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RADIOIMMUNOASSAY OF METALLOTHIONEIN

Health Effects Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
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RADIOIMMUNOASSAY OF METALLOTHIONEIN

by

Felix Friedberg
Department of Biochemistry
College of Medicine
Howard University
520 W Street, N.W.
Washington, D.C. 20059

Grant No. R-803201

Project Officer

Larry L. Hall
Toxic Effects Branch
Environmental Toxicology Division
Health Effects Research Laboratory
Research Triangle Park, N.C. 27711

U.S. ENVIRONMENTAL PROTECTION AGENCY
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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The goal of this project was to develop a radioimmunoassay for metallothionein. As this protein is involved with the transport of cadmium in biological systems and may in fact protect against cadmium poisoning, the ability to monitor the levels in the human population is of the utmost importance to our evaluation of the hazards of environmental cadmium exposure.

John H. Knelson, M.D.
Director,
Health Effects Research Laboratory

INTRODUCTION

Metallothionein, a cadmium and zinc containing (6-11%), cysteine-rich (30-35%) protein of low molecular weight was first described for equine renal cortex (1). This protein is devoid of histidine, aromatic amino acids, leucine and isoleucine. It is also present in large amounts in human kidneys as well as human and equine liver. After administration of salts of cadmium, zinc or certain other heavy metals, the metallothionein accumulates in the liver and kidney of a variety of animals in which normally it is not readily detectable.

The equine renal metallothionein exists in at least two types whose sizes and total metal contents are identical but which differ in at least 7 amino acids and in their cadmium/zinc ratio. The one with the higher ratio exhibits the following amino acid composition Cys_{20} Ser_8 Lys_7 Arg_1 Ala_7 Gly_5 Val_3 Asp_2 Asn_1 Glu_1 Gln_2 Pro_2 Thr_1 Met_1 $(Cd + Zn)_7$ (2).

The 20 cysteinyl residues are distributed along the entire chain but are closer to each other in the center portion. Fourteen form part of 7 Cys x Cys tripeptides. There are 3 Cys X-X-cys and 3 cys-cys sequences. (Cys X-X cys occur also at the iron-binding sites of many iron-sulfur proteins.) (The native protein contains no disulfide bonds.) The cysteine is associated particularly with serine, lysine and arginine.

Binding of Cd or Zn ion displaces 3 protons and formation of a trimercaptide has been suggested.

There are also two major variants of metallothionein in livers of Cd treated rats. But to complicate the picture, it has been reported that administration of Cu^{2+} to rats leads to the accumulation of a similar but different protein: "Cu-chelatin." The chelatin does contain histidine, phenylalanine, leucine, isoleucine and tyrosine (3). Other workers claim that even if a Cu^{2+} inducible protein for rat liver exists, administration of this metal results in appreciable amounts of the copper being held by copperthionein (4).

Among the many hypotheses for the functions of the metallothionein, the protective role suggested by Nordberg et al. (5) is the most likely one. They reported that while intravenously administered cadmium salts cause testicular necrosis in mice, animals excreting protein in the urine (induced by repetitive ingestion of the cadmium salts) showed no significant damage of testicular tissue. Another suggested role for metallothionein is its involvement in the transport of cadmium to the kidneys which was also proposed by Nordberg (6). Such transport, in excess, may result in the degeneration of the proximal renal tubular lining cells and cause proteinuria.

EXPERIMENTAL PROCEDURE

Rats were daily injected intraperitoneally with $CdCl_2$ (2.5 mg/kg body weight) over a period of one week. After sacrifice of the anesthetized animals, livers and kidneys were removed and frozen immediately. Approximately 1000 rats were sacrificed and isolation of the metallothionein from the accumulated tissues was performed as described below. Extraction and fractionation were done at 4°C.

The tissue (1:1 w/v) was homogenized in 0.001 M Tris-buffer (pH 8.6) (containing 0.25 M Sucrose) followed by centrifugation at 15,000 g for 1 hr. Next, the supernatant was centrifuged at 100,000 g for 2 hrs. Upon application of the supernatant resulting from the second centrifugation to a Sephadex G-75 column (equilibrated and eluted with 0.001 M Tris-buffer (pH 8.6) the emerging fractions comprising peak B (see Fig. 1) were pooled and lyophilized.

