

## ZINC REGULATION IN THE FRESHWATER CRAYFISH (INCLUDING SOME COMPARATIVE COPPER ANALYSES)

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In the Plymouth area, estuarine decapod crustaceans like *Carcinus maenas* and *Palaemon serratus* have much higher concentrations of zinc in the blood than marine species such as *Homarus vulgaris* and *Maia squinado*. These differences between species with different powers of osmotic and ionic regulation have not been explained, but they were the starting-point of the present work. This is a study of the freshwater crayfish *Austropotamobius pallipes pallipes* (Lereboullet) to see whether adaptations to fresh water include modifications in zinc regulation.

In *Homarus* and *Carcinus* the zinc concentrations in the body are controlled within fairly close limits. Unfed animals can achieve zinc balance when the concentration in the animal is more than  $10^4$  times that in sea water (Bryan, 1964, 1966, and unpublished). Balance is maintained by mechanisms regulating zinc absorption through the gills and zinc loss in the urine and across the body surface. Whereas in *Homarus* urinary losses of zinc are particularly important, losses across the body surface are more important in *Carcinus*. When animals are loaded with excess zinc, by feeding or by injection, the regulating processes become even more apparent. As *Homarus* is fairly closely related to the freshwater crayfish its method of zinc regulation has been used as a point of comparison throughout this work.

### MATERIALS AND METHODS

Crayfish were obtained from L. Haig and Co. Ltd., Beam Brook, Newdigate, Dorking, Surrey. They were kept in Plymouth tapwater to which a little sea water was added and most experiments were carried out in 0.1 % sea water. The radioisotope  $^{65}\text{Zn}$  (half-life = 245 days) was obtained from the Radiochemical Centre, Amersham. Additions of zinc to media or to animals were made with solutions of zinc sulphate. Concentrations of zinc and copper were measured by the dithizone method of Bowness, Morton, Shakir & Stubbs (1952). The tissues and body fluids studied were: whole blood, urine, main abdominal flexor muscle, hepatopancreas, stomach fluid, gills, excretory organs, gonads and shell (minus epidermis) from the pericardial region of the carapace. Fine pipettes were used to collect blood and urine but blunt Kirk-type pipettes were used for manipulating the stomach fluid. Dry weights and ash weights of tissues were measured before the ash was dissolved in 0.1 N sulphuric acid prior to analysis. Samples of shell ash were dissolved in a few drops of hydrochloric acid first. The addition of 0.1 N sulphuric acid then precipitates much of the shell calcium, but the zinc and copper remain in solution.

In whole crayfish  $^{65}\text{Zn}$  was detected with a ring of  $\gamma$ -sensitive GM tubes (20th Century G 12). Tissue samples were dissolved in nitric acid and counted with a well-type  $\gamma$ -scintillation counter.

Electrophoresis of  $^{65}\text{Zn}$ -labelled blood samples was carried out by the starch-gel method of Smithies (1959) using borate buffer and a voltage of 170 V. for 15 hr. Total protein in part of the gel was stained with nigrosine. Another part of the gel was stained for peroxidase activity to locate the haemocyanins by the method of Manwell & Baker (1963). Transverse strips of the remaining piece of gel were counted in the well-type  $\gamma$ -scintillation counter.

Borosilicate glass, silica and polythene apparatus was used throughout this work. Rubber usually contains zinc and must be avoided. The experimental temperature was 15° C.

## RESULTS

### *Zinc concentrations in tissues and body fluids*

#### (a) *In low-zinc solutions*

Zinc concentrations in crayfish from 0.1 % sea water or Plymouth tapwater containing less than 4  $\mu\text{g./l.}$  of zinc are summarized in Table 1. The least variable concentrations are found in the main abdominal flexor muscle. This is also characteristic of marine species such as *Homarus* (Bryan, 1964). As it is the closest marine relative of the crayfish, zinc concentrations in *Homarus* are shown in Table 1 for comparison. The majority of crayfish tissues and body fluids contain less zinc than those of *Homarus* but the hepatopancreas and stomach fluid are important exceptions. As a result, the whole-body concentrations of the two species are similar.

Table 1. *Mean zinc concentrations ( $\mu\text{g./g.}$  fresh weight) in six 25 g. male crayfish from media containing less than 4  $\mu\text{g./l.}$  of zinc*

(Results are not corrected for contamination of the extracellular spaces by blood. Comparable results for the lobster *Homarus* from Bryan (1964 and unpublished) are also given.)

Tissue	Crayfish		Lobster
	Range	Mean	
Blood	0.7-1.3	0.9	7.4
Urine	-	0.02*	2.2
Excretory organs	4-8	7	20
Stomach fluid	32-54	41	0.7
Hepatopancreas	45-177	109	34
Main abdominal flexor muscle	10-13	11.5	15
Gills	6-10	8	15
Shell	1-18	8	4.6
Vas deferens	-	14†	13
Ovary	-	26‡	50
Whole Body (mean of 2)	24-25	24.5	22

\* Estimated from the distribution of injected  $^{65}\text{Zn}$  between blood and urine.

† From three pooled samples.

‡ From four female crayfish.

