

## STUDIES ON FRESH-WATER OSMOREGULATION IN THE AMMOCOETE LARVA OF *LAMPETRA PLANERI* (BLOCH)

### III. THE EFFECT OF EXTERNAL AND INTERNAL SODIUM CONCENTRATION ON SODIUM TRANSPORT

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#### INTRODUCTION

The present investigation of the factors affecting sodium balance in a primitive fresh-water vertebrate was undertaken to provide a basis for comparison with the extensive and rewarding studies which have been conducted on fresh-water crustacea (Potts & Parry, 1964). Shaw (1959, 1961) has made detailed investigations of the kinetics of sodium transport in this group using isotopic methods of measuring flux rates, and has shown how variables like internal and external sodium concentration can affect sodium transport, and what effect this may have on sodium balance in different environmental situations.

Whole vertebrates have not been studied to the same extent from this point of view, and there is no detailed study of a particular animal, probably because vertebrates are prone to handling diuresis and the individual variation which this produces tends to cloud any relationships which may exist. Jørgensen, Levi & Ussing (1946) performed pioneer studies on whole axolotls using isotopes of sodium and chloride, and were able to measure flux rates for these ions. Contrary to expectations, injections of hypertonic sodium chloride gave increased rates of sodium influx and outflux which they attributed to a handling effect. Later, Jørgensen (1950) was able to demonstrate an increase in sodium influx in response to sodium depletion using frogs and toads, whilst Jørgensen, Levi & Zerahn (1954) showed that sodium and chloride uptake were activated independently when frogs were depleted of these ions. Meyer (1951) partitioned sodium balance in the goldfish and measured sodium flux rates across the gills; and later studies on the same animal by Maetz (1956) demonstrated that sodium influx increased as the external concentration rose from 0.1 to 0.7 mM-Na/l. Sodium outflux gave a more variable response, but there was a significant increase in the net flux of sodium into the animals as the environmental concentration of sodium increased.

Work on isolated frog skin (Ussing, 1954; Kirschner, 1955) has yielded a great deal of information on the kinetics of ion transport and there are clear demonstrations of the rate-limited nature of the sodium-transport mechanism when the external concentration of sodium is increased, beside the fact that a decrease of internal sodium concentration causes an increase of sodium influx (Kirschner, 1955). Indeed, the results from frog skin show a general similarity to those obtained from whole-animal studies on fresh-water crustacea, though a more detailed analysis reveals differences

in the mechanism (Potts & Parry, 1964), and it is sometimes difficult to decide whether the differences are real or a consequence of acknowledged differences in transport mechanisms which arise when tissues are isolated from whole animals.

A previous publication in this series dealt with basic parameters like the fluid and ionic compartments of ammocoete larvae (Bull & Morris, 1967), whilst a later report analysed the effects of low temperature and de-ionized water on sodium balance (Morris & Bull, 1968), because this method of ion depletion has been used extensively in the present investigations.

#### MATERIALS AND METHODS

Ammocoete larvae (3–5 g.) in the year previous to metamorphosis were used in these investigations. Details of the methods by which they were collected and identified, together with the methods of chemical analysis employed, are given in a previous publication (Bull & Morris, 1967). The methods of measuring sodium flux rates by means of the radioactive isotope  $^{24}\text{Na}$ , combined with conventional analyses, are described in Morris & Bull (1968).

#### THE EFFECT OF EXTERNAL SODIUM CONCENTRATION ON THE RATE OF SODIUM TRANSPORT

Two series of experiments were performed on separate groups of animals.

The first group consisted of six *sodium-depleted animals*. These were immersed in de-ionized water at 1° C. for 48 hr. and their sodium loss was recorded. The sodium flux rate at a particular external sodium concentration was then measured on individual animals. Each animal was immersed in 25 ml. of a solution which consisted of frog Ringer made up without the normal sodium chloride content and diluted 1:100 with distilled water (R–Na/100). Sufficient  $^{24}\text{Na}$  and sodium chloride were then added to give the required concentration and count rate. The methods used for measuring sodium flux rates have already been described (Morris & Bull, 1968), and in the present experiments measurements were made over a period of 2 hr. The experiments, involving a range of different external sodium concentrations, took over a month to complete, because the aim was to obtain results which could be related to individual animals and the animals had to be sodium-depleted before each experiment. During the periods between flux rate measurements, the animals were returned to individual Perspex containers containing a layer of mud overlaid by running tap water, and they maintained their weight and condition throughout the experiments.

The second group of experiments was performed on single *normal animals* and involved consecutive measurements of sodium flux rates in several different external sodium concentrations. Small amounts of sodium chloride were added to the environment to increase the sodium concentration before making new flux measurements, and, since the procedure was repeated several times,  $^{24}\text{Na}$  was also added to maintain the isotopic gradient between the external solution and the animal. The duration of each flux measurement was kept to a minimum (2 hr.) to avoid significant increases of the sodium content of the animal since this would have had the effect of lowering sodium influx (see below).

The results from a third series of experiments, where normal animals were allowed to rest for a number of days following flux rate measurements at different external concentrations, are not included here. The results showed that there was no significant difference in the relationship between sodium influx and external sodium concentration when they were compared with the previous group of animals, but there was a great deal of difference in sodium outflux. The increase in outflux which occurs is thought to be caused by the excessive handling which the animals have to withstand when this method is used. During consecutive measurements of flux rates animals only show sodium loss caused by diuresis during the initial stages of the experiments and because of this we have only reported the results of this type of experiment.

### Results

The results of experiments conducted on sodium-depleted and normal animals are given in Table 1, whilst Fig. 1 illustrates the relationships between external sodium concentration and sodium flux rates for a typical sodium-depleted individual.

