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THE INVOLVEMENT OF SODIUM TRANSPORT IN THE VOLUME REGULATION OF THE AMPHIPOD CRUSTACEAN, *GAMMARUS DUEBENI*

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INTRODUCTION

The maintenance of body volume forms an integral part of the overall osmoregulation of all metazoan animals. The fact that such regulation occurs in various invertebrates has long been experimentally established and it has been shown that the capacity to regulate the body-water content is particularly well developed in euryhaline species (Beadle, 1937; Ellis, 1937, 1939; Jørgensen & Dales, 1967; Schwabe, 1933; Nagel, 1934). The constancy of the fluid content of various marine animals (Vinogradov, 1953) implies that they too regulate their volume accurately in their normal environment though when placed in dilute media many stenohaline species show a sustained increase in volume (Dakin & Edmonds, 1931; Bethe, 1934; Krishnamoorthi, 1962).

The presence of the exoskeleton in arthropods limits the extent to which the volume can be changed but this does not absolve these animals from the need to regulate their fluid intake and output actively. A clear illustration of this is provided by the isopod *Asellus aquaticus*, where failure of volume regulation in unfavourable circumstances results in an uptake of water which can be sufficiently severe to cause the body to become cylindrical instead of dorso-ventrally flattened (Needham, 1947). Size increase at moult might also be expected to vary with the magnitude of the gradient between blood and medium in the absence of adequate volume-regulating mechanisms, but it has been shown that there is no such variation in the euryhaline crab *Callinectes sapidus* (Haefner & Shuster, 1964).

The most obvious responses of crustacea to changes in internal volume are variations in the volume of urine produced and in the amount of water taken in. Thus the urine volume of *Carcinus maenas* (Nagel, 1934; Shaw, 1961), *Pachygrapsus crassipes* (Gross & Marshall, 1960) and *Gammarus fasciatus* (Werntz, 1963) increases with the gradient maintained between blood and medium, and Kamemoto & Ono (1969) have demonstrated that such variations in the rate of urine flow in the crayfish *Procambarus clarkii* are in part controlled by a hormone emanating from the eyestalk. Water intake by drinking can also be varied according to need, at least in some forms. Thus *Neomysis integer* adjusts its drinking rate in different external salinities (Ralph, 1965).

Volume regulation is not solely a question of elimination or gain of water, however. The osmotic and ionic levels of the blood must be maintained despite fluctuations in volume. In mammals it has been clearly demonstrated that the mechanisms responsible

for determining the rates of loss of water and loss of sodium from the body are independent of each other but are governed by both the volume and the concentration of the blood (Farrell & Taylor, 1962, for review). Comparable studies on the interrelation of volume-regulatory and concentration-regulatory mechanisms in crustacea are practically non-existent, though it has been shown that a massive increase in the rate of active uptake of sodium accompanies volume increase at moult in *Gammarus duebeni* (Lockwood & Andrews, 1969). If the processes of volume regulation in crustacea are analogous to those of mammals it might be expected that changes in intermoult blood volume would also result in variations in sodium influx. This possibility has now been investigated and it is shown that a decrease in body volume will stimulate an increase in the influx of sodium.

METHODS

Animals

The *Gammarus duebeni* used in these experiments were obtained from salt-marsh pools at Redbridge on the River Test. In the laboratory they were kept, prior to experimental acclimatization, in approximately 20% sea water at room temperature (*c.* 15–18 °C). They were fed on Bemax and *Enteromorpha* and appeared normal in every way.

Equipment

In experiments involving the use of ^{22}Na the animals were counted individually in a test-tube containing 1 ml of solution and placed in the well of either an I.D.L. or an Ekco scintillation counter. A rubber piston was used to restrict the movement of the animals within the tube and so to ensure a constant geometry for the system.

Experimental media

For measurements of sodium influx the media used were composed of $2\frac{1}{2}$% sea water labelled with ^{22}Na plus sufficient mannitol (or sucrose) to make the solution isotonic with the blood.

The sodium concentration in the loading media is adequate to ensure that the sodium-transporting sites of the animal are tending towards full saturation (Shaw & Sutcliffe, 1961). The overall sodium concentration is, however, low by comparison with that in the blood of *Gammarus duebeni* (Lockwood, 1964) and hence this loading medium has the additional advantage that even if the animals take in small amounts of fluid by oral or anal drinking (Fox, 1952) this is unlikely to account for more than a small proportion of the total influx of ^{22}Na.

The sea water used for all dilutions had a salinity of 33%.

