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SODIUM REGULATION IN THE FRESHWATER MOLLUSC *LIMNAEA STAGNALIS* (L.) (GASTROPODA: PULMONATA)

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INTRODUCTION

The greater part of recent work on sodium regulation in freshwater invertebrates has concerned insect larvae and crustaceans whilst freshwater molluscs, although forming an important part of the fauna in most freshwater habitats, have received comparatively little attention.

Previous studies on ionic regulation in freshwater pulmonates have been largely confined to blood analyses of *Limnaea stagnalis* (Huf, 1934; Florkin & Duchâteau, 1950; van der Borght, 1962; Burton, 1968). Krogh (1939), however, demonstrated that *L. stagnalis*, depleted of salt by treatment with distilled water, could absorb chloride from dilute Ringer solutions. Picken (1937), investigating the mechanism of urine formation in invertebrates, found that the urine of *L. peregra* was more dilute than its blood or pericardial fluid, suggesting a reabsorption of ions during the passage of urine to the exterior. More recently the roles of the parietal and pleural ganglia of *L. stagnalis* and *L. limosa* in sodium and water balance have been studied (Hekstra & Lever, 1960; Lever, Jansen & de Vlieger, 1961; Lever & Joose, 1961; Chaisemartin, 1968*a*, *b*). Data on sodium regulation in other freshwater molluscs is equally sparse, although the mechanism of urine production has been examined in some detail in both prosobranchs (Little, 1965*b*; Monk & Stewart, 1966) and bivalves (Picken, 1937; Hiscock, 1953; Chaisemartin, 1968*a*). Again, Krogh (1939) has demonstrated chloride uptake, by *Paludina* (*Viviparus*) and several species of bivalves. The only investigation of sodium balance of freshwater molluscs using tracer techniques was carried out by Chaisemartin, Martin & Bernard (1968) on the bivalve *Margaritana margaritifera*. Sodium and chloride influx were found to be related in a non-linear manner to their external concentrations, reaching a near maximum level in external concentrations exceeding 1·0 mM/l.

In the present investigation sodium balance has been studied in *L. stagnalis* with the aid of the radioisotope ^{22}Na. *L. stagnalis* was selected for its comparatively large size and ease of culture in the laboratory.

MATERIALS AND METHODS

Materials

The experimental animals were bred in the laboratory in glass aquaria containing aerated Newcastle tap water at room temperature (15–20 °C). Snails were fed on cabbage, and micro-organisms were also available in the bottom sediment of the tanks. Fine sand was added to the tanks as *L. stagnalis* requires abrasive material for trituri-

tion of food in the gizzard (Noland & Carriker, 1946). Isotopes were obtained from the Radiochemical Centre, Amersham.

Methods

Experiments were carried out at 10 °C ± 1 °C on snails of 2–4 g total weight which had been adapted to the experimental temperature for at least a week. Animals were starved for several days before use in order to prevent excessive fouling of the experimental media by faecal material. An artificial tap water similar in its major ion concentrations to Newcastle tap water was used in all experiments. Its composition is shown in Table 1. Media of different sodium concentrations were obtained by varying the concentration of NaCl in the artificial tap water, the concentration of the other ions remaining unchanged.

Table 1. *Composition of artificial tap water*

Salt	Concentration (mM/l)
NaCl	0·350
KCl	0·044
$Ca(HCO_3)_2$	1·000
$Mg(HCO_3)_2$	0·400

Chemical analyses

The sodium and potassium concentrations of the blood and experimental media were determined with an EEL flame photometer after suitable dilution of the samples. The relatively high calcium concentration of the artificial media (1 mM/l) interfered with the measurement of sodium and was corrected for by the addition of 1 mM/l $CaCl_2$ to the sodium standards. Calcium concentrations were measured with a Unicam S.P. 900 flame spectrophotometer operated at a wavelength of 622 mμ. Chloride determinations were made with an Aminco-Cotlove chloride-titration instrument operated on the range suitable for samples containing 0–2 μM chloride. Depression of freezing point was measured by the method of Ramsay & Brown (1955), blood used in the determinations being frozen immediately after removal from the snail to prevent possible changes in freezing point.

To measure the total ion concentrations in fresh tissues the animals were first removed from their shells and, after weighing, dried in an oven at 100 °C. The dried tissue, in silica 'Vitreosil' crucibles, was then ashed at 450 °C in a muffle furnace; the ash was dissolved in a drop of concentrated HCl and diluted to a volume suitable for analysis.

Removal of blood samples

A small hole was bored in the shell with a dental drill and blood was withdrawn from the pulmonary sinuses into a fine Pyrex glass pipette. The blood was then discharged from the pipette under liquid paraffin and samples were withdrawn and diluted for analysis.

Measurement of sodium fluxes

Sodium influx measurements were made on individual snails, using ^{22}Na, in an apparatus similar to that used by Shaw (1960). Influx was calculated from the fall in the radioactivity of the external medium by the method of Shaw (1959). Flux measurements were carried out out over periods of up to 20 h during which time errors due to

backflux of tracer were less than 1%. Small samples of the external medium were removed at intervals for sodium analysis, in order to follow any net movements of sodium between animal and medium.

To measure the rate of sodium loss snails were placed in 100 ml. chambers through which a constant volume of sodium-free artificial tap water was recirculated. Samples of the medium, of 1 ml volume, were removed at half hourly intervals for sodium analysis. Each experiment was stopped before the external sodium concentration reached the minimum equilibrium concentration, and sodium loss over the period of measurement was linear with respect to time. Snails were acclimatized to this apparatus for several days before sodium loss rates were determined, since handling and disturbance were found to increase the rate of sodium loss from the snails. Flux measurements are given as μM-Na/g fresh tissue/hour.

