STUDIES ON ADAPTATION TO SALINITY IN *GAMMARUS* SPP.

1. REGULATION OF BLOOD AND TISSUES AND THE PROBLEM OF ADAPTATION TO FRESH WATER

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(With Two Text-figures)

INTRODUCTION

Invasion of brackish and fresh waters by marine animals has not always been accomplished by means of the same type of osmoregulatory mechanism. This is clearly shown by the great differences in the ability of fresh-water animals to produce a urine hypotonic to the blood (Table II) and to concentrate ions from the medium (Krogh, 1939, p. 203). As a result the blood concentration is maintained at very different levels in different species (Table II). It should therefore be of interest to investigate several species of the same genus which have become adapted to habitats of different salinity range and which, we can be reasonably certain, form an evolutionary series. For this reason we have chosen four species of the genus *Gammarus*. In the present work a survey is made of the relation between the external medium and blood, when the salinity of the former is rapidly changed. In addition, estimations of tissue chloride show that the chloride in the tissues can be regulated against changes in that of the blood and that this mechanism is as important as that for regulation of blood concentration in facilitating adaptation to changes of salinity.

In a later paper results of experiments will be recorded in which certain species are acclimatized by very gradual change to salinities not found in their normal habitats and outside the range tolerated when transfer is rapid. Another interesting problem is raised by the brackish water species *G. duebeni* which is apparently in the process of becoming adapted to fresh water and is actually the common inland water *Gammarus* of Ireland (Reid, 1939). Comparative experimental work on samples of *G. duebeni* from different habitats will be published in the second paper of this series. The *G. duebeni* used in the present experiments were taken from brackish water only. The value of *Gammarus* as material for this kind of work is obviously limited by their small size, but ultramicro-methods are now available by which some useful information can be obtained.

MATERIALS AND METHODS

G. locusta was supplied from Millport by Mr R. Elmhirst and was obtained by dredging. *G. obtusatus*[1] was collected under stones close to low-tide level at Cullercoats. Both these species were kept for at least 48 hr. in aerated sea water (540 mM.) before the experiments.

G. duebeni was collected from Meggies Burn, Blyth, Northumberland. The salinity of this stream is influenced by the daily tidal inflow but never becomes very low owing to saline water continuously pumped into it at its head from a coal mine nearby. Measurements made at irregular intervals during the past four years indicate that it fluctuates between about 10 and 50% sea water. They were kept at least 48 hr. in 50% sea water (270 mM.).

G. pulex collected from the Cor Burn, Northumberland, and kept in tap water.

The above stock cultures were fed with elm leaves, but feeding was stopped during the experiments. Dilutions of sea water were made with tap water and aerated by thorough shaking. No further aeration was provided during the 24 hr. taken for each experiment. To obtain the points in Fig. 1, about 25 animals were transferred direct from the culture to about 150 c.c. of dilute sea water of the required salinity, and the measurements were made after 24 hr. Except in the case of *G. pulex* (see below) only salinities were chosen in which the animals showed no signs of distress after this time.

Extraction of blood, after drying between filter papers, was effected by holding the animal between the thumb and forefinger of the left hand and pricking the dorsal vessel with a needle held in the right hand. A drop of blood immediately appears which can be sucked up into a waxed micropipette for transfer to the thermocouple loop for vapour-pressure determination, or directly into the graduated micropipette for chloride estimation.

Blood osmotic pressure was measured, to the nearest 2 mM./l., by Baldes's modification of the Hill vapour-pressure method (Baldes, 1934).

Blood chloride was estimated to the nearest 3 mM./l. by Wigglesworth's micro-method (Wigglesworth, 1938*a*). By the method adopted by Wigglesworth we found it impossible to obtain a stable coating of wax on the micropipettes and burettes. For the benefit of others who may find the same difficulty we suggest the following procedure by which we were invariably successful. With the object of removing possible moisture and adsorbed air from the inner surface, a column of absolute alcohol followed by one of xylol was drawn up the capillary. Immediately after discharging the latter, a column of molten wax was sucked up and discharged before the final coating operation. This was done as described by Wigglesworth except that the molten wax was as *cold* as possible.

