FURTHER STUDIES ON IONIC REGULATION IN THE MUSCLE FIBRES OF *CARCINUS MAENAS*

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INTRODUCTION

When a euryhaline crab like *Carcinus maenas* is living in dilute sea water, the blood concentration is markedly reduced and, as a consequence, all the animal's cells must be able to adapt themselves to these new conditions. In attempting to analyse this process of cell adaptation it is necessary to know the normal processes by which the ionic composition of the cell is maintained and the extent to which these are modified under the conditions of reduced blood concentration.

Earlier papers (Shaw, 1955*a*, *b*) described the normal ionic composition of the muscle fibres of *C. maenas* and the changes in composition which occurred when the crabs were adapted to diluted sea water. Under these conditions the reduction in blood concentration is reflected in alterations in the concentration of many of the muscle constituents. The muscle sodium and chloride concentrations are reduced in about the same proportion as their respective concentrations in the blood, but there is a marked retention of calcium, magnesium and potassium. In the case of calcium and magnesium there is reason to suppose that they are retained simply as a result of the almost complete impermeability of the fibre membrane, but this simple hypothesis is not likely to be an adequate explanation in the case of potassium. A more acceptable hypothesis is that the potassium ions are held in the fibre by the electrostatic charge of the non-chloride anion fraction to which the membrane is impermeable, and that these ions are selected in preference to sodium ions because of an active mechanism for the exclusion of the latter.

This paper describes investigations: (1) to determine the nature of the non-chloride anion fraction and the permeability of the muscle fibre membrane to this; (2) to measure the permeability of the fibre membrane to potassium and to explore the mechanisms for its retention; and (3) to throw some light on the mechanism of sodium exclusion.

MATERIAL AND METHODS

The supply and maintenance of the animals and the analytical methods were as described previously, except for additional estimations described below. In all analyses the muscle fibres were prepared free from extracellular fluid and the measurements are of intracellular concentrations.

Phosphate

Phosphate estimations were made on trichloracetic acid extracts of the muscle by the colorimetric method of Fiske & Subbarow (1925), as detailed in Delory (1949). The various phosphate fractions were determined as follows:

(*a*) *Inorganic phosphate.* The crabs were chilled to 0° C., the muscles quickly removed, weighed and transferred to ice-cold 8% trichloracetic acid and ground with sand. The extract was filtered in the cold and immediately neutralised. The phosphate content was measured directly, without precipitation of the inorganic phosphate, since arginine phosphate in the presence of ammonium molybdate is hydrolysed very slowly at room temperature (Baldwin & Yudkin, 1950).

(*b*) *Arginine phosphate.* A trichloracetic acid extract of muscle at room temperature was diluted to 2% acid and the phosphate ester hydrolysed in a boiling water-bath for 3 min.—solution contained orthophosphate derived from inorganic phosphate and arginine phosphate and the arginine phosphate was determined by difference.

(*c*) *Adenosine triphosphate.* The remainder of the hydrolysed extract was made normal with respect to hydrochloric acid and further hydrolysed for 10 min. in a boiling water-bath. This hydrolysed the last two phosphate radicals on the adenosine triphosphate molecule, and these were added to the phosphate already present in the solution. Again the ATP content was calculated by difference.

(*d*) *Other phosphate esters.* Hydrolysis was continued for 24 hr. in the normal acid solution. The total phosphate in the acid extract was also measured by neutralizing a known volume of the extract with sodium carbonate, drying and incinerating. The ash was taken up in acid and phosphate content measured. The phosphate content was also determined on trichloracetic acid extracts of dried muscle fibres.

Arginine

This was measured in the diluted trichloracetic extract of dried muscle by the colorimetric method of Macpherson (1946) modified by the replacement of the α-naphthol by hydroxyquinoline (Janus, 1956).

Tracer studies

The penetration of phosphate into the muscle was studied by the injection into the blood of crab-Ringer solution containing ^{32}P orthophosphate. Sodium and potassium penetration was followed by using Ringer containing ^{24}Na and ^{42}K respectively. The radioactivity was measured by the usual method using an end-window counter for sodium and potassium measurements and a liquid counter for phosphate. Counting errors were not greater than $\pm 3\%$.

Potential difference measurements

These were made with the aid of two calomel half-cells—the penetrating electrode being of the Ling & Gerard (1949) type. The potentials were recorded on a modified Pye pH meter.

RESULTS

(*a*) *Phosphate*

The measurements on the phosphate content of normal muscle fibres is shown in Table 1. The values for the phosphate compounds were derived from the assumption that the first phosphate fraction represents inorganic phosphate in the intact fibre, that the second fraction contained the additional phosphate liberated from arginine phosphate and the third fraction contained an additional two phosphate radicals derived from adenosine triphosphate. The extra phosphate which appeared after prolonged hydrolysis was then accounted for by the third phosphate from the ATP molecule and the fact that the total acid soluble phosphate is almost identical with the phosphate yielded by this prolonged hydrolysis indicates that phosphate compounds, other than inorganic phosphate, arginine phosphate and ATP are either completely absent or only present in traces.