After exposure to tracer-containing media the animals were rinsed in a salt-free solution of mannitol (or sucrose) of the same concentration as that in the labelled medium. A test of the rinsing procedure on animals previously placed in labelled medium for a period of only 1 min indicated that all superficial tracer is removed.

Acclimatization of animals

Except where otherwise stated, all the animals used were acclimatized to 50%, 55%, 70% or 100% sea water for a period of at least 48 h prior to use. They were not fed during the acclimatization time nor during the experiment itself.

RESULTS

The effect of fluid loss on sodium influx

The effect on the sodium influx of three methods of decreasing the fluid volume of the animals have been tested. These are: (1) placing the animals in hypertonic sucrose solutions, (2) placing them in isotonic sucrose or isotonic mannitol solutions, and (3) physically removing a blood sample with a fine-tipped cannula. In the first case osmotic withdrawal of water decreases the volume but at the same time the blood concentration is increased. In the second case water loss occurs as sodium leaks out of the body so that the volume declines but without marked change in the blood concentration. In the third case there is again a net depletion in volume but no change in blood concentration.

The effect on sodium influx of water loss accompanied by rise in blood concentration

Animals initially acclimatized to 50% sea water and then placed in a sucrose solution isosmotic with sea water show a subsequent decline in sodium influx by comparison with the influx prior to exposure to the high-sucrose solution (Table 1).

Table 1. *Influx of sodium before and after reduction of body-fluid volume by treatment with hypertonic sucrose solution*

(The loading medium prior to volume reduction was 2½% sea water with sucrose added to make it isosmotic with 53% sea water, and labelled with ^{22}Na. The same loading medium was used to test the influx into animals A–D after volume reduction. For animals E–H additional sucrose was added to the loading medium to make it isosmotic with 100% sea water.)

	Influx of sodium prior to reduction in blood volume (counts/100 sec)	Influx of sodium after reduction of blood volume (counts/100 sec)
A	7809	1717
B	8751	1218
C	9656	5874
D	16162	10585
Mean	10594	4848
E	8352	485
F	7549	1972
G	9961	2801
H	7203	3395
Mean	8268	2163

For this experiment the animals were placed initially in the loading medium for a period of 1 h. After counting they were transferred to sucrose isosmotic with sea water. Since this medium is more concentrated than the blood, water is lost from the body and at the same time the blood concentration is raised. After 2¾ h in this sucrose

medium the animals were re-counted, and then half of them (A–D) were replaced in the loading medium for an additional 1 h period. The other half (E–H) were put into loading medium to which additional sucrose had been added so as to make it isosmotic with sea water. In both sets of animals the influx of sodium was markedly smaller in the second period of influx than in the first (Table 1). The fact that this reduction occurs both when the loading medium is hypertonic to the blood and when it is hypotonic implies that the reduction is not due to water movements across the body surface preventing access of sodium to the transporting sites.

A repeat experiment showed a similar effect, the influx of sodium again declining after exposure of the animals to sucrose osmotically equivalent to 100% sea water for 2½ h (Table 2).

Table 2. *The effect of sodium influx of exposure of animals from* 50% *sea water to volume reduction accompanied by increase in blood concentration*

(The effect is produced by placing the animals into sucrose isosmotic with 100% sea water for 2½ h. $T = 18{\cdot}5 \pm 0{\cdot}5$ °C)

	Influx of sodium in 1 h prior to exposure to sucrose isosmotic with 100% sea water (counts/100 sec)	Influx of sodium in 1 h after exposure to sucrose isosmotic with 100% sea water (counts/100 sec)
	8946	8724
	11176	5760
	6604	6209
	8632	5040
	4921	2280
Mean	8063	5602

The effect of water loss to isotonic mannitol or sucrose solutions

Sodium is lost slowly to isosmotic sucrose solutions (Lockwood, 1964; Sutcliffe, 1967) and this loss in turn is accompanied by loss of water from the body. Thus there is a gradual weight loss when the animals are kept in a sucrose solution (Table 3) even if this medium is initially slightly hypotonic to the blood.

It is presumed that a similar loss of weight also occurs when animals previously acclimatized to other salinities are placed in isotonic non-electrolyte solutions.

By contrast with the results of the experiment in hypertonic sucrose, when animals previously acclimatized to 50%, 70% or 100% sea water are exposed to mannitol or sucrose solutions approximately isosmotic with their blood they subsequently show an increase in sodium influx when returned to the loading medium.