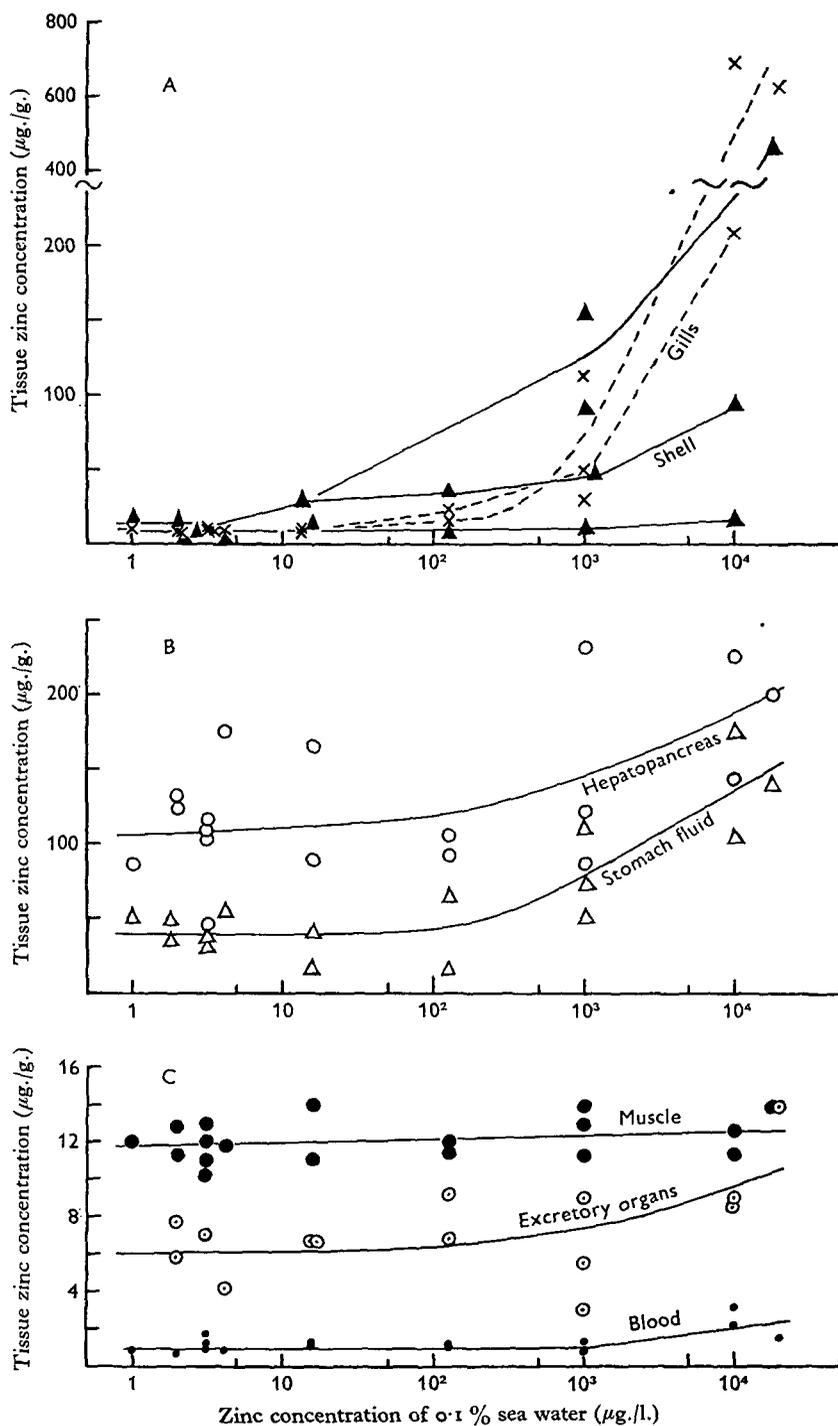


Fig. 1. Zinc concentrations in crayfish exposed to 0.1% sea water with different zinc concentrations for more than 32 days. In A, separate curves are shown for the shell and gills from different batches of animals.

(b) *In high-zinc solutions*

The toxicity of zinc to freshwater animals is usually highest in soft waters and is reduced by the calcium in hard waters (e.g. Jones, 1938). Crayfish were exposed to soft water (0.1 % sea water) containing up to  $2 \times 10^4$   $\mu\text{g./l.}$  of zinc for at least 32 days. Higher concentrations of zinc seem to be toxic. Fig. 1 summarizes the zinc concentrations in animals from different solutions. The main feature of this figure is the comparatively small effect which the highest external zinc levels have on the internal zinc concentrations. An external zinc concentration of  $10^3$   $\mu\text{g./l.}$  is equal to the concentration in the blood. It is only at external concentrations exceeding this that the concentrations in blood, hepatopancreas and stomach fluid are obviously increased. The zinc concentration of muscle is almost unaffected.

Concentrations of zinc in the shell and particularly in the gills increase markedly in the high zinc media. The toxicity of zinc to crayfish may depend on this accumulation of zinc by the gills. Crayfish were killed by  $10^5$   $\mu\text{g./l.}$  of zinc in 0.1 % sea water and the gills had an opaque white appearance which suggested that precipitation of protein had occurred. Zinc was absorbed by the shell to different degrees in different batches of animals. This is shown by the different curves in Fig. 1A. Animals from which the lower curve was obtained were exposed to 100 mg./l. of calcium for the first 10 days of the 33-day exposure period. However, in another batch of animals, where calcium was present for the full period, zinc concentrations similar to those in the middle curve were obtained. Reasons for this variability in zinc uptake by the shell have not been studied and may depend on factors such as the age of the shell and the type of water to which the animals were subjected before they were received.

*Absorption of zinc and  $^{65}\text{Zn}$  directly from water*

The results in Fig. 1 show that over a period of more than 32 days the internal zinc concentrations are only obviously increased when the medium contains more zinc than the blood. One possible reason is that zinc penetrates very slowly and so only in high-zinc media does a measurable amount enter. Alternatively, the animal may be able to excrete any additional zinc which enters and this will give the impression that very little is absorbed. Radioactive  $^{65}\text{Zn}$  was used as a tracer to settle this question.

Uptake of  $^{65}\text{Zn}$  by whole crayfish was followed in 0.1 % seawater containing between 4 and  $10^4$   $\mu\text{g./l.}$  of added zinc. More  $^{65}\text{Zn}$  was absorbed from low-zinc waters and most of this was associated with the gills and shell. Uptake of  $^{65}\text{Zn}$  by the shell seems to be a surface adsorption process and nearly all the activity can be removed from the outer surface with sandpaper. Uptake by the gills may also be largely an adsorption process. Variation in the degree of uptake of  $^{65}\text{Zn}$  by the shell and gills occurs between different batches of crayfish and is a major factor on which uptake by the whole crayfish depends. Curves for  $^{65}\text{Zn}$  uptake by whole crayfish from waters with different zinc concentrations are shown in Fig. 2A. Uptake is expressed as a concentration factor and is plotted against time. The concentration factor is the ratio  $^{65}\text{Zn/g. tissue} : ^{65}\text{Zn/g. water}$ . In the experiment shown in Fig. 2A the effect of 100 mg./l. of calcium (as chloride) on uptake was studied over the first 10 days. Calcium reduces  $^{65}\text{Zn}$  uptake, particularly in low-zinc waters, and is probably competing with the zinc for adsorption sites on the shell and gills. At the end of the experiment the  $^{65}\text{Zn}$

concentration factors and the zinc concentrations of the tissues were measured in each animal. If during the uptake process the tissues do not lose any zinc, the total amount of zinc absorbed by the body or individual tissues will equal the  $^{65}\text{Zn}$  concentration factor multiplied by the zinc concentration of the medium. These values for different external zinc concentrations are summarized in Fig. 2 B. They are very low in low-zinc waters and this is why it is only in high-zinc waters that increases in internal zinc concentrations can be detected by chemical analysis. When the values in Fig. 2 B for high-zinc waters were compared with estimates of zinc uptake coming

