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Effect of Chelating Agents on the Accumulation of Cadmium by the Barnacle *Semibalanus balanoides*, and Complexation of Soluble Cd, Zn and Cu

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ABSTRACT: The effect of the presence of the chelating agents humate, alginate and EDTA on the accumulation of dissolved cadmium from seawater by the barnacle *Semibalanus balanoides* (L.) has been investigated. Humate and alginate significantly reduced the accumulation of cadmium after 7 d but any reduction in accumulation after 15 and 30 d was not significant. EDTA significantly reduced cadmium accumulation throughout 30 d exposure. It is concluded that the amount of cadmium accumulated by the barnacles is a function of the level of free Cd^{2+} ions available in solution. Soluble Cd, Zn and Cu in the barnacles are bound to high (> 30,000 MW) molecular weight proteins and to low molecular weight compounds eluting at or below the lower limit of linear separation of the gel Sephadex G-50 (~ 1500 MW). There was no evidence for the binding of Cd, Zn and Cu to metallothionein-like proteins. The presence externally of dissolved cadmium complexed with low molecular weight metabolites extracted from barnacles, resulted in no change of cadmium accumulation by barnacles, indicating that the uptake of cadmium does not require the external binding of Cd ions with released metabolites before entry into the barnacle.

INTRODUCTION

It is well known that heavy metals like zinc, copper and cadmium are accumulated by many marine organisms (see Wright, 1978, for review) including crustaceans such as barnacles (Walker et al., 1975; Walker, 1977; Walker and Foster, 1979). Little is known, however, of the factors that affect the uptake, storage and elimination of metals by marine organisms (Coombs and George, 1978). Studies of the effects of chelating agents on metal accumulation may provide information on mechanisms of metal uptake, and Coombs and George (1978) have illustrated possible methods of the transport of heavy metals across the cell membrane, a process which may for example involve the binding of the metal ion to a hydrophobic ligand.

This study therefore investigates the effect of chelating agents on the accumulation of cadmium by the barnacle *Semibalanus balanoides*. The chelating agents used are alginate, humate and ethylenediaminetetra-acetic acid (EDTA). Alginates are polysaccharide components of the cell walls of large laminarian brown algae which are released into seawater by the breakdown of the algae. Alginates have an affinity for divalent metals such as cadmium (Muzzarelli, 1973) and thus are naturally occurring chelating agents. Humic acids are a major constituent of humic substances which are decomposition resistant compounds making up the bulk of the organic matter in most soils and waters (Schnitzer and Khan, 1972). Humic substances have an appreciable exchange capacity and can complex metal ions (Schnitzer, 1971). EDTA is a manufactured chelating agent used widely in washing powders and may be found in high levels in sewage effluents (Gardiner, 1975); it is a strong agent which readily chelates with divalent metals.

George and Coombs (1977) found that the presence of chelating agents increased the rate of accumulation and final tissue concentrations of cadmium in *Mytilus edulis*, and later commented (Coombs and George, 1978) that the results were in accord with a process of carrier-assisted diffusion and that 'whether the carrier is a metallothionein-like polypeptide secreted from the cell . . . is a question that awaits further investigation'. Metallothioneins are heavy metal-binding proteins with characteristic amino acid compositions and low molecular weights; they are typically induced by exposure to certain heavy metals such as cadmium (see Cherian and Goyer, 1978, for review). Metallothioneins were originally isolated from mammals and similar heavy metal-binding proteins have since been found in invertebrates such as limpets (Howard and

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Nickless, 1975, 1977a), mussels (Noël-Lambot, 1976) and crabs (Jennings et al., 1979; Olafson et al., 1979; Overnell and Trewhella, 1979). In an attempt to investigate the question posed above, cadmium bound to low molecular weight proteins, polypeptides or smaller organic molecules has been isolated from cadmium-exposed barnacles and used in turn as a cadmium source for accumulation by further barnacles.

The binding of metals to proteins and/or lower molecular weight compounds by marine invertebrates probably plays a role in the transport, storage, elimination and thus detoxication of accumulated heavy metals and so this study also investigates the complexation of soluble zinc and copper in addition to cadmium, by the barnacle *Semibalanus balanoides*.

MATERIAL AND METHODS

Effect of Chelating Agents on the Accumulation of Dissolved Cadmium

Large individuals of *Semibalanus balanoides* (L.) – at least 3 years old – attached to the shells of the mussel *Mytilus edulis* L., were collected from Southend-on-Sea, Essex, in late November 1978. The mussel shells were cleared of any tissue and both mussel and barnacle shells were scrubbed to remove traces of organic material. The barnacles were maintained starved at about 13 °C. Crisp and Patel (1960) and Ritz and Crisp (1970) have shown that during December in Britain the barnacle is in a non-feeding, non-moulting phase of anecdysis and so no complications arose from the loss with moulting of any cadmium adsorbed onto the exoskeleton, nor from the possible uptake of cadmium adsorbed onto any food source that might otherwise be required.

