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IN VIVO METAL SUBSTITUTIONS IN METAL SEQUESTERING SUBCELLULAR COMPARTMENTS: X-RAY MAPPING IN CRYOSECTIONS

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Abstract

Qualitative digital X-ray mapping techniques were employed to determine the distributions of essential and non-essential elements in three invertebrate "models": (1) Pb, Zn, Cd, Cu, Fe in thin cryosections of the hepatopancreas of the terrestrial isopod, *Oniscus asellus*; (2) Pb, Zn, Cd, Ca in thin cryosections of the chloragogenous tissue of the earthworm, *Lumbricus rubellus*; and (3) As in air-dried smears and thin cryosections of chloragogen in *L. rubellus*. Four general conclusions were drawn from the results of these studies: (a) non-essential elements can accumulate, distribute and be compartmentalized because they, or the organo-complexes that they form, act as "mimics" of essential elements with which they share to a greater or lesser extent certain chemical affinities; (b) thermodynamic considerations notwithstanding, the influence of biological factors on the sequestration and fates of certain elements (e.g., arsenic) is profound through modifications of redox states and organo-compound formation; (c) X-ray mapping, combined with anhydrous preparative procedures, yields unbiased information concerning the relative spatial distributions of several elements in structurally heterogeneous sampling "fields", although the morphological characterization of (occasionally unsuspected) subcellular compartments may be constrained by the intrinsic quality of the preparation; and (d) X-ray microanalysis yields co-distribution data, when integrated with biochemical information from other sources, which give strong pointers to the identity of binding ligands and of the valence state of sequestered cations.

Key Words: X-ray digital mapping, cryosections, metals, arsenic, detoxification, invertebrates

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Introduction

General background

Certain groups of detritivorous terrestrial invertebrates, such as isopods and lumbricid earthworms, are recognized as efficient accumulators of essential and non-essential metals (Morgan *et al.*, 1986, 1993; Morgan and Morgan, 1988; Hopkin, 1989). Whilst often sound, this observation may be regarded as an over-generalization, because: it does not accommodate the fact that a given group or species accumulates different metals with a wide range of efficiencies; and it does not acknowledge that two closely related species inhabiting the same locality may express substantial differences in their metal accumulation characteristics (Morgan *et al.*, 1993). In evolutionary terms, organisms are presented with at least four physiological options when they encounter a metal; these "options" can be conveniently referred to as: (i) regulation; (ii) exclusion; (iii) sequestration; and (iv) intrusion (Fig. 1).

The intention of the present paper was not explicitly to examine the physiology of animal metal relationships, but to look at interactions between essential and non-essential metals, and the fates of selected metals and a metalloid, within the cells of key terrestrial species. Accumulated metals are usually distributed unevenly between tissues (Hopkin and Martin, 1982a, 1984a; Dallinger and Wieser, 1984; Morgan and Morgan, 1990). Within the favoured "target" tissues, they are preferentially sequestered by particular cell types (Hopkin and Martin, 1982b, 1984b; Morgan, 1984; Morgan and Winters, 1987; Hopkin *et al.*, 1989; Hopkin, 1990a). Many metals are predominantly incorporated into discrete membrane-limited vesicles or granules (Brown, 1982; Morgan, 1984; Morgan and Winters, 1987; Nott, 1991, 1993) possibly representing functionally differentiated lysosomes (Dallinger, 1993). These compartments were presumably evolved to serve as storage or detoxification sites for essential metals. For example, the majority of the intracellular granules in the invertebrates have a significant calcium content, whose function in many cases is to maintain the cytosolic free Ca^{2+} concentration at low levels of about 10^{-7} M (Borle, 1988).

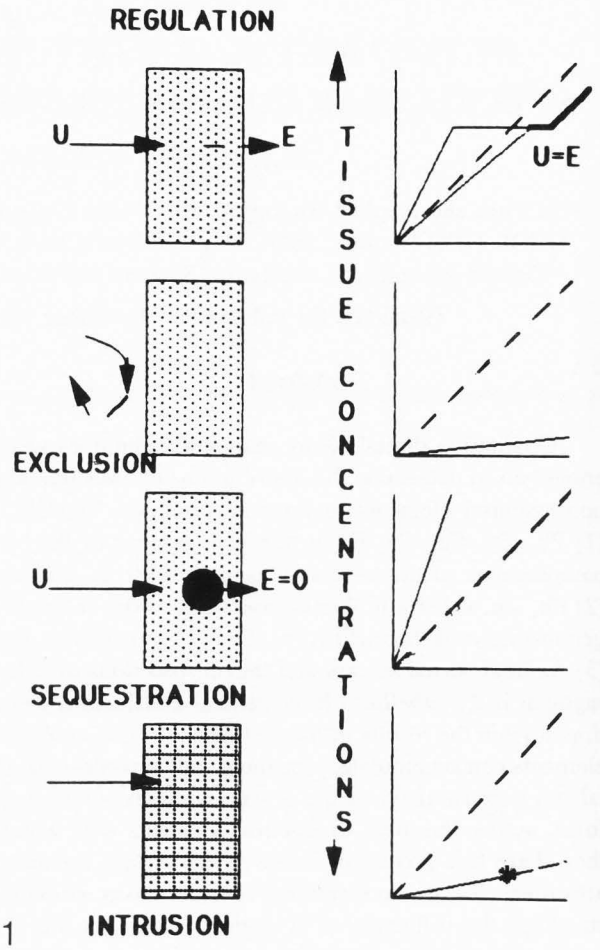


Figure 1 (at right). Schematic diagrams illustrating the main strategies in the responses of animals (represented by stippled rectangles) to metals. Each diagram is accompanied by a figure depicting the relationship between tissue metal concentrations and environmental metal concentrations {the broke line in each (hypothetical) figure is a unity line}. (**A**) "Regulation", where metals are fairly efficiently accumulated when environmental concentrations are low, tissue concentrations remain fairly constant (uptake, U ≈ excretion, E) over a wide range of environmental concentrations, and where regulatory capacity may be lost at high exposures (*). (**B**) "Exclusion", where a metal is effectively prevented from entering the body, or where the rate of excretion very nearly matches the rate of uptake. (**C**) "Sequestration", where metals that enter the body are accumulated within storage or detoxification compartments (large black symbol) within certain cell types, and where the rate of metal excretion is very low, even though the metal-rich vesicular structures may periodically be eliminated, i.e. there is a metal sequestration cycle. (**D**) "Intrusion", where a metal enters an animal and, because of its intrinsic reactivity and binding stability, interferes with biochemical processes (represented in the rectangular animal "model" by small open stars) such that it exerts direct toxicological effects (*) at low concentrations. These "models" were partly based on those designated "accumulators", "indicators" and "excluders" proposed by Baker (1981) for plants. [N.B. Depledge and Rainbow (1990) warn against a simplistic distinction between metal "regulators" and "non-regulators": a "regulator" organism may accumulate metal in a relatively unavailable form within a cell or tissue and be capable of drawing upon this store for physiological purposes under some circumstances; also, the ability to regulate one metal does not necessarily confer the ability to regulate another].

The intracellular distribution of metals is largely determined on the one hand by the nature of the organic matrix of the sequestration compartments, and on the other by the ligand-binding affinities of individual metals. Non-essential metals are absorbed, transported, and sequestered or excreted by organisms mainly because they or the complexes that they form mimic (i.e., behave as biochemical analogues) essential chemical species. Understanding metal-metal interactions, "biochemical mimicry" and substitutions is important to better understand the mechanisms of metal toxicity, to develop effective treatments for metal and metalloid intoxication, and to exploit the chemotherapeutic potential of metals and metallo-compounds.

Bio-inorganic considerations

For detailed discourses on pertinent aspects of bio-inorganic principles, which are beyond the scope of the present paper, reference should be made to: Wetterhahn-Jennette (1981); Taylor and Simkiss (1989); Da Silva and Williams (1991); Clarkson (1993); Streit and Stumm (1993); and Markich and Jeffree (1994).

Metal ions can be grouped according to certain shared thermodynamic properties as "hard" (Class A), "soft" (Class B), or borderline ions. In general, hard acids (cations) possess high affinities for hard bases, with an affinity sequence for ligands donating O > N > S; soft acids have high affinities for soft bases, in the donor sequence S > N > O (Da Silva and Williams, 1991). Partly for these reasons, certain non-essential ions can act in biological systems as analogues, and have been fairly widely used as physiological "probes" (Table 1) of essential elements. Furthermore, the observed interactions between, for example, Ca and Pb (Pounds, 1984; Fullmer, 1991; Fullmer and Rosen, 1990; Weiss and La Velle, 1991; Mielke and Heneghan, 1991), selenate and sulfate (Shennan and McNeillie, 1990), arsenate and phosphate (Alves, 1992), vanadate and phosphate (Karlish *et al.*, 1979), and the manifold intimate relationships between Class B metals and the cysteine-rich

Table 1. Selected examples of the use of non-essential elements as physiological "probes" for essential elements in studies involving electron probe X-ray microanalysis and/or cryopreparative procedures.

"Model" Element	Analogue (mimic) Element	Biological System	Reference
Ca	Sr	Calcium carbonate spherites in earthworm calciferous gland.	Morgan (1981)
Ca	Sr	Type I luminal concretions in the Malphigian tubules of Drosophila.	Wessing and Zierold (1992)
Ca	Sr	Epiphyseal growth plate.	Krefting *et al.* (1992)
K	Rb	Principal cells of frog skin epithelium, and rabbit urinary bladder.	Dörge and Rick (1990)
K	Rb, Cs, Tl	Frog skeletal muscle.	Edelmann (1986, 1988)
K	Cs	Frog skeletal muscle.	Edelmann (1989)
Cl	Br	Rat atrial myocardium.	Somlyo *et al.* (1988)
Cl	Br	Mitochondria-rich cells of toad skin.	Dörge and Rick (1990)

metallothionein proteins (Vasak, 1992; Winge, 1992) reflect chemical similarities. Analogues are similar, not identical, and the dissimilarities between "model" and "mimic" are biochemically important. For example, there is much evidence that vertebrate tissues discriminate against Sr in favour of Ca (Sugihira and Suzuki, 1989, 1991; Kobayashi *et al.*, 1991), and that the substitution of Cd and Ni for Zn in the finger-loop domains of DNA-binding proteins alters their transcription properties and may provide a mechanism for metal-induced genotoxicity, teratogenicity and carcinogenicity (Sundermann, 1990; Mankowski *et al.*, 1991). Whilst the classification of metals as "hard" and "soft" acids provides a useful first-order explanation of tissue and cellular distributions (Morgan and Winters, 1987; Taylor and Simkiss, 1989; Nott, 1993), clearly a more comprehensive description is required to circumscribe biological metal selectivity/discrimination. Da Silva and Williams (1991) listed eight simultaneously operational factors that determine selection: (i) ionic charge; (ii) ion radius; (iii) the liganding donor atom; (iv) preferential co-ordination geometry; (v) spin-pairing stabilization (transition metal ions); (vi) binding in clusters; (vii) control of the concentrations of metal and ligand; and (viii) transfer coefficients from water to proteins or membranes. The first six are thermodynamic factors, the last two biological factors.

