

# Metal-Binding Proteins as Metal Pollution Indicators

by H. F.-K. O. Hennig\*

The fact that metal-binding proteins are a consequence of elevated metal concentration in organisms is well known. What has been overlooked is that the presence of these proteins provides a unique opportunity to reformulate the criteria of metal pollution. The detoxification effect of metal-binding proteins in animals from polluted areas has been cited, but there have been only very few studies relating metal-binding proteins to pollution. This lack is due partly to the design of most experiments, which were aimed at isolation of metal-binding proteins and hence were of too short duration to allow for correlation to adverse physiological effects on the organism.

In this study metal-binding proteins were isolated and characterized from five different marine animals (rock lobster, *Jasus lalandii*; hermit crab, *Diogenes brevisrostris*; sandshrimp, *Palaemon pacificus*; black mussel, *Choromytilus meridionalis*; and limpet, *Patella granularis*). These animals were kept under identical metal-enriched conditions, hence eliminating differences in method and seasons. The study animals belonged to different phyla; varied in size, mass, age, behavior, food requirements and life stages; and accumulated metals at different rates.

It is possible to link unseasonal moulting in crustacea, a known physiological effect due to a metal-enriched environment, to the production of the metal-binding protein without evidence of obvious metal body burden.

Thus a new concept of pollution is defined: the presence of metal-binding proteins confirms toxic metal pollution. This concept was then tested under field conditions in the whelk *Bullia digitalis* and in metal-enriched grass.

## Introduction

Metal-binding proteins such as metallothioneins (MT) were originally isolated and characterized from equine kidney (1) and have extensively been studied in mammals (2). There is very little information available on MT in the other zoological groups, particularly in the invertebrates (3). The available information consists mainly of descriptions of the isolation of these proteins.

So far very little use has been made of these unique, relatively low molecular weight compounds. It has been suggested (4) that metal-binding proteins could be used to study cadmium resistance cells, but only recently have the properties of MT been used at all, in this case, as a promoter to control and induce gene activity (5-7).

The fact that metal-binding proteins are formed as the result of elevated metal concentrations in organisms has been well known for many years. What has been overlooked is the unique opportunity that MTs afford to reformulate the criteria for metal pollution. The detoxification effect of MT in animals from polluted areas has been mentioned (8-11), but only three studies relating metal-binding proteins to pollution have been published

(12-14). This lack is partly due to the design of most experiments which have been aimed at the isolation of proteins. Such experiments are performed for short time periods only and under severe metal toxic conditions. Little attention has been paid to adverse physiological effects on the organism by low metal concentrations and the production of metal-binding proteins. In humans, changes in toxic elements in blood serum or whole blood (15) were found to be sufficiently different to be of diagnostic significance. In invertebrates, an equation was derived (16) that correlates shifts in copper metabolism with adverse effects on the growth of crab larvae.

In this study, metal-binding proteins have been isolated from different marine animals that were kept under identical enriched conditions, so that differences in method and seasons were eliminated. The animals used differed in many aspects, e.g., different phyla, size, mass, age, behavior, food, and life stages. As expected, they also accumulated metals at different rates (see Hennig, manuscript in preparation). Furthermore, a physiological effect, e.g., abnormal moulting in crustacea, has been demonstrated in the absence of metal accumulation but in the presence of metal-binding proteins.

These findings were tested under field conditions, e.g., the whelk *Bullia digitalis* and metal-enriched grass, and a new definition of pollution is proposed.

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## Material and Methods

### Accumulation of Metals in Organisms

*Jasus lalandii* (rock lobster) was used in two separate experiments. Two adults were kept in each of twelve 10-dm<sup>3</sup> aquaria at 15 ± 1°C and dosed with industrial effluent. Details of the procedure are given elsewhere (17).

Juvenile rock lobsters (*J. lalandii*), hermit crabs (*Diogenes brevisrostris*), sandshrimp (*Palaemon pacificus*), black mussels (*Choromytilus meridionalis*), and limpets (*Patella granularis*) were kept in an open system containing one-week-old seawater and solutions of cupric sulfate and zinc chloride. This was dispersed at a rate of 0.2 dm<sup>3</sup>/hr in discrete portions, by means of a modified Mount and Brungs diluter (Hennig, manuscript in preparation). The resulting mixture from the diluter was monitored daily for metals and contained 15 µM/dm<sup>3</sup> zinc and 16 µM/dm<sup>3</sup> copper.

The field studies concentrated on the whelk *Bullia digitalis* which was collected at Koeberg, South Africa. These whelks have an unexplained high cadmium concentration (18).

"Kikuyu" grass (*Cornisetum clandestinum*) was obtained from CSIR Water Research Bellville, South Africa. This grass was dosed with 320 t/ha dried sewage sludge and came from site A5<sub>4</sub>.

### Metal Determination of Organisms

After digestion of the organisms, the residues were analyzed by flame atomic absorption spectroscopy using standard conditions (19). Background correction was used for those elements with resonance lines of wavelength shorter than 280 nm. After the accumulation experiments, the organisms were frozen (-10°C) in plastic bags.

Background metal concentrations were determined for the animals used in the above experiments (Hennig, manuscript in preparation).

### Isolation of Metal Binding Proteins

Partially thawed organisms were dissected or scraped from their shells and an appropriate amount of 25 mM phosphate buffer (pH 7.0) was added to give a 50% (w/v) homogenate. This was prepared by blending the mixture at full speed in ice twice for 1 min in a Du Pont Omni-mixer. The resulting homogenate was centrifuged at 4°C for 3 hr at 30,000g in a Sorval RC5 Superspeed refrigerated centrifuge with rotor SS 34. Supernatant material (10 cm<sup>3</sup>) was decanted and applied to a Sephadex G-75 column (2.6 × 100 cm) kept at 12 ± 2°C, and protein fractions (5 mL) were eluted with 20 mM Tris buffer (pH 8.6) (13). The column was standardized by using the following molecular weight markers: bovine albumin (68,000 daltons), ribonuclease (13,700 daltons), cytochrome c (12,500 daltons), and tryptophan (204 daltons). Concentrations of metals were monitored in the resulting frac-

tions by direct aspiration into the flame of an atomic absorption spectrophotometer (detection limits: Cd = 0.006 µg/mL; Cu = 0.050 µg/mL; Zn = 0.009 µg/mL). The absorbance of each fraction at 280 nm was monitored with a rapid sampling I.S.C.O. absorbance monitor, and absorbance at 250 nm and 280 nm was measured using a Beckman spectrophotometer with slit width 0.5 mm and path length 10 mm.

Fractions emerging at the elution volume of 10,000 to 12,000 daltons (peak II) were pooled and freeze-dried.

### Protein Purification

The freeze-dried material was dissolved in 1 mL of 20 mM Tris buffer and applied to a DEAE-Sephadex A-25 column (0.8 × 14 cm). The column was washed with 100 mL equilibration buffer, and the freeze-dried material was applied. This was then eluted with a linear gradient of Tris buffer, 250 mL each of 20 mM and 600 mM at a pH of 8.6. Fractions (5 mL) were monitored for absorbance at 280 nm and for cadmium, copper, and zinc by atomic absorption spectrometry. The fractions from the metal peaks were pooled separately, scanned from 320 nm to 220 nm, and freeze-dried.

