INTERACTION BETWEEN Zn^{2+} Co^{2+}, Mn^{1+} WITH HEMOCYANIN FROM *CARCINUS MAE NAS*

par

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Résumé

Chez *Carcinum maenas,* la concentration de ions métalliques est plus élevée dans l'hépatopancréas (325.2 µg/g de poids frais) qui est surtout un organe digestif. Le deuxième tissu où la concentration de métaux est la plus forte est le carapace (283.5 µg/g de poids frais). On a supposé que le rejet périodique de l'exosquelette pourrait être un moyen d'éliminer l'excès de métaux car *Carcinus maenas,* comme nombre d'autres Crustacés, change son exosquelette plusieurs fois pendant l'année (Varagnolo, 1071).

Lus branchies accumulent en quantité significative des ions métalliques; cet organe, avec sa surface étendue (1.360 mm^2/g de tissu frais) représente la barrière entre les milieux intérieur et extérieur.

Introduction

Le fer est amplement présent dans l'épilhelium des branchies, riche en mitochondries en raison de l'existence de la pompe Na^+ — K^+ — Adénosine — Triphosphatasique. Le zinc a été évalué en quantité élevée dans le muscle où il y a l'activité lactico et glutamate déshydrogénase et même l'anhydrase carbonique. La concentration de Mn^{2+} et de Co^{2+} est pauvre dans les tissus de *Carcinus maenas.*

Il est aussi rendu compte dans ce travail des résultats concernant l'interaction de l'hémocyanine avec quelques ions métalliques. Récemment dans notre laboratoire l'hypothèse que l'hémocyanine peut être la protéine qui transporte les métaux de l'hémolymphe au lieu de leur utilisation métabolique a été proposée.

Les expériences *d'équilibre* de dialyse à 4 °C en Tris-Hcl I = 0.1, pH 8.0 entre l'hémocyanine et les ions Zn^{2+}, Co^{2+} et Mn^{2+} ont démontré la présence de 4-5 sites pour le Zn^{2+}, de 2-3 sites pour le Mn^{2+} et de 7-8 sites pour le Co^{2+} par mole de protéine.

Les constantes de liaison entre l'hémocyanine et les ions ci-dessus ont été calculées égales à $2.7 \times 10^3 M^{-1}$ pour le Co^{2+}, $7.8 \times 10^3 M^{-1}$ pour le Mn^{2+} et $1.6 \times 10^4 M^{-1}$ pour le Zn^{2+}.

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MATERIAL AND METHODS

Carcinus maenas were collected in the Venetian lagoon near the harbour of Chioggia (Venice) between June and September and quickly transferred to the laboratory here they where kept in aquariums at 15 °C, well aerated and fed twice a week with clams. Animals were starved one week before experimentation.

Hemocyanin preparation

Hemocyanin (Hc) was prepared from the hemolymph extracted from the base of the carapace in living animals.

Hc was first filtred through a cheesclolh and then centrifuged. Supernatant was diluted 1:1 with Tris-HCl buffer I=0.1 pH 7.5 and Hc precipitated by ammonium sulphate at 52 per cent saturation.

After centrifugation the precipitate was solubilized in Tris-HCl buffer and the Hc sedimented by centrifugation. The pellet was redissolved in the same Tris-HCl buffer and the protein concentration kept at 60-70 nig/ml. After dialysis against Tris-buffer, He was mantained in 25 per cent sucrose at 30°C. Before using, He was dialysed to avoid any sucrose contamination.

Determination of protein concentration

Concentration has been determined speelrophotometrieally in NaOH ().„i M. The absorplion was calculated by using the Lambert-Beer formula :

$$A = \varepsilon CL$$

A = Absorption at 278 nm

L = Light pathway in the cuvette

ε = Extinction coefficient which is equal to 1.41 ml mg-1 cm-1 for C. *maenas* He in NaOH 0.3 M

C = Concentration in mg/ml

Spectra registration have been carried out by using a Perkin-Elmer mod. 576 spectrophotometer.

Spectroscopic properties of native hemocyanin

Functional properties of He are Expressed by a correct rale between the absorptions at 278 and 337 nm which is equal to:

$$\frac{E_{337nm}}{E_{278nm}} = 0.206 \text{ in } C. \textit{ maenas}$$

Determination of metal ions concentration

Metals ions bound to the protein were determined by using an atomic absorption spectiophotometer Perkin-Elmer mod. 4000 or by Ihe PIXE (proton-induced X-ray emission) technique.