Specific objectives

Bio-inorganic theory is sufficiently well established to elicit predictions. However, intact biological systems are extraordinarily complex, so that the canon of theory cannot be readily applied to *in vivo* states. Accurate measurements of subcellular (competing) cation pools on the one hand, and precise definitions of accessible binding ligands on the other, are fraught with difficulties. The electron microprobe does, however, permit multi-element measurements in microvolumes that are both morphologically defined and of useful dimensions.

The specific aims of the present paper were two-fold. First, to explore the distributions of accumulated non-essential metals in two familiar invertebrate mineralized systems, the isopod hepatopancreas and the earthworm chloragogenous tissue using a combination of anhydrous and cryopreparative procedures and, in each case, X-ray mapping. Each application will be described as a brief "case history" with its own introduction and will serve to determine to what extent data obtained by X-ray analysis concerning metal compartmentation and substitutions are amenable to interpretation in the light of known bio-inorganic theory. Second, the case histories will be used to illustrate the utility of X-ray (element) mapping in ecotoxicological studies.

Materials and Methods

Animals and sampling sites

Woodlice (Crustacea: Isopoda), *Oniscus asellus*, were sampled from a disused Pb/Zn mine, whose associated soil is contaminated not only with the primary metals but also Cd (Morgan and Morris, 1982), at Draethen, South Wales (Ordnance Survey Map Ref. = ST 217877).

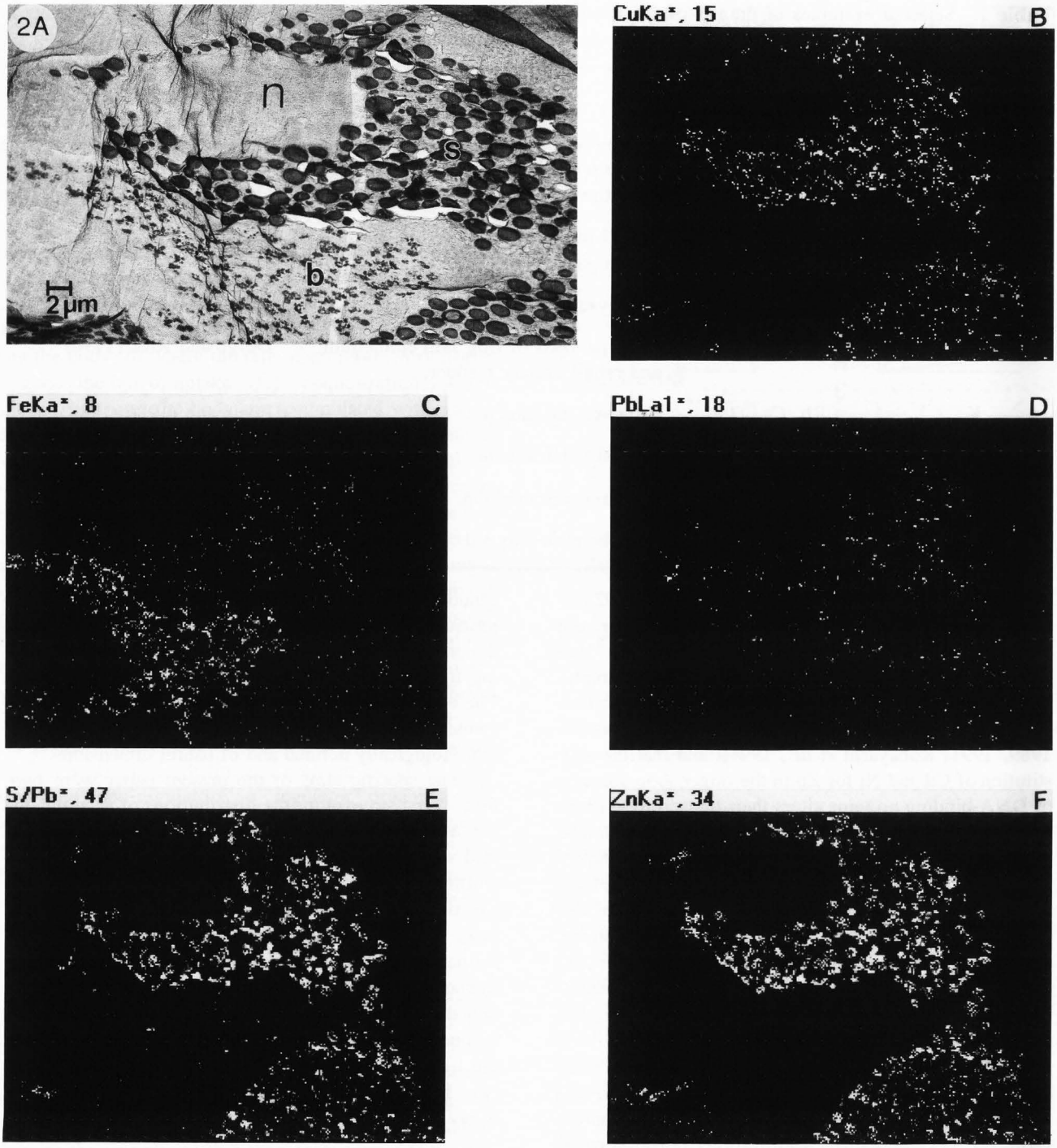
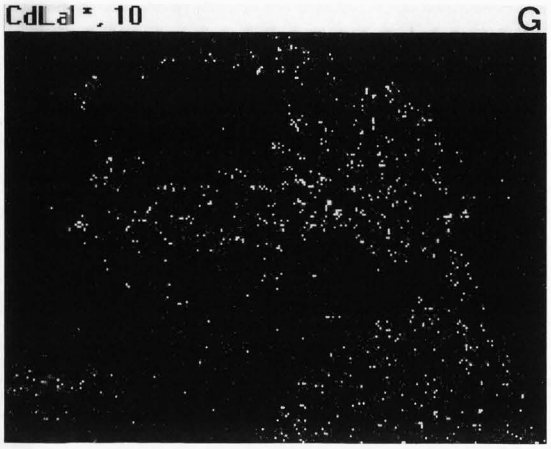
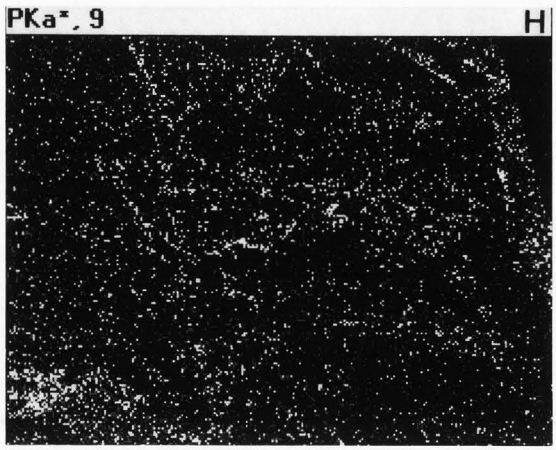
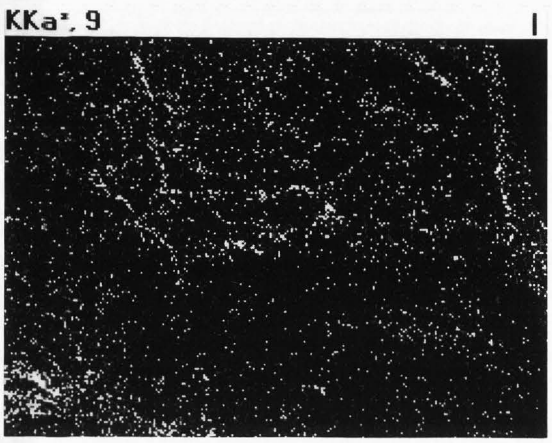
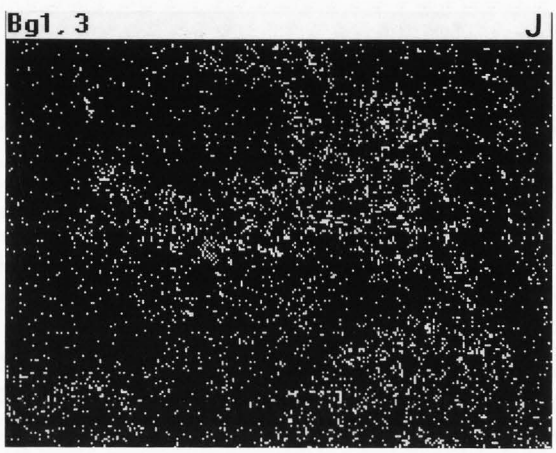


Figure 2. (**A**) Transmission electron micrograph of part of an ultrathin, freeze-dried, cryosection of the hepatopancreas of the isopod, *Oniscus asellus*, sampled from a Pb + Zn + Cd-contaminated site. Three "S"-cells (s) and one "B"-cell (b) are seen in the micrograph field; n: nucleus. (**B-J**; **B-F above, G-J on the facing page**) X-ray distribution {peak (P) - background (b)} maps for Cu Kα (**B**), Fe Kα (**C**), Pb Lα1 (**D**), S/Pb {**E**; there was no deconvolution of the contribution of Pb (M) signal to the S (K) signal}, Zn Kα (**F**), Cd Lα (**G**), P Kα (**H**), K Kα (**I**), and background (**J**; in the energy band from 5.5 to 6.0 keV). Note that although the available software did not permit deconvolution of S (Kα) peak from its overlapping Pb (M) peaks, the much higher signals recorded for combined S/Pb in the granules of the "S"-cells (Fig. 2E), compared with the Pb (Lα) signals in the same granules (Fig. 2D), indicates that these granules are definitely sulphur-rich and that Pb is accumulated by "S"-cell and "B"-cell granules.









Earthworms (Annelida: Oligochaeta: Lumbricidae), *Lumbricus rubellus*, were sampled from the Draethen site (see above) and maintained on native soil in the laboratory.

Earthworms, *Lumbricus rubellus*, were sampled from two arsenic-contaminated metalliferous sites in Cornwall, S.W. England: Luckett (SX 387736) and Bissoe (SW 771414).

