VARIATIONS OF COPPER CONCENTRATIONS IN *CARCINUS MAENAS*

by

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Résumé

1. — La teneur en cuivre des tissus du crabe *Carcinus maenas* a été cherchée. Les valeurs obtenues, en γ/g. de poids frais des tissus, sont : carapace, 9,6 ; branchies, 38,5 ; intestin antérieur, 10,0 ; intestin, 6,9 ; muscle, 9,6 ; cœur < 3 ; sang, 15-150 ; hépato-pancréas, 20-125.

2. — D'après des analyses bi-mensuelles du sang et de l'hépatopancréas réparties sur deux ans, le sang a une teneur en cuivre supérieure à celle de l'hépatopancréas.

3. — Il existe une variation annuelle de la teneur en cuivre du sang, avec un minimum au moment de la mue. La chute de la teneur en cuivre avant la mue est graduelle.

4. — Il n'y a pas d'apparence de transfert du cuivre de l'hépatopancréas dans le sang après la mue. Au lieu de cela, l'accroissement de la teneur en cuivre dans les deux tissus est interprété comme une absorption active du cuivre du milieu par l'animal.

5. — Le cuivre du sang n'est pas considéré comme existant sous forme d'ion puisque il n'est pas en équilibre libre avec un dialysat.

6. — Les animaux placés dans des solutions riches en cuivre l'accumulent dans l'hépatopancréas et non dans le sang.

INTRODUCTION

The presence of copper in animal tissues was first demonstrated when Harless (1847) showed its combination with the blood protein of the snail. Subsequent work has indicated that invertebrates generally contain appreciably higher concentrations of copper than either vertebrates or plants. Invertebrates are also able to accumulate copper within their bodies, as shown by Melvin (1931) in many insects, and it is now generally accepted that copper is assimilated from the environment and concentrated in the tissues. Little information is at present available as to the mode of transport of copper in the body, or on its accumulation in marine animals, despite its widespread use as a toxic agent in antifouling paints. We have made a study of seasonal variation in the copper levels of an estuarine crab, *Carcinus maenas,* subjected to the variations occurring in a typical industrial estuary—Southampton Water. The copper levels in the haemolymph

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have been paralleled by studies of the seasonal variations in the hepatopancreas, as this organ is known to be an active metabolising site in *Carcinus*. (Munday and Thompson 1961.)

Copper levels are correlated where possible with the stage of ecdysis of *Carcinus maenas* as defined by Drach (1939).

METHODS

Animals

Carcinus maenas were taken from near and at the water intakes of the electricity generating station at Marchwood, Southampton. This station on the south bank of a mile wide estuary receives industrial effluent from Southampton Docks and also from industrial factories on both sides of Southampton Water.

Animals were collected at regular intervals and maintained in the laboratory in estuarine (80%) sea-water aquaria for periods of up to seven days before sampling. Only active animals in good condition were used for sampling and assay studies.

All analyses of haemolymph recorded in this paper were carried out on samples taken before clotting had commenced to avoid any possibility that this would affect the copper levels.

Sampling

Haemolymph was collected by a finely drawn glass micro-pipette inserted through the arthrodial membrane at the base of walking legs (Robertson 1937, 1939, and Morrison 1952). The haemolymph was placed in a small watch glass and duplicate 0.2 ml samples then pipetted accurately into a pyrex boiling tube.

The hepatopancreas was removed, well washed in Plymouth sea water to remove all traces of haemolymph, and dried on absorbant tissue paper. The wet weight was accurately determined by weighing on a small glass cover-slip. The coverslip and tissue were then dried in an electric oven (at 110°C) overnight, and the dry weight determined. Hepatopancreas tissue samples were taken in triplicate, whenever possible.

Copper Estimations

The micro method of analysis used for estimation of the bound copper was that of Eden and Green (1940) with minor modifications to suit the various tissues. By this method a wide concentration range of copper varying between 1 - µg could be accurately estimated for very small samples.

All reagents were AR quality and the 0.5% ethyl ammonium-diethyl - dithiocarbamate solution (D.D.C.) was freshly prepared with glass distilled water each week and stored in a dark glass-stoppered

bottle in a refrigerator. Standard copper solutions were kept in a dark refrigerator to prevent bacterial growth. Iron interferes with the colour reaction of the D.D.C. copper complex and has to be removed before estimation of the copper. Analytical reagent, ammonium citrate—frequently used for this purpose—contains minute amounts of copper, and instead a 4% AR sodium pyrophosphate solution, which forms a complex with iron in alkaline solutions was used. Phosphate precipitation may be prevented by avoiding complete evaporation of the acid residue before addition of the ammonium hydroxide.

