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MeV-ION MICROPROBE ANALYSES OF WHOLE *DROSOPHILA* SUGGEST THAT ZINC AND COPPER ACCUMULATION IS REGULATED STORAGE NOT DEPOSIT EXCRETION

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Summary

We examined *Drosophila* spp. using a penetrative ion microprobe technique that allows us to quantify element contents in whole organs and organisms. Comparatively non-penetrative techniques, such as electron microscopy, could not have been used to make many of these measurements because material is lost during sectioning. We found that zinc was accumulated predominantly within a single organ: in the main segments of both the anterior and posterior Malpighian tubules. In contrast to zinc, iron and copper were more generally distributed throughout the body. Zinc concentrations as high as 2.8 % of dry mass were measured in cell-sized volumes of the Malpighian tubules. The large quantities of zinc (approximately 2×10^{-8} g in 8-day-old male adults) were sequestered by an unidentified mechanism. We found less than 1 % of the estimated amount of consumed zinc and copper in the abdomen of flies fed food containing several hundred parts per million dry mass of these metals. Our results are inconsistent with the detoxification hypothesis that predicts that a large proportion of the heavy metals passing through the gut are absorbed and stored permanently. We found for both zinc and copper that the quantity in the abdomen was not proportional to the concentration of these metals in the consumed food but was, instead, relatively invariant. For these reasons, we suggest that regulated biological availability, not detoxification, may be the primary benefit of zinc and copper storage.

Key words: detoxification, metallothionein, PIXE, STIM, heavy metals, toxins, metabolism, *Drosophila melanogaster*, *Drosophila hydei*.

Introduction

Heavy metals can be essential as well as toxic. For example, while high dosages of zinc are usually toxic, zinc is a component of at least 300 enzymes and also plays an important role in gene regulation because a major category of transcription factor – zinc finger proteins – chelate zinc in a way that promotes a protein structure that binds DNA (Coleman, 1992). The mechanisms of zinc sequestration and regulation in arthropods are unknown: metallothioneins in *Drosophila melanogaster* bind copper and cadmium; zinc, however, neither binds to nor induces the synthesis of metallothioneins (Maroni and Watson, 1985). In addition to its well-known roles in proteins, zinc is used in large quantities by many arthropods and annelids in structures such as claws and mouthparts. The form of these accumulations is unknown and of interest because the volume fractions of zinc are thought to be too low to affect the mechanical properties as zinc–biominerals and too high for metalloproteins (measured concentrations reach 25 % of dry mass; Schofield, 1997). It is also not known how such large quantities of zinc are made available for incorporation into the cuticle during molting. Considering that heavy metals are employed in many essential roles, at times in large quantities, the assumption by most previous investigators that the accumulation of heavy metals is deposit excretion – a detoxification mechanism – now seems questionable. Here, we suggest that accumulation plays a more complex role as part of a system for regulating the biological availability of heavy metals.

Zinc, iron and copper were first reported in insects in certain types of storage vacuoles in the Malpighian tubules (the excretory organs of insects) of the housefly *Musca domestica* (Sohal *et al.* 1976). It has been suggested that these metals are accumulated in intracellular storage vacuoles as a detoxification mechanism, because they could be reabsorbed into the hemolymph if they were excreted from the Malpighian tubule into the gut (Maddrell, 1977). It was reasoned that metal ions are small in comparison with the molecules of organic toxicants and would therefore diffuse more readily through the cells of the hindgut. Recently,

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zinc has been identified as a prominent component of storage vacuoles in cells of Malpighian tubules in *Drosophila hydei* larvae (Zierold and Wessing, 1990). *Drosophila* have four Malpighian tubules, two anterior and two posterior. Each anterior tubule is joined with a posterior tubule at their proximal ends and connected from this 'Y' junction to the gut by a short duct, the ureter. In the experiments of Zierold and Wessing (1990), cross sections of tubules from near the ureters were analyzed using an electron microprobe. Zinc concentrations averaged approximately 20 000 p.p.m. (wet mass) in storage vacuoles in cells of the anterior tubules. In contrast, measured concentrations were more than an order of magnitude lower in storage vacuoles located in the posterior tubule cells.

Maddrell (1977) suggested a comparison of consumed and accumulated quantities of metals as a test of the hypothesis that metals are sequestered in Malpighian tubule cells as a detoxification mechanism. Experiments reported here make this comparison and test some predictions of the detoxification hypothesis. Our results lead us to believe that zinc and copper accumulations are not simply a detoxification mechanism, and we suggest that storage aids in the regulation of the biological availability of these metals. We use a new MeV ion microprobe technique (Schofield and Lefevre, 1993) to determine the zinc content and distribution in whole *Drosophila* and in dissected organs. This penetrative technique does not require sectioning, in contrast to the electron probe techniques used in the earlier experiments (Zierold and Wessing, 1990). We are not aware of other techniques that could have been used for the measurements of element quantities that are reported here. The proton probe techniques employed here are also more sensitive than electron probe techniques by 2–3 orders of magnitude (Legge *et al.* 1988), with sensitivity decreasing for thicker samples. The distribution of zinc that we observed in the Malpighian tubules did not completely coincide with the distribution previously reported from electron probe microanalysis (Zierold and Wessing, 1990). Instead of an asymmetric distribution, we found that both the anterior and posterior Malpighian tubules contain comparable quantities of zinc in larval *D. hydei*. However, we found that zinc concentrations varied unpredictably by an order of magnitude along the tubules, and we think that the previous investigators may have inadvertently taken sections of the anterior tubules from high-concentration regions and sections of the posterior tubules from low-concentration regions.

