement across the gill were measured in sodium-depleted *Carassius auratus*. Sodium uptake and ammonia excretion are considerably enhanced by sodium depletion. In this respect sodium-depleted fish behave as ammonia-loaded fish.

2. A decrease of the external pH by one unit results in a 50–60 % decrease in both influx and efflux of sodium. A transient augmentation of the rate of ammonia excretion is observed upon acidification, suggesting an increased permeability of the gill to the free-base form of ammonia.

3. The ionic balance sheet of the movements of the monovalent ions across the gill was drawn up when a permeant co-ion for sodium was absent from the external medium. In both acid and alkaline media the ionic balance was achieved in accordance with the law of electroneutrality of external and internal solutions.

4. Net Na^+ uptake was only found to be correlated to NH_4^+ excretion if H^+ ion movements across the gill were taken into account.

5. Ammonia-gradient experiments were performed to test the ability of the gill to excrete ammonia against a gradient of ammonia partial pressure. Ammonia excretion is independent of this gradient in alkaline media when sodium ions are present. In these conditions ammonia moves in the ionic form. In acid media, with Na present in the external medium, ammonia movement is correlated with the $p\mathrm{NH_3}$ gradient but uphill movement still occurs. Ammonia moves in both the free-base and the ionized forms. In the absence of external sodium, movements of ammonia obey the

law for passive transfer and ammonia moves exclusively in the free-base form, even though the external medium is alkaline.

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