alternative pathway and binds to some serum proteins such as α_2-macroglobulin and α_1-antitrypsin[10]. It could be concerned with the immune reaction of the hosts. Further immunological studies are under way.

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Metallothionein induced in the earthworm[1]

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Summary. One of the 3 different molecular weight cadmium-binding proteins induced in the earthworm was characterized as a metallothionein; this characterization was based on a high cysteine and cadmium content, low molecular weight, heat-stability, and mercaptide bonding.

The earthworm, *Eisenia foetida*, shows a marked tolerance to cadmium and accumulates the metal to a degree related to its concentration in composted sewage sludges[4]. The accumulated cadmium was mostly found in the cytosol fraction; this is known to be usual in animals, including mammals[5]. The distribution profile of cadmium among the soluble proteins, however, was quite different from that in mammals; cadmium was not only found in the metallothionein fraction (apparent mol. wt of about 10,000), but also in higher and lower mol. wt fractions (estimated mol. sizes of 63,000–70,000 and less than 2000, respectively)[6]. A lower mol. wt cadmium-binding protein than the mammalian metallothionein has been isolated from a fungus, *Neurospora crassa*, and characterized as a metallothionein with a mol. wt of 2000[7]. This is the first observation of the induction of cadmium-binding proteins with 3 different mol. wts. The present communication reports some characterizations of the earthworm cadmium-binding proteins and defines one of the cadmium-binding proteins as a metallothionein from its amino acid composition, metal content, and other features accepted as being characteristic of metallothioneins[5].

The earthworm, *Eisenia foetida*, was grown for 60 days in a composted sewage sludge which contained 400 µg Cd/g dry wt. The earthworms were homogenized after discharging excreta, and heat-unstable proteins were removed from the homogenate by heating at 80 °C. Typical gel filtration chromatograms of the heat-denatured supernatant on Sephadex G-75 and SW 3000 columns are shown in figure 1. Most of the zinc distributed in the high mol. wt fraction was removed by heat-treatment without affecting the distribution profile of cadmium. The highest mol. wt cadmium-binding protein (EW-I) on a Sephadex G-75 column was eluted as a broad peak at a retention time of 17.2 min from an SW cokumn. On the other hand, the other 2 cadmium-binding proteins separated on a Sephadex G-75 column, EW-II and -III, were separated into several peaks on an SW column due to the cation exchange chromatographic property of the column with elution at alkaline pH[8].

Further separation of the 3 cadmium-binding proteins (EW-I, -II, and -III) was carried out on a DEAE Sephadex A-25 column, one of the most usual procedure for separation of metallothionein into isometallothioneins. EW-I could not be eluted from the column using conditions the same as, or more drastic than those used for the separation

Amino acid composition, total sugar and metal contents of earthworm cadmium-binding protein-IIB (EW-IIB)[a]

Amino acid composition	Mole %	Residues[b] / Molecule
Half-cystine	15.4	14
Aspartate	9.8	9
Threonine	5.7	5
Serine	6.1	6
Glutamate	8.5	8
Glycine	17.0	15
Alanine	10.0	9
Valine	4.6	4
Methionine	-	-
Isoleucine	2.2	2
Leucine	5.1	5
Tyrosine	-	-
Phenylalanine	Trace	Trace
Lysine	8.6	8
Histidine	-	-
Arginine	3.0	3
Proline	4.0	4
Metal content (mole %): Cd 93.9, Zn 2.8, Cu 3.3		
SH/metal ratio: 2.4		
Total sugar: -		

[a] EW-IIB was hydrolyzed in 6 N HCl at 110 °C for 20 h. Half-cystine and methionine were analyzed after performic acid oxidation. Values are expressed as percent of the total number of residues. Total sugar and metal contents were determined by the H_2SO_4-phenol assay[9] and atomic absorption spectrophotometry, respectively.
[b] Isoleucine was assumed to be 2 residues per molecule.