Table 1. *The concentrations of acid-soluble phosphate compounds*

Phosphate fractions	Concn. (mM./kg. fibre water)	S.D.	No. of measurements
1. Free phosphate in cold TCA extract	23	3	8
2. Total free phosphate after 3 min. boiling 2 % TCA	82	5	14
3. Total free phosphate after 10 min. boiling N-HCl	100	5	14
4. Total free phosphate after 24 hr. in N-HCl	109	9	8
5. Total acid-soluble phosphate	108	8	11
Phosphate compounds			
1. Inorganic phosphate	23	—	—
2. Arginine phosphate	59	—	—
3. Adenosine triphosphate	9	—	—
Arginine	81	4	14
Potassium	146	12	48

The total arginine content of the fibres shows a very close correspondence to the sum of inorganic phosphate and phosphagen-phosphate. Since arginine phosphate is known to be a labile compound readily broken down during muscle activity (Meyerhof & Lohmann, 1928*a*), it is by no means impossible that the normal method of estimating inorganic phosphate by extraction in the cold leads in fact to phosphagen breakdown, and that in the intact fibre the phosphate is actually all combined with the arginine in the form of the phosphagen.

The phosphate and arginine analyses are in general agreement with other analyses which have been reported for crustacean muscle. Robertson's (1957) analyses for the muscles of *Nephrops norvegicus* give values for intracellular concentrations of phosphate compounds (calculated from his figures, using 11·5% as extracellular volume and assuming absence from the blood) of 21·6, 84 and 13·7 mM./kg. fibre water respectively for inorganic, arginine phosphates and ATP. The value for

arginine phosphate is somewhat higher than in *Carcinus*, but this may almost certainly be associated with the higher intracellular potassium concentration. The inorganic phosphate concentrations are similar, and these values are also close to those found by Eggleton & Eggleton (1928) for crab muscle, and the percentage of phosphagen phosphate to inorganic and phosphagen phosphate in *Carcinus* (72%) is not very different from the value of 66% found by Meyerhof & Lohmann (1928*a*) for the abdominal muscle of *Astacus fluviatilis*. The arginine content of the muscles of a number of American crabs has been measured by Arnold & Luck (1933). In three species the concentration varied from 771 to 855 mg. arginine per 100 g. of muscle. This would correspond to a concentration of about 70 mM./kg. of intracellular water and would thus be only slightly smaller than the concentration found in *Carcinus* muscle.

Table 2. *The electrostatic equivalence of the potassium and the acid-soluble phosphate compounds in the muscle*

Substance	Mean concn. in muscle (mM./kg. water)	Equivalent concn. at pH 7 m-equiv./kg. water)
Arginine phosphate	82	84·5
Adenosine triphosphate	9	33·4
Total negative charges	—	117·9
Potassium	146	146
Excess negative over positive charges	—	28·1

It is generally supposed that phosphate compounds in muscle make up a large proportion of the total anions, and in some vertebrate muscles like rat muscle (Conway, 1950) and frog muscle (Boyle & Conway 1941) a good agreement has been found between total cations and measured anions. Potassium ions and the organic phosphate compounds make the most important contributions to the cation and anion fraction respectively. There seems little doubt that the same is broadly true of crustacean muscle. In *Carcinus* muscle sodium is largely balanced by an equivalent quantity of chloride (Shaw, 1955*a*). The contribution of the phosphate compounds to the remaining anion fraction can be computed from their concentrations (Table 1) and from a knowledge of the pH of the fibre interior. The latter has been measured directly by means of an intracellular glass electrode by Caldwell (1954), and found to be about 6·9. The results are shown in Table 2, where the number of charges per g. molecule were calculated from the pK values of the various phosphate compounds given by Meyerhof & Lohmann (1928*b*) and by Conway (1950). The values for potassium concentration in the fibres is rather higher than found previously (Shaw, 1955*a*). Assuming that all the arginine is combined with phosphate as phosphagen, there is an approximate balance between the positive charge of the potassium ions and the negative charges of the phosphate compounds. The excess of positive charges of some 28 mM./kg. fibre water could easily be accounted for by the negative charge on the cell proteins, and it is probably not necessary to postulate the presence of other small anion molecules.

Robertson's (1957) analysis of whole muscle of *Nephrops* also show a satisfactory agreement between the potassium and phosphate fractions.

With the identification of the main anion fraction within the muscle fibre it is now possible to explore the behaviour of this fraction under circumstances where the blood concentration is greatly reduced. Measurements of the concentrations of the various phosphate fractions were made on muscle of crabs which were kept in dilute sea water at concentrations down to 40% normal sea water.