Specimen preparation

Fresh pieces of hepatopancreas and chloragogen were mounted on aluminium pins, and plunged into liquid N_2-cooled, magnetically-stirred, propane in a home built device. Cryosections were cut at a nominal thickness of 90 nm on glass knives in a Reichert FC4E Ultracut cryomicrotome at -125°C. The sections were mounted on 200 mesh titanium grids with a hair probe, flattened using a perspex/brass device and freeze-dried externally in a liquid N_2-cooled transfer chamber under the vacuum (10^{-4} torr) of a coating unit (Winters and Morgan, 1988).

Simple air-dried smears of fresh chloragogen were prepared by gently "brushing" forceps-held pieces of tissue over the coated surfaces of 200 mesh titanium grids. The smeared grids were stored over silica gel until required for analysis.

Analytical procedures

Qualitative X-ray "speed maps" (Morgan *et al.*, 1994a) were determined in selected areas of cryosectioned hepatopancreas of woodlice from Draethen, cryosectioned chloragogen of earthworms from Bissoe, and smeared chloragogen of earthworms from Luckett.

Analyses (e.g., see Figs. 2, 6 and 7) were performed in a JEOL JEM-1210 transmission electron microscope (TEM) without a scanning TEM (STEM) unit, and equipped with a Link ATW Pentafet detector (138 eV resolution) and a Link "ISIS" analyser (Oxford Instruments), under the following conditions: accelerating voltage, 120 kV; filament heating current, 10 μA; condenser aperture, 200 μm; spot size "1" in "fine probe" mode producing a narrow convergent beam ~100 nm in diameter which was scanned using the Link ISIS system; objective aperture withdrawn; the detector was positioned to minimize dead time at a count rate of ~5000 counts/s; total acquisition times of 30 or 60 minutes. Some static probe analyses were also performed post-mapping on individual compartments.

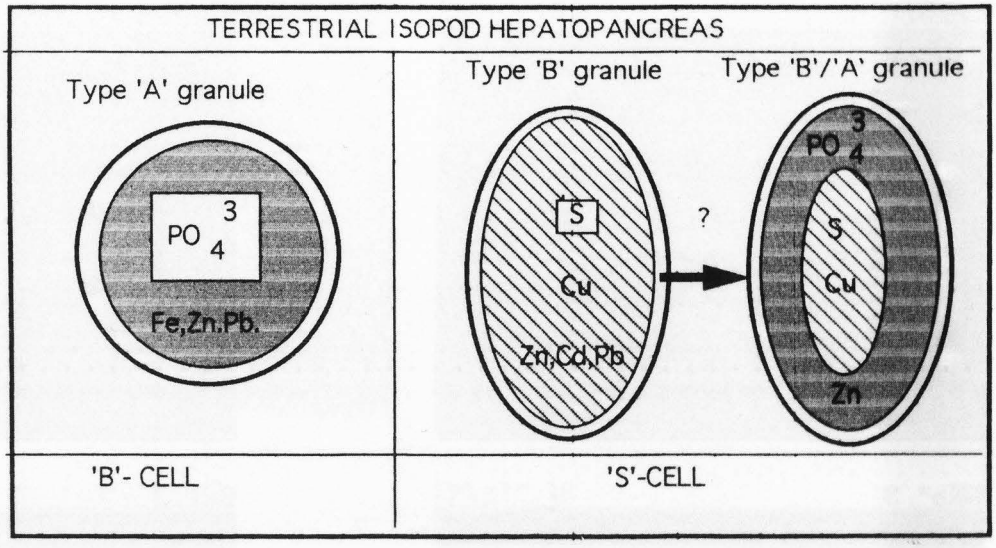


Figure 3. A simplified schematic diagram based on Figure 5 in Hopkin (1990a) to illustrate two of the three metal sequestration pathways described by Hopkin and others in the "B"- and "S"-cells of the isopod, *Procellio scaber*.

Some analyses (e.g., Fig. 4) were performed using a JEOL 2000 EX TEM operated in the STEM mode and equipped with a Link Pentafet Plus detector (premium grade 30 mm active area) and a Link "ISIS" analyser. Accelerating voltage used was 200 kV and conditions were optimized to produce the maximum count rate using the free lens control and a final dwell time of < 1 second/pixel (resolution 128 x 128 pixels and total acquisition time 1½ hrs). Maps were obtained using the speedmap software and additional static probe analyses were also performed on individual compartments.

Results and Discussion

Application 1: Metal compartmentation in the isopod hepatopancreas

The hepatopancreas of terrestrial isopods consists of two specialized epithelial cell types. Both contain characteristic metal sequestering compartments: the "B"-cells contain multivesicular bodies with oxygen donating, phosphate bearing, ligands that normally have high Fe contents; the "S"-cells contain vesicles with a more homogeneous matrix with sulphur donating ligands and normally have high Cu contents. The compartmentation of "extraneous" metals, derived from contaminated environmental materials, between and within the two cell types has been fairly extensively studied (Hopkin and Martin, 1982b; 1984b; Morgan and Winters, 1987; Dallinger and Prosi, 1988; Prosi and Dallinger, 1988; Hopkin *et al.*, 1989; Hopkin, 1990a; Morgan *et al.*, 1990; Hames and Hopkin, 1991). Although Hopkin *et al.* (1989) capitalized on the several advantages of X-ray mapping for studying element compartmentation (Morgan *et al.*, 1994b), they and others, apart from a cursory study of "S"-cell (Cu) granules by Morgan and Winters (1987), analysed chemically fixed preparations. The validity of data derived from such preparations, even when they apply to relatively insoluble mineralized structures, must be viewed with circumspection (Morgan, 1980; Nott, 1993).

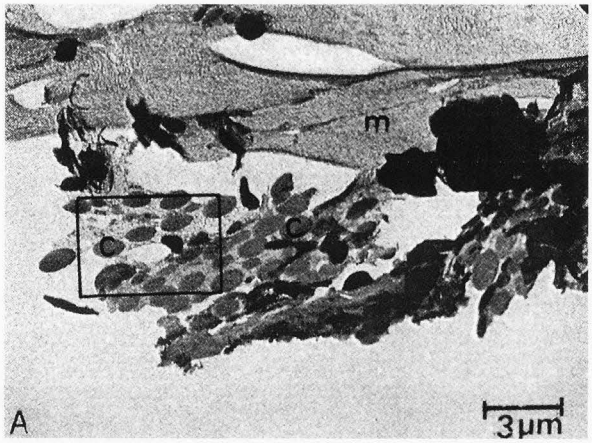
We present, for the first time, X-ray mapping data, albeit of low resolution, obtained from unfixed cryosectioned "B"- and "S"-cells. Figure 2 illustrates the distributions of accumulated elements in representative cells of the woodlouse, *Oniscus asellus*, hepatopancreas. Cu was focally distributed with sulphur in the "S"-cells; Fe was predominantly confined within focal deposits to the "B"-cell, although P was relatively homogeneously distributed across the cytoplasms and nuclei of both cell types. Cd and (especially) Zn appeared to be mainly accumulated within the sulphur bearing granules of the "S"-cells. Pb was accumulated by granular deposits in both the "S"- and "B"-cells.

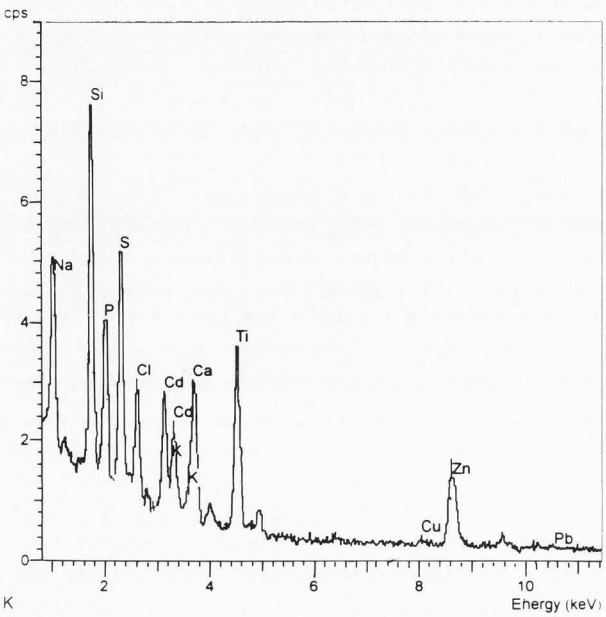
Hopkin *et al.* (1989) studied the distribution of metals in two related woodlouse species, *O. asellus* and *Porcellio scaber*, but they did not explicitly state which species their maps referred to. This distinction is important, because Hopkin (1990b) showed that differences in net assimilation of zinc by these isopods reflected differences in the extent to which metals are distributed between the two cell types in their hepatopancreas. "B"-cells effectively have a short residence time and regularly eliminate their mineralized contents; "S"-cells have much longer residence periods and are involved in metal storage (Hames and Hopkin, 1991). Although not directly comparable, because of differences in specimen preparation {although Hopkin *et al.* (1989) stated that the composition of isopod granules was identical in cryopreparations and chemically-fixed preparations} and analytical protocols, our observations on *O. asellus* differ in some respects from those emanating from the Hopkin group (Hopkin *et al.*, 1989; Hopkin, 1990a); see, Figure 3. The compartmentation of Cd and Pb in both studies

were similar: Cd accumulated in S-donating (Type B) granules confirming its status as a soft acids, soft base-seeking, metal; the occurrence of Pb in both Type A (oxygen-donating) and Type B granules confirms its "borderline" status (Nieboer and Richardson, 1980; Da Silva and Williams, 1991). Zinc is also a borderline metal and apparently does not qualitatively discriminate between Type A or Type B granules (Hopkin, 1990a), although it does form a phosphate-associated (Type A material) segregated rim around a Cu/S-rich (Type B material) in some "S"-cell granules (Hopkin *et al.*, 1989). Our studies on *O. asellus* indicated that Zn behaves more like a soft acid, because it was restricted to sulphur-bearing granules, and did not seem to be segregated within them as a phosphate salt (Fig. 2F).

The above observations raise a number of intriguing issues. Type B granules in the "S"-cells are probably components of the lysosomal system (Dallinger and Prosi, 1988; Prosi and Dallinger, 1988), and although Hopkin *et al.* (1989) suggested that their sulphur-bearing matrix was composed of the metal-binding, cysteine-rich, metallothionein, there is no evidence of the existence of such metalloproteins in the isopod hepatopancreas (Dallinger and Prosi, 1988; Donker *et al.*, 1990). However, Dallinger (1993) recently described a cadmium-binding glycoprotein of relatively low cysteine content and with a probable cytosolic distribution in the hepatopancreas of *P. scaber*. How can these various and apparently disparate observations be reconciled? Interestingly, Cu-metallothionein has been isolated from the livers of the human foetus, Bedlington terriers, and pigs, but the technique of immunocytochemistry has failed to detect metallothionein in lysosomes in these tissues (Bremner, 1992). It is possible that lysosomal metallothionein is somehow rendered inaccessible to antibodies (Bremner, 1992), or that the protein is rapidly hydrolysed within the acidic contents of the organelle such that antigenicity is lost and the released metals are precipitated as sulphur-containing salts (George, 1983).