Measurement of potential difference

Measurements of the potential difference between the blood and the external medium were made using a Vibron Electrometer Model 33B-2 and two calomel:saturated KCl electrodes. The snail was suspended from a glass rod, attached to its shell with Sira wax, so that its head and foot were immersed in the medium and the shell was above the water. One electrode was placed in the external solution (artificial tap water) and the other was in contact with the blood via a bridge filled with a *Limnaea* Ringer solution, the tip of which was inserted into the blood through a small hole drilled in the second whorl of the shell. Measurements were made at 20 °C.

Measurement of blood volume

Measurements of the extracellular fluid volume were obtained by an isotope-dilution method. A snail was placed in a small volume of artificial tap water labelled with ^{22}Na and allowed to absorb a measured amount of radioactivity over a period of several hours. The animal was then removed from the labelled solution, washed in distilled water and blood samples were taken for radioactive assay. The dilution of the ^{22}Na absorbed into the blood was used to calculate the sodium space of the fresh tissues on the assumption that sodium was confined to the extracellular fluid. Standard counting procedures were observed, each sample being dried on a planchette with a drop of 1 M dextrose as a spreading agent, and counted on an I.D.L. low-background counter.

Terminology

The terms influx, efflux, uptake, net uptake and loss rate are used as described by Shaw (1959).

Experimental vessels

Experiments were carried out in either Perspex chambers or Pyrex glass beakers. Samples of diluted blood and of the external medium were stored in Polythene containers.

RESULTS

The composition of the blood and tissues

The concentrations of the major ions in the blood and in fresh tissues of *L. stagnalis* are given in Table 2. The measurement of the calcium concentration in fresh tissue

was made on snails of 1–2 g total weight whilst other values in Table 2 were for animals of 2–4 g. The tissue calcium concentration was later found to increase with increasing size, an animal of 1–2 g total weight containing about 26·6 mM-Ca/kg tissue water whilst animals of 3–4 g contained 60–100 mM-Ca/kg tissue water. The concentration of sodium, potassium and calcium in the blood and of sodium and potassium in the total fresh tissue remained fairly constant over the size range examined.

Table 2. *Composition of the blood and total fresh tissues of* Limnaea stagnalis *adapted to artificial tap water*

Measurement	Blood (mM/l ± S.E.)	Tissues (mM/kg tissue water ± S.E.)
Δ as NaCl	70·4 ±4·0 (6)	—
Sodium	57·0 ±1·02 (24)	37·2 ± 2·1 (13)
Potassium	1·84 ± 0·16 (7)	14·7 ± 1·7 (12)
Calcium	4·91 ± 0·1 (57)	26·6 ± 2·3 (21)
Chloride	43·9 ±2·5 (8)	—
Total cations	68·7	—
Na Space as % fresh tissue wt.	65·7 ±3·5 (9)	—

Brackets denote number of observations.

Sodium balance in artificial tap water

After transfer to artificial tap water containing 0·35 mM-Na/l from aquarium water most snails lost sodium to the medium for several days. This was followed by a period of net sodium uptake and a steady state was eventually reached at the original concentration of 0·35 mM-Na/l. A similar response was observed when *L. stagnalis* was adapted to artificial tap water containing 0·1 and 0·2 mM-Na/l. At these concentrations a degree of hysteresis was observed, more sodium being reabsorbed than was originally lost. A second period of net loss followed absorption, however, sodium equilibrium finally being reached at the initial sodium concentration. In view of this, care was taken to ensure that each experimental animal was in sodium balance with the medium before steady-state flux measurements were made.

Sodium influx

The sodium influx was measured using snails adapted to artificial tap water containing 0·35 mM-Na/l, over a range of external sodium concentrations from 0·05 to 0·4 mM-Na/l. Influx data is presented in Fig. 1. The relationship of sodium influx to the external sodium concentration was found to be non-linear, the influx levelling off at higher sodium concentrations. In certain other freshwater animals the relationship between sodium influx and the external sodium concentration can be characterized by the Michaelis–Menten equation:

$$\text{Influx} = K\left(\frac{C}{K_m + C}\right),$$

where K is the maximum rate of sodium transport, C the external sodium concentration and K_m the external sodium concentration at which half the maximum influx is obtained (Shaw, 1959; Stobbart, 1965; Shaw & Sutcliffe, 1961; Sutcliffe, 1967*a*, *b*). Such a relationship may also be true of *L. stagnalis*, this being more evident in the data for depleted snails shown in Fig. 4. The variability of the data presented (Fig. 1, 4) is

large, however, and the Michaelis curves shown are necessarily of an approximate nature; other similar curves could also be fitted to the data. Influx from artificial tap water containing 0·35 mM-Na/l was 0·132 μM-Na/g/h, equivalent to a turnover of blood sodium of 0·35 %/h. Half-saturation of the influx mechanism was reached at an external sodium concentration of 0·25 mM-Na/l. Some measurements of sodium influx have been made using snails adapted to artificial tap water containing 0·1 and 0·2 mM-Na/l. The results are shown in Table 3. Influx from artificial tap water containing 0·2 mM-Na/l into snails adapted to this solution was similar to the rate measured

