CONF-8508108--1

CONF-8508108--1

DE85 017303

Submitted to:

Second International Meeting on Metallothionein, August 21-24, 1985, Zurich, Switzerland.

CADMIUM RESISTANCE IN DROSOPHILA: A SMALL CADMIUM BINDING SUBSTANCE

K. Bruce Jacobson[1], M. W. Williams[2], L. J. Richter[1], S. E. Holt[2], G. J. Hook[3], S. M. Knoop[1], F. V. Sloop[3], and J. B. Faust[2]

[1]Biology Division, [2]Health and Safety Research Division,

[3]University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences,

Oak Ridge National Laboratory*, Oak Ridge, Tennessee 37831, USA

---

*Operated by the Office of Health and Environmental Research, U.S. Department of Energy, under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.

CADMIUM RESISTANCE IN DROSOPHILA: A SMALL CADMIUM BINDING SUBSTANCE

K. Bruce Jacobson[1], M. W. Williams[2], L. J. Richter[1], S. E. Holt[2], G. J. Hook[3], S. M. Knoop[1], F. V. Sloop[3], and J. B. Faust[2]

[1]Biology Division, [2]Health and Safety Research Division, [3]University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences, Oak Ridge National Laboratory*, Oak Ridge, Tennessee 37831, USA

Abstract: A small cadmium-binding substance (CdBS) has been observed in adult Drosophila melanogaster that were raised for their entire growth cycle on a diet that contained 0.15 mM $CdCl_2$. Induction of CdBS was observed in strains that differed widely in their sensitivity to $CdCl_2$. This report describes the induction of CdBS and some of its characteristics.

Introduction

The physicochemical characterization of metal ions is an important step in understanding the mechanism by which metal ions interact with macromolecules and cause toxic effects. We wish to develop sufficient understanding of the toxicity mechanisms to be able to predict the effects of metal ions. The correlation of thermodynamic properties of metal ions with their toxic effects in animals was reviewed (1). Recognizing the need for a uniform set of toxicity data, we evaluated 14 divalent cations for their effects on the mouse and Drosophila melanogaster. The order in terms of decreasing toxicity was Cd > Hg > Cu > Pt > Zn > Ba > Be > Ni > Pb > Pd > Co > Mn > Mg > Sr for the BALB/c male mouse and Cd > Hg > Ni > Cu > Co > Ba > Sr > Zn > Mn > Be > Mg for an inbred strain of Drosophila, v; bw, (2). The similarities and differences for the two organisms were discussed (2) and, since Cd was the most toxic in both, its toxic effects were studied further in Drosophila.

For Drosophila grown at 25°, the three larval stages are completed in 4.5 days and the pupal stage in another 4.5 days. The imago or adult ordinarily lives for 3-4 months but its reproductive activity diminishes after 3-4 weeks of age. The resistance of adults to dietary Cd was evaluated by including various concentrations of $CdCl_2$ in the diet and maintaining 0 to 1 day old male adult flies on the media for 4 days.

A number of inbred strains of Drosophila were examined for their resistance to Cd by determining the metal-ion concentration in the medium that killed 50% of male adults in four days (LC50). The v; bw strain was the most resistant (LC50 = 3.3 mM) and the wild type strain Austin was one of the

most sensitive (LC50 = 1.3 mM) (3). When cadmium toxicity in different aged male adults was compared, survival was greatest with the one-day-old fly, markedly less for the one-week-old fly and least for the 2 and 4-week-old adults (4). Females were more sensitive than males in some strains but not in others (4). The sensitivity of the pre-adult stages was evaluated by maintaining all stages of the v; bw strain on medium that contained $CdCl_2$ and measuring the number of adults that arose from 15 pairs of parents. The concentration of $CdCl_2$ that reduced the yield of the v; bw strain by 50 percent was 1/50 the LC50 for the 4 day test on adults (4). One of our major goals is to determine the basis for differences in resistance among different genotypes and for the different stages in the life cycle. The occurrence of a cadmium binding protein in Drosophila larvae that had been exposed to Cd or Cu was reported by Maroni and Watson (5) and they report further studies on this topic in this volume. The present report describes the induction and some properties of a cadmium binding substance (CdBS) that we find in adult Drosophila.

