Effects of Heavy Metals on Drosophila Larvae and a Metallothionein cDNA

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Drosophila melanogaster larvae reared on food containing radioactive cadmium retained over 80% of it, mostly in the intestinal epithelium. The majority of this radioactivity was associated with a soluble protein of less than 10,000 molecular weight. Synthesis of this cadmium-binding protein was induced by the metal as demonstrated by incorporation of radioactive cysteine. Most copper ingested by larvae was also found to associate with a low molecular weight, inducible protein, but some of it was found in an insoluble fraction. Zinc was unable to, or very inefficient at, binding or inducing the synthesis of a similar protein.

A *D. melanogaster* cDNA clone was isolated based on its more intense hybridization to copies of RNA sequences from copper-fed larvae than from control larvae. This clone showed strong hybridization to mouse metallothionein-I cDNA at reduced stringency. Its nucleotide sequence includes an open-reading segment which codes for a 40-amino acid protein; this protein was identified as metallothionein based on its similarity to the amino-terminal portion of mammalian and crab metalloproteins. The ten cysteine residues present occur in five pairs of near-vicinal cysteines (Cys—X—Cys). This cDNA sequence hybridized to a 400-nucleotide polyadenylated RNA whose presence in the cells of the alimentary canal of larvae was stimulated by ingestion of cadmium or copper; in other tissues this RNA was present at much lower levels. Mercury, silver, and zinc induced metallothionein to a lesser extent. Whether (any of) the protein(s) discussed above correspond(s) to that coded by this RNA sequence has not yet been determined.

Introduction

Copper, zinc, and cadmium are metals of great importance in biological processes. Copper and zinc are enzymatic cofactors (*1*), and while cadmium is not known to be required in any physiological role, its importance derives from the fact that it is extremely toxic. The cellular mechanisms affected by cadmium seem to be multiple and may include phenomena ranging from plasma membrane transport to transcription of DNA (*2*). At least in mammals, the three metals share a characteristic ability to bind to metallothionein (MT), although cadmium and zinc are most frequently associated with the protein. The function of MT is not well understood, but it is suspected of being important either in protecting the organism from the toxic effects of cadmium or in the homeostatic storage of zinc (*3*). We decided to study metallothionein in the fruit fly with the expectation that a combination of genetic and molecular analyses would increase our understanding of the physiological function and regulation of this protein. As far as we are aware, there have been no reports of the presence of metallothionein in insects. Poulson et al. (*4*) observed that copper

accumulation by the larvae of *Drosophila melanogaster* was roughly proportional to the medium concentration. This histochemically detectable copper was found to be preferentially stored in a small region of the middle midgut although the use of a radioactive tracer showed the presence of large amounts in all regions of the midgut. Filshie et al. (*5*) observed the presence of copper in naturally fluorescing microbodies in cuprophilic cells of the middle midgut. Tapp (*6*) and Tapp and Hockaday (*7*) confirmed these observations by X-ray microanalysis and detected the presence of sulfur in the same structures; they also demonstrated, cytochemically, acid phosphatase in them. Thus, these organelles are cytolysosomes, as had been suspected from morphological evidence. Sohal et al. (*8*) observed similar organelles in the midgut epithelium of the adult house fly. It has been suggested that these microbodies play a role in the excretion of excess metals (*9*).

Jacobson et al. (*10*) described several changes in the protein and tRNA patterns of *Drosophila melanogaster* following the ingestion of cadmium. More recently, these authors have detected a very low molecular weight (ca. 3000) macromolecule capable of binding cadmium in adult flies (*11*).

In this paper we review various aspects of the uptake and fate of cadmium, copper and zinc in *Drosophila me-*

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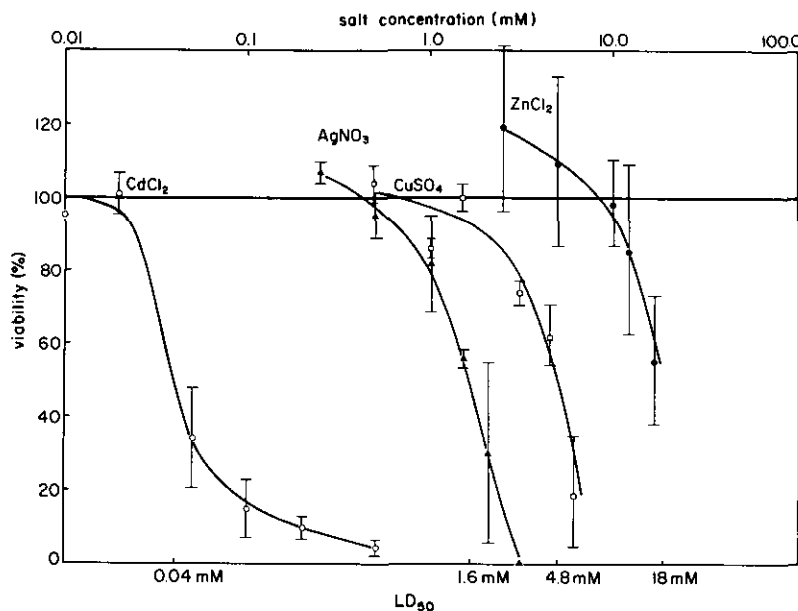
FIGURE 1. Relative viability in media with one of four metal salts. Each data point represents an average of ratios between the number of larvae that reach pupariation at a given salt concentration and the number of larvae that reach pupariation in unsupplemented medium, n = 3. Vertical bars, 95% confidence interval (*12*).

lanogaster larvae. The results presented here are consistent with the idea that Cd^{2+} and Cu^{2+} (but not Zn^{2+}) induce the synthesis of MT-like protein(s) and that the major fraction of those two metals associates with this protein. We also describe the isolation and properties of a Drosophila cDNA derived from a gene that responds to metal treatments by accumulation of the corresponding RNA. By sequence analysis we showed that this cDNA probably codes for a metallothionein. Details of the experimental methods have been published elsewhere (*12, 13*).