The rate-limited curve for sodium influx shown by these animals is similar to that observed for isolated vertebrate tissues (Ussing, 1949; Kirschner, 1955; Frazier, Dempsey & Leaf, 1962), and for whole crayfish by Shaw (1959). Kirschner (1955) derived a theoretical equation describing the relationship for frog skin, and Shaw (1959) found that a similar equation also fitted conditions in the crayfish. The equation has the same form as that derived by Michaelis, expanded later by Briggs & Haldane (Baldwin, 1957) which relates the rate of enzyme action to substrate concentration. We have followed the symbols used by Kirschner (1955) since we shall be referring to his theoretical treatment in the discussion which follows, in which case the equation becomes

$$M_i = \frac{M_{i\max} [Na_0]}{K_s + [Na_0]},$$

where  $M_i$  is the rate of sodium influx for a particular external sodium concentration ( $Na_0$ ), whilst  $M_{i\max}$  (the maximum influx) and  $K_s$  (the half-saturation concentration) are characteristic of particular transport situations.

In order to simplify the statistical analysis of our data, we tested the correspondence between the influx equation and the regression line calculated from the expression  $Y = \log X$ . The results (Fig. 1) show that there was a close correspondence over the range of external concentrations used in our experiments and, since the deviation was far less than that shown by the experimental results, we used the regression of  $Y = \log X$  in our statistical analysis. It is emphasized that the results gave an equally good fit employing the influx equation cited above, and there are sound theoretical arguments to expect this relationship (Kirschner, 1955).

The regressions for the effect of external sodium concentration on sodium influx given by individual sodium-depleted animals gave probabilities of  $P < 0.05$  for the majority of the regressions. This result is encouraging considering the small number of degrees of freedom, and at this stage the data was pooled and analysed by multiple regression and analysis of variance techniques (Brownlee, 1949; Snedecor, 1956), so that the effect of weight could be assessed.

The results of the more complex analysis (Table 2) show that regressions for

Table 1. *The effect of external sodium concentration on sodium influx and sodium outflux in normal and sodium-depleted ammocoete larvae. † indicates diuretic values which were not included in the statistical analysis summarized in Table 2*

<i>Sodium-depleted animals</i>				
Animal and weight (g.)	Extl. Na concn. (mm/l.)	Na influx ( $\mu\text{M/hr.}$ )	Na outflux ( $\mu\text{M/hr.}$ )	Na lost in depletion period ( $\mu\text{M}$ )
1 1·62	0·343	0·541	0·008	4·316
	0·356	0·851	0·361	8·681
	0·367	0·455	0·347	7·091
	0·539	0·770	0·278	9·966
	0·739	1·134	0·416	4·851
	1·232	0·780	0·513	7·121
2 1·64	0·175	0·452	0·127	7·828
	0·337	0·666	0·066	8·656
	0·343	0·659	0·236	3·515
	0·416	0·488	0·098	6·341
	0·550	0·679	0·117	12·047
	0·722	0·592	0·170	2·842
3 1·57	1·122	0·849	0·317	9·735
	0·149	0·332	0·116	3·777
	0·326	0·261	0·091	5·849
	0·343	0·388	0·174	8·781
	0·370	0·472	0·309	2·578
	0·506	0·516	0·202	2·574
4 3·57	0·840	0·769	0·569	4·326
	1·067	0·560	0·383	4·805
	0·152	0·608	0·111	4·000
	0·306	0·971	0·185	6·620
	0·356	1·085	0·502	8·540
	0·378	0·904	0·304	2·130
5 4·06	0·495	1·157	0·477	3·390
	0·695	0·839	0·515	2·960
	1·122	1·151	0·396	5·230
	0·165	1·082	0·364	12·490
	0·312	1·441	0·634	10·490
	0·352	1·063	0·686	9·660
6 2·12	0·383	0·985	0·478	7·000
	0·500	1·662	0·789	8·250
	0·616	1·251	0·446	11·950
	0·670	1·092	0·427	8·540
	0·968	1·702	1·007	7·990
	0·155	0·418	0·102	4·420
<i>Normal animals</i>	0·321	0·705	0·434	5·040
	0·323	0·659	0·373	2·210
	0·374	0·533	0·238	2·980
	0·456	0·558	0·131	2·460
	0·642	0·791	0·121	4·290
	0·979	0·998	0·732	6·740
Animal and wt. (g.)	Extl. Na concn. (mm-Na/l.)	Influx of Na ( $\mu\text{M/hr.}$ )	Outflux of Na ( $\mu\text{M/hr.}$ )	
1 3·83	0·110	0·629	0·198	
	0·230	0·887	0·411	
	0·360	1·071	0·486	
	0·540	1·182	0·759	
	0·750	1·089	0·713	

Table 1 (cont.)

Animal and wt. (g.)	Extl. Na concn. (mm-Na/l.)	Influx of Na ( $\mu\text{M/hr.}$ )	Outflux of Na ( $\mu\text{M/hr.}$ )
2	0.067	0.294	0.343
3.42	0.238	0.784	0.527
	0.370	0.477	0.243
	0.583	0.513	0.378
	0.800	0.803	0.268
3	0.077	0.338	0.731†
3.30	0.270	0.533	0.194
	0.370	0.390	0.710
	0.590	1.143	1.028
	0.790	1.068	1.255
4	0.093	0.400	0.898
3.05	0.271	0.736	1.565†
	0.400	1.077	0.908
	0.583	0.439	0.439
	0.730	1.098	0.911
5	0.066	0.297	0.844†
2.92	0.264	0.546	0.358
	0.387	0.486	0.486
	0.594	0.544	0.544
	0.772	0.794	0.669
6	0.193	0.349	0.844†
3.27	0.409	0.983	0.225
	0.546	1.053	1.962
	0.847	1.058	0.811
	1.079	1.475	1.111
7	0.069	0.257	1.275†
2.80	0.271	0.435	0.435
	0.616	0.574	1.532
	0.792	0.458	0.458

