Osmotic Regulation in Aquatic Animals
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MOLLUSCA

From the point of view of osmotic conditions and behaviour there is no essential difference between the molluscs and the invertebrates so far studied, although the general level of organization is higher in the molluscs. The molluscs possess a body cavity of varying volume and a fairly efficient circulatory system maintaining always a flow of haemolymph along the respiratory surfaces. These are in the larger forms often highly developed, which means that the ratio surface/volume is not a simple function of size, but that even in larger forms the surface area per unit volume may be considerable. The kidneys represent an advanced stage compared with the Annelida. The coelomic cavity into which they open by a ciliated nephrostome is reduced to comprise only the pericardial sac which is shut off entirely by thin walls from the body cavity proper. The powerful cilia in the proximal part of the kidney tubule reduce the pressure in the pericardial cavity and cause filtration into it from the haemolymph. The urine formed in this way is modified during its passage down the tubule by the action of cells, but details regarding the function are not available. The kidneys are usually well developed both in marine and in fresh-water forms. It is evident therefore that their general function is not osmoregulatory, but by reabsorption of ions from the walls they *may* produce a dilute urine in the fresh-water molluscs.

The large majority of molluscs are provided with an external shell consisting mainly of $CaCO_3$. They are generally able also to resist lack of O_2 for long periods, and by virtue of this faculty they can shut themselves off from the surrounding medium for periods of days and in some cases even months. In the snails the shell permanently protects a large part of the surface against diffusion and osmotic inflow of water. "No molluscs without an external shell are found in fresh water" (Ellis, 1926).

The molluscs are mainly marine and only two classes, the Pelecypoda or bivalves and the Gastropoda are represented in fresh water. Within these classes the migration of forms from the sea through estuaries into rivers has been going on from the earliest

geological periods and is still in progress, so that a study of the adaptive modifications ought to be feasible. Ellis, who gives a number of examples, refers especially to the small snails of the genus *Hydrobia*, common in brackish water and with representatives also in fresh water. One species, *H. jenkinsii*, was confined entirely to brackish water until near the end of the nineteenth century. In 1893 it was first observed in an inland locality in England, and subsequently it has become abundant in rivers, streams and canals over most of England, Wales and Ireland. In continental Europe the species appears to be mainly confined to brackish water, but is reported recently from a few fresh-water habitats. In this species there must be an active regulation, and it would be interesting to see if the fresh-water individuals represent a physiological race with a more highly developed power of osmotic regulation.

MARINE MOLLUSCS

Like other marine invertebrates these are generally in osmotic equilibrium with the surrounding sea water (Fredericq, 1901; Bottazzi, 1908) and remain so when the concentration of this is slowly varied, at least within certain limits compatible with their normal life. Because rapid changes in concentration of the outside medium are followed by corresponding changes in weight of the animals exposed, the osmotic adaptation was, until the work of Bethe, taken to be due to semipermeability of the surfaces concerned. Dilution of the medium causes swelling and concentration shrinkage, and a new equilibrium, which is due mainly to osmotic gain or loss of water, is established generally in a few hours. Quinton (1904) maintained strongly that marine invertebrates (*Aplysia* especially) were permeable to chlorides, but his analyses were not sufficiently convincing. Bethe (1929), experimenting on the sea hare *Aplysia*, varied the concentration of single ions in artificial sea water, while maintaining isotonicity, and observed variations in the same direction in the blood. These variations were completely reversible, and it is significant that both increases and decreases in Ca concentration could be obtained, so that there can be no reason to assume the production of any abnormal permeability.

When transferred to an isotonic mixture of sea water with cane sugar *Aplysia* will shrink at a fairly rapid rate, because salt leaves the body and sugar does not enter in any appreciable amount (Bethe, 1930), and when such an animal is brought back in time into normal sea water it will swell, and in some cases it is noted that the original weight is even exceeded (Fig. 15). This permits the assumption that the skin is not quite impermeable to cane sugar.

