OSMOREGULATION IN THE MUSCLE FIBRES OF *CARCINUS MAENAS*

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(*Received* 29 *July* 1958)

INTRODUCTION

In considering the general problem of the penetration of marine animals into brackish water and ultimately into fresh water, it is clear that even in animals, like *Carcinus maenas*, which are able to maintain the concentration of their body fluid significantly above that of their environment in dilute sea water, all the cells of the organism must be able to adapt themselves to considerable changes in the composition of their surrounding fluid. In *Carcinus*, for example, living in 40% sea water the blood concentration drops to about 60% of normal. The process of cellular adaptation to blood dilution is quite unknown. It is, however, possible to visualize several ways by which this might come about. On the one hand, the cells may take up large amounts of water during blood dilution, and adaptation may take the form of increased tolerance to cell hydration. On the other hand, the cells may maintain their normal composition by the maintenance of the cellular osmotic pressure—a process which would involve the removal of water entering by osmosis. Although it has been generally assumed that cells are always isosmotic with their surrounding fluid, processes for the removal of water are known in the Protozoa and probably exist in the cells of fresh water sponges and coelenterates. It has also been suggested that certain mammalian organs contain cells which are hyperosmotic to the body fluids, although this still requires confirmation (Opie, 1949; Robinson, 1950). Finally, the process of adaptation may involve a reduction in the cell osmotic pressure by the removal of some of the osmotically active constituents sufficiently rapidly to prevent the excessive intake of water.

From previous work on the ionic composition of the muscle under different conditions of blood composition (Shaw, 1955*a*, *b*, 1958) some of these possibilities can be eliminated. In the first place, the change in water content of the muscle fibres in crabs from dilute sea water is not sufficient to account for the behaviour of the fibres on the basis that they are simple osmometers. In the second place, there is no evidence to show that the concentrations of the muscle ions are regulated to any extent; they behave passively with respect to water movements.

Two possibilities remain—either that the osmotic pressure of the fibre is maintained above that of the blood or that the osmotic activity of the fibre is reduced by the removal of some part of the non-ionic fraction of the muscle. This latter fraction is quite extensive—the muscle ions account for only about one-third of the total

osmotic pressure (Shaw, 1955*a*). In the decapod Crustacea this fraction has long been associated with the presence of large quantities of nitrogenous substances. Thus Duchâteau & Florkin (1955) find from 4427 to 5708 mg. amino acids per 100 g. muscle in marine *Eriocheir*. Kermack, Lees & Wood (1955) find a similar range of free amino acids together with trimethylamine oxide and betaine in the lobster, and Robertson (1957) finds 500 mg./kg. muscle amino-N and 100 mg./kg. trimethylamine oxide in *Nephrops* muscle. In *Carcinus* muscle, Lewis (1952) identified glycine, alanine and taurine by qualitative chromatography.

The possibility of regulation of the concentration of these substances is apparent from the fact that in freshwater decapods like *Astacus* (Camien, Sarlet, Duchâteau & Florkin, 1951) and *Eriocheir*, adapted for long periods to fresh water (Duchâteau & Florkin, 1955) the concentrations of some of the free amino acids may be much lower than in the marine forms. Lack of information on the water content of the muscle of the latter makes it impossible to know the cause of the reduction in concentration.

This paper describes investigations directed towards (i) the elucidation of the mechanisms by which adaptation to reduced blood concentration is achieved in the muscle, and (ii) the distinction between the maintenance of osmotic pressure and the reduction of osmotic activity.

MATERIAL AND METHODS

Experimental details with regard to the crabs followed those in previous papers (Shaw 1955*a*, *b*). The muscles used were, as before, the extensor and flexor of the carpopodite of the chela. Muscles were prepared as groups of single fibres, and in such a way that all analyses refer to the composition of the muscle fibres—not to the whole muscle, including trapped blood.

Freezing-point depression. Measurements on blood and on single muscle fibres were made by means of the technique of Ramsay (1949) as modified by Ramsay & Brown (1955). For making measurements on individual fibres, crabs were cooled in a cold room at $-3°$ C., muscles were removed and single fibres dissected out in liquid paraffin at this temperature. The fibres were introduced into glass capillaries and then frozen at $-80°$ C. and stored at this temperature until required for measurement. In this way it was hoped that autolytic changes occurring in the fibre after removal were reduced to a minimum.