100 ml of Tris-HCl buffer, 1= 0.1 and pH= 8 containing 10 mM of glycine and **various** concentrations of metal ions were put in cylinders where 3 ml of He (2 mg/ml) were dialysed at 4 °C for 48 hours.

He was previously dialysed against EDTA (20 mM) in the same Tris-buffer. The metal ions concentration has been determined inside and outside the dialysis bag.

RESULTS

Metal ions distribution in the tissues of Carcinus

In tables IA and IB, data on the concentration of various metal ions in the different organs of *Carcinus maenas* are reported. In the hemolymph metal ions are almost exclusively bound to hemocyanin, as it can be seen in the graph concerning the PIXe analysis (fig. **1).**

TABLE IA

Hemo-

	Gills	Carapace	Deferent	Heart	Epidermis	Hepato-pancreas	Muscle	lymph (*)	Plasma (*)
Cu	**28.52**	**21.50**	13.23	20.53	**17.86**	144.68	36.68	43.63	6.92
Zn	**21.89**	**55.82**	17.38	**36.94**	83.21	40.51	115.48	22.08	0.88
Fe	184.51	127.17	12.30	12.85	30.17	135.27	5.12	1.24	0.48
Mn	12.33	54.87	5.11	2.10	4.07	1.88	8.52	0.37	
Co	1.19	**15.13**	**5.16**	3.12	2.97	1.48	**2.60**	1.27	0.38
Cd	**1.39**	**9.02**	**2.70**	2.70	14.78	1.42	1.84		

Tab. IA : µg/g fresh weight
(*)µg/ml

TABLE IB

HEPATOPANCREAS		325,2	µg/g	fresh weight
CARAPACE		283,5	µg/**g**	fresh weight
GILLS		249,8	**µg/g**	fresh weight
MUSCLE	(leg)	174,2	**µg/g**	fresh weight
EPIDERMIS		153,0	**µg/g**	fresh weight
DEFERENT		85,8	µg/g	fresh weight
HEART		**72,2**	**µ**g/g	fresh weight
HEMOLYMPH	(Proteln+plasma)	68,5		µg/ml
		59,9		µg/ml
PLASMA	8,3			µg/g

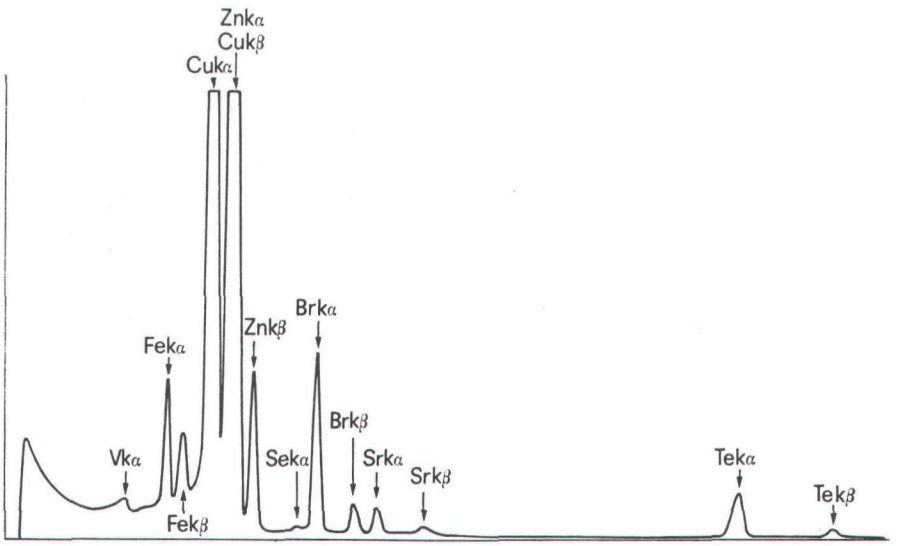

FIG. 1
PIXE Elemental analysis of *Carcinus maenas* hemocyanin.

Equilibrium dialysis

Treatment of the results from the interaction between small organic molecules or metal ions with macromolecules in a steady state, concerns the determination of C and *v*. C is the molar concentration of the free small organic molecules or metal ions and *v* represents the average number of the organic molecules or metal ions bound per mole of macromolecules (Scatchard, 1949). In the experiments reported in this paper, the total number of metal ions was determined both inside and outside of the dialysis membrane.

