METALLOBIOCHEMISTRY

dinitrophenyl (DNP) moiety at 360 nm] are analyzed for their amino acid content after hydrolysis. By this technique the unhydrolyzed PC molecule and the peptide lacking the carboxyl-terminal glycine are not separated.[18] Fast atomic bombardment has been used as an elegant alternative method for fingerprinting and sequencing of PC peptides.[7]

Reconstitution of Phytochelatin–Metal Complexes

Principle

The metal-free PC peptides can be reassociated with heavy metal ions simply by mixing metal ions with the reduced peptides. Extra care should be taken to avoid oxidation of the sulfhydryl groups during reconstitution. We performed the reconstitution in an anaerobic chamber under a reducing atmosphere [5% hydrogen and 95% nitrogen (v/v) over palladium catalyst]. For most purposes, working under a stream of nitrogen with nitrogen-purged solutions is sufficient, particularly with high concentrations of peptide material.

Procedure

The metal-free peptides (10 mg) are dissolved in 10 m*M* Tris-HCl, pH 8, the pH is readjusted, and the sample is reduced with $NaBH_4$ as outlined under Isolation of Metal-Free Phytochelatin Peptides, above. A second aliquot of $NaBH_4$ (1 mg) is added and the incubation continued for another 15 min. The excess reducing agent is destroyed by acidification with 6 *N* HCl to pH 1–2 and 25 μmol of Cd^{2+} is added, corresponding to a ratio of metal to sulfhydryl group of approximately 1:1.5. Tris base (2 *M*) is added to the solution to a final concentration of 0.2 *M*. The pH of the PC sample is adjusted to pH 8 using 6 *N* NaOH. The PC-Cd complex precipitates during the process of neutralizing the acidic solution but redissolves easily under slightly alkaline conditions. The reconstituted complex is incubated for another hour and then removed from the anaerobic chamber. The Cd-PC is chromatographed on a gel-filtration column (Sephadex G-50, 1.5 × 50 cm) in 10 m*M* Tris-HCl, pH 8, and 0.1 *M* KCl (flow rate, 20 ml/hr and 4 ml/fraction). The fractions strongly absorbing UV at 254 nm are desalted using a Sephadex G-25 column (1.5 × 25 cm) and lyophilized. Starting from 10 mg of metal-free peptide we finally obtained 9.2 mg of Cd-PC complex.

[18] W. Gekeler, E. Grill, E.-L. Winnacker, and M. H. Zenk, *Arch. Microbiol.* **150**, 197 (1988).

Properties of Phytochelatin–Metal Complexes

Phytochelatin peptides form complexes with Cd^{2+} of M_r 2.5K and 3.6K.[2] In fission yeast a Cd-PC complex of M_r 1.8K was observed.[19] These complexes appear to consist of a mixture of the PC peptides of various lengths. The metal-to-sulfur ratio is 1:2 for complexes with ions of cadmium,[20] zinc, and lead,[2,13] whereas a 1:1 ratio was determined for copper complexes.[13,21] In addition, acid-labile sulfide was identified in the complexes isolated from yeast[21,22] and plants[7,18,23] capable of forming Cd–S crystalline structures.[24] The level of sulfide varied widely, from below 1 to 30% of total sulfur in the complexes isolated from plants.[18] We also observed small amounts of cysteine and GSH in metal complexes. These findings could reflect different phases of PC complex catabolism in which PC-Cd molecules are processed similarly to GSH–S conjugates.

Acknowledgments

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[22] A. Murasugi, C. Wada, and Y. Hayashi, *J. Biochem. (Tokyo)* **93**, 661 (1983).
[23] D. N. Weber, C. F. Shaw, and D. H. Petering, *J. Biol. Chem.* **262**, 6962 (1987).
[24] C. T. Dameron, R. N. Reese, R. K. Mehra, A. R. Kortan, P. J. Carroll, M. L. Steigerwald, L. E. Brus, and D. R. Winge, *Nature (London)* **338**, 596 (1989).