Amino acid nitrogen. This was measured on tungstic acid and trichloracetic acid extracts of fresh or dried muscle by means of Folin's method (Folin, 1922, as modified by Danielson, 1933). Examination of standard solutions of expected nitrogenous compounds showed that the method gave quantitative results for glycine, alanine, taurine and the α-amino group of arginine, but was negative for trimethylamine oxide and betaine.

Trimethylamine oxide. This was reduced to ammonia by acid stannic chloride (Kermack *et al.* 1955). The ammonia was removed by diffusion and estimated by a micro-conductimetric method (Shaw & Staddon, 1958).

Non-protein nitrogen. By micro-Kjeldahl digestion and estimation of ammonia as above.

RESULTS

(*a*) *Total osmotic pressure*

Measurements of the freezing-point depression of single muscle fibres of crabs from normal and diluted sea water are shown in Fig. 1. It is clear that the measurements are consistent with the view that the total osmotic pressure of the muscle

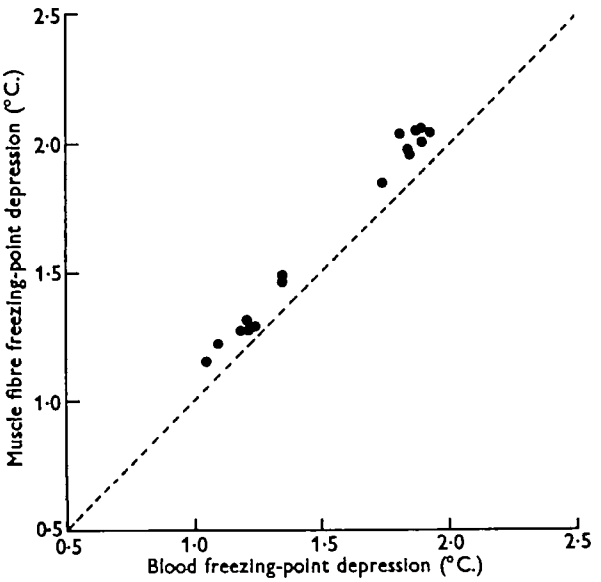

Fig. 1. The relation between the freezing-point depression of the blood and of the muscle fibres.

fibres falls in proportion to that of the blood when this is diluted. It is, however, noteworthy that in every case the fibre appears to be slightly hyperosmotic to the blood—generally about 7–8% higher. This was invariably found, both in the normal crabs and in those from dilute sea water. There was no evidence that this difference increased as the blood was diluted. It is important to consider whether this difference can be regarded as normal or whether it is an artifact of the technique. Although the temperature during preparation and measurement was kept below zero, it has been shown that autolysis may take place at 0° C., even in mammalian tissues (Conway, Geoghagen & McCormack, 1955). If autolysis did occur the most likely substances to be involved would be the labile phosphate esters—arginine phosphate and adenosine triphosphate. The concentrations of these substances in the muscle (Shaw, 1958) is just about sufficient to give the observed increase in concentration if they were broken down. The following observations add weight to this suggestion. If the fibres are separated and kept in liquid paraffin at room temperature for some hours before measurement, there is no increase in the apparent freezing-point depression. Further, if the fibres are dried (this is known

to bring about the breakdown of phosphate; Shaw, 1958) and then extracted with distilled water the water extract again (when corrected to same water content) shows the same degree of hyperosmosticity.

These considerations make it probable that the hyperosmosticity arises by autolysis after the removal of the fibres and that, *in vivo*, the muscle fibres are in osmotic equilibrium with the blood. In any event, the intracellular osmotic pressure always changes in proportion to a change in the blood concentration. There is no evidence for the maintainence of the fibre osmotic pressure in diluted blood.

THE COMPOSITION OF THE MUSCLE FIBRE

The inorganic ions of the muscle, together with the organic phosphate anions, only account for a relatively small proportion of the total osmotic pressure of the fibre (Shaw, 1955*a*, 1958). The remainder of the osmotic activity is due almost entirely to the presence of organic nitrogenous compounds. Some of these have been identified qualitatively as the amino acids, glycine and alanine, and the related taurine (Lewis, 1952). A quantitative analysis of the main nitrogenous compounds is shown in Table 1. The largest part of this fraction is accounted for by the

Table 1. *The concentrations of non-protein nitrogen-containing substances in the muscle fibres*