Oxidation of the tissues was effected by perchloric acid and the residual acid removed by sulphuric acid. Both acids were added slowly in the cold to sample tubes and the temperature gradually increased to the maximum after 15 minutes. This prevented spontaneous combustion of the perchloric acid. Oxidation proceeds for

TABLE I

% recovery of added copper from *Carcinus maenas* blood

Volume of blood sample 0.2 ml. Copper concentration in blood, before addition of standard amounts of copper, varies between 9-15 μg/ml. Means ± standard errors. Number of results in parenthesis.

Copper added (μg)	% copper recovered
5	102.7 ± 1.04 (6)
10	100.5 ± 0.62 (5)
15	101.1 ± 0.48 (4)
20	98.1 ± 0.62 (5)

several hours and after the perchloric acid has evaporated leaves a colourless fuming liquid containing soluble copper sulphate. This is neutralised with ammonium hydroxide and after some hours the excess ammonia evaporates. The residues were then transferred to small 30 ml screw-topped bottles by rinsing with the sodium pyrophosphate solution and two washings of glass distilled water. The efficacy of these final washings was routinely checked by addition of a few drops of D.D.C. reagent to the final washing.

The copper-diethyl dithiocarbamate complex was shaken and after a few minutes to develop its yellow colour, was then extracted by shaking for 30 seconds with isoanyl alcohol. The alcohol-copper-complex layer readily separated and if the copper concentrations were too great for the volume of alcohol added, a dark brown precipitate appeared at the bottom of the alcohol layer. This readily dissolved if the volume of alcohol was increased. The colour intensity of the solutions was measured by a Hilger Absorptiometer with violet filter, and compared with copper standards containing 0-100 μg total, using various volumes of amyl alcohol for extraction. The efficacy of this method was checked by adding concentrations of copper varying between 5 μg - 20 μg to 0.2 ml haemolymph samples from the crab. The percentage recovery is shown in Table I.

RESULTS

1. The concentration of copper in the various tissues of the crab.

The tissues of the crab *Carcinus maenas* were removed and analysed for their copper content. The values obtained expressed as μg copper per gm wet weight of tissues, were as follows: Carapace 9.6; gill 38.5; foregut 10.0; intestine 6.9; muscle 0.6; heart (washed) less than 3.0 The hepatopancreas and the blood showed considerable variation in their copper content, depending upon the time of year and the moult condition of the crab. Hepatopancreas values varied between 15-150 μg/gm whilst those for the haemolymph were 20-125 μg/gm. The blood and hepatopancreas had values that were in general much higher than those for the other tissues; the high value of the gills most probably being due to the blood contained in them.

These results obtained for *Carcinus* agree with those of Zuckerkandl (1959) for *Maia* where he also found that the haemolymph and hepatopancreas were the two main copper containing tissues. However, we found slightly higher values in the other tissues compared with Zuckerkandl (1959) in that he reports less than 4 μg copper per grm wet weight for all other *Maia* tissues studied.

2. Seasonal variations in the copper concentration of the haemolymph and the hepatopancreas.

The seasonal variations in the copper concentration of the haemolymph and the hepatopancreas of the crab throughout the year were determined by taking a number of crabs each week and carrying out duplicate determinations of the copper content of the two tissues of each individual animal. The results were then tabulated and a frequency histogram plotted to determine the nature of the distribution of the various concentration values. A representative histogram is shown in Fig. 1. This shows the copper concentration in the haemolymph and the hepatopancreas in the months July-August, 1959. There can be seen to be a wide spread of the copper concentrations, the highest concentration in the haemolymph was 160 μg/gm wet weight whilst the highest for the hepatopancreas was 175 μg. The maxima are clearly similar. On the other hand there is a marked difference in the distribution of the copper values between the two tissues; in general it can be seen that the hepatopancreas levels are lower than those of the blood. (Fig. 1.) The mean values reflect this and for the hepatopancreas are 57.0 μg/g ± 0.43, and for the haemolymph 88.46 μg/ml ± 0.36. Table 2 summarises the values for the copper content of the haemolymph and the hepatopancreas over the whole November 1957 to September 1959 period. The values are again given in terms of μg copper per gram

