J. Exp. Biol. (1967), **47**, 485–494
With 1 *text-figure*
Printed in Great Britain

STUDIES ON FRESHWATER OSMOREGULATION IN THE AMMOCOETE LARVA OF *LAMPETRA PLANERI* (BLOCH)

I. IONIC CONSTITUENTS, FLUID COMPARTMENTS, IONIC COMPARTMENTS AND WATER BALANCE

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(*Received* 10 *July* 1967)

INTRODUCTION

Apart from the studies of Hardisty (1956), there is very little information about the mechanism of freshwater osmoregulation in the brook lamprey, a common representative of primitive agnathan vertebrates. The present series of papers deals with various aspects of this problem, starting with some of the ionic constituents of whole animals and with the ionic content of their plasma and muscle. The extent of various fluid compartments such as volumes of blood and plasma, and sodium, chloride and inulin spaces have also been measured in an attempt to relate the concentration of ions to their location within the body.

Later papers will deal with the effect of temperature on these parameters and with a variety of factors which appear to affect sodium balance in whole animals. The emphasis throughout this work has been on the measurement of the concentration and movements of monovalent ions, because they seem to be mainly responsible for the osmotic pressure exerted by the body fluids in this group of cyclostomes (Hardisty, 1956; Robertson, 1954; Morris, 1960; Bull & Morris (present studies)).

MATERIALS AND METHODS

Ammocoete larvae were collected from the source of the River Manifold in Derbyshire by an electrical fishing method employing pulsed direct current. The animals were kept in the laboratory in plastic tanks containing a 3–4 in. layer of mud covered by 6–7 in. of running tap-water. The animals were taken from the Manifold spawn in the summer following metamorphosis, a feature which is characteristic of *Lampetra planeri*. They have also been identified by a key devised by MacDonald (1960).

Plasma samples were collected from animals anaesthetized in a 1:20,000 (w/v) solution of M.S. 222 (Sandoz). Blood was collected from an incision into the heart by means of heparinized pipettes made from Pyrex capillary (1·5 × 80 mm.). The blood was withdrawn from one end of the pipette before sealing this off by heat, after which the sample was centrifuged for 2 min. at 4000 r.p.m. to separate plasma and red cells. The haematocrit values of the blood were obtained by comparing the relative lengths of the two columns. Later the capillary tube was cut and broken at the junction of the plasma and red blood cells; the part of the tube containing plasma was

then sealed at both ends and stored at −20° C. until required. No visible haemolysis occurred in samples prepared in this way, and a single animal of 3–4 g. yielded 3–4 tubes of blood (0·2 ml.).

Whole-animal and muscle digests were prepared from animals which had been starved in tap water for a day or so. Animals were rinsed in distilled water and drained on cigarette paper, then strips of muscle were taken from the mid-dorsal musculature. Whole animals and muscle samples were minced before digesting in 0·1 N nitric acid at room temperature (5 ml. acid/0·2 g. tissue). Reaction vessels were shaken frequently during the 3 weeks required to digest the tissues completely, and the fat which resulted was then extracted with carbon tetrachloride which was later removed by centrifugation.

Dry weights were obtained by heating minced tissues for 48 hr. at 105° C. and the water content was calculated from the dry and fresh weights.

Fat content was determined by measuring the difference in weight in dried tissue before and after extraction in ether for 12 hr. at 40° C.

Sodium and potassium were measured by means of an E.E.L. flame-photometer using reference standards which were matched to give approximately the same ionic composition as the samples.

Chloride was measured by means of a potentiometric method, the details of which are given in Morris (1965) for samples of greater volume than 0·5 ml. The amount of serum available for chloride analyses amounted to 0·015 ml., so that the original method had to be modified to suit the smaller volume. The samples were held in the form of a drop on a silver–silver-chloride electrode manufactured from a small loop of silver wire. Glacial acetic acid (0·005 ml) was drawn into a graduated pipette followed by an aliquot of undiluted serum. The contents of the pipette were transferred to the electrode loop together with the washings from the pipette. An indifferent electrode of platinum wire contacted 0·01 N silver nitrate contained in an Agla syringe burette and the sample drop was stirred during the titration by means of a fine jet of compressed air.

