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Zinc sequestration by earthworm (Annelida: Oligochaeta) chloragocytes

An in vivo investigation using fully quantitative electron probe X-ray micro-analysis

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Summary. The elemental compositions of chloragosome "granules" in the earthworm *Lumbricus rubellus* living in non-polluted and heavily Zn-polluted soils were determined by fully quantitative electron probe X-ray microanalysis. P, Ca, S and Zn were the major elemental components of the chloragosomes. The in vivo accumulation of Zn by the chloragosomes was accompanied by diminished chloragosomal Ca concentrations. Zn was apparently bound by at least two ligand pools (Pool 1 = uncharacterised; Pool 2 = P-containing ligands, binding approximately 45% and 55% of the Zn, respectively) in the "control" chloragosomes. In Zn-contaminated chloragosomes, most (~70%) was bound by P-containing ligand(s) but some (<1%) was also bound by S-containing ligands. It is suggested that the sequestration of Zn in chloragosomes results in the detoxification of the metal by accumulative immobilisation.

Introduction

Discrete metal-rich intracellular "granules" or "inclusion bodies" of considerable morphological and compositional diversity occur in certain tissues of invertebrates belonging to most of the major phyla (Simkiss 1976; Brown 1982; Morgan 1984; Morgan and Winters 1987).

One granule type that has received extensive attention is an organelle unique to the chloragogenous tissue of oligochaete annelids: the so-called chloragosome granule (e.g. Prentø 1979; Wróblewski et al. 1979; Morgan 1982; Morgan and Morris 1982; Morgan and Winters 1982). Earthworm chloragosomes are compositionally distinguishable from "typical" calcospherites (Simkiss 1976) because only 20% of their dry mass consists of inorganic material (Roots 1960; Urich 1960) compared with ~ 90% (value calculated from the data of Howard et al. 1981) in most other types. Furthermore, the organic matrix of chloragosomes is a highly complex mixture of carbohydrates (Fischer 1971a), amino acids (Fischer 1971b), lipids (Roots 1957, 1960; Roots and Johnston 1966) and some redox pigments such as riboflavin, thiamine, carotene and metalloporphyrins (Roots and Johnston 1966; Fischer 1977). Thus, chloragosomes bear some resemblence to the pigmented lipochrome-containing granules (cytosomes) found in molluscan tissues (Zs Nagy 1977).

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The major element constituents of chloragosomes of most earthworm species inhabiting uncontaminated soils are Ca, P, Zn and S (Prentø 1979; Morgan 1982; Morgan and Winters 1982; Morgan 1985), with trace quantities of K, Cl, Fe and Mn.

Chloragosomal Zn is, for a number of reasons, the focus of particular attention in the present paper. First, although in vitro incubation experiments with isolated chloragosomes (Ireland 1978), and observations on the chloragosomes of earthworms living in Pb-polluted microhabitats (Ireland and Richards 1977; Morgan and Morris 1982; Morgan 1984), indicate that the granules have a high affinity for Pb and possibly detoxify the metal by accumulative immobilisation, only limited evidence exists (Morgan 1985) that Zn is accumulated within chloragosomes above very high base-line levels of about 10000 μg/g dry weight (Morgan and Winters 1982; Morgan 1985) in earthworms from Zn-contaminated soils. It is, therefore, pertinent to ask whether chloragosomes are capable of sequestering Zn when available in excess? Second, unreliable (Morgan 1984) semiquantitative electron probe X-ray microanalysis (EPXMA) suggests that Zn is probably not bound to oxygen-donating, phosphorus-containing ligands within the chloragosome matrix (Prentø 1979; Morgan 1981). The "borderline" ligand-affinity status of Zn (Nieboer and Richardson 1980; Simkiss 1981) does not, however, preclude its binding to phosphorus groups. We investigated the binding characteristics of Zn by a combination of fully quantitative EPXMA, and statistical analysis of the data by partial correlation and multiple regression. Finally, it is of general importance to increase our understanding of how biological matrices sequester, regulate and release Zn, because it is an essential metal with wide-ranging cellular functions (Williams 1984; Wensink et al. 1987).