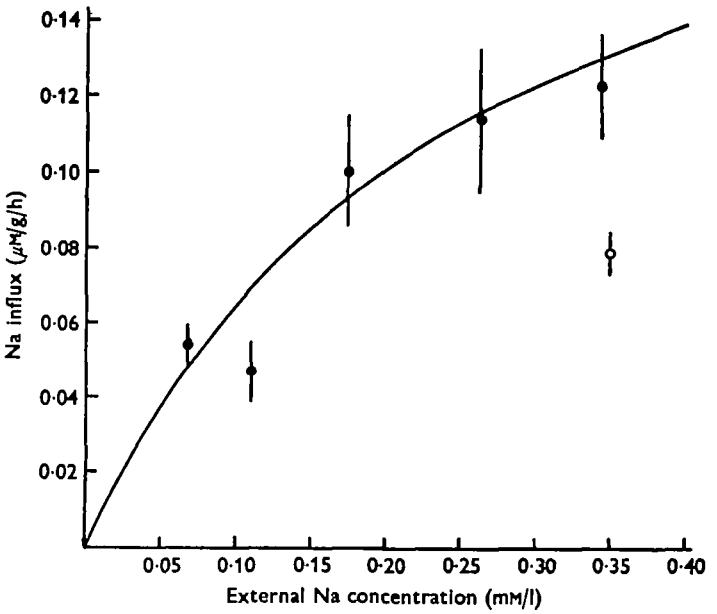

Fig. 1. Sodium influx into snails adapted to artificial tap water containing 0·35 mM-Na/l. The curve represents the equation

$$\text{Influx} = 0{\cdot}225\left(\frac{C}{0{\cdot}25+C}\right),$$

●, mean sodium influx; ○, mean sodium loss rate from snails adapted to 0·35 mM-Na/l. The vertical lines represent standard errors.

Table 3. *Sodium influx into animals adapted to low sodium concentrations*

Adaptation conc. mM-Na/l	Na influx from adaptation conc. μM/g/h ± S.E.	Na influx into snails adapted to 0·35 mM-Na/l μM/g/h
0·1	0·110 (2)	0·065
0·2	0·090 ± 0·012 (5)	0·101

Brackets indicate number of observations.

from the same external concentrations into snails adapted to 0·35 mM-Na/l. The sodium influx from artificial tap water containing 0·1 mM-Na/l was 0·110 μM/g/h, about 40 % greater than the influx from the same external concentration into snails adapted to 0·35 mM-Na/l, but somewhat lower than the influx into depleted snails from the same concentration. However, further measurements would be necessary before any firm conclusions could be drawn from these results.

Measurements of potential difference

The potential difference between the blood of *L. stagnalis* and the external medium has been measured. The media used were artificial tap water solutions of a range of sodium concentration (0–0·35 mM-Na/l). The results are shown in Table 4. No significant change in the potential difference occurred when the concentration of sodium in the medium was reduced. The measured potential difference between the blood and artificial tap water containing 0·35 mM-Na/l of −16·4 mV (blood negative) would support a blood sodium concentration of only 0·67 mM-Na/l. The normal blood sodium concentration, however, is 57 mM-Na/l (Table 2) and this must, therefore, be maintained by an active transport of sodium into the blood.

Table 4. *Potential difference between the blood and media containing 0–0·35 mM-Na/l*

External conc. mM-Na/l	Potential difference mV ± S.E.
0	−17·0 ± 1·7 (9)
0·10	−16·4 ± 4·6 (7)
0·20	−16·6 ± 2·0 (7)
0·35	−16·4 ± 1·4 (9)

Table 5. *Blood sodium concentration and sodium loss rate in snails adapted to a range of sodium concentration*

Adaptation conc. (mM-Na/l)	Sodium loss rate μM/g/h ± S.E.	Blood Na conc. mM/l ± S.E.	Reduction of loss rate %
0·35	0·079 ± 0·006 (17)	57·0 ± 1·02	—
0·20	0·070 ± 0·010 (11)	50·4 ± 1·88	11·4
0·10	0·054 ± 0·009 (8)	49·3 ± 1·95	31·6
0·05	0·048 ± 0·023 (6)	45·2 ± 6·35	39·2
0·025	0·037 ± 0·006 (7)	36·0 ± 3·15	46·8

Brackets indicate number of observations.

The electrochemical gradient between the blood and medium (artificial tap water) was even less favourable for the passive uptake of chloride than for sodium. *Limnaea stagnalis* however, maintains a blood concentration of about 43 mM-Cl/l in a medium containing only 0·4 mM-Cl/l. In addition Krogh (1939) showed that a net uptake of chloride was possible, by salt-depleted snails, from media containing more than 0·56 mM-Cl/l. Clearly the uptake of chloride ions by *L. stagnalis* must also be by an active transport process.

Sodium loss

The rate of sodium loss from snails adapted to artificial media containing between 0·05 and 0·35 mM-Na/l has been measured. The results are shown in Fig. 2 and Table 5. The value for sodium loss from snails adapted to 0·025 mM-Na/l in artificial tap water has been taken as equal to the influx, at the same external concentration, into snails adapted to their minimum balance concentration. As the possibility exists that the influx may include a small exchange component, the true rate of sodium loss from snails adapted to 0·025 mM-Na/l may be slightly lower than the influx value given in

Table 5. The difference between sodium loss rates from snails adapted to 0·025 mM-Na/l and to 0·35 mM-Na/l is highly significant ($P < 0.001$). The difference between loss rates from snails adapted to 0·35 and 0·1 mM-Na/l was also significant ($P < 0.05$) but no significant difference was found between sodium loss rates from snails adapted to 0·05 and 0·35 mM-Na/l ($P > 0.1$), variability shown by snails adapted to 0·05 mM-Na/l being large. The blood sodium concentration of snails adapted to 0·35 mM-Na/l was significantly higher than in snails adapted to artificial media containing either 0·1 or 0·2 mM-Na/l ($P < 0.01$ in each case). Animals adapted to 0·025 mM-Na/l also show a significantly reduced blood sodium concentration ($P < 0.001$). From this data