Freezing-point depression of individual plasma samples was measured using the method devised by Ramsay & Brown (1955).

Blood-volume measurements were made on individual animals. Blood samples were taken by the capillary method described above and one of these was used to determine the haematocrit value, whilst a second sample was used to prepare a standard diluted with a known amount of distilled water. The blood eluted from the minced tissues, together with that contained in the haematocrit, was then diluted to a known volume with distilled water and, after extracting the fat, its haemoglobin content was compared with that of the standard in a Unicam SP 500 spectrophotometer at a wavelength of 540 mμ.

The *sodium space* of animals was assessed by an isotopic method using ^{24}Na. Animals were left for 2–3 hr. in tap water containing isotope and the quantity of isotope which had entered the animal was then calculated from measurements of the count rate of the environment at the beginning and end of the experiment. The count rate of a known volume of serum was determined from a freshly drawn blood sample at the end of the experiment so that the volume occupied by the isotopic sodium could be calculated. Count rates were measured from samples dried on planchettes by infra-

red lamps and the radiation was detected by means of an end-window Geiger–Müller tube connected through a quench unit to a decatron scaler and counter.

Sucrose and inulin spaces were determined by a similar method to that detailed above using the appropriate ^{14}C labelled compounds (0·01 ml. of 0·05 μc. in Ringer solution) which were injected into the heart by means of an Agla syringe. Blood samples were withdrawn for counting 1 hr. following the injection. Two methods of counting were used. The first method was essentially the same as that used for sodium except that a thin end-window (Mullard MX 123) was used. The second method is described by Davies & Cocking (1966). In our experiments samples were weighed on glass-fibre disks (Whatman GF/A), diluted, dried and immersed in 0·1 ml. of N 215 scintillator (Nuclear Enterprises) contained in bottles selected for low background. Count rates were measured in a liquid scintillation detector coupled to a decatron scaler and counter.

RESULTS

Water and fat content

Estimations of water and fat content were obtained from a series of individuals in July and November (Table 1) so that measurements of ion concentration could be expressed in terms of water content (Table 2).

Table 1. *Water and fat content of ammocoete larvae and their tissues*

(Standard errors follow mean values and the figures in parentheses are the number of animals on which the observations are based.)

	Water (% fresh wt.)	Fat (% dry wt.)	Water (% fat-free fresh wt.)
		November	
Whole animal	79·76 ± 0·58 (6)	11·38 ± 1·64 (6)	81·64 ± 0·54 (6)
Muscle	80·17 ± 0·82 (6)	16·54 ± 3·86 (6)	82·95 ± 0·76 (6)
Blood	85·38 ± 0·80 (6)		
		July	
Whole animal	78·21 ± 0·50 (6)	23·48 ± 2·55 (6)	82·52 ± 0·25 (6)
Muscle	74·25 ± 3·36 (6)	31·48 ± 9·85 (6)	82·28 ± 0·59 (6)
Blood	85·59 ± 1·25 (6)		

These results show that there is no significant difference in the water content of ammocoetes and their tissues in July and November ($P > 0·05$) but there is a significant variation in fat content in whole animals ($P < 0·001$). Hardisty (1956) obtained an apparent seasonal variation in water content which he believed to be caused by the seasonal difference in fat content. He was able to reduce the variability in water content by expressing this in terms of fat-free fresh weight, and our data support his conclusions. Hardisty's results also indicated a peak of fat storage in May which steadily fell off with the approach of winter, and the significant variation in fat content in whole animals (Table 1) is presumably a reflexion of this. The higher fat content of muscle compared with whole animals indicates that the muscles are the chief sites of fat storage in ammocoetes.

Ionic constituents

The results of a series of analyses of the main osmotically active ions in normal animals are given in Table 2.