The chelating agents to be tested were EDTA (ethylenediaminetetra-acetic acid, disodium salt; BDH Ltd., Poole, Dorset, UK), sodium alginate (Type IV, origin *Macrocystis pyrifera;* Sigma Chemical Co., Poole, Dorset, UK) and humic acid (technical grade, Aldrich Chemical Co. Ltd., Gillingham, Dorset, UK). Artificial seawater (Instant Ocean, Aquarium Systems Inc., Ohio) was used in all experiments in an attempt to eliminate the presence of unknown dissolved organic molecules which might act as chelating agents.

Stock solutions of each chelating agent were therefore made up in Instant Ocean (100 μM for EDTA and 1.05 g l^{-1} for sodium alginate). Humic acid was more insoluble and so a saturated solution of humic acid in Instant Ocean with excess solid was vacuum filtered through a 3 μm filter and the filtrate (calculated to contain 1.1 μg humic acid ml^{-1}) was used as the stock solution. Such high concentrations of chelating agents were used to ensure that the chelating agent under test would be present in excess in the final experimental solution. A stock solution of 10 μg ml^{-1} Cd in the form of Cd Cl_2 in distilled water was also prepared. Experimental vessels (acid washed beakers) contained 98 ml Instant Ocean, 1 ml stock solution of one chelating agent (or 1 ml Instant Ocean alone in the case of the control) and 1 ml of the cadmium stock solution, giving a final concentration of 0.1 μg Cd ml^{-1}. The experiment was set up with 15 vessels each containing approximately 20 barnacles representing each of the 4 experimental conditions, viz: uncomplexed dissolved cadmium; dissolved cadmium complexed with EDTA (Cd-EDTA); dissolved cadmium complexed with sodium alginate (Cd-alginate) and dissolved cadmium complexed with humic acid (Cd-humate). The temperature was maintained at 13 °C and the vessels were continuously aerated. Experimental solutions were changed after 2, 5, 12, 19 and 26 d to restore regularly the concentrations of cadmium and chelating agents to initial levels. After 7, 15 and finally 30 d, barnacles from 5 vessels in each experimental condition were sacrificed for cadmium analysis, as was an initial sample to record a starting cadmium concentration. The bodies of the barnacles were dissected out and deep frozen before being dried at 50 °C, weighed and digested in concentrated nitric acid (Aristar grade, BDH) at 100 °C before being made up to a known volume with distilled water. Cadmium and also calcium levels were measured on a Varian AA 375 atomic absorption spectrophotometer using background correction. Lanthanum chloride (1 % w/v in sample solutions) was added to reduce interference during calcium measurement.

The cadmium concentrations of the barnacles have been compared statistically using parametric tests. The data were not normally distributed and were therefore transformed by application of Taylor's power law. Transformed means have been compared using Student's *t*-test after initial F-tests have confirmed that significant values of t have not been produced by significant differences in the respective variances.

Complexation of Soluble Cd, Zn and Cu

Large individuals of *Semibalanus balanoides* (at least 3 years old) were collected from two sites, Dulas Bay, Anglesey, and Southend-on-Sea, Essex. Dulas Bay receives water from Afon Goch, a river known to contain extremely high levels of particular metals such as zinc (5550 μg l^{-1}) and copper (2850 μg l^{-1}), and also a relatively high level of cadmium (3.9 μg l^{-1}) (Foster, 1976). Barnacles from this site have therefore been exposed to extremely high concentrations in the field whereas levels of heavy metal exposure at Southend-on-Sea were considered likely to be more typical of

coastal water. Preston et al. (1972), for example, have recorded mean metal levels in the North Sea as 3.1 μg l^{-1} zinc, 0.48 μg l^{-1} copper and 0.41 μg l^{-1} cadmium. The barnacles from Southend-on-Sea were attached to mussel shells which were cleaned of all traces of organic material by scrubbing. The Southend barnacles were then divided into three batches of approximately 200 barnacles each. Each batch was exposed to either zinc (1 μg ml^{-1} for 27 d, copper (1 μg ml^{-1} for 27 d) or cadmium (1 μg ml^{-1} for 50 d). The chloride of each metal was dissolved in Instant Ocean to give the required final concentration and all vessels were aerated. Barnacles were exposed to cadmium for the longer period to ensure that measurable levels of absorbed metal would be achieved. The experimental media were changed weekly to restore original metal concentrations.

The bodies (thorax + prosoma) of the barnacles exposed in the laboratory, control barnacles from Southend-on-Sea and barnacles from Dulas Bay were dissected from their shells and stored deep frozen before being homogenised in an approximately equal known volume of 0.025 M phosphate buffer (pH 7.0). The homogenate, which was kept below 10 °C at all times, was divided into two aliquots. One aliquot was dried at 50 °C, weighed and acid digested in Aristar HNO_3 at 100 °C. The other aliquot was centrifuged at 25,000 g for 3 h at 4 °C and the residue dried at 50 °C, weighed and acid digested. Aliquots of the supernatant were separated by Sephadex gel chromatography using a column of 75 × 2.6 cm containing Sephadex G-50 superfine (separating proteins of molecular weights between 1500 and 30,000) or a column of either 60 × 2.6 cm or of 40 × 1.6 cm containing Sephadex G-75 (separating proteins of MW 3000–70,000). Samples were eluted with 0.02 M Tris-HCl buffer (pH 8.6), at approximately 9 ml h^{-1}, the temperature being maintained at 4 °C. Columns were calibrated with proteins of known molecular weight viz: dextran blue, ovalbumin, chymotrypsinogen A and cytochrome C. The optical absorbance of the column effluent was monitored at 280 nm (Uvicord II, L.K.B.) and recorded with a 6-channel chopper-bar recorder (L.K.B.), and fractions were collected in an Ultrorac fraction collector (L.K.B.). The metal contents of fractions and acid digests were measured by atomic absorption spectrophotometry as in the previous experiment.