[40] Sulfur-Containing Cadystin–Cadmium Complexes

By NORIHIRO MUTOH and YUKIMASA HAYASHI

Cadystins[1] are heavy metal-chelating peptides whose structural formula is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2,3,4, \ldots$).[2,3] They are synthesized in some fungi[1,4] and in plants[3,5,6] on exposure to heavy metal salts. They form

[1] A. Murasugi, C. Wada, and Y. Hayashi, *J. Biochem. (Tokyo)* **90**, 1561 (1981).
[2] N. Kondo, K. Imai, M. Isobe, T. Goto, A. Murasugi, C. W. Nakagawa, and Y. Hayashi, *Tetrahedron Lett.* **25**, 3869 (1984).
[3] E. Grill, E.-L. Winnacker, and H. M. Zenk, *Science* **230**, 674 (1985).

complexes with the heavy metal ions by sequestering them from the intracellular environment. The fission yeast mutants that cannot synthesize cadystins are hypersensitive to heavy metal salts.[7] Among the heavy metal ions, cadmium ion is the strongest inducer for cadystin synthesis.[8-10] Cadystin–cadmium complexes formed in the fission yeast *Schizosaccharomyces pombe*, in which cadystins with n equal to 2 or 3 are dominant, are called Cd-BP1 and Cd-BP2.[1] Cd-BP1 contains inorganic sulfur in addition to cadystins and cadmium.[11,12] Cd-BP2 is composed of cadystins and cadmium and no other components are found in the complex. By definition, Cd-BP1 means the cadystin–cadmium complexes containing inorganic sulfur. Because Cd-BP1's have greater Stokes radii than Cd-BP2's, they can be separated from each other by Sephadex G-50 column chromatography.[1] Cd-BPs can also be separated from cellular proteins or other low-molecular-weight components by gel-filtration chromatography. Cd-BP1 is composed of several species of complexes and each complex has a specific UV spectral property.[13,14] They are nanometer-scale quantum semiconductor particles. Electron transitions seen in ultraviolet (UV) spectra depend on the particle size of the Cd–S core,[15] although the exact structure of the complexes is uncertain. The larger Cd-BP1 has a higher molar ratio of cadmium to cadystin and of labile sulfur to cadmium. The cadmium-to-cadystin ratio of Cd-BP2 is lower than that of any species of Cd-BP1. Inorganic sulfur stabilizes the complexes and the complex containing more inorganic sulfur is more stable than the complex containing less inorganic sulfur.[14]

[4] R. K. Mehra, E. B. Tarbet, W. R. Gray, and D. R. Winge, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 8815 (1988).
[5] J. C. Steffens, D. G. Hunt, and B. G. Williams, *J. Biol. Chem.* **261,** 1389 (1986).
[6] W. Gekeler, E. Grill, E.-L. Winnacker, and H. M. Zenk, *Z. Naturforsch. C: Biosci.* **44,** 361 (1989).
[7] N. Mutoh and Y. Hayashi, *Biochem. Biophys. Res. Commun.* **151,** 32 (1988).
[8] Y. Hayashi, C. W. Nakagawa, and A. Murasugi, *Environ. Health Perspect.* **65,** 13 (1986).
[9] E. Grill, E.-L. Winnacker, and M. H. Zenk, *FEBS Lett.* **197,** 115 (1986).
[10] N. Mutoh, C. W. Nakagawa, M. Kawabata, and Y. Hayashi, unpublished observation.
[11] A. Murasugi, C. Wada, and Y. Hayashi, *J. Biochem. (Tokyo)* **93,** 661 (1983).
[12] Y. Hayashi, C. W. Nakagawa, D. Uyakul, K. Imai, M. Isobe, and T. Goto, *Biochem. Cell Biol.* **66,** 288 (1988).
[13] R. N. Reese, R. K. Mehra, E. B. Tarbet, and D. R. Winge, *J. Biol. Chem.* **263,** 4186 (1988).
[14] R. N. Reese and D. R. Winge, *J. Biol. Chem.* **263,** 12832 (1988).
[15] C. T. Dameron, R. N. Reese, R. K. Mehra, A. R. Kortan, P. J. Carroll, M. L. Steigerwald, L. E. Brus, and D. R. Winge, *Nature (London)* **338,** 596 (1989).