Further purification of B resulted from rechromatography utilizing Sephadex G-50. The same Tris-buffer was employed. The eluant labeled M_2 (see Fig. 2) was pooled and lyophilized after desalting by passage through Sephadex G-25.

Finally, the material was applied to a DEAE Sephadex A-25 column. Elution by a linear gradient Tris-buffer pH 8.6 (0.05 0.25 M) allowed resolution of two fractions Q and R (see Fig. 3). This result is in agreement with that reported by Kimura et al. (7) who claim that both Q and R represent metallothionein and imply that further purification is not necessary.

Because of difficulty in reproducibility, in later experiments, DEAE cellulose rather than DEAE Sephadex A-25 was utilized and the linear gradient obtained with Tris-buffer (pH 8.6) was extended on both sides (0.01 0.4 M). Two peaks were resolved (DEAE I and II; see Fig. 4). In turn, each peak was rechromatographed on DEAE cellulose. Thereafter, the protein labelled DEAE I showed a single band when examined by 7.5% polyacrylamide disc gel electrophoresis while that labelled DEAE II exhibited sometimes one and sometimes two bands when applied to the gel. The final yield for each protein (DEAE I and II) was approximately a couple of mgs/100 g of liver.

We evaluated also the modification suggested by Cherian (8), i.e., heating and ammonium slufate treatment as means of facilitating the preparation of metallothionein. When we heated the material at 70 C for 20 sec., the DEAE I peak was not affected but the DEAE II was much smaller than when the heat treatment was not applied.

The following instruments were employed in this project:

Atomic Absorption Perkin Elmer 360
Gilford Model 220 Spectrophotometer
Baird Atomic Scaler 135, plus Scintillation Detector 8109
LKB Fraction Collector 7000
Serval RC-2 Centrifuge
New Brunswick Freeze Dryer B67
Beckman L2-65 Ultracentrifuge

All chemicals were Fisher Scientific products.

Rats (both males and females, 100-200 grams) were primarily of Sprague-Dawley strain even though some mutants have also been used by us. They were kindly furnished by N.I.H.

CONCLUSION

eliminary amino acid analysis (see Table I) suggests that our
ein" appears similar in composition to Riordan and Gower's (9)
ns isolated from copper loaded liver of rats (where the cysteine
out four times less than that of metallothionein), it must be
that these workers used a mixture of three proteins for their

ection of $CuSO_4$ into rats, Bremner and Young (4) isolated "copper
ilar in composition to that of "zinc thioneins" as described by
avies (10) and unlike that of the copper chelatin described by
(3).

ile workers do not agree whether "copper thioneins" and "zinc
rat are identical, we find in preliminary analysis that our
appears to have a composition similar to that of copper chelatins
those who claim that copper thioneins and zinc thioneins are
teins.

RECOMMENDATION

Any radioimmunoassay is as specific as the antigen that was used. Today, many laboratories employ antibodies to antigens which were not properly analysed and characterized and the radioimmunoassay does not really test what the investigator claims it does. We are not referring here to impurities carried by the antigen (they can be adsorbed), but to the identification of the main components.

We recommend that development of a radioimmunoassay should not be attempted until the putative metallothionein is clearly characterized. There are too many kinds of metallothionein induced by the injection of different metals according to the reports in the literature. They must be properly identified first.

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TABLE I

Amino Acid Composition of Putative Rat Metallothionein

Amino Acid Composition (Neutral and Acidic Amino Acid only)	Composition in Relative Moles
Asp	9.1
Thr	11
Ser	9.8
Glu	12
Pro	
Gly	11.3
Ala	4.4
Half Cys	8.5
Val	3.3
Met	4.1
Ileu	4.3
Leu	3.6
Tyr	1.8
Phe	3.4