Results and Discussion

Effect of Metals on Larval Development and the Localization of Cd^{2+}

The effect on larval viability of food supplemented with different concentrations of four metal salts: $CdCl_2$, $CuSO_4$, $ZnCl_2$, and $AgNO_3$ is shown in Figure 1. The LC_{50} for cadmium was highly dependent on the medium on which larvae were reared; thus, on standard corn meal, molasses, and killed-yeast medium or on Instant Drosophila Media (IDM) supplemented with live yeast, larvae could tolerate concentrations of $CdCl_2$ ten times higher than when they were grown on IDM with yeast extract (YE).

Larvae labeled with the radioactive isotope $^{109}Cd^{2+}$ were dissected in Ephrussi and Beadle's salt solution, and individual organs were transferred to vials for gamma-counting. Even after a 24 hr "chase period," more than 95% of the radioactivity was recovered in the alimentary canal (Table 1). These results were confirmed

Table 1. Distribution of $^{109}Cd^{2+}$ among the major larval organs.

Organ	CPM[a]	Percent[b]
Midgut	16,947 ± 8914	96.0 ± 3.8
Dissection fluids[c]	867 ± 567	2.8 ± 2.2
Fat body/sal. glands	107 ± 98	0.4 ± 0.6
Carcass[d]	90 ± 88	0.4 ± 0.5
Malpighian tubules	72 ± 114	0.3 ± 0.5

[a] Average counts per minute and 95% confidence interval; n = 9.

[b] Percent of recovered counts in different organs and 95% confidence interval. Approximately 90% of the counts present in the whole larvae could be accounted for after dissection.

[c] The dissection fluid (50 μL) includes hemolymph and remnants of cells accidentally broken.

[d] Carcass includes the body wall, nervous system, and imaginal discs.

by autoradiography of alimentary canals from larvae treated with radioactive cadmium and fixed in glutaraldehyde. The experiments showed that silver grains occurred along the whole extent of the midgut, with a region in the middle midgut showing a slightly higher concentration. This segment of elevated radioactivity corresponds to the site of cuprophilic cells.

To determine whether cadmium is concentrated in subcellular organelles, larvae were treated with cadmium, copper, or both, extracts were prepared, and lysosomal pellets and the corresponding supernatants were assayed for the metals. While a significant proportion of the total copper was found in the particulate fraction, practically none of the cadmium was (Table 2). Copper-binding lysosomes have a distinctive ultrastructure (Fig. 2); although we were able to detect large numbers of the characteristic lysosomes in the middle midgut of copper-treated larvae, similar structures were not evident in the same cells of cadmium-treated larvae.

Table 2. Distribution of Cd and Cu between soluble and particulate fractions.[a]

Treatment	Cd, %		Cu, %	
	In pellet	In supernatant	In pellet	In supernatant
Cd (0.01 mM)	2.3	97.7	—	—
Cd (0.01 mM) + Cu (1.50 mM)	2.4	97.6	—	—
Cu (3.00 mM)	—	—	16.2	83.8

[a] Results are averages of two extracts. Several experiments gave similar results.
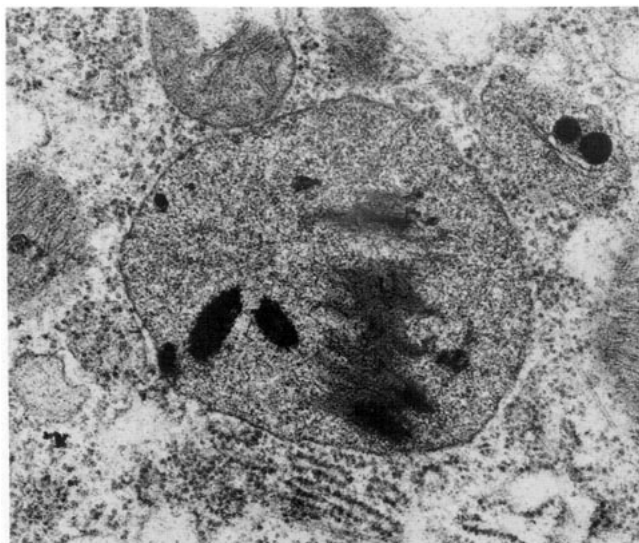

FIGURE 2. EM photomicrograph of a segment of a cuprophilic cell of the larval midgut (5). The larvae had been reared in food supplemented with 5 mM CuSO₄; this treatment leads to the accumulation of vesicles which include darkly staining bodies and stacks of membranous material. The vesicles in general and the dark inclusions in particular contain high concentrations of copper and sulfur as determined by scanning EM microanalysis (data not shown). The tissue was prepared using standard methods, fixation was with glutaraldehyde and osmium tetroxide and staining with lead and uranium salts.

FIGURE 3. Accumulation of cadmium by second instar larvae. Treatment was with $^{109}Cd^{2+}$ at $CdCl_2$ concentrations of (●) 0.015 mM or (▲) 0.10 mM; (○, △) Cd levels in larvae transferred to unsupplemented food after 24 hr labeling (12).