Materials and methods

Media

Larvae of *Drosophila hydei* and larvae and adults of *Drosophila melanogaster* were raised on either standard cornmeal, molasses and yeast medium or on instant *Drosophila* medium (Carolina Biological Supply; Beaverton, Oregon, USA). Samples of the media were assayed by atomic absorption analysis. The standard medium contained 11 p.p.m. iron, less than 0.3 p.p.m. copper and 140 p.p.m. zinc. The instant medium contained 36 p.p.m. iron, 0.2 p.p.m. copper and 300 p.p.m. zinc (all by dry mass). Water in the media is thought to have been the source of the high zinc content. Different batches of medium were prepared using water from the same source. Typical iron, copper and zinc concentrations in fruit are in the tens of parts per million (by dry mass; Holland *et al.* 1996). A number of adults were placed on instant medium prepared with 5 mmol l^{-1} $CuSO_4$ (approximately 800 p.p.m., dry mass, copper in the medium) or with 5 mmol l^{-1} $ZnCl_2$ (approximately 800 p.p.m. zinc, dry mass) for 1 week and then placed on unsupplemented instant medium for 1 day prior to examination.

Rate of food consumption

The approximate rate of food consumption was calculated in order to compare the quantity of metal consumed with the quantity accumulated. The time required to exchange the contents of the gut was measured following the method of Maroni and Watson (1985). Adult flies were placed on medium containing powdered charcoal. At a range of times thereafter, flies were dissected to determine the distance that the dark food had progressed through the gut. By 65 min after being placed on charcoal food, 55 % of the flies dissected contained black food throughout the gut. Maroni and Watson (1985) reported similar results (1 h) for larvae. In order to calculate the rate of consumption, the volume of food in the gut was estimated by measuring the length and diameter of the food column visible within the gut. We estimated that an average of approximately 10^{-4} cm^3 of food was consumed per hour, or approximately 4×10^{-5} g dry mass. While this rate is a rough approximation, the actual rate of consumption could be less than this value by more than an order of magnitude without affecting our conclusion that only a small fraction of the zinc and copper consumed was stored.

Sample preparation

Adults and third-instar larvae were either examined whole or dissected for clarity. Whole flies were freeze-substituted with acetone followed by vacuum evaporation (Fujita *et al.* 1987). Dissections were arranged on 6 μm thick lexan plastic and allowed to dry in air.

We investigated the possibility that freeze-substitution with acetone caused metals to be leached from or redistributed within the specimens. To investigate leaching of metals from the flies, a number of flies were raised together and then either dried whole in air or freeze-substituted with acetone. The iron, copper and zinc contents of flies subjected to the two different treatments are compared in Table 1. Although the metal content of the flies is quite variable and the sample size small, we can conclude that, on average, less than half of the content of any of these metals could have been leached by the acetone treatment (95 % confidence level, two-sample *t*-test). Wilcoxon's two-sample rank test (Snedecor and Cochran, 1989) gives the same confidence level for copper and zinc, although not for iron.

To determine whether freeze-substitution redistributed metals within the flies, the metal distribution in dissected dried flies was compared with the distribution in whole freeze-substituted flies. No differences in the distribution of iron, copper or zinc were observed.

Table 1. *Does freeze-substitution with acetone leach iron, copper or zinc from whole* Drosophila*?*

Fly	Treatment	Content of whole fly (ng)*		
		Iron	Copper	Zinc
1	Dried	6.5	0.82	5.5
2	Dried	1.8	0.91	8.0
3	Dried	4.6	0.79	7.6
4	Dried	4.3	0.59	4.5
5	Dried	5.8	0.83	9.2
Mean	Dried	4.6	0.79	7.4
6	Freeze-substituted	2.6	0.71	5.5
7	Freeze-substituted	4.2	0.57	7.0
8	Freeze-substituted	4.0	0.49	6.1
Mean	Freeze-substituted	3.6	0.59	6.2

Metal contents were measured using PIXE; Dried indicates that flies were air-dried at room temperature.

*These flies were younger and not fed on the same medium as the flies in Table 3.

All flies were male.

Elemental analysis

Specimens were analyzed using a scanning ion microprobe using protons accelerated across a voltage drop of 4 MV (Lefevre *et al.* 1983). Two ion beam techniques were used: PIXE (proton induced X-ray emission) and STIM (scanning transmission ion microscopy). In combination, these two techniques can be used to measure the depth-averaged concentrations of a number of elements simultaneously in biological specimens (Schofield and Lefevre, 1993). PIXE data yield the quantity of an element in the sampled volume, while STIM data give a value for the total quantity of material in this volume.