Methods

Binding ^{109}Cd to CdBS in vitro: Extracts were prepared by homogenization of adult or larval forms of Drosophila with 5 ml of 20 mM Tris·HCl pH 8.6 - 5 mM 2-mercaptoethanol - 0.25 M sucrose per gram fresh weight, centrifugation at 17,000 xg for 30 min, and storage at -20°C. Two procedures were employed to bind ^{109}Cd to CdBS: (A) An exchange process and (B) acid displacement. In the exchange process aliquots of the extract were incubated with 50 µM $^{109}CdCl_2$ (100,000 cpm) in 200 µl of 9 mM Tris·HCl pH 8.6 - 22 mM NaCl - 2.25 mM 2-mercaptoethanol - 1.35 mM NaN_3. After 45 min at 40° a measured volume was analyzed for CdBS. In the acid displacement procedure 200 µl aliquots of the extract were added to 500 µl of 14 mM Tris·HCl pH 8.6 - 33 mM NaCl - 4 mM 2-mercaptoethanol - 2 mM NaN_3, then the solution was adjusted to pH 2 and to 26 µM $CdCl_2$ (200,000 cpm); after 10 min 0.25 volumes of 1 M acetate pH 5 were added. After 10 min 1 M NaOH was used to adjust the pH to 7.5 and the resulting solution was analyzed for CdBS. Proteins that were larger than CdBS and bound ^{109}Cd precipitated and were removed by centrifugation at the pH 5 step.

Binding ^{109}Cd to CdBS in vivo: Labeling of larvae was done by growth in medium that contained 0.015 mM $CdCl_2$ and 1.5 µCi/ml of ^{109}Cd (5). Five ml of this medium was placed in each of four vials along with 50-100 larvae of v; bw. After one or two days at 25°C third instar larvae were collected and stored at -80° until extracted as above. Aliquots of the extract were chromatographed on Sephadex G-50 and the fractions were analyzed for ^{109}Cd.

To label cadmium binding substances in adults, 3 female and 1 male v; bw were placed in a vial of medium that contained 0.15 mM $^{109}CdCl_2$ (0.5 µCi/ml of ^{109}Cd). These parents were removed and discarded after 10 days; after time for development adult offspring were collected each day and stored at -80°C until the CdBS was analyzed.

Quantitation of Cd Binding Substance: Two procedures were used to estimate the amount of CdBS in the extract: Sephadex G-50 and Chelex-100 chromatography.

A Sephadex G-50 column (1.5 × 113 cm) was equilibrated and operated in 20 mM Tris·HCl pH 8.6 - 5 mM 2-mercaptoethanol - 50 mM NaCl - 3 mM sodium azide at 4° at a flow rate of 1 ml/min. An extract that had been labelled with ^{109}Cd was added. Fractions (2.5 ml) were collected and 1 ml aliquots were mixed with 10 ml of BBOT in a solution of 33.3% Triton X-100 - 66.7% toluene and the ^{109}Cd was counted in a beta-detecting scintillation counter using settings for ^{32}P.

Chelex-100 (BioRad) was slurried and placed in pasteur pipettes that were plugged with glass wool. The 1.5 ml of resin was washed with 3 ml 1N HCl, 8 ml H_2O, 3 ml 1N NaOH and 8 ml H_2O before sample application. ^{109}Cd-labeled CdBS was washed through the Chelex-100 column with three successive aliquots of 500 µl water. The washes were pooled in a glass vial and counted in a scintillation counter as described above.

Mouse Metallothionein: A BALB/c female mouse was injected i.p. on days 1, 2 and 3 with 0.2 ml of 1.64 mM $CdCl_2$ in 0.15 M NaCl. On day six the liver was removed and an extract made as above for Drosophila. ^{109}Cd was bound to metallothionein by the exchange process described above.