Materials and methods

Preparation of air-dried chloragosome smears for electron probe X-ray microanalysis (EPXMA). Mature (clitellate) specimens of *Lumbricus rubellus* were collected by digging and handsorting from abandonded metalliferous mine sites at Llantrisant (O.S. Grid reference = ST 048822; "total", i.e. conc. HNO_3-extractable, soil Zn = 2050 μg/g dry wt.); Draethen (ST 217877; "total" soil Zn = 16400 μg/g dry wt.); and an unpolluted "control" site, Dinas Powys (ST 146723; "total" soil Zn = 190 μg/g dry wt.). Worms were transferred to the laboratory in their native soil. The animals were rinsed clean of any adhering soil particles in tap water, and

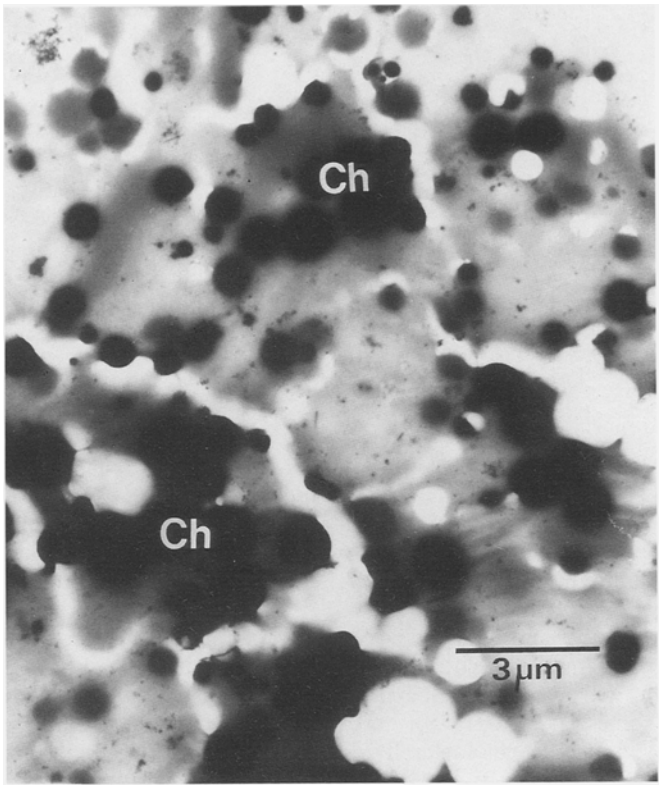

Fig. 1. Transmission electron micrograph of an air-dried chloragocyte smear of *L. rubellus* from Dinas Powys. In air-dried smears, the chloragosomes of Dinas Powys, Llantrisant and Draethen animals appeared morphologically similar. *Ch* = chloragosome

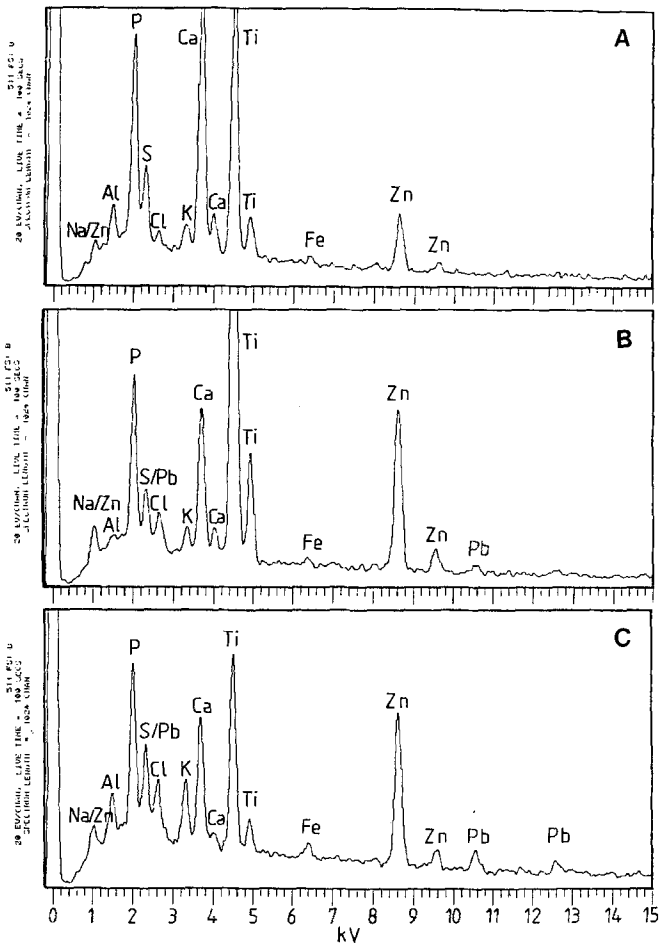

Fig. 2A–C. Representative X-ray spectra of individual chloragosomes (air-dried smears) of *L. rubellus* from Dinas Powys (control), Llantrisant and Draethen. The *Al, Ti* and *Fe* peaks were derived from the specimen holder

maintained for 2 days in the dark at 10° C in plastic petri dishes containing moist Whatman No. 1 filter paper discs. To prevent coprophagy, the filter paper was changed daily.