We can now attempt to bring at least some of the available data on metal distributions in the isopod hepatopancreas together if not in a rational model, then perhaps in a speculative but heuristic one. First, the absence of Zn from "B"-cell granules in our population of *O. asellus* (Fig. 2) could reflect cellular (i.e. membrane) discrimination, or ligand discrimination with the metal expressing a tendency toward soft-acid behaviour. Alternatively, the absence of Zn from the mapped "B"-cell could reflect the trophic status in which this particular cell was arrested. Certainly, there is evidence that the morphology and composition of these absorptive cells are highly variable (Storch, 1984; Hames and Hopkin, 1991). Second, the incorporation of Cd, Zn and Pb into the Cu-containing sulphur-bearing matrix of "S"-cell granules is thermodynamically improbable, whatever the nature of the (organic?) matrix. Copper binds more avidly, and forms more stable complexes with S-donating ligands, including cysteine, than most other divalent cations (Fig. 2.8, Da Silva and Williams, 1991). Incidentally, the affinity constant of Cu for binding sites in vertebrate and invertebrate metallothioneins is significantly higher than those for Cd > Pb > Zn (Vasak, 1992). Consequently, if Cu ions were in excess over the total available sulphur-bearing ligand concentration, all other metals would fail to bind. Da Silva and Williams (1991) eloquently described how metals can successfully "compete" for binding sites under "unfavourable" conditions. In the case of forming sulphur-bearing "S"-cell granules, Cd, Pb, and Zn are incorporated if the concentration of ligand exceeds the concentration of available Cu ions. Cation availability could, for example, be determined by transport selectivity across the limiting membrane of the organelle. Morgan and Winters (1987) measured much reduced Cu concentrations in the "S"-cell granules of isopods inhabiting a Pb/Zn-contaminated site compared with "controls" from a relatively clean site, which implies transmembrane selectivity. What appears to be a substitution of Cu from the granule matrix by metals that form less stable products, is the result of what Da Silva and Williams (1991) term "selection by transfer coefficients from water to proteins or membranes" and, within the granules, "selection by the control of metal and ligand concentrations". Finally, the formation of $ZnPO_4$ deposits around the submembrane periphery of some "S"-cell granules may, as suggested by Hopkin *et al.* (1989), be a maturation phenomenon missed in our maps because the cell, and therefore, the granules that it contained, were fairly young. It would be interesting to establish whether or not the incidence of these compositionally biphasic granules is higher in the oldest "S"-cells in the proximal end, compared with the younger cells in the mid and distal regions, of the hepatopancreatic tubules (Hames and Hopkin, 1991). But, how is phosphate delivered to a Type B ("S"-cell) granule? Are phosphatases implicated? Why is Zn but not Pb precipitated in the "rims" of these hybrid A/B granules? Maybe the sulphur-bearing ligands of older granules have become metal-saturated, the intrusion of more metals would displace Zn, the metal forming the least stable complexes, from the core matrix. Free intragranular Zn^{2+} ions would need to be actively pumped back into the sequestration compartment against a steep concentration gradient to prevent leakage into the cytoplasm. If such a membrane pump exists in "S"-cell granules, then inorganic phosphate could be generated which captures Zn^{2+} ions at the intragranule periphery, a "model", analogous to intramitochondrial calcium phosphate deposition in necrotic cells.





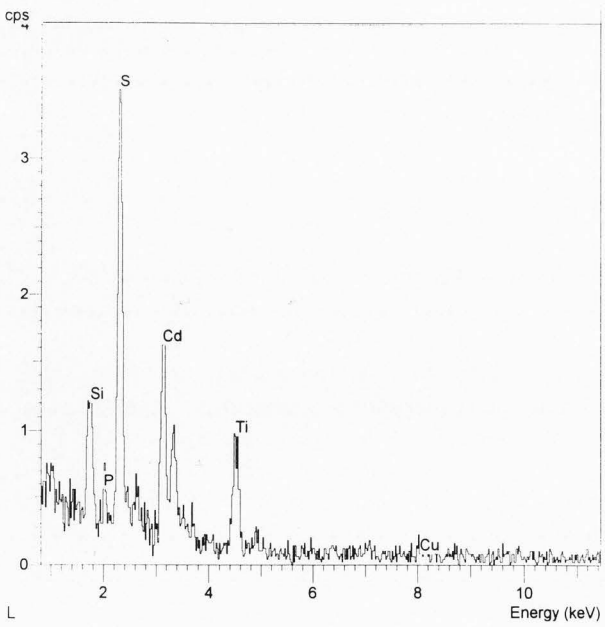


Figure 4 (A, K, and L at left; B-J on the facing page at right). (**A**) Scanning transmission electron micrograph of an unfixed, ultrathin, frozen-dried cryosection of earthworm, *Lumbricus rubellus*, chloragogenous tissue, showing a muscle layer (m) and attached chloragocytes with their characteristic ovoid chloragosome (c) granules. (**B**) Secondary electron image of the area enclosed within the rectangle in A; this is the area scanned and across which element distribution was determined by X-ray maps for P Kα (**C**), S Kα (**D**), Cd Lα(**E**), Ca Kα (**F**), background (**G**; in the energy band from 15.5 to 16.45 keV), Cu Kα (**H**), Zn Kα (**I**), and Pb Lα (**J**). Maps marked with an asterisk (4E, 4F, 4H, 4I and 4J) are background subtracted. Note that the contribution of Pb (M) signals to the S (K) signal was not subtracted, so that the sulphur map is strictly a combined S/Pb map; however, scrutiny of the X-ray spectra, derived by static probe analysis of a chloragosome dense "cap" (**K**) and a cadmosome (**L**), indicate that the Pb contribution in the 2.3 keV region of the spectrum is probably negligible. Note also the presence of an electron-dense, metal-enriched compartment ("d" in 4B) which may be a confluent degradation derivative of the two major sequestration pathways: Type A granules, the oxygen-donating, phosphate bearing chloragosome ("c" in 4B) containing Ca, Pb, and Zn; and Type B granules, the sulphur-donating Cd-binding compartment(s) (Fig. 4B).

Application 2: Metal compartmentation in earthworm chloragocytes

Metal accumulation and compartmentation in earthworm chloragocytes has been extensively studied in conventionally-fixed and anhydrous cryopreparations (for a review, see, Morgan *et al.*, 1993). The data presented in Figure 4 are consistent with our recent findings (Morgan *et al.*, 1994b) on the distributions of Ca, Pb, Zn and Cd in the cells of *L. rubellus* inhabiting a calcareous soil naturally contaminated with the three heavier metals.

From a bio-inorganic viewpoint, the interesting feature of the mapping data is that the two borderline metals, Pb and Zn, were accumulated in the oxygen-donating ligands within the chloragosome granules, so that both metals express predominantly hard-acid tendencies within this cellular environment. Zinc was virtually non-detectable in the sulphur-donating cadmosome matrix (Fig. 4I; Morgan *et al.*, 1994b). Although by no means certain, it is likely that the cadmosome contains Cd-metallothionein, or a degradation product of the metalloprotein. Morgan *et al.* (1989) reported that the metal binding sites of metallothionein isolated from earthworms exposed to a Cd- and Zn-contaminated soil were occupied almost exclusively by Cd; the Zn content of the protein was low. It is not difficult to predict the circumstances under which metals, such as Cu and Hg,

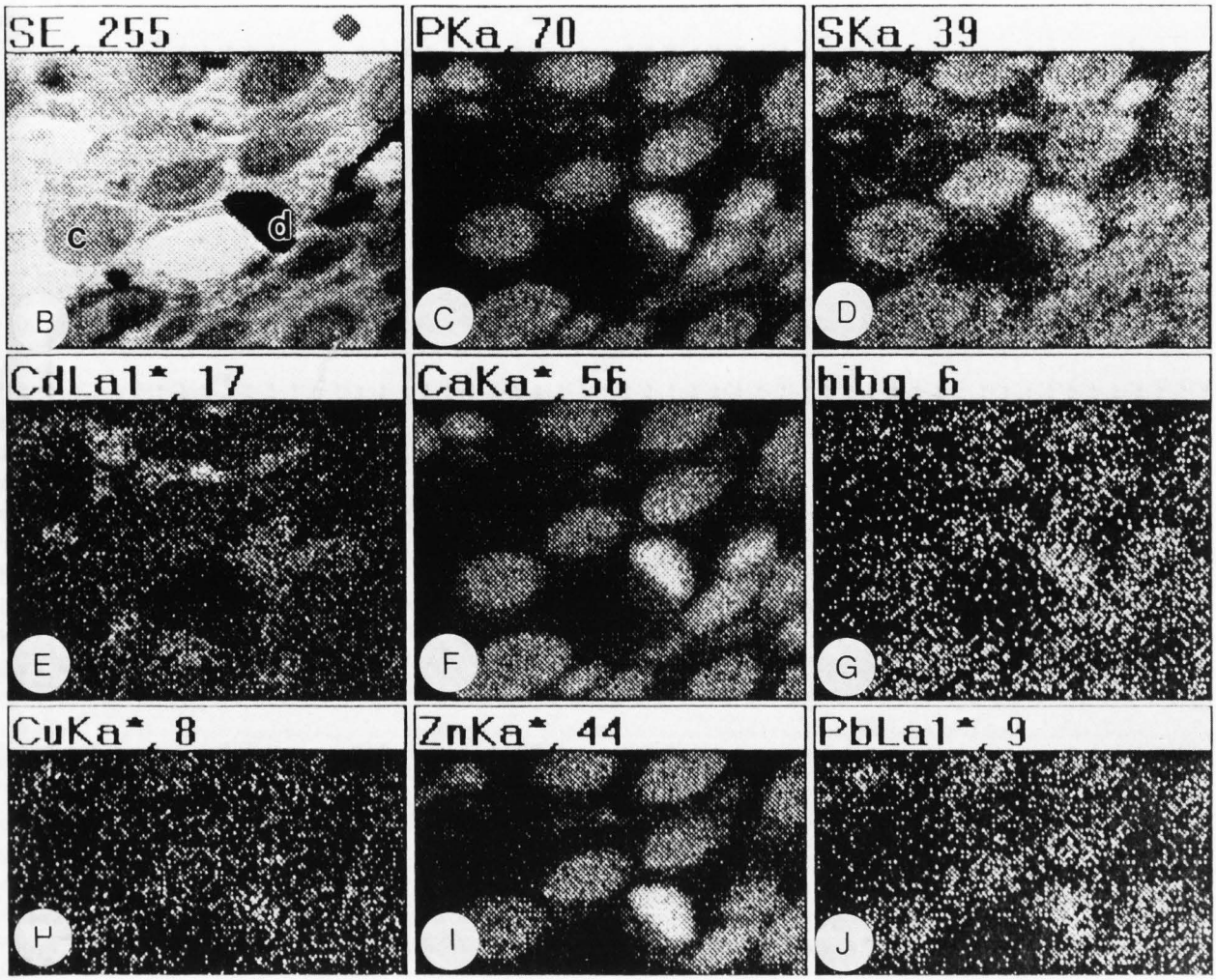


Table 2. Arsenic and metal concentrations (μg/g dry weight) in two Cornish metalliferous soils.*

Site	As	Pb	Zn	Cd	Fe	Mn	Cu
Luckett	21000	630	370	1	12.7×10^4	502	870
Bissoe	2200	140	150	10	70.6×10^4	120	530

*Pressed soil briquets were analysed by quantitative X-ray fluorescence spectroscopy - the preparative and analytical details were essentially as described by Winters and Morgan (1988).