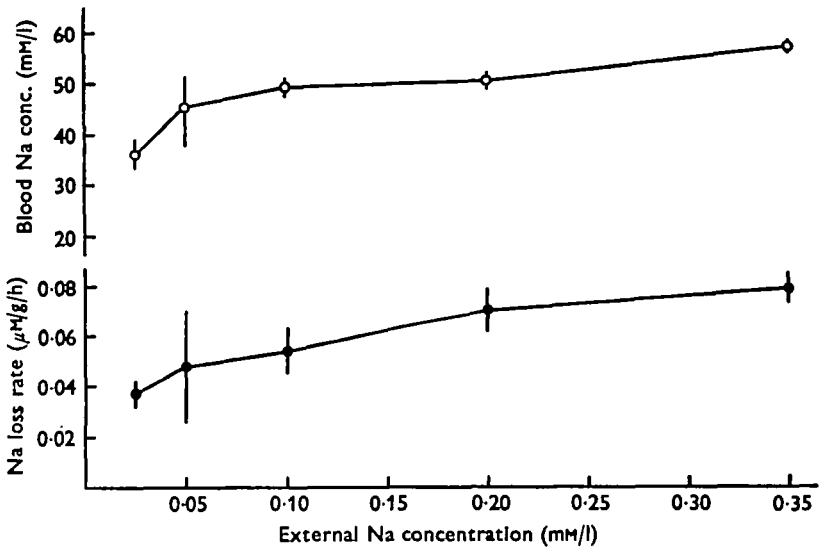

Fig. 2. The relationship between blood sodium concentration and sodium loss rate in snails adapted to a range of external sodium concentration. ●, Mean sodium loss rates; ○, mean blood sodium concentrations. The vertical lines indicate standard errors.

(Fig. 2, Table 5) it would appear that blood sodium concentration and the sodium loss rate decline in a more or less parallel fashion when snails are adapted to low external sodium concentrations. It has not been possible to measure the urinary sodium loss rate of *L. stagnalis* as the urinary pore is rather inaccessible and the snail is very sensitive to handling or mechanical stimulation. Attempts to measure passive sodium loss using isosmotic sucrose solutions (Sutcliffe, 1967*a*) did not yield consistent results. It is difficult, therefore, to tell whether or not control of the urinary sodium loss plays a significant part in the regulatory mechanism.

Snails in sodium balance with the medium show no net gain or loss of sodium so the rate of sodium uptake must therefore equal the rate of sodium loss. However, the sodium influx from artificial media containing 0·35 mM-Na/l into snails adapted to this solution was greater than the loss rate from snails adapted to the same concentration (Table 6). It follows therefore that sodium influx at this external concentration is not a true measure of the rate of sodium uptake. The discrepancy between sodium influx and loss rate may be explained by the presence of an exchange component of sodium influx whereby exchange of external for internal sodium ions occurs on a

1 : 1 basis. The exact size of this exchange component must remain in doubt as variation in influx and loss data was considerable. Also, without further data it is not possible to characterize the exchange process as due to an 'exchange diffusion' of the type envisaged by Ussing (1947) and Levi & Ussing (1948) or due to a pump-linked exchange mechanism. Exchange components of sodium influx have been described in a number of other freshwater animals, e.g. *Astacus* (Shaw, 1959, 1960), *Asellus* (Lockwood, 1960) and *Aëdes* (Stobbart, 1959).

Table 6. *The exchange component of sodium influx into snails in equilibrium with artificial tap water containing* 0·35 *mM-Na/l*

Na loss rate	0·079
Na influx	0·132
Na exchange component	0·053

All values as μM-Na/g/h.

Table 7. *Some values for the minimum equilibrium concentration of sodium*

Sodium conc.	0·017	0·030	0·021	0·012	0·040
(mM-Na/l)	0·030	0·044	0·006	0·030	
Mean	0·025				

Table 8. *Reduction in blood volume (measured by weight changes) during adaptation to the minimum equilibrium concentration*

	Original weight lost (%)					
Changes of sodium-free water	No. of animal					
	1	2	3	4	5	Mean
1	7·3	3·8	3·0	0	3·9	3·6
2	7·9	6·1	6·8	0	8·5	5·9
3	12·1	6·0	9·2	1·3	11·9	8·1
4	18·8	11·4	14·3	10·5	8·1	13·0

Sodium balance in depleted snails

Snails were depleted of sodium by adaptation to their minimum sodium equilibrium concentration in the manner described by Shaw (1959) and Little (1965*a*). 100 ml volumes of sodium-free artificial tap water were employed. Some values for the minimum sodium equilibrium concentration are shown in Table 7. Adaptation to the minimum balance concentration involved a reduction of blood sodium concentration to 36 mM-Na/l (Table 5) a value significantly lower than in normal snails. *L. stagnalis* also showed a reduction in blood volume during each exposure to sodium-free media (Table 8), the cumulative reduction being about 13% of the initial fresh tissue weight. The blood sodium concentration of snails adapted to their minimum equilibrium concentration was at approximately the expected level considering the measured sodium losses during depletion and the reduction in blood volume. Fig. 3 shows a set of sodium balance curves for a typical snail, indicating the manner in which sodium balance was achieved in each change of sodium-free artificial tap water. A large initial loss of sodium to the medium occurred in the first volume of sodium-free water (curve 1) but much of this sodium was regained before the snail reached sodium balance with the medium. In subsequent volumes of sodium-free artificial tap water (curves 2–5)

relatively small amounts of sodium were lost and the equilibrium concentrations were close to the minimum values given in Table 7. The initial loss of sodium to the medium before net uptake began (Fig. 3, curve 1) averaged about 23% of the total body sodium