Fig. 1 shows the relation between the total acid soluble phosphate of the muscle and the blood sodium concentration. There is a marked fall in the phosphate concentration as the blood level falls, but at the lower concentrations the fall is less marked. This type of relationship is similar to that found for the behaviour of the intracellular potassium under similar circumstances (Shaw, 1955*b*). In the latter

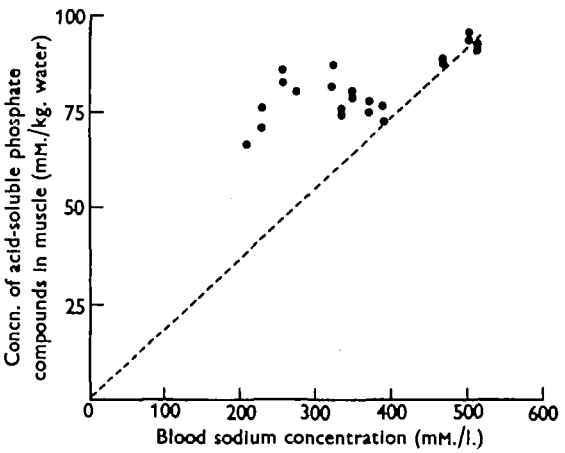

Fig. 1. The relation between the sodium concentration of the blood and the concentration of acid-soluble phosphate compounds in the muscle.

case the fall in concentration could be explained by the increase in water content of the muscle resulting from the dilution of the blood, and a similar explanation is adequate in the case of the muscle phosphate. When the acid-soluble phosphate is calculated on a dry-weight basis, and this is related to the blood sodium concentration (Fig. 2), it can be seen that there has been no significant loss of phosphate from the muscle to the diluted blood.

The simplest explanation of these findings is that the muscle fibre membrane is impermeable to the organic phosphate compounds and also to inorganic phosphate. The fact that the correlation between the arginine content of the muscle and the easily hydrolysed phosphate fraction is very high at all blood dilutions adds further evidence for the view that all the arginine is normally combined (Table 3). If permeability to any of these substances is assumed then active processes would have to be evoked to explain their retention. The concentrations of these compounds in the blood are very low, and their electrochemical potentials are in each case much

higher inside the fibre than outside. Such active processes would also have to be capable of regulation under conditions of blood dilution.

A formal demonstration of the impermeability of the fibre membrane to all of these substances has yet to be made, but tracer studies on the behaviour of inorganic phosphate lend further support to this hypothesis. Sodium orthophosphate labelled with ^{32}P was made up in crab-Ringer solution and injected into a number of crabs and in such a concentration as to only increase the normal blood phosphate level by about 0·2 mM./l. The crabs were kept out of water, but in a damp environment to prevent loss of radioactivity by diffusion into the surrounding sea water, for periods up to 12 days. At intervals during this period a crab was removed and an analysis made of a trichloracetic acid extract of the muscle for phosphate and total radioactivity. This was compared with the phosphate content and radioactivity of a trichloracetic acid extract of the blood.

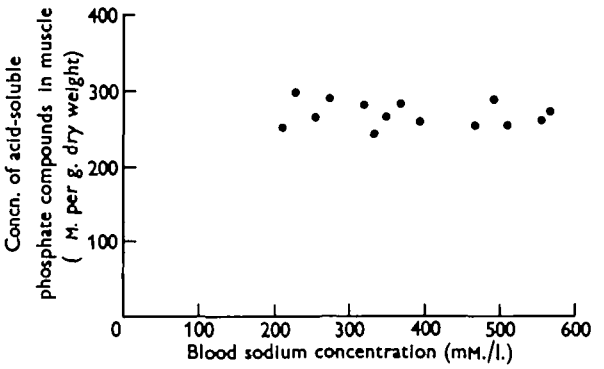

Fig. 2. The relation between the sodium concentration of the blood and the amount of acid-soluble phosphate compounds per g. dry weight of the muscle.

Table 3. *The muscle concentrations of arginine and the total inorganic phosphate after hydrolysis in 2% trichloracetic acid*

Muscle no.	Arginine concn. (mM./kg. water)	Inorganic phosphate after TCA hydrolysis (mM./kg. water)
1	87	85
2	83	86
3	80	82
4	78	82
5	78	81
6	76	74
7	67	67
8	69	68
9	61	65
10	66	68

The ratio of the radioactivity in a given volume of muscle fibre water to the radioactivity in the same volume of blood is shown in Fig. 3 plotted against the time after the injection of the phosphate solution. After a few hours this ratio reaches about 10, then levels off and shows no further increase for the remainder of the 12 days.

Since the blood concentration is in all cases less than 0·5 mM./l. phosphate the ratio represents less than a 5% exchange with the total acid-soluble phosphate. If instead of the total phosphate, the 'inorganic' phosphate alone is considered then the ratio would indicate some 20% exchange with the cell inorganic phosphate. From studies on vertebrate muscle, however, there is every reason to believe that the phosphate radicals of the various compounds (with the exception of the third phosphate of ATP) are readily exchangeable with each other (see, for example, Hevesy, 1947) and the separation into the various fractions would be misleading. Furthermore, the rapid uptake of the radioactive phosphate within a few hours and the subsequent constancy does not suggest a permeability phenomenon but probably some kind of absorption.