Substance	mean concn., mM./kg. fibre water	S.D. ± mM/kg.	No. of measurements
α-Amino-N	516	47	15
Trimethylamine oxide-N	90	20	10
Arginine α-Amino-N	82	—	—
Arginine total N	328	—	—
Adenosine tri-phosphate total N	45	—	—
Non-arginine α-Amino-N (by difference)	434	—	—
Total non-protein N by addition	897	—	—
Total non-protein N by Kjeldahl	990	36	6
Unknown N compounds (by difference)	93	—	—

compounds containing amino nitrogen. These will include the above-mentioned substances, together with arginine, either occurring in the free state or liberated from its combination with phosphate. Trimethylamine oxide is also an important constituent, as was found also in lobster muscle (Kermack *et al.* 1955) and in *Nephrops* (Robertson, 1957). When all measured nitrogen is added up, together with

the contribution from adenosine triphosphate, the total falls somewhat short of the measured total non-protein nitrogen, estimated by the Kjeldahl method. The deficit amounts to 94 mM./kg. fibre water. This nitrogen may well be present largely in the form of betaine. This substance was found in concentrations of this order in the lobster muscle (Kermack *et al.* 1955), and, in view of the general agreement in the composition of the other known nitrogenous substances, it is a reasonable assumption this substance may also be present in *Carcinus* muscle.

Table 2. *The osmotically active components of the muscle fibre*

Substance	Concn., mM. or mg. ions/kg. fibre water
α-Amino-N compounds (excluding arginine)	434
Trimethylamine oxide	90
Unknown N-compounds (possibly betaine)	93
Potassium	146
Arginine- and inorganic phosphate	82
Adenosine triphosphate	9
Calcium	5
Magnesium	17
Sodium	54
Chloride	53
Total	983
Blood osmolar concn. (from freezing-point (m-osm./kg.))	1000

Making use ot these analyses of the organic nitrogen compounds, together with the analyses of inorganic ions and phosphate compounds previously described (Shaw 1955, 1958), it is possible to see to what extent the concentrations of these substances can account for the total osmotic activity of the fibre. These concentrations are all listed in Table 2, and the sum of these (in mM./kg. water or mg. ions/kg. water, whichever is appropriate) can be compared with the osmotic activity of the blood at the same time, as measured by the freezing-point depression. The correspondence between the two is extremely good and the difference can scarcely be significant. However, in summing the concentrations of the muscle constituents in terms of osmotic activity, the assumption has been made that in every case the osmotic coefficient can be taken as unity. This is unlikely to be the case for the free ions—but even taking this into consideration the discrepancy between the muscle and blood is not likely to be greater than about 50mM./kg. water. It seems certain, therefore, that, together with the muscle ions, the organic nitrogenous compounds make up a very large part, if not the whole, of the total muscle osmotic activity.

MUSCLE COMPOSITION OF CRABS FROM DILUTE SEA WATER

With the identification of a very large fraction of the osmotically active components of the muscle it is now possible to study the regulation of the total osmotic activity of the muscle fibre in terms of the variations of the concentrations of the individual

constituents. The behaviour of the various muscle ions under conditions of blood dilution have already been described (Shaw, 1955*b*, 1958). We can now look at the composition of the muscle in crabs from dilute sea water with respect to changes in the concentrations of the nitrogenous fraction. Analyses of these compounds in muscles from crabs living in 40% sea water is shown in Table 3. The measurements of the concentrations of arginine and adenosine triphosphate are taken from Shaw (1958). It is evident that, by comparison with the similar analyses for the muscle of crabs from normal sea water (Table 1), every nitrogen-containing substance shows a marked and significant fall in concentration; and that this is especially true of the amino-N compounds, the concentration of which is approximately halved.

Table 3. *The concentrations of the nitrogenous compounds in the muscles of crabs from* 40% *sea water*

Substance	Concn., mM./kg. fibre water	S.D.	No. of measurements
Total non-protein N α-Amino N	611	44	6
α-Amino N	255	47	13
Trimethylamine oxide	58	10	6
Arginine	64	—	—
Adenosine triphosphate	8	—	—
Total arginine N	256	—	—
ATP-N	40	—	—
α-Amino N (excluding arginine)	191	—	—
Unknown N compounds (by difference)	66	—	—

In a similar study of the behaviour of the muscle ions during blood dilution (Shaw, 1955*b*, 1958), it was found that in dilutions of sea water at least down to 40% the changes in the concentration of the major ions (potassium and organic phosphates) could be explained solely on the grounds of the dilution of the muscle contents by the osmotic intake of water, consequent upon the dilution of the blood.