The concentration inside the dialysis bag is the total amount of three plus bound metal ones, while outside of the dialysis membrane only the free metal ions are measured, therefore protein bound metal ions are calculated as the difference between the total metal ions and the free metal ions; hence:

$$\bar{v} = \frac{[\mathrm{Hc\text{-}M^{n+}}]}{[\mathrm{Hc}]}$$

Hc from C. *maenas* has a generally accepted molecular weight for the minimal functional subunit of 75.000 D (Van Holde, 1983) even if the question concerning the number of subunits per whole molecule is still a matter of discussion (Ghiretti *et al.,* 1983).

Equilibrium dialysis data are not corrected for a possible Donnan effect, since the buffer has an ionic strenght high enough to avoid such an effect.

Figures 2 A, B, and C report the result of the equilibrium dialysis between CO^{2+}, Mn^{2+} and Zn^{2+} with Hc. These data are graphically reported as a log of the free metal ion concentration (log $[M^{n+}]$) with respect to *v*.

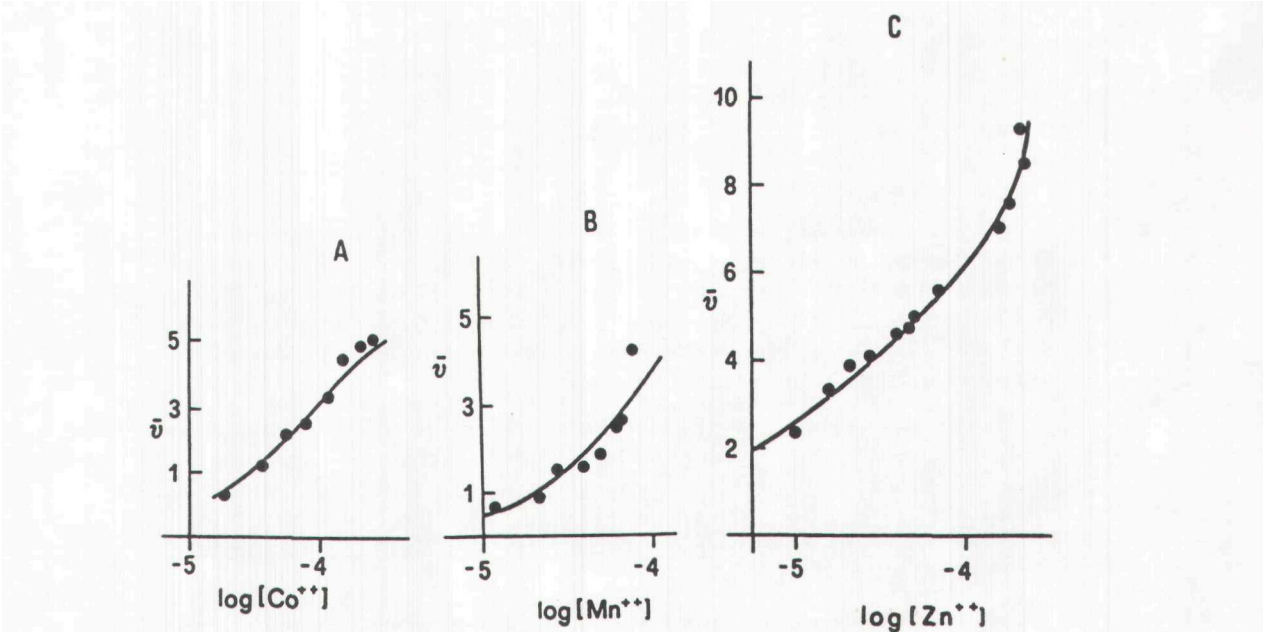

FIG. 2 (A, B, C)
Co^{2+}, Mn^{2+} and Zn^{2+} binding to *Carcinus maenas* hcmocyanin in TRIS-HC1 buffer I=0.1, pH 8.0, t=4 C.

Figures 3 A, B and C report the above data by using the Scatchard graphic method (Scatchard, 1949); these data are finally summarized in table II.