The amino acid analyses were carried out in a Beckman automatic amino acid analyzer (Model 119) on samples

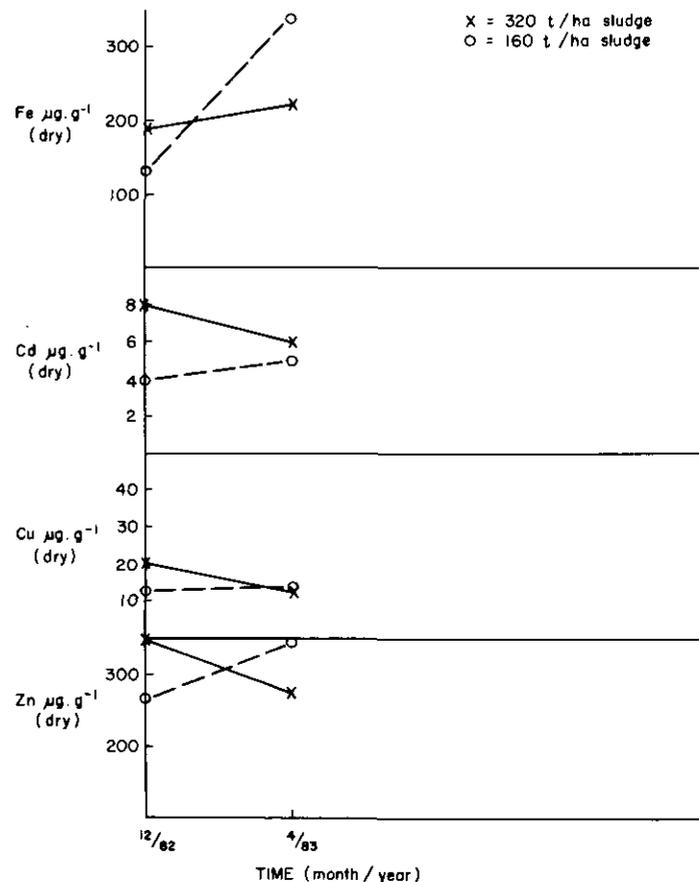


FIGURE 1. Metal concentrations in Kikuyu grass.

carboxymethylated and hydrolyzed in 5.7 N HCl for 24 hr at 110°C in tubes that had been evacuated and then sealed under nitrogen.

## Results

### Metal Accumulation

No difference in metal concentrations could be detected by the digestion method either in the digestive gland or tail meat of dosed crayfish. Zinc and copper levels were elevated in dosed hermit crabs. Zinc concentration was found to be particularly high.

The metal concentration of both copper and zinc were found to be higher in the dosed sandshrimps than in the control animals. The dosed mussels seemed to show only an elevated zinc level; all other measured metals were found to be well within the background levels.

Limpets kept in enriched media accumulated more copper and zinc than did the control animals. It should, however, be noted that zinc levels in limpets from the field (Koeberg) were very much higher than those from other locations (Hennig, manuscript in preparation). The zinc-enriched limpets from the experiment had just started to accumulate metals during the study time (60 days).

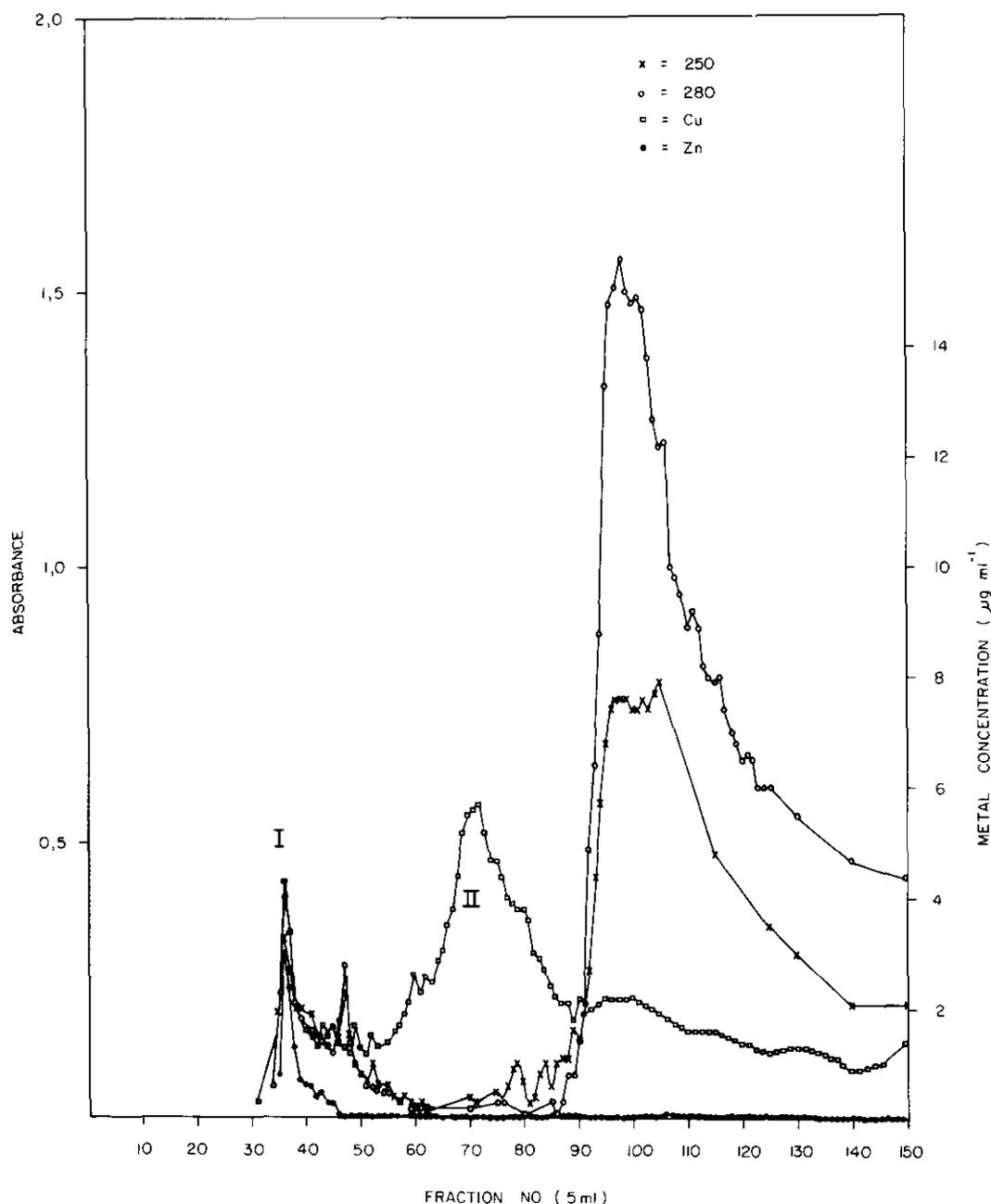


FIGURE 2. Elution profiles from Sephadex G-75 column chromatography of supernatant fractions from crayfish (*Jasus lalandii*) digestive gland. 250 = absorbance at 250 nm; 280 = absorbance at 280 nm.

In summary, the digestion method for metal determination in animals kept under identical conditions thus showed: no accumulation of metals in crayfish; very much elevated zinc levels and raised copper levels in hermit crabs; raised zinc and copper levels in shrimps and limpets; and only elevated zinc levels in mussels.

*Bullia* from the field (Koeberg) were compared with whelks from two other regions and were found to contain elevated cadmium and zinc levels. Copper and strontium concentrations could also be higher in the Koeberg animals.

Grass was grown for 8 months and harvested every 4

months, with the relevant metal concentrations shown in Figure 1. Elevated levels were found for cadmium (control =  $1 \pm 0 \mu\text{g/g dry}$ ), copper (control =  $8 \pm 0 \mu\text{g/g dry}$ ), and zinc (control =  $91 \pm 8 \mu\text{g/g dry}$ ), while the level of iron (control =  $238 \pm 23 \mu\text{g/g dry}$ ) showed no deviation from that in untreated grass.

### Column Chromatography

Typical Sephadex G-75 elution profiles obtained with supernatant material from crayfish digestive gland are shown in Figure 2. Two metal peaks could be identified.