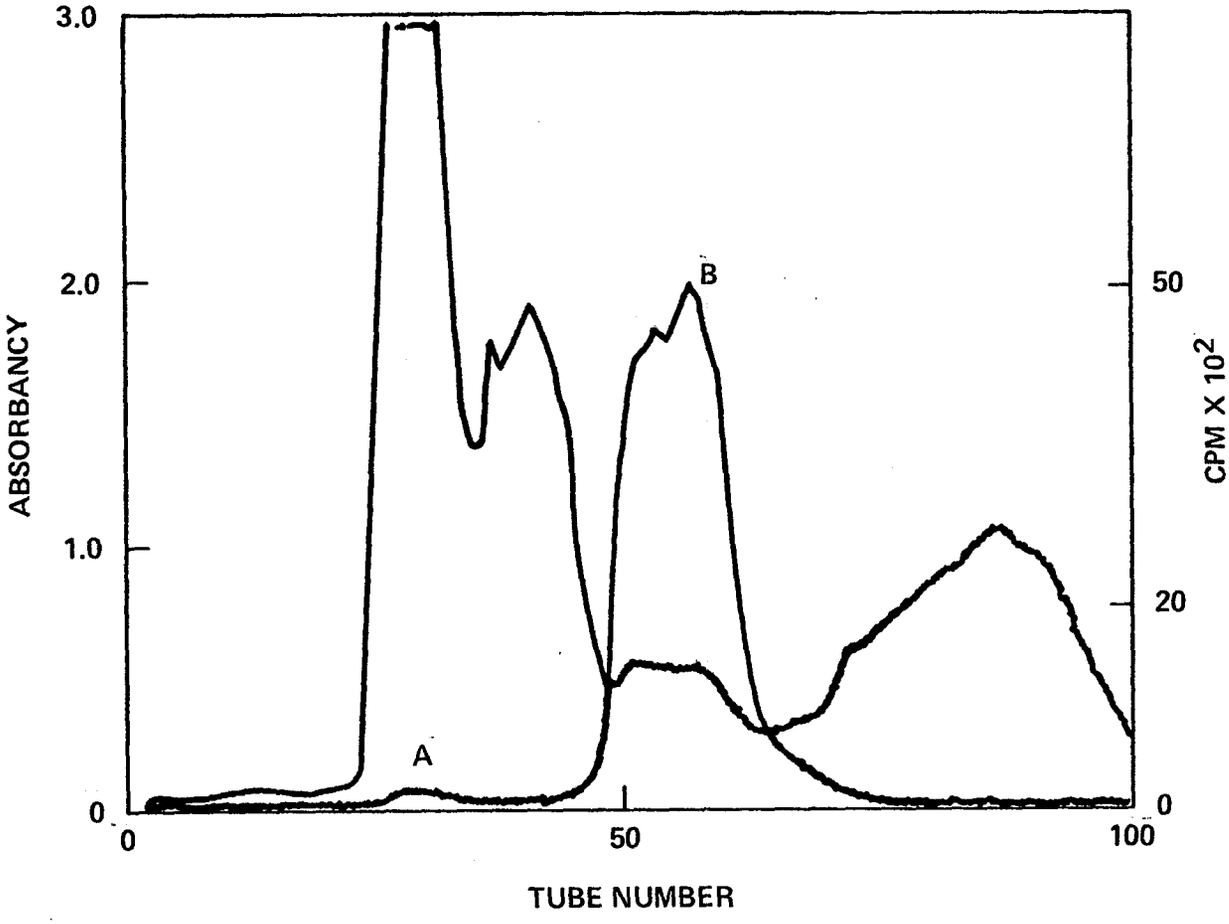

Fig. 1. Liver supernatant (after centrifugation) applied to a Sephadex G-75 column (2.5 x 100 cm). Flow rate .15 ml/hr. Elution volume: 10 ml/tube. Elution buffer: 0.001 M Tris-HCl (pH 8.6).

Radioactivity (resulting from radioactive cadmium chloride) given by the curve showing A and B. The other curve represents absorbance measurements.