The only comparable figures for whole animals are those of Hardisty (1956), whose figure of 24 mM-Cl/kg. water (converted from 132 mM-Cl/kg. dry tissue) compares favourably with the results obtained from both groups of animals in our analyses. Hardisty, however, found a seasonal variation in the total chloride content of the

Table 2. *The monovalent ion content of normal ammocoetes and their tissues*

(Standard errors follow the mean values for estimations expressed in mM/kg. wet weight, whilst figures in italics represent concentrations in mM/kg. water. Values for fat and residue are given in g./kg. water.)

	Whole animals	Serum	Muscle
		November	
Na	26·04 ± 1·49 (5) *32·64*	99·42 ± 2·80 *116·44*	18·98 ± 0·63 *23·67*
K	61·73 ± 2·31 *77·39*	6·39 ± 0·76 *7·48*	89·37 ± 5·29 *111·47*
Cl	20·13 ± 1·50 *25·23*	81·22 ± 0·31 *95·12*	8·76 ± 0·34 *10·92*
Δ		113·65 ± 1·29	.
Fat	*2·89*	*2·89*	*4·11*
Residue	*22·48*	*22·48*	*19·02*
		July	
Na	28·01 ± 1·42 (5) *35·81*	97·39 ± 2·48 *113·78*	16·16 ± 0·84 *21·76*
K	59·29 ± 2·92 *75·80*	7·83 ± 1·07 *9·14*	76·46 ± 1·73 *102·97*
Cl	20·86 ± 1·62 *26·67*	80·25 ± 0·80 *93·76*	9·11 ± 1·06 *12·26*
Δ	.	119·90 ± 5·07	.
Fat	*6·58*		*9·90*
Residue	*21·21*		*15·65*

ammocoete. Our results show no significant seasonal variation of the means on applying Student's *t* test to the ionic analyses (Na: $P > 0{\cdot}3$; Cl: $P > 0{\cdot}7$; K: $P > 0{\cdot}5$).

The ionic composition of ammocoete blood also shows no significant seasonal differences in monovalent ion content. The values for sodium and chloride are somewhat lower than in the adult river lamprey from which Robertson (1954) obtained values of Na, 119·6; K, 3·2; Cl, 95·8 mM/kg. water, but they are consistent with the lower total osmotic pressure of ammocoete blood (113–120 mM/l. NaCl compared with 135–150 mM/l. NaCl in adult river lampreys (Hardisty, 1956; Morris, 1956)). Sodium and chloride appear to contribute roughly the same proportion of osmotically active constituents in both animals, i.e. Na, 81–90%; Cl, 67–71%. The main differences in our analyses and those of other workers are that our potassium values are nearly twice as high as those obtained by Robertson (1954) from *Lampetra fluviatilis* in spite of the precautions we took to avoid the leakage of potassium from blood. Our analyses also differ from those of Hardisty (1956) in that he records very low chloride levels from

ammocoete serum (58 mM/l. Cl) which represent only 53% of the recorded total osmotic pressure.

The analyses on muscle are in keeping with those of serum and whole animals in that there is no significant seasonal variation.

Fluid compartments

The results of studies on the fluid compartments of ammocoetes taken in the July of the year preceding metamorphosis are summarized in Table 3, together with a series of recent measurements taken from other aquatic vertebrates. The values for blood and plasma volume and extracellular space as measured by sucrose and inulin are of the same order as those given by Thorson (1959) for adult *Petromyzon marinus* in spite of the large size difference between the animals. Thorson found that large blood volumes were characteristic of more primitive groups of animals like the lampreys and elasmobranchs, presumably because both groups of animals have large blood sinuses which are absent in other fish groups.

Table 3. *The body-fluid compartments of ammocoete larvae (July) compared with those of other aquatic vertebrates*

(For further explanation see text.)