Tissue chloride. The method used was based on Patterson's cold nitric acid digestion method for blood (Patterson, 1928). The main advantage of this method is its extreme rapidity. Five to ten *Gammarus* were dried on filter paper, cut in two,

[1] A species allied to *G. marinus*, kindly identified by Mr G. M. Spooner of Plymouth.

and the blood drained off by pressing between two filter papers. After weighing (100–200 mg.) they were transferred to a hard glass test-tube ($\frac{3}{4} \times 6$ in.) containing a drop of distilled water, and were ground to a paste by means of a bulb-ended

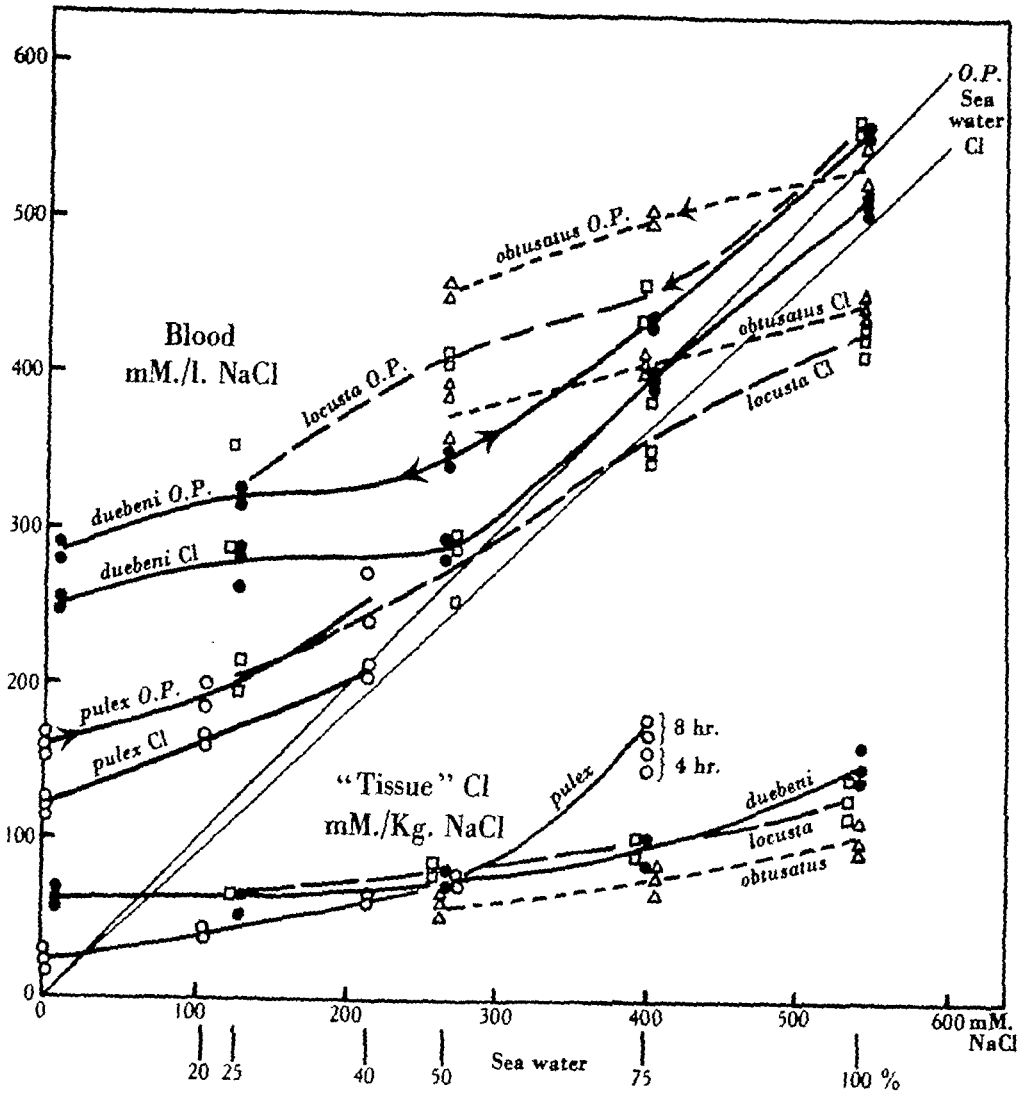

Fig. 1. Blood osmotic pressure (*O.P.*), blood chloride (Cl) expressed as mM. NaCl/l., and tissue chloride expressed as mM. NaCl/kg. wet weight, 24 hr. after direct transfer to various concentrations of sea water. The direction of transfer is indicated by the arrows on each osmotic pressure curve. With the exception of the highest concentration to which *G. pulex* was submitted (400 mM., see text), the range of external concentrations chosen for each species was that which could be withstood without apparent harm for at least 24 hr.