The animals were individually dissected along their dorsal surface to expose the chloragogenous tissue immediately posterior to the clitellum. From this region, a small piece of chloragogenous tissue was removed and lightly smeared (Fischer and Trombitás 1980) over individual, carbon-coated celloidin films covering titanium grids (200 mesh). Each grid was allowed to air-dry (17° C), carbon-coated, and stored in a desiccator. The advantages of this preparative technique is that it is extremly simple and rapid, particularly compared with the definitive anhydrous preparative technique of cryomicrotomy (e.g. Morgan and Winters 1982). The element composition of air-dried chloragosome smears has been found to compare favourably with that from cryo-sectioned material (Morgan 1984; Winters and Morgan 1988); thus it appears that during the air-drying procedure the element composition of major intra-cellular components are not seriously altered (Chandler and Battersby 1976). This is a fundamental advantage over processing procedures that involve the exposure of tissue to aqueous media which tend to extract and redistribute cellular elemental constituents (Morgan 1980). Two grids were prepared from each animal, and five animals used for each site sample. No less than ten granules, each approximately 1.0 µm in diameter, were analysed from each animal.

Microprobe analysis. Analysis was performed in a transmission electron microscope (Philips EM 300) equipped with a LINK 30 mm^2 energy dispersive X-ray spectrometer interfaced with a LINK 860 Series 2 microprogrammed computer. The normalised microscope operating conditions were: (*a*) accelerating voltage, 80 kV; (*b*) counting (live) time, 100 s; (*c*) specimen tilt angle, 21°, and (*d*) output count rate approximately 1000 counts per second.

The beam current, accurately measured by a "Faraday cup" prior to the analysis of each granule, was adjusted to be in the range of 0.2 to 0.3 nA. The probe diameter was standardised to approximately 0.75 µm so that it was always less than the diameter of the granules selected for analysis.

Spectra obtained from chloragosomes were quantitively processed by LINK QUANTEM/FLS software. The quantitative procedure is based on the Hall Continuum Method (Hall 1979; Hall and Gupta 1982, 1983). The technique compares peak-to-continuum ratios from the specimens with those from known concentrations of standards of each element. The measured continuum counts are corrected for contributions from holder, grid and support film, together with absorption of characteristic X-ray emissions caused by local variations in the mass thickness of the specimen. Overlapping element peaks were deconvoluted by the Filtered Least Squares technique (Hall and Gupta 1982), in which filtered peak profiles (stored in a library of reference spectra which were acquired by analysing pure salts) were fitted to the filtered spectrum.

Biological thin-film standards were made from aqueous salt solutions dispersed in an aminoplastic (Roos and Barnard 1984), the matrix of which resembles biological soft tissue because of its low atomic number composition (Roos and Barnard 1984; Roos and Morgan 1985), and permits homogeneous doping with relatively high amounts of different elements (Roos and Barnard 1984). The measured chloragosomal elemental concentrations were all within the standard concentration ranges.

Table 1. Concentrations of P, Ca, S, Zn and Pb (m*M*/kg dry wt., mean ±SE) in chloragosomes of *L. rubellus* from uncontaminated (Dinas Powys) and Zn-contaminated (Llantrisant and Draethen) sites. The chloragosome data was obtained from five individual worms from each site. The statistical analysis was performed in each case by comparison with the appropriate Dinas Powys values

Site	Element concentration [m*M*/kg dry wt.]				
	P	Ca	S	Zn	Pb
Dinas Powys ($n=59$)	1760±45	1173±42	501±22	149±12	nd
Llantrisant ($n=60$)	1860±77 N.S.	873±39 ***	432±26 N.S.	533±32 ***	57±6 ***
Draethen ($n=61$)	1682±91 N.S.	774±42 ***	537±47 N.S.	520±33 ***	90±8 ***

n = number of chloragosomes analysed, nd = not detected
*** $p<0.001$
N.S. = not statistically significant ($p>0.01$)

Statistics. The chloragosomal element concentrations were normally distributed without the need for data transformation (Morgan 1987), and differences in element concentrations were assessed by the Mann-Whitney *U* test. Partial correlation and multiple regression were used to describe element relationships within the chloragosomes. Values obtained from the statistical procedures were regarded as significant at $p<0.01$.