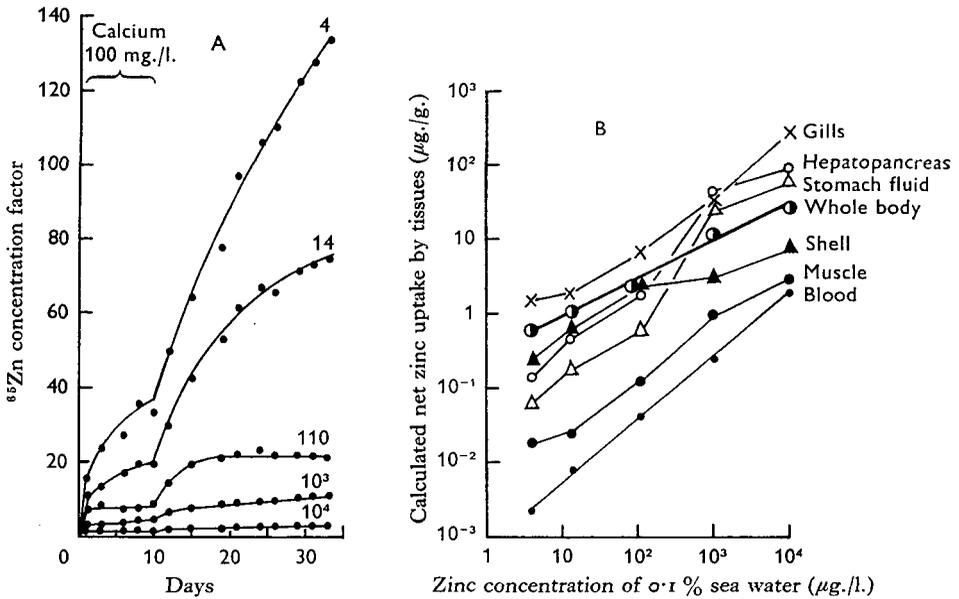


Fig. 2. A, Uptake of  $^{65}\text{Zn}$  from 0.1% sea water by whole crayfish. Concentrations of Zinc in the medium in  $\mu\text{g./l.}$  are shown with each curve. The effect on uptake of 100 mg./l. of calcium is also shown. B, Amount of Zn absorbed in 33 days by tissues of animals from A ( $^{65}\text{Zn}$  concentration factor of tissue  $\times$  external zinc concentration in  $\mu\text{g./g.}$ ) at different external zinc concentrations.

from the chemical data, the agreement was quite good except in muscle tissue. In muscle the zinc concentration seems to remain constant and so presumably the absorbed zinc is being returned to the blood. Apart from this, it appears that the additional zinc in the blood, hepatopancreas and stomach fluid of animals from high-zinc media is not being excreted from the body. The reason for the failure to remove this excess zinc will be seen in the section on zinc loss.

To summarize: zinc uptake by the crayfish from solution is probably due largely to surface adsorption on the shell and gills. This can vary between different batches of animals and is influenced by the calcium concentration of the water. Some zinc slowly penetrates into the body, although it is not known whether this occurs solely over the body surface or to some extent through drinking. Most of the extra zinc which is absorbed by the internal tissues of crayfish in high-zinc media is not excreted.

*Absorption of zinc and  $^{65}\text{Zn}$  from the stomach fluid*

In view of the low permeability of the body surface the digestive system is almost certainly the main route for zinc absorption.

(a) *Absorption of zinc*

In a group of crayfish some of the stomach fluid was replaced with a 1 mg./ml. zinc solution to give 100  $\mu\text{g.}$  per 10 g. of animal. Internal zinc concentrations at subsequent time-intervals are shown in Fig. 3. The main changes are that the concentration in the stomach fluid falls whereas that in the hepatopancreas rises. Zinc

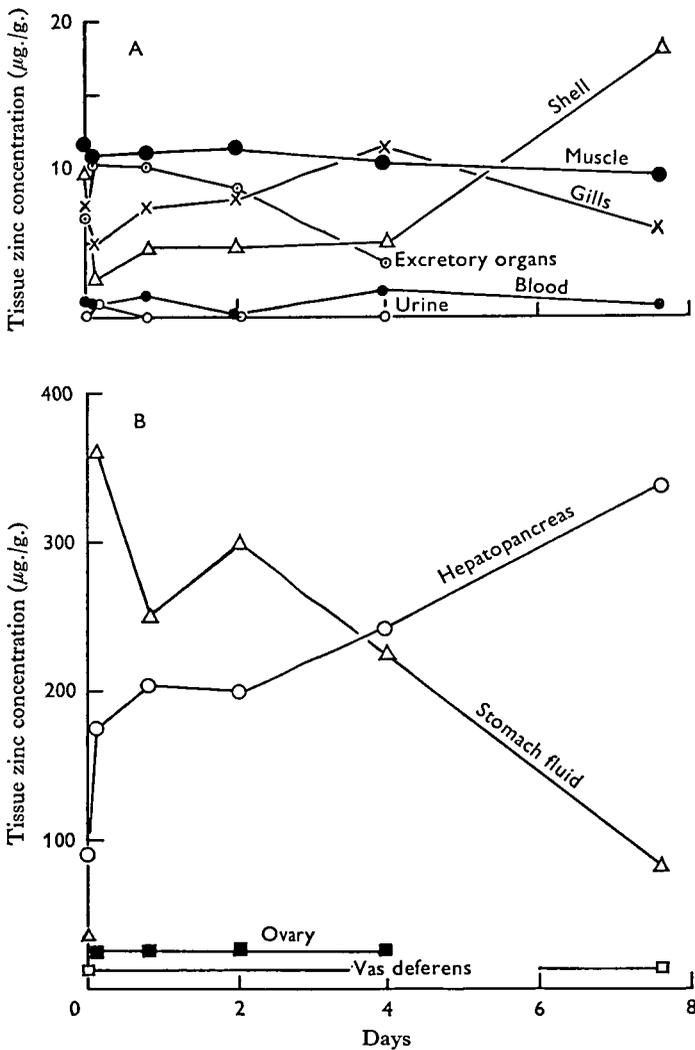


Fig. 3. Changes in zinc concentration in tissues and body fluids following the introduction of 100  $\mu\text{g.}$  of zinc/10 g. of animal into the stomach fluid. Values at zero time are from control animals.

seems to be absorbed directly by the hepatopancreas and there is no evidence that it penetrates into the blood first or influences the concentrations in other tissues.