Animals from each salinity were placed in the appropriate isotonic loading medium for 30 min (or 1 h). After counting they were transferred to isotonic sucrose or mannitol for 2¼–2½ h. Finally they were given a further 30 min (or 1 h) in the loading medium. Two sets of controls were run with each experiment, in order to ensure that the observed increase in uptake of sodium by the experimental animals was not due to the small drop in blood sodium concentration which the latter experienced. Where the experimental animals had previously been acclimatized to 70% sea water, controls acclimatized to 70% and 55% sea water were used. For experimental animals from 100% sea water the controls were from 70% and 100% sea water. Control animals

were put through the same experimental procedure as the test animals except that they were returned to their acclimatization medium between periods in the tracer medium instead of being placed in isotonic sucrose or mannitol. Analysis of samples of blood taken at the end of the experiment indicated that the sodium concentration of the blood of the experimental animals from 70% drops slightly during the experiment but it nevertheless remains *higher* than that of the control animals from 55% sea water (Table 4). Similarly the blood of experimental animals from 100% sea water does not drop to the level of controls from 70% water (Table 5). Despite the fact that the blood concentration remains higher than that of the controls, the experimental animals show a markedly greater sodium influx in the second period in the tracer medium than in that prior to exposure to isotonic mannitol. Neither of the batches of control animals shows this increase in sodium flux (Tables 6, 7). Furthermore, the absolute sodium influx shown in the second period of loading by the experimental animals exceeds that of the controls despite the lower blood concentration of the latter.

Table 3. *Loss of weight by animals from* 50% *sea water when placed in a sucrose solution slightly hypertonic to the blood* (17·1 *g plus* 100 *ml water*)

(All values in milligrammes)

	Start	1 h	2 h	3 h	4 h	5 h	6 h
	91·8	90·8	88·8	87·0	85·8	85·5	85·0
	86·7	87·0	87·8	86·1	86·2	87·4	87·5
	84·0	83·0	84·1	81·4	81·3	80·8	81·3
	96·5	95·0	94·0	93·2	94·0	93·4	92·2
Mean	89·75	88·95	88·7	86·9	86·8	86·8	86·5

Table 4. *Terminal blood sodium concentrations of the four sets of animals whose sodium influx data is combined in Table* 6

(All values m-equiv/l Na)

Experimental animals from 70% sea water	Control animals from 70% sea water	Control animals from 55% sea water
328 ± 13 ($n = 6$)	327 ± 18 ($n = 5$)	298 ± 11 ($n = 8$)
320 ± 13 ($n = 7$)	326 ± 3 ($n = 5$)	292 ± 18 ($n = 9$)
329 ± 17 ($n = 4$)	366 ± 5 ($n = 4$)	319 ± 13 ($n = 4$)
328 ± 30 ($n = 7$)	336 ± 13 ($n = 4$)	309 ± 23 ($n = 8$)

Table 5. *Terminal blood sodium concentrations of the animals whose sodium influx data is given in Table* 7

(All values m-equiv/l Na.)

Experimental animals from 100% sea water	Control animals from 100% sea water	Control animals from 70% sea water
475 ± 7 ($n = 5$)	498 ± 17 ($n = 4$)	366 ± 2 ($n = 4$)

Additional experiments were undertaken using isotonic sucrose solution instead of mannitol in order to check that the results previously described were not due to some action of mannitol *per se*. Comparable increases in influx of sodium to those found in mannitol were observed, however, after volume reduction in isotonic sucrose both with

animals previously acclimatized to 100% sea water (Table 8) and with those acclimatized to 50% sea water (Table 9).

The count of 60 μl of the loading medium used with the animals from 50% sea water was 11986 in 100 sec. The increase in count in the second period of loading could therefore be explained if (1) the animals had drunk a volume of the medium equivalent to some 50 μl each, or (2) that sodium influx across the body surface was larger in the second than in the first loading period. Measurement of the drinking rate under comparable circumstances indicates that the first explanation is highly improbable.

Table 6. *The effect on sodium influx of reduction in volume of body fluid without gross change in blood concentration in animals acclimatized to 70% sea water*

(The volume change is produced by placing the animals in a mannitol solution (12 g mannitol made up to 100 ml with de-ionized water) for 2½ h, T = 16 °C. The medium for sodium uptake was 12 g mannitol made up to 100 ml with ^{22}Na-labelled 2½% sea water.)