For PIXE, X-rays of characteristic energy are counted for each element of interest. The possible extremes of X-ray attenuation within the specimen and of variations in the ratio between the number of X-rays produced and the quantity of the element present (due to slowing of the beam protons) are calculated. These calculated extremes are used in determining the PIXE measurement uncertainty. In cases where more precise measurements of local concentrations and element quantities are desired, tomography can be employed to reduce these uncertainties greatly (Schofield, 1995). When there was substantial overlap between the zinc and copper peaks in the X-ray data, the X-ray tallies were decreased by the appropriate fraction.

For STIM, a detector is used to measure the residual energy of single protons that have passed through the specimen. The projected density is calculated from these data. The resolution currently available with the STIM–PIXE technique is of the order of several micrometers.

Results and discussion

Zinc distribution

Whole freeze-substituted flies were examined to ascertain the general distribution of zinc in the abdomen of a fly. Fig. 1A is a STIM image showing a sagittal view of the abdomen of an adult male *D. melanogaster*. Fig. 1B was made using PIXE and shows that zinc X-rays appear to originate mainly from

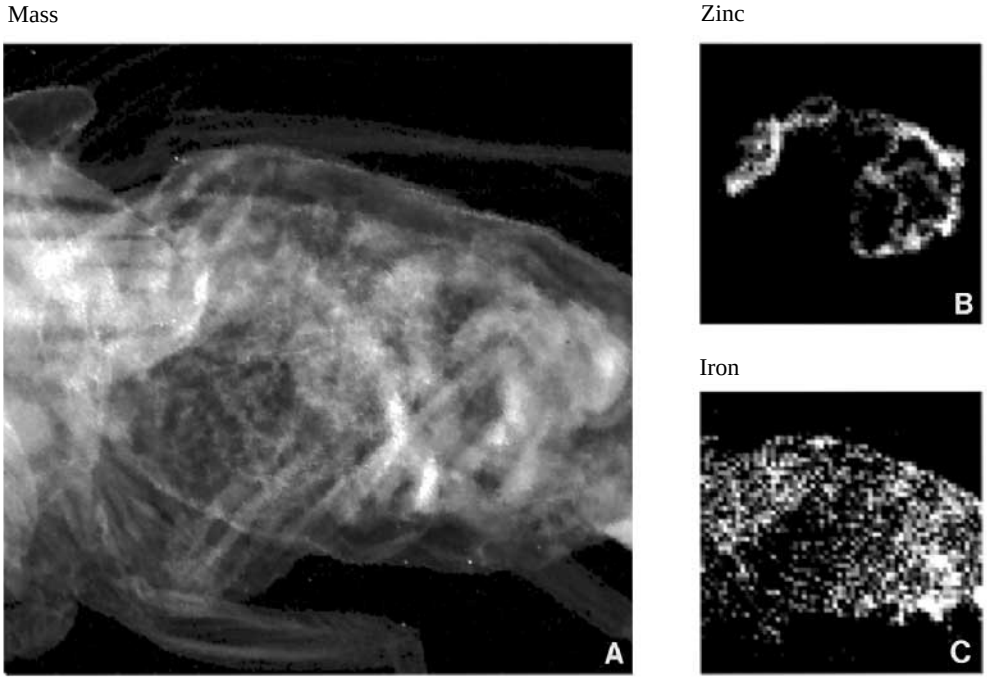

Fig. 1. STIM- and PIXE-derived images showing a portion of the abdomen and thorax of an adult male *Drosophila melanogaster* in a sagittal view (anterior to the left, dorsal to the top). This fly was reared on instant medium that was not supplemented with zinc or iron. (A) A STIM image obtained from measured energy losses of 4 MeV protons. Whiter shades indicate larger proton energy losses and, therefore, larger projected (areal) mass densities. The legs are visible at the bottom of the image, the thoracic muscles appear as horizontal striations at the upper left, and the wing as a horizontal line towards the top. (B) A PIXE image of the same field as A. Whiter shades correspond to greater zinc X-ray yields. Zinc X-rays apparently originate mainly from tubules in the dorsal and posterior regions of the abdomen. (C) A PIXE image in iron X-rays. Iron is distributed in a more general pattern than zinc. Averaging element densities over the whole abdomen shows that the contents of zinc and iron are roughly equal in the abdomen, although their spatial distributions are different. Frame size, 1 mm×1 mm.