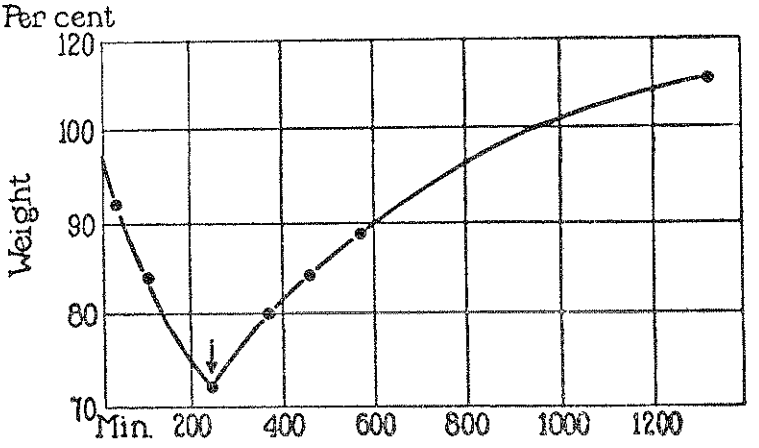

Fig. 15. Loss of weight of *Aplysia* in one part isotonic cane sugar and three parts sea water and recovery (↓) in sea water. (Bethe.)

In later experiments (1934) Bethe, replying to an objection raised by Schlieper (1929), made experiments on *Aplysia* after ligaturing the mouth, and again found a definite passage of ions (Cl^-, $SO_4^{=}$, Ca^{++} and Mg^{++}) through the integument. A few experiments made on the nudibranch gastropod *Doris* in three-quarters sea water showed an increase in weight beyond what would be expected if it was semipermeable, while in all other forms studied the increase was much smaller, due to the simultaneous exchange of salt. The abnormal behaviour of this animal, which Bethe does not attempt to explain, ought to be investigated further. It shows other peculiarities to be referred to below.

In spite of the permeability of integuments the ionic composition of the blood need not be identical with that of the sea water with

which molluscs are in osmotic equilibrium, but shows differences which may in some higher forms become pronounced. Duval (1925) compared the Cl content of the blood in several species with the Cl concentration of the sea water with which they were in equilibrium, and I can supplement his figures with a few more. Calculated in mM./litre we find:

Table IV

			Blood Cl	Water Cl	$\frac{\text{Blood Cl}}{\text{Water Cl}}$
Bivalves	*Ostrea edulis*	Krogh	423	424	0·997
	Mytilus edulis	Krogh	526	532	0·99
Gastropoda	*Buccinum undatum*	Duval	543	569	0·96
	Patella vulgata	Krogh	348	363	0·96
Cephalopoda	*Sepia officinalis*	Duval	550	599	0·92
		Duval	536	591	0·91
		Duval	536	585	0·92

Bethe and Berger (1931) give figures for some other ions calculated on the basis of Cl = 100. They find:

Table V

	Na	K	Ca	Mg
Sea water	87	1·8	2·1	9·3
Aplysia	91	1·9	2·2	8·8
Mytilus	83	1·7	2·2	9·0*
Doris	98	2·9	2·4	10·6

* This figure is taken from a determination by Krogh and Wernstedt (1938).

While some of the differences found may be accidental there can be little doubt, especially with regard to the Cl concentrations, that they express the beginning of regulation processes for which the kidneys are probably responsible. Bottazzi (1908) found the urine of *Octopus vulgaris* slightly hypotonic (Δ 2·24° C.) compared with the blood (Δ 2·296° C.).

In the molluscs ionic concentrations within tissue cells are definitely different from those in the blood and often much lower.