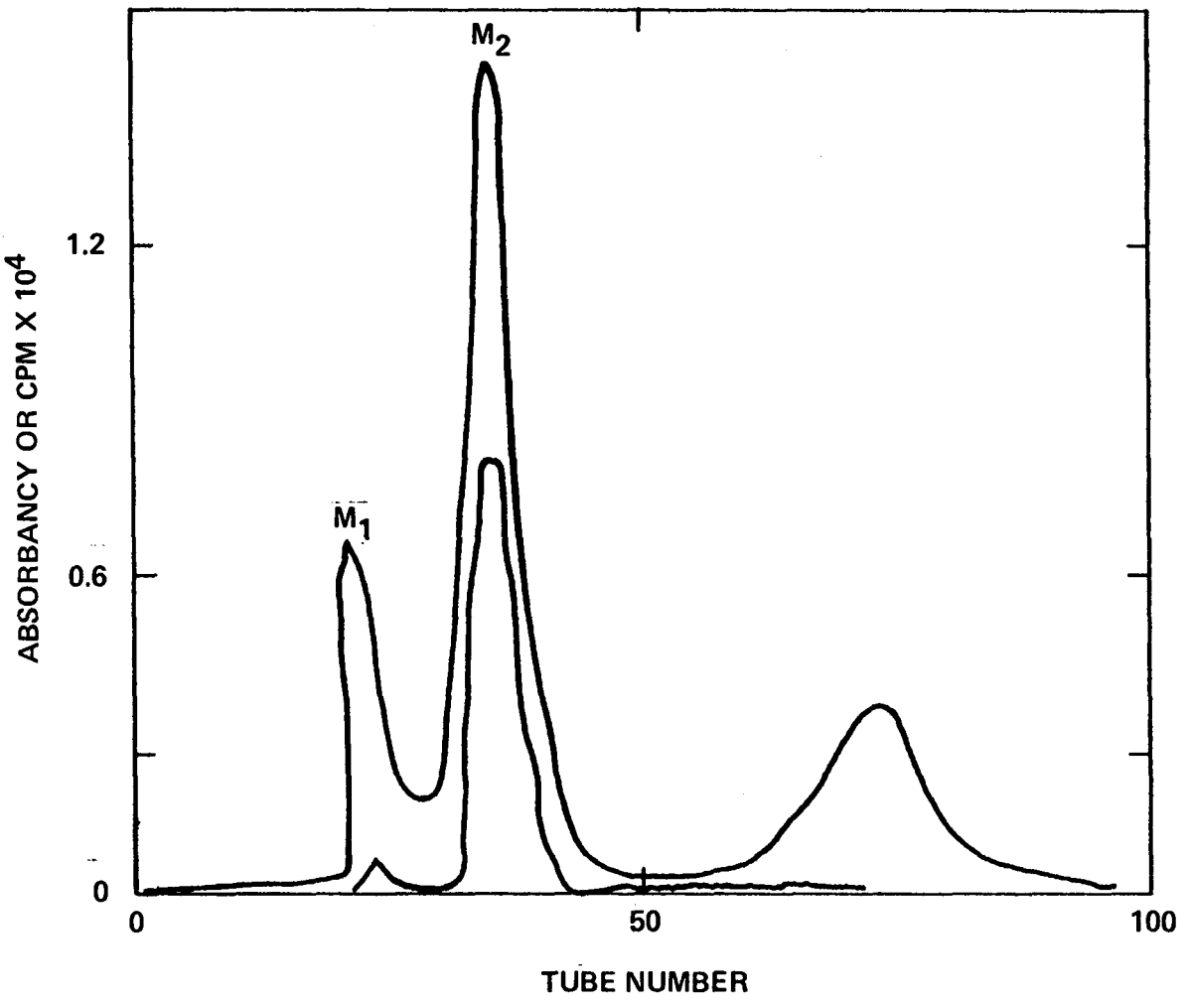

Fig. 2. Purification of B (liver) (lyophilized fraction from Sephadex G-75) on a Sephadex G-50 column (2.5 x 60 cm). Equilibrated and eluted with 0.001 M Tris-HCl (pH 8.6). Elution volume: 5 ml/tube. Upper curve gives absorbance at 252 nm. Lower curve indicates radioactivity (cadmium).