Average influx of sodium (c.p. 100 sec)

Experimental animals from 70% sea water		Control animals from 70% sea water		Control animals from 55% sea water	
Before exposure to isotonic mannitol	After exposure to isotonic mannitol	First period in tracer	Second period in tracer	First period in tracer	Second period in tracer
2848 (n = 17)	5354 (n = 27)	2827 (n = 23)	2159 (n = 23)	5181 (n = 29)	3273 (n = 20)

Mean individual ratios of sodium influx in the second period of loading to that in the first

Experimental animals from 70% sea water	Control animals from 70% sea water	Control animals from 55% sea water
2·04 ± 0·95 (n = 27)	0·87 ± 0·4 (n = 23)	0·73 ± 0·29 (n = 29)

Table 7. *The effect on sodium influx of reduction in volume of body fluid without gross change in blood concentration in animals acclimatized to 100% sea water*

(The volume change is produced by placing the animals in a mannitol solution (16 g mannitol made up to 100 ml with de-ionized water) for 2½ h, T = 16 °C. The medium for sodium uptake was 16 g mannitol made up to 100 ml with ^{22}N-labelled 2½% sea water.)

Average influx of sodium (c.p. 100 sec)

Experimental animals from 100% sea water		Control animals from 100% sea water		Control animals from 70% sea water	
Before exposure to isotonic mannitol	After exposure to isotonic mannitol	First period in tracer	Second period in tracer	First period in tracer	Second period in tracer
2461 (n = 5)	6581 (n = 5)	1496 (n = 4)	1470 (n = 4)	4047 (n = 4)	3434 (n = 4)

Mean of individual ratios of influx of sodium in the second relative to the first period of loading

Experimental animals from 100% sea water	Control animals from 100% sea water	Control animals from 70% sea water
2·24 ± 0·73 (n = 5)	1·05 ± 0·55 (n = 4)	0·95 ± 0·29 (n = 4)

Table 8. *The effect of sodium influx on reduction of volume in body fluid without gross change in blood concentration of animals acclimatized to* 100% *sea water*

(Volume reduction was effected by exposing the animals to sucrose isosmotic with 100% sea water for 2½ h, $T = 18{\cdot}5 \pm 0{\cdot}5$ °C. The medium for sodium uptake was ^{22}Na-labelled 2½% sea water plus sucrose osmotically equivalent to 100% sea water.)

	Net influx of sodium in 1 hr before volume reduction (counts/100 sec)	Net influx of sodium in 1 hr after volume reduction (counts/100 sec)
	2539	6560
	1451	3282
	3054	4803
	4189	7457
	1732	2813
Mean	2593	4983

The results of this experiment may be compared directly with those in Tables 2 and 9 as all were carried out at the same time.

Table 9. *The effect on sodium influx of reduction in volume of body fluid without gross change in blood concentration in animals acclimatized to* 50% *sea water*

(The volume change is produced by placing the animals in a sucrose solution (17·1 g + 100 ml of water) for 2½ h, $T = 18{\cdot}5$ °C. The medium for sodium uptake was 17·1 g sucrose plus 100 ml of ^{22}Na-labelled 2½% sea water).

	Net influx of sodium in 1 h before volume change (counts/100 sec)	Net influx of sodium in 1 h after reduction in volume (counts/100 sec)
	4046	14588
	6624	25220
	9722	19507
	5012	11486
	3093	10049
Mean	5699	16170

The drinking rate

An approximate measure of the rate at which *Gammarus* drinks its medium both under normal conditions and under the conditions of the influx experiment has been obtained by determining the amount of the dye amaranth which is ingested in a 1 h period. Animals previously acclimatized to 50% sea water were exposed to three experimental procedures: (1) direct transfer to 50% sea water containing amaranth at a concentration of 2·42 g %; (2) direct transfer to unlabelled loading medium containing amaranth; (3) transfer to rinsing medium plus amaranth for 2½ h followed by 1 h in unlabelled loading medium plus amaranth. Eight animals were used in each experiment.

At the end of a 1 h period in the amaranth solutions the animals were weighed and then homogenized in de-ionized water. The homogenate was filtered off and the volume was made up to 10 ml and adjusted to pH 9 with buffer. The optical density of the dye in the homogenate was then compared with that of standards prepared by homogenizing untreated animals in known dilutions of the dye. The results were as follows:

No dye was detectable (less than 5 μl of the medium adsorbed) in the animals transferred to 50% sea water plus amaranth.

Uptake of the medium was more than 5 μl but less than 10 μl in both of the other two treatments.

The total wet weights of the animals used in the three experiments were 0·273, 0·251, 0·247 g respectively.