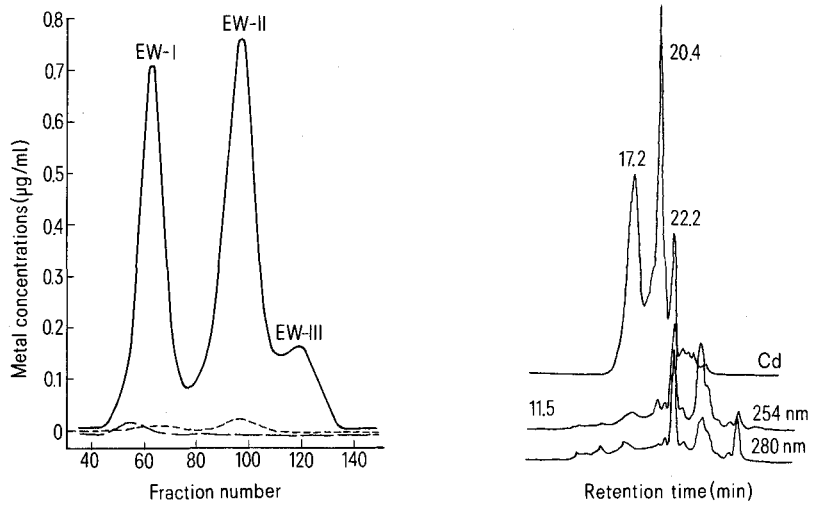

Figure 1. Gel filtration chromatography of heat-treated supernatant from cadmium-exposed earthworms on Sephadex G-75 and SW 3000 columns. The cadmium-exposed earthworms were homogenized in 4 volumes of 10 mM Tris-HCl buffer solution (pH 7.4) using a polytron homogenizer in an atmosphere of nitrogen and under icewater cooling. The homogenate was centrifuged at 10,000 × g for 20 min at 4 °C, and then the supernatant was heated at 80 °C for 10 min. The denatured proteins were removed by ultracentrifugation at 180,000 × g for 60 min at 4 °C. The heat-treated supernatant (40 ml) was applied on a Sephadex G-75 column (5 × 80 cm) (left panel) and the column was eluted with 10 mM Tris-HCl buffer solution (pH 8.6). 10-ml fractions were collected and the metal concentrations were determined in each eluate on an atomic absorption spectrophotometer (Hitachi 170-50A). The heat-treated supernatant was also analyzed on a high speed liquid chromatograph (Toyo Soda HLC-803A) which was equipped with an SW column (TSK GEL SW 3000, 7.5 × 600 mm with a precolumn of 7.5 × 100 mm) (right panel). A 0.1-ml portion of the supernatant was applied on an SW column and the column was eluted with 50 mM Tris-HCl buffer solution (pH 8.0 at 25 °C, 0.1% NaN_3) at a flow rate of 1 ml/min. The concentration of cadmium in the eluate was continuously monitored by directly connecting the outlet of the column to a nebulizer tube of an atomic absorption spectrophotometer. Left panel; Cd ——, Zn– – –, and Cu ------.

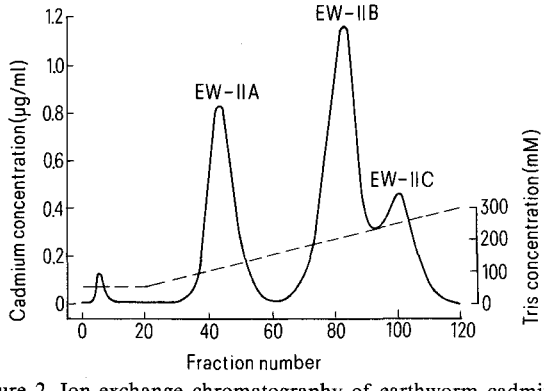

Figure 2. Ion-exchange chromatography of earthworm cadmium-binding protein-II (EW-II) on a DEAE Sephadex A-25 column. EW-II fraction on a Sephadex G-75 column (fig. 1) was analyzed on a DEAE Sephadex A-25 column (1.6 × 20 cm) by elution with a linear concentration gradient of Tris-HCl buffer (pH 8.6) from 50 to 300 mM. 3-ml fractions were collected and the concentration of cadmium was determined in each eluate on an atomic absorption spectrophotometer.