(b) Absorption of  $^{65}\text{Zn}$

Uptake of  $^{65}\text{Zn}$  in the situation described in the last section has been compared with uptake in normal animals where the zinc concentrations of the stomach fluid and hepatopancreas are in equilibrium. This will show whether the animal is able to limit the uptake of excess zinc and whether much exchange of zinc occurs between the hepatopancreas and the other tissues. Results in Fig. 4 show that, if anything,  $^{65}\text{Zn}$

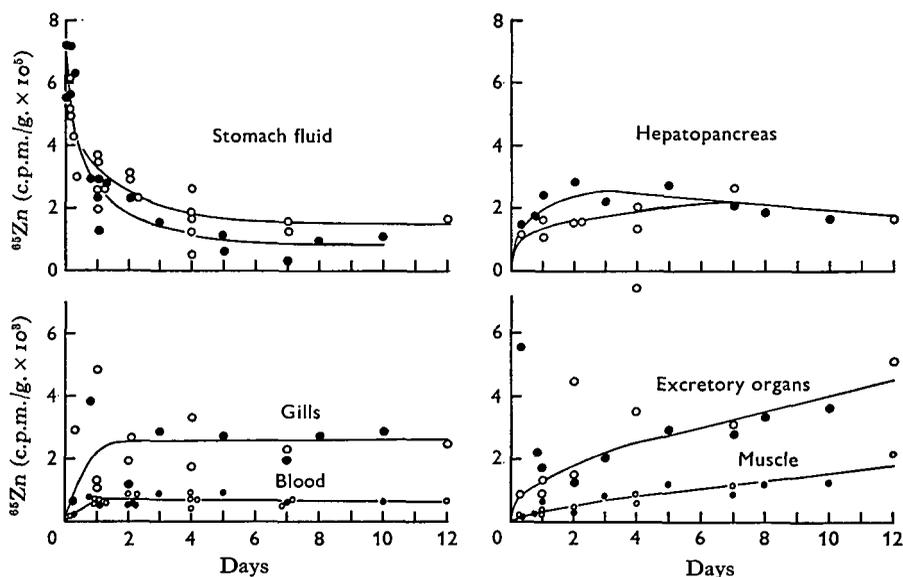


Fig. 4. Uptake of carrier-free  $^{65}\text{Zn}$  from the stomach fluid (open symbols) and uptake of  $^{65}\text{Zn}$  with  $100\ \mu\text{g.}$  of carrier per  $10\ \text{g.}$  of animal from the stomach fluid (closed symbols).  $0.25\ \mu\text{c}$  of  $^{65}\text{Zn}$  was given per  $10\ \text{g.}$  of animal and a tissue activity of  $5.1 \times 10^5\ \text{cpm/g.}$  is equivalent to  $1.0\ \mu\text{c/g.}$

is absorbed more rapidly from the stomach fluid into the hepatopancreas in the presence of excess zinc. Much smaller amounts of  $^{65}\text{Zn}$  reach the other tissues and despite some variations similar results are given by both experiments. There is certainly no suggestion that the crayfish can slow down the absorption of excess zinc from the stomach fluid.

*Loss of zinc and  $^{65}\text{Zn}$*

Ten crayfish were injected with carrier-free  $^{65}\text{Zn}$  and four were also injected with  $100\ \mu\text{g.}$  of zinc per  $10\ \text{g.}$  of body weight. Table 2 shows that during the next 7 days between 1% and 6% of the  $^{65}\text{Zn}$  was lost into flowing Plymouth tapwater from both normal and zinc-loaded animals. Subsequently  $100\ \mu\text{g.}$  of zinc were placed in the stomachs of animals nos. 1 and 3 but did not increase the slow rate of loss of  $^{65}\text{Zn}$ . These results support the evidence from page 285 that excess zinc is not readily removed—at least in unfed animals.

In unfed crayfish some faeces are produced but carry very little zinc. Large amounts of faeces were produced by feeding seven of the ten injected crayfish with earthworms

for 24 h. Faeces were collected in a polythene bag covering the abdomen of each animal. During the 2 days after feeding was started there was a sharp drop in the  $^{65}\text{Zn}$  activity of each animal and this could almost wholly be accounted for by  $^{65}\text{Zn}$  in the faeces (Fig. 5). Subsequent feeding had a much smaller effect on  $^{65}\text{Zn}$  losses. The effect of a second meal may depend on time being allowed for  $^{65}\text{Zn}$  in the hepatopneas to become available for removal. Table 2 compares the percentage loss of

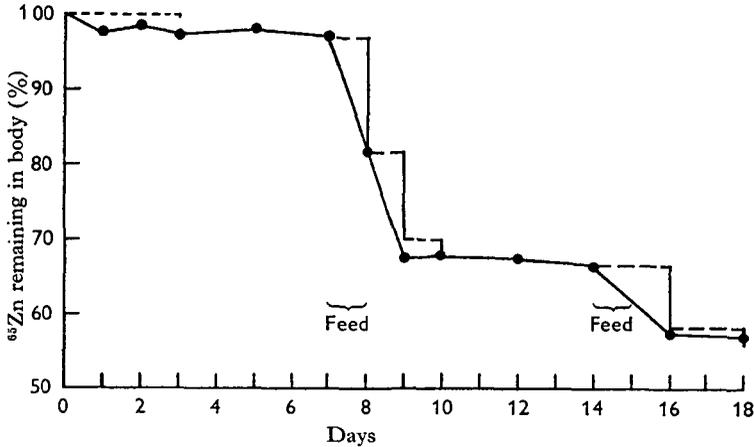


Fig. 5. The effect of two 24 hr. periods of feeding, and the subsequent production of faeces, on the loss of injected  $^{65}\text{Zn}$  into inactive Plymouth tapwater. Horizontal broken lines show the periods over which faeces were collected and the vertical lines show how much  $^{65}\text{Zn}$  was in the faeces. In this animal  $100\ \mu\text{g.}$  of Zn per  $10\ \text{g.}$  of animal had been injected with the  $^{65}\text{Zn}$ .