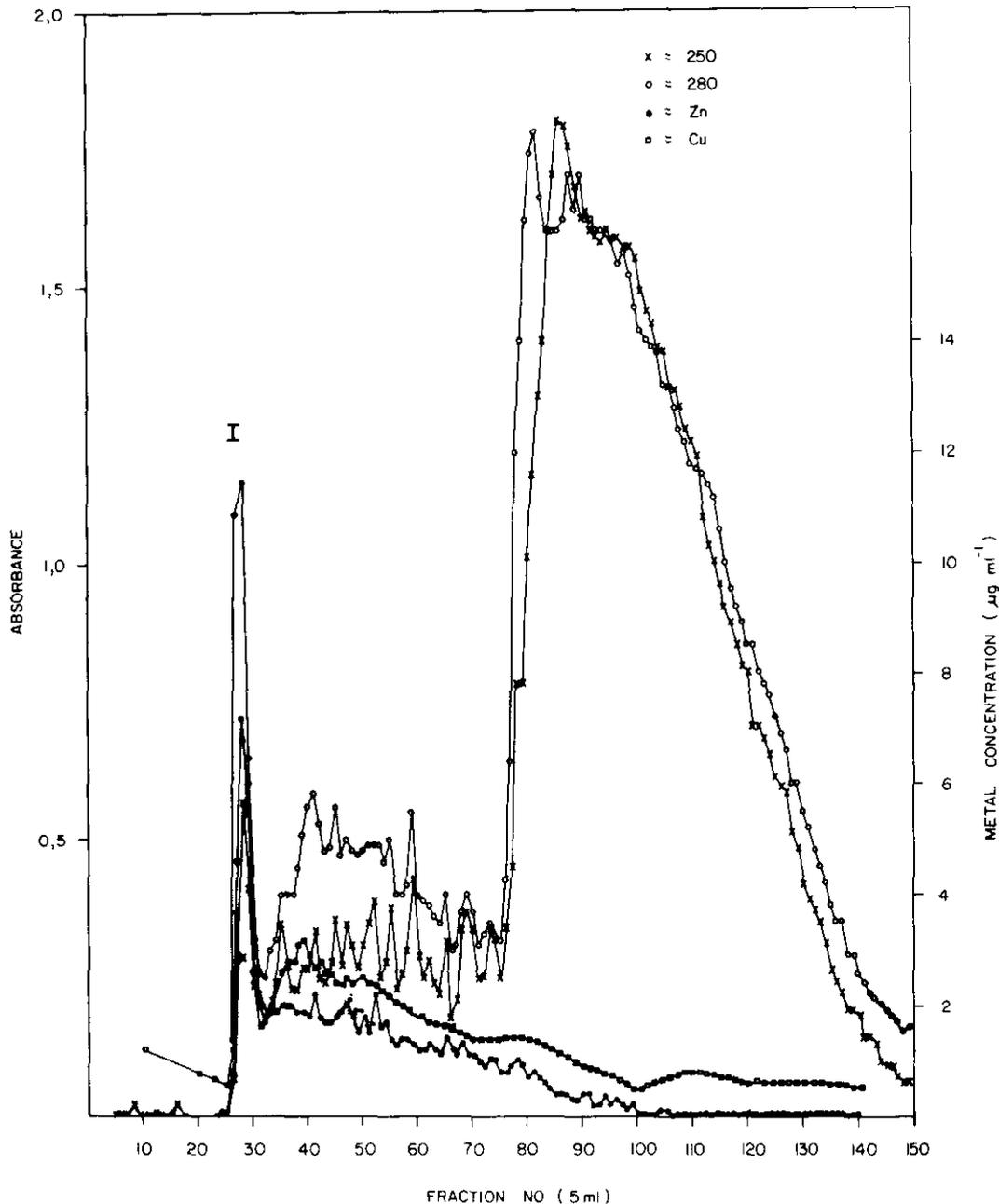


FIGURE 3. Elution profiles from Sephadex G-75 column chromatography of supernatant fractions from crayfish (*Jasus lalandii*) tail meat. Absorbance as in Fig. 2.

The high molecular weight protein peak (I) contains both copper and zinc, while the metal-binding peak (low molecular weight protein) contains mainly copper.

The elution profiles obtained from crayfish tail meat are shown in Figure 3. Only a metal peak associated with high molecular weight material (Peak I) was present.

In Figure 4 the elution profiles obtained from hermit crabs are shown. High molecular weight material was found (peak I), and a peak of metal-binding protein (peak II) containing copper and zinc was eluted.

The supernatant fractions of ten shrimps were eluted.

A typical profile is shown in Figure 5. The absorbance is a function of the protein concentration and in this case not much material was available hence the absorbance at 280 nm was small. One high molecular weight peak (peak I) and two low molecular weight peaks (peaks II) were observed.

The elution profiles obtained for black mussels are shown in Figure 6. Three peaks were obtained: peak I (higher molecular weight material), peak II (metal-binding low molecular material), and peak III (very small molecular weight fractions.). Only zinc was found to be bound to any protein.

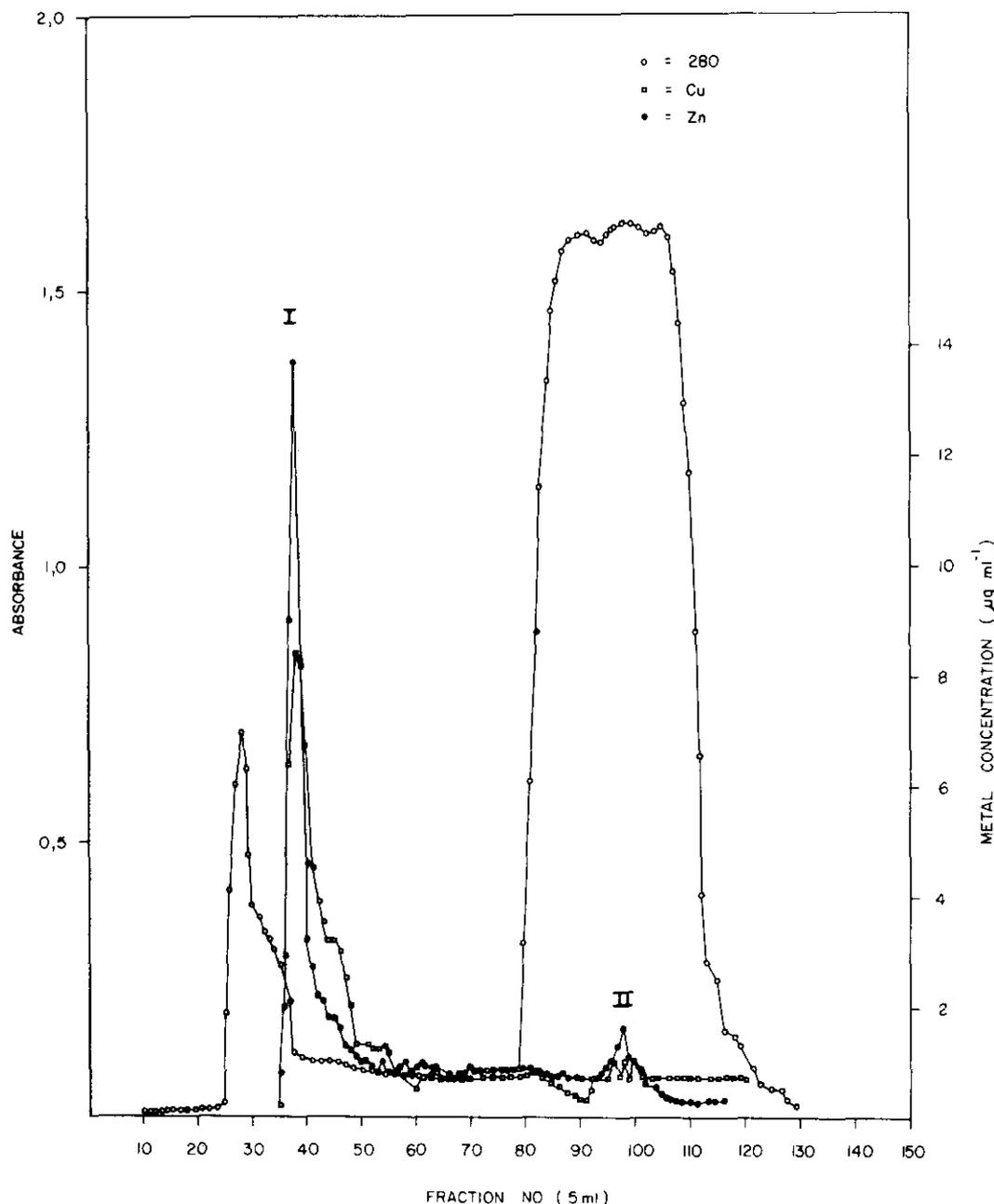


FIGURE 4. Elution profiles from Sephadex G-75 column chromatography of supernatant fractions from hermit crab (*Diogenes brevisrostris*). Absorbance as in Fig. 2.