Uptake and Accumulation of $^{109}Cd^{2+}$

It was established that third instar larvae take approximately 1 hr to exchange the contents of their gut by feeding medium containing finely ground charcoal to larvae and observing the passage of black food along the intestinal tract. Third instar larvae were fed for 1 hr on IDM-YE containing $^{109}Cd^{2+}$, washed thoroughly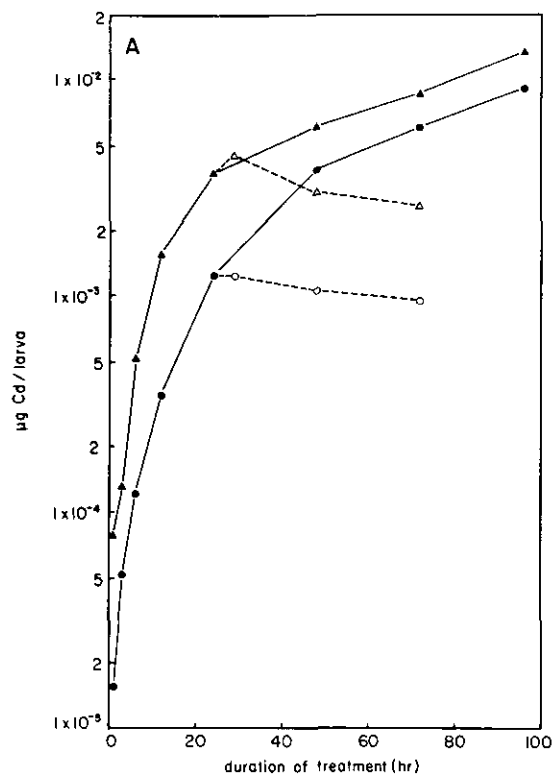, and their radioactivity was measured. The same larvae were then fed nonradioactive food for a 1-hr or a 3-hr period, and their radioactivity was again measured. The results summarized in Table 3 establish that a high proportion of radioactive cadmium ingested is retained by these larvae: 85% after 1 hr and 80% after 3 hr. These experiments also show that pretreatment of larvae with cadmium chloride does not enhance their ability to retain $^{109}Cd^{2+}$.

Second instar larvae, transferred to medium supplemented with $^{109}Cd^{2+}$, began to incorporate radioactivity immediately and continued the incorporation for 4 or 5 days, until pupariation occurred (Fig. 3). This figure also shows that cadmium was bound in a stable fashion: there was a very slow rate of loss in larvae that were transferred to cadmium-free medium after 24 hr of treatment (open symbols). A more realistic picture of cadmium binding was obtained by discounting the effect of growth from the total counts accumulated per individual. The amount of soluble protein in crude extracts was used as

Table 3. Retention of $^{109}Cd^{2+}$ by larvae.[a]

Pretreatment	Chase time hr	Radioactivity in single larvae		% retained
		After labeling	After chase	
0.015 mM CdCl₂	1.0	205 ± 51	168 ± 53	82.3 ± 8
None	1.0	488 ± 41	445 ± 41	86.0 ± 12
0.015 mM CdCl₂	3.0	368 ± 175	281 ± 117	75.2 ± 9
None	3.0	308 ± 223	263 ± 203	85.3 ± 7

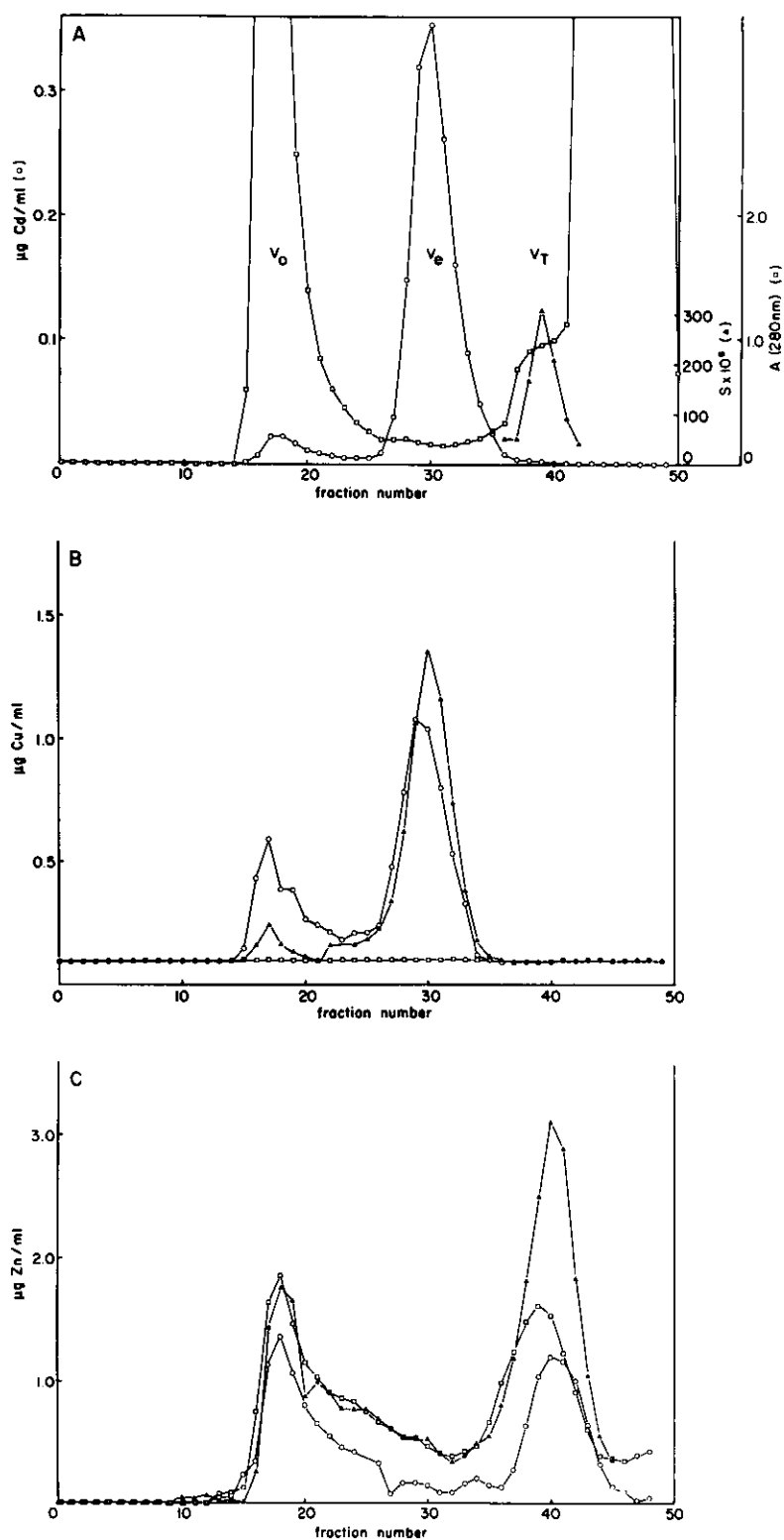[a] Mean and 95% confidence interval of five observations for each treatment.