The results suggest that the animals drink the medium to some extent when the body volume is depleted. The volume taken up, however, represents at most only about one twenty-fifth of the animal's weight and is inadequate to explain the large increases in sodium uptake which occur in comparable circumstances. The rise in influx of sodium after volume reduction is therefore presumed to result, at least in part, from an increase in influx across the body surface.

The gross uptake of sodium after decrease in body volume

The preceding experiments imply that sodium influx is increased after the animal has suffered a water loss to isosmotic sucrose, or mannitol. It does not, however, indicate whether or not the increase in influx represents a passive or active movement of sodium. Evidence that there is a net uptake of sodium by animals with a depleted blood volume has been obtained by the use of animals previously loaded in a labelled medium until they had reached a steady state. (Provided that all media to which the animal is subsequently exposed contain either no sodium or sodium at the same specific activity as that in the original labelled medium, then any change in the animal's count represents a net change in its total sodium content.)

Table 10. *The half time of sodium loss in* 50% *sea water at* 18·5 °C (*hours to the nearest half-hour*)

10·5	7·0	5·5	8·0	10·5	10·5	6·5	4·0	5·0	Mean 7·5 ± 2·5

The animals were loaded in 50% sea water labelled with ^{22}Na for 68 h at 18 ± 1 °C. (For an average animal this represents 8 half-times for sodium exchange (Table 10).) After rinsing and counting, the experimental animals were retained in fresh isotonic sucrose medium for 2½ h in which time they lost, on average, 15·9% of their total sodium. They were transferred finally to a test medium composed of the original 50% sea water loading medium diluted to 2½% sea water with the sucrose solution.

Control animals, after the initial count, were transferred back to the original labelled 50% sea water for 2½ h instead of being put in the sucrose rinsing medium. At the end of this time they were recounted and transferred to the test medium. Both the controls and the experimental animals were recounted after 1 h in the test medium.

All the experimental animals showed a rise in their total count after the 1 h period. With one exception, all the control animals lost sodium to the same medium (Table 11). This indicates that the experimental animals increased their total sodium content after losing sodium in isotonic sucrose solution.

The effect of removal of blood on sodium influx

The same technique used in the experiments already described has been extended to measure the influx of sodium before and after removal of blood.

Table 11. *Increase in total body sodium when animals are placed in a medium containing sodium after they have suffered a reduction in body volume without change in blood concentration*

(Column A, net count of individuals loaded to a steady state in ^{22}Na-labelled 50% sea water (s.w.) and then exposed to a sucrose solution (17·1 g sucrose plus 100 ml water) for 2½ h; B, net count of the same animals after a further 1 h in the test loading medium; C, net count of control animals loaded to a steady state in the ^{22}Na-labelled 50% sea water; D, count of the controls after 1 h in the test-loading medium. The test-loading medium in this experiment was 5 ml of the labelled 50% sea water diluted to 100 ml plus 17·1 g sucrose, T = 20 °C.)

	Counts/100 sec. Net count after 2½ h in isosmotic sucrose (A)	Counts/100 sec. Net count after further 1 h in 2½% s.w. plus sucrose (B)		Net count after 2½ h in labelled 50% s.w. (C)	Net count after further 1 h in 2½ s.w. plus sucrose (D)
	8564	8812		27067	25551
	19082	19268		35053	31583
	19387	21220		28786	27109
	21895	23567		30219	39524
	7990	9500		14764	12290
	12197	14482		14687	13773
	26797	27914	Mean	26262	24971
	14777	16247			
Mean	16336	17626			

After preliminary exposure to the labelled medium for 1 h, blood was removed by puncture through a dorsal intersegmental membrane and the animal was then counted to determine the proportion of the count removed. The average amount removed in the animals shown in Table 12 was equivalent to 10·4% of the total body sodium and in Table 13 to 8·2% of the total body sodium.

In considering the effect on influx of blood removal the fact that technical difficulties would be expected to preclude the manifestation of full active uptake by the experimental animals must be taken into account. The most important of these technical points is that it takes between 1½ and 3 h for the maximum uptake rate to be mobilized (unpublished data) whereas the period in the loading medium after removal of blood is of only 1 h duration. A longer period in the loading medium is not permissible since this would entail further volume reduction. Unfortunately there is no medium into which the animal can be placed to allow time for mobilization of reserve uptake capacity after blood removal which does not itself influence the subsequent influx. Distilled water, isotonic sucrose and 50% sea water are all ruled out for this purpose. It must be accepted therefore that mobilization of increased uptake capacity will be only partial in the experimental animals for which data is presented.