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which have relatively high binding affinities (compared with Cd) with sulphur-donating ligands (Da Silva and Williams, 1991; Vasak, 1992), would be incorporated into so-called "cadmosome" vesicles; it is less easy to envisage Zn incorporation (Fig. 5).

Application 3: Arsenic distribution in earthworm chloragocytes

Metal concentrations in the arsenious soils from the Luckett and Bissoe mine sites in S.W. England are presented in Table 2. Note that the "total" arsenic concentrations differed by about an order of magnitude between the two locations.

A recent preliminary study (Morgan *et al.*, 1994a) concluded that arsenic is sequestered not within the phosphate-rich chloragosome granules in *L. rubellus* from Luckett, but within a distinct sulphur-bearing compartment in the same cells. This finding has been confirmed by X-ray distribution mapping of smeared chloragocytes of Luckett worms (Fig. 6), and of cryosectioned

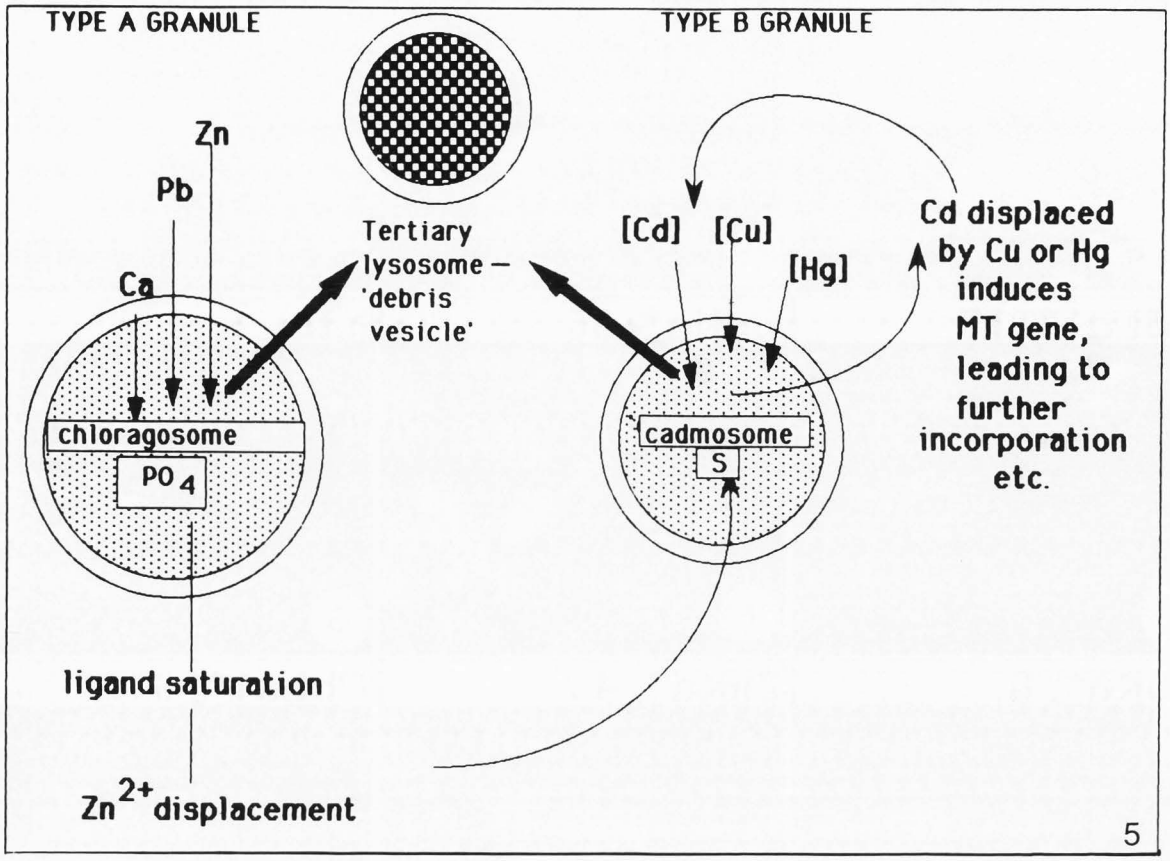

Figure 5. A hypothetical scheme illustrating some of the metal relationships of the two major metal-sequestering compartments in earthworm chloragocytes. Ca, Pb and Zn accumulate in the chloragosome matrix (Type A granules) where there is competition for available ligands (L), the outcome of which depends on the relative metal (M) concentrations transported across the limiting membrane, and on the relative stabilities of the M-L complexes. Cd induces the synthesis of sulphur-containing metallothionein (MT), and the Cd-MT complex or a derivative forms the matrix of a "cadmosome". Hg could presumably behave in much the same way as Cd, assuming that it is capable of inducing MT-gene expression; or Hg could displace Cd from the Cd-induced cadmosomal matrix, because it usually forms a more stable complex with soft bases. Cu would behave like Hg, and for the same reasons (Cosson *et al.*, 1991), in the presence of pre-formed cadmosomes. However, we have acquired preliminary evidence (Marino and Morgan, 1995) suggesting that Cu is not able to induce MT synthesis in earthworm tissues, so could not form "cuprosomes" unless MT was induced by another agent. In the event of Zn being displaced from metal-saturated chloragosomes, it is possible that the metal could induce MT synthesis as it is certainly capable of doing so in diverse vertebrate systems; it is unlikely that it would be incorporated into mature cadmosomes by substitution/displacement both because of adverse ligand-stability properties, and because the concentrations of Cd-induced ligands will probably be approximately proportional to the Cd concentrations such that there will be no "excess" available for the lower affinity interaction.

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chloragocytes of Bissoe worms (Fig. 7). Element maps derived from the tissue smear were more convincing than those derived from cryosections (compare: Figs. 6B-6H with 7B-7H). There are two factors that contribute to this difference. First, the smear was inevitably thicker than the section and, consequently, generated more intense local X-ray signals. Second, the net arsenic concentration in the smeared tissue was probably much higher than that in the cryoprepared tissue, because Luckett worms (smeared films) were exposed to a higher level of arsenic contamination than their Bissoe (cryo) counterparts (Table 2). Static probe analyses (Figs. 6I and 7I) showed that the arsenic accumulating compartments were sulphur-bearing and contained low

phosphorous contents.

Technical and bio-inorganic generalizations can be drawn from the arsenic distribution studies. The arsenic/sulphur-rich compartment, although spatially discrete, does not present any striking morphological features that might assist in its prior localization in smeared-tissue (Fig. 6A) or cryo preparations (Fig. 7A). Digital X-ray mapping, in this instance, provided an efficient unbiased sampling and compartment characterization based solely on compositional data (Morgan *et al.*, 1994b). The maps, of course, provide a correlation of spatial elemental chemistry with the general morphology of the scanned area. Even with fairly unsophisticated mapping facilities, the outputs facilitate the subsequent static probe analyses of noteworthy locations. Unbiased sampling of fairly large cellular areas is a major asset when seeking to determine the subcellular distribution of "unfamiliar" elements like arsenic. In this specific case, the chemistry of the metalloid might lead to the expectation of its co-distribution with phosphate (see below) in the chloragosome granules. Since static probes are usually positioned on identifiable structures, the localization of arsenic sequestration sites by this approach in cryosections, and in specimens prepared by alternative anhydrous protocols, would be laborious if not improbable.

Arsenic possesses a highly variable chemical behaviour, and can form many inorganic and organic compounds differing in their toxic potential (Vahter and Marafante, 1993). The metalloid can occur in either the pentavalent or trivalent states, and it is methylated only from the trivalent form (Fig. 8; Irgolic, 1986). With one remarkable exception, the marine polychaete *Tharyx marioni* (Gibbs *et al.*, 1983), there is little evidence that arsenic accumulates strongly in the tissues of aquatic and terrestrial organisms. However, there is ample evidence that plants (MacNair and Cumbes, 1987; Ullrich-Eberius *et al.*, 1989; Meharg and MacNair, 1991a, 1991b; Watkins and MacNair, 1991) and microorganisms (Silver and Misra, 1988; Silver *et al.*, 1989a, 1989b, 1993; Silver and Walderhaug, 1992) can evolve resistance mechanisms to withstand arsenic toxicity. "Mimicry" between arsenate and phosphate (Clarkson, 1993) has been implicated to a greater or lesser extent in the majority of arsenic resistance mechanisms. In higher and lower plants, resistance has been achieved by modification of arsenate influx via phosphate uptake systems. In prokaryotes, the mechanism differs fundamentally: phosphate pumps do not discriminate against arsenate, but the cells have acquired an arsenate-specific efflux pump.

Based on the well-documented interactions between arsenate and phosphate, and the probability that the surface litter layers inhabited by the epigeic earthworm species that we studied contain arsenic largely in the oxidized arsenate form, we predicted that arsenic would be accumulated within phosphate-bearing chloragosome granules (Morgan *et al.*, 1994a). X-ray mapping data refute this hypothesis. Arsenic appears to be reduced, either in the lumen of the earthworm gut or in one of the body tissues, prior to its subsequent methylation to a relatively easily excreted organoarsenical (Nielsen and Uthus, 1984). However, the trivalent form is much more toxic than arsenate because it has a high affinity for sulphur-donating ligands (Nielsen and Uthus, 1984; Irgolic, 1986) and can potentially inhibit the activities of a number of enzymes.