Results

The electron-dense chloragosomes of *L. rubellus* were easily identifable in the air-dried chloragocyte smear preparations (Fig. 1), and these organelles subjectively appeared similar in animals from the three sites. The smear preparations precluded any conclusions regarding their detailed morphology, i.e. the concentric patterns seen in chloragosomes in conventionally prepared thin sections and cryosections (Prentø 1979; Morgan 1981; Morgan and Winters 1982).

Representative X-ray spectra from individual chloragosomes in animals from each of the three sites are shown in Fig. 2. The major elemental constituents were P (5%–6% dry wt.), Ca (3%–5%), S (1%–2%) and Zn (1%–3%) (Table 1). Small concentrations of Pb (1%–2%) were detected in the chloragosomes of Llantrisant and Draethen animals, but Pb was not detected in Dinas Powys chloragosomes (Table 1). Llantrisant and Draethen chloragosomes contained significantly higher concentrations of Zn and Pb, but significantly lower concentrations of Ca than those of Dinas Powys animals (Table 1). No significant differences in chloragosomal P and S concentrations were found.

Significant positive correlations were found between chloragosomal P and Ca in the three populations of *L. rubellus* (Table 2). There were, however, striking differences between the slopes of the calculated P:Ca regression curves in the chloragosomes of the "control" and Zn-exposed animals (Fig. 3). The P:Ca relationships were supported by the coefficients of partial correlation (Table 3).

Second-order partial correlation (Table 4) demonstrated a strong positive relationship between P and Zn in the three groups, and also indicated that the positive correlations found between Zn and Ca in the Llantrisant and Draethen chloragosomes (Table 2) were due to other coincident chloragosomal elemental relationships. Indeed, the second order partial correlations between Zn and Ca were significantly negative, as found in the chloragosomes of Dinas Powys ("control") animals (Tables 2 and 4). Third-order partials, by the further addition of Pb, substantiated the Zn:Ca findings for these two groups (third-order partial correlations = −0.608 and −0.368 for Llantrisant and Draethen, respectively; $p<0.01$ or better).

Partial correlation showed that there was only a weak relationship between Zn and S in "control" chloragosomes (Table 4). By contrast, significant positive (partial) correlations were found in the Llantrisant and Draethen granules (Table 4).

Stepwise multiple regression (Table 5) demonstrated that 70% of the Zn in Dinas Powys chloragosomes was explained by Ca (negative variable) and P (positive variable). The regression model was not significantly improved by the further addition of S. In the Llantrisant and Draethen chloragosomes, P and S (both positive variables) and Ca (negative variables) explained 96% and 91% of the variability in chloragosomal Zn, respectively (Table 5), with P alone explaining over 88% in each of the two groups (Fig. 4).

Discussion

The elemental composition of chloragosomes of *L. rubellus* from Dinas Powys corroborates the previous findings in this species from other uncontaminated sites (Morgan 1982;

Table 2. Linear correlation coefficients between P, Ca, Zn and S concentrations within individual chloragosomes of *L. rubellus* from Dinas Powys, Llantrisant and Draethen

	Dinas Powys ($n=59$)				Llantrisant ($n=60$)				Draethen ($n=61$)			
	P	Ca	Zn	S	P	Ca	Zn	S	P	Ca	Zn	S
P	–	0.704 ***	0.078 N.S.	−0.168 N.S.	–	0.870 ***	0.955 ***	0.929 ***	–	0.781 ***	0.939 ***	0.797 ***
Ca		–	−0.533 ***	0.212 N.S.		–	0.747 ***	0.822 ***		–	0.641 ***	0.591 ***
Zn			–	−0.346 *			–	0.935 ***			–	0.820 ***
S				–				–				–

n = number of chloragosomes analysed
*** $p<0.001$; * $p<0.01$
N.S. = not statistically significant ($p>0.01$)