Stress caused by damage during removal of blood may also cause delay in the onset of increased influx in some cases. However, despite these points, the data in Table 12 illustrate that on average the increase in influx in the second period in the loading medium is considerably larger in the experimental animals from which blood was removed than in the controls. This same effect is also shown in a similar experiment, the results of which are given in Table 13.

In order to assess the statistical significance of this apparent difference between the controls and animals from which blood had been taken the test for standard error of difference of proportions has been applied.

Table 12. *The effect of removal of a volume of blood on the influx of sodium*

(Column A, influx of sodium in a 1 h period prior to removal of a volume of blood; B, net increase in count in 1 h after removal of blood; C, control animals, influx of sodium in a 1 h period; D, influx of sodium in a second 1 h period. The loading medium was 17·1 g sucrose plus 100 ml of ^{22}Na-labelled 2% sea water, T = 14 °C.)

Experimental animals			Unsampled control animals		
Counts/100 sec (A)	Counts/100 sec (B)	Ratio	Counts/100 sec (C)	Counts/100 sec (D)	Ratio
1120	2921	2·61	715	1383	1·93
1429	2806	1·96	1596	2063	1·29
1783	4143	2·32	1376	1985	1·44
524	2012	3·83	854	1884	1·20
860	1528	1·66	577	1125	1·95
1165	2862	2·30	462	708	1·56
934	2164	2·26	904	1749	1·93
920	3124	3·18	1861	2807	1·51
1096	2605	2·39	1256	1823	1·64

Table 13. *Increased influx of sodium after removal of a volume of blood from animals initially acclimatized to* 60% *sea water*

(Column A, influx of sodium in a 1 h period; B, influx of sodium in a 1 h period after removal of blood; C, influx of sodium in unsampled control animals; D, influx of sodium in a second 1 h period in unsampled control animals. The loading medium was ^{22}Na-labelled 5% sea water with sucrose added to make it isosmotic with 64% sea water.)

Experimental animals			Unsampled controls		
Counts/100 sec (A)	Counts/100 sec (B)	Ratio 2nd/1st	Counts/100 sec (C)	Counts/100 sec (D)	Ratio 2nd/1st
10900	11750	1·08	5200	7670	1·47
8850	16760	1·89	9400	16900	1·79
8220	19000	2·32	10150	12800	1·26
8800	26350	2·99	6500	6810	1·05
4000	17050	4·26	8850	12600	1·42

The standard error of difference of proportions is given by the espression

$$\sqrt{\left[Pq\left(\frac{1}{N_1}+\frac{1}{N_2}\right)\right]},$$

where N_1 and N_2 in this case are the numbers of experimental and control animals respectively. The value P is given by the expression

$$P = \frac{P_1N_1+P_2N_2}{N_1+N_2},$$

where P_1 is the fraction of the experimental sample with an influx ratio exceeding the arbitrarily chosen level of 2·0 and P_2 is the comparable fraction in the case of the control animals. The value of q is $1-P$.

On this basis the standard error for the difference of proportions for the values in Table 8 is 0·16 and the difference of proportions $(P_1 - P_2)$ is 0·55. The difference of proportions exceeds the standard error by more than a factor of three and the difference in the populations may therefore be regarded as significant. A t test on the sample also indicates significance at better than the 1% level. The same test cannot be applied to the data in Table 9 because of the smaller size of the sample. In this case, however, the populations can be shown to be distinct by the Mann–Whitney U test for small samples. The probability of the medians of the controls and experimental animals being the same in this case is 0·048, or less than 5%.

The effect of isotonic mannitol on the Na/Cl ratio

The effect of the experimental technique on the sodium-to-chloride ratio was checked by analysis of the blood of animals from 100% sea water which had been placed in isotonic mannitol (16 g/100 ml) for 3 h at 16 °C. Table 14 indicates that the Cl/Na ratio after this treatment does not differ significantly from that of untreated controls maintained in sea water.

Table 14. *The effect of exposure to isotonic mannitol on the Cl/Na ratio of animals acclimatized to* 100% *sea water*

(Each value represents the mean of readings for the blood of four animals.)

	Na (m-equiv/l)	Cl (m-equiv/l)	Cl/Na
		Controls in sea water	
	495	508	1·025
	487	509	1·045
	477	493	1·023
Mean	486	503	1·034
		Experimental animals	
	470	498	1·060
	456	473	1·037
	467	478	1·024
Mean	464	483	1·040

DISCUSSION

Previous studies on the active uptake of sodium by crustacea have shown that changes in blood concentration influence the rate of transport. A fall in blood concentration below a given level stimulates an increased rate of ion uptake in both freshwater animals such as *Austropotamobius* (Shaw, 1959) and brackish-water forms such as *Carcinus* (Shaw, 1961). The results described in the present paper imply that variation in the fluid volume of an animal may also influence the rate of sodium uptake.