Purification of Cd-BP1

Strains and Growth Condition

Schizosaccharomyces pombe L972h$^-$ or its derivatives are used. Virtually any growth media for yeast culture may be used. A typical rich medium is YPD medium [2% (w/v) polypeptone, 1% (w/v) yeast extract, 2% (w/v) glucose]. Cells are grown at 30° with vigorous shaking. *Schizosaccharomyces pombe* is more sensitive to cadmium ion at higher temperature (37°); however, cadystins are induced at this temperature. Cadystins are induced by adding cadmium salt, usually $CdCl_2$, to a logarithmically growing culture to a final concentration of 0.5 mM. Although a high concentration of cadmium inhibits the growth of *S. pombe*, cadystins can be induced at up to 4 mM $CdCl_2$ (we have not tried concentrations of cadmium ion higher than 4 mM). The lowest cadmium concentration we have tested was 50 μM; cadystins are induced at this concentration of cadmium, although the amount of cadystins synthesized was reduced. To obtain a large amount of Cd-BP1, incubation with cadmium ion is continued for 17 hr because this longer incubation leads to greater accumulation of Cd-BP1 in *S. pombe*.[11] Cells are harvested by centrifugation at 3000 g at 4° for 5 min and washed twice with buffer A (50 mM Tris-HCl, pH 7.6, 100 mM KCl, 1 μM $CdCl_2$). Cell pellets can be stored at −70° for at least 1 year without any change in cadystin–cadmium complexes.

Preparation of Cell Extract

Cells are disrupted by grinding with quartz sand. Three grams (wet weight) of cell pellet is ground with 6 g of acid-washed quartz sand for 10 min with a mortar and a pestle. Three milliliters of buffer A is added to extract water-soluble components. The extract is centrifuged at 5000 g at 4° for 5 min to remove cell debris and quartz sand. The precipitate is reextracted with 3 ml of buffer A, which has been used to wash the quartz sand in the mortar. The cell extract can be further clarified by centrifugation at 100,000 g at 4° for 60 min. The cell pellet used to prepare the cell extract can be scaled up (the largest pellet we have used had a 50 g wet weight).

Sephadex G-50 Column Chromatography

In order to purify Cd-BP1 and Cd-BP2, cell extract is applied to a Sephadex G-50 (superfine) column. A 2 × 60 cm column is used. Three milliliters of cell extract can be applied to this column without any interfer-

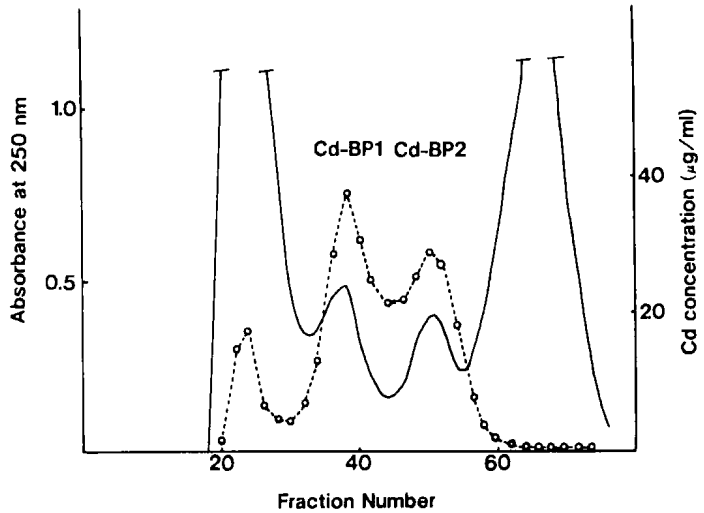

FIG. 1. Sephadex G-50 column chromatography of cell extract of *S. pombe* grown in YPD medium containing 0.5 m*M* $CdCl_2$. —, Absorbance at 250 nm; O–O, Cd^{2+} concentration.

ence in the separation of Cd-BP1 and Cd-BP2. Each 2-ml fraction is collected and the absorbance at 250 nm is recorded. Cd-BP1 is eluted around fraction 40 and Cd-BP2 is eluted at fractions 48–52 (Fig. 1). Most proteins are eluted in the void volume and low-molecular-weight components are eluted after fraction 55. Cadmium concentration in each fraction can be determined by atomic absorption spectrophotometry. Almost all of the cadmium ions are eluted with Cd-BP1 and Cd-BP2, but small fractions of cadmium ions are eluted in the void volume and are probably bound nonspecifically to cellular proteins. Cadmium ions not complexed with cadystins or proteins are undetectable in the induced cells. A significant cadmium peak is observed in the glutathione region (at about fractions 55–60) when cadmium ions are mixed with the extract of uninduced cells and chromatographed. Cd-BP1 and Cd-BP2 are pure enough for most experiments at this stage.