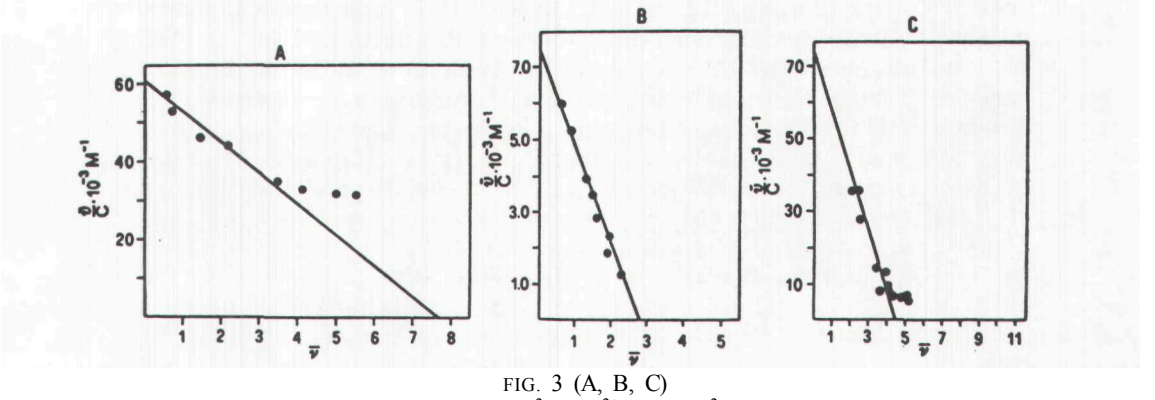

FIG. 3 (A, B, C)
Scatchard plot: binding data of Mn^{2}+, Co^{2+} and Zn^{2}+ to *Carcinus maenas* hemocyanin.

TABLE II

	n	Ka (M^{-1})
Co^{3}+	2-3	$2.7.10^{3}$
Mn^{2}+	7-8	$7.8.10^{3}$
Zn^{2}+	4-5	$1.6.10^{4}$

n=binding sites Ka = binding constant

DISCUSSION

Many papers appeared in the last decade on the concentration of the different metals in crustacea (Van Holde, 1983; Hiyama and Shimizu, 1964; Fowler, 1977; Knauer, 1970; Sidwell *et al.*, 1977; Sidwell *et al.*, 1978). Studying the concentration factors several authors (Martin, 1974) established a distinction among the metal ions and divided them into two groups:

1) In the first group are those metals present in small quantity in marine environments which even if they are strongly concentrated in animals, they are still present in small quantities, such as: copper, zinc, iron, manganese (Bryan, 1964; Ball and Meyerhoff, 1940; Ghidalia *et al.*, 1972; Martin, 1974; Hiyama, 1964; Ogura, 1965; Marlin *et al.*, 1977).

2) The second group includes magnesium, calcium, sodium, potassium which are «structural components» and are responsible for many different physiological functions such as osmoregulalion (Spaargaren and Ceccaldi, 1984).

In the last twenty years many papers appeared on the presence of metal ions in crustacea (Adams *et al.*, 1982), but few concern themselves with metal ions metabolism. It is interesting to point out that besides in the hepatopancreas, which is above all, but not only, a digestive organ (Gibson and Barker, 1979) the higher concentration of metal ions has been determined in the carapace (lab. IA and B). It has been hypothesized that the periodic elimination of the carapace during moulting, can represent an easy way to eliminate the excess of metal ions. The third organ which significantly accumulates metals are the gills, which represent the most important barrier between the internal and the external environments and therefore is of fundamental importance to the ionic exchange especially for that which concerns the osmoregulalory processes. Iron concentration is particularly high in the gills as reported by other authors (Martin, 1973) when an epithelium particularly rich in mitochondria is present (Zatta and Milanesi, 1984). It is responsible for an active transport in relation to the osmoregulalion (Towle, 1981).

Zinc is concentrated in the muscle tissue, where, a high lactic and glumatic dehydrogenase activity (Arvy, 1959) has been ascertained. Bryan reported in *Homarus* (Bryan, 1967) a higher zinc concentration in the leg muscles with respect to the abdomenal muscle and the author hypothesized that zinc concentration is lower in the muscles which can contract rapidly.

Manganese and cobalt are present in low concentration in *C. maenas.* In *Homarus,* Bryan reported (Bryan and Ward, 1965) an active accumulation of M^{2+} in the carapace and even in *C. maenas* manganese accumulates in higher concentration in the exoskeleton.

Equilibrium dialysis experiments between Hc and Zn^{2+}, CO^{2+} and Mn^{2+} have been carried out at pH=8. At this pH value aminic and guanidinic groups are all protonated and conseguentlyare are not available for metal ion dictation. Imidazolic groups on the contrary are completely deprotonated and therefore available for metal binding. Brower (Brower *ci al.*, 1982) eslablihed by titrating He with zinc, a process of «self association» at an initial metal concentration equal to 1.8 X 10^{-5}M.

This result can correspond to the deviation from the linearity of the curve shown in the Scatchard plot (fig. 3C). According to Brower (Brower *et al.*, 1982) zinc, from a physiological point of view, has the capability, to increase the O_2 affinity for Hc, stabilizing in a way not yet completely understood, the quaternary structure of the protein.