The first to emphasize this fact was L. Fredericq (1901), who determined the content of soluble salt in the tissues of marine animals and found it generally much lower than in sea water, while

the osmotic concentrations were identical. Fredericq's actual figures are probably in many cases too low. He gives, for instance, the salt content of the adductor muscle of *Ostrea* as 1 %, while recent determinations of alkali and chloride on oysters from water of the same salinity indicate a content of alkali chloride of 1·5 %. In principle, however, Fredericq was right, and analyses on different molluscs have revealed the presence of considerable quantities of small organic molecules in tissue extracts. Thus Kelly (1904) found 5 % taurin in *Mytilus* muscles and 4·8 % in those of *Pecten opercularis*. The taurin formula is $H_2NCH_2CH_2SO_3H$, and the molecular weight 125. 5 % corresponds osmotically to 200 mM. of a soluble salt. These results were qualitatively confirmed by Mendel (1904) for gastropod muscles and by Henze (1905) for *Sepia*. Kelly also demonstrated the presence of glycin in the muscles of *Pecten irradians*.

In several tissues in molluscs the salt concentration exceeds that of sea water which is possible only if salts are present as indiffusible (probably solid) compounds. Examples are given in the adjoined Table VI recalculated from the figures of McCance and Shackleton (1937). They are included here because in some of the organs the sum of K and Na alone exceeds the total base in sea water, so that a definite fraction of these bases must be present in an unionized state:

Table VI. *Concentration mM./kg. water*

Name	Organ	Water g./kg.	Na	K	Ca	Mg	Total mE.
Littorina littorea	Foot and gut	695	428	156	294	270	1712
,,	Gonad and liver	640	475	170	356	334	2025
Patella vulgata	Whole animal	747	270	152	111	37	718
Buccinum undatum	Foot and gut	732	255	145	26	64	580
,,	Gonad and liver	738	454	280	68	56	982
Aplysia punctata	Whole animal	860	320	71	34	55	569
Sea water		967	462	10·0	10·9	54·3	603

Very comprehensive analyses were made on the nudibranch *Archidoris britannica* by McCance and Masters (1937). In this

animal an enormous excess of Ca and Mg was found, and this was located as solid concrements in the body wall (along with strontium and fluorine) which are of no further interest from our point of view. When the animals were prepared for analysis the viscera were removed and thereafter the body wall was allowed to secrete mucus. On one occasion 80 g. body wall secreted 24·1 g. mucus in about 1½ hr. As first secreted the mucus is very viscous, but after 2 hr. or so becomes much more fluid. Viscera, body wall and mucus were separately analysed, and I give the results for the major constituents recalculated as mM./kg. water:

Table VII

	Na^+	K^+	Ca^{++}	Mg^{++}	Total base mE.
Viscera	209	179	65	65	648
Body wall	293	42	—	—	—
Mucus	442	13·6	13·7	58·5	600
Sea water	462	10·0	10·9	54·3	602

	Cl^-	F^-	$HPO_4^{=}$	$SO_4^{=}$	CO_3	Total acid mE.
Viscera	391	—	13·0	27	—	635
Body wall	405	300	24	5·3	—	—
Mucus	512	—	—	—	56	568
Sea water	545	—	—	29	2	605

Regarding the body wall total concentrations cannot be given, because unknown amounts both of cations and anions are present in a non-diffusible state. The composition of the viscera shows a normal preponderance of K^+ and $HPO_4^{=}$, and the total concentrations are so close to those of sea water that it can safely be concluded that only a few per cent of the ions can be indiffusible. The mucus finally, which has been elaborated and excreted from the body wall without circulation or any addition of fluid, is in its inorganic composition almost identical with sea water, showing only a slight excess of K^+, Ca^{++} and Mg^{++} and a corresponding deficit in Na^+. There is also a Cl^- deficit which is mainly made up by bicarbonate. Is it conceivable that we have to do mainly with intercellular fluid pressed out by muscular contractions and to which is added a small amount of secretion, or is the mucus in its totality a secretion pro-

duct from cells which one would expect to have a very different composition?