Action of Extracted Metabolites as Chelating Agents

Southend-on-Sea *Semibalanus balanoides* exposed to cadmium in the previous experiment, were used to provide a cadmium source for further absorption studies. The bodies of the cadium-exposed barnacles were treated as described in the previous experiment and two aliquots of the resulting supernatant were passed through the Sephadex G-75 column. All fractions from the two elutions that contained cadmium bound to low molecular weight metabolites (< 3000 MW), were pooled and the total amount of complexed cadmium was measured. The pooled fractions were then diluted in Instant Ocean to give a final concentrations of 0.07 μg Cd ml^{-1} in the experimental vessels. It is not certain that all the extracted metabolites did dissolve in the Instant Ocean as a small amount of precipitation was observed. The concentration of 0.07 μg Cd ml^{-1} was used rather than 0.10 μg Cd ml^{-1} as in the previous experiment with chelating agents, because it was necessary to make up a minimum volume for the experiment and the initial amount of bound cadmium available was limited. A solution of 0.07 μg Cd ml^{-1} as $CdCl_2$ in 'Instant Ocean' was made up as a control with the addition of the same volume of 0.02 M Tris-HCl buffer as was present in the pooled fractions above.

Semibalanus balanoides were collected from Southend-on-Sea as in the first experiment and similarly cleaned. 50 barnacles were placed in each of six acid-washed vessels, three of which contained 60 ml of 0.07 μg ml^{-1} cadmium bound to low molecular weight proteins with the remaining three vessels containing 0.07 μg ml^{-1} uncomplexed cadmium as the control. The temperature was maintained at 13 °C and the vessels were continuously aerated. The barnacles were exposed to the cadmium for 10 d, the solutions being changed after 5 d. The bodies of the barnacles were prepared as described in the first experiment and cadmium levels measured.

RESULTS

Effect of Chelating Agents on the Accumulation of Cadmium

The effect of the chelating agents EDTA, humate and alginate on the accumulation of cadmium from solution by *Semibalanus balanoides* is shown in Figure 1 and Tables 1 and 2. Barnacles accumulated cadmium from solution under all situations over the whole period of 30 d. The barnacles exposed to cadmium complexed with EDTA accumulated significantly less cadmium over the experimental period than barnacles exposed to uncomplexed cadmium or to cadmium complexed with humate or alginate. After 7 d the barnacles exposed to cadmium complexed with either humate or alginate had accumulated significantly less cadmium than those exposed to non-complexed cadmium, but

Table 1. *Semibalanus balanoides*. Means ± 1 standard deviation of accumulated cadmium concentrations ($\mu g\ g^{-1}$ dry wt) of barnacle bodies showing the effect of the chelating agents EDTA, alginate and humate on the accumulation of Cd from solution (0.1 μg Cd ml^{-1} for up to 30 d)

Form of complexing of dissolved Cd	Time (d)			
	0	7	15	30
Uncomplexed Cd	10.62	42.18 ± 7.12	58.27 ± 13.22	119.33 ± 22.14
Cd-EDTA	10.62	22.84 ± 4.27	22.77 ± 4.54	35.32 ± 8.15
Cd-alginate	10.62	30.04 ± 6.40	43.30 ± 9.54	111.83 ± 8.95
Cd-humate	10.62	30.43 ± 5.97	52.41 ± 6.51	103.61 ± 34.36

Table 2. *Semibalanus balanoides*. Student's *t*-test comparisons of transformed means of cadmium concentrations of bodies of barnacles exposed to complexed and uncomplexed dissolved cadmium (8 degrees of freedom in most cases). Data have been transformed by application of Taylor's power law to ensure normal distribution and thus validity for the use of parametric tests. Previous use of *F*-tests has confirmed that significant differences in variances have not produced the significant values of *t* observed. • significant difference (i. e. $P < 0.05$ that the observed difference in means would have occurred by chance if the means were from the same population); •• highly significant difference ($P < 0.01$); ••• very highly significant difference ($P < 0.001$). The difference between means is not significant at the 5% level when no asterisks are shown

Comparison of forms of complexing of dissolved Cd	Time of exposure (d)		
	7	15	30
Uncomplexed Cd v. Cd-EDTA	5.64•••	6.44•••	7.91•••
Uncomplexed Cd v. Cd-alginate	2.64•	2.05	0.51
Uncomplexed Cd v. Cd-humate	2.92•	0.76	0.91
Cd-humate v. Cd-alginate	0.14	1.83	0.62
Cd-humate v. Cd-EDTA	2.41•	7.66•••	4.72••
Cd-alginate v. Cd-EDTA	1.93	4.73••	8.11•••