Electron probe X-ray analysis detects elements, but is unable to directly distinguish between bound and ionized forms or to identify different valency states. However, the observation that arsenic is co-distributed with sulphur and not phosphorus in earthworm chloragocytes provides strong indirect evidence, that the compartmentalized arsenic is in a trivalent state. Similarly, the observed co-distribution of Cu with sulphur in the cuprosomes of isopod "S"-cells (Figs. 2B and 2E) suggests that the metal is bound in the monovalent, and not the divalent state, since the former is a softer acid than the latter (Da Silva and Williams, 1991). The absence of a spin resonance signal in freeze-dried hepatopancreas preparations confirms this proposal (Morgan and Rowlands, unpublished) because Cu^{+}, unlike Cu^{2+}, is not paramagnetic. Interestingly, in this context, metallothioneins bind copper in the Cu^{+} state (Vasak, 1992; Winge, 1992).

Unfortunately, the electron microprobe cannot fully characterize the arsenic sequestering molecule other than to show that it is sulphur bearing. Given the richness of arsenic chemistry, the sequestering compartment may contain one or more sulphated arsenicals (Irgolic, 1986; Vahter and Marafante, 1993). An intriguing alternative is that, like cadmium (Morgan *et al.*, 1989), arsenic may be bound to a metallothionein. Recent published studies (Lo, 1992; Huang *et al.*, 1993; Scott *et al.*, 1993; Wang *et al.*, 1993) not only show that the sulphydryl-containing peptide, glutathione, protects cells against arsenite toxicity, but there is a steadily growing body of evidence that arsenic is capable of inducing rodent metallothionein-gene expression (Maitani *et al.*, 1987; Albores *et al.*, 1992; Kreppel *et al.*, 1993). The possibility, therefore, exists that arsenic induces metallothionein synthesis in earthworm chloragocytes, and that the arsenic-metallothionein complex is compartmentalized within vesicles homologous with "cadmosomes". We are currently investigating these possibilities in our laboratory, against a background of no literature evidence that metallothioneins bind arsenic.

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We thank: Ms. D. Janes and Mr. Peter Fisher for

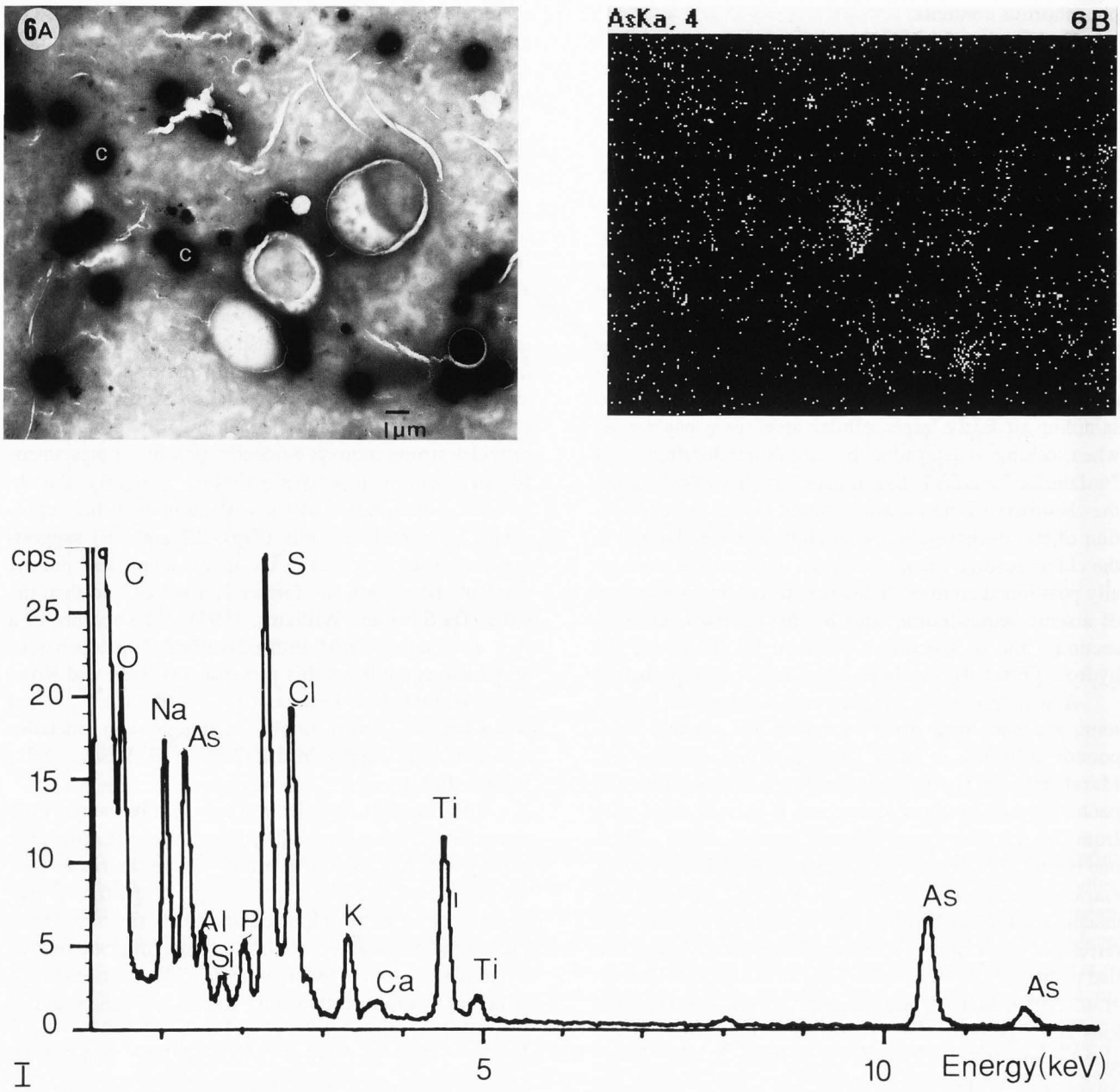

Figure 6. **(A)** Transmission electron micrograph of an air-dried smear of the chloragogenous tissue of an earthworm, *L. rubellus*, from the Luckett arsenious site. The numerous electron dense granules (c) are chloragosomes. X-ray distribution maps (**B above, C-H on the facing page**) for As Kα (**B**), S Kα (**C**), Ca Kα (**D**), P Kα (**E**), Zn Kα (**F**), background (**G**; in the energy band from 5.5 to 6.0 keV), K Kα (**H**). [No Pb was detected either by mapping or by static probe analyses, so the potential problems associated with the overlap between As (Kα) and Pb (Lα) at approximately 10.55 keV did not arise. This is not surprising given the relatively low soil Pb concentrations]. **(I, above)** X-ray energy spectrum derived from an arsenic- and sulphur-bearing compartment analysed by static probe, but previously located by X-ray mapping (Figs. 6B and 6C) because it has no easily distinguishable morphological features. Note: the area represented in the X-ray maps is slightly larger than that depicted in the micrograph Figure 6A.

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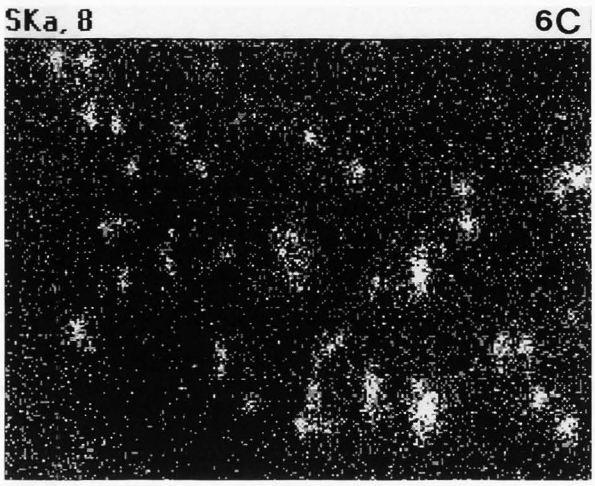
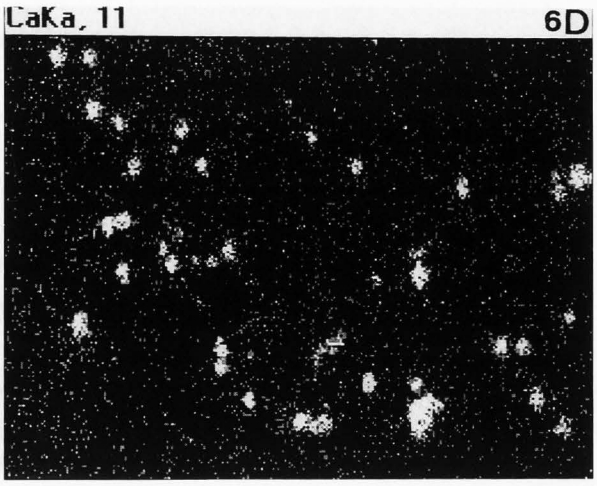

SKa, 8
6C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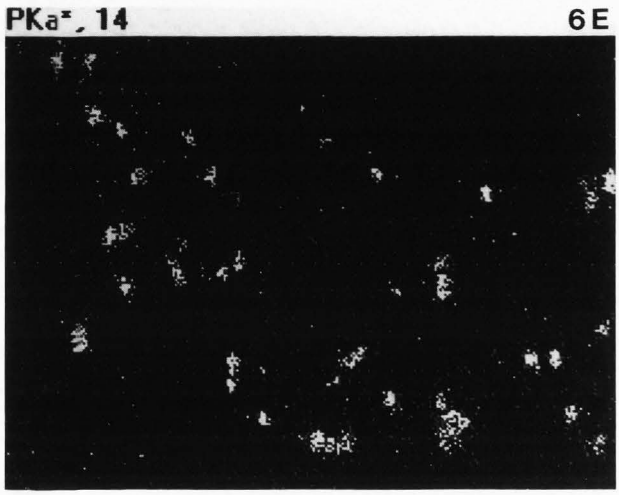
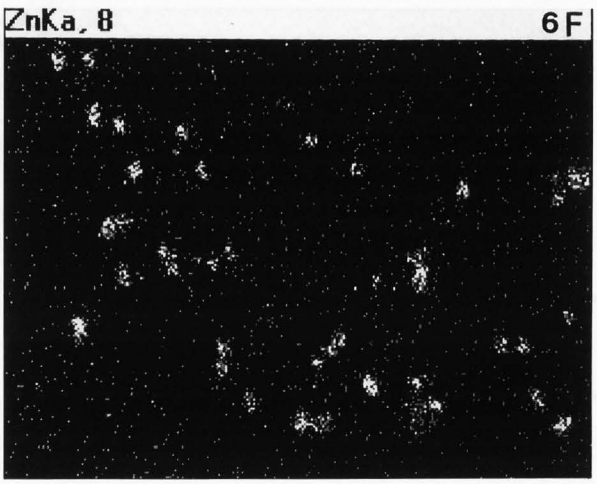
6D