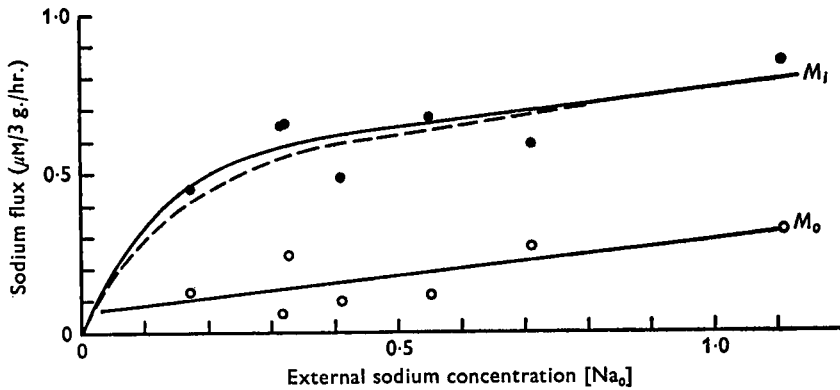


Fig. 1. The relationship between sodium influx ( $M_i$ ) and external sodium concentration based on a  $Y = \log X$  regression equation (upper continuous line) compared with the relationship deduced from the Kirschner influx equation (broken line). The lower continuous line ( $M_o$ ; open circles) shows the corresponding outflux values obtained from the same sodium-depleted ammocoete larva.

sodium influx ( $Y_i$ ) on external concentration ( $X_1$ ) based on the relationship  $Y_i = \log X_1$  are highly significant, and that weight ( $X_2$ ) also has a significant effect on sodium influx in both sodium-depleted and normal animals. In both cases the regression equations for animals of 3 g. weight are given. The equations were used during tests for non-parallelism and elevation differences between the regressions for normal and sodium-depleted animals (Snedecor, 1956). The tests for non-parallelism gave a value of  $P = > 0.2$ , indicating that there was no significant difference between the slope of the two regressions, whilst tests for elevation differences gave a high degree of significance ( $P = < 0.001$ ). Thus the curves were similar in character, though of differing magnitude.

Table 2. *Table of multiple regression equations and their significance*

The equations relate the effect of external sodium concentration ( $X_1$  in mM/l.) and weight ( $X_2$  in g.) on sodium influx ( $Y_i$  in  $\mu\text{M/hr.}$ ) and sodium outflux ( $Y_o$  in  $\mu\text{M/hr.}$ ) in sodium-depleted and normal ammocoete larvae

		Probabilities		
		$X_1$ on $Y$	$X_2$ on $Y$	$X_1 + X_2$ on $Y$
<i>Sodium influx on external concentration and weight</i>				
Sodium-depleted animals	$Y_i = 0.539 \log X_1 + 0.254X_2 + 0.365$ $Y_i = 0.539 \log X_1 + 1.125$ for a 3 g. animal	0.001 —	0.001 —	0.001 —
Normal animals	$Y_i = 0.582 \log X_1 + 0.409X_2 - 0.340$ $Y_i = 0.581 \log X_1 + 0.885$ for a 3 g. animal	0.001 —	0.01 —	0.001 —
<i>Sodium outflux on external concentration and weight</i>				
Sodium-depleted animals	$Y_o = 0.616X_1 + 0.130X_2 - 0.302$ $Y_o = 0.611X_1 + 0.088$ for a 3 g. animal	0.001 —	0.001 —	0.001 —
Normal animals († values in Table 1 omitted)	$Y_o = 0.611X_1 - 0.164X_2 + 0.896$ $Y_o = 0.611X_1 + 0.405$ for a 3 g. animal	0.05 —	0.02 —	0.02 —

The analysis of sodium outflux ( $Y_o$ ) in sodium-depleted animals (Table 2) gave a highly significant relationship between outflux ( $Y_o$ ) and external sodium concentration ( $X_1$ ) and between outflux and weight ( $X_2$ ) which was found to fit a  $Y_o = X_1 + X_2$  relationship better than the log form. Normal animals gave non-significant results because of variability, most of which arises in the initial measurements when the animals are most likely to suffer from handling diuresis. When these values are omitted (results marked † in Table 1), the relationship between sodium loss and external concentration becomes significant and fits a  $Y_o = X_1 + X_2$  type of equation best, though the relationship between sodium loss ( $X_1$ ) and weight ( $X_2$ ) is negative and not very significant in this case. Tests for non-parallelism in the regressions for sodium loss between sodium-depleted and normal animals are barely significant ( $P = 0.05$ ) but there is a significant difference in elevation ( $P = 0.01$ ), indicating that the regressions could have a similar slope but differ in magnitude.

Fig. 2. illustrates the sodium flux rates which are obtained when different values of external sodium concentration ( $[\text{Na}_0]$ ) are substituted into the regression equations

for 3 g. animals, whilst Table 3 shows the half-saturation constant ( $K_s$ ) and the maximum influx rate ( $M_{i\max}$ ) calculated from the sodium influx curves derived from sodium-depleted and normal animals by the method of Linweaver & Burke (1934).

