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FISH MERCURY-BINDING THIONEIN RELATED TO ADAPTATION MECHANISMS

by

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1. Introduction.

Cadmium and mercury have been shown to induce in kidney and liver of several species of mammals and birds (1,2) the synthesis of a particular type of low molecular weight protein known as metallothioneins mainly characterized by their high content in cysteine and by the virtual absence of aromatic amino acids residues. Other investigations carried out on plaice (3) have also demonstrated that animals exposed to sublethal doses of cadmium reacts to the intoxication by producing a low molecular weight protein which also seems to belong to the metallothionein family.

It has been proposed that metallothioneins could act as protective agents preventing for example in the kidney of cockerel chick (4) the inhibition by cadmium ions of the activation process of vitamin D. One of us has shown that eels adapted to sea water are able to stand continuous intoxication at sublethal

doses of $HgCl_2$ by developping adaptation mechanisms which suppress within a few days the misfunctionning of the gill and restores the NaCl balance in the animal (5). Moreover such intoxicated eels become resistant to usually lethal doses of mercuric chloride (6).

We report here that such adaptation mechanisms can be identified as being related to the appearance in different organs of the eel of metallothionein like proteins.

2. Material and methods.

2.1. *Biological material.*

Previous to any experiment, fresh water eels are adapted to sea water for at least eight days. Control and test fishes are each individually placed in polyethylene bags containing 10 l. of continuously oxygenated sea water poisoned in the latter case with 0.4 ppm of mercury added as $HgCl_2$. In both cases, clean and polluted waters are replaced every day during two weeks.

2.2. *Extraction and chromatographic procedure.*

Liver, kidney, gill and muscle tissues are homogenized in three volumes of 0.5 M sucrose using a Polytron Homogenizer. The extracts are centrifuged without delay in a Beckman L-2 Ultracentrifuge at 100000 g and 4°C. The clear supernatants are frozen until used or immediately chromatographied on Sephadex G 75 columns (105 x 4.8 cm) or (50 x 5 cm) equilibrated in NH_4HCO_3 0.05 M. The elution is monitored automatically at 254 nm using a L.K.B.

Uvicord 4701 A and manually at 215 nm using a Spectrophotometer Zeiss PMQ II.

2.3. *Mercury determination.*

The amount of mercury is determined by flameless atomic absorption spectrometry according to previously described method (5) and using the Coleman Mercury Analyzer System MAS 50.

2.4. *Amino acid analyses.*

Amino acid analyses are made in duplicate using the procedure of Benson and Patterson (7) and a Beckman amino acid analyzer Model 120 B. The performic acid oxydized proteins (8) are hydrolysed under vacuum at 107°C during 24 hrs in constant boiling HCl.

2.5. *Ultraviolet absorption.*

The ultraviolet spectra of the native and of the Hg free protein are taken in 0.05 M NH_4HCO_3 using a double-beam spectrophotometer Hitachi Perkin-Elmer Model 124.

The mercury free sample is obtained by 24 hrs dialysis against two liters of 0.05 M NH_4HCO_3, 1 mM dithiotreitol and 0.1 mM EDTA. This process is repeated twice. The sample was finally dialysed against two batches of two liters of 0.05 M NH_4HCO_3.

3. Results and discussion.

Fig.1 shows the typical distribution of mercury in the various fractions obtained after chromatography on Sephadex G 75

of the whole extracts corresponding to four organs of chronically intoxicated eels. In the control samples, the concentration in mercury is not shown because it falls down the detection limits of the analytical method. Exception being for the muscle sample, most of the mercury is found in a retarded fraction (Kav = 0.4) showing weak absorbance value at 215 nm and having an elution volume characteristic of substances having a molecular weight close to 10000 daltons. This is corroborated by the fact that in the muscle sample there is a clearly visible protein peak, eluted in the same volume, which has been identified as well known muscle proteins, called parvalbumins (9, 10, 11) exhibiting molecular weights between 11000 - 12000 daltons. Note that no mercury is found in the salts fraction.

The mercury carrying retarded fractions of the liver extracts have been pooled and lyophylized in order to further analyze the mercury carrier. Fig.2 represents the U.V. spectra obtained from residual material solubilized in NH_4HCO_3 0.05 M. The figure shows an unusual protein spectrum, quite similar to those produced by metallothionein (12). Removal of most part of the mercury (70%) by dialysis of the solution against chelating agents produces an alteration of the spectrum, also typical of metallothionein characterised by a drastic decrease of the absorbance value around 280 nm (13).