Table 2. Loss of injected  $^{65}\text{Zn}$  into Plymouth tapwater from normal crayfish and from animals injected with  $100\ \mu\text{g.}$  of zinc per  $10\ \text{g.}$  animal

Normal crayfish					Zinc-loaded crayfish				
No.	Sex	Wt. (g.)	Loss in first 7 days (%)	Loss in 6-7 days after feeding (%)	No.	Sex	Wt. (g.)	Loss in first 7 days (%)	Loss in 6-7 days after feeding (%)
1	♂	18	3	—	2	♂	17	6	—
3	♂	20	3	—	—	—	—	—	—
4	♂	25	2	29	5	♂	25	3	31
6	♂	20	1	22	7	♂	20	2	38
8	♂	10	3	44	—	—	—	—	—
9	♀	9	6	36	10	♀	12	5	58

$^{65}\text{Zn}$  before and after the normal and zinc-loaded crayfish were fed. More  $^{65}\text{Zn}$  was lost by the three loaded animals than by the corresponding normal animals, and this type of result has been confirmed in the next section. Binding of  $^{65}\text{Zn}$  by the faeces is very tight. When faeces were ground up with tapwater and filtered through an oxid membrane filter of  $0.5\text{--}1.0\ \mu$  pore size, all the  $^{65}\text{Zn}$  remained with the particles and large molecules on the filter. When  $^{65}\text{Zn}$ -tapwater was filtered in the same way only about 20% of the activity was retained by the filter.

Clearly, the production of faeces is of much greater importance as a means of zinc loss than any other process. This is probably why in unfed crayfish excess zinc which is absorbed from high-zinc solutions cannot be removed.

#### Feeding and uptake and loss of zinc

If zinc or  $^{65}\text{Zn}$  is placed in the stomach of a crayfish without food, most of it is absorbed by the hepatopancreas. In marine species such as the lobster *Homarus* most of the zinc is absorbed even if food is present (Bryan, unpublished). The production of faeces is not necessary for the removal of excess zinc in the lobster. In the crayfish the question is raised as to how much zinc or  $^{65}\text{Zn}$  can be absorbed from food before it is removed in the faeces.

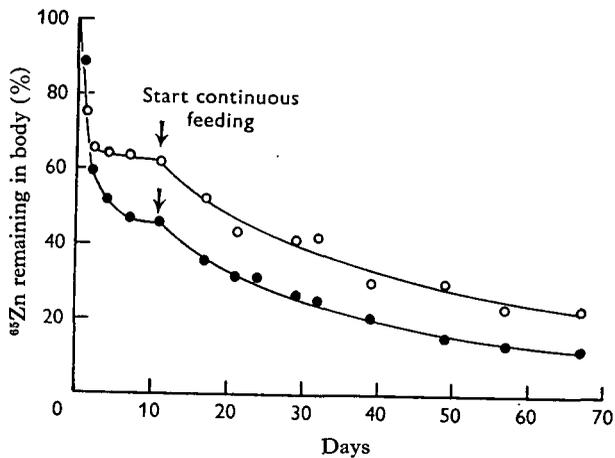


Fig. 6. Loss of  $^{65}\text{Zn}$  into inactive Plymouth tapwater from a crayfish which had been fed with earthworms plus  $^{65}\text{Zn}$  (open symbols). In the other crayfish (closed symbols)  $100\ \mu\text{g}$ . of Zn per 10 g. of animal were also included with the food. After 11 days continuous feeding with inactive earthworms was started.

Six crayfish were fed with earthworms and  $^{65}\text{Zn}$  and three were also given  $100\ \mu\text{g}$ . of zinc per 10 g. of animal. More  $^{65}\text{Zn}$  was lost into inactive Plymouth tapwater in the faeces of the zinc-loaded crayfish (Fig. 6). However, appreciable amounts of zinc and  $^{65}\text{Zn}$  were absorbed in both types of experiment. In other experiments four animals were given food with  $^{65}\text{Zn}$  and two were given 1 and 2 mg. of calcium per 10 g. of animal in addition. The animals with calcium retained 52% and 55% of the  $^{65}\text{Zn}$  whereas the others retained 42%. Possibly the calcium reduces the tendency for  $^{65}\text{Zn}$  to attach to food and faeces and so more is absorbed.

When no more faeces are produced the loss of  $^{65}\text{Zn}$  almost ceases. A continuous loss of  $^{65}\text{Zn}$  was induced by feeding the crayfish with inactive earthworms every 4–5 days (Fig. 6). Loss is roughly exponential and has a half-time of about 33 days in the normal animal and 27 days in the zinc-loaded animal.

These results show that, although there is some limitation on the absorption of excess zinc from food, appreciable amounts can still be absorbed by the hepatopancreas and will be retained until further feeding on low-zinc food provides more faeces for zinc removal.

*The hepatopancreas and zinc regulation*

Three crayfish were each injected with  $100 \mu\text{g.}$  of zinc per  $10 \text{ g.}$  of body weight and the internal zinc concentrations at subsequent intervals are shown in Fig. 7. A  $10 \text{ g.}$  crayfish normally contains about  $250 \mu\text{g.}$  of zinc and the injection of more than  $100 \mu\text{g.}$  seems to be toxic. During the loss of zinc from the blood the concentrations in the gills and muscle are temporarily increased. But eventually nearly all the zinc is absorbed by the hepatopancreas and some is transferred to the stomach fluid. Very