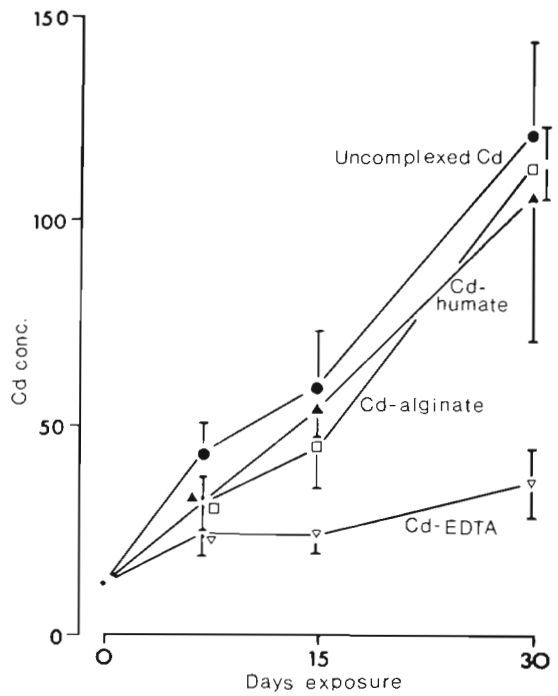

Fig. 1. *Semibalanus balanoides*. Increasing cadmium concentrations (μg Cd g^{-1} dry wt) in barnacles exposed to 0.1 μg Cd ml^{-1} for 30 d in the presence of complexing agents EDTA, alginate or humate, or dissolved cadmium in the absence of any added complexing agent

after 15 d and 30 d there was no significant difference in the accumulation under any of these three situations. Barnacles accumulating cadmium complexed with humate never significantly differed in cadmium concentration from those accumulating cadmium complexed with alginate.

A relationship between cadmium uptake and calcium metabolism has been observed in another crustacean, the crab *Carcinus maenas*, by Wright (1977b) and so the calcium concentrations of the barnacle bodies were also measured. No significant correlation (positive or negative) was found between cadmium and calcium concentrations of the barnacle bodies (r = 0.159 for 57 degrees of freedom using transformed data).

Complexation of Soluble Cd, Zn and Cu

Barnacles from Dulas Bay: Barnacles collected from Dulas Bay would have experienced extremely high levels of zinc and copper, and appreciable levels of cadmium in the field. Table 3 shows the zinc, copper and cadmium concentrations of the *Semibalanus balanoides* collected from Dulas Bay (and also from Southend-on-Sea) and compares these concentrations with others reported in the literature. As can be seen the zinc concentration of the Dulas Bay barnacles,

Table 3. Comparison of zinc, copper and cadmium concentrations ($\mu g\ g^{-1}$ dry wt) of barnacles in the present study and of others as reported in the literature. –: not measured, n. d.: not detectable

	Zn	Cu	Cd		References
Semibalanus balanoides					
Southend-on-Sea, Essex, UK	27 837	232	10-28	Body	This study
Dulas Bay, Anglesey, Wales	113 250	3232	60	Body	This study
Dulas Bay, Anglesey, Wales	50 280	3750	n. d.	Body	Walker (1977)
Dulas Bay, Anglesey, Wales	82 000	2470	–	Body	Walker, personal communication
Menai Strait, N. Wales	1220-19 230	175-290	–	Body	Walker, personal communication
Menai Strait, N. Wales	–	170	–	Body	Walker (1977)
Menai Strait, N. Wales	5140	–	–	Soft tissue	Walker et al. (1975)
Aberffraw, Anglesey, Wales	–	370	–	Body	Walker (1977)
Aberystwyth, Wales					
Constitution Hill	4500 ± 90	–	–	Body	Ireland (1973)
Constitution Hill	3000-12 000	100-300	–	Body	Ireland (1974)
Alltwen	23 100 ± 1100	–	–	Body	Ireland (1973)
Alltwen	7000-30 000	100-700	–	Body	Ireland (1974)
Alltwen	8850	–	–	Soft tissue	Walker et al. (1975)
Castle Point	8300 ± 300	–	–	Body	Ireland (1973)
Castle Point	10 515	–	–	Soft tissue	Walker et al. (1975)
R. Ystwyth estuary	17 190	–	–	Soft tissue	Walker et al. (1975)
Woods Hole, USA	–	104	–	Soft tissue	Clarke (1947)
Buzzard's Bay, USA	–	68	–	Soft tissue	Clarke (1947)
Balanus amphitrite					
La Jolla, California	910	–	–	Body	Alexander & Rowland (1966)
Venice, N. Adriatic	–	96-109	–	Soft tissue	Barbaro et al. (1978)
Grado, N. Adriatic	–	41- 54	–	Soft tissue	Barbaro et al. (1978)
Rio Tinto Estuary, Spain	1780-3300	550-600	12	Soft tissue	Stenner & Nickless (1975)
Balanus perforatus					
Portugal	40-60	8	6	Soft tissue	Stenner & Nickless (1975)
Balanus eburneus					
Cotuit, USA	–	8-16	–	Soft tissue	Clarke (1947)
Balanus crenatus					
R. Conwy Estuary, Wales	14 010	–	–	Soft tissue	Rainbow (unpublished)
Chirona hameri					
Irish Sea	910	–	–	Soft tissue	Rainbow (unpublished)
Chthamalus stellatus					
Portugal, S. W. Spain	100-237	6-10	6	Soft tissue	Stenner & Nickless (1975)
Pollicipes mitella					
E. Coast, Hong Kong	370	–	–	Soft tissue	Rainbow (unpublished)
Pollicipes polymerus					
R. Columbia, USA	1490-2090	–	–	Body	Alexander & Rowland (1966)
Lepas anatifera					
N. Atlantic	690	–	–	Soft tissue	Walker et al. (1975)
N. Atlantic	393	–	–	Soft tissue	Bertrand & Vladesco (1923)