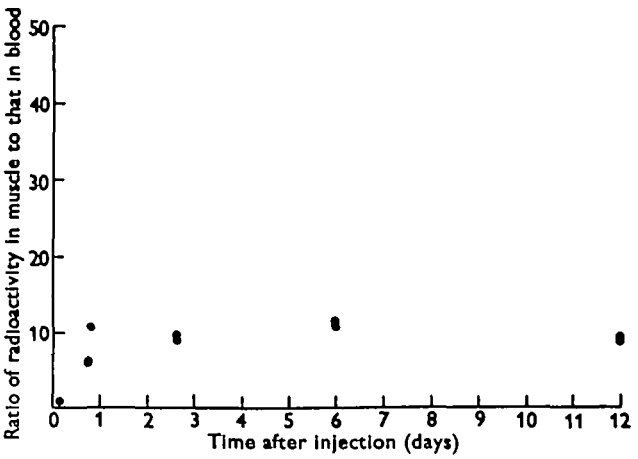

Fig. 3. The penetration of ^{32}P, as inorganic orthophosphate, into the muscle.

The behaviour of *Carcinus* muscle with respect to radioactive phosphate resembles in many respects that of frog skeletal muscle (Causey & Harris, 1951). In this case the radioactivity was shown, by autoradiography, to be located at the surface of the fibres. Generally speaking, no convincing evidence has been brought forward to show the permeability of any skeletal muscle to phosphate (Harris, 1956, p. 184).

(*b*) *Potassium*

The possibility that potassium was held in the muscle fibre because of the impermeability of the membrane to this ion was soon ruled out by a study of the potassium exchange between the blood and muscle, using ^{42}K as a tracer. In the first series of experiments crabs were kept in artificial sea water (made according to Pantin, 1946, p 64), but containing some ^{42}K introduced as an isotonic solution of the chloride. The crabs remained in this solution for 48 hr., which allowed ample time for full equilibration of the tracer between the sea water and the blood. At the end of this time muscles were removed and weighed. The total radioactivity and the potassium content of the muscle were measured and these were compared with the activity and potassium concentration of the blood on an equal volume basis. The

percentage of the muscle potassium which had exchanged was calculated from $P = 100A_iK_o/A_oK_i$, where A_i and A_o are the activities per kg. water of the muscle and the blood respectively, and K_o and K_i are the concentrations of potassium in the blood and muscle. The results of these measurements are shown in Table 4. It is clear that the whole of the muscle potassium is exchangeable with the potassium of the blood, and this contrasts in a striking manner with the behaviour of the phosphate compounds. The exchangeability of the potassium is in general accord with the results which have been obtained for vertebrate muscle. Although in the latter case low percentage exchanges have been recorded the exchange is generally complete when experimental conditions are adequate (see, for example, Creese, 1954).

Table 4. *The exchangeability of the muscle potassium*

Crab series no.	Muscle	Muscle potassium concn. (mM./kg. water)	Percentage of of potassium exchanged
1	L. extensor	134	101
	L. flexor	145	105
2	L. extensor	123	127
	L. flexor	124	123
3	R. extensor	126	97
	R. flexor	117	102
4	L. extensor	128	98
	L. flexor	122	100
5	L. extensor	126	90
	L. flexor	139	90
6	L. extensor	133	99
	L. flexor	121	99
			103*

* Mean exchange

The rate at which potassium ions penetrate into the muscle was also studied. In these experiments a number of crabs were each injected with about 1 μc. of ^{42}KCl (this raised the blood potassium concentration by about 2mM/l.) and they were then kept out of water but in a damp environment for periods of up to 6 hr. At known times after the injection blood and muscle samples were taken from individual crabs and the percentage of the muscle potassium which had exchanged was calculated as before. These results are shown in Fig. 4. If the blood and muscle potassium concentrations are constant and there is a constant proportion of radioactive to normal ions in the blood, then the rate of penetration should follow a simple relation, $P = 100(1 - e^{-kt})$, where P is the percentage of the potassium exchanged, t, the time and k, the exchange constant. The measurements of the blood activity showed that after the initial mixing of the injected material (which took about 3 min.) the level remained fairly constant during the experimental period, so that the relation could be used to give, at least, an approximate indication of the rate of exchange. The experimental data (Fig. 4) is represented approximately by such a curve with the exchange constant $= 0{\cdot}4$ hr.$^{-1}$.

It is interesting to find that a similar value has been found by Keynes & Lewis (1951) for the rate of exchange of potassium in the isolated nerve of *Carcinus*—they

find values of 0·49 hr.$^{-1}$ for the whole nerve and 0·37 hr.$^{-1}$ for the isolated 30 μ axon. The diameter of even the largest nerve axons is, however, much less than that of the muscle fibres, so that although the internal potassium concentration of the nerve is higher, the potassium flux through the axon membrane must be appreciably smaller than through the muscle fibre membrane. Keynes & Lewis calculated that the axon membrane flux was in the order of 22×10^{-12} M./cm.^{2}/sec.—a similar calculation for the muscle fibre, using an exchange constant of 0·4 hr.$^{-1}$ and a fibre diameter of 300 μ allows a rough estimate of the flux as 130×10^{-12} M./cm.^{2}/sec. It may be of some significance that this flux is of a different order of magnitude from that which has been found in amphibian muscle fibres. Thus, for example, in the frog toe muscles and sartorius muscle the membrane fluxes are only 4·5 and 12×10^{-12}M.cm.^{2}/sec. respectively (Keynes, 1954). The large difference between the