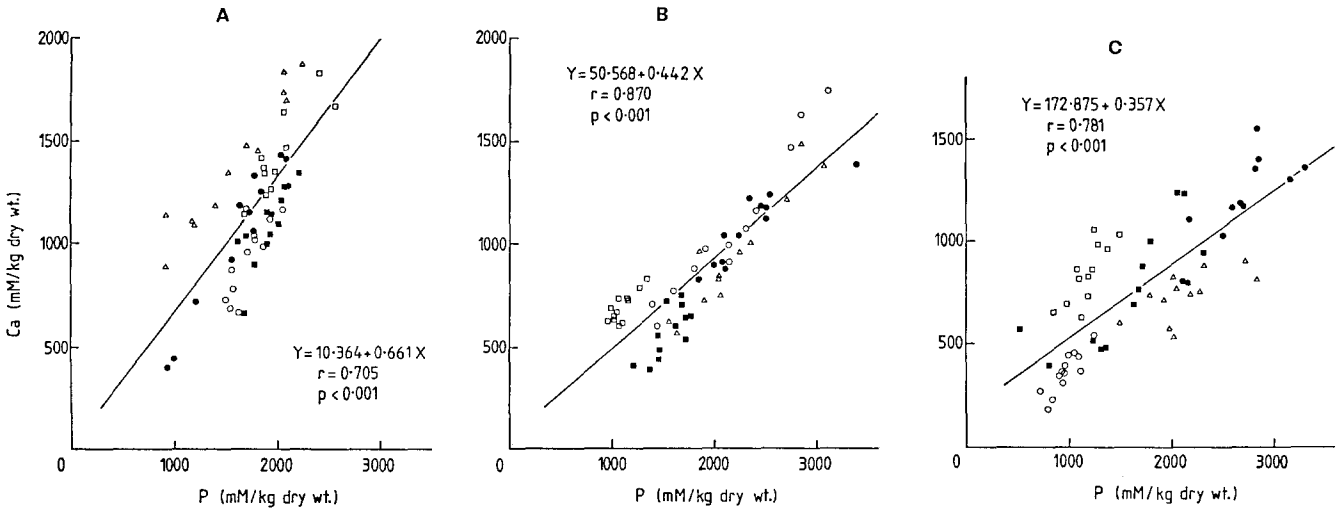

Fig. 3A–C. The relationships between P and Ca (m*M*/kg dry wt.) in individual chloragosomes of *L. rubellus* from Dinas Powys (**A**), Llantrisant (**B**) and Draethen (**C**) in air-dried chloragocyte smears. The different symbols represent chloragosomes analysed from different individual animals

Table 3. Coefficients of partial correlation (first-, second-, and third-order) of the relationships between P and Ca within individual chloragosomes of *L. rubellus* from Dinas Powys, Llantrisant and Draethen

Site	Metal(s) accounted for						
	Zn	S	Pb	Zn, S	Zn, Pb	S, Pb	Zn, S, Pb
Dinas Powys ($n=59$)	0.891	0.770	–	0.906	–	–	–
Llantrisant ($n=60$)	0.796	0.503	0.660	0.771	0.667	0.362*	0.666
Draethen ($n=61$)	0.667	0.636	0.535	0.670	0.587	0.554	0.589

n = number of chloragosomes analysed
* $p<0.01$, otherwise all coefficient values are $p<0.001$

Table 4. Coefficients of first- and second-order partial correlation between Zn and other major elements (P, Ca and S) in chloragosomes of *L. rubellus* from Dinas Powys, Llantrisant and Draethen

Site	Metal(s) accounted for								
	Ca	S	Ca, S	P	S	P, S	P	Ca	P, Ca
	Zn:P			**Zn:Ca**			**Zn:S**		
Dinas Powys ($n=59$)	0.766	0.030 N.S.	0.747	−0.838	−0.498	−0.817	−0.340 *	−0.274 N.S.	0.114 N.S.
Llantrisant ($n=60$)	0.932	0.662	0.834	−0.578	−0.109 N.S.	−0.681	0.434	0.848	0.590
Draethen ($n=61$)	0.914	0.824	0.839	−0.427	0.338 *	−0.426	0.344 *	0.713	0.343 *

n = number of chloragosomes analysed
$p<0.001$, except * $p<0.01$
N.S. = not statistically significant ($p>0.01$)

Morgan 1985). It has been suggested that Zn participates in respiratory control and in processes underlying tissue growth, development and regeneration (Wróblewski et al. 1979; Morgan 1981, 1984). Furthermore, fluctuations in chloragosomal Zn have been shown to occur in *Allolobophora (Apporectodea) longa* undergoing diapause (Morgan 1984), indicating that the metal is a primary or secondary mediator of regulatory activities during this discrete physiological resting state.