It is now necessary to see to what extent the changes in the other muscle constituents can be explained on the same grounds. To do this the concentration of these substances in the muscles of the 40% sea-water crabs were compared with the concentrations which would be expected on the assumption that water intake was the only factor determining their distribution. Water intake was calculated from the mean difference in water content of the muscles of normal crabs as compared with the muscles of crabs from 40% sea water. The results of these calculations are shown in Table 4. It is clear that in all cases the concentrations are below those expected from the change in water content and this is particularly marked in the case of the amino-N compounds. Here the drop in concentration is some 160mM./kg. greater than can be accounted for by water intake. This must mean that either these substances are removed from the fibre during blood dilution or they are combined

with other muscle constituents and no longer extractable with trichloracetic acid. Another possible explanation that these substances were combined to form simple soluble peptides was found to be untenable. In the normal muscle, hydrolysis of the acid extract with N-HCl showed the absence of simple acid-soluble peptides. In the muscles from the 40% sea-water crabs there was likewise no increase in α-amino-N on hydrolysis.

Table 4. *The effect of the increased water content on the concentrations of the nitrogenous substances in the muscles of crabs from* 40% *sea water*

(Concentrations in mM./Kg. fibre water)

Substance	Mean concn. in muscles of crabs from 100% sea water	Mean concn. in muscles of crabs from 40% sea water	Concns. for 40% crabs calculated from change in water content	Loss not accounted for by change in water content
α-Amino-N compounds (excluding arginine)	436	191	355	164
Trimethylamine oxide	90	58	73	15
Unidentified N compounds	93	66	76	10
Water content (%)	74·0	77·8	—	—

The significance of the fall in α-amino-N concentration now becomes apparent. The measurements of the freezing-point depression of the fibre (Fig. 1) show that in 40% sea water (blood mean $\Delta = 1{\cdot}2°$ C.) the osmotic activity of the fibre must fall from 1000 m-osm./kg. water to 650 m-osm./kg. water. The change in water content accounts for a fall of 200 m-osm. The difference is made up quantitatively by the loss of free α-amino-N compounds.

There is clearly a process operating for the reduction of osmotic activity within the fibre by the removal of free nitrogen-containing substances, chiefly amino acids and taurine. It is interesting to establish if this process is reversible, inasmuch as normal muscle concentrations are regained on the return of the crabs to normal sea water.

The demonstration that the concentration of the nitrogenous substances in the muscles of crabs which have been taken down into dilute sea water and then returned to normal sea water is unaltered, invites the criticism that in such animals the concentrations of these substances were, in fact, never changed, except by water movements. To meet this objection, measurements were made on individual crabs. as follows. A crab was adapted to 40% sea water, one chela was removed and its muscles analysed. The wound was allowed to heal and then the crab was returned to full-strength sea water. Finally the other chela was taken and the muscle analyses compared. The results of these measurements are shown in Table 5. The results are fully consistent with concept of the process as a reversible one. In all cases additional free α-amino-N compounds are made available, and the amount added (mean 173 mM./kg.) is comparable with the amount that was lost in the previous experiments (Table 4).

Table 5. *The concentrations of substances containing α-amino nitrogen in muscles of crabs from 40% sea water and also in muscles after transference of the crabs to full-strength sea water*

Concn. in muscle of crab from 40% sea water (mM./kg. water)	Concn. in muscle after transfer to 100% sea water (mM./kg. water)	Water content of muscle from 40% sea water (%)	Water content of muscle from 100% sea water (%)	Concn. expected from change in water content	Amount added (mM./kg. water)
271	508	76·9	72	348	160
286	537	76·5	73·7	332	205
266	533	80·3	74·2	377	156

DISCUSSION

It has often been suggested that free amino acids and other similar compounds play an important part in the osmotic activity of the cells of marine invertebrates. The analyses presented here and also the analysis of whole muscle of *Nephrops* by Robertson (1957) show that this is certainly the case. In *Carcinus* they account for over 60% of the total osmotic activity. Further it is now clear that in *Carcinus* these substances do not play an entirely passive role in the maintenance of cellular osmotic pressure but are actively concerned in the regulation of the osmotic activity. In discussing the adaptation of the muscle cells to diluted blood, from the point of view of the behaviour of the muscle ions (Shaw, 1955*b*) it was suggested that an important part of cell adaptation was the restriction of water uptake. It is now clear that this is not done by the maintenance of the intracellular osmotic pressure. The process involves the reduction of the internal osmotic pressure by the removal of osmotically-active components from the muscle. The process of reduction of internal osmotic activity achieves the same result, in terms of cellular hydration, as the maintenance of a high blood concentration.