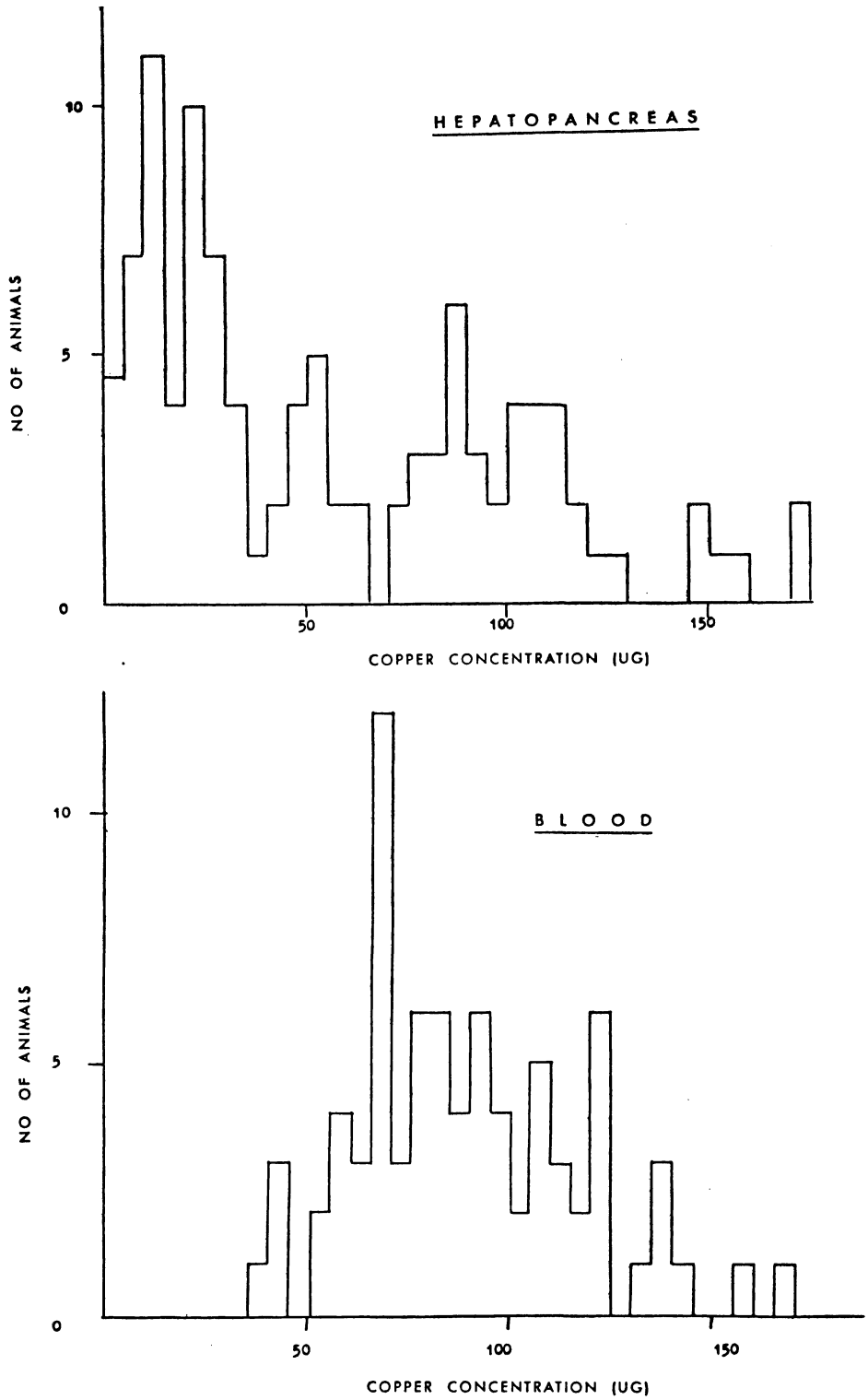

FIG. 1. — Histogram showing the copper concentration in the hepatopancreas and haemolymph of *Carcinus maenas, July-Aug.* 1959.

wet weight of tissue. Means are quoted together with their standard error. The number in brackets refers to the number of animals used; the variation being due to the availability of the crabs. Of the eleven pairs of monthly figures, only one set, March-April 1959, had no significant difference between the haemolymph and the hepatopancreas copper content. In two others, November 1957 and April-May 1958, the hepatopancreas had the higher copper content. In the other eight readings the haemolymph was significantly greater than the hepatopancreas. Thus the first conclusion that can be

TABLE 2

Seasonal variation in hepatopancreas and haemolymph Copper concentrations of *Carcinus maenas*

Copper expressed as μg copper/grm wet weight for hepatopancreas and as μg/ml for haemolymph.
Number of animals sampled in parenthesis.
Means ± standard error.