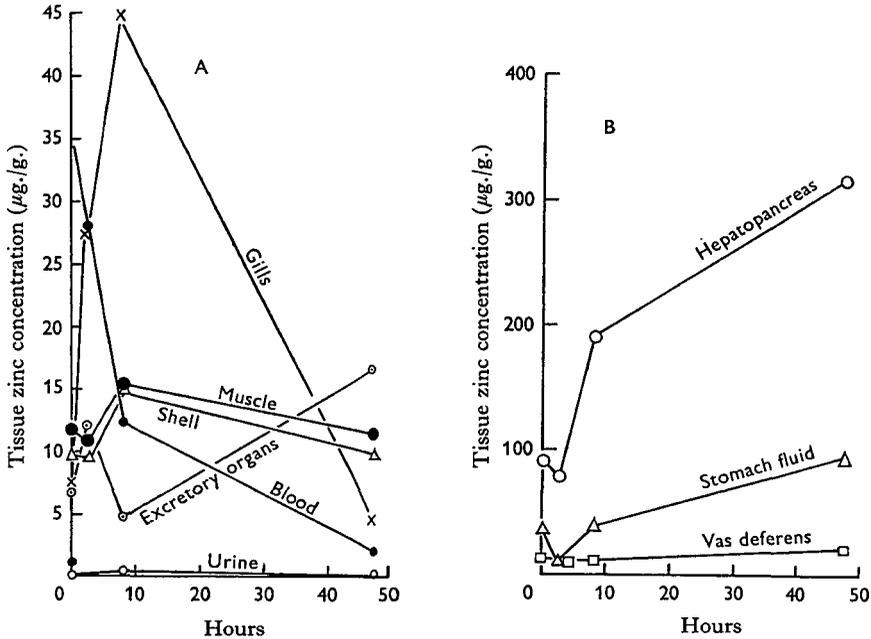


Fig. 7. Changes in zinc concentration in tissues and body fluids following the injection into the blood of  $100 \mu\text{g./10 g.}$  of animal. Values at zero time are from control animals.

little of the excess zinc is excreted in the urine and regulation of the zinc level in the blood is largely the role of the hepatopancreas. Zinc in the blood is bound to blood proteins (see p. 293). It is not known how the excess zinc is transferred from the proteins to the hepatopancreas until the blood concentration is about  $2 \mu\text{g./g.}$

Excess zinc is removed from the hepatopancreas with the aid of the faeces. For the production of faeces to constitute a regulatory system there must be a mechanism whereby comparatively little zinc is removed when the concentration in the hepatopancreas is low and much more zinc is removed when it is high. In Fig. 8 the zinc concentrations of the hepatopancreas and stomach fluid are compared in animals where the two levels were probably in equilibrium. The concentrations are not proportional to each other and the hepatopancreas/stomach-fluid ratio decreases with increasing concentrations. If the amount of zinc bound to the faeces is proportional to the zinc concentration in the stomach fluid, this system will ensure that zinc removal is regulated.

How zinc enters the stomach fluid from the hepatopancreas is uncertain. Some

may enter in association with proteins in the digestive juices but some may also enter as inorganic zinc. When the stomach fluid was removed from a crayfish every day for 15 days its zinc content fell from 47 to 7  $\mu\text{g./g.}$  and the protein in the fluid was reduced to a very low level. An injection of 100  $\mu\text{g.}$  of zinc per 10 g. of animal was then given and 4 days later the stomach-fluid protein content was still low but the zinc concentration was 37  $\mu\text{g./g.}$  There is thus no evidence that zinc enters the stomach fluid in association with the digestive juices. Nor is there evidence that zinc in the stomach fluid is bound by proteins. Most of the zinc and  $^{65}\text{Zn}$  was easily separated from the stomach fluid by dialysis against sea water. Nor was there any evidence of zinc binding to any specific proteins when  $^{65}\text{Zn}$ -labelled fluid was separated by starch-gel electrophoresis.

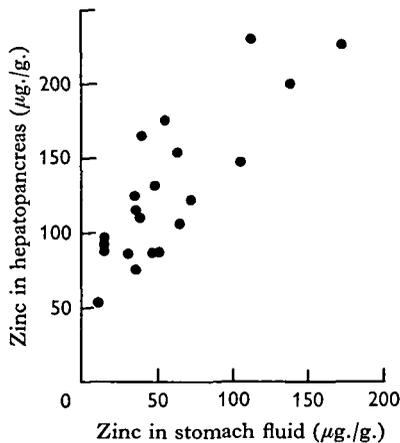


Fig. 8. A comparison of zinc concentrations in the stomach fluid and in the hepatopancreas when the system is in equilibrium.

The ability of the faeces to bind zinc so tightly presumably resides in the binding capacity of undigested organic and inorganic material from the food and perhaps in bacteria.

#### *Copper in relation to zinc*

Copper was determined in many of the samples which were used for zinc analyses. The results are summarized in Table 3. Most of the copper in the body lies in the blood and hepatopancreas. Concentrations are very variable and this may be related to the different phases of the moult cycle as it is in *Maia squinado* (Zuckerandl, 1960). Most of the copper in other tissues is probably due to the contamination of the extracellular spaces by the blood. If it is assumed, as by Robertson (1961), that copper in crustacean muscle is all due to contamination with blood, the extracellular space can be calculated. This assumption seems to be quite reasonable in the crayfish because, as Fig. 9 shows, low muscle concentrations of copper are related to low blood concentrations. The extracellular space of the main abdominal flexor muscle was 12% in the crayfish compared with 7% in *Homarus* (Bryan, 1964). Corrected zinc concentrations in muscle fibres are 13  $\mu\text{g./g.}$  in the crayfish and 15  $\mu\text{g./g.}$  in *Homarus*.

The blood in *Homarus* contains about 10 times as much copper as zinc and both concentrations increase as the solid content of the blood rises. Changes in solid

content of the blood are largely due to changes in blood protein and the most abundant proteins are haemocyanins. These not only contain copper but are also able to bind zinc (Bryan, 1964, and unpublished). The relationship between copper concentration and solid content minus ash content in the blood of the crayfish is similar to that in the blood of *Homarus* (Fig. 10). However, the concentration at which zinc is regulated does not increase very markedly with the solid content of the blood. This may be a result of good regulation by the hepatopancreas. But a second possibility is that zinc is associated with proteins other than haemocyanins, which do not vary so much in concentration between different animals.

Table 3. *Tissue copper concentrations and solid and ash contents*

Tissue	No. of samples	Solid (%)	Ash (%)	Copper		
				Range	$\mu\text{g./g.}$	$\pm$ S.D.
Blood	20	3.8	1.36	6-59	32	15
Urine	9	$\sim 0.1$	v. low	0.9.3*	1.6	2.9
Excretory organs	11	11.7	1.08	0-11	2.4	3.9
Stomach fluid	12	12.2	1.53	0-30	10	8
Hepatopancreas	12	17.6	1.48	21-690	335	180
Main abdominal flexor muscle	12	16.6	1.19	0.8-8.0	3.9	2.1
Gills	12	10.3	2.02	3-42	26	13
Shell	10	76.4	54.1	0-6.7	2.5	2.2
Vas deferens	5	24.9	1.14	0.8-4.1	1.2	1.5
Ovary	4	50.5	1.45	48-54	50	3
Whole body	2 ♂	28.6	14.6	16-18	17	—

\* Exceptional value and suggests possible contamination by blood.