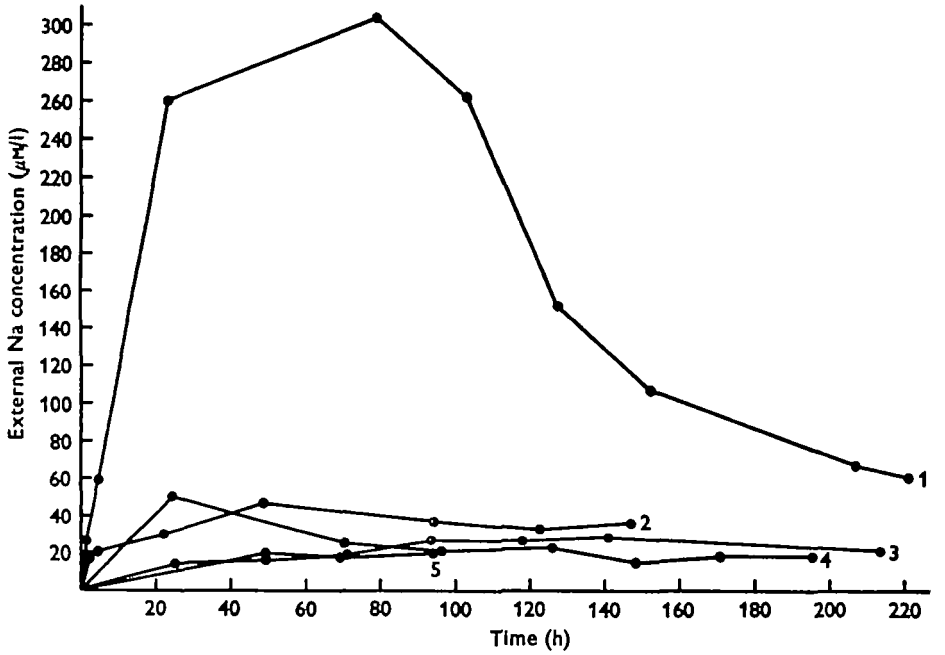

Fig. 3. Sodium balance in sodium-free artificial tap water by a single snail. Curves 1–5 show how sodium balance was achieved in five successive 100 ml volumes of sodium-free medium.

Table 9. *Sodium loss and blood volume reduction of snails placed in* 100 *ml of sodium-free artificial tap water*

Maximum sodium loss (mM)	Maximum sodium loss as % total sodium	Blood sodium conc. at point of max. sodium loss (mM/l)	Reduction of fresh tissue weight at point of max. sodium loss (%)
0·031	39·8	44·0	13·6
0·024	34·0	48·0	12·8
0·009	10·7	55·2	5·3
0·020	26·6	50·4	15·2
0·011	13·4	43·2	2·9
0·012	11·5	55·2	2·7
0·021	27·8	44·9	11·8
0·015	20·2	53·6	9·0
0·090	12·8	50·4	2·5
0·012	17·6	53·6	6·0
0·011	10·5	—	—
0·031	37·4	—	—
0·018	24·9	—	—
0·014	21·9	—	—
0·016	20·5	—	—
0·014	21·8	—	—
0·036	52·6	—	—
0·016	16·1	—	—
0·012	12·5	—	—
0·016	25·2	—	—
Mean 0·017 ± 0·002 S.E.	22·9 ± 1·9 S.E.	49·9 ± 1·5 S.E.	8·2 ± 1·6 S.E.

(Table 9). The period of initial loss varied between 24 and 94 h in the snails examined, the total amount of sodium lost increasing with time. At the peak of sodium loss to the medium a reduction in blood volume of about 8% (calculated from weight changes) occurred and there was a significant fall in blood sodium concentration ($P < 0.001$) from 57 to 49·9 mM-Na/l (Table 9). This fall in blood concentration (12·5%) was of the magnitude expected from the measured sodium losses and blood volume reduction. The maximum sodium net uptake rate, during resorption of sodium following the initial period of loss, was measured and data for several animals is presented in Table 10. The mean maximum net uptake rate was similar to the rate of net sodium uptake by fully depleted snails in the same external medium (Fig. 4). Thus the sodium uptake mechanism of *L. stagnalis* was near maximally stimulated by the initial loss of sodium. During recovery of lost sodium from the balance solution the rate of sodium net uptake fell off more rapidly than expected from the fall in external sodium concentration. Presumably, therefore, the gradual restoration of blood sodium concentration acted to reduce the rate of sodium uptake.

Table 10. *The rate of net uptake of sodium after the period of initial loss during sodium balance in sodium-free water*

Initial sodium loss as % total body Na	Max. rate of net uptake of sodium during resorption (μM/g/h)	Net uptake from the same ext. conc. by depleted snails (μM/g/h)
10·5	0·038	0·076
37·4	0·186	0·157
24·9	0·073	0·132
21·9	0·084	0·100
20·5	0·047	0·120
21·8	0·054	0·114
52·6	0·253	0·184
16·1	0·081	0·100
12·5	0·064	0·076
Mean 24·3 ± 4·4 S.E.	0·098	0·117

Values for net uptake of sodium by depleted snails were taken from Fig. 4.