Table 1 summarizes the results of the amino acid analysis made on the mercury containing fractions isolated from liver extracts on a large size Sephadex G 75 column. The number of residues is calculated assuming that histidine and phenylalanine residues are unity. This amino acid composition is typical of metallothionein, with an extremely low level in aromatic amino-acid residues, and a high content in cysteine residues which seems however relatively low when compared to previous figures determined from described mammalian metallothioneins (13). This could be due either to a significant difference between the amino acid compositions of mammal and fish metallothioneins or to the presence of some proteinic contaminants which however would amount to nearly 50% in order to explain the lowering of the cysteine content from about 20 to 10%. This would not certainly give the U.V. spectra shown in fig.2. Another interesting feature is the presence of 10% of proline residues. Such a high content has already been found in other metallothioneins and of course in even higher amount in highly elongated proteins such as collagen. That might perhaps indicate that metallothioneins are also quite asymetrical molecules and that the proline residues could be distributed at regular intervals along the polyeptide chain inducing it to affect an elongated, possibly helicoidal structure, such that the -SH groups are well apart from each other preventing any internal recombination as disulfide bridges.

In order to test the possible role of these metallothionein like proteins in adaptation mechanisms developed by chronically intoxicated eels, we have compared the Hg distribution in the different proteinic fractions obtained from gills of chronically (8 days - 0.4 ppm) and acutely (5h - 10 ppm) intoxicated eels. This procedure generates animals either with intact or completely disturbed NaCl balance (5). In chronically intoxicated eels the total amount of Hg is on an average 120 µg per g of tissue. It is distributed as follows : 23 µg in the insoluble fraction and 97 µg in the supernatant from which 78 µg were found to be bound to the retarded protein fraction (fig. 3a). In the acutely poisoned animals, one gram of tissue contains 61 µg Hg ; 46 µg are found in the insoluble fraction and the rest in the supernatant exclusively at the level of high molecular weight proteins (fig. 3b). The total absence of mercury in the low molecular weight fraction indicates that metallothionein does not exist in detectable amount in control fish since mercury would displace any other metal bound to the sulfhydryl groups of the metallothionein (12).

From these results, we conclude that mercury most probably induces the synthesis of a low molecular weight protein containing a high content of cysteine amino acid residues. This component can be identified as a Hg-thionein which protects the gill and other organs of the eel against injuries caused by mercury absorption.

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Table 1

Amino acid composition of Hg binding protein in eel liver

amino acid	N_o of residues/molecule	
	calculated	assumed
Lys	9.60	10
His	1.10	1
Arg	1.70	2
Asp	8.08	8
Thr	6.50	7
Ser	7.60	8
Glu	10.20	10
Pro	10.20	10
Gly	11.10	11
Ala	11.20	11
+Cys (half)	8.60	9
Val	4.80	5
‡Met	0.83	1
Ile	3.10	3
Leu	4.60	5
Tyr	0.00	0
Phe	1.06	1
Trp	-	-
Total	100.30	102.0