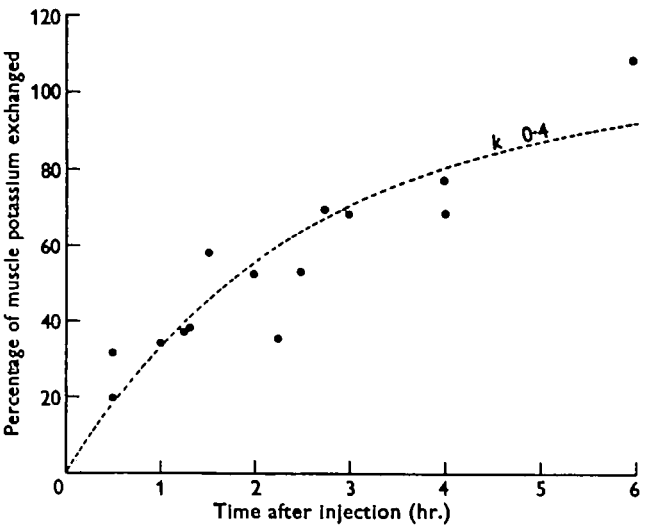

Fig. 4. The penetration of ^{42}K into the muscle.

permeability of the muscle fibre membrane to potassium in *Carcinus* and in the frog is confirmed by measurements of the transverse resistance of the membrane by an electrical method (Fatt & Katz, 1953). Their results show that *Carcinus* muscle has a low membrane resistance—a mean of 165 ohm.cm.^{2}—compared with the higher values which have been found for frog muscle (Fatt & Katz, 1951; Castillo & Machne, 1953) which range from 1500 to 4300 ohm.cm.^{2}.

The demonstration of the relatively high permeability of *Carcinus* muscle fibre membrane to potassium ions means that an explanation of the retention of this ion in the muscle must be sought among mechanisms which promote a dynamic steady state between potassium ions in the blood and the fibre. The simplest condition is where the electrochemical potential of the potassium ions is the same on either side of the fibre membrane and it was suggested previously that this is the case (Shaw, 1955*a*). This point has been re-investigated in view of the fact that (*a*) the muscle

potassium concentration was higher in the present series of crabs, and (*b*) the previously recorded membrane potentials were rather lower than had been recorded in the same muscles by Fatt & Katz (1953). In the present experiments membrane potentials were measured by the technique of Fatt & Katz, using a crab-Ringer solution for bathing the isolated muscle preparation. This technique gave better visibility than previously and had the advantage that any solutes leaking out from damaged fibres could be washed away by the large volume of solution. Damaged fibres were more easily seen and potential difference measurements were made only on those fibres which appeared completely undamaged. In this way many of the previously recorded low potentials were eliminated and this resulted in an increase in the mean recorded membrane potential. Values of this potential recorded from several muscles are shown in Table 5 and the mean value of 70mV. shows a close agreement with that found by Fatt & Katz.

Table 5. *The effect of reduced blood potassium concentration on the muscle potassium concentration and on the membrane potential*

(Concentrations in mM./kg. water).

Blood K concn. (K_o)	Muscle K concn. (K_i)	Muscle Na concn.	Ratio K_i/K_o	Membrane potential (mV.)	'Equilibrium' potential (mV.)
(*a*) Muscles of crabs from normal sea water					
12·0	164	—	13·6	73	—
12·8	146	—	11·4	72	—
12·6	142	—	11·2	70	—
12·3	146	—	11·8	71	—
11·7	144	—	12·3	67	—
(*b*) Muscles of crabs from potassium-reduced sea water					
10·0	143	—	14·3	78	74
10·3	150	—	14·5	67	74
9·5	150	—	15·8	73	77
9·5	137	—	14·4	76	74
9·2	137	—	15·0	70	75
8·8	138	—	15·6	77	76
9·0	138	—	15·3	75	76
7·6	127	—	16·7	78	78
7·6	135	—	17·8	82	79
7·8	126	—	16·2	—	—
6·7	137	—	20·5	85	83
4·9	100	61	20·4	—	—
6·4	128	72	20·0	76	82
4·0	110	74	27·5	75	89
5·5	115	65	21·0	78	83
5·2	130	78	25·0	79	88
5·8	111	65	19·2	82	82

The nature of the distribution of the potassium is best tested by comparing the membrane potential with the 'equilibrium potential' derived from the expression, $E = 58 \log C_i f_i / C_o f_o$ where C_o and C_i are the blood and muscle potassium concentrations respectively, and f_o and f_i, their activity coefficients. The calculation can only be made if the ratio, f_i/f_o is assumed to be unity. The mean value for the equilibrium potential, calculted in this way, comes out to be 63 mV., compared with the membrane potential of 70 mV. As it is unlikely that the discrepancy between

the two can be due to experimental error, if the values are accepted as they stand it would be necessary to postulate the presence of some active process transporting potassium ions out of the fibre.