When specimens of *Gammarus duebeni* are placed in isotonic mannitol or sucrose solutions they lose sodium down the concentration gradient, but the blood concentration declines only slightly. Thus animals previously acclimatized to 70% sea water have been observed to lose an average of 13% of their total body sodium during $2\frac{1}{2}$ h in isotonic mannitol at 16 °C whilst their blood sodium concentration declined by only 3·2% in this time. Similarly animals acclimatized to 100% sea water lost 23% of

their sodium in 2½ h in isotonic mannitol and the blood sodium concentration fell by 4·6%. It is presumed that the difference between the loss of total body sodium and decline in blood concentration is due largely to a loss of water from the body. This assumption receives support from the observation (Table 3) that the weight of animals declines in isotonic sucrose. The average weight loss during 3 h in sucrose is 3·2% or, in terms of the total body water, some 4·0%. Since there is little change in concentration during this withdrawal of water no cellular osmotic changes should be initiated, and it is reasonable to suppose that the major part of the loss is from the blood. Assuming that the blood volume is of the order 25–35% of the total body water then the observed loss represents an average loss of blood volume of some 11–13%. These values are subject to various sources of error and cannot be regarded as more than a rough measure of the change in blood volume. They indicate, however, that the volume change is considerable. Exposure to conditions similar to those which cause such a loss of water without gross change in blood concentration also results in an increase in the rate of sodium influx. Animals acclimatized to various salinities in the range 50–100% sea water all show increases in the rate of sodium influx (generally by a factor of 1·5–3·5 times) after a period in isotonic non-electrolyte solutions. No such increase occurs in control animals returned to their acclimatization medium between periods in the loading medium. Repetition of experiments using animals which had been in sea water labelled with ^{22}Na until they had reached a steady state indicated that there was a net increase in total body sodium during the period of influx following reduction of volume. The change in influx of sodium is therefore presumed to represent an increase in the rate of active transport, not just an increase in diffusion or exchange diffusion.

Physical removal of blood by pipette is followed by smaller, but also significant, increase in sodium influx. The probable reason for the smaller size of the response has already been discussed.

By contrast with experiments in which blood is withdrawn physically or when the blood volume is reduced in isosmotic sucrose, removal of water accompanied by rise in blood concentration depresses the influx of sodium. Thus when animals acclimatized to 50% sea water are placed for a period in sucrose isosmotic with 100% sea water the influx of sodium subsequently declines. This decline is not due to any direct inhibiting effect of the high concentration of sucrose since animals acclimatized to 100% sea water and then put into sucrose isosmotic with 100% sea water subsequently show an increase in influx, similar to that shown by animals in 50 and 70% sea water after reduction in blood volume.

Measurements of the drinking rate (oral and anal) of animals in isotonic sucrose solutions indicate that the amount of sodium taken in by these means is too small to account for more than a small part of the observed increase in sodium influx. Consequently much of the increased uptake of sodium must occur across the body surface. It is pertinent therefore to seek the nature of the stimulus which initiates an increased rate of sodium uptake after the animals have spent a period in isotonic mannitol or sucrose solutions. Since the influx is greater in the experimental animals which have suffered fluid loss than in control animals which have either significantly lower or higher blood concentrations, it is apparent that the stimulus cannot be the absolute level of blood sodium concentration. Another potential stimulant of increased sodium uptake

is a change in the Cl/Na ratio (Shaw, 1964), but in the present experiments this too can hardly be the effective factor since the period in isotonic mannitol produces no detectable variation in the Cl/Na ratio (Table 14). There remain the possibilities that the animals regulatory systems are able to detect and respond to variations in the total sodium content of the body or, more likely (if analogy may be drawn with vertebrates), that reduction in body-fluid volume initiates an increase in the rate of active sodium transport.