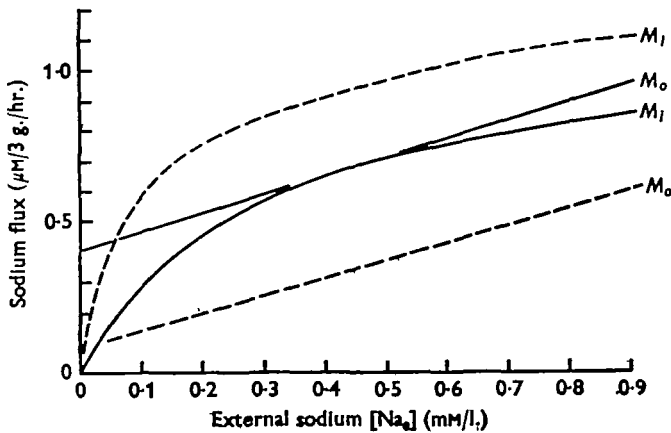


Fig. 2. The relationship between sodium influx ( $M_i$ ), sodium outflux ( $M_o$ ) and external sodium concentration ( $[Na_0]$ ) in normal and sodium-depleted ammocoete larvae. The curves are derived from the regression equations for 3 g. animals (Table 2) and the results for sodium-depleted animals are shown by broken lines.

Table 3. A comparison of the characteristics of sodium transporting mechanism from different freshwater animals

$M_{i\max}$  = maximum influx ( $\mu\text{M/g./hr.}$ )  
 $K_s$  = half-saturation concentration (mm/l.)

Animal	$M_{i\max}$ $\mu\text{M/g./hr.}$	$K_s$ mm/l.	Minimum equilibrium conc. mm/l.	Normal environmental conc.	Author
<i>Gammarus pulex</i>	7.5	0.15	0.06	within the range 0.3–2 mm/l.	Shaw & Sutcliffe, 1961 Shaw, 1961 Kirschner, 1955
<i>Astacus</i>	0.15	0.2–0.3	0.04		
<i>Rana</i> (skin)	—	17	—		
<i>Lampetra planeri</i> (Ammocoete)					
Sodium-depleted	1.35	0.13	—	0.3 mm/l.	Present series and Morris & Bull, 1968
Normal	1.08	0.26	0.005–0.03	—	

Discussion

Considering the effect of external concentration on sodium influx first, it is clear that both sodium-depleted and normal animals show a rate-limited relationship which fits the equation derived by Kirschner for frog skin, though the values of  $M_{i\max}$  and  $K_s$  ( $F_{\max}$  &  $S$  in Shaw, 1959) are nearer the values obtained from fresh-water crustacea than those measured for amphibian skin (Table 3). This confirms the conclusions reached from previous studies (Morris & Bull, 1968) which showed that the ammocoete has a very efficient sodium-uptake mechanism. The present studies (Fig. 2) indicate that the mechanism would be capable of maintaining sodium balance against loss rates of 0.8  $\mu\text{M}/3$  g. animal/hr. at the normal concentration of its environment (0.3 mm/l.).

The significant difference in elevation between the regressions relating sodium uptake and external concentration in sodium-depleted and normal animals implies differences in  $M_{tmax}$  and  $K_s$  in response to lowered sodium content (Table 3, Fig. 2) in ammocoetes. According to previous measurements on a similar group of animals in July, the plasma sodium content of the normal group amounts to 97.7 mM-Na/kg. water (Bull & Morris, 1967), whilst that of sodium-depleted animals can be calculated to be 94 mM-Na/kg. water from the sodium loss rates recorded during the experiments. The calculations involved are detailed later in this paper when further evidence is given that sodium influx is inversely related to the blood sodium level and the significance of these changes will be dealt with then.

*Sodium loss* increases as the environmental concentration of sodium increases (Fig. 2) and the trend is the same in both groups of animals, although there is a marked difference in the rates of sodium loss at any given environmental concentration. If one assumes that internal sodium remains relatively constant at 97.7 mM-Na/kg. body water for normal animals and at 94 mM-Na/kg. body water for sodium-depleted animals, then the diffusion gradient from the blood to the environment actually reduces with increased environmental concentration and, considering diffusion loss ( $M_{do}$ ) alone one would expect the following relationship

$$M_{do} = K([Na_t] - [Na_0]),$$

where  $K$  is a permeability constant. This situation, where sodium loss decreases in spite of increased diffusion losses, has also been found in isolated frog skin (Kirschner, 1955) and in the whole crayfish (Shaw, 1959; Bryan, 1960), and two alternative suggestions have been advanced to account for the unexpected relationship. Kirschner (1955) and Bryan (1960) believe that sodium losses are reduced because internal sodium, diffusing from the animal through the tissues, is back-transported by the sodium-transport mechanism and hence the system behaves like a leaky pump which has a limited transport capacity. In consequence, at high external sodium concentrations, when the transport mechanism is saturated with sodium ions from the outside, the system has less capacity to transport those arising from outward diffusion. At low external sodium concentrations, when the mechanism is transporting a few sodium ions from outside inwards, the system has a greater capacity for back-transporting lost ions and so sodium losses decrease.

Shaw (1959) believes that the effect is an artifact caused by the isotopic method which may give rise to high assessments of uptake rates resulting from exchange diffusion. Since loss rates are calculated from isotopic influx rates and net transport, the artifact could give rise to a high assessment of loss rate which would decrease as the environmental sodium decreased. He tested this situation in the crayfish by comparing sodium outflux at high external concentrations under normal circumstances and after partially inhibiting sodium influx by means of carbon dioxide. Outflux values were expected to increase following inhibition if the effect was due to back-transport and decrease if an artifact was involved. Shaw obtained a decreased outflux when influx was inhibited and so concluded that true loss rates in the crayfish were obtained at low values of external sodium concentration. Against this, however, is the fact that Shaw noted that sodium loss was reduced by 20% when crayfish were transferred from fresh water to distilled water, whilst Bryan (1960) obtained values



which were 36% less from crayfish maintained in distilled water than those obtained from animals in 2 mM-Na/l. solutions. These observations are more readily explained by an unsaturated transport mechanism, back-transporting lost ions.