FIGURE 4. Sephadex G-50 elution profile (A) of $^{109}Cd^{2+}$ from an extract of larvae treated with the radioactive metal for 48 hr and chased for another 24 hr: (□) absorbance at 280 nm; (○) micrograms cadmium (determined from measurements of radioactivity); (▲) conductivity measurements (this peak represents the elution volume of NaCl). V_o, void volume; V_e, elution volume of the Cd-binding protein; V_T, total volume of the column. (B, C) Elution profiles, in the same column used for (A), of extracts from larvae treated with (○) 1 mM $CuSO_4$, (□) 5 mM $ZnCl_2$, or (▲) both. Each sample was assayed for Cu (B) and Zn (C) (12).

an estimate of larval size. Figure 5A (below) shows a plot of cadmium/soluble protein (w/w) as a function of time. The rate of cadmium uptake was greater than the rate of protein synthesis for the first 24 hr; after 24 hr, however, the ratio remained constant, indicating that the incorporation of cadmium, from that time on, simply kept pace with growth. The ratio was 7 times higher for larvae treated with 0.1 mM $CdCl_2$ than for those in 0.015 mM salt.

Molecular Associations of Cadmium, Copper, and Zinc

A crude extract from larvae labeled with $^{109}Cd^{2+}$ was subjected to gel filtration chromatography in a bed of Sephadex G-50 (Fig. 4A). A small amount of radioactivity eluted with the bulk of macromolecules in the void volume of the column (V_0), while the rest was delayed in the column (V_e), indicating a carrier with molecular weight below 10,000. No $^{109}Cd^{2+}$ eluted at V_T, the position where free Cd^{2+} should occur.

To observe the rate of appearance of the main cadmium-binding peak, crude extracts of second instar larvae which had been fed on IDM-YE supplemented with $^{109}Cd^{2+}$ (0.015 mM) for different times were run on a G-50 column. The proportion of total radioactivity at each of the three positions was measured (Fig. 5B): after short treatments most of the radioactivity appeared as free Cd^{2+}, but by 12 hr the cadmium-binding peak (CBP) accounted for approximately 90% of the radioactivity. The distribution seemed to be stabilized by 24 hr. A similar result was obtained with larvae treated with 0.1 mM $CdCl_2$.

Extracts were also prepared from larvae reared on food containing one of the following supplements: 1 mM $CuSO_4$, 5 mM $ZnCl_2$, or both. Extracts fractionated on a Sephadex G-50 column were assayed for copper and zinc by atomic absorption. The results are shown in Figure 4; Figure 4B shows that most of the copper from larvae treated with copper or copper plus zinc appeared in the CBP region, while Figure 4C shows that zinc appeared either in the V_0 peak with the bulk of proteins or with very low molecular weight species, probably as a free ion.

Homogenates of copper-fed larvae were also separated into cytosol and particulate fractions. Sephadex G-50 chromatography showed the majority of the metal in the cytosol fraction associated with a peak of mobility comparable to CBP. The copper in the particulate fraction could not be solubilized by treatments which should release membrane-bound material (0.1% Triton X-100 or sonic probe).

Induction of ^{35}S-Cysteine Incorporation by $CdCl_2$, $CuSO_4$, and $ZnCl_2$

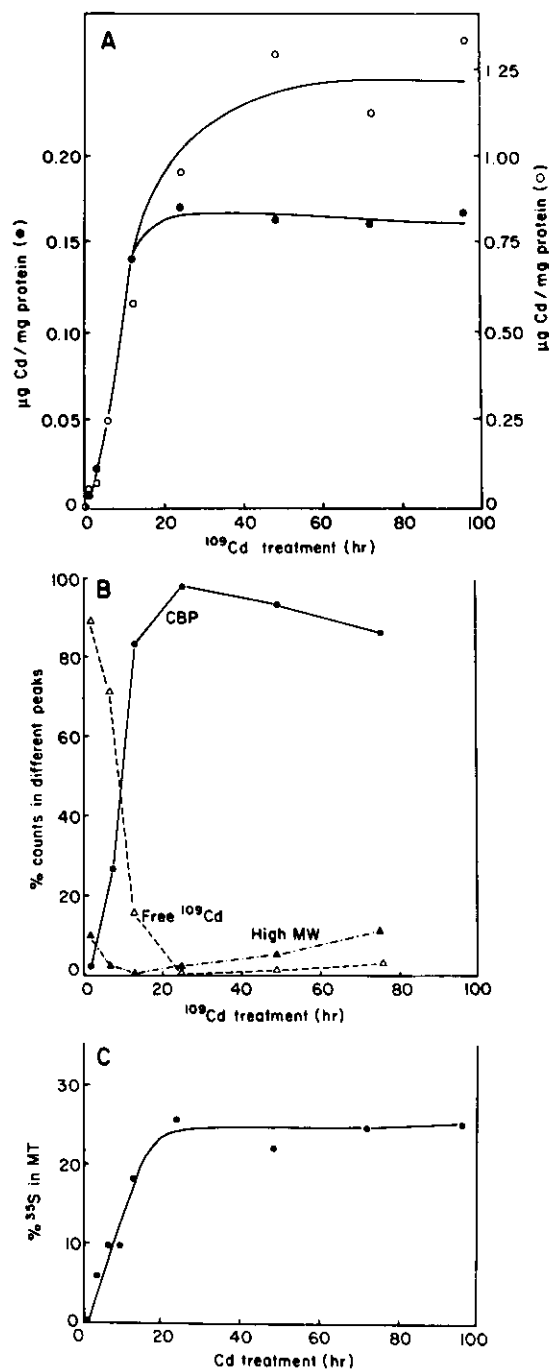Crude extracts of larvae labeled with ^{35}S-Cys, with or without $CdCl_2$ treatment, were prepared and run in