Sodium influx in depleted snails

Simultaneous measurements of sodium influx and net uptake have been made over the range of external sodium concentration 0·025–0·5 mM-Na/l using snails in equilibrium at their minimum sodium balance concentration. The results are shown in Fig. 4. After each measurement at high external sodium concentrations the snails were redepleted by treatment with sodium-free artificial tap water to ensure that the animals remained maximally stimulated with respect to the uptake of sodium ions. The relationship between sodium influx and the external sodium concentration again shows saturation kinetics. The sodium influx from 0·35 mM-Na/l in artificial tap water was approximately twice that of snails in sodium balance with the medium at the same external concentration. Half-saturation of the uptake mechanism again occurred at an external concentration of about 0·25 mM-Na/l. In addition, sodium uptake exceeded sodium loss making possible a net uptake of sodium of 0·192 μM-Na/g/h from artificial tap water containing 0·35 mM-Na/l. The sum of sodium loss rate and net uptake rate

was about 10% less than the influx value, in artificial tap water (0·35 mM-Na/l) (Table 11). Thus the exchange component of sodium influx, demonstrated in steady-state snails adapted to 0·35 mM-Na/l, was also present in depleted snails. The divergence of influx and net uptake curves (Fig. 4) implies that the size of this exchange component increases as the external concentration rises. In a few cases sodium influx and net uptake have been followed over longer periods. As sodium was regained by the snails the influx and net uptake rate were gradually reduced until steady-state conditions were reached, although in a few animals a degree of hysteresis was observed.

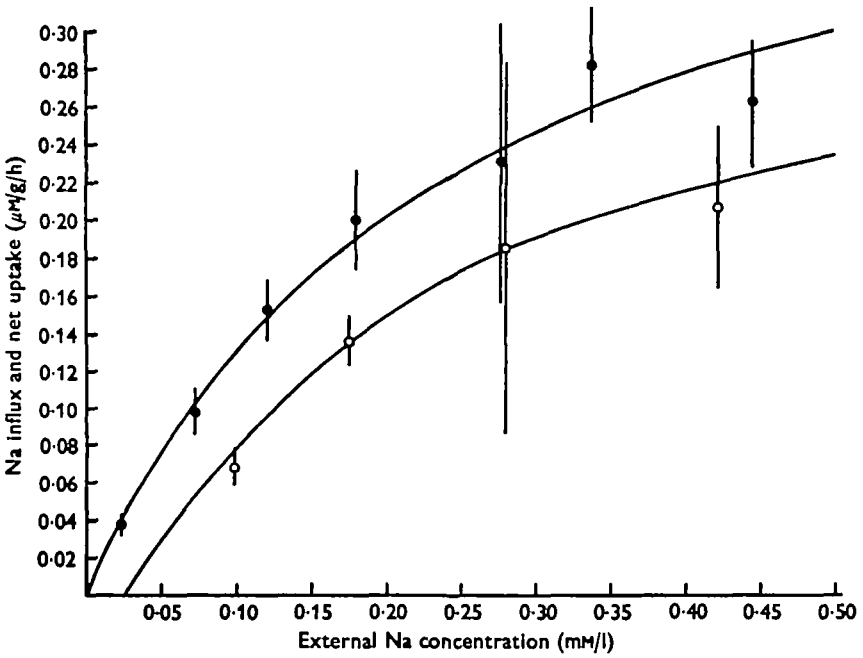

Fig. 4. Sodium influx and net uptake in snails depleted of sodium by adaptation to their minimum sodium balance concentration. ●, Mean sodium influx; ○, mean sodium net uptake. The curve through the influx values represents the equation:

$$\text{Influx} = 0{\cdot}42\left(\frac{C}{0{\cdot}25+C}\right).$$

Vertical lines represent standard errors.

Table 11. *The exchange component of sodium influx in sodium-depleted snails in artificial tap water* (0·35 *mM-Na/l*)

Loss	0·037
Influx	0·262
Net uptake	0·203
Net uptake + loss	0·240
Exchange component	0·022

All values as μM-Na/g/h.

The effect of reduction in blood volume on sodium uptake

Reduction of blood volume of *L. stagnalis* was effected by stimulating blood loss from the haemal pore. This connects the circulatory system with the cavity of the lung (Lever & Bekius, 1965), and blood released into the lung is expelled via the pneumo-

stome. Blood loss was stimulated by prodding the sole of the foot gently with blunt forceps, after the foot had been in contact with moist filter paper for several minutes. This caused the snail to retract violently into its shell, expelling blood from the haemal pore. This technique was adapted from the method of Lever & Bekius (1965). Blood loss had no apparent ill effects, and the snails generally resumed crawling within 15 min of being replaced in water. A series of experiments was carried out to determine the effects of a reduction of blood volume on sodium influx and net uptake. Blood eliminated from the animal was collected in sodium-free artificial tap water and the snail was allowed to resume crawling in this medium to wash traces of blood from the mantle cavity and body surface. The medium was then analysed to determine the amount of sodium lost from the blood. This generally amounted to about 40% of the total body sodium, equivalent to 0·45 ml of blood or a reduction in blood volume of about 35%. The snails were then transferred to artificial tap water (0·35 mM-Na/l) labelled with ^{22}Na. Sodium influx was constant throughout the period of measurement and sodium net uptake was apparent within 1 h of transfer to the labelled medium. The data for sodium influx and net uptake is shown in Table 12. Both influx and net