which were very large specimens several years old, is the highest on record but does agree with the results of Walker who also detected very high concentrations in Dulas Bay barnacles (Walker, 1977 and personal communication) and found that the zinc content of the body of *S. balanoides* continues to increase during the life of the barnacle (Walker and Foster, 1979). The copper concentration of the same barnacles again agrees well with the results of Walker (1977 and personal communication) and like the zinc concentration is extraordinarily high because of the elevated ambient levels of these two metals in Dulas Bay. There are few cadmium concentrations of barnacles reported in the literature, and the level measured in barnacle from Dulas Bay is again the highest recorded.

Figure 2 shows the elution profile of the supernatant of barnacle bodies after after passage through Sephadex G-75 (separating proteins of MW 3000–70,000). Most soluble zinc is bound either to larger proteins of molecular weight above 70,000 or to low molecular compounds below about 3000 MW. The supernatant extracted from further such barnacles was therefore passed through Sephadex G-50 superfine which separates proteins of molecular weight between 1500 and 30,000 MW (Fig. 3). Although the resolution was not as clear as in the case of the Sephadex G-75 elution, this profile confirms that soluble zinc is again mostly bound to both high molecular weight protein and low molecular weight material. The low molecular weight compounds peak near the lower resolution

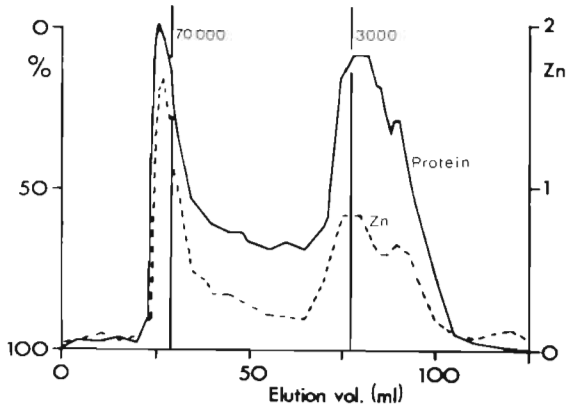

Fig. 2. *Semibalanus balanoides*. Elution profile of supernatant of homogenised barnacles from Dulas Bay, Anglesey, after passage down a 60 × 2.6 cm Sephadex G-75 column. Protein levels indicated by % transmittance when monitoring optical absorbance at 280 nm, except at low molecular weights where other compounds such as free amino acids will also absorb. Vertical lines: elution volumes corresponding to proteins of high (70,000) and low (3,000) molecular weight. Zinc concentration is in μg ml^{-1}

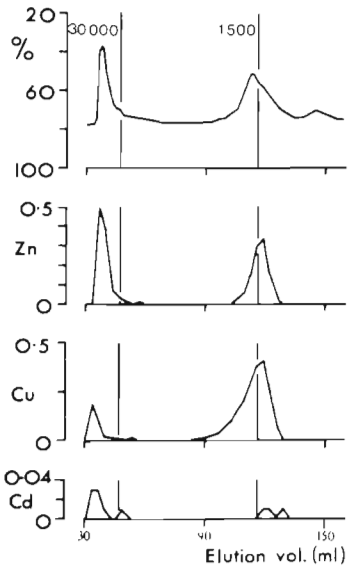

Fig. 3. *Semibalanus balanoides*. Elution profile of supernatant of homogenised barnacles from Dulas Bay, Anglesey, after passage down a 75 × 2.6 cm Sephadex G-50 superfine column. Protein levels indicated by % transmittance at 280 nm and all metal concentrations are in μg ml^{-1}. Vertical lines: elution volumes corresponding to proteins of 30,000 or 1,500 MW

limit (1500 MW) of the gel, and so the exact molecular weight of 2291 at the top of the peak is only an approximate value. The low MW peak of the zinc profile correlates with compounds of MW 2178. The G-50 eluted fractions were also monitored for soluble copper and cadmium which each had a similar distribution to that for zinc, although in the case of both copper and cadmium the metal peak corresponding to the lower MW material is the higher peak. The copper and cadmium levels peaked in the fraction after the zinc peak, both the former metals therefore corresponding to compounds of about 1914 MW. Since fractions eluted in this region of the profile are so close to the lower limit of linear separation of the gel, small, possibly insignificant, differences in elution volume correspond to relatively large differences in the calculated molecular weight of the compounds eluted. It appears therefore that soluble zinc, copper and cadmium are bound to metabolites eluting approximately simultaneously at an elution volume corresponding to compounds of about 2000 MW.