Thus in *Carcinus*, as far as the muscle fibre is concerned, changes in the state of water content of the cell are restricted by two complementary processes—firstly, by the maintenance of the blood osmotic pressure above that of the environment, when this is diluted; and secondly, by a reduction of the osmotic activity within the cell itself. Thus, for example, in 40% sea water, the total effect of these two processes is to present to the cell conditions equivalent to a reduction in the concentration of the sea water to only 80% of the normal value. There is no reason to believe that the internal regulatory process is any less important than that operating on the blood. Indeed, in many brackish water animals with limited or no powers of osmoregulation (for example, *Arenicola*) the process of cell adaptation must be the dominant one and the development of such powers must play an important part in the penetration of marine animals into brackish waters.

The study of the process of cell adaptation shifts the emphasis from the importance of the maintenance of osmotic pressure, *per se*, to that of the water content of the

cell in relation to the osmotically inactive cellular components. Our present knowledge of the fine structure of cells, with their complicated array of thin membranes and vesicles, makes it apparent that any change in the water content could easily lead to disruption and distortion of these structures.

The question of the mechanism by which the reduction of the osmotic pressure exerted by the acid-soluble nitrogen compounds is effected in *Carcinus* muscle is an interesting one. It does not follow that these substances are removed from the fibre, it is only necessary that their osmotic activity be reduced. The fact that the process is reversible and that the blood concentrations of these substances are always low (less than 5 mM/l.) makes it a little difficult to see where the substances would be stored when they are temporarily removed from the fibre. The possibility that these substances are rendered osmotically inactive by their combination with large molecules of the cell, such as proteins, must be seriously considered and this would be worthy of further investigation.

As far as *Carcinus* is concerned one cannot consider the possession within the muscle of large amounts of nitrogenous substances which act as an osmotic reserve as a special adaptation for life in brackish water. Analyses of muscles of other, purely marine, decapod Crustacea, such as the lobster and *Nephrops*, show that these substances are present in these animals in much the same order of concentration. *Carcinus* has simply exploited an already existing situation.

The reason for the occurrence of the large concentrations of these nitrogenous compounds in the first place in the muscles of marine animals is more obscure. It is noteworthy that the ionic composition of the muscles of the animals in sea water is not very different in the concentrations of the main ionic constituents from that found in many terrestrial and freshwater animals. It is conceivable that there is an optimum concentration for ions in an efficiently operating striated muscle fibre, and it is possible that this is achieved in the marine forms by the addition of the organic nitrogen compounds.

Finally, it must be remembered that dilution of the blood affects all cells of the organism, not only the muscle fibres, and it does not follow that all cells behave in the same manner. Cells of the hepatopancreas of the lobster also contain large amounts of free amino acids (Kermack *et al.* 1955), but on the other hand the nerve fibres of *Carcinus* show a very different composition pattern from that of the muscle (Lewis, 1952). For this cell type, although free amino acids are present they are characterized by the large amounts of acidic acids, aspartic and glutamic in contrast to the muscle. These acids are balanced electrostatically by a much higher concentration of potassium. In this cell, adaptation to dilute blood must involve either a much greater change in water content than in the muscle or a substantial reduction in the intracellular potassium concentration. It seems reasonable to suppose that different cells may possess different powers of adaptability, and it may be that the death of the whole organism in very dilute sea water may be the result of the failure of one particular cell type to adapt itself to the new conditions.

SUMMARY

1. Measurements have been made of the freezing-point depression of single muscle fibres of *Carcinus maenas* and of the concentrations of the non-protein nitrogenous components of the muscle.

2. When the blood is diluted the osmotic activity of the muscle fibres always falls in proportion. The fibres are probably always in osmotic equilibrium with the blood.

3. The osmotic activity of the fibres can be accounted for in terms of the concentrations of the muscle ions together with nitrogen-containing compounds, such as free amino acids, taurine and trimethylamine oxide. These organic substances account for over 60% of the total osmotic pressure.

4. In crabs from dilute sea water the concentrations of the nitrogenous compounds are reduced below the level expected from the increase in water content of the muscle. It is suggested that the muscle fibre can prevent excessive water intake by the removal of nitrogenous substances, thus reducing the internal osmotic activity. The process is reversible.

5. The importance of the mechanism in relation to the adaptability of the cell to reduced blood concentration is discussed.

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