glass rod. 3 c.c. of conc.HNO_3 were added and stirred thoroughly with the rod. After a few minutes a clear yellow solution containing skeletal debris was obtained. The rod was washed into the tube with the least possible distilled water from a wash

bottle. 1 or 2 c.c. (according to the amount of Cl present) of 0·25 % $AgNO_3$ were added and the mixture shaken, followed by 3 c.c. of pure acetone. After thoroughly cooling under the tap the excess $AgNO_3$ was titrated with 0·05 *N* NaSCN from a Bang 2 c.c. microburette, using 6 drops of saturated iron alum as indicator. The NaSCN was previously standardized by substituting for the tissue 1 c.c. of *c.* 8·4 mM. NaCl, this being the only originally accurate standard required. The method was tested on a piece of fresh fish muscle (haddock), and the following seven results were obtained: 33, 31, 34, 34, 33, 33, 31 mM./kg.Cl. These figures give a standard deviation of ± 1·6 %.

DISCUSSION OF RESULTS

The main results are shown in Fig. 1. These curves require no detailed description since they are largely self-explanatory. We will therefore proceed to discuss their more significant features.

(i) *The marine species*, G. obtusatus *and* locusta

Both of these can withstand considerable dilution of the medium, and over their entire tolerance range maintain their blood concentration at a higher level than *G. duebeni*, which can actually survive much greater dilution. In fact, the degree of dilution which each of these three species can tolerate bears an inverse relation to the osmotic pressure of the blood. In view of the wider tolerance range of *G. locusta* than that of *G. obtusatus* it is interesting to note that *locusta* has often been recorded from brackish water, whereas *obtusatus* has never been found except on shores inundated by pure sea water (private communication from Mr G. M. Spooner).[1] It is well known that *G. marinus* commonly occurs in brackish water at the mouths of streams and estuaries. Though its tolerance range has not, so far as we know, been determined, Widmann (1935) was able to acclimatize it to 186 mM. sea water, in which its blood concentration was 470 mM. Its osmoregulatory mechanism is thus even more effective than that of *G. locusta* (Fig. 1). But since some marine animals such as *Arenicola marina* can survive in 25 % sea water with no mechanism for maintaining hypertonic body fluids (Krogh, 1939, p. 47,) the possession of such by the marine *Gammarus* species does not appear to be functionally significant.

(ii) *The brackish water species*, G. duebeni

This species can be transferred direct from 100 to 2 % sea water and vice versa without harm. It will be shown in a subsequent paper that adaptation to extreme dilutions and to fresh water depends upon the composition of the water. From the form of the blood curve (Fig. 1) it is evident that there is a critical external concentration (259–350 mM.) below which the osmoregulatory mechanism comes into play, and above which the hypertonicity of the blood is relatively slight.

[1] Howes (1939) found an abnormal form of *G. locusta* in a brackish lagoon in Essex, of which the salinity fluctuated between 21·5 and 27·9 % (*c.* 370–480 mM.).

(iii) *The fresh-water species*, G. pulex

The blood concentration is here much lower than in the other species. Widmann (1935) measured the freezing-point of the blood of *G. pulex* in fresh water, by accumulating blood samples from a large number of animals. The figures do not entirely agree with ours. She found seasonal differences in blood concentration ranging from 185 mM. in August to 265 mM. in February. Our figures, obtained in April, were lower than these (142–165 mM.). The reasons for this seasonal fluctuation, which we have not yet confirmed, are quite obscure, but we are not at present concerned with them since all our experiments were done at the same time of year on animals with a similar level of blood concentration.

G. pulex can withstand sudden transfer to 20% sea water (107 mM.), but in 215 mM. a few died after 24 hr., though the majority remained normal. This species showed more individual variation than the others. In 50% sea water (270 mM.) all had practically stopped moving after 24 hr. The blood concentration is raised by increasing the salinity of the medium, and a critical point seems to be reached when the concentration of the medium approaches that of the blood (Fig. 1). Widmann (1935) acclimatized *G. pulex* by gradual addition of sea water during 17 days to 155 mM. sea water. At this season (May) the blood concentration in fresh water was *c.* 220 mM. and at the end of the experiment it had risen to 283 mM. These figures would fall on a curve parallel with ours but at a higher level.