In ammocoete larvae, Morris & Bull (1968) have shown that the situation is complicated by the fact that animals lose calcium when they are immersed in de-ionized water and this promotes ion losses which may be abnormal. It is possible to overcome this difficulty by comparing the rate of sodium loss to de-ionized water to which calcium has been added, and under these conditions there is a significant increase in sodium loss ( $P = < 0.001$ ) when the transport mechanism is inhibited by low temperature (Morris & Bull, 1968). This indicates that back-transport may play an important

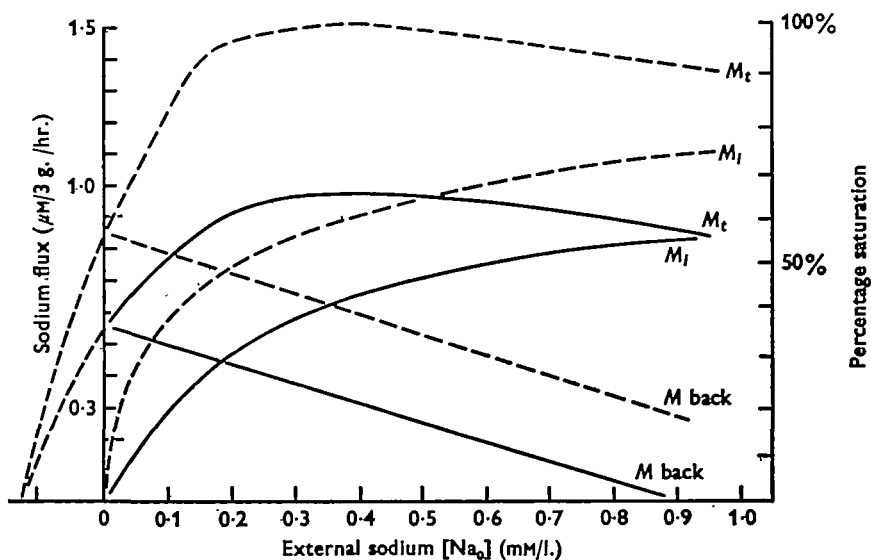


Fig. 3. The relationship between sodium influx ( $M_i$ ), calculated back-transport of sodium ( $M_{back}$ ) total sodium influx ( $M_t = M_i + M_{back}$ ) and external sodium concentration ( $[Na_0]$ ) in normal and sodium-depleted ammocoete larvae. Parameters are calculated from the regression equations (Table 2) and the results from sodium-depleted animals are shown by broken lines.

part in the mechanism of sodium transport in ammocoetes. Moreover, it seems significant that sodium-depleted animals have much lower loss rates than normal animals (Fig. 2) and that there is a parallel increase in sodium influx when the external sodium concentration is greater than 0.2 mM. This may imply that the increase in the total activity of the system brought about by sodium depletion results in increased sodium influx accompanied by an increase in back-transport of about the same magnitude.

Provided one can obtain a measure of the diffusion outflux of sodium ( $M_{do}$ ) it is then possible to assess the amount of back-transport which occurs in various circumstances. In the present experiments back-transport should be minimal at high external sodium concentrations coupled with low transport rates so that the best assessment of diffusion outflux ( $M_{do}$ ) will be given by sodium loss from normal animals at an external concentration of 0.9 mM-Na/l. (Fig. 2). The loss rate amounts to 0.955  $\mu$ M-Na/3 g. animal/hr. at this concentration and this compares favourably with loss rates

of  $0.837 \mu\text{M-Na}/3 \text{ g. animal/hr.}$  obtained from animals whose sodium transport had been inhibited in de-ionized water in previous experiments (Morris & Bull, 1968).

Fig. 3 illustrates the relationship between back-transport ( $M_{\text{back}}$ ), sodium influx ( $M_i$ ), total influx ( $M_t$ ) and the concentration of external sodium ( $[\text{Na}_0]$ ) of sodium-depleted and normal animals. This has been derived from the data contained in Fig. 2 by the following calculations:

In *normal animals*, the diffusion outflux of sodium ( $M_{do}$ ) will be proportional to a permeability constant ( $K$ ) and the diffusion gradient across the animal so that

$$M_{do} = K ([\text{Na}_i] - [\text{Na}_0]).$$

Substituting values of  $0.955 \mu\text{M-Na}/3 \text{ g. animal/hr.}$  for  $M_{do}$ ,  $97.5 \text{ mM-Na/kg. body water}$  for  $[\text{Na}_i]$ , and  $0.9 \text{ mM-Na/l.}$  for  $[\text{Na}_0]$ , the value of  $K$  becomes  $0.00989$ , and from this it is possible to evaluate  $M_{do}$  at varying values of  $[\text{Na}_0]$ .