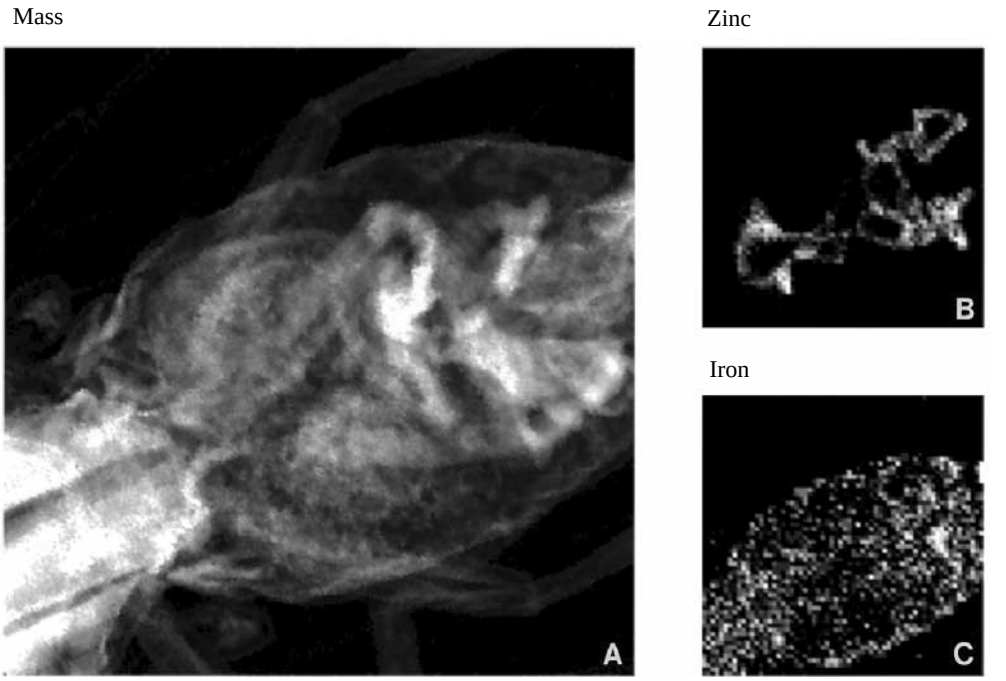

Fig. 2. STIM and PIXE images of the fly shown in Fig. 1 in a dorsal–ventral view. (A) A STIM image shows the muscles of the thorax at the lower left and the contents of the abdomen at the center and upper right. (B) A PIXE image in zinc X-rays. The zinc is located in tube-like arrays with the same distribution as in the Malpighian tubules. (C) A PIXE image in iron X-rays. The distribution of iron is more general than that of zinc, although iron is somewhat localized at the projected edges of the abdomen. Frame size, 1 mm×1 mm.

tubule-shaped structures. Fig. 1C is also a PIXE image of this region, but in iron X-rays. While this field contains similar quantities (within a factor of approximately 2) of zinc and iron, the iron is much more generally distributed than is the zinc.

Fig. 2 shows the same fly as in Fig. 1 but in a dorsal–ventral view. Once again, the zinc appears to be localized in tubules (Fig. 2B) but the iron is more generally distributed (Fig. 2C). The tubules extend along the dorsal and posterior sides of the abdomen and exhibit an approximate bilateral symmetry. Their location is consistent with that of the Malpighian tubules (Miller, 1965).

To determine whether Malpighian tubules were in fact the organs that accumulated zinc, we dissected out the internal organs from the abdomens of several adult and one larval *D.*

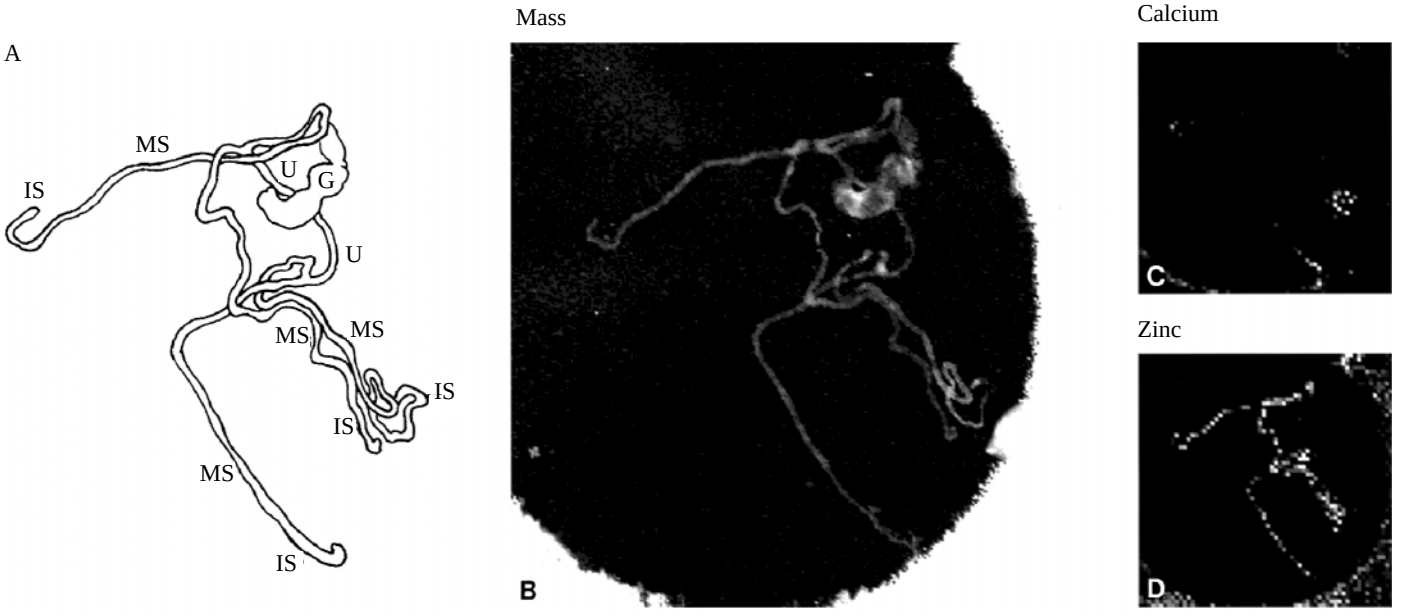

Fig. 3. The dissected Malpighian tubules of an adult *D. melanogaster*. (A) A sketch of the Malpighian tubules with a small portion of the gut attached. G, gut; IS, initial segment or distal tip; MS, main segment; U, ureter. (B) A STIM image showing the projected mass density of the dissected tubules. At the edges of the image is the circular metal frame that holds the lexan film and the sample. (C) A PIXE image in calcium X-rays showing that calcium is localized mainly at the distal segments of the Malpighian tubules. The edge of the circular metal frame is partially evident as noise in the corners of the images. (D) A PIXE image showing that very few zinc X-rays originate at the ureters and that the quantity of zinc declines in the distal segment where calcium content is high. Frame size, 3 mm×3 mm.