(iv) *Blood chloride*

The curves for blood chloride (Fig. 1) are mainly parallel with those for osmotic pressure. This suggests that the change in total concentration is due to salt exchange and not to movement of water under osmotic forces. The absence of any significant change in total water content was also shown by weight measurements on *G. pulex* and *duebeni* following a sudden change in external salinity. These are given in Table 1. The difference between total osmotic pressure and chloride concentration (non-Cl fraction) is relatively constant for each species, but there is an obvious difference between the bloods of the two marine species and those of *G. duebeni* and *pulex*. The approximate average concentrations of the non-Cl fractions are as follows: *G. locusta* 120, *obtusatus* 95, *duebeni* 30, and *pulex* 35 mM. It remains to be seen whether a low non-Cl fraction in *G. duebeni* and *pulex* is in any way connected with their ability to live in low salinities.

Table I

G. duebeni Previously kept 24 hr. in 540 mM. sea water. Transferred to 10 mM. 10 specimens, original wt. 376 mg.		*G. pulex* Previously kept in tap water. Transferred to 215 mM. sea water. 20 specimens, original wt. 638 mg.	
Time hr.	Wt. % original	Time hr.	Wt. % original
0·6	100	9·5	99
1·2	100	22·5	98
9·2	98	48·0	98
22·5	97	—	—

(v) *Tissue chloride*

The blood curves in Fig. 1 present a problem which cannot be entirely solved by reference to blood-regulating mechanisms. Why should the two marine species begin to succumb in dilute media before the blood concentration has fallen to a level normally maintained by *G. pulex*? And, conversely, why should *G. pulex* be unable to withstand a blood concentration as high as the lowest level maintained successfully by the brackish and marine species? Regulation of the blood is of course important only in so far as it leads to the maintenance of healthy tissues. Tissues in general, though in osmotic equilibrium with the blood, are known to differ considerably from it in the concentration of individual ions, a condition which is essential for the normal functioning of the cells. It may therefore be suspected that the intracellular mechanism for maintaining these differences depends upon a certain range of blood concentration which differs in each species. For this reason we estimated tissue chloride, which in most tissues is at a much lower concentration than in the blood. In order to detect a breakdown in the intracellular regulatory mechanism it is necessary to measure the tissue Cl of animals in salinities which they are unable to withstand, but at the same time the concentration of the blood must not be allowed to depart from the range which is normal for other species. There are practical difficulties in this, since from unhealthy animals succumbing to the effects of low salinity treatment, it was impossible to obtain a discrete drop of blood which could be picked up in a micropipette. No doubt this is due to a loss of hydrostatic pressure. Though the blood could not be extracted in the manner necessary for vapour pressure or chloride estimations, it was readily drained off as a preliminary to tissue chloride estimation (see section on methods). For this reason we could not determine the blood concentration of the matine species in water of salinity lower than the tolerance range. But with *G. pulex*, in salinities above the normal tolerance range, the blood concentration could be predicted with certainty as being at least within the range withstood by *G. duebeni* (Fig. 1).

The tissue Cl concentration found for *G. pulex* in fresh water is of an order to be expected from previous work on the muscles of fresh-water animals (Table II). The differences are obviously related to the differences in blood chloride. Scholles (1933) found 152 mM./kg. Cl in the muscle of *Eriocheir sinensis* in sea water, which is almost identical with our average figure for *G. duebeni* in sea water. Though the blood and tissue Cl concentrations are not exactly comparable since they were estimated per litre and per kg. respectively, yet, with the greatest possible allowance for the lower water content of the tissues, it is evident that a very large blood tissue Cl gradient is maintained within the tolerance range of all species. *G. pulex* was presumably evolved from a brackish water ancestor, but it is now less able than *G. duebeni* to maintain a low tissue Cl when the blood Cl is rapidly raised. On transfer to 40% sea water (215 mM.) the blood Cl is increased by about 56% and the tissue Cl by about 140%. This is not sufficient to immobilize the animals (only healthy ones were analysed), but in 50% sea water (270 mM.) the still more rapid increase of tissue Cl is associated with obviously deleterious effects on all the