The supernatant material of limpets eluted is shown in Figure 7. Two peaks containing zinc were found; the high molecular weight material (peak I) also contains copper. The material from the field studies are shown in Figure 8 for *Bullia* and Figure 9 for grass. In the case of *Bullia*, a high (peak I) and very small (peak III) protein peak was observed. The metal-binding protein peak (peak II) contained all the cadmium and some copper. In the grass, all the copper was associated with the higher protein fraction (peak I), but separate low molecular weight zinc peaks (peaks II and III) were isolated.

The ultraviolet absorption spectrum for the DEAE-

Sephadex A-25 column solutions for crayfish digestive gland is shown in Figure 10a. The absorbance of the material is dependent on the concentration of the protein and hence is therefore relative. Ultraviolet absorption spectra of the other materials—hermit crab (Fig. 10b), sandshrimp (Fig. 10c), black mussel (Fig. 10d), and limpet (Fig. 10e)—are also shown.

The absorption characteristics of the field samples are shown for the cadmium peak (peak II) of *Bullia* (Fig. 11a) and for the zinc peak (peak III) of *Bullia* (Fig. 11b), while Figure 12 shows the grass peak (peak III).

The results presented here for two different experi-

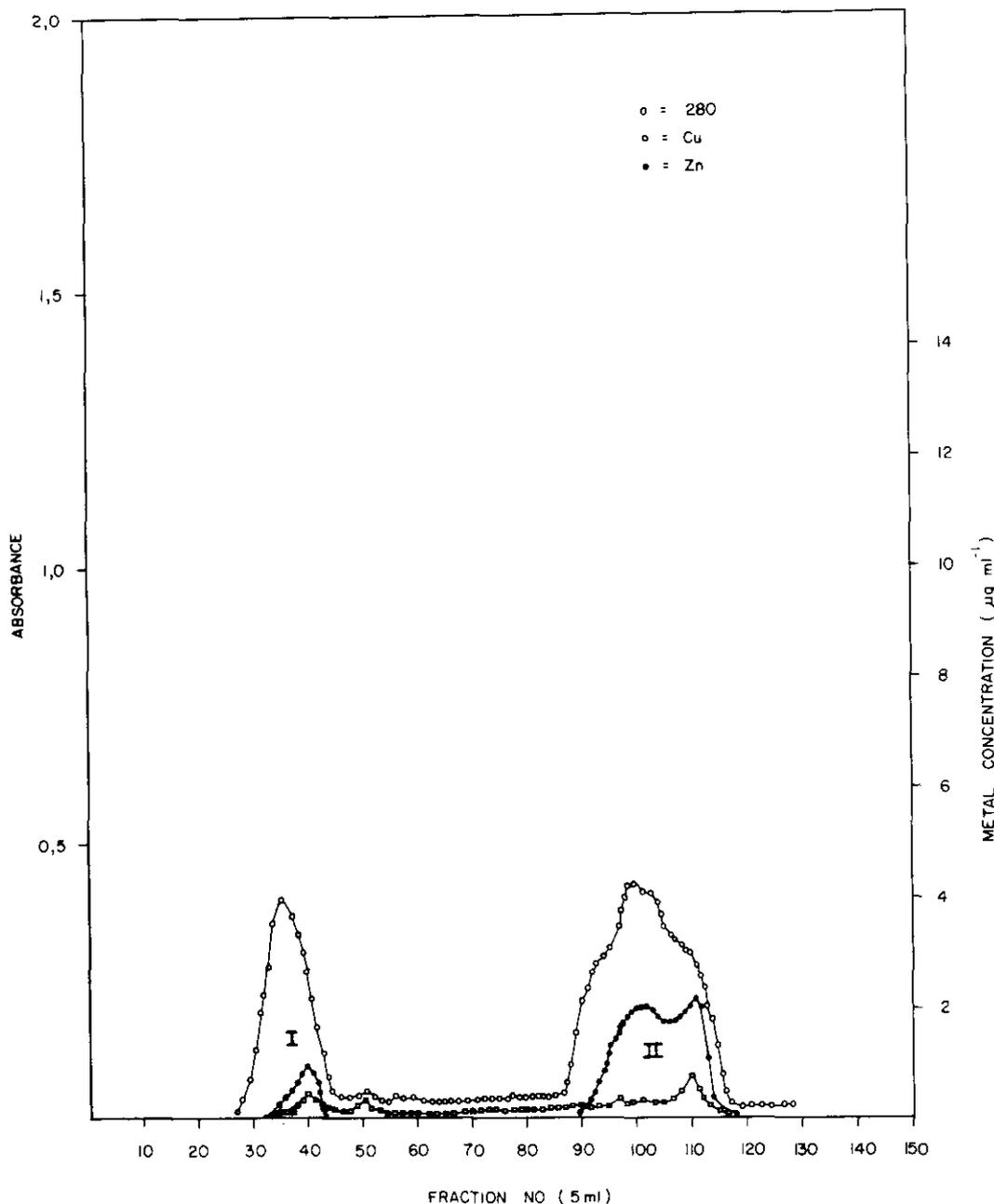


FIGURE 5. Elution profiles from Sephadex G-75 column chromatography of supernatant fractions from sandshrimp (*Palaemon pacificus*). Absorbance as in Fig. 2.

ments, five different animals, and two field organisms have been simplified in Table 1, to make comparison easier. For instance, no metal was found to have accumulated in crayfish digestive gland, but metal-binding proteins were isolated. In hermit crabs, the digestion method suggested very high levels of zinc, but only very low levels of metal-binding protein was found.

In shrimps, the digestion method showed slightly greater copper and zinc concentrations, but a very much higher level of zinc metal-binding protein level was found. In mussel, the higher zinc levels were confirmed by the isolation of metal-binding proteins. In limpets, the diges-

tion method showed raised metal levels of copper and zinc, but metal-binding proteins for only zinc were isolated.

In *Bullia*, the high levels of cadmium determined by the digestion method were not reflected in the quantities of metal-binding proteins isolated, but copper- and zinc-binding proteins were present.