From the point of view with which we are here concerned, viz. the distribution of diffusible ions, the analyses so far given are clearly insufficient. In the whole organism or in a piece of tissue there is a certain amount of fluid which is outside the living cells and which is in the molluscs identical with the haemolymph. Inside the cells there is a certain amount of water holding certain ions in solution, and it is the concentrations of these ions which we desire to know. In addition there may be, both outside and inside cells, certain structures holding salts in indiffusible combinations which tend to complicate the analytical problems.

A preliminary attempt has been made to determine total osmotic concentrations by the vapour-pressure method as well as concentrations of single diffusible ions both in the haemolymph and in the intracellular fluid of mussels (*Mytilus edulis*) in equilibrium with varied concentrations of sea water. In order to determine the total volume of haemolymph a known quantity of a substance which was supposed not to penetrate into cells was injected and its concentration determined after a suitable interval for its distribution. The first experiments were made with thiocyanate which has been found suitable in higher animals (Lavietes, Bourdillon and Klinghoffer, 1936; Krogh, 1938), but the volume thus determined turned out in *Mytilus* to be the total amount of water in the body. It is probable therefore that thiocyanate penetrates into all cells in *Mytilus*. Experiments with thiosulphate gave results which were consistent and probable, but there is no proof that the volume so determined corresponds really to the extracellular fluid. Assuming that it does the concentration of thiosulphate in haemolymph and in press juice from *Mytilus* muscles was compared and gave the result that 12 % (11–13) of the juice was extracellular and 88 % intracellular.

Analyses were made of chloride, sulphate, total alkali and potassium in sea water, haemolymph and press juice from muscles from three animals which had been kept for many days, respectively, in about 10, 25 and 33 $^{o}/_{oo}$ sea water. The results obtained on the muscle juice were recalculated on the assumption that 12 % of

the juice was haemolymph, and all concentrations are given in mM./kg. of water:

Table VIII

Dry substance g./l.			Cl			SO_4		
W	Bl	M	W	Bl	M	W	Bl	M
10	26	192	152	158	55	—	—	—
25	29	246	432	419	110	22·5	19·4	13·4
33	38	234	590	554	284	30	26·2	42
Na			K			Osmolar concentration		
W	Bl	M	W	Bl	M	W	Bl	M
135	126	27	3·4	16·6	90	189	230	232
390	367	84	8·8	14·2	115	—	410	410
508	504	120·5	11·6	20·3	136·5	585	592	585

W = sea water; Bl = blood water; M = muscle water.

The results given in Table VIII show that the total concentrations and single ions in the haemolymph follow with only small differences the sea water in which the animal lives. The water content of muscle is practically the same in 25 and 33 °/₀₀ sea water, but definitely higher in 10 °/₀₀. In 25 °/₀₀ sea water the sum of Na and K in the muscles is 199. Separate determinations of Ca and Mg have given the quantities per kg. water as 4·1 mM. Ca and 16·5 mM. Mg as against 11·2 and 44·3 in the corresponding sea water. The total base is therefore 240 mE. in the muscle as against 510 in the water, so that more than half of the osmotic concentration must be made up probably by organic molecules. The variations in the single ions, observed when the outside concentrations are changed, are very remarkable; but the material is insufficient for a discussion.

The chemical analyses give a slight and the vapour-pressure determinations a definite indication of an active osmotic regulation of haemolymph and tissues in *Mytilus* in 10 °/₀₀ sea water, but we have not succeeded in confirming this by direct experimentation (Conklin and Krogh, 1938).

FRESH-WATER MOLLUSCS

Determinations of the ionic composition of the blood in *Limnaea stagnalis* from an Hungarian pond near the Balaton lake containing

at the time 3·5 mM. Cl/litre were made by Huf (1934), who found that narcosis with ether would cause a loss of salts. This loss affected the different ions to a very different extent, and the concentration of K was even increased. I am inclined to look upon this as a kind of regulation by which K was given off from the tissues. The figures, recalculated into mM./litre, are given in Table IX:

Table IX

	Cl	Na	K	Ca	Mg	Total kation mE.
Fresh animals	42·6	47·5	2·8	3·05	4·8	66
Narcotized animals	31·3	39	3·0	2·35	3·95	54·4

Florkin (1938) finds that *Anodonta* preserves a constant weight in media which are hypotonic or isotonic to its own blood and loses weight rather rapidly in hypertonic media, from which he draws the tentative conclusion that the animal's surface is impermeable for water in one direction, but freely permeable from inside out!