Specific Strains of Drosophila Constructed from Austin and v; bw: The v; bw and Austin strains of D. melanogaster underwent a series of genetic crosses with strains that contained balancer chromosomes. The final matings produced 6 strains that carried assorted chromosomes from the two original strains in 6 combinations. These crosses were designed to combine a single chromosome of one strain with the 2 chromosomes from the other strain so that the alleles for resistance to cadmium could be localized if they were restricted to one chromosome. Only the 3 major chromosomes of D. melanogaster were considered in these crosses and the fourth minor chromosome was not controlled. The details of these matings will appear elsewhere (6).

Results

Cadmium Resistance of Six Specific Strains: The v; bw and Austin strains were used since they represent the extremes of LC50 values for cadmium. The Austin chromosomes (designated as 1', 2' and 3') and the v; bw chromosomes (designated as 1, 2 and 3) were assorted genetically into the 6 combinations shown in Table I. The resistance to cadmium of each of the 6 strains was evaluated four times in the usual toxicity test with male flies. The LC50 of v; bw and Austin differ by a factor of ~3 but absolute values differed from time to time (3). The averages of a large number of LC50s are 3.3 mM for v; bw and 1.3 mM for Austin.

Testing the 6 genotypes with the assorted chromosomes also showed variation of the LC50s from test to test (Table I) but in each case the values could be arranged in two groups. The groups differed by the origin of X-chromosome (chromosome 1). If the origin of the X-chromosome was Austin the flies were more sensitive to cadmium than those for which the origin was v; bw. Statistical analysis confirmed the difference between those groups (6). The fact that the difference between the two groups was less than the difference between the two parent strains indicates that the resistance-factor on the X-chromosome may not be the only one involved.

Table I. Genetic localization of cadmium resistance factor(s)

Strain	Chromosome Combination	LC50 (mM)				
		Experiment				
		I	II	III	IV	Average
A	1 2' 3'	1.7	1.6	3.0	2.7	2.25
B	1 2 3'	2.0	2.3	3.7	3.2	2.8
C	1 2' 3	1.6	1.7	3.4	3.0	2.4
D	1' 2 3	1.2	1.5	2.4	2.0	1.8
E	1' 2' 3	1.0	1.5	2.8	1.9	1.8
F	1' 2 3'	1.1	1.3	2.0	2.2	1.65

Characterization of CdBS. Comparison of the CdBS of Drosophila to methallothionein (MT) of the mouse was performed by chromatography on Sephadex G-50. In Figure 1 the position of the MT peak is shown by an arrow along with a typical peak of CdBS. By this criterion CdBS of Drosophila may be less than one-half the molecular weight of mouse metallothionein. The last peak of the chromatogram is a cadium-mercaptoethanol complex.

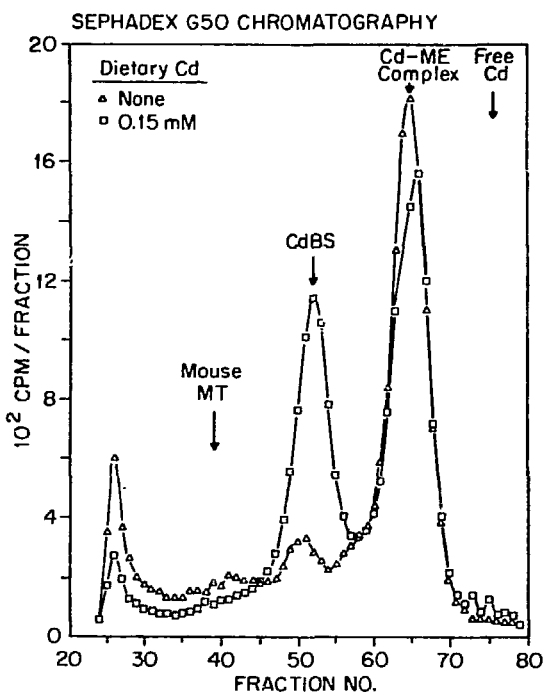

Fig. 1. Chromatography of CdBS on Sephadex G-50. Extracts from v; bw grown on normal medium (△) or medium that contained 0.15 mM $CdCl_2$ (□) were labeled with ^{109}Cd by the exchange procedure.