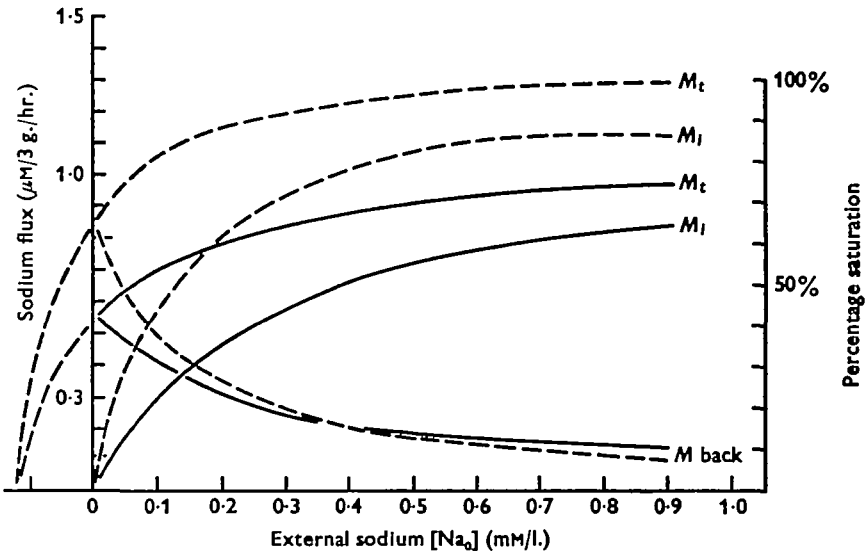


Fig. 4. The relationship between sodium influx ( $M_i$ ), back-transport of sodium ( $M_{\text{back}}$ ), and total sodium transport ( $M_t = M_i + M_{\text{back}}$ ) and external sodium concentration ( $[\text{Na}_0]$ ) obtained by substituting the values of  $K_p$  and  $M_{\text{max}}$  obtained from normal and sodium-depleted animals (broken lines) into the Kirschner equations. For further explanation see text.

Once these values have been established it is possible to calculate the other parameters shown in Fig. 3 as follows: When  $[\text{Na}_0] = 0.9 \text{ mM-Na/l.}$  we assume that there is very little back-transport, so that the total influx ( $M_t = M_i + M_{\text{back}}$ ) equates to the sodium influx ( $M_i$ ) and this amounts to  $0.858 \mu\text{M-Na}/3 \text{ g. animal/hr.}$

At lower external sodium concentrations, the back-transport ( $M_{\text{back}}$ ) increases and the following example illustrates the method of calculation of back-transport and total influx.

When  $[\text{Na}_0] = 0.25 \text{ mM-Na/l.}$ ,  $M_{do} = 0.962 \mu\text{M-Na}/3 \text{ g. animal/hr.}$  and the measured loss rate ( $M_o$ ) =  $0.558 \mu\text{M-Na}/3 \text{ g. animal/hr.}$

Since  $M_o = M_{do} - M_{\text{back}}$  it is possible to calculate the back-transport ( $M_{\text{back}}$ ) which amounts to  $0.404 \mu\text{M-Na}/3 \text{ g. animal/hr.}$

$M_t = 0.535 \mu\text{M-Na}/3 \text{ g. animal/hr.}$  at  $0.25 \text{ mM-Na/l.}$ , so that since  $M_t = M_i + M_{\text{back}}$  the total sodium influx ( $M_t$ ) has a value of  $0.939 \mu\text{M}/3 \text{ g. animal/hr.}$

Assuming that there is no change of permeability in *sodium-depleted animals*, then  $M_{do}$  amounts to  $0.00989 \times (94.0 - 0.9) = 0.920 \mu\text{M-Na}/3 \text{ g. animal/hr.}$  at  $[\text{Na}_0] = 0.9 \text{ mM/l.}$ , and it is possible to construct a second set of curves using similar calculations. The maximum capacity of the system can be deduced from the total influx curve for sodium-depleted animals (Fig. 3). This amounts to  $1.503 \mu\text{M-Na}/3 \text{ g. animal/hr.}$  and occurs at an external concentration of  $0.4 \text{ mM-Na/l.}$  and taking this to represent a fully saturated system it is possible to express the results in terms of percentage saturation of the system (Fig. 3).

Kirschner (1955) derived a series of theoretical equations for the parameters we have measured. It has not been possible to test the equation for diffusion outflux ( $M_{do} = K ([\text{Na}_i] - [\text{Na}_0])$ ) and it has already been pointed out that sodium influx from external sources of sodium fits the Kirschner equation

$$M_i = \frac{M_{i\text{max}} [\text{Na}_0]}{K_s + [\text{Na}_0]}$$

quite well (Figs. 1 and 4).

Kirschner also derived an equation for the back-transport of sodium from internal sources, which is

$$M_{\text{back}} = \frac{A[C_t]}{1 + [\text{Na}_0]/K_s}$$

where  $A$  is a constant and  $[C_t]$  represents the concentration of the total amount of carrier. It is possible to test this against the present series of results since  $M_{\text{back}} = A[C_t]$  when  $[\text{Na}_0]$  is zero. The results of substituting the values from normal and sodium-depleted animals are shown in Fig. 4, which shows that the expected values for back-transport are less than those calculated for ammocoetes, particularly in the case of sodium-depleted animals at higher values of  $\text{Na}_0$ . Attempts to fit the sodium-loss data to an exponential relationship gave a regression equation of  $Y_0 = 0.43 \log X_1 + 0.55$  for sodium-depleted animals with a  $P = 0.01$ , which is much less significant than the straight-line relationship (Table 2).

It is difficult to assess the significance of the increase of back-transport calculated for ammocoetes relative to the values forecast from the Kirschner equations, but it is worth noting that Kirschner based his findings on the assumption that a single carrier mechanism was operating in frog skin. In ammocoetes the gill cells responsible for sodium uptake (Morris & Bull, unpublished) can consist of three to four layers of cells, separated by extracellular space, so that the Kirschner equations can only apply to the outer border of cells in contact with the environment. Thus sodium transport in the ammocoete may be the result of sequential sodium movements located in the membranes of a series of cells and this may explain the relatively high rate of back-transport.

#### THE EFFECT OF INTERNAL SODIUM CONCENTRATION ON SODIUM INFLUX AND OUTFLOW

These investigations involved measuring sodium flux rates from individual ammocoetes in an environment of constant ionic composition between periods of sodium

loss to de-ionized water at 10° C. The flux rates were measured over a period of 1 hr. on six normal individuals immersed in solutions containing 0.3 mM-Na/l. at 10° C. Because of the effect of other ions, principally calcium, on the rates of sodium loss and uptake (Morris & Bull, 1968), we added the remaining ionic constituents of frog Ringer diluted 1:100 with distilled water (*R-Na/100*). The same experimental sequence was used for each animal and consisted of an initial measurement of sodium flux, then a 2 hr. period of ion depletion in de-ionized water, followed by a second measurement of sodium flux. After a further 2 hr. period of ion depletion, a final measurement of sodium flux was made. The animals were killed immediately and the serum was analysed for sodium concentration.