melanogaster. These organs were placed directly onto lexan plastic discs, and the location of each organ was sketched. The organs were allowed to dry in air onto the plastic and were examined without further treatment. The zinc content of the Malpighian tubules greatly exceeded that found in any other organs, including the internal reproductive apparatus of both males and females, the entire digestive tract, the fat body, the epidermis and the muscles. Fig. 3 shows the Malpighian tubules and a portion of the midgut – other organs also produced dramatically smaller zinc X-ray signals similar to those of the gut.

Images of several undissected adult *D. melanogaster*, similar to Figs 1B and 2B but including images of the head and thorax, showed no other zinc accumulations comparable with those in the Malpighian tubules. A whole pupa in its puparium was also examined. No concentrations comparable with those in the Malpighian tubules of the larva were found, although the general concentration of zinc in the pupa was higher than in the organs other than the Malphigian tubules of the adults examined. Apparently, zinc was reabsorbed from the larval Malpighian tubules and dispersed. The possibility that zinc was redistributed deserves further examination since it may indicate that the stored zinc plays a role in metamorphosis. Since we had shown that the mouthparts of many arthropods contain large quantities of zinc (Schofield, 1997), we scanned the cephalopharyngeal skeleton of a *D. melanogaster* larva, but found no accumulation of zinc comparable with that in the Malpighian tubules.

A dissected set of Malpighian tubules from an adult *D. melanogaster* which had been reared on standard medium was examined to determine the distribution of zinc and calcium within this organ (Fig. 3). The position of the dissected tubules on the holder were sketched (Fig. 3A) for comparison with the STIM image (Fig. 3B). Examination of the tubules in calcium X-rays showed that calcium is accumulated in the distal (initial) segments at the end of each of the tubules (Fig. 3C). This confirms earlier work that showed that calcium is present in concretions that fill the lumen of the distal segments of *Drosophila* Malpighian tubules (Sohal *et al.* 1976; Wessing and Eichelberg, 1978). It is likely that the calcium accumulation shown here is located in these concretions.

Fig. 3D shows the distribution of zinc in these tubules. Very little zinc was found in the ureters, the region between the gut and the splitting of the tubule pair. This is consistent with the lack of intracellular storage vacuoles in the proximal region of the ureter (Sohal *et al.* 1976; Wessing and Eichelberg, 1978; Zierold and Wessing, 1990). The distal segments of the tubules also contain less zinc than the main segments of the tubules, although the difference is less pronounced than the difference between the main segments and the ureters. The region of low zinc content in the distal segments overlaps with the region of high calcium content, suggesting cellular specialization along the tubule (contrast Fig. 3C,D).

Zinc concentrations

To compare our data with previous reports (Zierold and Wessing, 1990), the zinc concentration and distribution were determined in the proximal portion of the Malpighian tubules of a third-instar *D. hydei* larva (Fig. 4). Zinc was found both in the anterior pair of tubules and in the posterior pair. The ureter contained comparatively little zinc, especially in the proximal portion.

To make quantitative comparisons of zinc concentrations, we examined each pixel of the 64×64 pixel PIXE image. The areal (projected) density of zinc obtained from the PIXE data was divided by the total areal density calculated from the STIM data. This zinc concentration is a mean value for the volume traversed by the beam and so is depth-averaged. The zinc concentration varied considerably in the main portions of the tubules. Over the field of this figure, the zinc concentration in the anterior tubules varied from 28 000±7000 p.p.m., dry mass, to 2000±1000 p.p.m. (the ranges delineate a 68 % confidence interval). Each of the measured concentration values is for a volume of the specimen that projects onto a single pixel of Fig. 4B. These volumes are large in comparison with the size of intracellular zinc-containing vacuoles, which were measured individually by Zierold and Wessing (1990) using electron probe microanalysis and compared with the surrounding cytoplasm; the sampled volumes for PIXE in the current experiments are approximately the size of an entire Malpighian tubule cell.