PKa*, 14
6E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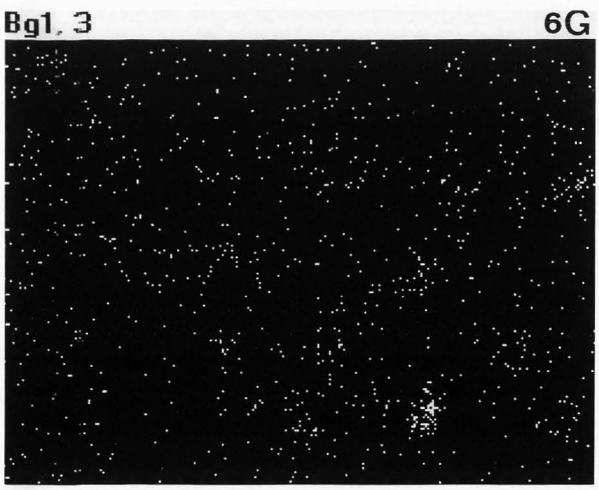
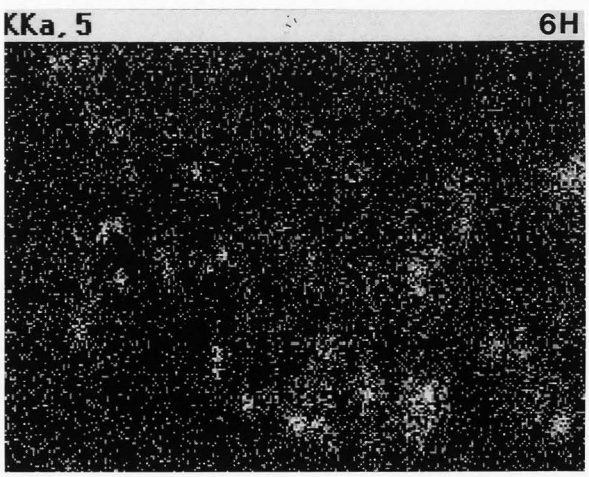

Bg1, 3
6G


KKa, 5
6H

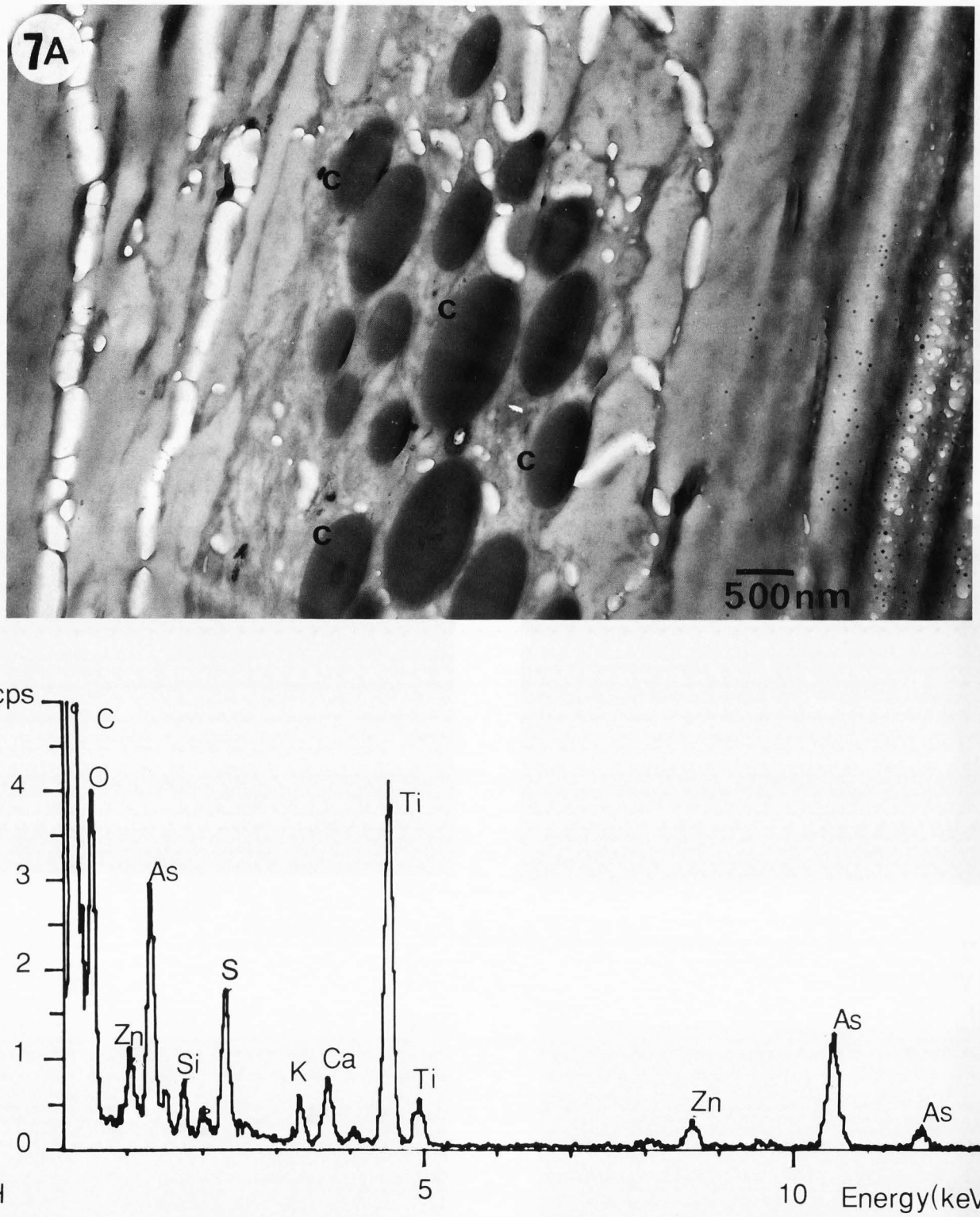
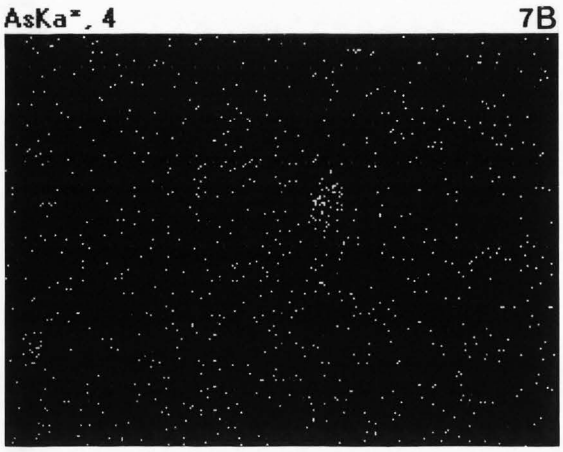
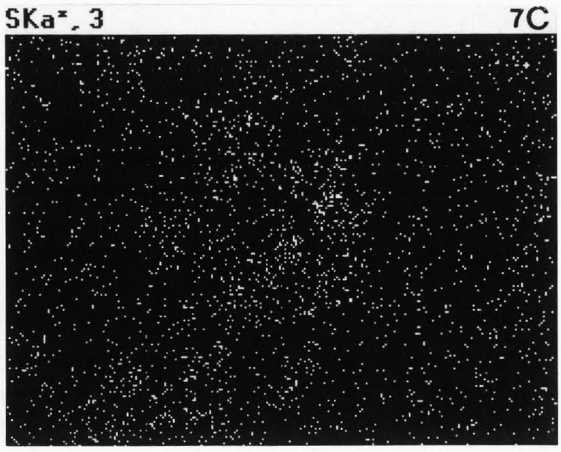


Figure 7. (**A**) Transmission electron micrograph of an unfixed, ultrathin cryosection of the chloragogenous tissue of an earthworm, *L. rubellus*, from the Bissoe arsenious site. The ovoid electron dense granules (c) are chloragosomes. X-ray distribution maps (**B to G on the facing page**): As Kα (**B**), S Kα (**C**), Ca Kα (**D**), P Kα (**E**), Zn Kα (**F**), background (**G**; in the energy band from 5.5 to 6.0 keV). (**H**) X-ray energy spectrum derived from an arsenic-rich compartment identified by X-ray mapping (Fig. 7B). Note that this compartment has a relatively high sulphur content, but a fairly low phosphorus content.