DEAE-Toyopearl 650M Column Chromatography

Cd-BP1 and Cd-BP2 can be purified further by DEAE-Toyopearl 650M (purchased from Tosoh, Tokyo, Japan) ion-exchange chromatography. The ion-exchange resin is packed in a 1 × 2 cm Sepacol Mini-pp disposable column (purchased from Seikagaku Kogyo, Tokyo, Japan).

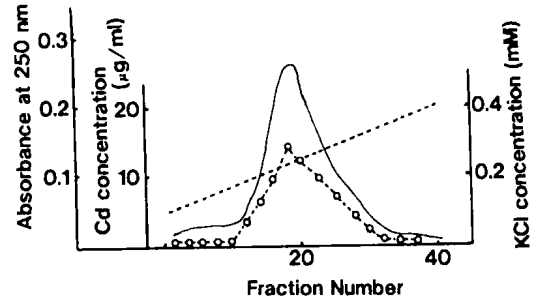

FIG. 2. DEAE-Toyopearl 650M column chromatography of Cd-BP1 obtained from Sephadex G-50 column chromatography. —, Absorbance at 250 nm; O–O, Cd^{2+} concentration.

Cd-BP1 and Cd-BP2 fractions obtained from Sephadex G-50 column chromatography are diluted with equal amounts of 1 m*M* Tris-HCl, pH 7.6, 1 μM $CdCl_2$ and applied to the ion-exchange column. Cd-BPs are eluted by a KCl gradient of 100 to 400 m*M* in 25 m*M* Tris-HCl, pH 7.6, 1 μM $CdCl_2$. Cadmium salt is added to the buffer to stabilize the complexes. In some cases, crude cell extract is directly applied to the ion-exchange chromatography column to purify the cadystin–cadmium complexes.[13,16] Cd-BP1 is eluted at a KCl concentration of about 230 m*M* (Fig. 2) and Cd-BP2 is eluted at a concentration of about 180 and 240 m*M*.[12] The broad peak of Cd-BP1 is derived from the heterogeneity of Cd-BP1 (described below). The largest Cd-BP1 obtained from a cadmium-hypersensitive mutant complemented with a recombinant plasmid, which has a 1.5-kilobase (kb) AT-rich sequence of *S. pombe* genomic DNA with unknown function, is eluted at 280 m*M* KCl.[10]

Characterization of Cd-BP1

Optical Properties of Cd-BP1

Cd-BP1 is composed of heterogeneous complexes and they are nanometer-scale semiconductor particles.[15] Each complex has a unique cadmium-to-inorganic sulfur ratio and shows a specific UV absorbance spectrum in the range of 260–320 nm.[13,14] Complexes with higher Stokes radii have optical transitions at longer wavelengths. The UV spectrum can be measured from the sample obtained by either Sephadex G-50 column chromatography or DEAE-Toyopearl 650M column chromatography. Salt

[16] A. Murasugi, C. Wada, and Y. Hayashi, *Biochem. Biophys. Res. Commun.* **103**, 1021 (1981).

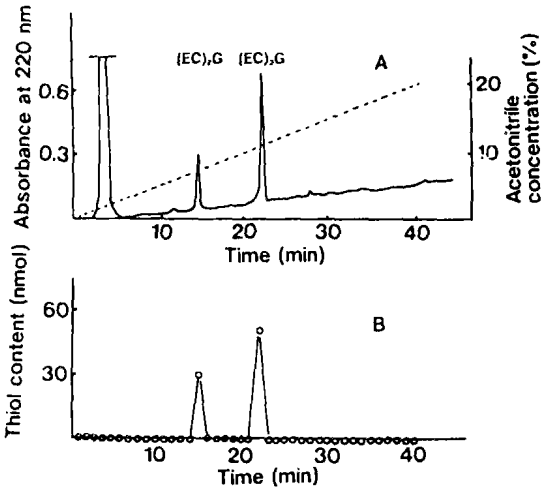

FIG. 3. HPLC profile of cadystins in Cd-BP1 obtained from ODS80 TM HPLC column. (A) Absorbance at 220 nm. (B) Thiol content.

has no effect on the spectral properties of Cd-BP1 in the range used by these preparations.[17] The UV spectrum is changed by lowering the pH because of the dissociation of the complexes,[12,13,16] and the original shoulder at 265 nm in the UV spectrum is not obtained after reneutralization in the presence of excess cadmium ion.