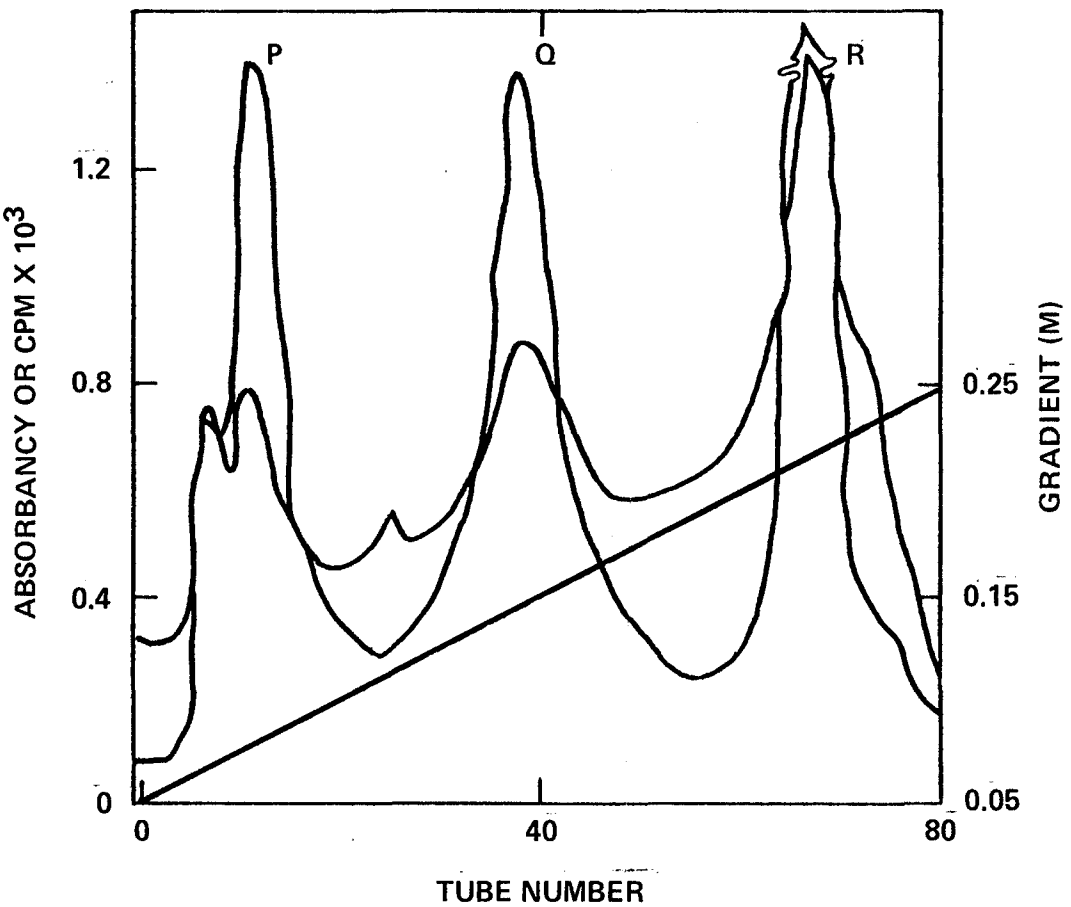

Fig. 3. Further purification of M_2 using DEAE-A25 Sephadex column (1.5 x 45 cm). Equilibrated and sample applied with 0.05 M Tris-HCl (pH 8.6). The linear salt gradient was established by gradually mixing 200 ml 0.05 M Tris-HCl. Higher curve: radioactivity. Lower curve: absorbance.