Barnacles from Southend-on-Sea: *Semibalanus balanoides* from Southend-on-Sea contained what seemed at first sight a surprisingly high concentration of zinc (Table 3), although not by any means as high as that of the barnacles from Dulas Bay. The high zinc level is explained by the fact that the barnacles selected for analysis were the largest available to ensure the presence of measurable levels of trace metals, and were therefore several years old and Walker and Foster (1979) have shown that *S. balanoides* continues to accumulate body zinc throughout its lifetime. Moreover the barnacles were collected at a time of the year when the body weight is low and so the concentration of body zinc would be relatively high (Walker and Foster, 1979). The copper concentration of these barnacles is in line with other copper concentrations recorded in barnacle tissues (Table 3). The cadmium concentration of the Southend *Semibalanus balanoides* is higher than the few cadmium concentrations reported (probably again as a result of the age of the barnacles analysed and the time of collection) but lower than the concentration measured in Dulas Bay barnacles.

As described in 'Material and Methods', Southend-on-Sea *Semibalanus balanoides* were exposed to high levels of zinc, copper and cadmium in the laboratory and accumulated metal levels are shown in Table 4. Barnacles exposed to copper and cadmium showed 3097 % and 2639 % respective increases in exposed metal concentration over that of the non-exposed control. The smaller percentage increase (32 %) in zinc concentration in the zinc-exposed barnacles over the non-exposed control is probably simply a reflection of the high concentration of zinc already accumulated in the barnacles.

Figure 4 shows the elution profile of the supernatant extracted from the bodies of the non-exposed control barnacles collected from Southend-on-Sea, after passage through Sephadex G-50 Superfine. As in the case of the barnacles from Dulas Bay, zinc is bound to high molecular weight proteins and to compounds at about the lower limit of linear separation of the gel. In this

Table 4. *Semibalanus balanoides*. Zinc, copper and cadmium concentrations ($\mu g\ g^{-1}$ dry wt) in the bodies of barnacles from Southend-on-Sea exposed to zinc, copper or cadmium (1 $\mu g\ ml^{-1}$ for 27 d in the case of Zn and Cu, 50 d in the case of Cd) and in the bodies of non-exposed control barnacles. Figures in parentheses indicate the relative proportions of each metal extracted by homogenisation in 0.025 M phosphate buffer (pH 7.0) (i. e. insoluble/soluble)

Condition	Zinc	Copper	Cadmium
Non-exposed control (% insoluble/% soluble)	27 837 (53/47)	232 (39/61)	28 (81/19)
Zinc exposed (% insoluble/% soluble)	36 737 (46/54)	308 (77/23)	40 (39/61)
Copper exposed (% insoluble/% soluble)	31 510 (35/65)	7417 (6/94)	41 (3/97)
Cadmium exposed (% insoluble/% soluble)	35 482 (28/72)	142 (74/26)	767 (46/54)

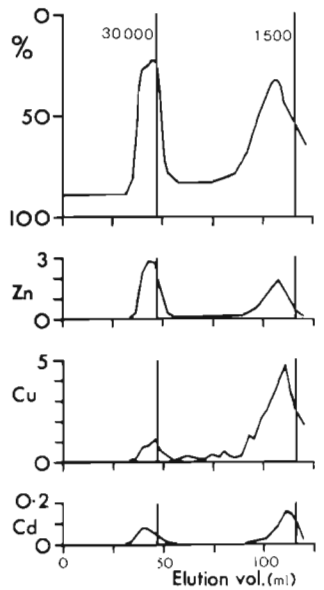

Fig. 4. *Semibalanus balanoides*. Elution profile of supernatant of homogenised barnacles from Southend-on-Sea, Essex, after passage down a 75 × 2.6 cm Sephadex G-50 superfine column. Details as for Figure 3

case the second zinc peak corresponds to compounds of molecular weight below the 1500 MW mark. Copper shows a similar distribution to zinc, peaking in the same fractions although in the case of copper the peak corresponding to the lower molecular weight compounds is the larger peak (as was the case even more distinctly in the Dulas Bay barnacle profiles). Some cadmium is bound to high molecular weight proteins in the void volume but other levels measured are at the sensitivity limit of the technique and may not be significant. Thus zinc, copper and cadmium are all bound to high molecular weight proteins, and measurable levels of zinc and copper are also bound to lower molecular weight compounds at about 1500 MW.

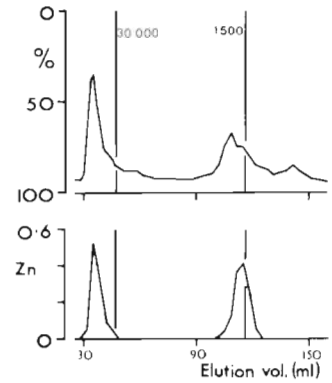

Fig. 5. *Semibalanus balanoides*. Elution profile of supernatant of homogenised barnacles collected from Southend-on-Sea, Essex, then exposed to 1 $\mu g\ ml^{-1}$ Zn for 27 d in the laboratory, after passage down a 75 × 2.6 cm Sephadex G-50 superfine column. Details as for Figure 3