Table II. *Fresh-water animals*

Blood and urine figures converted where necessary to mM./l., tissue Cl quoted as mM./kg. wet weight

	Blood		Urine	Tissue	Authority
	O.P.	Cl	*O.P.*	Cl	
Crustacea:					
Eriocheir sinensis	318	280	330	43 (muscle)	Scholles (1933)
Telphusa fluviatile	340	—	342	—	Schlieper & Herrmann (1930)
Potamobius fluviatilis	238	189	47	37 (muscle)	Scholles (1933)
Cambarus clarkii	188	117	19	—	Lienemann (1938)
Gammarus pulex	160	120	—	25	This paper
	185–263	—	—	—	Widmann (1935)
Asellus aquaticus	265–302	—	—	—	Widmann (1935)
Daphnia magna	68	—	—	—	Fritsche (1916)
Insecta:					
Sigara distincta	183	—	—	—	Claus (1937)
Aedes aegypti (larva)	138	40	—	—	Wigglesworth (1938*b*)
Aedes detritus (larva)	140	35	—	—	Beadle (1939)
Mollusca:					
Anodonta cygnea	16	—	10	—	Picken (1937)
Limnea peregra	73	—*	54	—	Picken (1937)
Fish:					
Amia calva	158	—	21	—	Smith (1932)
Cyprinus carpio	147	—	—	—	Duval (1925)
Amphibia:					
Frog	115	76	—	—	Macallum (1926)
	129	—	50†	—	Botazzi (1908)
	—	—	—	19 (muscle)	Hill & Kupalow (1930)

* Wigglesworth's observations make it practically certain that the urine is hypotonic to the blood.

† Overton (1904; quoted by Krogh, 1939, p. 155) found that at 0° C. the osmotic pressure of the urine is less than one-tenth that of the blood.

animals. So rapid is the penetration of chloride into the tissues in 75% sea water (400 mM.) that in 4 hr. the tissue Cl had already reached the level finally reached by *G. duebeni* in 540 mM. sea water. After 8 hr. a further rise had occurred, and the animals had almost ceased moving. It has been maintained that in frog muscle the chloride is confined to the extracellular fluid which is in equilibrium with the blood (Eggleton *et al.* 1937). Whether or not this is true of *Gammarus* tissues is unimportant in the present connexion. The conception of a blood/tissue Cl gradient is equally valid, and a higher percentage increase in the tissues as a whole than in the blood can only be explained as due to intracellular penetration of chloride.

On the assumption that the tissues remain isotonic with the blood, it is possible to calculate from the data in Fig. 1 the percentage of the total tissue osmotic pressure due to Cl. The results are plotted in Fig. 2 against blood Cl. No previous correction was made to the tissue Cl figures (estimated per kg.) for the water content of the tissues, which may vary, but presumably to the same extent in each

species over the same range of blood concentration. These curves illustrate clearly the importance for adaptation to changing salinity of the mechanism for regulating tissue composition against changes in the blood. The two species *G. duebeni* and *locusta*, which have the widest tolerance range, have the greatest powers of regulation, whilst the tissues of *G. pulex* can be maintained only with a relatively low blood chloride.[1] Whether the restricted range of *G. obtusatus* is due to inability to adapt its tissues to a low blood concentration, or to insufficient power to maintain hypertonic blood in dilute sea water cannot be decided from the data available.