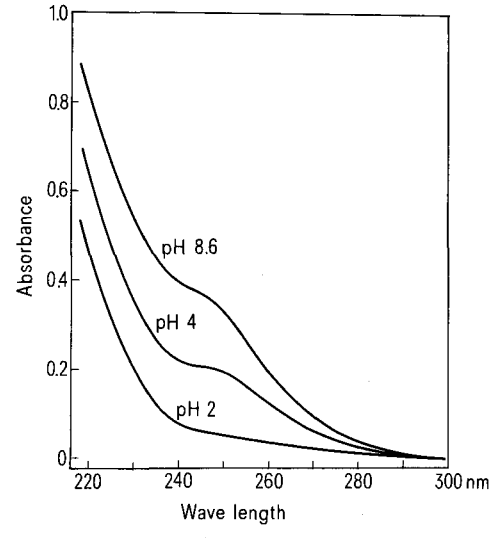

Figure 3. pH-dependent change of absorption spectra of earthworm cadmium-binding protein-IIB (EW-IIB). UV-absorption spectra of EW-IIB were measured in 10 mM Tris-HCl buffer solution (pH 8.6) on a Shimadzu MPS-5000 spectrophotometer. The pH was adjusted by adding 1 N HCl solution.

of mammalian metallothioneins (concentration gradient of Tris-HCl buffer solution to 300 mM at pH 8.6 or 7.4). On the other hand, the other 2 cadmium-binding proteins (EW-II and -III) were separated into isoproteins on a DEAE Sephadex A-25 column under conditions the same as those used for the separation of mammalian metallothioneins, as suggested by the elution profile on an SW column in figure 1. Figure 2 shows a typical elution profile of EW-II on an anion exchange column. EW-II was separated into 3 isoproteins and the 3 isoproteins were tentatively labelled EW-IIA, -IIB, and -IIC at the eluted order. The former 2 isoproteins, EW-IIA and -IIB, correspond to a cadmium peak at a retention time of 20.4 min on an SW column in figure 1. The 2 isoproteins were not separated on an SW column. The 3rd isoprotein, EW-IIC, corresponds to a cadmium peak at a retention time of 19.4 min on an SW column in figure 1. As the smallest mol. wt cadmium-binding protein, EW-III, was separated into many peaks on a DEAE Sephadex A-25 column with elution under the conditions used for the separation of mammalian metallothioneins, further characterizations were not performed in the present study.

Although EW-IIA and -IIC were still contaminated with minor proteins, EW-IIB was shown to be pure on a gel slab of 7.5% w/v polyacrylamide with Tris-glycine pH 8.3 as electrode buffer. Amino acid analysis of the purified EW-IIB was carried out along with assays of total sugar[9] and metal contents. The amino acid composition listed in the table indicated a high half-cystine content and no aromatic amino acids in the cadmium-binding protein, EW-IIB. The absence of sugars and the cysteine-to-metal ratio of 2.4 also coincided with the accepted metallothionein characteristics. However, there are several characteristics which distinguish the earthworm cadmium-binding protein (EW-IIB) from the established metallothionein concept for mammalian metallothioneins; namely, a) low cysteine residue percent (15.4% compared to 33%), b) lack of methionine, c) low serine residue percent (6.1% compared to 14%), d) high level of acidic amino acid residues, and e) longer polypeptide chain. The longer polypeptide chain of EW-II was already suggested by co-chromatography with rat liver metallothionein on a Sephadex G-75 column[6]. The characteristic properties of EW-IIB are rather similar to the cadmium-binding proteins isolated from mussels[10] and blue green algae[11].