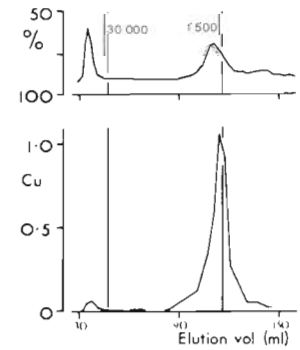

Fig. 6. *Semibalanus balanoides*. Elution profile of supernatant of homogenised barnacles, collected from Southend-on-Sea, Essex, then exposed to 1 $\mu g\ ml^{-1}$ Cu for 27 d in the laboratory, after passage down a 75 × 2.6 cm Sephadex G-50 superfine column. Details as for Figure 3

The distribution of extracted zinc from the bodies of barnacles exposed to zinc in the laboratory (Fig. 5) was very similar to that of the control barnacles (Fig. 4). This is not surprising considering the relatively small increase in the zinc concentration of the barnacles' bodies brought about by zinc exposure in the laboratory (Table 4). The second zinc peak in this case corresponds to compounds of 1585 molecular weight.

The distribution of extracted copper from the bodies of barnacles exposed to copper in the laboratory (Fig. 6) is different from that of control barnacles (Fig. 4). Much of the newly accumulated copper is now bound to the second copper peak (corresponding to compounds of 1648 MW). This copper profile is now closer to that of the Dulas Bay barnacles which have been exposed to very high copper levels in the field. The second copper peak is now much the larger and probably contains most of the extra copper accumulated (note that 94 % of the copper content of the bodies of

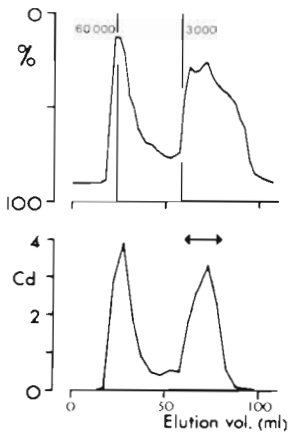

Fig. 7. *Semibalanus balanoides.* Elution profile of supernatant of homogenised barnacles collected from Southend-on-Sea, Essex, then exposed to 1 μg ml^{-1} Cd for 50 d in the laboratory, after passage down a 40 × 1.6 cm Sephadex G-75 column. Protein levels indicated by % transmittance at 280 nm and cadmium concentration is in μg ml^{-1}. Vertical lines: elution volumes corresponding to proteins of 60,000 or 3,000 molecular weight. Horizontal line: position of fractions used in subsequent experiment as part of chelated cadmium source

copper-exposed barnacles was extractable/soluble – see Table 4).

The distribution of soluble cadmium from the bodies of barnacles exposed to cadmium is shown in Figure 7. The second peak is now almost as large as the first (Fig. 4) and is below the lower limit of linear separation of the gel (in this case corresponding to compounds of 3000 MW). It is probable that this peak is directly equivalent to the peak seen on the Dulas Bay profile (Fig. 3) where the cadmium is apparently bound to compounds eluted at the same time as those binding zinc and copper. Sephadex G-75 was chosen in this case since fractions from this elution and from a second identical elution, were to be used in turn as chelating agents in the following experiment and Sephadex G-75 had given the better resolution in the initial separation of supernatants of barnacles from Dulas Bay (Figs 2 and 3).

It may be concluded, therefore, that the barnacles from Dulas Bay and from Southend-on-Sea show similar elution profiles for zinc, copper and cadmium and that exposure to increased metal concentrations appeared to cause relatively more metal to be bonded to the lower molecular weight compounds at about the lower limit of linear separation of Sephadex G-50 (viz 1500 MW).

Action of Extracted Metabolites as Chelating Agents

As described in 'Material and Methods' and shown in Figure 7, Sephadex G-75 fractions containing cadmium bound to low molecular weight compounds (< 3000 MW) as isolated from the bodies of *Semibalanus balanoides* exposed to cadmium, were used as a source of dissolved cadmium for absorption by further barnacles. Table 5 shows that barnacles exposed to cadmium chelated in this way reached a mean cadmium concentration of 56.18 ± 14.39 μg g^{-1} dry wt, whilst control barnacles exposed to uncomplexed cadmium reached 49.28 ± 7.38 μg Cd g^{-1} dry wt. There is no significant difference (Student's t test on transformed data; t = 0.68, 4 degrees of freedom) between these means and thus it can be concluded that the binding of cadmium to extracted metabolites has not affected the accumulation of cadmium by the barnacles.