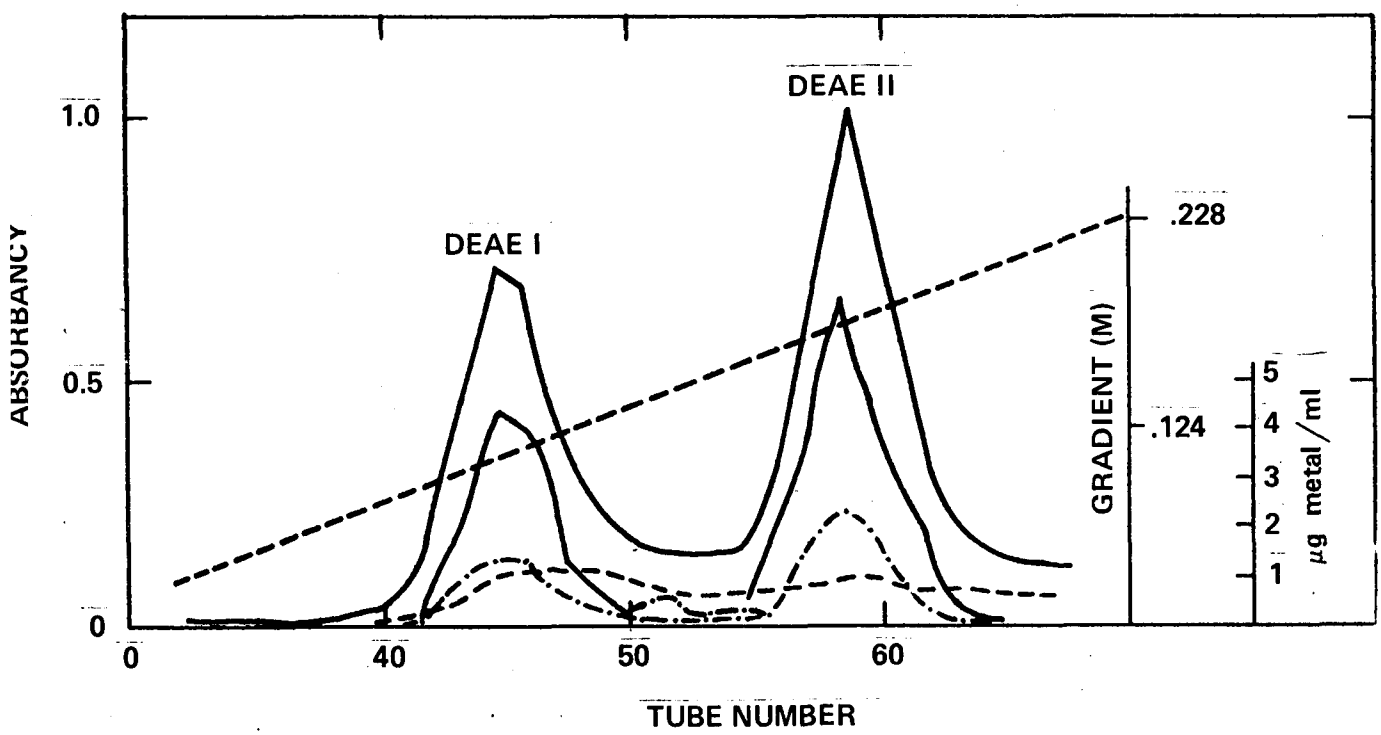

Fig. 4. Further purification of M_2 using a DEAE cellulose column (2.2 x 5 cm) equilibrated with 0.01 M Tris-HCl buffer (pH 8.6). The limiting buffer for the linear salt gradient was 0.4 M.

Solid curves:

Highest peaks: Absorbancy 250 nm

Peaks just below those highest peaks: Cadmium determined by Atomic Absorption

Stipled peaks:

-·-·-·-·- Zinc determined by Atomic Absorption

- - - - - - - Absorbancy: 280 nm

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15. SUPPLEMENTARY NOTES

16. ABSTRACT

The goal of this project was to develop a radioimmunoassay for metallothionein. Since this protein is involved with the transport of cadmium in biological systems and may in fact protect against cadmium poisoning, the ability to monitor the levels in the human population is of the utmost importance to our evaluation of the hazards of environmental cadmium exposure.

While researchers do not agree whether "copper thioneins" and "zinc thioneins" of rats are identical, this study found in preliminary analysis that zinc thionein appears to have a composition similar to that of copper chelatins.

It is recommended that development of a radioimmunoassay should not be attempted until the putative metallothioneing is clearly characterized. There are too many kinds of metallothionein induced by the injection of different metals according to the reports in the literature. They must be properly identified first.

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