Diamond (1965) and Curran (1960) have discussed various theories by which water could be caused to move across an epithelium separating two solutions of equal tonicity by means of an active transport of inorganic ions. Furthermore, Copeland (1968) has suggested that the fine structure of the posterior gills of the land crab *Gecarcinus lateralis* is compatible with the assumption that these gills may take up water by utilizing a mechanism comparable with Diamond's local osmosis hypothesis. In the foregut of the same animal measurement of the sodium and water fluxes across the gut wall also indicate that fluid movement is probably isosmotic (Mantel, 1968). If a similar isosmotic transfer of water can occur across the body surface of *Gammarus* then it would be possible for the animal to restore lost fluid volume by increasing the rate of active transport. In the present experiments if the solution moved across the body surface is isotonic with the body fluids (when the body fluids are isosmotic with the media) the animals should be able to replace a fluid loss of 4·5% in 1–8 h at the observed rates of increment in sodium influx. Lockwood & Andrews (1969) have suggested that a similar isosmotic fluid uptake is responsible for the expansion of *Gammarus* after moult.

Gammarus duebeni is almost isotonic with its medium at high salinities but hypertonic in salinities less concentrated than 50% sea water. Consequently if such a form is to make use of isosmotic water transport for volume regulation in high salinities it must also be able to vary the relative rates of water and sodium entry when it is regulating the blood hypertonic to the medium. It is of interest therefore to note that preliminary experiments indicate that the water flux is lower in animals acclimatized to dilute salinities than it is in sea water (A. P. M. Lockwood, C. B. E. Inman & N. C. Platt, unpublished). A similar result has been found for the brackish-water crab *Rhithropanopeus harrisi* (Smith, 1967). Whether or not such changes in permeability at the body surface can be related to the observation of Mantel (1968) that extracts of the thoracic ganglionic mass increase water permeability of the foregut remain to be studied, though this would appear a promising approach.

In the case of *Gammarus* a plausible explanation for the observation that volume reduction stimulates increased active sodium uptake can be made in terms of the problem faced by the animal in its environment. *Gammarus duebeni* is primarily an inhabitant of salt-marsh pools and streams running across the shore or salt-marsh. In these habitats it is liable to be exposed both to rapidly changing salinities and to the possibility of damage and haemorrhage as a result of being abraded between the stones under which it lives. Loss of blood by haemorrhage would reduce the blood volume without stimulating receptors responsible for monitoring blood concentration. In such circumstances the presence of a mechanism monitoring blood volume and activating increased sodium uptake on volume decrease would clearly be advantageous as a means of expediting the return of total sodium and blood volume to their normal levels.

However, if such a mechanism were to operate in a situation where water withdrawal raised the blood concentration then it could result in an excess of sodium being taken into the body. It is probably for this reason that water withdrawal accompanied by a rise in blood concentration does not result in a rise in sodium uptake. How the stimulus of reduction in blood volume is overridden remains uncertain, but analogy can perhaps again be drawn with the situation in mammals where the systems responsible for concentration regulation and volume regulation mutually interact in their control of the rate of sodium excretion. Whichever system is most stressed has the dominating influence.

A mechanism initiating increased sodium uptake following a change in volume, if generally present in invertebrates, may be of considerable significance with respect to development of the active transport processes in brackish-water and freshwater forms. Evolutionary selection can only act on existing processes and, up till the present, no adequate reason has been suggested as to why the archetypical marine ancestors of current non-marine types should have had sodium-transporting systems at the body surface on which selective processes could act. A volume-controlling mechanism involving changes in the rate of sodium uptake could provide the *raison d'être* for the presence of sodium-transporting sites at the body surface. In this latter context an interesting analogy may be made with concept that transport of inorganic ions across the body surface from the medium to the enteron may be involved in the osmotic regulation of *Chlorohydra* and *Pelmatohydra* (Marshall, 1969).

SUMMARY

1. The effect on sodium influx of reduction in the fluid volume of the amphipod *Gammarus duebeni* has been investigated.

2. Removal of water and sodium by exposure to isotonic sucrose or mannitol results in no significant change in blood concentration but is followed subsequently by a marked increase in sodium influx.

3. The increased influx is due, at least in part, to increased active uptake of sodium.

4. Physical removal of blood by pipette stimulates a greater degree of increased uptake in some individuals by comparison with the controls. For technical reasons the increases as measured are smaller than in §2 above and in some individuals there is no response.

5. When the blood concentration is caused to rise at the same time as body volume is reduced there is subsequent decrease in sodium influx.

6. It is concluded that a mechanism is present which initiates an increase in sodium uptake on reduction of blood volume. This mechanism may be of value in replacing fluid loss resulting from haemorrhage, if water uptake accompanies sodium uptake.

7. The evolutionary significance of such a mechanism is discussed in relation to development of active transport mechanisms in present-day freshwater and brackish-water forms.

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