Table 5. *Semibalanus balanoides.* Cadmium concentrations (μg g^{-1} dry wt) of bodies of barnacles exposed for 10 d either to cadmium complexed with low molecular weight metabolites previously isolated from cadmium-exposed barnacles of the same species or to uncomplexed cadmium. Each sample consists of 50 barnacles

Sample	Cd complexed with metabolites	Uncomplexed Cd
Sample 1	42.22	42.26
Sample 2	48.68	55.28
Sample 3	56.94	70.99
Mean	49.28	56.18
Standard deviation	7.38	14.39

DISCUSSION

When barnacles accumulated cadmium from solution the presence of chelating agents had variable effects. The presence of either humate or alginate significantly decreased the accumulation of cadmium after seven days but the observed reduction was not significant after fifteen and thirty days exposure. The presence of EDTA significantly reduced the accumulation of cadmium by the barnacles at all exposure times. These results differ from those of George and Coombs (1977) on the accumulation of cadmium by the mussel *Mytilus edulis* in the presence of the same chelating agents, when the chelating agents significantly increased cadmium accumulation. The present results do however agree with those of Fowler and Heyraud (1974; quoted by Wright, 1978) who found that complexation of ^{65}Zn substantially reduced its concentration by the shrimp *Lysmata seticaudata.*

The accumulation of cadmium by crustaceans at least and its toxicity to them would appear to be related to the level of free cadmium ion available. Mantoura et al. (1978) have produced interesting results on the complexation of cadmium along a model estuary. At 35 ‰ salinity the level of free Cd^{2+} is less than approximately 6 % of the total cadmium present

(Fig. 6; Mantoura et al., 1978), the majority of cadmium being present as complexed $CdCl^+$ or $CdCl_2^\circ$. At lower salinities the percentage of free Cd^{2+} rises to reach 100 % at 0 ‰ salinity. Mantoura et al. (1978) also found that there was no complexation of cadmium with the humic substances used at salinities higher than 5 ‰. Engel and Fowler (1979) showed that the toxicity of cadmium to the grass shrimp *Palaemonetes pugio* increased as salinity was decreased and concluded that their results could be interpreted in terms of the toxicity of cadmium being a function of free cadmium ion concentration, in turn controlled by the chloride concentration of the water. Experiments on the effect of an added chelating agent (nitrilotriacetic acid) on the toxicity of cadmium to the same shrimp at different salinities (Sunda et al., 1978) supported this interpretation. Other authors have also found that low salinities increase cadmium toxicity and accumulation in the case of marine organisms. Frank and Robertson (1979) for example established that cadmium was more toxic to the blue crab *Callinectes sapidus* at reduced salinities. Wright (1977a) similarly found that the crab *Carcinus maenas* accumulated cadmium faster in seawater of reduced salinity, and both Phillips (1976) and George et al. (1978) have shown that *Mytilus edulis* accumulates more cadmium when the salinity is decreased.

The present results may therefore be interpreted as a decreased accumulation of cadmium by the barnacles when the availability of free cadmium ions is decreased. Complexation with humate had almost no effect which suggests that the humic substance used in the experiment did not complex to any great extent, if at all, with the cadmium ions in seawater of normal salinity. This would agree with the results of Mantoura et al. (1978) remembering that it is by no means certain that the humate used in either case would be identical. It is likely that commercially available humate and alginate will also not be identical with naturally occurring material owing to the nature of the extraction and purification procedures used (Muzzarelli, 1973). Complexation of alginate to cadmium in this experiment would presumably have released a level of free cadmium ions similar to that released by complexation with humate. EDTA on the other hand is a strong chelating agent and would seem to have further strongly reduced the available free cadmium ions, already reduced as a result of complexation with inorganic chloride (Mantoura et al., 1978). The results of George and Coombs (1977), however, on the effect of chelating agents on the accumulation of cadmium by *Mytilus edulis*, can not be satisfactorily explained in terms of this interpretation although the effect of lower salinities increasing cadmium accumulation (George et al., 1978) would be expected.

The soluble moieties of metals like cadmium, zinc and copper are bound to proteins and to lower molecular weight compounds in the marine invertebrates that have been investigated (Howard and Nickless, 1975, 1977a, b, 1978; Noël-Lambot, 1976) even if metallothionein-like proteins are absent as in the case of oysters (Coombs, 1974; Howard and Nickless, 1975). The present study showed that all three metals were bound to proteins of high molecular weight and to compounds of molecular weight at or below the lower limit of linear separation (1500 MW) by Sephadex G-50. These low molecular weight compounds complexing with the heavy metals may be polypeptides or even smaller molecules such as betaines or amino acids. Taurine and homarine for example, have been implicated in the complexation of zinc and copper in oysters (Coombs, 1974; Howard and Nickless, 1977b) and similar compounds may also be involved in barnacles. There was no evidence of metal binding to proteins of intermediate molecular weight – the expected position of metallothionein-like heavy metal binding proteins, in contrast to the situation in another crustacean – the shore crab *Carcinus maenas* (Rainbow and Scott, 1979).

Coombs and George (1978) wrote that the role of metallothionein-like polypeptides in the uptake of heavy metals like cadmium by marine organisms awaited further investigation. In this study the external complexation of cadmium with extracted metabolites did not affect the accumulation of the dissolved metal by the barnacles. It can be concluded therefore that the uptake of cadmium by *Semibalanus balanoides* does not require the initial binding of cadmium to metabolites released into solution by the barnacles, although it has not been proved that such metabolites play no role in the uptake of cadmium, either when incorporated into cell membranes or within the cell as a later stage in the transfer of cadmium from the outside to the inside of the absorbing cells. The solution in seawater of cadmium complexed with extracted metabolites presumably made available an equal level of free cadmium ion for accumulation by barnacles, as was produced by the normal equilibrium in seawater of cadmium ions with inorganic chloride complexes.

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