FIGURE 5. Plots of (A) μg Cd/mg soluble protein accumulated at different times after transferring larvae to medium containing (●) 0.015 mM and (○) 0.10 mM $CdCl_2$ (data from Fig. 3); (B) changes, with time, in the proportion of ^{109}Cd which is present in each of the main peaks in which it may appear after G-50 liquid chromatography. Second instar larvae were transferred to radioactive food and, at various times, samples were frozen and later tested, by column chromatography, as in the experiment in Figure 4A (C). Accumulation of ^{35}S in a chromatographic peak with the same elution volume as CBP. The data were from a series of chromatographic profiles as the one in Figure 4 and were expressed as a percent of the total radioactivity incorporated into macromolecules, i.e., (radioactivity in V_e/radioactivity in V_T and V_e × 100) (12).

Table 4. ^{35}S-Cys incorporated in two chromatographic peaks.

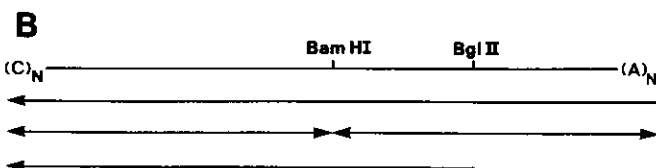| Treatment	^{35}S-Cys incorporation, %[a]	
	CBP peak	High MW peak
0.05 mM CdCl$_2$	25	75
1.00 mM CuSO$_4$	30	70
5.00 mM ZnCl$_2$	<5	>95

[a] The values were obtained from column profiles as those in Figure 5. Larvae had been treated with the metals for 48 hr before the extracts were prepared.
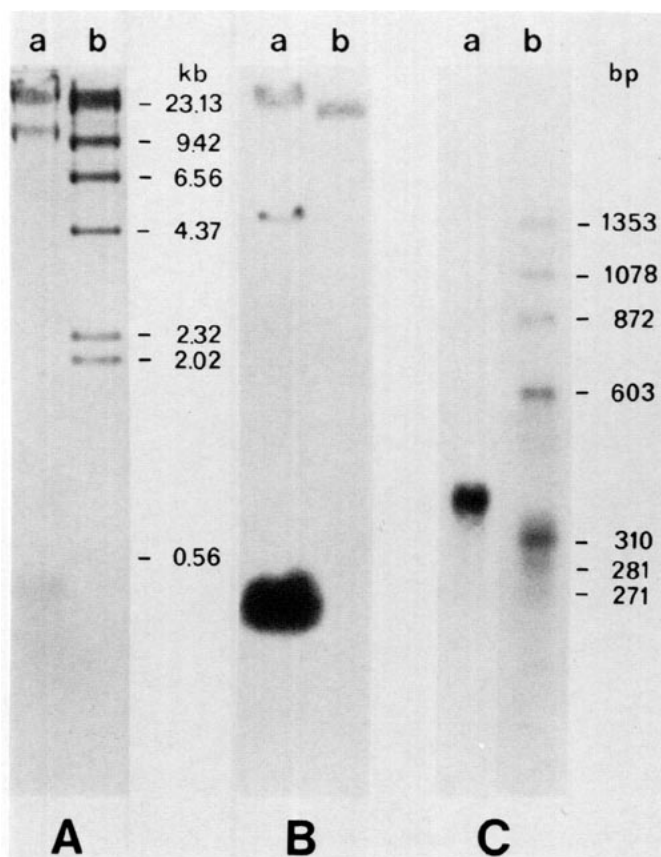
FIGURE 6. Photograph and autoradiograph of cDm51 insert and complementary RNA: (A) ethidium-bromide-stained agarose gel: (lane a) cDm51 digested with Eco RI (lane b) lambda DNA digested with Hind III; (B) autoradiograph of a Southern blot of the gel in Figure 6A hybridized to the mouse MT probe at reduced stringency; (C) (lane a) northern blot of total nucleic acid from Cd-treated larvae, run in a denaturing gel with radioactive cDm51 used as a hybridization probe; (lane b) end-labeled Hae III fragments of ΦX174 used as size markers (13).

A

B

FIGURE 7. Primary structure of MT cDNA obtained by the dideoxynucleotides sequencing method (16): (A) nucleotide sequence of the anti-sense strand of cDm51 with the predicted protein (dot marks are spaced every 100 nucleotides; some restriction enzyme sites and a polyadenylation site are marked by horizontal lines); (B) strategy used for the sequencing experiments (13).

a Sephadex G-50 column as before; there was considerable radioactivity present in the CBP peak from larvae treated with Cd^{2+} but not in the control group. The kinetics of incorporation, determined from samples chromatographed at different times after the beginning of Cd^{2+} treatment, is given in Figure 5C. This shows the fraction of ^{35}S in the CBP peak plotted as a function of time. During the first 24 hr this fraction rose rapidly, then it leveled off at approximately 25%.

CuSO$_4$ and ZnCl$_2$ were tested using a procedure similar to the one described for CdCl$_2$. Copper induces the incorporation of radioactive Cys into a molecule which

elutes in the same position as the CBP, but zinc does not. Table 4 summarizes these results.

Isolation of a cDNA Clone from a Metal-Inducible Gene

The results presented so far suggested the occurrence, in Drosophila, of a metal-inducible, metal-binding protein, and we proceeded to attempt to isolate the corresponding nucleic acid.