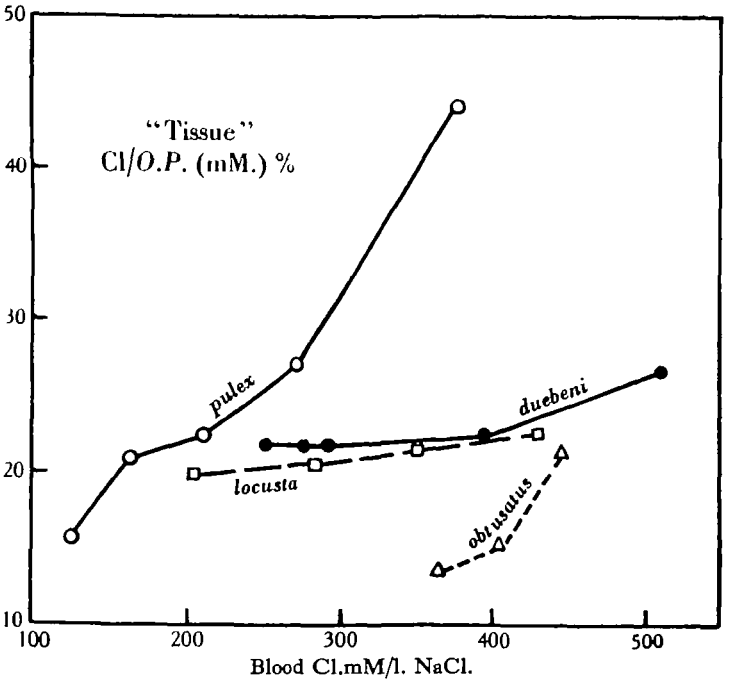

Fig. 2. The relation between blood chloride (mM. NaCl/l.) and the ratio tissue Cl/tissue osmotic pressure (%), calculated from the data in Fig. 1. It was assumed that the tissues remain isotonic with the blood, and no correction was made for the water content of the tissues, whose Cl was estimated as mM. NaCl/kg. wet weight.

Owing to the roughness of the method for obtaining tissues for Cl estimation, further speculation on the meaning of the curves in Fig. 2 would be useless. It would, however, be worth while doing similar work on large animals such as crabs, from which a sufficient quantity of a single tissue could be got for estimations of several ions.

[1] The highest percentage Cl reached by *G. pulex* may actually be greater than that calculated (44 %), since the animals had been in 400 mM. sea water for only 8 hr. It is possible that the tissues had not yet become isotonic with the blood or that the latter had not yet come into equilibrium with the external medium, both of which were assumed in making the calculation.

ADAPTATION TO FRESH WATER

It is remarkable that fresh-water animals differ so greatly in the concentration of their bloods. We have collected as much data as possible to illustrate this (Table II). These variations may be due in part to mere quantitative differences between the osmoregulatory mechanisms. But that these mechanisms differ also in kind is clearly shown by the available data for urine concentrations (Table II). The urine is hypotonic to the blood in all except the crabs, *Eriocheir sinensis* and *Telopusa fluviatile*, in which it is iso- or slightly hypertonic to the blood. It is surprising that these two species, in which the excretory organs do not therefore function for osmoregulation, happen to be those with the highest blood concentration (over 300 mM.). It is known that *Carcinus moenas* also produces an isotonic urine (Schlieper & Herrmann, 1930). In dilute sea water it maintains a strongly hypertonic blood (of the same order as that of *G. locusta*), and, though apparently incapable of adaptation to pure fresh water, interpolation of the blood concentration curve would give a concentration in fresh water of about 300 mM. (Duval, 1925). If a high blood concentration in dilute sea water is associated with iso- and a low blood concentration with hypotonic urine production, we should expect from our results (Fig. 1) that the marine *Gammarus* species are of the former and *G. pulex* of the latter class. There is, of course, no direct evidence on this point, but Schwabe (1933), from a comparison of the structure of the excretory organs of *G. locusta* and *pulex*, deduced that only *G. pulex* can produce an hypotonic urine. A similar investigation of *G. duebeni* would be of interest.

We suggest the following as a working hypothesis to explain the facts: adaptation to fresh water has proceeded by two main stages. The first was accomplished by a mechanism which is very effective in maintaining a high blood concentration. The excretory organs do not play a part in this mechanism, which probably entails active absorption of ions from the medium. By this means the tissues are not subjected to a very great change in blood concentration. The second stage was marked by a lowering of the blood concentration with a consequent lessening of both blood/external medium (O.P.) and blood/tissue Cl gradients to levels more easily maintained. This was accompanied by the development of the renal salt reabsorption mechanism, which further facilitated adaptation to fresh water. The attainment of this final condition entails the inability to withstand transfer back to more than low concentrations of sea water, for the reason that the tissues have lost the power of maintaining a high blood/tissue Cl gradient, which is essential for resistance to a raised blood concentration (e.g. *G. pulex*, Fig. 1). Duval (1925) noted that not only *Eriocheir sinensis*, which is also found in brackish water, but also *Telphusa fluviatile*, a purely fresh-water form, can be transferred from fresh water direct to sea water without harm, the blood of both becoming ultimately isotonic with the medium. Both of these maintain high blood concentrations and, as shown by *Eriocheir* (Table II), high blood/tissue Cl gradients. On the other hand, most fresh-water animals have a low blood concentration and presumably, as in the cases

of *G. pulex* and the frog (Table II), a low blood/tissue Cl gradient. This, according to our argument, would account for their restricted salinity tolerance range. *Potamobius fluviatilis* has an intermediate blood concentration level (Table II). It is therefore significant that Bogucki (1934) found it to withstand a salinity as high, but not higher than *c.* 370 mM., in which its blood concentration was approaching that of the medium.