Picken (1937) made very interesting and valuable experiments on *Anodonta cygnea* and *Limnaea peregra*.* He determined by means of the vapour-pressure method the molar concentration of blood, pericardial fluid (a filtrate from the blood) and urine. In the bivalve the blood concentration is very low, 16·2 mM. on an average, with variations between 10 and 20, while in the snail it is much higher (73 mM.). The pericardial fluid is stated to have the same molar concentration as the blood. The actual figures are lower, especially in *Limnaea*, but it must be admitted that the difference is not statistically significant. Florkin (1935), who took samples of urine close to the pericardium, found the concentration equal to that of the blood. The concentration of the final urine is definitely lower in both species, viz. 10 as the average for *Anodonta* and 54 in *Limnaea*. A reabsorption of osmotically active substances must therefore take place during the passage of the urine along the nephridial tube.

In special experiments Picken measured the rate of filtration from the haemolymph into the pericardium of *Anodonta*. He found rather large variations, but for animals of an average weight of

* *L. peregra* is a form which penetrates into brackish water.

50 g. (without the shell) the average filtration was about 1 ml. in 5 min. or six times the animal's weight per day. This must correspond roughly to the osmotic uptake of water, and the animal should therefore lose 0·300 × 10 mM. = 3 mM. of osmotically active substances per day through the urine. Picken makes calculations (admittedly rough) of the possible intake of food and comes to the conclusion that it is improbable that the salts of the food can cover the loss. He points out moreover that *Anodonta* is able to live for months in aquaria with running tap water, containing very little in the way of micro-organisms,* and finds the conclusion inevitable that *Anodonta* must be able to absorb salt through the outer surface from fresh water, in spite of the extremely low concentrations normally found there.

This conclusion is verified by work done about the same time in my laboratory, but not yet published in a paper. We tested *Limnaea stagnalis*, *Paludina vivipara*, *Dreissena*, *Anodonta* and *Unio* with about millimolar salt solutions after treatment with distilled water for periods of a few days up to 1 month.

Six *Limnaea stagnalis* with an aggregate weight of 26 g. reduced the Cl concentration of 45 ml. 0·01 Ringer solution in 8 hr. from 1·16 to 0·56, taking up Cl at a maximum rate of 4·3 μM/hr. In subsequent experiments Cl was absorbed from $CaCl_2$, but at a slower rate, and the concentration was never reduced below 0·9 mM. There is reason to believe that calcium was not absorbed at all.

Eight *Paludina*, washed out only 3 days and weighing 33 g., at first failed to absorb Cl from 0·01 Ringer, but after 11 hr. took up a little. Washed out for 4 days more they reduced the Ringer from 1·1 to 0·105 mM. Cl and absorbed at a maximum rate of 5·7 μM./hr. The Cl concentration in the blood of two animals after the experiment was 17·3 and 17·4 mM., while in two animals from tap water it was 22 and 27·6 mM.

Ten *Dreissena*,† washed out for 3 days and weighing 23 g., reduced 40 ml. NaCl solution from 0·74 to 0·15 in 9 hr., but this was clearly the limit to which they could attain, since the concentration rose

* Florkin (1938) observed that *Anodonta* in running tap water would keep up a normal concentration for nearly a year, but later it would drop slowly to about 18 mM. after 20 months.

† *Dreissena* is a form which has invaded fresh water from the Caspian Sea.

during the next 8 hr. to 0·22. In some later experiments *Dreissena* took up Cl from NH_4Cl, but the total amount was small only.