In the grass exposed to sludge, the following results were obtained by the digestion method: the cadmium level in grass exposed to sludge was five times that in the control: the copper level in sludge enriched grass was twice that in the control; and the zinc level in sludge enriched grass was three times that in the control. Al-

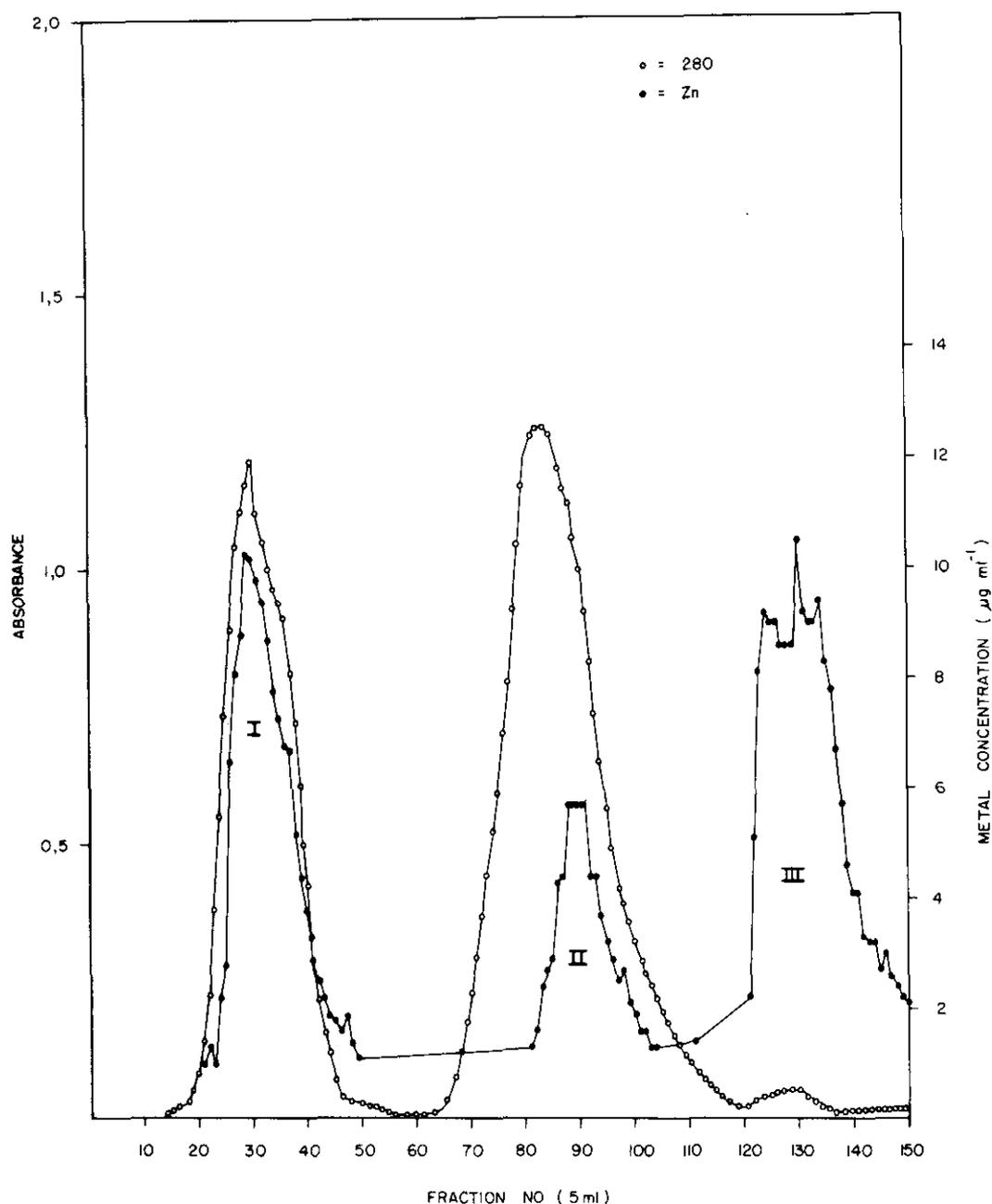


FIGURE 6. Elution profiles from Sephadex G-75 column chromatography of supernatant fractions from black mussel (*Choromytilus meridionalis*). Absorbance as in Fig. 2.

though all three metal concentrations were elevated, only zinc-binding proteins were found.

## Discussion and Conclusions

Until now, metal determination in biological systems has been based on the acid digestion method. This method has been employed in the "Mussel Watch Programme" (20), and in many other baseline studies (Hennig, manuscript in preparation). It has been shown that this approach has many drawbacks (21-24); for instance, differences in phyla, size, mass, age, food, and life stages

have usually been ignored. Some of these flaws can be overcome by sampling metal indicator species or organisms. This could be acceptable for baseline studies, although extrapolating should be avoided (24). For a pollution study the digestion method of metal determination is totally inadequate. For such a study the following questions are of major importance: (1) Is an organism under stress even although no metal accumulation can be measured? (2) Suppose metal accumulation can be detected, does it influence the normal physiology or behavior of the organism in any way?

In order to be able to answer the first question, a

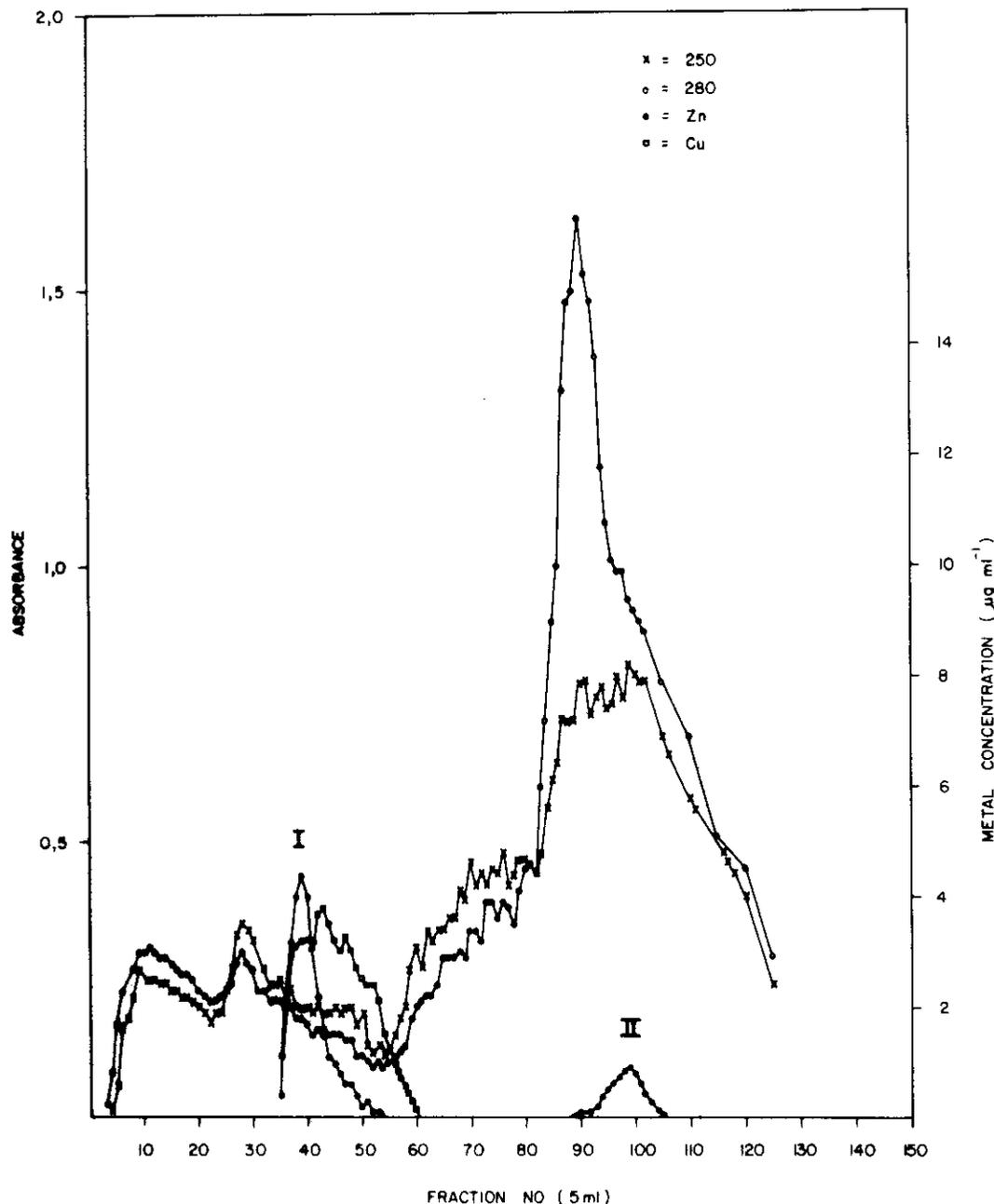


FIGURE 7. Elution profiles from Sephadex G-75 column chromatography of supernatant fractions from limpets (*Patella granularis*). Absorbance as in Fig. 2.