Duval (1925), in comparing *Telphusa fluviatile*, the carp, and *Anodonta cygnea*, did in fact conclude that the degree of salinity which a fresh-water animal can withstand depends upon the level of its blood concentration. Whilst confirming this we would suggest further that high blood concentration is associated with an intracellular mechanism for maintaining a high blood/tissue Cl gradient, and that it is upon this that the tolerance range primarily depends. The inequality in the distribution of ions is certainly not confined to Cl, but tissue Cl was chosen for the ease with which it is estimated. The way is clear, by carefully controlled experiments, for further testing of this hypothesis. If the seasonal differences in blood concentration of *G. pulex* found by Widmann (1935) can be confirmed, it would be of great interest to determine whether these are associated with differences in the salinity tolerance range. There are several recorded experiments in which, by very gradual change over several months, purely marine animals have been acclimatized to fresh water and vice versa, and Sexton (1928) succeeded by these means in adapting *G. pulex* to sea water and *G. locusta* to fresh water. The physiological mechanism by which this is accomplished is obviously of great interest, and we are starting investigations on this point.

There are of course several animals of fresh-water origin which can live in extremely saline water. But, as investigations on two of such animals show (*Artemia salina*, Medwedewa, 1927; *Aedes detritus* larva, Beadle, 1939), a special mechanism has been evolved whereby the blood concentration is maintained at a level characteristic of purely fresh-water animals. By these means they can withstand a salinity several times that of sea water, conditions which are intolerable to most marine animals. A similar mechanism also exists in a few brackish water animals, by which the blood concentration is kept at a high level (e.g. 330–350 mM. in *Palaemonetes varians*, Pannikar, 1939), though hypotonic to the medium when this is pure sea water. Whether such animals can withstand an abnormally high salinity has not apparently been determined.

SUMMARY

1. Four species of *Gammarus* were studied: the fresh-water *G. pulex*, the brackish water *G. duebeni*, and two normally marine species *G. locusta* and *obtusatus*, the former of which has also been recorded from brackish water.

2. The relation between osmotic pressure and chloride of the blood and of the external medium, after sudden transfer to salinities which could be withstood for at least 24 hr., is shown in Fig. 1.

3. The changes in blood osmotic pressure are due to salt and not to water movements.

4. The marine species *G. obtusatus* and *locusta* maintain a very hypertonic blood in dilute sea water and can withstand 50% (270 mM.) and 25% (135 mM.) sea water respectively.

5. The brackish water *G. duebeni* has a tolerance range from pure sea water to water containing a trace of salt, but is not as well adapted to fresh water as *G. pulex*.

6. For a wide salinity tolerance range two mechanisms are necessary, (*a*) for regulating the blood concentration within certain limits, and (*b*) for maintaining a low intracellular concentration of certain ions (e.g. Cl) in spite of changes in blood concentration. Defection of the latter mechanism can alone account for the inability of *G. pulex* to withstand direct transfer to more than about 40% sea water (115 mM.).

7. On the basis of this work and that of others on other animals the following hypothesis is suggested. Adaptation to fresh water has proceeded by two main stages: (*a*) Probably by active ion absorption, a high blood concentration is maintained (as in *Eriocheir sinensis* and *Telphusa fluviatile*) and is associated with a large blood/tissue Cl gradient. Such animals can still be transferred suddenly to a high concentration of sea water. (*b*) Evolution of the renal salt-reabsorption mechanism, and lowering of both blood concentration and blood/tissue Cl gradient to levels more easily maintained (as in *G. pulex* and most fresh-water animals). The consequent loss of power to maintain a large blood/tissue Cl gradient entails inability to withstand transfer to more than low concentrations of sea water, unless, as in certain species, a special mechanism is evolved for preventing the blood concentration from rising.

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