The first specimens of *Anodonta* tested were very large, weighing from 250 to 300 g. After washing for 6 days they failed to absorb. Nine days later a slight and doubtful absorption from mM. NaCl was noticed in single periods, but sometimes the concentration would rise again. A test with mM.$CaCl_2$ made after about 1 month's treatment with distilled water was entirely negative. It was thought at the time that the animals might be too old. The Cl concentration in the blood was determined and found to be 9·1 mM. Later experiments show that *Anodonta* can absorb Cl from millimolar solutions, but that the concentration cannot be reduced very far. Small individuals absorb with more energy than large ones and resist the distilled-water treatment better. In a small number of experiments some absorption of Cl^- from NH_4Cl with excretion of NH_3 was observed and also absorption of Na^+ from $NaHCO_3$.

Unio pictorum. From one fresh *Unio* weighing 54 g. of which the shells made up 15·4 g., 22 g. haemolymph was obtained showing a Cl concentration of 31 mM. The dry substance of the body was only 4·4 %. Four individuals with an aggregate weight of 70 g. reduced 200 ml. 1·11 mM. NaCl to 0·15 in 50 hr. with a maximum uptake of 21 μM./hr. In experiments with mM. $CaCl_2$ they took up Cl slowly, but lost Ca at the same time.

It is to be concluded from these experiments, carried out in collaboration with Agnes Wernstedt, that the fresh-water molluscs studied are able to absorb Cl^- and Na^+ from dilute solutions, but are probably unable to reduce the concentration below 0·1 mM. If this holds for all individuals they should be unable to live in water with Cl contents below 3 mg./l.

According to the experiments on fresh-water molluscs now described we find actively absorbing cells, both in the external surface and in the kidney tubule, able to take up salts from dilute solutions. As they stand Picken's experiments would place the kidney tubules as being much less effective than the cells in the surface, but I venture to predict that this is only because they were not put to a crucial test. When animals are prevented from absorbing salts from outside, the kidneys will probably be able to

produce a urine which is almost salt free. Otherwise the process of washing out with distilled water would be much more rapid than is actually the case.

Philippson, Hannevart and Thieren (1910) and later Duval (1925) studied the behaviour of *Anodonta* in balanced salt solutions. Below 67 mM. the molar concentration of the blood is higher than that of the surrounding solution, but at higher concentrations there will be a complete equilibrium as shown in the curve, Fig. 28, p. 95. No regulation is possible by which the osmotic pressure of the blood can be kept below that of the external medium.

Although the problems of shell formation in molluscs do not, strictly speaking, come within the scope of the present monograph it may be appropriate briefly to draw attention to them. The $CaCO_3$, forming almost the whole of the inorganic material of the shell, must come from the water either directly or through the food. The Ca concentration in sea water is 10·9 mM. In fresh waters it is generally much lower, and, for instance, in the Danish lake Furesö about 1·1 mM. There are soft waters with Ca contents of 0·1 mM. and below. Molluscs are found in all these waters, and certain bivalves like *Margaritana margaritifera* can accumulate very large amounts of $CaCO_3$ in their shells in very soft water. In a suggestive study by Galtsoff (1934) it is shown that an oyster, showing at the beginning of May a "meat" weight of 5 g. with a 60 g. shell, grows until the middle of November, during a period when the temperature is always above 10° and food is regularly absorbed, to 15 g. "meat", while the shell reaches 100 g. Until the next May no food is taken and the weight drops to about 13 g., while the shell continues to grow at the same rate and reaches 135 g. This shows that Ca is taken up only to a slight extent with the food and mainly through the integument. The 75 g. $CaCO_3$ absorbed corresponds to 750 mM. Ca or the quantity present in 70 l. sea water or 7000 times the mean weight of the animal. It is almost inconceivable that such a quantity can be obtained from the water without a special mechanism for absorbing Ca.