There are, however, at least two reasons why the derivation of this equilibrium potential may be misleading. In the first place, the assumption that the ratio of the activity coefficients is unity is probably not valid. The ionic strength, upon which the coefficients depend, will not be the same in the blood and the muscle. In the latter, only about one-third of the total osmotic pressure is made up of ions (Shaw 1955*a*). In the second place, it is quite possible that the muscle ions are not uniformly distributed throughout the muscle (this is discussed below). If potassium is restricted to the inner parts of the fibre, then the measurement of its internal concentration, within that region, would be too low. Both these factors would have the effect of increasing the calculated equilibrium potential and they would be of the right order to raise it up to the measured membrane potential. It is difficult to decide to what extent these corrections should be applied, but in view of them it is clear that further evidence would be required to establish the existence of a mechanism concerned with the active transport of potassium. In any event, the active transport of potassium ions would represent only a small fraction of the total potassium flux, and until more critical measurements can be made it is simplest to accept as a first approximation that the potassium ions are distributed according to the electrochemical gradient.

However, it must be clearly stated that the acceptance of the passive distribution of the potassium ions as a working hypothesis does not imply that these ions cross the fibre membrane by simple diffusion process, nor does it characterize the membrane potential as a simple diffusion potential.

The question of the retention of potassium in the muscle under conditions of blood dilution can now be considered. It was shown previously (Shaw, 1955*b*) that, in crabs from dilute sea water, the muscle potassium concentration was reduced in proportion to the intake of water into the fibres. There was generally no loss of potassium from the muscle except, perhaps, at the more extreme dilutions. The behaviour of the muscle potassium in general resembles closely that of the acid-soluble phosphate compounds and, in fact, as is shown in Fig. 5, there is always a very close correlation between the muscle potassium concentration and the total concentration of the acid-soluble phosphates, both in the normal muscle and in the muscles from diluted blood.

Under conditions of extreme blood dilution the ratio of muscle potassium to blood potassium is greater than normal (Shaw, 1955*b*). It was suggested that if the membrane potential was constant, some active component was concerned with the retention of potassium. Again, however, the detection of the presence of an active mechanism turns critically on the measurements of the membrane potential and on the calculation of the equilibrium potential. More favourable conditions for the analysis of this problem can be found by reducing the blood potassium concentration, but keeping the total blood concentration constant. In this way dilution of the muscle potassium by the intake of water is prevented and large initial muscle-

to-blood potassium ratios can be achieved. These conditions were realized by keeping crabs in artificial sea water with a reduced potassium concentration, for periods of a week or longer. At the end of this period, in each crab the membrane potentials were measured in the muscles from one chela and the muscles of the other chela were analysed for potassium content and, in some cases, sodium content. The equilibrium potential for the potassium ion (E') was calculated on the assumption that this ion is passively distributed in the normal muscle, and hence, from the expression $E' = 58 \log C_i/C_o + E_c$, where E_c is the small potential (7 mV.) which must be added to the first term to make E' equal to the measured membrane potential in the normal muscle. The results of these analyses are shown in Table 5.

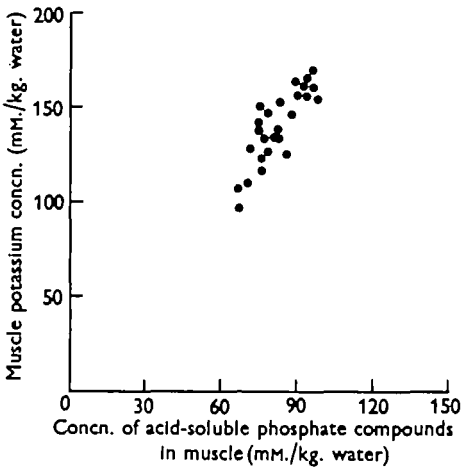

Fig. 5. The relation between the concentration of potassium and of the acid-soluble phosphate compounds in the muscle.

From the results of the effects of reduced blood potassium concentrations several important conclusions can be drawn. The muscle potassium concentration is not wholly maintained; it shows a significant fall which is most marked (a drop of 25%) when the blood potassium concentration is lowest. Despite this, however, the ratio K_i/K_o, is increased from the normal value of about 12 to a value of 20 or more. The loss of potassium is accompanied by the entry of sodium. In most cases the increase in sodium concentration (from the normal of 54 mM./kg. fibre water) is not as great as the fall in potassium concentration and some anions are presumably also lost. Finally, the membrane potential of the muscles is significantly increased. It is obvious that if the distribution of potassium remains passive, then the new membrane potential must be equal to the equilibrium potential (E'). In many cases this is so; in some, particularly at the lower blood potassium concentrations, the equilibrium potential is somewhat higher than the measured membrane potential. The possibility therefore that there is some small active component in the potassium influx cannot therefore be ruled out. However, it is certain that if any active mechanism for the uptake of potassium is present, it can account for only a small fraction of the total

potassium influx and is quite inadequate to maintain the normal muscle potassium concentration under these conditions. This applies even more forcibly in the case of the blood dilution, where the K_4/K_0 ratios do not generally exceed about 17.