A cDNA library was prepared from polyadenylated RNA of larvae which had been treated with copper sulfate; this library would be expected to be enriched in sequences coding for metal-binding proteins. Duplicate nitrocellulose filter-lifts of the cDNA library were hybridized to DNA probes derived from either Cu^{2+}-induced RNA or control RNA (plus-minus test) in order to identify clones that hybridized preferentially to the former. Since Cd^{2+} is capable of inducing a heat-shock protein in rat embryo cultures (14), control polyadenylated RNA was isolated from heat-shocked larvae in order to avoid selecting heat-shock protein clones. Of the approximately 10,000 plaques screened using this procedure, some showed preferential hybridization to the RNA from induced larvae. Of these, 19 were picked and plaque-purified for further analysis.

These 19 phage stocks were retested by hybridization, under conditions of reduced stringency, with the mouse MT-I cDNA probe (15). Four of the plaques gave a stronger signal than the vector and proved to carry in-

serts that were approximately 400 bp long. One of them (cDm51) was chosen for further study and is shown in Figure 6.

When cDm51 was used as a probe, 17 of the 19 plaques which displayed differential hybridization in the plus-minus test showed hybridization to it. On filter lifts of plates of the cDNA library, 148 out of approximately 10,000 plaques hybridized to cDm51.

cDm51 Sequence

The strategy and results of sequencing experiments are shown in Figure 7. We used the diagonal-traverse search computer program of White et al. (17) to compare

cDm51 and human MT-2 (18) sequences. A search in which the program was set to identify regions of at least 50 bases long, in which 58% were the same in the two species, revealed four such segments. These four segments overlap partially and taken together they span the nucleotides corresponding to amino acid residues 11 to 39 in the Drosophila sequence and 2 to 38 in the human sequence. If the stringency of the matches was relaxed to 50%, the search also found similarities between the Drosophila sequence and the C-terminal portion of the human message. Neither of these comparisons detected similarities outside of the coding region of the cDNAs. Figure 8 shows a visual alignment of the amino acid sequences of cDm51, human metallothionein II and crab metallothionein II (19).
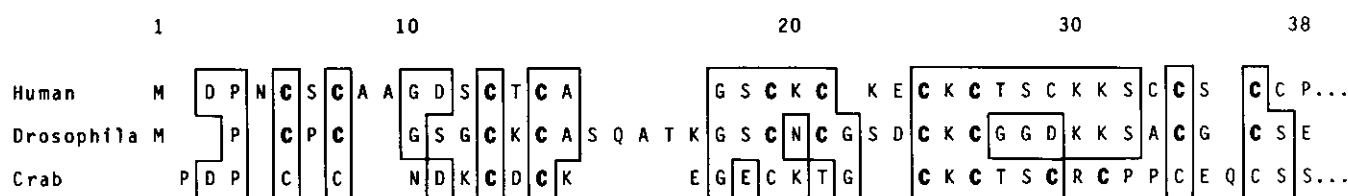

FIGURE 8. Alignment of three MT sequences: human MT 2, Drosophila, and crab MT 2. The numbers refer to positions in the human sequence. Only the amino-terminal portions of the human and crab proteins are shown. Gaps have been assumed in order to increase the number of matches. Identical residues are boxed in; Cys—X—Cys (X represents any residue other than Cys) groups are in bold face. See text for references. A,Ala; R,Arg; N,Asn; D,Asp; C,Cys; E,Glu; Q,Gln; G,Gly; K,Lys; M,Met; P,Pro; S,Ser; T,Thr (13).
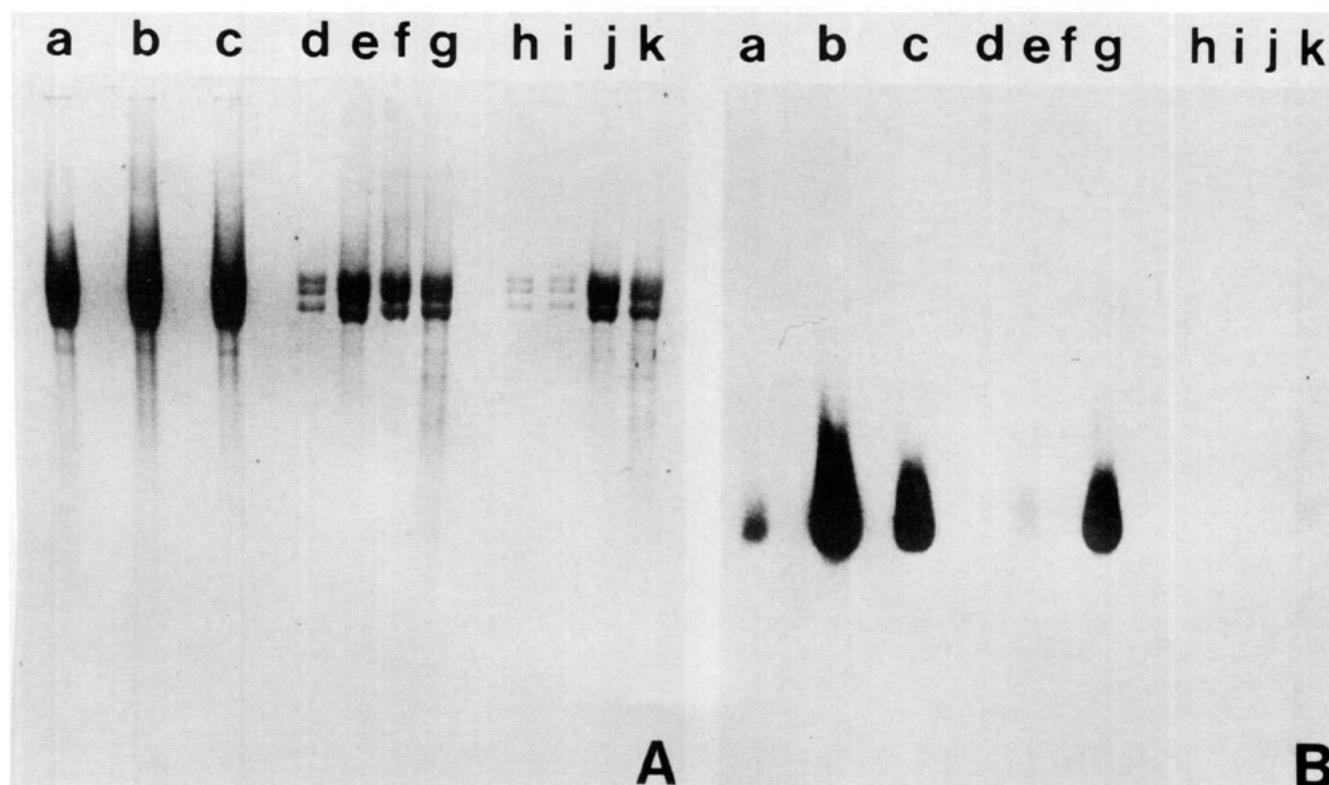