Date	Hepatopancreas	Haemolymph
Nov. 57)	73.0 ± 0.68 (69)	60.8 ± 0.47 (61)
Dec. 57) Jan. 58)	23.1 ± 0.93 (23)	53.9 ± 1.81 (21)
Apr. 58) May 58)	53.1 ± 1.29 (35)	37.9 ± 0.57 (35)
July 58) Aug. 58)	35.1 ± 0.32 (58)	61.3 ± 0.50 (41)
Sept. 58) Oct. 58)	42.1 ± 0.65 (42)	79.4 ± 0.55 (36)
Nov. 58) Dec. 58)	42.8 ± 1.43 (27)	89.2 ± 1.55 (27)
Jan. 59) Feb. 59)	41.9 ± 0.92 (25)	72.0 ± 1.80 (23)
Mar. 59) Apr. 59)	58.2 ± 0.68 (56)	55.1 ± 0.75 (46)
May 59) June 59)	41.6 ± 0.43 (76)	78.2 ± 0.72 (50)
July 59) Aug. 59)	57.0 ± 0.43 (101)	88.5 ± 0.36 (72)
Sept. 59)	42.0 ± 0.46 (58)	65.2 ± 0.62 (40)

drawn from these results is that in general the haemolymph contains more copper than does the hepatopancreas.

The second point concerns the variability of the copper content. One of the major problems that faced us was the nature of this variability; was it due to individual differences between the animals or was there a seasonal variation correlating with the yearly ecdysis cycle? Our results in Table 2 give the copper levels over two successive years and it is striking that there is no marked copper accumulation or loss in any given month. Thus the hepatopancreas values for July-August 1958 were 35.1 μg/g ± 0.32, and for July-August 1959, 57.0 μg/g ± 0.43. The results for the haemolymph show no better agreement (61.3 μg/ml ± 0.50 and 88.5 μg/ml ± 0.36 respectively). *Carcinus* in both years tended to ecdyse in the months March-April

and thus the variation between successive July-August series was not due to any variation in the animal's ecdysis pattern.

The third point that these figures shows is that there is a significant difference between the copper levels in the haemolymph and the hepatopancreas in successive months. This must mean that something of importance was happening to the copper levels of crabs throughout the year but that the absolute level was not comparable between the same months of different years. Figure 2 shows this variation in graphical form and it can be seen that there is, in general,

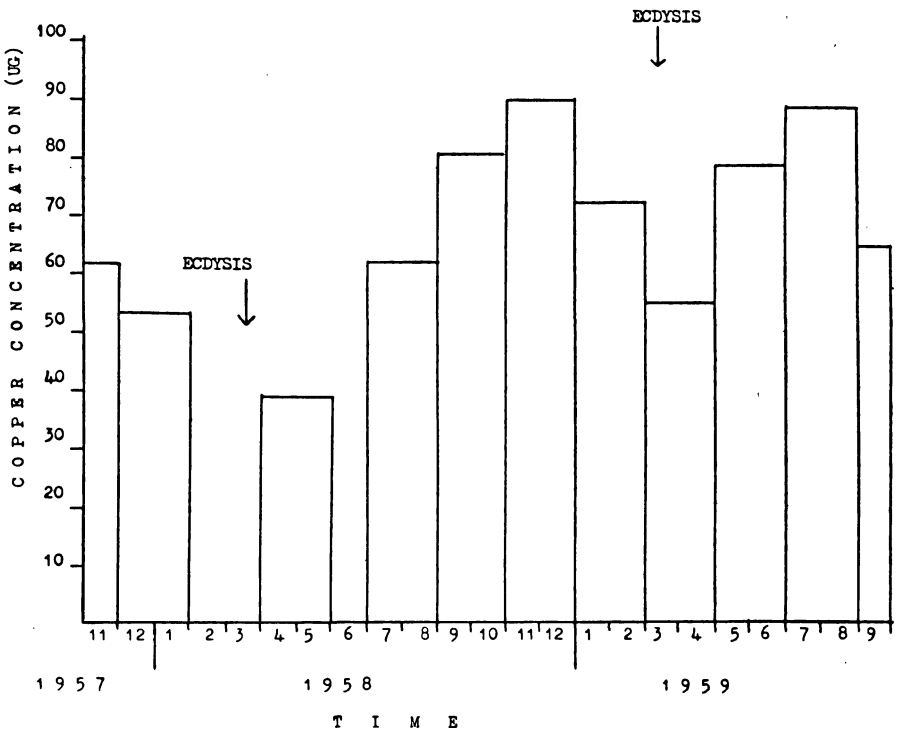

FIG. 2. — Histogram of the copper concentration in *Carcinus maenas* collected throughout the time.
November 1957 to September 1959.
The values shown are the values in the blood.

a lower value of copper in the haemolymph at the time of ecdysis. A possible interpretation of this will be discussed in the following section.