### Results

The results of these experiments are shown in Table 4 which contains assessments of the total freely exchangeable sodium corresponding to particular measurements of flux rate at various stages of each individual experiment. These have been calculated

Table 4. *The effect of sodium loss on sodium influx in ammocoete larvae*

Calculated values are shown in italics and sodium loss measurements are quoted for periods beginning at each influx determination.

Animal (g.)	Na loss ( $\mu\text{M}$ )	Total int. exchangeable Na ( $\mu\text{M}$ )	Na loss % initial total sodium	Plasma Na ( $\mu\text{M/l.}$ )	Na influx ( $\mu\text{M/3 g./hr.}$ )
N	0.0	73.07	0.0	96.0	0.23
2.91	1.83	71.24	2.4	93.8	0.29
	1.85	69.39	4.9	91.4	1.08
	0.19	69.20	—	91.1	—
P	0.0	82.11	0.0	108.0	0.46
2.91	1.23	80.88	1.5	106.3	0.41
	3.76	77.12	6.1	101.5	1.38
	0.12	77.00	—	101.5	—
Q	0.0	88.90	0.0	102.5	0.15
3.31	2.46	86.44	2.8	100.0	0.24
	3.58	82.86	6.8	95.7	0.46
	0.86	82.00	—	95.0	—
R	0.0	96.66	0.0	94.3	0.42
3.90	1.64	95.02	1.7	92.8	0.65
	10.38	84.64	12.5	82.5	1.00
	+0.66	85.30	—	83.3	—
S	0.0	101.60	0.0	95.5	0.50
4.09	0.60	100.94	0.5	95.0	0.56
	1.99	98.95	2.5	93.0	0.61
	0.35	98.60	—	92.4	—
T	0.0	115.07	0.0	89.3	0.19
4.94	1.85	113.22	1.6	88.0	0.25
	0.95	112.27	2.5	87.1	0.45
	+0.23	112.50	—	87.2	—

from the plasma sodium level at the end of the experiment using the average value of sodium space (314.7 ml./kg. body water equivalent to 26.09% of the body weight) found previously (Bull & Morris, 1967). Knowing the amount of sodium lost during each experimental period, which includes the loss during flux measurements, it was

then possible to calculate the total internal exchangeable sodium level corresponding to various stages of the experiment and from this the expected changes of blood concentration.

*Discussion*

Table 4 shows that there is an increase in sodium influx as the total internal sodium decreases. In the majority of animals sodium losses amount to 4–6% of the total exchangeable sodium and the loss brings about an increase of 2 to 3 times the original rate of sodium influx, though there is a great deal of individual variation in this respect. However, the value of using internal exchangeable sodium as a measure of

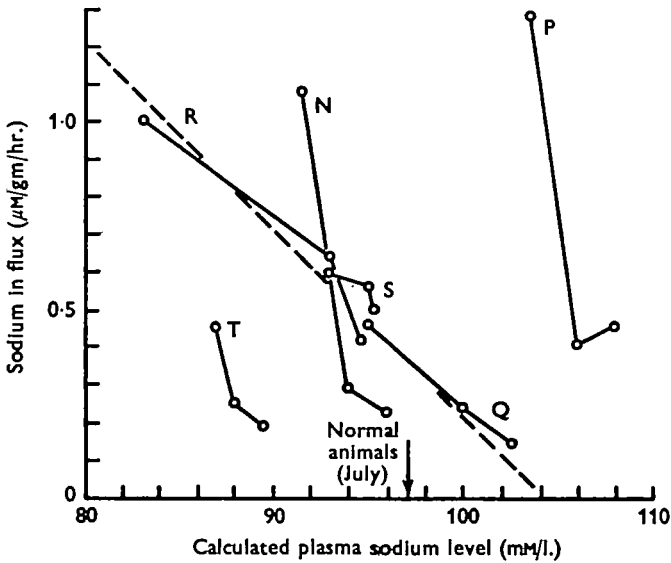


Fig. 5. The effect of sodium depletion on sodium influx in individual ammocoete larvae. Sodium losses have been converted into expected changes of blood sodium concentration, and the broken line represents the regression equation  $Y = 0.05X + 5.22$  obtained from animals N, Q, R and S.

sodium balance is open to question, since this not only varies with the weight of the individual and thus makes direct comparison difficult, but it seems unlikely that total sodium could play a direct part in controlling the rate of sodium uptake, whether this is exercised directly at the transporting cells or centrally by nerves or hormones. It seemed more likely that animals respond to changes in the level of sodium circulating in the blood, and Fig. 5 shows the relationship between sodium influx and the calculated change in blood concentration. There is a reduction in variability compared with the results obtained by employing total exchangeable sodium. The majority of individuals appear to give a linear relationship between plasma sodium and sodium influx in spite of the fact that the animals varied considerably in size. The regression is non-significant when the results from all of the individuals are pooled, but it is highly significant ( $P = 0.001$ ) when animals P and T are omitted. The regression equation takes the form

$$Y = -0.05X + 5.22,$$

where  $Y$  is the sodium influx ( $\mu\text{M}/3 \text{ g. animal/hr.}$ ) and  $X$  the plasma sodium concentration in  $\text{mM/l.}$  The results from previous experiments (sodium transport at different external concentrations) give slightly higher values at  $0.3 \text{ mM/l.}$  than those forecast by these relationships, but the results correspond sufficiently well to illustrate that the rate of sodium influx is dependent on both the external and internal sodium content.