FIGURE 9. Photograph (A) and autoradiograph (B) of total nucleic acid samples run on an agarose denaturing gel. cDm51 was used as hybridization probe. Because of the small and well-defined size of the nucleic acid hybridizing to the probe, we believe this to be RNA and not DNA. Each lane was loaded with the nucleic acid extracted from three larvae or from organs of three larvae: (a) untreated whole larvae, (b) whole larvae treated with 0.5 mM CuSO$_4$ or (c) 0.1 mM CdCl$_2$. The next four lanes correspond to organs from larvae treated with 0.1 mM CdCl$_2$: (d) salivary glands, (e) fat bodies, (f) carcasses and (g) alimentary canals. The last four lanes have, in the same order, tissues of untreated larvae (13).

Table 5. Level of MT RNA in larval tissues.

	MT-RNA, cpm ± SE[a]				
	Whole larvae	Alimentary canal	Fat body	Salivary glands	Carcass
Untreated	19 ± 11	33 ± 6	9 ± 2	2 ± 2	8 ± 3
0.10 mM Cd	106 ± 12	849 ± 75	57 ± 5	33 ± 6	14 ± 6
0.50 mM Cu	180 ± 22	882 ± 56	249 ± 12	87 ± 1	48 ± 6

[a] Averages from three replicate dot hybridizations. The amount of nucleic acid per dot (2.5 μg) was within the range (2–20 μg) showing a linear relationship between the number of counts retained after hybridization to labeled cDm51 and the amount of nucleic acid on the filter (data not shown).

Metal Induction

An RNA that hybridized strongly with cDm51 was present in the total nucleic acids from untreated, cadmium-treated (0.1 mM) or copper-treated (0.5 mM) larvae as indicated by gel electrophoresis and filter hybridization. However, the amount of this RNA was much higher in extracts of metal-treated than untreated larvae (Fig. 9). Similar results were obtained with polyadenylated RNA.

Northern blot analysis of nucleic acid extracted from organs dissected from uninduced or cadmium-induced larvae showed that most cDm51-complementary RNA was in the alimentary canal (Fig. 9). Tissue-specific induction was also measured using dot blots (Table 5) that showed that although 80% of the RNA in question was in the alimentary canal, other tissues had measurable amounts of it.

A profile of the time course of induction of cDm51-complementary RNA was obtained by dot blot analysis of nucleic acid extracted from larvae exposed to copper sulfate (5.0 mM) or cadmium chloride (0.1 mM) for various lengths of time (Fig. 10).

The RNA size was determined to be 400 bases by filter hybridization of northern blots (Fig. 6C). Similar northern blots of polyadenylated RNA showed that cDm51-complementary RNA was present in copper-treated but not in heat-shocked larvae. Third instar larvae were also treated with other metals: cadmium and copper ions are effective inducers at 0.1 mM while mercury, silver and zinc require higher concentrations.

Conclusions

The results presented demonstrate that Drosophila larvae exposed to cadmium ions at a sublethal concentration retain the metal very efficiently (up to 85% of the amount ingested). Most of this retained metal (>95%) remains in the midgut epithelium (with a slightly higher concentration in the region of the cuprophilic cells of the middle midgut) and practically all of it is found in the cytosol fraction. Within 24 hr, 90 to 95% of the cadmium ingested by larvae becomes bound to a low molecular weight cysteine-containing protein that does not appear to be present prior to metal treatment; i.e., the synthesis of this protein seems to be induced by the metal.

Although the synthesis of CBP starts soon after the initiation of cadmium treatment, it takes several hours for the protein to reach a level high enough to bind most
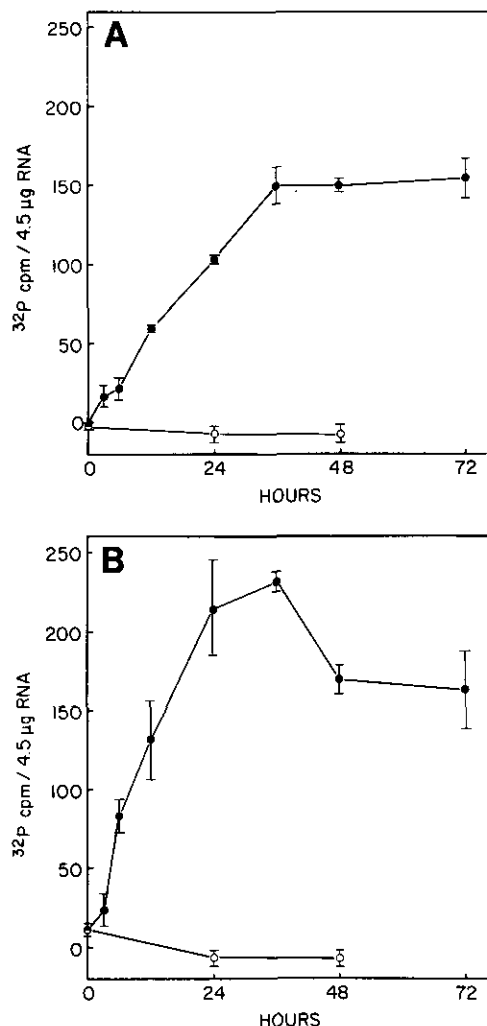