A series of experiments was performed to characterize CdBS. Evidence for the presence of one or more SH groups was obtained by performing Sephadex G-50 chromatography in the presence and absence of 2-mercaptoethanol. In its absence a major peak, presumably a dimer of CdBS (fraction 23), appeared earlier in the elution than CdBS itself (fraction 30) as shown in Fig. 2. The absence of the dimer in Fig. 1 may be due to the exposure of the sample to Cd^{2+} prior to chromatography.

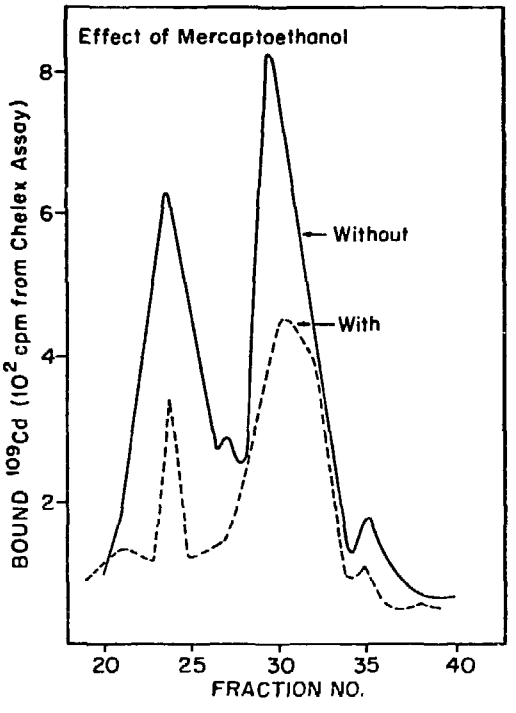

Fig. 2. CdBS chromatography with and without 2-mercaptoethanol. The column of Sephadex G-50 (2.5 x 85 cm) was operated at 22°C and 10 ml fractions were collected and assayed by the exchange procedure to label CdBS with ^{109}Cd; the Chelex-100 assay was used to detect bound ^{109}Cd.

CdBS is stable at 100° for 30 min. After the coagulated protein was removed, more than 50% of the CdBS was recovered in its usual place on Sephadex G-50.

These characteristics of CdBS support those of Maroni and Watson (5) who first reported a cadmium-binding protein from the larvae of Drosophila. Since we tested the cadmium resistance on adults, we repeated the *in vivo* labeling experiments of these authors (5) to compare the CdBS from extracts of adults with those from larvae. On Sephadex G-50 column slightly longer than used in Fig. 1, the CdBS eluted at fraction numbers 54-55 for adults labeled *in vivo* (Fig. 3A), for larvae labeled *in vivo* (Fig. 3B) and for larval extracts labeled *in vitro* (Fig. 3C); this also corresponds to the position of CdBS from adults labeled *in vitro* and agrees with the K_d = 0.58 found before (5). The peak at 74 represents complexes of Cd with