Mean zinc concentrations in the field of this image were calculated in order to compare the anterior and posterior tubules and the gut. The mean concentration for the main segment of the two anterior tubules was 12 000±2000 p.p.m., dry mass; for the two posterior tubules, it was 8000±2000 p.p.m. The mean concentration of zinc in the portion of the gut shown in Fig. 4 was found to be 280±50 p.p.m., dry mass, which was comparable to the zinc concentration in the food. The relatively few zinc X-rays originating in the gut are not indicated with the gray scale used for Fig. 4B. Zinc concentrations decreased

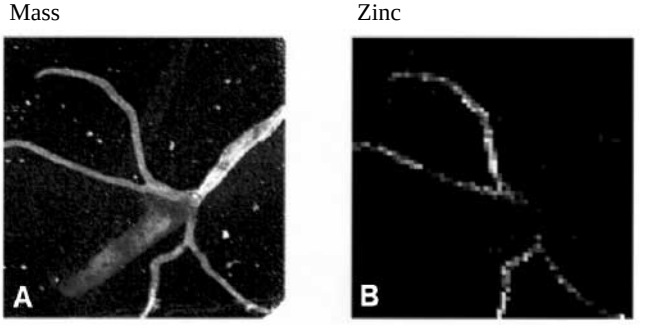

Fig. 4. A portion of the gut and Malpighian tubules of a third-instar *D. hydei* larva. (A) A STIM image showing part of the midgut at the lower left and part of the hindgut at the upper right. Proximal portions of Malpighian tubules are shown; the anterior tubules are at the upper left, the posterior tubules are at the lower right. (B) A PIXE image in zinc X-rays showing that the distribution of zinc in the main segments is not homogeneous and that relatively little zinc is located in the gut or the ureters, the common duct that connects the tubule pairs to the gut. Frame size, 2 mm×2 mm.

Table 2. *Zinc in the dissected Malpighian tubules and gut of* Drosophila

		Malpighian tubules			
			Main segments		
Organism	Gut	Ureters	Anterior	Posterior	Distal tips
D. hydei					
Larva 1	280±50	–	12000±2000	8000±1000	
Larva 2	30±10	–	7000±1000	6000±1000	–
D. melanogaster					
Larva 1	210±60	–	3000±1000	4000±1000	
Larva 2	–	–	+	+	
Adult 1	–	–	+	+	–
Adults 2 and 3	–	–	+*		
Adults 4 and 5	–		+*		

Entries indicate either mean zinc concentrations (parts per million of dry mass) from STIM–PIXE or, when only PIXE was employed, the presence (+) or absence (–) of large quantities of zinc.

*Not known whether the scanned portions were anterior or posterior tubules.

Values are mean zinc concentrations, measured using the STIM–PIXE technique, at 10 or more locations spread evenly along the scanned portion of the structure indicated ± the absolute measurement uncertainty at a confidence level of 68%, not the statistical spread of local values.

across the ureter from the high values of the main segments to the low values of the gut.

Several other dissections were examined to test the generality of these observations. Results for all of the dissections are included in Table 2. Mean concentrations were calculated from data at ten or more locations spaced at even intervals along the portion of the organ that was scanned. The 68 % confidence intervals for the absolute concentration in the pooled sampled volumes are given, as an indication of the uncertainty associated with the technique, and not the standard deviation for ten individual measurements. When concentrations were not calculated, observational data were included in the table. Main segments of all tubules contained elevated concentrations of zinc. In contrast, the zinc concentration in all the ureters and distal tips of the tubules examined was substantially lower.

Differences between zinc and copper accumulation

For a quantitative comparison between zinc accumulation and copper accumulation, a number of adults were placed on food containing 5 mmol l^{-1} $ZnCl_2$ or 5 mmol l^{-1} $CuSO_4$ (approximately 800 p.p.m., dry mass, added metal). After 1 week, flies were placed on unsupplemented food for 1 day and then freeze-substituted with acetone. In Table 3, the abdominal contents of these metals are compared for these flies as well as for flies raised on unsupplemented medium.

Maroni and Watson (1985) found low-molecular-mass proteins, thought to be metallothioneins, that bound cadmium and copper in *D. melanogaster*. Zinc, however, did not bind to or induce a similar protein. Our observations support these earlier results because we found no correlation between zinc and sulfur distributions. Metallothioneins are characterized by a ratio of sulfur (in cysteine) to metal of approximately 3:1. Nevertheless, Table 3 shows that the fly abdomens contained more zinc than copper. The flies must employ an effective sequestration mechanism other than the metallothionein mechanism.

Some copper may have been sequestered in a manner similar to zinc. Copper has been shown to accumulate in storage vacuoles in the epithelium of the larval midgut (Filshie *et al.* 1971; Tapp and Hockaday, 1977). Two of several hundred storage vacuoles examined using an electron

Table 3. *Food concentrations and abdominal accumulations of zinc and copper in adult male* Drosophila melanogaster

	Medium (p.p.m.)*		Abdomen (ng)		
	Zinc	Copper	Zinc	Copper	*N*
Instant	300	0.2	33±13	4.0±2.0	5
+ 5 mmol l^{-1} $ZnCl_2$	1100	0.2	38±15	6.0±3.0	6
+ 5 mmol l^{-1} $CuSO_4$	300	800	36±10	25±10	7

*Parts per million dry mass

Instant medium concentrations were determined by atomic absorption analysis; increases due to doping were calculated. Abdominal contents were determined using PIXE.