Most (~60%) of the Zn is accumulated within the posterior alimentary canal (i.e. intestine and encapsulating chloragogenous tissue) in earthworms from uncontami-

Table 5. Multiple regression equations of the relationships between the concentrations of Zn (**Y**) and other elements (**Ca, P** and **S**) in chloragosomes of *L. rubellus* from three sites

Dinas Powys (control)	
Y= 321.937−0.147 **Ca**	$R^2=0.284$
*Y= 91.368−0.326 **Ca**+0.294 **P**	$R^2=0.704$
Llantrisant	
*Y= −214.759+0.402 **P**	$R^2=0.913$
Y= −200.276+0.529 **P**−0.286 **Ca**	$R^2=0.942$
Y= −130.756+0.387 **P**−0.305 **Ca**+0.489 **S**	$R^2=0.962$
Draethen	
*Y= − 51.421+0.340 **P**	$R^2=0.881$
Y= − 19.173+0.407 **P**−0.187 **Ca**	$R^2=0.903$
Y= − 2.172+0.351 **P**−0.175 **Ca**+0.126 **S**@	$R^2=0.914$

* Best fit as determined by the *F*-test; R^2 = % variability in chloragosomal Zn concentration accouted for
All equations significant at $p<0.001$ (*T*-test) except @, where $p<0.01$

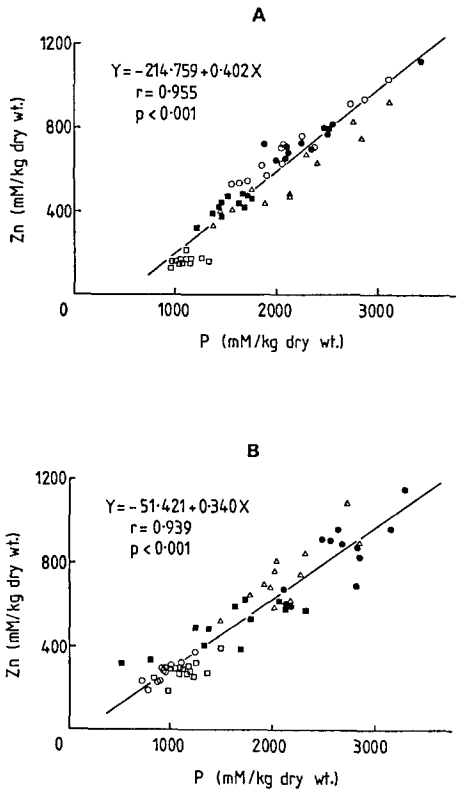

Fig. 4A and B. The relationship between Zn and P (m*M*/kg dry wt.) in individual chloragosomes of *L. rubellus* from Llantrisant (**A**) and Draethen (**B**) in air-dried chloragocyte smears. The different symbols represent chloragosomes analysed from different individual animals

nated environments (Morgan 1987). The high Zn concentrations recorded in the chloragosomes of earthworms from the Zn-contaminated Llantrisant (~35000 µg/g dry wt.) and Draethen (~34000 µg/g dry wt.) sites reflect the high (geometric) mean whole-worm Zn concentrations (1300 µg/g and 3000 µg/g dry wt., respectively), much (>80%) of which is sequestered in the posterior alimentary canal (Morgan 1987; Morgan et al. 1988). The present work cannot furnish direct evidence that this tissue accumulation and subcellular immobilisation process is necessarily a detoxification mechanism, but the possibility is strongly suggested. Earthworm chloragosomes may, therefore, be capable of both storing/releasing Zn to meet diverse physiological requirements and sequestering the metal when available in excessive quantities due to environmental contamination.