The absence of aromatic amino acids in EW-IIB accounts for the UV-absorption spectra in figure 3. The decrease of the shoulder around 250 nm with the decrease of pH value agrees with the dissociation of cadmium from the protein by the cleavage of mercaptide bonds.

The present communication revealed the amino acid composition and some characteristic properties of only 1 of the isoproteins among the 3 different mol. wt cadmium-binding proteins. The isoprotein characterized has properties consistent with the metallothionein concept.

The unique induction of 3 different mol. wt cadmium-binding proteins in the earthworm facilitates further studies not only on biochemical and biological functions of the inducible cadmium-binding proteins but also on evolutionary aspects.

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Growth inhibition and occurrence of cleft palates due to hypervitaminosis A[1]

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Summary. DNA synthesis in palatal processes obtained from fetal rats was 36% lower in the presence of excess vitamin A, but vitamin A-exposed maxillary explants showed only a modest decrease. Scanning electron micrographs of fetuses exposed to hypervitaminosis A in utero demonstrated both decreased head size and stunted palatal processes. The results suggest that cleft palates occur in this model system because the palatal processes are more sensitive to the actions of vitamin A than are the surrounding maxillary tissues.

The production of cleft palates by giving excess vitamin A to pregnant laboratory animals has been well documented. Nanda reported an 86% occurrence of cleft palate in Wistar albino rats when 40,000 IU/day of retinyl palmitate was given on days 9–13 of gestation[2] and a 74% rate following 60,000 IU/day on days 10–13[3]. Myers et al.[4], Yarington and Shrivers[5], and Nanda[2,3] all reported that palatal tissue from fetuses of vitamin A-treated mothers and palatal tissue cultured in vitamin A-containing medium appeared stunted in comparison to palatal tissue of controls. That palatal shelves might not come into contact at the proper time due to reduced size was offered as an explanation for palatal clefting. This explanation was supported by the work of Lorente and Miller[6] who showed that vitamin A-exposed palatal processes cultured in contact are able to fuse, implying that vitamin A affects events occurring prior to fusion. Sauer and Evans[7] compared DNA synthesis in control and vitamin A-treated maxillary explants of Wistar albino fetal rats and showed that maxillary explants were also sensitive to the growth-inhibiting effects of vitamin A. The present study examines differential responses of tissues to hypervitaminosis A. The aims of the study were: 1. to compare the effects of vitamin A in vitro on rates of DNA synthesis in palatal processes and in maxillary explants having the palatal processes removed; 2. to evaluate the palatal morphology of 16-day and 19-day rat fetuses treated with excess vitamin A in utero using the scanning electron microscope.

Materials and methods. A) In vitro. Palatal processes and maxillary explants with palatal processes removed were dissected from Wistar albino rat fetuses (Charles River Laboratories, Wilmington, Mass.) and cultured in NCTC-135 medium (Grand Island Biological Co., Grand Island, N.Y.) containing 10% fetal calf serum, 50 units/ml penicillin, 50 µg/ml streptomycin and 50 µg/ml sodium ascorbate using techniques reported by Nanda[2]. Gestational day 16 was chosen because the palatal shelves on day 16 are horizontal above the tongue but not yet fused in this strain of rat. In each of 8 experiments, 20 tissue samples were cultured (table 1): 5 control explants with palatal processes removed, 5 vitamin A-treated explants with palatal processes removed, 5 control pairs of palatal processes and 5 vitamin A-treated pairs of palatal processes. After a 24-h stabilization period of culture in either control medium or medium containing 30 IU vitamin A palmitate (Grand Island Biological Co.) per ml, ^{3}H-thymidine (New England Nuclear Corp., Boston, Mass., 15 Ci/mmole) was added to the medium to give a concentration of 1 µCi/ml and the