better, more sensitive assay for metal is required. The second question needs some definition of the influence on the "normal." From laboratory studies (2,3) and field studies (8,12,25) increasing metal accumulation in an organism takes place as follows: (a) metal concentration in the body fluids increases; (b) metal concentration in specific organs increases; (c) specific detoxification by metal-binding protein may occur, and MT are produced to chelate the excess metal from the body fluids; (d) metal concentration exceeds the production of chelates and metals "spill over" into the enzyme pool; (e) obvious toxic effects are observed, and the organism is visibly poi-

soned; (f) the condition of (e) continues until the death of the organisms.

The first two steps (a) and (b) could be due to variations within the same species; hence "deviation from the normal" starts with the third step (c). It is proposed that the production of a protein not specifically geared to growth or reproduction, as is the case of metal-binding protein, constitutes a significant "deviation from the normal" and is therefore a powerful indicator of pollution.

So far it has been difficult to link pollution and the presence of metal-binding proteins or MT. At low pollution levels some physiological change or effect of pollution

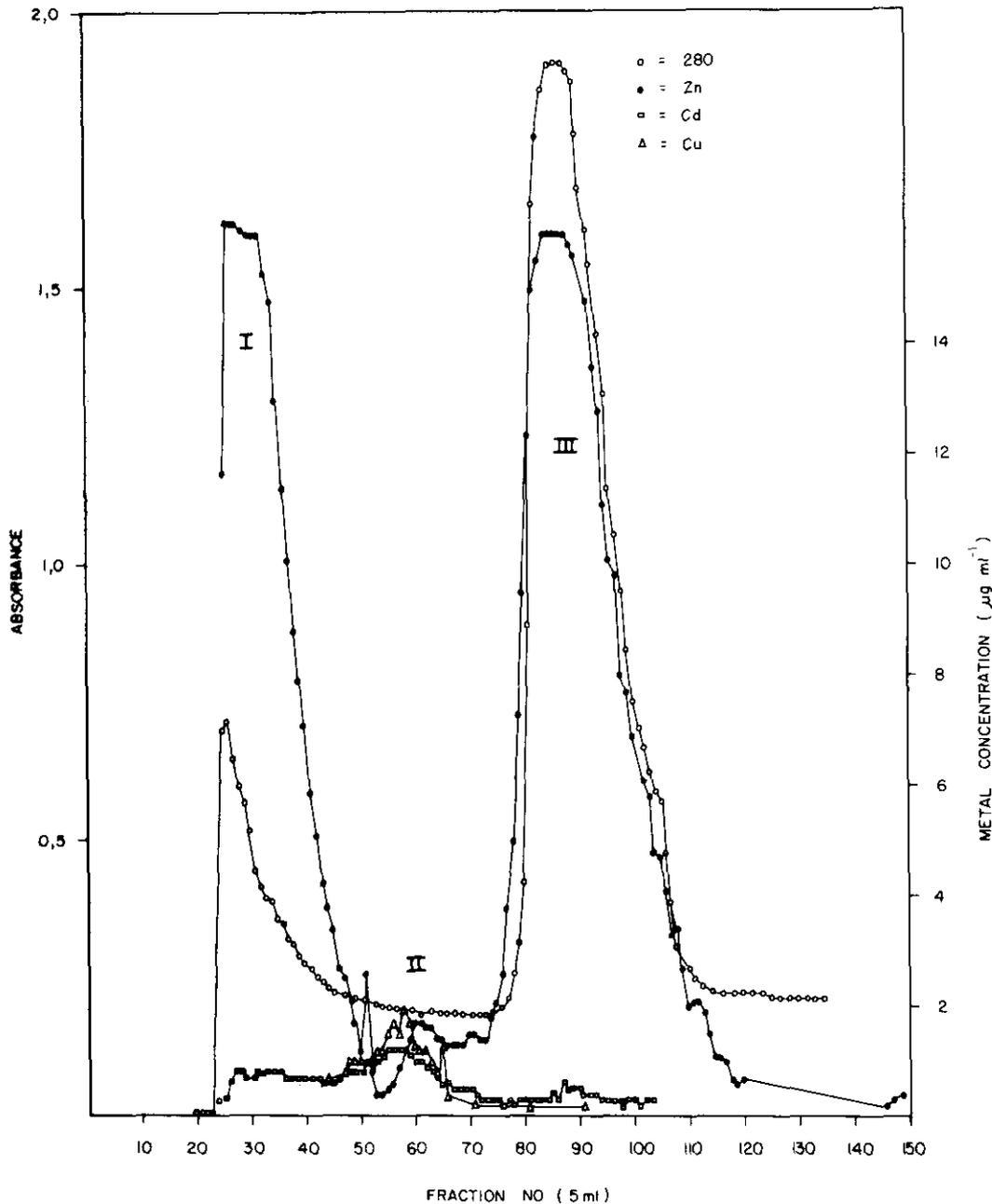


FIGURE 8. Elution profiles from Sephadex G-75 column chromatography of supernatant fractions from whelks (*Bullia digitalis*) collected from Koeberg. Absorbance as in Fig. 2.

had to be found. In this study it has been shown that no metal accumulation in crayfish could be detected by digestion method, but Hennig has shown that these crayfish moulted out of season and, furthermore, metal-binding proteins were found in these animals. This established the link between the presence of metal-binding protein in an organism which showed no detectable metal accumulation but showed obvious pollution symptoms.

If the above argument holds, the assessment of metal pollution may be simplified to: "The presence of metal binding proteins confirms toxic pollution." If this criterion is then applied to the other organisms in this study, the

following conclusions can be drawn for identical environmental conditions (Cu, 16 M/dm<sup>3</sup>; Zn, 15 M/dm<sup>3</sup>). In crayfish, copper had reached pollution levels while the animals could detoxify zinc at this level. These conclusions could not have been drawn from the determination of metals by the acid digestion method. The determination of metals in hermit crabs suggested toxic levels for zinc. This cannot be confirmed by the relative amounts of metal-binding proteins. Shrimp showed considerably higher toxic zinc levels by metal-binding protein than was to be expected by the digestion method. Both methods confirmed toxic pollution by zinc in mussels. Limpets accu-