AsKa*, 4
7B


SKa*, 3
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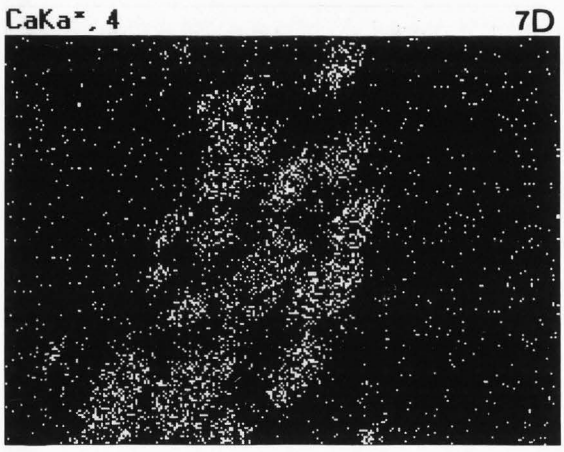
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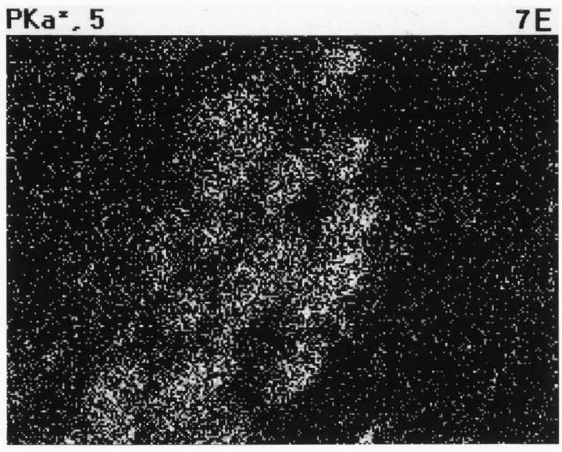
7D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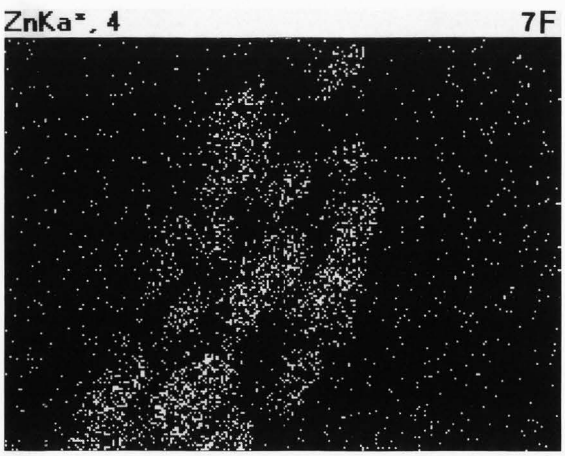
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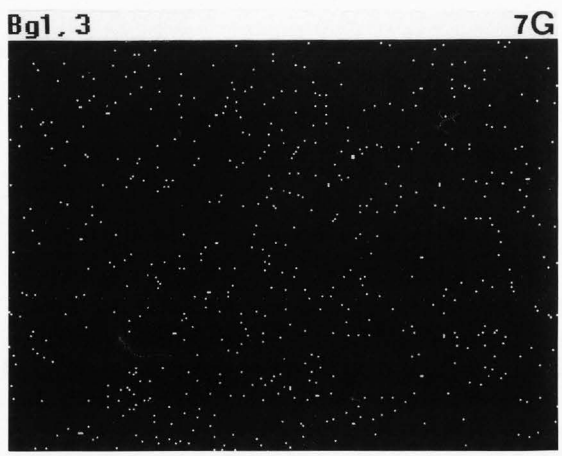
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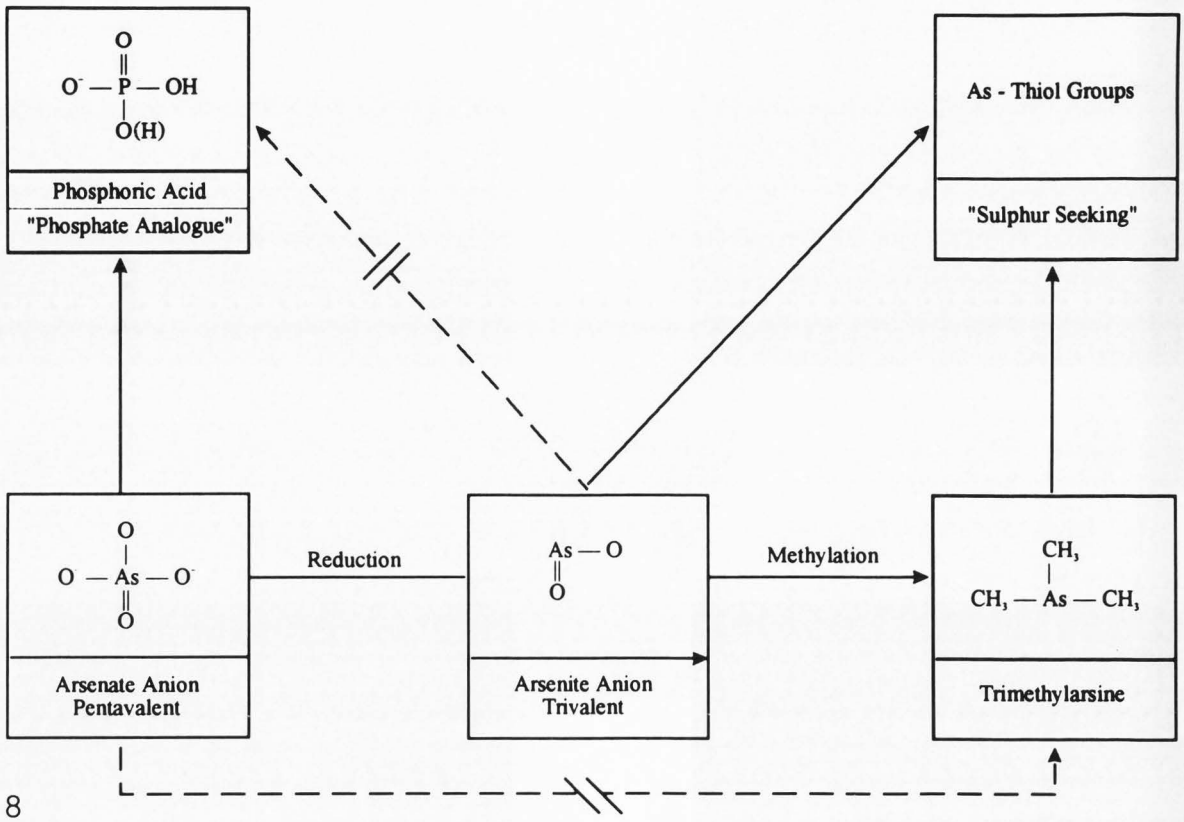

Figure 8. A schematic diagram illustrating, in very simplified form, the interactions between the pentavalent (As^{5+}), trivalent (As^{3+}), and methylated forms of arsenic, together with the phosphate-seeking tendencies of the arsenate anion and the thiol-seeking tendencies of the arsenite anion. [Adapted from information in: Nielsen and Uthus (1984), and Clarkson (1993)].

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Discussion with Reviewers

J.A. Nott: Physiological options for processing a metal are not mutually exclusive (Fig. 1). The most obvious example in molluscs and crustaceans is "sequestration" in lysosomes and phosphate granules which are subsequently excreted via the gut ("regulation"?).

Authors: These are highly pertinent comments concerning the four metal relationships "models" presented in Figure 1. These strategies are not always discrete and, therefore, not always readily distinguishable.

J.A. Nott: The impression is given in **Abstract** that "biological factors" are not constrained by thermodynamic considerations. This impression is repeated in **Introduction** (Bio-inorganic consideration).

Authors: It was certainly not our intention to give the impression that metal selectivity or discrimination by biological systems is predominantly determined by either what Da Silva and Williams (1991) describe as "biological factors", or by thermodynamic considerations. On the contrary, we hoped to make it abundantly clear in the **Introduction** that the various biological and thermodynamic factors are interdependent co-determinants. This was the purpose of the phrase "eight simultaneously operational factors that determine selection". We reinforce the principle by stating that "intact biological

systems are extraordinarily complex, so that the canon of (bio-inorganic) theory cannot readily be applied to *in vivo* states".

J.A. Nott: Intracellular granules do not always have a significant calcium content. In the molluscs, Monodonta spp. and Rucina spp., the granules are magnesium phosphates.
Authors: Quite so! The composition of other magnesium phosphate-rich molluscan granules have been described by Nott (1993). The iron-accumulating "B"-cell granules of terrestrial isopods are yet another example of phosphate granules without a significant calcium content (see: **Application 1**).

K. Zierold: How do the exogeneous metals (e.g., As, Cd, Pb, Zn) interfere with the physiological ion distribution in the cells?
Authors: The question specifically refers to physiological ions, presumably those that participate in dynamic functional events. Strictly, therefore, the answer is, we do not know, because we have not measured ion concentrations outside the metal-sequestering compartments. There is some evidence that exogenous metal accumulation in the mineral-rich compartments {e.g., earthworm chloragosomes: Morgan AJ *et al.* (1989) The subcellular accumulation of toxic heavy metals: qualitative and quantitative X-ray microanalysis. In: Electron Probe Microanalysis, Applications in Biology and Medicine. Zierold K, Hagler HK (eds.). Springer-Verlag, Berlin. pp. 59-72) displaces certain endogenous metals. It may be hypothesized that if these metal "sinks" act as efficient detoxification sites, then the free exogenous ion concentrations are minimized, and their direct cytotoxic effects are also minimized. This does not preclude the possibility that they exert indirect toxicological effects via the displaced ions (Ca^{2+} cytotoxicity incurred by Pb accumulation, for example). However, even though the earthworm chloragocytes have a prodigious capacity to sequester Pb, there is some evidence (Morgan *et al.*, 1993) indicating that Pb ions interfere with haem synthesis within these cells. This implies that Pb sequestration/detoxification is not absolutely efficient in this cell type, and implies that systematic studies of metal-induced ion disturbance in invertebrate cells are warranted.

G.M. Roomans: On how many cells and how many animals are your data in this paper based? Do you feel that this is sufficient to be representative for a whole animal population, and a sufficient basis for your theories about metal handling by the species investigated?
Authors: The fact is that our data were of a qualitative nature, and were based on a somewhat limited number of replicates. We only wish to claim that, in this paper, we have established nothing more than hypotheses, some of which, at least, are worthy of further testing.

G.M. Roomans and K. Zierold: It is evident that you need quite high elemental concentrations (see maps of As and spectra in Figs. 6 and 7) to get a signal in a map. That means that absence of an element in a map might still mean that it occurs in a fairly high concentration. Have you taken this into account in your theories? This also means that your mapping technique is quite insensitive. Do you have data about minimal detectable levels? How stable were your cryosections during X-ray mapping? Have your observed drift, shrinkage or radiation damage?
Authors: These are all pertinent observations and questions. Whilst we acknowledge that the analytical technique is insensitive, and that the toxicological implications of this constraint is profound, we can (obviously) comment on the element relationships within those subcellular compartments where the local concentrations exceed the detectable limits of our analytical system under the selected conditions of operation. The key issue is whether or not we are inadvertently, but inevitably, overlooking important cytochemical information because of the high (but presently unknown) minimum detectable concentrations. Sensitivity could be improved significantly by increasing the number of counts collected per spectrum; this being most practically achieved by increasing the aquisition times. Protracted mapping periods can lead to distortions due to drift and shrinkage, for example. However, analytical routines are available for correcting the effects of drift, although we have found in our limited experience with quantitative mapping (total aquisition times $>$ 18 hours) of carefully freeze-dried cryosections, that drift, shrinkage and radiation damage problems did not appear excessive.

J.A. Nott: It is stated in **Results and Discussion** (**Application 1**) that excess Cu ions would saturate all S-bearing ligands, and that Cd, Pb and Zn would fail to bind. Would it not be the case that all the different metal ions would bind with the ligand in ratios which would reflect the relative affinity constants?
Authors: This is a complex question that stretches our competence to respond constructively. We would suggest that Dr. Nott may well be correct, providing the ligands were equally accessible to the competing ions (that is membrane selection effects were not extant) and if the concentrations of the ions were identical. Ligand binding is, as Da Silva and Williams (1991) imply, a function of relative affinity constants and relative ion concentrations in the immediate vicinity of ligand.