Discussion

The experimental results reported above show that the majority of copper in the crab is localised in the blood and hepatopancreas. The copper in the blood is mainly bound to the protein and amino acids since dialysis experiments that we have carried out on freshly

drawn *Carcinus* haemolymph failed to give any copper in the dialysate. Hence the copper in the blood must be chelated or bound in some manner. (It is interesting to record that even if 50 μg of copper tartrate is added to 0.5 ml of *Carcinus maenas* haemolymph, it is still not possible to obtain repeatable dialysate values for copper even after three days of dialysis against distilled water.)

The precise state of the copper in the hepatopancreas is not clear. There is a certain amount of blood removed together with the hepatopancreas, but the hepatopancreas can at times in the normal animal have a higher concentration of copper than the surrounding blood. In November 1957 our readings show that the hepatopancreas had 20% more copper than the blood. This indicates that the hepatopancreas is capable of concentrating and storing the copper. In general, however, the blood had a higher copper content than did the hepatopancreas, and consequently the concentrating capacity of the hepatopancreas is not frequently used.

We have confirmed the ability of the hepatopancreas to store copper by placing animals in sea-water containing added copper. Thus the average monthly values of copper in the hepatopancreas of animals taken freshly from the Solent was 41.6 μg/g. The average values for animals kept for 24 hr in sea water containing 12.5 mg% copper tartrate was 82 μg/g. That for animals kept for 48 hr in this solution was 95.3 μg/g. Animals kept for 72 hrs in 50 mg% copper tartrate solution had hepatopancreas values for copper of 130 μg/g. The blood of all these animals had copper values that fell within or below the normal range (78.2 ± 0.72 μg/ml).

The main problem presented by our experimental results was the nature of the variation of the copper content in *Carcinus*. We found considerable individual variation between animals, even though precautions were taken to obtain animals in approximately the same stage of the moult cycle, and of similar size. Even with the individual variations discounted, we found a marked monthly variation in the copper content of both the blood and hepatopancreas. The picture is somewhat similar to that described by Zuckerkandl (1959) where he extracted blood from individual specimens of *Maia* throughout the moult cycle and showed that the copper content in the haemolymph rapidly increased prior to ecdysis. This haemolymph copper comes from the hepatopancreas in *Maia,* and the copper level fell rapidly as the animal passed to Stage B after ecdysis. The whole cycle took approximately 9 months. Our crabs moulted over the months March-April, and we found a definite rise in the copper concentration of the blood at this time. But our results differ in that we appear to obtain a fall in the copper content of the blood prior to the advent of ecdysis. Furthermore, the levels as traced over two years, were not identical. Thus in 1958, the post ecdysis crabs had a level of 37 μg/ml, whilst in 1959, the crabs had a value of 55 μg/ml. Our results, however, confirm Zuckerkandl's (1959) general conclusion that the copper concentration in the blood is low at moulting. We differ in that our results suggest that the haemolymph concentrations begin to fall before ecdysis, and also the hepatopancreas copper content does not oppose the pattern of the haemolymph copper content. Zuckerkandl (1959) found that as the blood copper rose over the

initial seven months toward the onset of the next moult, so the hepatopancreas copper content decreased. We found that in general hepatopancreas copper content increased as the blood copper concentration increased. This suggests *Carcinus* is taking up copper from the sea water rather than translocating it around the body. This is what happens in the last three weeks of the moult cycle of *Maia* when both hepatopancreas, and blood copper increased (Zuckerkandl 1959).

These studies on the variation in copper content of the tissues of *Carcinus maenas* in its natural habitat, have provided us with background information with which it is hoped to study the nature of the toxic action of copper poisoning in Crustacea.

Acknowledgments

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Summary

1. The tissues of the crab *Carcinus maenas* were analysed for their copper content. The values we obtained were carapace, 9.6; gill 38.5; foregut 10.0; intestine 6.9; muscle 9.6; heart, less than 3; blood 15-150; hepatopancreas 20-125; the values being in μg copper/g. wet weight of tissue.

2. Throughout bi-monthly analyses of both the blood and hepatopancreas of the crab over two years, the blood had a higher copper content than the hepatopancreas.

3. There is a yearly variation in the blood copper content, with a minimum at the time of ecdysis. The fall in copper content before ecdysis being gradual.

4. There is no indication of the transfer of copper from the hepatopancreas to the blood after ecdysis. Instead the increase in copper in both tissues is interpreted as an active uptake of copper by the animal from the environment.

5. The copper in the blood was not considered to be in an ionic state since it was not in free equilibrium with a dialysate.

6. Animals placed in copper rich solutions, accumulated copper in the hepatopancreas, not the blood.

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