Chloragosomes possess significant cation-exchange capacities (Fischer 1973, 1977), and the available evidence suggests that an exchange mechanism exists within the chloragosome whereby the accumulation of chloragosomal Zn accompanies diminished chloragosomal Ca concentrations. A similar phenomenon has been found to occur for Pb incorporation into chloragosomes (Ireland 1978; Morgan and Morris 1982; Morgan 1984; Morgan 1985, 1987, 1988). Since an analogous "exchange" is also prevalent in "control" chloragosomes (i.e. negative Zn:Ca correlations in earthworms from non Zn-polluted soils) it may be an integral part of the physiological functioning of chloragosomes (Prentø 1979; Fischer and Trombitás 1980; Morgan 1982), which has been effectively exploited for the sequestration of potentially toxic concentrations of Zn.

Whether the accumulation of Zn and loss of Ca from individual granules represents a disturbance of the Ca metabolism of earthworms is unclear; it would be pertinent to measure morphometrically whether the amount of Ca in the cloragosomes is preserved by increasing the number of chloragocytes or the number of chloragosomes per cell. Interestingly, these Zn:Ca relationships have also been recorded in chloragosomes of *Apporectodea caliginosa* (Morgan 1987), a species which is eco-physiologically different from *L. rubellus*, particularly with respect to its calcium metabolism (Piearce 1972).

The significant positive correlations between chloragosomal P and Ca concentrations of *L. rubellus* confirmed Morgan's (1982) semiquantitative EPXMA findings on chloragosomes in this species. This P:Ca relationship has also been documented from chloragosomes of several other earthworm species (Prentø 1979; Morgan 1981, 1982; Morgan and Morris 1982; Morgan 1987), and it has been suggested that Ca is bound to P-containing ligands (Prentø 1979; Morgan 1982), possibly in the form of inorganic $CaHPO_4$ and organic $R\text{-}OPO_3Ca$ (or Ca-polyphosphate) complexes (Prentø 1979).

The analysis of the present fully quantitative microprobe data by (second order) partial correlation demonstrates that earthworm chloragosomes contain at least two Zn pools. In control (Dinas Powys) animals, Pool 1 consists of a chloragosomal Zn fraction (~45%) which is not associated with P-containing ligands. This observation may explain the poor correlation (assessed by linear regression) between Zn and P recorded by Morgan (1981) in a semiquantitative study of chloragosomes of *L. terrestris*. Pool 2 is a Zn-P complex, which assumes increasingly greater importance when the chloragosomes contain elevated Zn concentrations (~70% in the Zn-contaminated populations studied). The volume of Zn-Pool 2 is effectively expanded in Zn-contaminated granules by the displacement of Ca from binding sites on P-rich ligand(s); it is conceivable that the volume of Zn-Pool 1 is similar in control and contaminated granules. Since the concentrations of chloragosomal P in the three earthworm populations are not significantly

different, and considering the strong relationship between P and Ca within chloragosomes, it reasonable to suppose that the P-containing ligands binding Ca and "excess" Zn are probably identical. The P-rich ligand(s) are at present uncharacterised, although they are probably not inorganic phosphate because Prentø (1979) found only a weak relationship between phosphate and Zn in *L. terrestris* from an uncontaminated environment.

Sulphur may also play a role in Zn-sequestration when there is a high chloragosomal concentration of the metal. Under "normal" uncontaminated conditions (as in Dinas Powys granules), however, S is not strongly associated with Zn. It seems that chloragosomal S, which incidentally is not associated with chloragosomal P (data not shown), is redeployed under conditions of elevated Zn metabolism, from a primary structural/metabolic role in normal granules to one where it assumes a Zn binding function. This may implicate the presence of an inducible, S-containing Zn-binding ligand. Whether such a S-ligand is a metallothionein-like protein is highly speculative, although earthworm Cd-metallothionein has been isolated and characterised from the posterior alimentary canal of *L. rubellus* (Morgan et al. 1988). Preliminary evidence (Morgan and Morgan, unpublished) suggests that the Cd-metallothionein complex is not located within the chloragosomes, but this does not preclude the possibility that Zn-binding metallothionein occurs in the granules.

In general, metal sequestration strategies adopted by earthworm chloragocytes are analagous to, for example, those found in the midgut parenchyma cells of the barnacle, *Balanus balanoides* (Walker 1977), i.e. within a single cell type (chloragocyte) different metals are accumulated within discrete compartments, with Zn (and Pb) being specifically accumulated within the chloragosome whilst Cd is accumulated within a separate S-rich structure, the so-called cadmosome (Morgan and Morris 1982; Morgan 1987). Such compartmentation reflects the ligand affinities of these metals.

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