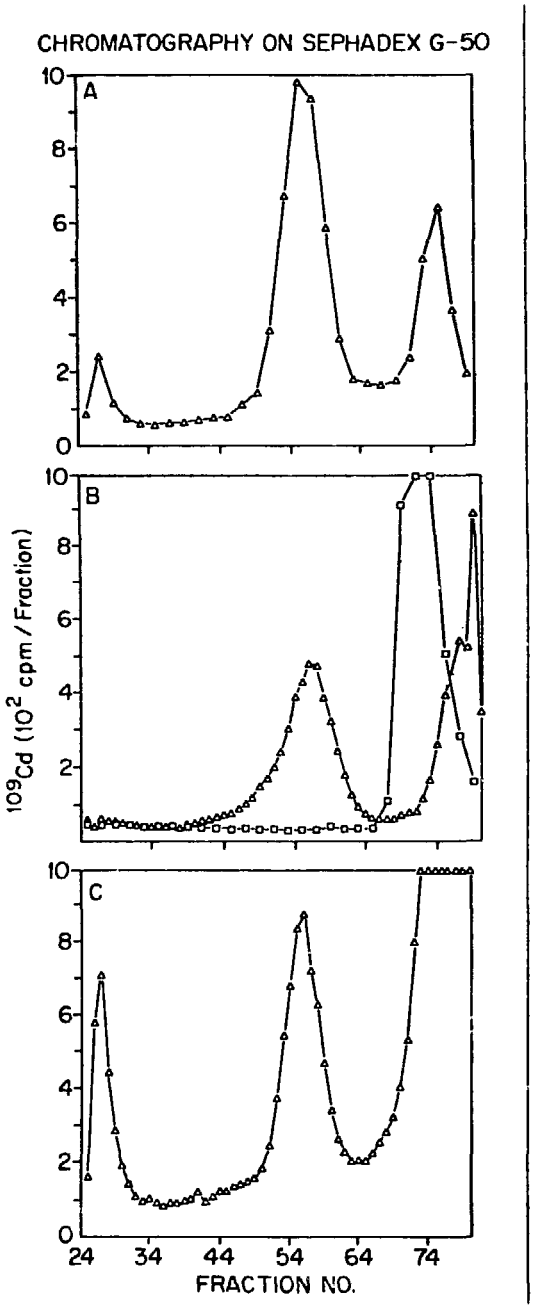

Fig. 3. Comparison of CdBS from adult and larvae forms of vi bw. Adults that had been raised on ^{109}Cd (A), larvae that had spent 48 hrs on 0.015 mM $^{109}CdCl_2$ (B), and larvae that had spent 48 hrs on unlabeled 0.015 mM $CdCl_2$ (C) were extracted. In A and B the supernatants contained ^{109}Cd while in C the extract was labeled with ^{109}Cd by the exchange procedure. In B the extract was either applied directly (△) or after adjusting to pH 2 (□).

mercaptoethanol. Fig. 3B shows the result of adjusting the acidity of the larval extract to pH 2 prior to chromatography; the Cd was completely dissociated from CdBS. We found no difference between the CdBS of larvae and adults.

The Chelex-100 assay for CdBS measures the presence of substances that bind ^{109}Cd and prevent its adsorption to the Chelex-100 resin. CdBS and the larger proteins (FN 27 of Fig. 3C) both accomplish this. The latter become insoluble at pH 5 and are removed when the ^{109}Cd is bound by the acid displacement method. The quantity of CdBS measured in the Chelex-100 assay was greater than 90% that found on Sephadex G-50 when it was labeled by the acid displacement method.

Induction of CdBS. The CdBS was induced when Drosophila was raised on medium containing $CdCl_2$. The level of CdBS in adults increased as the $CdCl_2$ in the medium was increased. As shown in Figure 4, the responses of the resistant and sensitive strains, v; bw and Austin respectively, were equivalent except that at the highest $CdCl_2$ concentration the sensitive strain produced less CdBS than it did at lower concentrations. This experiment was repeated in its entirety but the analysis for CdBS was by Sephadex G-50 chromatography. With one exception the Austin produced slightly more CdBS than v; bw at each level of $CdCl_2$ in the diet. At the highest tolerable concentration Austin made a distinctly larger amount as opposed to the lesser amount shown in Fig. 4.