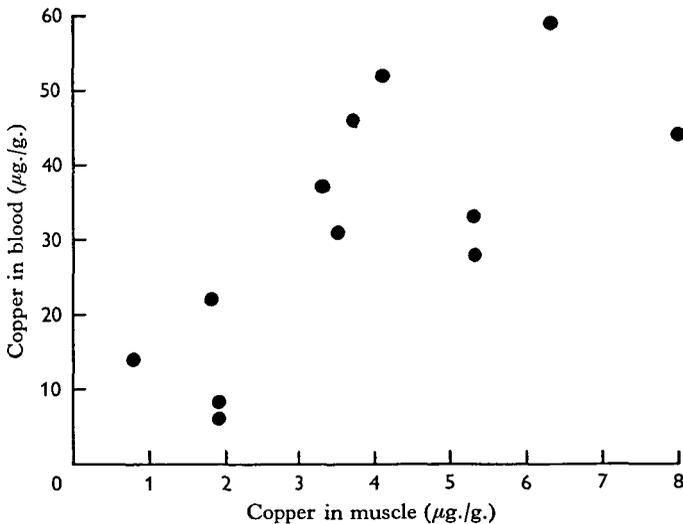


Fig. 9. A comparison of copper concentrations in muscle and blood.

Carrier-free  $^{65}\text{Zn}$  was added to samples of whole blood, and starch-gel electrophoresis was used to separate the proteins. Four of the protein bands were particularly obvious after staining with nigrosine (Fig. 11) but part of band 1 and all of band 3

did not show peroxidase activity. This shows that there is an appreciable amount of non-haemocyanin protein in the blood. The distribution of  $^{65}\text{Zn}$  in the gel shows that zinc is mainly associated with bands 1-3 where the non-haemocyanin proteins occur.

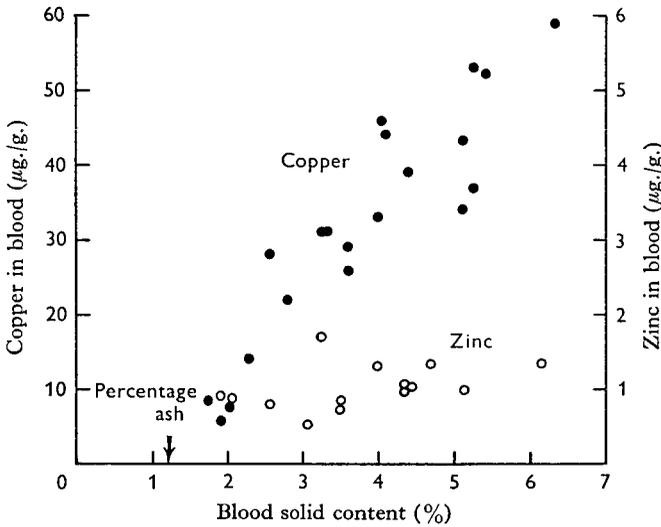


Fig. 10. Relationship between concentrations of copper and zinc in the blood and the solid content of the blood. Animals were from waters containing less than 100  $\mu\text{g./l.}$  of zinc.

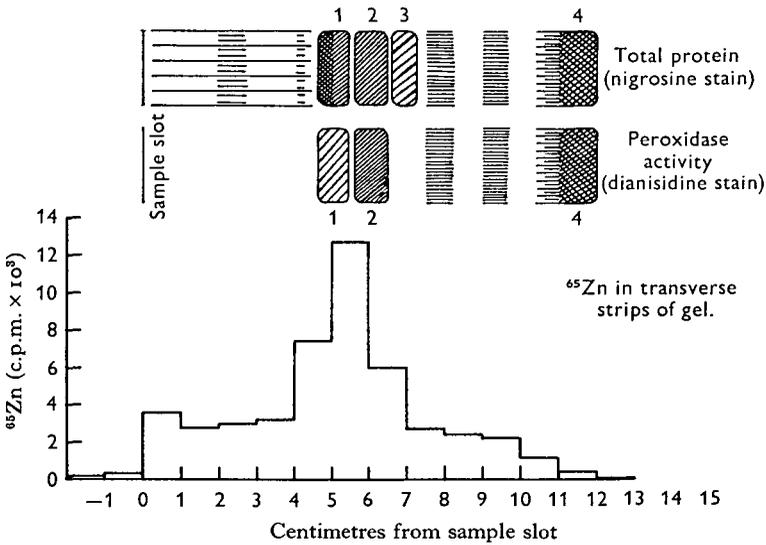


Fig. 11. Diagram of separation of proteins in  $^{65}\text{Zn}$ -labelled blood by starch-gel electrophoresis. This shows total protein distribution, the peroxidase activity of the haemocyanins and the distribution of  $^{65}\text{Zn}$  throughout the gel.

Very little  $^{65}\text{Zn}$  is associated with band 4 (fast haemocyanin), and so a large change in the amount of this protein would change the blood concentration of copper but not that of zinc.

Confirmation of the ability of blood proteins to bind zinc was obtained by dialysing  $^{65}\text{Zn}$ -labelled blood in 0.4 cm. bore 'Visking' tubing against a small volume of 50% sea water. This has roughly the same osmotic pressure as the blood. After 29 days at 5° C. equilibrium seemed to be reached when the blood contained 300 times as much  $^{65}\text{Zn}$  as an equal weight of water. Thus the blood can passively maintain its zinc concentration of about 1  $\mu\text{g./g.}$  in 50% sea water containing only 3  $\mu\text{g./l.}$  of zinc.

#### DISCUSSION

The process of zinc regulation in the freshwater crayfish differs from that in the marine lobster *Homarus* in several ways. These differences may represent changes which have occurred during the evolution of the crayfish into a fresh-water animal. Most crayfish tissues contain less zinc than those of the lobster, but the concentrations in the hepatopancreas and stomach fluid are much higher. Concentrations in the main abdominal flexor muscles are similar in the two species and this suggests that the amount of zinc in these muscles is important. The constancy of zinc concentrations in muscle under different conditions also supports this idea. In both species it is the tissue responding least to changes in the blood concentration of zinc, although zinc exchange occurs (Bryan, 1964, and unpublished).