Table 12. *The effect of blood volume reduction on sodium influx and net uptake*

	Ext. sodium conc. (mM/l)	Sodium influx (μM/g/h) ± S.E.	Sodium net uptake rate (μM/g/h) ± S.E.	Sodium lost (μM)
	0·246	0·469	0·280	35·5
	0·284	0·404	0·258	19·8
	0·298	0·380	0·217	24·8
	0·301	0·270	0·202	21·0
	0·270	0·294	0·216	27·0
Mean	0·280	0·363 ± 0·036	0·235 ± 0·015	25·6

Table 13. *The exchange component of sodium influx into blood-depleted snails in artificial tap water*

Loss	0·079
Influx	0·363
Net uptake	0·235
Net uptake + loss	0·314
Exchange component	0·049

All values as μM/g/h.

uptake were considerably greater than into sodium-depleted snails in media of the same sodium concentration. Flux and net uptake measurements were based on the fresh tissue weight of the snails before blood loss and must, therefore, represent minimal values. An exchange component of sodium influx, similar to that found in steady-state snails, was apparent during these measurements (Table 13). The sodium loss rate has been taken as normal for snails adapted to 0·35 mM-Na/l although with a reduced blood volume the rate of filtration of primary urine from the blood and thus the rate of sodium loss may well be lower than this.

In a second series of experiments the relative rates of recovery of water and sodium, after blood loss, were examined by following weight changes and net sodium uptake. Snails were depleted of blood as before and the uptake of sodium and water from

artificial tap water containing 0·35 mM-Na/l was then followed. In these experiments there was a considerable time lag (up to 48 h) before net uptake of sodium began. During this period there was an initial recovery of weight (water) which resulted in a small but significant reduction in blood sodium concentration ($P < 0.02$) of about 4 mM-Na/l. After this initial weight recovery no further increase occurred until net uptake of sodium began, recovery of sodium and water then proceeding in a more or less parallel fashion (Fig. 5).

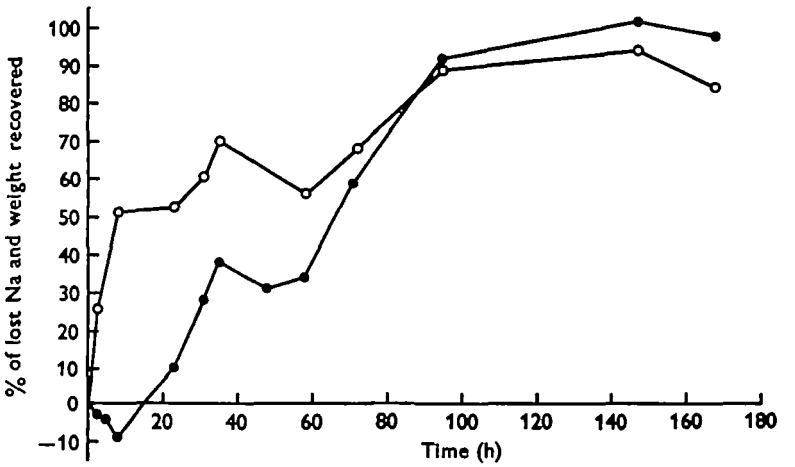

Fig. 5. The recovery of sodium and water by a single snail after an experimental reduction in blood volume. ●, Sodium; ○, Weight.

Table 14. *The available data for blood ion concentrations in* Limnaea stagnalis

Na (mM/l)	K (mM/l)	Ca (mM/l)	Mg (mM/l)	Cl (mM/l)	HCO_3 (mM/l)	Δ mM-NaCl/l	Σ Cations (m-equiv/l)	Source
57	1·8	4·9	—	43·9	—	70·4	68·7	Present study
47·4	2·8	3·0	4·8	42·6	—	—	65·8	Huf, 1934
—	1·2	7·1	2·1	—	—	—	—	Florkin & Duchâteau, 1950
31·2	1·2	7·1	2·1	27·2	—	—	50·8	Duchâteau & Florkin, 1954
57	—	4·5	—	—	—	—	—	van der Borght, 1962
50 (Oct.)	1·5	5·6	2·3	—	—	—	67·3	Burton, 1968
49 (Sept.)	2·0	4·0	1·5	—	—	—	62·0	Burton, 1968
60	2·96	4·1	—	53·0	15·8	—	71·2	Chaisemartin *et al.* 1967

DISCUSSION

The measurements of blood ion concentrations made in this investigation agree quite closely with other recent measurements (van der Borght, 1962; Chaisemartin, Mouzat & Sourie, 1967; Burton, 1968) although earlier analyses are less consistent (Huf, 1934; Florkin & Duchâteau, 1950). The available data is shown for comparative purposes in Table 14. The variability of these early measurements may be due in part

to the use of less accurate techniques of ion measurements or possibly to the snails not being in ionic equilibrium with the medium. Burton (1968) claimed that significant seasonal variations in blood ion concentrations occurred, but made determinations on only two to four animals at each season. The value for the freezing-point depression of the blood, equivalent to 70·4 mM-NaCl/l, agrees well with the values given for *L. stagnalis* by Picken (1937) of 66 mM/l and by Chaisemartin *et al.* (1967). Potts & Parry (1964) give a similar value for *L. peregra*. Reasonable agreement also exists between the sum of the cations and the total blood concentration even making an allowance of 4 m-equiv/l for magnesium. The concentration of bicarbonate in the blood has been measured by Chaisemartin *et al.* (1967), and using their value the sum of the anions is 59·7 m-equiv/l revealing an anion deficit of about 10 m-equiv/l. This suggests that either some of the cations may be bound or that other anions were present. A small proportion of the blood calcium may in fact be bound to organic molecules (0–6%) (van der Borght & van Puymbroeck, 1964). It would appear likely, however, that the major part of the anion deficit might be explained by the presence of organic anions in the blood. The value given by Chaisemartin *et al.* (1967) for chloride is about 10 mM/l higher than found in the present study and the concentration of organic anions in their animals must, therefore, be low.