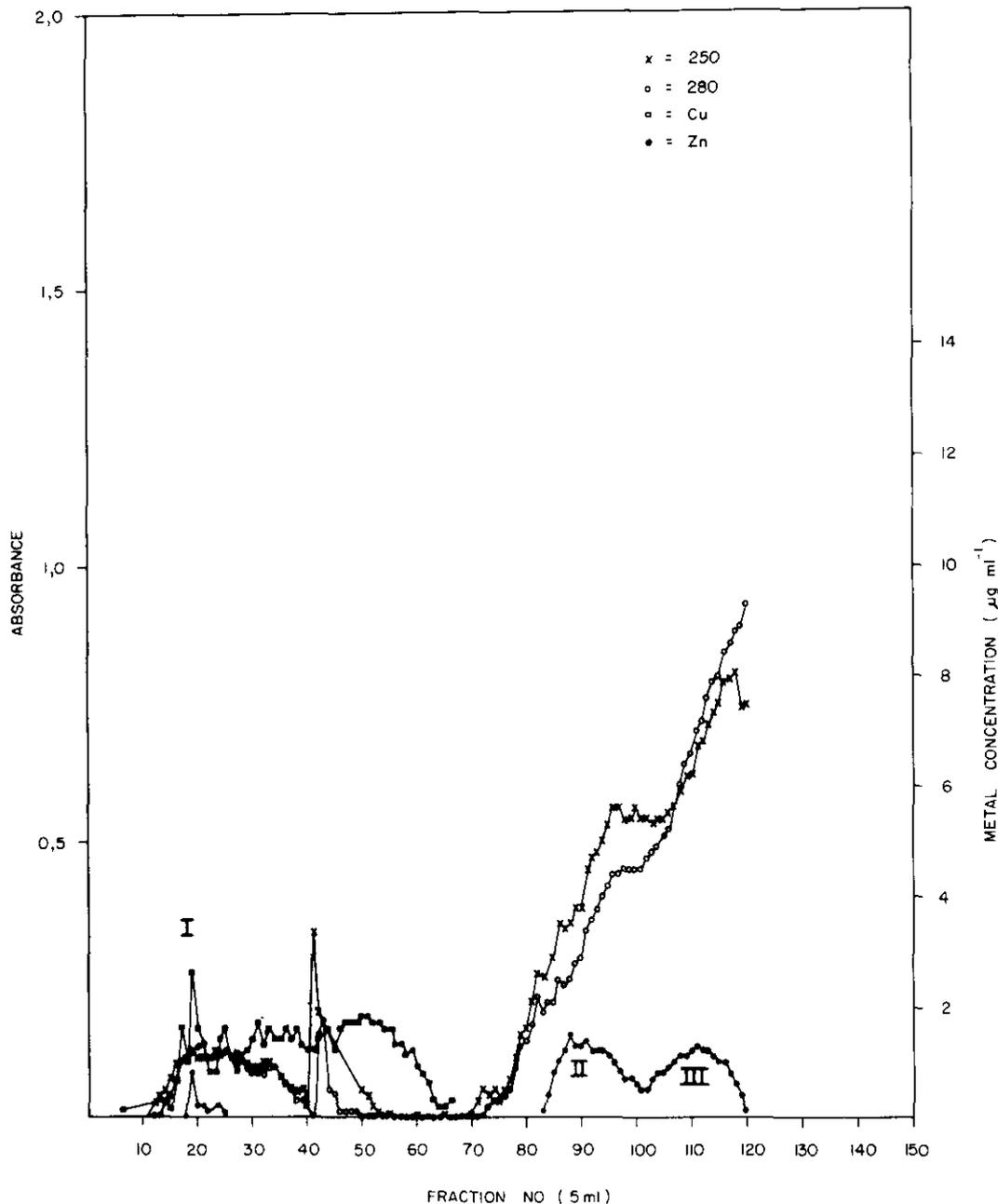


FIGURE 9. Elution profiles from Sephadex G-75 column chromatography of supernatant fractions from grass grown in 320 t/ha sludge.

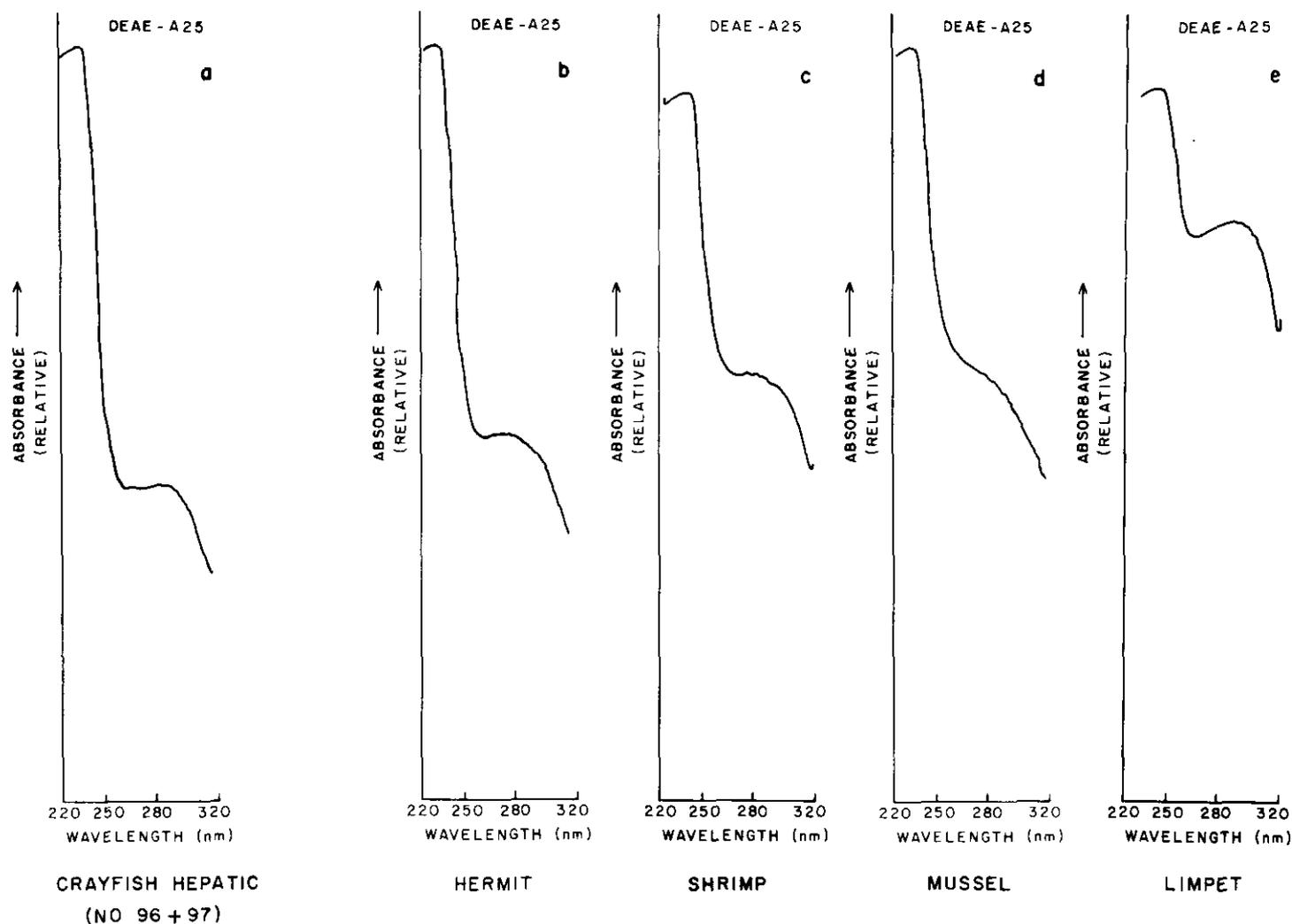


FIGURE 10. Ultraviolet absorption spectrum of the low molecular weight binding protein (pH 8.6) from: (a) crayfish digestive gland (4.121 mg); (b) hermit crab (9.490 mg); (c) sandshrimp (8.097 mg); (d) black mussel (5.924 mg), and (e) limpet (13.805 mg).

mulated copper and zinc but only the zinc was shown to be toxic. In the whelk *Bullia*, analysis of the metal-binding protein showed toxic levels of both cadmium and zinc. This result was unexpected, but it suggests the possibility that intracellular zinc accumulation may involve an active transport mechanism. It appeared that the cad-

mium exposure may have stimulated zinc transport into the soft tissues of the whelk. Similar results have been obtained by other workers (26), and these suggest that a unique mechanism for metal uptake may exist in some marine organisms.