Values are means ± standard deviation of values for individuals.

microprobe contained high concentrations of zinc instead of copper (Tapp and Hockaday, 1977). The zinc-containing granules were indistinguishable in appearance from the copper-containing granules. The storage vacuoles containing copper (and occasionally zinc) in the midgut epithelium (Filshie *et al.* 1971; Tapp and Hockaday, 1977) and the vacuoles containing zinc in the Malpighian tubules (Zierold and Wessing, 1990) are similar in ultrastructure. These similarities suggest the possibility that the mechanisms of copper accumulation in the midgut and of zinc accumulation in the Malpighian tubules are also similar. Maroni and Watson (1985) noted the presence of copper in the particulate fraction of homogenates and suggested that this fraction of the copper (15–25 %) was not associated with metallothionein. Perhaps this fraction of copper is sequestered in a manner similar to zinc.

The different distribution patterns of copper and zinc are demonstrated in Fig. 5, which shows an adult *D. melanogaster* fed food containing 5 mmol l^{-1} $CuSO_4$ (approximately 800 p.p.m., dry mass, copper) and approximately 300 p.p.m. zinc (not supplemented) and chased with unsupplemented food containing less than 1 p.p.m. copper. The PIXE images in the top row (Fig. 5A–C) show sagittal views in iron, copper and zinc X-rays, respectively. The bottom row of PIXE images (Fig. 5D–F) are dorsal–ventral views, again in iron, copper and zinc X-rays, respectively. The copper images have been corrected for overlap of copper and zinc X-ray peaks. Copper contrasts with zinc in that it is distributed generally and not only in the Malpighian tubules. The general distribution of copper in copper-fed flies, including its appearance in the Malpighian tubules, has been noted previously (Marchal-Segault *et al.* 1990). The localized zinc distribution pattern suggests specialized regulation of zinc and indicates that sequestration mechanisms for copper and iron discriminate against zinc.

Only a small proportion of consumed zinc and copper was accumulated in flies fed diets rich in metal

Maddrell (1977) has suggested that the amount of metals consumed by flies should be compared with the accumulated quantity as a test of the hypothesis that Malpighian tubules store metals as a detoxification mechanism. Our blackened food studies indicate that 8-day-old adults fed on food containing 300 p.p.m., dry mass, zinc have consumed roughly 2.3×10^{-6} g of zinc since eclosion. The fly shown in Fig. 1 (fed on 300 p.p.m. zinc) had accumulated only $2.1\times10^{-8}\pm0.8\times10^{-8}$ g of zinc in its abdomen; this is less than 1 % of the estimated quantity consumed.

In his detoxification hypothesis, Maddrell (1977) suggested that heavy metals are permanently stored in, rather than excreted from, the Malpighian tubules because they would be reabsorbed from the hindgut if excreted and, presumably, would return to the Malpighian tubules. For this cycling of metals to be problematic, a substantial proportion of the metals passing through the gut would have to be absorbed. Thus, the detoxification hypothesis predicts that a substantial proportion of the consumed zinc would be absorbed and stored. Our finding that less than 1 % of the consumed zinc was stored is thus inconsistent with the detoxification hypothesis.

Since a food concentration of 300 p.p.m. of zinc is high relative to typical concentrations in fruit (tens of parts per million; Holland *et al.* 1996), it is possible that the storage mechanism was saturated. However, if the storage mechanism were saturated, then it is unlikely that storage is a very important detoxification mechanism because the flies fed on this undoped medium appeared normal and healthy, and zinc is fatally toxic only at considerably higher dosages (larvae, Maroni and Watson, 1985; adults, R. M. S. Schofield, J. H. Postlethwait and H. W. Lefevre, unpublished observations).

Our observations of other flies also did not support the hypothesis that storage is necessary to prevent re-uptake of toxic