FIGURE 10. Time course of MT RNA accumulation. Dot blots were prepared (4.5 μg nucleic acid/dot) from larvae treated with (A) 0.1 mM CdCl₂ or (B) 0.5 mM CuSO₄ for different lengths of time as indicated: (●) metal treated; (○) untreated control. Data points are means for three filters, and vertical bars give SE (13).

of the free cadmium ingested by the larva. Since cadmium is highly toxic, the lag in CBP production may be responsible for the diminished larval growth observed during the first 12 hr of cadmium treatment. When growth resumes an equilibrium is quickly reached: after 24 hr, cadmium and CBP accumulation advances in step with larval growth and the ratio of cadmium to soluble protein remains constant until pupariation. The accu-

mulation of cadmium (and therefore CBP) per milligram soluble protein is approximately proportional to the Cd^{2+} concentration in the medium, at least between 0.015 and 0.10 mM.

We have found that copper also induces ^{35}S-Cys incorporation into a macromolecule having the same V_e as CBP. Most of the copper assimilated by larvae growing in medium with 1.0 mM Cu^{2+} is bound to this cytosol protein, but 15 to 25% of the metal occurs in a particulate fraction resistant to mild detergents and sonication. In agreement with several published reports (6–9) we have detected copper within gut epithelium lysosomes by X-ray microanalysis. We further noticed that the highest copper concentrations are in dense intralysosomal inclusions (data not shown); similar mineralized concretions have been found in adult Musca domestica (8) and in mollusks (20). It seems likely that these lysosomal concretions within cuprophilic cells contain the fraction of insoluble copper while the soluble fraction is distributed between the bulk of proteins and metallothionein.

The results of our experiments dealing with zinc ions demonstrated similarities as well as differences in the responses of Drosophila and mammals to this metal. As in mammals, zinc was the least toxic of the tested ions, four times less toxic than copper and 500 times less toxic than cadmium. On the other hand, zinc, even at a concentration 100 times greater than that used for cadmium, does not have the ability to induce MT synthesis in Drosophila to any detectable extent, nor does it bind to Drosophila MT in significant amounts when the protein is induced by copper. This outcome was somewhat unexpected because zinc is known to both induce and bind MT in mammals (e.g., comparable levels of induction are obtained in HeLa cells if the concentration of zinc is five times higher than that of cadmium (21). Zinc was the only one of the three metals tested in our study that was found, to any great extent, in fractions corresponding to very low molecular weight compounds, probably in the form of free ions.

The 399 bp Drosophila cDNA fragment, cDm51, includes 338 bp in addition to poly(C) and poly(A) tails. There is one open reading frame beginning at position 124 which encodes a 40-amino acid, cysteine-rich, protein; a polyadenylation site (AATAAA) (22) starts 31 bp upstream of the poly-A tail. The amino acid composition and the arrangement of all 10 cysteine residues in Cys—X—Cys groups make the protein predicted from cDm51 bear strong resemblance to mammalian MTs (3). Computer-aided comparison of nucleotide sequences indicates that there is a great deal of similarity between Drosophila's cDm51 and the N-terminal portion of the structural region of human metallothionein-2 cDNA (18). It is on this basis, as well as its inducibility with Cu^{2+} and Cd^{2+}, that we conclude that the cDm51 sequence corresponds to that of a Drosophila MT.

The Cys—X—Cys groups occur in strikingly similar positions in the insect and crab sequences: in Drosophila the groups start at positions 3, 9, 20, 26, and 36 and in Scylla (19) at positions 9, 20, 25, and 37. Four of the first

five Cys groups in mammalian MT are also in comparable positions. This concordance is likely to reflect functional constraints and it may represent a combination of homology (common ancestry) and convergent evolution. The alignment of Figure 8 includes mismatching two of the Cys—X—Cys groups of Scylla with those of the other two MTs, but the remaining similarities seem compelling enough to validate it. This alignment also provides hints of an evolutionary appearance and disappearance of Cys—X—Cys groups that might have led to those mismatches (near positions 20, 30, and 35) and yet kept the spacing of these groups in the primary structure constant. To test this hypothesis and assess which similarities are the consequence of common ancestry and which are the result of convergent evolution will require the sampling of more taxonomic groups. All 20 cysteine residues in mammalian MT seem to be involved in the binding of divalent cations (23), but only 14 of them are in Cys—X—Cys groups. Drosophila's sequence is the only one known to date in which all cysteines are arranged in Cys—X—Cys groups.

Treatment of larvae with any of several metal ions leads to significant increases in the level of specific RNA(s) complementary to the MT clone cDm51. Given the sequence organization of cDm51 and the evidence from mammalian systems (24), we assume this RNA to be MT-mRNA. Copper and cadmium are the most effective inducers, leading to a 15- to 20-fold increase in the relative concentration of these sequences, but zinc, mercury, and silver also produce detectable increases. Nearly 80% of MT-mRNA is found in the alimentary canal, which represents approximately 10% of the whole larva, on the basis of soluble protein in extracts (25) or 20%, on the basis of total nucleic acids (unpublished observations). The average representation of cDm51 in the cDNA library of Cu-induced larvae is 1.5% (148/10,000). Therefore, assuming an unbiased sampling of the RNA population in the cDNA library, one can estimate that MT-mRNA sequences represent 6 to 12% of the polyadenylated sequences in the alimentary canal of induced larvae. We have no evidence yet to ascertain whether the protein coded for by cDm51 is present, by itself or with others, in the peak of CBP observed after gel filtration chromatography of extracts from metal-treated larvae.

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