Compartment	*L. planeri* ammocoete (3 g.)*	*P. marinus* adult (190 g.)†	Elasmobranch†	Freshwater teleost‡
Haematocrit (blood cells as a % of whole blood)	38·61 ± 1·43 (6)	33·0	18·3	32
Blood volume (% body wt.)	7·97 ± 0·66 (6)	8·5	6·6	2·8
Plasma volume (% body wt.)	4·84 ± 0·48 (6)	5·5	5·4	1·8
Extracellular volume (% body wt.)				
(*a*) Inulin sp.	24·41 ± 1·26 (5)			
(*b*) Sucrose sp.	22·3 (2)	23·9	21·2	14·0
Interstitial sp. (% body wt.)	19·57	18·4	15·8	12·2
Muscle blood volume (% wet wt.)	6·33			

*Present series. †Thorson, 1959. ‡Thorson, 1961.

Ionic compartments

It is possible to assess the 'space' occupied by ion species by two different methods. The values obtained by dividing the total amount of ion within the animal by its concentration in serum (extracellular fluid) give an assessment of the total space occupied by a particular ion. Thus the total sodium space for ammocoetes works out to be 314·7 ml./kg. tissue water, whilst chloride gives a lower value of 285 (Table 4). It is also possible to measure the ion space by isotopic dilution, in which case the value obtained represents the exchangeable space for a particular ion. The exchangeable sodium space amounts to 303 ml./kg. tissue water. The difference between the values obtained by these two methods of assessment will represent the amount of bound or non-exchangeable sodium and this is most probably located in the tissues, because any bound sodium in the serum will have been measured by the techniques

employed in these analyses. The volume of the extracellular space of the whole animal has also been measured and amounts to 24·41 % body weight (Table 3) when assessed by the inulin space. This value is equivalent to 284 ml./kg. tissue water, so that it is possible to calculate the proportions of ions in the cells and serum of the whole animal (Table 4 and Fig. 1). The most striking feature of these results is that the values for chloride space and extracellular space are almost identical, so that if one assumes that the same relationship applies to muscle the ionic distribution between muscle cells and their extracellular fluid can also be calculated (Table 5 and Fig. 1).

Table 4. *Ionic compartments in whole ammocoete larvae (July)*

(Calculated values are shown in italics and further explanation is given in the text.)

	Ionic content (mM/kg. water)	Concentration (mM/kg. water)	'Space' (ml./kg. water)
Total Na	35·81	113·78	*314·7*
Exchangeable Na	*34·40*	113·78	303
Bound Na	*1·41*	.	.
Total Cl	26·67	93·76	*285*
Extracellular space			284
Extracellular Na	*32·39*	113·78	284
Intracellular Na	*3·42*	*4·78*	716
Bound Na	1·41	*2·02*	716
Total K	75·80	.	.
Extracellular K	*3·20*	9·14	284
Intracellular K	72·60	*101·2*	716
Extracellular Cl	*26·58*	93·76	284
Intracellular Cl	0·09	0·13	716

Table 5. *Ionic compartments in ammocoete muscle (July)*

(Calculated values are shown in italics and further explanation is given in the text.)

	Ionic content, (mM/kg. water)	Concentration (mM/kg. water)	'Space' (ml./kg. water)
Total Cl	12·26	93·76	*130·8*
Total Na	21·76	113·78	*191·0*
Extracellular Na	*14·88*	113·78	130·8
Intracellular Na	6·88	*7·92*	869·2
Total K	102·97	.	.
Extracellular K	1·12	9·14	130·8
Intracellular K	101·85	*117·2*	869·2

One of the points which requires comment in these analyses is that the tissues in whole animals are 17 mM/kg. lower in cationic content than the blood or the intracellular component of muscle (Fig. 1). In muscle cells the total monovalent ions are almost the same as those in the extracellular space and, since muscle accounts for about 75 % of the total weight of the animal, this implies that the deficit in whole animals is confined to the remaining tissues and, because of this, will be greater than the amount calculated. The deficit may have arisen as a result of the methods employed, because in these experiments any free water such as that contained in the urine

or gut fluid would be included in the calculations as part of the intracellular space of the animal, and in consequence the tissue ions would appear to be more dilute than they actually were.