It is abundantly clear that the really important factor which is responsible for the retention of potassium in the muscle is the rigid exclusion of sodium ions. The efficiency of this mechanism, whatever form it may take, is maintained during blood dilution and only begins to show signs of break-down at very low blood potassium concentrations.

(*c*) *Sodium*

The exchangeability of the muscle sodium with sodium of the blood was measured by injecting a number of crabs, each with a small amount of crab-Ringer solution containing about 20 μc. of ^{24}Na. The animals were subsequently kept out of water but in a damp environment, and at known times after injection muscles were removed, separated into individual fibres and washed to avoid contamination with blood. The percentage of sodium exchanged was calculated from measurements of sodium concentration and radioactivity per unit volume of water, as described for the similar experiments with potassium. The results are shown in Table 6. The exchange between the blood and muscle sodium is very rapid and complete. The difference between the mean value for the exchange ($97 \pm 2{\cdot}7\%$) and a complete exchange is not significant.

Table 6. *The exchangeability of the muscle sodium*

Crab series no.	Muscle	Time after injection (hr.)	Muscle sodium concentration (mM./kg. water)	Percentage sodium exchanged
1	L. extensor	0·5	48	104
	R. extensor	0·5	59	87
2	L. extensor	1·0	50	102
	R. extensor	1·0	58	78
3	L.extensor	1·5	57	98
	R. extensor	1·5	62	97
4	L. extensor	2·0	64	105
	R. extensor	2·0	57	98
5	L. extensor	3·2	48	100
	R. extensor	3·2	56	102
				97*

* Mean exchange

Even an approximate measurement of the rate of penetration of ^{24}Na into the muscle was impossible by this method. Only 3 min. after the injection as much as 70% of the sodium had exchanged and the exchange was complete in 10 min. Since it took several minutes for the injected-sodium to become evenly distributed throughout the circulating blood, the bulk of the exchange occurred at a time when the blood activity was far from constant. For this reason it is not possible to measure the exchange constant but, on the other hand, it is quite clear that the penetration rate is extremely rapid.

A widely accepted view holds that low muscle sodium concentrations are maintained by the active extrusion of sodium ions from the muscle fibre. For a steady state, the rate of sodium extrusion (sodium efflux) must equal the rate of sodium penetration (sodium influx). If, in *Carcinus* muscle, the rate of penetration of ^{24}Na is a measure of the normal sodium influx then a simple calculation shows that this theory is untenable—the energy requirement for the extrusion of sodium at the same rate and against the electrochemical gradient would be far too great. Other explanations must be looked for to account for the rapid exchange.

The explanation which immediately springs to mind is that an exchange diffusion, such as has been suggested by Ussing (1947), occurs between labelled and non-labelled ions. This type of interaction may be regarded as an artifact of the tracer technique and will lead to estimates of ion fluxes which are too large. Exchange diffusion will not affect net movements of the ions in question.

To test for the presence of exchange diffusion, net movements of the muscle sodium were induced by perfusing chelae with a Ringer solution containing 50% or more of isotonic dextrose or choline chloride. As shown in Table 7, a rapid loss of sodium resulted and after 10 min. perfusion some 40–50% of the muscle sodium was lost. On these grounds it seems unlikely that exchange diffusion can account for more than a small part of the rapid sodium exchange.

Table 7. *The loss of sodium from muscles perfused with sodium-reduced Ringer*

Perfusing fluid	Na concn. of perfusing fluid (mM./l.)	Perfusion time (min.)	Na concn. of muscle after perfusion (mM./kg. water)	Na concn. of normal muscle (mM./kg. water
Dextrose-Ringer	246	10	31	53
	246	15	32	53
	246	30	40	57
	27	15	21	57
	27	10	23	53
	27	10	18	83
Choline-Ringer	230	4	29	60
	230	7	35	63

The loss of sodium in the perfused muscle is quite compatible with the behaviour of the muscle sodium under conditions of blood dilution (Shaw, 1955*b*), where the fall in muscle sodium concentration is in proportion to the decrease in blood sodium concentration.

How, then, can these facts be reconciled with the maintenance of a low muscle sodium concentration? No certain answer can be given—it is possible, however, to erect a hypothesis which is in accord with the facts, such as they are. One may suggest that the muscle sodium is not uniformly distributed throughout the fibre, in association with all the other muscle ions, but is situated in a special region or compartment which is outside the boundary across which the membrane potential is developed. This special region would be in close contact with the blood and the sodium within would be at approximately the same concentration as in the blood.

The relatively small volume of the region would account for the fact that measurement of sodium concentration on the basis of the fibre as a whole leads to a value much less than that of the blood.

The idea that a muscle fibre may consist of more than one region is, of course, by no means new. Several authors have reached the same conclusion with regard to the behaviour of sodium ions in amphibian muscle. Conway (1954) first suggested that the muscle sodium was divisible into several fractions and, later (Conway & Carey, 1955) that a rapidly exchanging fraction was located in the sarcolemma. Since then, Simon, Shaw, Bennet & Muller (1957), Edwards & Harris (1957) and Harris (1957) have all brought forward additional evidence which suggests that the muscle is divisible into at least two compartments, one of which contains the bulk of the muscle sodium. Harris locates the rapidly exchanging sodium in an outer annulus surrounding the interior of the fibre.