+ determined as cysteic acid

‡ determined as methionine sulfone

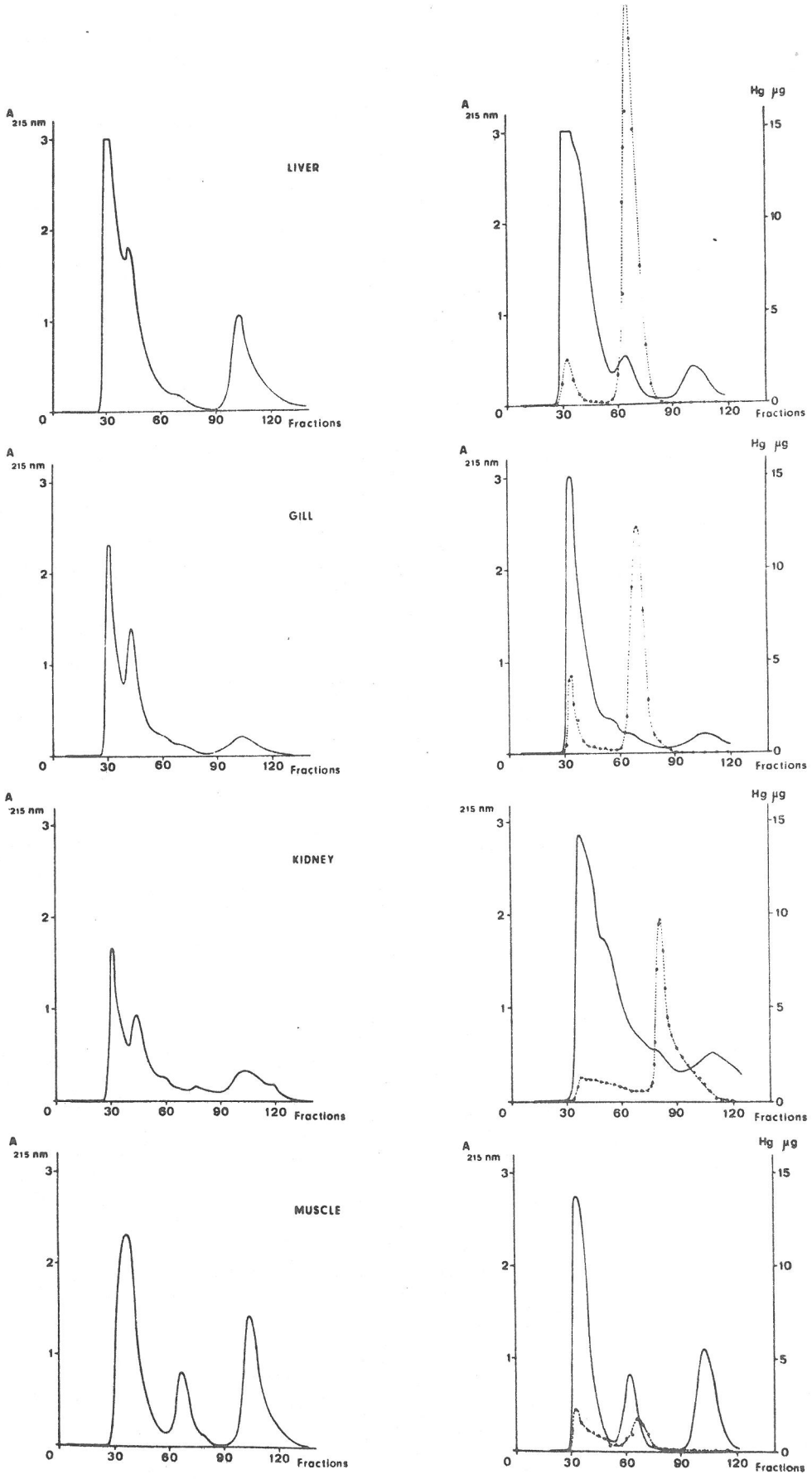

Fig.1. : Elution profiles on Sephadex G 75 columns (5 x 50 cm) of the extracts of different eel tissues. Left side, control fish ; right side, intoxicated fish. Hg concentration is expressed in µg/9 ml fractions (dotted line).

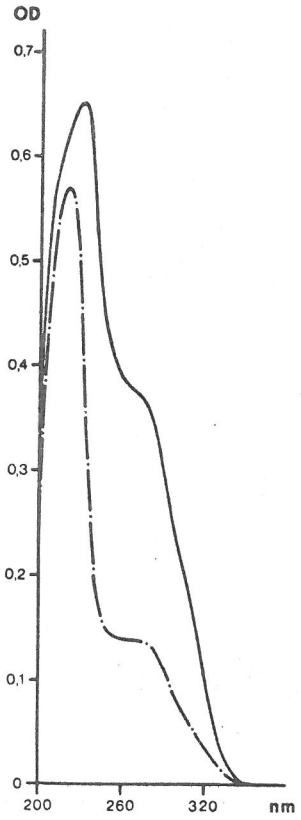

Fig.2. : Ultra-violet absorption spectra in NH_4HCO_3 0.05 M of the Hg binding protein of eel liver in presence (——) and absence (—·—) of mercury.

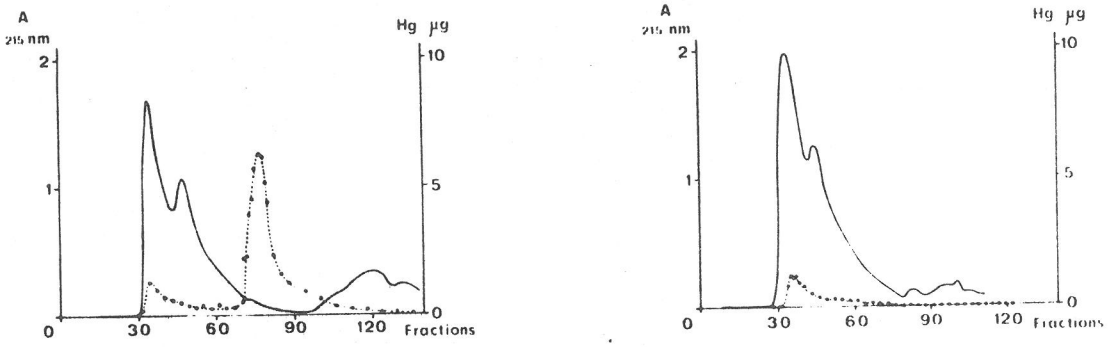

Fig.3. : Elution profiles on Sephadex G 75 columns (5 x 50 cm) of the gill extracts prepared from 1 g gill tissue of chronically (a) and acutely (b) intoxicated eels. Hg concentration is expressed in µg/9 ml fractions.