Blood contains less zinc in the crayfish than in any marine decapod crustaceans which have been examined. This is slightly surprising because, in the Plymouth area at least, blood contains far more zinc in estuarine than in marine species and it was thought that this trend might continue into fresh water. The blood concentration of zinc seems to be closely controlled and does not increase in animals having high concentrations of blood protein and of copper as it does in *Homarus*. Zinc is bound to proteins in the blood, but binding to the variable haemocyanin component may be less important than in *Homarus*.

Low zinc concentrations have been found consistently in the excretory organs and urine. This system appears to be unimportant in regulating zinc, although it certainly prevents zinc loss. The permeability of the body surface to zinc is low and as a result zinc losses are small. Similarly, very little zinc can be absorbed across the body surface from solution and only when the external concentration exceeds that of the blood for more than 32 days can an increase be detected in the zinc concentrations of the internal tissues. Much lower external concentrations influence the levels of zinc in the shell and gills because considerable surface adsorption seems to occur.

In contrast, *Homarus* can absorb and lose zinc across the body surface, apparently in a controlled manner. Losses in the urine are closely controlled and can be appreciable.

Both species are likely to obtain excess zinc from food and in the crayfish this is virtually the only source of zinc. Excess zinc is absorbed rapidly from the stomach fluid in *Homarus*, partly at least via the hepatopancreas, and causes the concentrations to increase in all tissues except muscle, gonads and shell. Uptake from the stomach fluid of the crayfish is slower, and all the zinc is absorbed by the hepatopancreas without affecting the other tissues. Some zinc may penetrate directly from the stomach into the blood but must be removed so rapidly by the hepatopancreas that no obvious change in blood concentration is seen. Further evidence that the hepatopancreas regulates the blood concentration of zinc was found by injecting excess zinc into the

blood. All the excess zinc is absorbed by the hepatopancreas in a few days and some of it is transferred to the stomach fluid. When excess zinc is injected into *Homarus* some is removed in the urine and some is lost across the body surface. But, as in the crayfish, an appreciable amount is removed by the hepatopancreas. However, whereas in the crayfish excess zinc in the hepatopancreas and stomach fluid is lost in the faeces, in *Homarus* excess zinc in the hepatopancreas is eventually lost via the blood and excretory organs or body surface.

It seems possible that as the crayfish evolved into a freshwater animal, having a low permeability and modified excretory organs, it became necessary to increase the importance of the hepatopancreas as a controlling organ and to re-route losses to the alimentary canal.

Although this work is not particularly concerned with the toxic effects of zinc, some tentative conclusions may be drawn. In 0.1 % sea water (calcium = 0.4 mg./l.) the zinc concentration above which unfed animals could not survive indefinitely lay between  $2 \times 10^4$  and  $10^5$   $\mu\text{g./l.}$  Because the crayfish is rather impermeable to zinc it seems unlikely that death is caused by large amounts of zinc reaching the blood and internal tissues. Injections of zinc raised the blood concentration of zinc by a factor of 35 without effecting the animal, although rapid injections of greater than 100  $\mu\text{g./10 g.}$  animal were sometimes fatal. This lack of effect probably depends on the capacity of the blood proteins to bind zinc and render it non-toxic. The absorption of excess zinc from the stomach fluid can occur, but the hepatopancreas seems to act as a buffer and protects the blood and other internal tissues. It seems likely that death in high-zinc waters is related to the high adsorption of zinc, perhaps coupled with absorption by the gills. The reason for this toxicity is not known but there are a number of possibilities. Adsorption of zinc on to the gills may reach such a high level that there is sufficient penetration to inhibit respiration, to inhibit processes of ionic regulation or even to bind to gill or blood proteins and cause them to precipitate. Certainly after death the appearance of the gills suggested that precipitation had occurred. Competition from calcium in hard waters reduces the degree of adsorption of zinc by the shell and gills. This would be expected to reduce the toxicity of zinc.

Contamination of the crayfish as a result of radioactive pollution will be of two fairly distinct types. First, there is surface contamination. This appears to be due to the adsorption of  $^{65}\text{Zn}$  from solution on to the gill and shell surfaces. Uptake of this type varies between different batches of animals but quite high levels are sometimes reached. Contamination is reduced as the zinc and calcium concentrations of the medium are increased. The second type of contamination is internal and is largely a result of feeding on contaminated food. Although an increase in the amount of zinc in the food can reduce the degree of  $^{65}\text{Zn}$  absorption, an appreciable amount can still be taken up before the remainder is lost in the faeces. Once production of faeces has ceased, very little of the absorbed  $^{65}\text{Zn}$  is lost until inactive food is eaten and more faeces are produced.

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## SUMMARY

1. Zinc concentrations in the freshwater crayfish *Austropotamobius pallipes pallipes* normally range from about 1  $\mu\text{g./g.}$  in the blood to 100  $\mu\text{g./g.}$  in the hepatopancreas.
2. The permeability of the body surface to zinc is very low. Long exposure to concentrations exceeding that of the blood is required to increase the internal tissue zinc concentrations appreciably.
3. Much of the zinc which is absorbed from solution appears to be adsorbed on to the gill and shell surfaces.
4. Most of the body zinc is obtained from food.
5. The hepatopancreas is the principal organ of zinc regulation. It can absorb excess zinc from the stomach fluid and can remove excess zinc if this is injected into the blood.
6. Very little of the excess zinc in the hepatopancreas can be lost in the urine or across the body surface. Zinc is lost only when the animal feeds and faeces are produced to which it can bind.
7. As the amount of zinc in the food increases, a smaller percentage of it is absorbed by the hepatopancreas and more is lost in the faeces.
8. Regulation of zinc seems to depend on changes in the hepatopancreas/stomach-fluid ratio. These alter the availability of zinc for removal in the faeces according to the concentration in the hepatopancreas.
9. There is no close relationship between the behaviour of zinc and copper although zinc is bound to blood proteins some of which are haemocyanins.
10. Differences in the methods of regulation between the freshwater crayfish and the marine lobster may represent changes which have occurred during the penetration of the crayfish into fresh water.

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