#### GENERAL DISCUSSION

Shaw (1959) has pointed out how changes of external and internal sodium concentration can alter sodium influx in crustacea and the way in which these variables can interact to give a system which is self-regulating when a change in ion balance takes place. He visualizes a series of curves, like those in Fig. 2, in which each curve represents the way in which sodium influx and environmental sodium are related at different levels of internal sodium. He then argues that factors which are likely to bring about a change in sodium balance, such as increased ion loss or a change in environmental concentration, set in train a series of self-compensating changes. Thus if sodium loss increases, this reduces the internal sodium content which in turn causes an increase in sodium influx. Then, because the new influx rate increases the internal sodium concentration, sodium influx gradually decreases as sodium balance is restored. These considerations also apply to ammocoetes because of the basic similarities between the two systems.

Shaw (1961) also maintains that sodium balance in crustacea can be described by the expression

$$\frac{M_{t_{\max}} [\text{Na}_0]}{K_s + [\text{Na}_0]} = K([\text{Na}_t] - [\text{Na}_0]),$$

i.e.  $\text{influx} = \text{diffusion outflux}$

and he points out that it is the low value for the half-saturation constant ( $K_s$ ) of the transport mechanism rather than a low permeability constant ( $K$ ) which enables an animal to maintain balance in low sodium concentration (Table 3), and this is presumably why only certain crustacea manage to maintain such low minimum equilibrium concentrations. In ammocoetes, where sodium balance involves the same equation for influx though not for outflux, we have the additional factor of back-transport to consider, so that at sodium balance

$$\text{influx} = \text{measured outflux} (\text{diffusion outflux} - \text{back-transport})$$

and, since the amount of back-transport will be increased by a low half-saturation value, this becomes an important method of sodium conservation and adds even more weight to importance of  $K_s$ . Emphasis on an efficient transport and conserving mechanism could be especially important to animals like the ammocoete where the soft skin is readily damaged causing increase in permeability. In spite of this it is possible to obtain extremely low minimum equilibrium values from ammocoetes which have been carefully adapted (Morris & Bull, 1968).

A further difference between the ammocoete and crustacea seems to be that both *Eriocheir* and *Astacus* respond to salt depletion by an increase in carrier activity or concentration ( $M_{t_{\max}}$ ), whilst ammocoetes show changes in both  $M_{t_{\max}}$  and  $K_s$ . The

affinity of carrier for sodium increases by a factor of two in sodium-depleted animals (Table 3), and since the capacity for sodium transport is much greater in ammocoetes than is suggested by influx measurements alone (Fig. 3), the maximum rate of influx measured from the total sodium transport curves rises from the normal value of  $1.08 \mu\text{M-Na}/3 \text{ g. animal/hr.}$  to  $1.35 \mu\text{M-Na}/3 \text{ g. animal/hr.}$  in sodium-depleted animals.

It is worth examining the variables concerned during sodium transport in the ammocoete from the point of view of possible controlling mechanisms, since there is a great deal of interest in this topic at the present time (Maetz, 1968). The fact that in ammocoetes the level of internal sodium affects both the amount of sodium carrier and its affinity for sodium provides the basis for two complementary controlling mechanisms. The first of these, a change in sodium affinity of the carrier molecule in response to changes in the level of internal sodium, might explain the short-term response of the self-regulating mechanism, whereas long-term changes, such as the gradual adaptation of animals to low minimum equilibrium concentrations of sodium (Bull & Morris, 1968), might be brought about by synthesizing more carrier. The third variable which might contribute to the controlling mechanism is permeability and it seems likely that a change in permeability may bring about far-reaching changes in the characteristics of the system when back-transport is operating. This comes about because the level of external sodium appears to govern the ratio of influx to back-transport (Fig. 3), presumably because sodium is lost by diffusion at a constant rate and this then competes for the carrier system with sodium ions which are already present externally. It is interesting in this respect to find that the rates of influx and back-transport are equal at an external concentration of  $0.18 \text{ mM-Na/l.}$  (Fig. 3) and that this presumably represents the concentration of sodium at the external surface brought about by diffusion loss. A similar value can be obtained from Fig. 3, by extrapolating the total sodium transport curves to their origin, this then gives the true concentration range for the whole system, and the internal contribution lies between  $0.1$  and  $0.2 \text{ mM-Na/l.}$  It follows from this that permeability changes will not only affect diffusion loss directly, but should also alter the ratio of sodium uptake to back-transport. Thus a decrease in permeability may have the effect of reducing the concentration of ions available for back-transport, leaving the carrier free to increase sodium uptake. This may explain why some posterior pituitary hormones which affect the permeability of lower vertebrate tissues may also have an effect on sodium transport (Maetz, 1968).

#### SUMMARY

1. Sodium influx in ammocoete larvae increases exponentially with external sodium concentration ( $0.1-1.0 \text{ mM/l.}$ ) and sodium-depleted animals show a 20% increase compared with normal animals.
2. Sodium loss decreases as the environmental concentration decreases, although the reverse situation is expected from considering diffusion outflux alone.
3. It is argued that part of the sodium loss is back-transported by the transport mechanism and this accounts for the reduced sodium loss from sodium-depleted animals whose sodium carrier activity is increased. The curves relating back-transport to environmental sodium differ from those derived by Kirschner for isolated frog skin.

4. Sodium influx increases as sodium loss increases, indicating a self-regulating mechanism whose features are discussed. In the ammocoete, the sodium carrier mechanism appears to change in affinity for sodium (short-term response) and can also change in concentration (long-term response), and it is suggested that these features, together with permeability changes, may form the basis of the controlling mechanism for sodium balance.

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