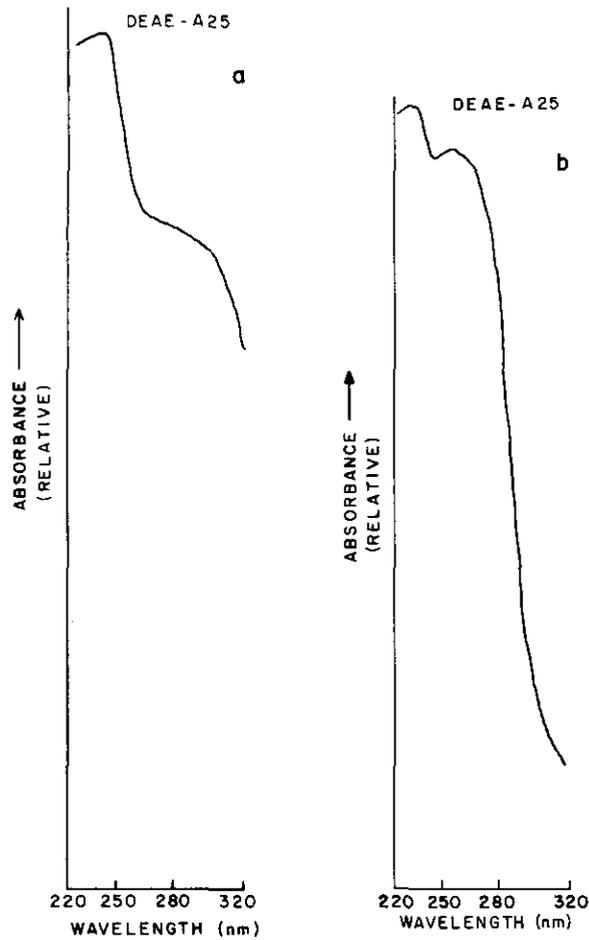
So far, very few reports have dealt with naturally oc-

Table 1. Diagrammatic presentation of results obtained from different experiments and field organisms with respect to different methods of determination and isolation.

Organism (effluent)	Metal of determination <sup>a</sup>		
	Total digestion	Metal-binding proteins	Isolation <sup>b</sup>
Crayfish (industrial)	Not detectable	Peak II Cd + Cu + Zn	Three fractions Cd + Cu + Zn
Crayfish (Cu + Zn)	Not detectable	Peak II Cu	Cu fraction (V <sub>0</sub> )
Hermit crabs (Cu + Zn)	>>Zn > Cu	Peak II Zn, Cu	Zn fraction (Cu)
Shrimp (Cu + Zn)	>Cu >Zn	Peak II >>Zn >Cu	Zn fraction
Mussel (Cu + Zn)	>Zn	Peak II Zn	Zn fraction
Limpet (Cu + Zn)	>Cu >Zn	Peak II Zn	Zn fraction (Cu)
<i>Bullia</i> (field)	>>Cd >Zn	Peak II Cd Zn Cu	Two fractions Cd + Zn (Cu)
Grass (sludge)	>>Zn >>Cd >Cu	Peak III Zn	Cu, Zn (V <sub>0</sub> ) fraction

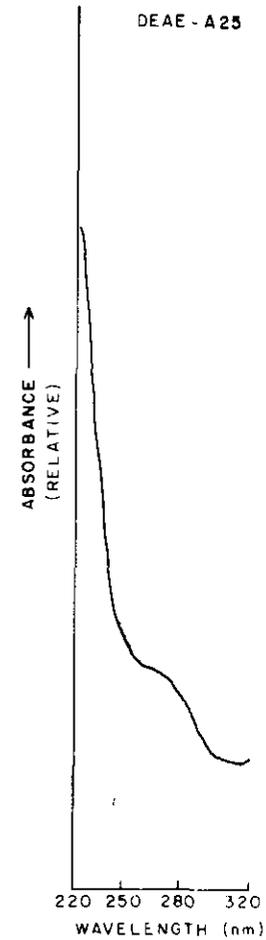
<sup>a</sup>>> = high metal concentration; > = lower metal concentration.

<sup>b</sup>V<sub>0</sub> = Elution at V<sub>0</sub> of DEAE-Sephadex G-25 column. (Metal) = Peak (V<sub>0</sub>) isolated, but not further analyzed.



BULLIA (Cd PEAK)

BULLIA (Zn PEAK)



GRASS (PEAK 2 NO 2 + 3)

FIGURE 11. Ultraviolet absorption spectrum of the low molecular binding protein (pH 8.6) from (a) Bullia (peak II-Cd, 10.481 mg); (b) Bullia (peak III-Zn, 13.137 mg).

FIGURE 12. Ultraviolet absorption spectrum of the low molecular binding protein (pH 8.6) from grass, Peak II (19.838 mg).

Table 2. Amino acid analysis of isolated metal-binding protein from six invertebrates.

	Species					
	<i>Janus lalandii</i>	<i>Diogenes brevisrostris</i>	<i>Palaemon pacificus</i>	<i>Choromytilus meridionalis</i>	<i>Patella granularis</i>	<i>Bullia digitalis</i>
Lysine	23.2	27.7	25.5	27.4	41.7	18.0
Histidine	—	—	—	—	—	—
Arginine	0	0	1.6	0	0	0.6
Cysteic acid	—	—	—	—	—	—
Aspartic acid	4.2	5.5	9.6	8.7	3.4	8.4
Threonine	3.2	1.7	4.1	2.8	1.9	2.4
Serine	7.7	8.5	9.5	9.9	6.7	6.3
Glutamic acid	9.8	9.8	10.4	13.6	6.8	10.4
Proline	3.1	—	6.2	—	—	5.4
Glycine	16.1	26.5	21.0	20.4	14.8	22.2
Alanine	5.5	9.7	8.4	7.3	5.3	7.3
Valine	1.2	1.2	1.1	0	1.5	4.6
Methionine	—	—	—	—	—	—
Isoleucine	2.5	2.5	2.6	3.6	1.6	5.0
Leucine	1.7	4.0	4.9	5.7	1.3	7.4
Tyrosine	—	0.6	0.9	—	0.4	0.2
Phenylalanine	0.2	—	2.1	0.9	0.4	0.2

curing metal-binding protein (27–30), but it shows that this method can very well be applied to the environment.

A very convincing argument for the proposed new definition of pollution can be found in the study of grass grown in sludge. Cadmium levels increased drastically and the absolute levels of copper were very high, yet no ill effect or pollution could be found. The grass was obviously able to cope with these high levels. Zinc, on the other hand, had a toxic effect.

From the evidence presented here, it is argued that metal-binding protein determinations should replace metal analyses in pollution work. This circumvents and largely eliminates differences due to animal size, mass, age, behavior, food, or life stage. Furthermore, so far only metal-binding proteins for cadmium, copper, mercury, and zinc have been reported, but that does not mean they do not exist for other metals.

The low molecular weight (Figs. 2–9) and absorption spectra (Figs. 10–12), together with the amino acid compositions (Table 2) of the isolated metal-binding protein from the seven organisms presented here, suggest that these proteins all belong to the family of metallothioneins.

Induced metallothioneins have been isolated from a vast variety of organisms, ranging from humans to earthworms, bacteria, and plants. It can therefore be assumed that metal-binding proteins are produced by all animals in some form or other. Fin fish, for instance, appear to be so sensitive to metals that small amounts of metalloproteins are found in so called “control” animals. Fortunately, fin fish are rarely used as metal pollution indicators organisms.

In conclusion it is hoped that the great interest and enormous effort devoted to metal determination in biological systems will be redirected to a more meaningful analysis of metal-binding protein.

Financial support from the South African National Programme for Environmental Sciences is gratefully acknowledged. I also wish to thank the Center for Biochemical Engineering, Duke University, Durham, North Carolina, for their support.

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