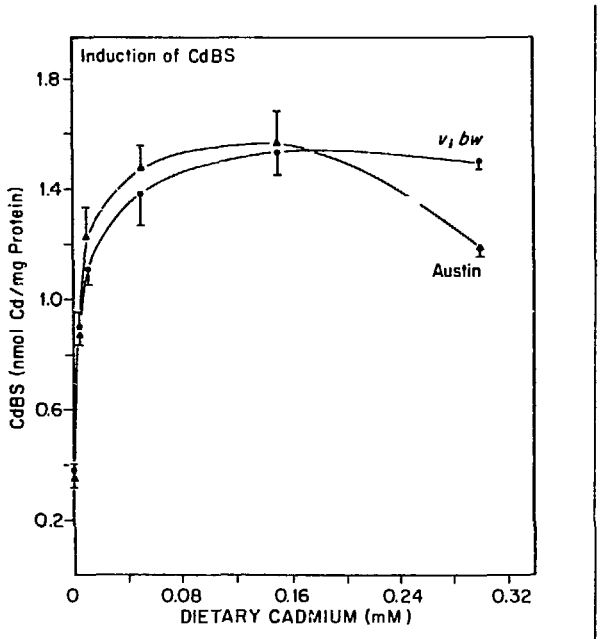

Fig. 4. Induction of CdBS in adults by raising v; bw on standard medium with different concentrations of $CdCl_2$ added. The CdBS was labeled with ^{109}Cd by acid displacement and assayed by the Chelex-100 procedure.

Purification of CdBS. The CdBS peak was purified on Sephadex G-50 and then on a C18 reversed phase column (0.46 × 30 cm, Analytical Services, Inc.) that was equilibrated with 0.1% trifluoroacetic acid (TFA). A gradient of acetonitrile, 0-40% with TFA constant at 0.1% was used to elute four peaks. The last peak was the only one that bound $^{109}Cd^{2+}$, according to the Chelex-100 assay, and it had little or no absorption at 280 nm; the molecule seems to be deficient in aromatic amino acids.

Discussion

By genetic procedures a major factor for resistance to cadmium toxicity was shown to be located on the X-chromosome of Drosophila. By biochemical procedures we have confirmed the presence of a cadmium binding substance reported earlier (5) that is induced similarly in the resistant and sensitive strains. Based on the similar induction for Austin and v; bw the question arises whether CdBS plays a role in determining resistance to cadmium toxicity. Since no appreciable difference in the induction of CdBS could be found between the v; bw and Austin strains the structure of CdBS from these two strains is being examined to determine if they differ. A cadmium binding peptide in Neurospora crassa has been found that is much smaller than metallothionein but the two still have sequence homology (7). We shall determine the sequences of the Drosophila CdBS as isolated from the resistant and sensitive strains.

Acknowledgement

Research was supported by the Office of Health and Environmental Research, U.S. Department of Energy, under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc. LJR was a science fellow from Earlham College with the Great Lakes College Association (Fall 1983); SEH and SMK were science fellows from Cantenary College with the Southern Colleges University Union (Spring 1984 and 1985, respectively); and JBF was a science fellow from Virginia Polytechnical Institute and State University with the Oak Ridge Associated Universities (Summer 1984).

References

1. Jacobson, K. B., and Turner, J. E., (1983) Toxicology 16, 1-37
2. Williams, M. W., Hoeschele, J. D., Turner, J. E., Jacobson, K. B., Christie, N. T., Paton, C. L, Smith, L. H., Witschi, H., and Lee, E. H., (1982) Toxicol Appl Pharmacol 63, 461-469
3. Christie, N. T., Gosslee, D. G., Bate, L. C., and Jacobson, K. B., (1983) Toxicology 26, 295-312
4. Jacobson, K. B., Opresko, L., Owenby, R. K., and Christie, N. T., (1981) Toxicol Appl Pharmacol 60, 368-378
5. Maroni, G., and Watson, D., (1985) Insect Biochem 45, 55-63
6. Christie, N. T., Williams, M. W., and Jacobson, K. B., (1985) Biochem Genetics, in press.
7. Lerch, K., (1980) Nature 234, 368

K. Bruce Jacobson, Biology Division, Oak Ridge National Laboratory, P. O. Box Y, Oak Ridge, Tennessee 37831 USA.