Urine output

Osmotic water is removed as a dilute urine in cyclostomes and fishes so that the ionic composition of the body fluids remains relatively constant (Morris, 1956; Potts & Parry, 1964). Because of the difficulty of collecting urine from small animals like ammocoete larvae, an indirect method was devised. The method involved measuring

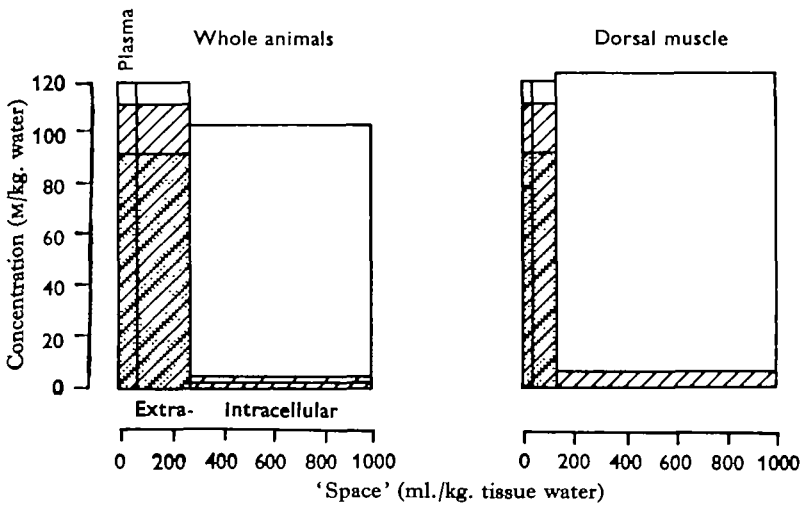

Fig. 1. Ion and water compartments in whole ammocoetes and their muscle. (For further explanation see text.) □, K ▨, Na; ▩, bound Na; ▦, Cl.

the change of weight which took place when animals were transferred abruptly to sea-water solutions which had been diluted to give the same osmotic pressure as the blood. Under these circumstances the fact that the animals lose weight can only be attributed to urine production, and the pattern of weight loss with time suggests that there is a gradual reduction of urine output in response to the new osmotic circumstances. Thus the initial rate of weight loss will be the best measure of urine output. A situation of this type has already been analysed for adult lampreys (Morris, 1956), where direct measurements of urine output were shown to correspond with initial weight losses obtained from animals kept in mildly hypertonic solutions. This method seems likely to be more accurate than many since it does not involve the use of anaesthetics or depend upon mechanical or surgical interference. However, like most methods of measuring urine output the present method can be affected by handling diuresis, and this is apparent when the frequency of weighings is increased. Because of this, ammocoetes were weighed before immersion in an isotonic solution containing 99 mM/l. NaCl and 7 mM/l. KCl, and only reweighed once after a period of 2 hr. to assess urine output.

Using this method, we obtained a mean value of 198 ml. per kg. per day with a standard error of $\pm 20{\cdot}5$ from a series of ten animals whose weights varied between 2 and 3 g. This output is higher than that obtained for adult river lampreys (Morris,

1956), which gave 155·8 ± 9·9 ml. per kg. per day, and for marine lampreys (Sawyer, 1955), where the urine output amounted to 159 ml. per kg. per day. Hardisty (1956) calculated urine flows of 1300 ml. per kg. per day from nine brook lampreys by following the increase of weight which resulted from ligaturing the urinary papillae. This result is very high in comparison with those detailed above and suggests that the method employed may have caused handling diuresis in the animals.

DISCUSSION

These studies emphasize that the ammocoete larva of *Lampetra planeri* is a typical freshwater vertebrate which shows a high degree of homoeostasis with regard to its water and ion content. These factors show remarkably little seasonal or individual variation whether one considers the whole animal, its blood or muscle, the latter tissue making the greatest contribution to the bulk of the animal (Tables 1, 2). Our findings differ from those of Hardisty (1956), who recorded a seasonal variation of the total chloride content of the ammocoete, which was lowest in June (132 mM-Cl/kg. dry wt.) and rose to a maximum in November (250 mM/kg. dry wt.). The values which he obtained were inversely related to fat content. We suggest that Hardisty's findings may have arisen because he expressed his results in terms of a nutritional variable (fat-extracted dry weight, which is presumably mainly protein) and that this may be the factor which is subject to seasonal variation, in much the same way as fat seems to be. This view is supported by the fact that Hardisty noted that starvation is accompanied by increased chloride content in his animals; a result which would be expected if our interpretation is correct.