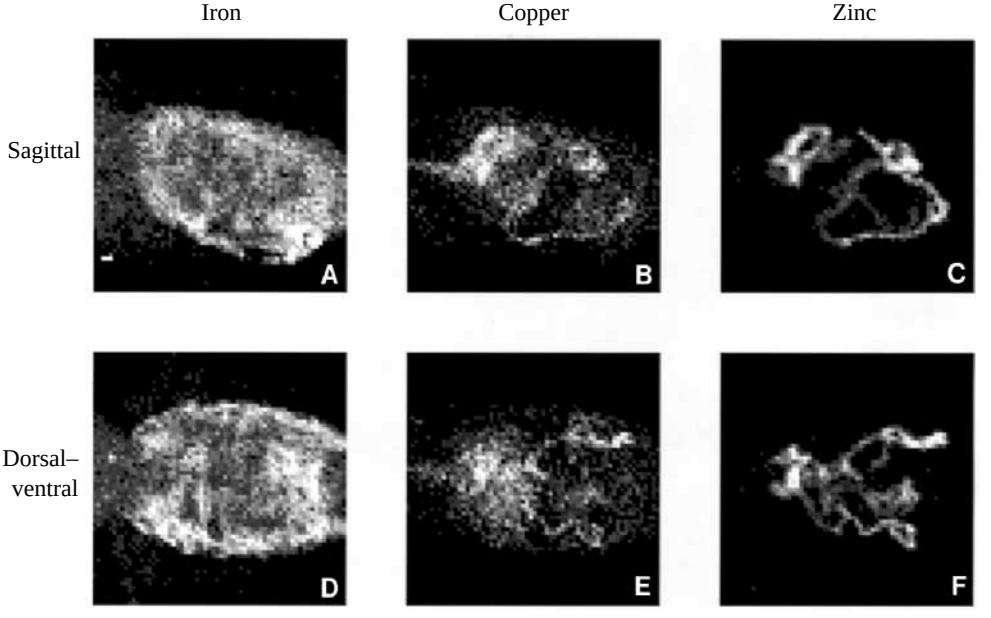

Fig. 5. PIXE images of the abdomen of an adult *D. melanogaster* that was fed instant medium supplemented with 5 mmol l^{-1} $CuSO_4$. (A,B,C) Sagittal views with the anterior of the fly to the left and the dorsal surface at the top. (D,E,F) Dorsal–ventral views with the anterior of the fly again at the left. (A,D) In iron X-rays; (B,E) in copper X-rays; (C,F) in zinc X-rays. Approximately twice as much zinc as copper is present in this field. The iron content is greater than that of zinc even though iron concentrations in the food were lower than zinc concentrations by approximately an order of magnitude. Frame size, 1 mm×1 mm.

elements. The flies reported in Table 3 had all accumulated less than 1 % of the estimated quantities of zinc consumed. As with zinc, less than 1 % of the consumed copper was accumulated in flies fed food containing approximately 800 p.p.m., dry mass, copper. However, approximately three times the estimated quantity of copper consumed since eclosion was found in flies fed food containing approximately 0.2 p.p.m. copper, suggesting that copper had been retained from the larval stage.

Accumulated quantities of zinc and copper were not proportional to food concentrations

If metals were permanently stored to remove them from the metabolic pool (deposit excretion), we would expect the quantity of metal in the fly to be roughly proportional to the quantity of metal consumed. If it is assumed that the metals diffuse through the gut wall into the body cavity, the rate of diffusion given by the diffusion equation is proportional to the concentration difference between the lumen of the gut and the hemolymph. If the concentration of metal in the hemolymph is low as a result of efficient sequestration of the metal or if the metal concentration in the hemolymph is approximately proportional to that in the gut, then the diffusion rate is approximately proportional to the concentration in the gut. Therefore, if metals diffused into the hemolymph, were sequestered and permanently stored, then the quantities of these metals accumulated in a given period would be proportional to the quantity consumed. Table 3 shows that this proportionality was not observed for either copper or zinc. The copper content of the abdomen was relatively invariant, varying by only a factor of 5, while the copper concentration in the medium changed by a factor of more than 2500, from approximately 0.2 p.p.m. (more than an order of magnitude lower than typical concentrations in fruit) to 800 p.p.m. The quantity of zinc accumulated was also relatively invariant, although the concentration of zinc in the food was only varied by a factor of approximately 4. The possibility that food intake was greatly reduced for zinc- or copper-supplemented food must be considered, but this seems unlikely because supplementation did not reduce the quantities of the unsupplemented metal accumulated (Table 3). The observed lack of proportionality between the medium concentrations and the quantities of metal accumulated is thus inconsistent with deposit excretion. Our observations are, instead, consistent with the hypothesis that flies actively regulate their zinc and copper contents, attempting to maintain stores that are independent of metal concentrations in their diets.

Sequestration may serve to make zinc and copper biologically available

The ability to take up and locally to sequester large quantities of zinc is widespread among the arthropods. Zinc accumulations have been reported in the region immediately surrounding the midgut of an ant (Schofield *et al.* 1988). The zinc concentration in this region, which includes the Malpighian tubules, reached 5000 p.p.m., dry mass, (Schofield, 1990), comparable to the concentrations we found in fly Malpighian tubules here. It was suggested that this intestinal accumulation might be related to the high concentrations (40 000 p.p.m., dry mass) of zinc found at the cutting edge of the ant mandibles. Such cuticular accumulations of zinc have been found in the mandibles of insects (Hillerton and Vincent, 1982), the tarsal claws of insects (Schofield, 1990), the fangs of spiders (Schofield and Lefevre, 1989), the chelicera, pedipalpi, tarsal claws and stings of scorpions (in concentrations reaching 250 000 p.p.m., dry mass) and the mechanical structures of a number of other organisms (Schofield, 1997), although not in fruit flies. Even though cuticular accumulations of zinc are not present in fruit flies, the mechanisms of zinc uptake and storage may well be similar to those in these other arthropods.

While we are unaware of arthropods that use large quantities of copper, the relative invariance of stored quantities of copper with respect to food concentrations prompts the suggestion that storage of both copper and zinc is beneficial in regulating the biological availability of these metals.

In conclusion, when flies were fed on food containing several hundred parts per million of zinc or copper, a minor fraction of the metal consumed was stored. The site-specific storage of zinc in the Malpighian tubules contrasted markedly with the more dispersed accumulations of iron and copper. This specificity in metal accumulation is more suggestive of regulation for biological availability than of deposit excretion. More compelling, the failure to observe a proportionality between the quantities of zinc and copper estimated to have been consumed and the quantities accumulated is inconsistent with deposit excretion and is, instead, consistent with the hypothesis that storage is regulated, perhaps to control biological availability. The technique demonstrated here promises to be of further use in the study of the metabolism of minor element because, in contrast with alternative techniques, whole organisms can be imaged and element quantities in specified volumes or organs can be measured.

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