The sodium uptake mechanism in *L. stagnalis* is estimated to be half-saturated at an external sodium concentration of about 0·25 mM-Na/l and would reach near saturation in media containing 1·5–2·0 mM-Na/l. These low values indicate a high affinity of the uptake mechanism for sodium ions, a feature characteristic of other freshwater animals e.g. *Astacus* (Shaw, 1959), *Gammarus pulex* (Sutcliffe, 1967*a*), *Triops* (Horne, 1967). The sodium uptake mechanism of *Margaritana margaritifera* appears to be half-saturated and near-saturated at external sodium concentrations of 0·04 mM-Na/l and about 1·0 mM-Na/l respectively (Chaisemartin *et al.* 1968). These values are somewhat lower than those obtained for *L. stagnalis* and this may be related to the extremely low blood sodium concentration of *Margaritana* (Chaisemartin *et al.* 1968). No apparent change in the affinity of the sodium uptake mechanism of *L. stagnalis* occurred when the sodium influx was increased as a result of sodium depletion. Similar observations have been made on other freshwater animals (e.g. Shaw, 1959; Horne, 1967).

Snails placed in media of low external sodium concentration or in sodium-free artificial tap water showed a large initial loss of sodium to the medium amounting to 10% of total body sodium for snails in artificial tap water containing 0·1 mM-Na/l and 23% for snails in sodium-free media. In each case this sodium loss caused increased activity of the sodium uptake mechanism resulting in a period of sodium net uptake. The sodium loss required to stimulate the sodium uptake mechanisms of some other freshwater invertebrates is much lower than in *L. stagnalis*, *Astacus pallipes*, for example, showing a detectable increase in the sodium influx after a loss of only 1% of total body sodium (Shaw, 1959). Sodium loss in *L. stagnalis* was accompanied by a loss of water which reduced the expected fall in blood sodium concentration. It may be, therefore, that sodium uptake in *Limnaea* is stimulated by a relatively small fall in blood sodium concentration although a large sodium loss is necessary to achieve this on account of changes in blood volume. Alternatively there may be a considerable time lag between the detection of sodium loss from the blood and the actual stimulation of increased sodium transport. The rate of sodium uptake is certainly affected by blood

sodium concentration since the sodium influx and net uptake rate decrease as sodium is regained. Similarly during resorption of lost sodium by snails in balance experiments net uptake was found to be related to internal as well as external sodium concentrations.

The increase in the sodium influx and net uptake rate brought about by an experimentally induced reduction in blood volume was considerably greater than that caused by adaptation of snails to their minimum sodium balance concentration. Snails in balance at their minimum equilibrium concentration showed a small reduction in blood volume about 13%) and a considerable fall in blood sodium concentration. Snails experimentally depleted of blood (35% blood volume reduction) showed a small reduction in blood sodium concentration where net uptake was delayed. It is probable that both sodium depletion and a sudden fall in blood volume may cause increased sodium transport. The control of blood volume may be related, to some extent, to ion regulation since sodium loss has been shown to be accompanied by a fall in blood volume. Similarly sodium and water uptake, after blood loss, occur in a parallel fashion and it may be that sodium uptake is initiated by a fall in blood concentration brought about by an initial uptake of water.

It has been demonstrated that the pleural ganglia of *L. stagnalis* affect water balance probably via a hormonal substance causing diuresis (Lever *et al.* 1961). Recent work by Chaisemartin (1968*b*) using *L. limosa* suggests that this diuretic hormone acts by increasing the rate of filtration of urine from the blood. Some evidence also exists for an antidiuretic hormone produced by the cerebral ganglia of *L. stagnalis* but this requires confirmation (Lever & Joose, 1961). In *L. limosa* the parietal ganglia appear to affect the permeability of the body wall to sodium (Chaisemartin, 1968*b*) but again this requires confirmation. In the present investigation the primary response of *L. stagnalis* to sodium loss was an increase in the sodium influx. The sodium influx has been shown to be linked to the blood sodium concentration and it seems likely that increased sodium transport may be brought about by a hormonal mechanism in response to a change in blood concentration or volume. The presence of such a hormone has not yet been established experimentally.

SUMMARY

1. Sodium regulation in normal, sodium-depleted and blood-depleted snails has been investigated.

2. *Limnaea stagnalis* has a sodium uptake mechanism with a high affinity for sodium ions, near maximum influx occurring in external sodium concentrations of 1·5–2 mM-Na/l and half maximum influx at 0·25 mM-Na/l.

3. *L. stagnalis* can maintain sodium balance in media containing 0·025 mM-Na/l. Adaptation to this concentration is achieved mainly by an increased rate of sodium uptake and a fall of 37% in blood sodium concentration, but also by a reduction of the sodium loss rate and a decrease in blood volume.

4. A loss of 23% of total body sodium is necessary to stimulate increased sodium uptake. This loss causes near maximal stimulation of the sodium uptake mechanism.

5. An experimentally induced reduction of blood volume in *L. stagnalis* increases sodium uptake to three times the normal level.

6. About 40% of sodium influx from artificial tap water containing 0·35 mM-Na/l into normal snails is due to an exchange component. Similar exchange components of sodium influx were also observed in sodium-depleted and blood-depleted snails in the same external sodium concentration.

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