Our findings on the ionic compartments of whole animals during July indicate that nearly all the chloride is confined to the extracellular space and there is very little chloride within the cells themselves (Table 4 and Fig. 1). The ratio of sodium to potassium for the extracellular fluid is 12·5:1, whilst the tissues show the inverse relationship which is so characteristic of many vertebrates, giving a potassium to sodium ratio of 21·2:1. It is difficult to assess the significance of the bound sodium within the tissues of whole animals, particularly as we have no corresponding measurements for muscle, but there is the possibility that the bound sodium may be confined to particular tissues.

The main features of the muscle analyses are the lack of intracellular chloride and the decreased extracellular volume. It is also worth noting that the potassium to sodium ratio in these cells amounts to 14·8:1, a value which is much higher than that recorded for other vertebrates. Gordon (1959) obtained a value 12·8 for the same ratio from brown trout whole muscle, whilst the ratios for intracellular striated muscle from the frog (Conway & Hingerty, 1946) and the rat (Conway, 1945) are 8·1 and 9·5 respectively. These comparisons indicate that the ammocoete has extremely well developed powers of ionic regulation at the cellular level; a situation which is very different from that in the marine Cyclostome *Myxine glutinosa* where both Robertson (1960, 1963) and Bellamy & Chester Jones (1961) found that the sodium content exceeded the potassium content in the muscle.

We conclude from this work that salt and water balance in the ammocoete larva is a well developed and closely controlled mechanism with regard to the extra-

cellular fluid and the contents of the cells themselves, which has been evolved as a result of life in fresh water. In this respect the ammocoete shows features which are usually associated with more recent freshwater vertebrates like the teleosts, and contrasts markedly with the feebly developed powers of osmoregulation in related *Myxine glutinosa* (Robertson, 1963; Morris, 1965) where there is no freshwater stage in the animal's life-history.

SUMMARY

1. Measurements have been made of a number of basic parameters related to osmoregulation in the ammocoete larva of the brook lamprey (*Lampetra planeri* Bloch).

2. There is no significant variation in the water content of whole animals or their tissues between animals analysed during November and July, but the fat content rises in July and the majority of fat storage takes place in the muscles.

3. Analyses have been made of the major osmotically active ions in whole animals, in muscle and in blood. Unlike previous investigators we found no significant seasonal variation in any of these components. The freezing-point depression of ammocoete blood (113–119 mM/l. NaCl) is lower than that of adult river lampreys (140 mM/l. NaCl), but apart from higher potassium values, monovalent ions contribute nearly the same proportion to the total osmotic pressure in both animals. The muscle analyses indicate that the ammocoete has a higher ratio of potassium to sodium (14·8:1) than other vertebrates.

4. Measurements of the fluid and ionic compartments have been made on whole animals and on muscle using isotopic dilution techniques and conventional analyses. The extracellular space of muscle is about half that of whole animals (24·4% body weight) and contains all the chloride and 69% of the sodium within whole muscle. Potassium almost completely replaces sodium within the cells and part of this may be bound sodium in some tissues.

5. The urine output of the ammocoete amounts to 198 ml. per kg. per day and is similar to the values previously obtained from other lampreys in fresh water.

6. The high potassium to sodium ratio within cells, together with the lack of seasonal variation and low variability in ionic analyses, is taken as an indication of the presence of very efficient controlling mechanisms operating on extracellular fluids and within the cells themselves.

Some of this work forms part of a thesis submitted by J. M. Bull to the University of Nottingham for the degree of Ph.D. Our thanks are due to Professor E. J. W. Barrington for his encouragement and interest, and to the Science Research Council for financial support.

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