It has been previously seen that metal ions are almost completely bound in the hemolymph the hemocyanin (Tab. I). Expriments with the complex Hc-Zn^{65}, show rapid transfer of Zn^{65} from the Hc to the tissues, indicaling that Hc can act as a metal transport protein from the hemolymph to the sites of metabolic utilization (Zalla, 1984).

Attempts to demonstrale an active zinc transport in the gill of *C. maenas* gave no concrete results (fig. 6), at least using only NaN_3 as inhibitor for the phosphorylative oxidation processes (Zalla, 1984).

Even though the data reported appear to be very promising, concerning the metal metabolism in crustacea, they must he considered as preliminay, and much more work must be done for a more complete understanding

Sommario

Nel Carcinus maenas le concentrazioni degli ioni metallici è più elevata nell'epatopancreas (325,2µg/g di peso *fresco*) che è principalmente un organo digestivo. Il secondo sito di maggior accumulo dei metalli è il carapace (283,5µg/g peso *fresco*) che è stato ipotizzato essere un mezzo per eliminare l'eccesso dei metalli in quanto il *Carcinus maenas* al pari degli altri crostacei cambia il proprio ososehcletro più volte all'anno (Varagnolo, 1971). Anche le branchie accumulano in modo specifico ioni metallici; quest'organo con la sua ampia superficie (1360 mm^2/g di tessuto fresco) rappresenta la barriera tra l'ambiente esterno e quello interno.

Il t'erro è ampiamente presente nell'epitelio delle branchie, che è ricco di mitocondri associati con la pompa Na + -K + -ATPasica.

Lo zinco è stato determinato in quantità maggiore nel tessuto muscolare dove sono presenti gli enzimi lattico e la glutammico deidrogenasi nonché l'anidrasi carbonica.

Scarsa risulta la conccntrazione del Manganese e del Cobalto nei tessuti di *Carcinus m.*

In questo lavoro sono riportati pure i dati riguardanti l'interazione dell'emocianina con alcuni ioni metallici. Recentemente è stata avanzata l'ipotesi nel nostro laboratorio che l'emocianina possa essere la proteina di trasporto degli ioni metallici dall'emolinfa ai luoghi di utilizzo metabolico.

Esperimenti di equilbrio di dialisi a 4 °C in Tris-HCl I = 0,1 pH = 8 tra l'emocianina e lo Zn^{2+}, Co^{2+}, Mn^{2+} hanno dimostrato la presenza di 4-5 siti per lo Zn^{2+}, 2-3 siti per il Mn^{2+} e 7-8 siti per il Co^{2+}, per mole di proteina.

Le costanti di legame fra emocianina e i suddetti ioni metallici sono state calcolate uguali a 2,7 x 10^3M^{-1} per il Co^{2}, 7,8 x 10^3M^{-1} per il Mn^{2}+ e 1,6 x 10^4M^{-1} per lo Zn^{2+}.

Summary

In *Carcinus maenas* metal ions arc present in greater concentration in the hepatopancreas (325.24 µg/g ww) which is chiefly a digestive organ. The second important site for metal accumulation is the carapace (283.51 µg/g wet weight (ww) which has been hypothesized to be a way of eliminating metal excesses, since C. *maenas* as do other crustacea changes the hexoskeleton more than once a year (Varagnolo, 1971). Even in gills the accumulation of metal ions is significant; this organ with its large surface (1.360 mm^2/g ww) is the barrier between the internal and external environment. Iron is largely present in the epithelium of gills, which is rich in motochondria associated with the sodium-potassium «pump».

Zinc has been found in great quantity in the muscle tissue where the activity of zinc-enzymes such as lactic and glutamic dehydrogenase and carbonic anhydrase is higher.

Manganese and cobalt are sarcely represented in the tissues of *C. maenas.* In this paper data concerning the interaction between the copper respiratory pigment hemocyanin and some metal ions are reported. Recently it has been suggested in our laboratory that hemocyanin can even act as a metal carrier from the haemolymph to the sites of metabolic utilization (Fig. I).

Experiments of equilibrium dialysis at 4 C in Tris-HCl l=0.1 pH=8.0 between hemocyanin and Zn^{2+}, Co^{2+} and Mn^{2+} demonstrated: 4-5 sites for Zn^{2+}, 2-3 sites for Mn^{2+} and 7-8 sites for Co^{2+} per mole of protein (Fig. 2A, H, C). The binding constants have been calculated to be $2.7x10^3M^{-1}$ for Co^{2+}, $7.8X10^3M^{-1}$ for Mn^{2+} and $1.6x10^4M^{-1}$ for Zn^{2+} (Fig. 8A, B, C).

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