The two concentric compartments suggested in Harris's hypothesis would seem applicable to *Carcinus* muscle, and, here, the sodium would be confined to the outer compartment and rigidly excluded from the inner. If the sodium concentration in the outer compartment was the same as in the blood, in a muscle fibre of 300 μ in diameter, the outer annulus would be about 7 μ in thickness. This is considerably thicker than the sarcolemma and there is no obvious correlation with any morphological location. The mechanism of exclusion of sodium from the inner compartment still remains unknown, but this, presumably is an energy-requiring process which works less efficiently at low blood potassium concentrations.

DISCUSSION

In attempting to describe the processes of ionic regulation in the normal muscle fibre of *Carcinus*, one must be careful to avoid accepting too simple a picture. It is noteworthy that in amphibian striated muscle, despite over 10 years of intensive research by many workers, the position still requires clarification.

In *Carcinus*, from normal sea water, each muscle fibre contains large amounts of relatively indiffusible organic phosphate compounds. These are negatively charged and the charge is approximately balanced by the high concentrations of potassium ions. The retention of potassium ions appears to be largely electrostatic—if an active process is concerned in potassium transport, it can account for only a very small part of the total potassium flux. The retention is a consequence of the permeability of the membrane to potassium and the efficient exclusion of sodium from the fibre. Undoubtedly it is the mechanism by which sodium exclusion is achieved which is the most important single process of ionic regulation in the muscle fibre. This is true wherever the muscle sodium is located—if, however, the freely-exchangeable sodium is indeed present in the outer layers of the fibre, then the process of sodium exclusion must lead to a practically complete absence of sodium from the fibre interior.

The distribution of the muscle sodium requires further investigation, as does the behaviour of the muscle chloride ions.

Despite the fact that a complete and certain picture of the processes of ionic regulation in the normal muscle fibre cannot yet be given, it is possible to attempt to explain the effect of dilution of the blood upon the composition of the muscle.

Dilution of the blood results in the intake of water into the muscle, and this brings about a fall in the concentration of both the muscle phosphates and the potassium—there is no attempt to maintain the normal concentrations of these substances. The behaviour of the phosphate compounds appears to be purely passive and the same is probably true of the potassium ions. There may be some minor active component in the potassium influx but this is quite insufficient to maintain the normal potassium concentration in the fibre.

The fact that during moderate blood dilution the blood potassium concentration falls roughly in proportion to the fall in muscle potassium concentration insures that the ratio of muscle to blood potassium, and also the membrane potential, remain substantially unaltered. This parallel fall in muscle and blood potassium concentrations may be purely fortuitous—but it is clear that the regulation of blood potassium concentration, as well as the concentrations of sodium and chloride, in animals from dilute sea water is of great importance for the maintenance of cell potassium levels.

Sodium ions remain excluded from the muscle and there is no evidence of interference with this mechanism under moderate dilutions of the blood. Changes in the muscle sodium concentration can best be explained as due to the rapid loss of sodium from the outer layers of the fibres.

Under conditions of extreme blood dilution, such as is found in animals living in less than 30% sea water where survival is poor, additional changes in the muscle composition are observed. The very low blood potassium concentration leads to higher muscle-to-blood potassium ratios and a consequent additional loss of potassium from the fibres. The low potassium also appears to interfere with the normal sodium exclusion mechanism and this, in turn, may lead to the penetration of some sodium into the fibre interior.

In relation to the general problem of the adaptation of the muscle to reduced blood concentrations, these studies show that, as far as the muscle ions are concerned, the cell behaves rather passively. The presence of special regulatory processes concerned with the maintenance of the normal ionic composition of the cell has not been revealed.

SUMMARY

1. The muscle fibres of *Carcinus maenas* contain 91 mM./kg. fibre water of acid soluble phosphate compounds, of which arginine phosphate is the most abundant. These compounds behave passively under conditions of blood dilution and the phosphate radicals do not exchange freely with inorganic phosphate in the blood.

2. Muscle potassium exchanges freely and completely with the blood potassium. The exchange constant is about 0·4 hr.$^{-1}$. In the normal fibre potassium ions are approximately in equilibrium with those in the blood. Potassium is lost from the muscle when the blood potassium concentration is reduced, but both the muscle-to-blood potassium ratio and the membrane potential are increased. The behaviour

of potassium appears to be largely passive but there may be some small active component in its retention.

3. Muscle sodium exchanges very rapidly and completely with blood sodium. The exchange is complete in 10 min. Sodium is also lost rapidly from the muscle to Ringer solutions with a reduced sodium concentration. It is suggested that the bulk of the muscle sodium is situated in a region distinct from the true fibre interior and that sodium ions are rigidly excluded from the latter.

4. These results are discussed in relation to the adaptation of the muscle fibre to reduced blood concentrations.

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