Assays for Components of Cd-BP1

Reversed-phase HPLC is used to determine the content of each homolog of cadystins in Cd-BPs. Trifluoroacetic acid (TFA) (5%, v/v) is added to a Cd-BP solution. After centrifugation and filtration to remove insoluble materials, the sample is applied to an ODS80 TM HPLC column (Tosoh) and eluted by a linear gradient of 0–20% (v/v) acetonitrile in 0.05% TFA at a flow rate of 1 ml/min. Absorbance at 220 nm is recorded. (γ-Glu-Cys)$_2$-Gly and (γ-Glu-Cys)$_3$-Gly are eluted at about 8 and 13% acetonitrile, respectively (Fig. 3). For the determination of thiol content, a 1-ml fraction is collected; 50 μl 1 *M* K_2HPO_4 and 7 μl of thiol reagent, 5,5′-dithiobis(2-nitrobenzoic acid)[18] (4 mg/ml in 45 m*M* phosphate buffer, pH 7.0), are added. Absorbance at 412 nm is recorded and calibrated against a standard curve for the reduced form of glutathione.

When Cd-BP1 is acidified to release cadmium from the cadystin–cadmium complexes, an odor resembling that of H_2S is usually noticed.

[17] Y. Hayashi and M. Kawabata, unpublished observation.
[18] G. L. Ellman, *Arch. Biochem. Biophys.* **82**, 70 (1959).

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This acid-labile sulfur in Cd-BP1 can be determined according to King and Morris,[19] using an iodometrically standardized Na_2S solution. The labile sulfur in purified ferredoxin can be determined by this method. In the determination of the inorganic sulfur content in Cd-BP1, it is necessary to compensate for the inhibitory effect of cadmium ion on the color development of the reaction. With a cadmium concentration of 10–20 μg/ml, color development is reduced to 80% of that obtained using a sample without cadmium.

Cadmium content is determined by atomic absorption spectrophotometry.

Reconstitution of Cd-BP1

Cadystin–cadmium complexes are dissociated by lowering the pH of the solution, i.e., below pH 5. The exact pH required to dissociate a complex depends on the size of complex.[12,16] Cd-BP1 can be reconstituted from cadystin, cadmium, and inorganic sulfur (Na_2S) by incubating them together. When the complex is reconstituted with an excess amount of Na_2S at room temperature, a complex is formed having a large Stokes radius. A complex with a small Stokes radius is reconstituted by incubating with a stoichiometric amount of Na_2S at low temperature (4°) for 16 hr.[14] Complexes with large radii can be formed by incubating complexes with small radii with an excess amount of Na_2S.[11,14] *In vivo* formation of Cd-BP1 depends on the concentration of cellular cadmium and sulfide production per cell also increases with the increasing concentration of cadmium ions in the culture medium.[20] This indicates that the reconstitution condition mimicks the formation of Cd-BP1 *in vivo*. Cd-BP2 can be reconstituted easily from cadystin and cadmium by mixing in acidic solution and neutralization by alkaline solution.

[19] T. E. King and R. O. Morris, this series, Vol. 10, p. 634.
[20] A. Murasugi, C. W. Nakagawa, and Y. Hayashi, *J. Biochem. (Tokyo)* **96**, 1375 (1984).

[41] Cadystins: Small Metal-Binding Peptides

By Y. HAYASHI, M. ISOBE, N. MUTOH, C. W. NAKAGAWA, and M. KAWABATA

Cadmium-binding peptides (Cd-BP1 and Cd-BP2) were induced in fission yeast on exposure to cadmium ion.[1] Metal-chelating peptides, cadystins, were first observed in these Cd-BPs as the peptide component having the general structure (γ-Glu-Cys)$_n$Gly ($n = 2,3,4, \ldots$).[2,3] These peptides were also detected in plants, other yeast, and protozoa as the peptide component of metal complexes, although the trivial names given were different from each other: phytochelatin,[4] γ-Glu peptide,[5] poly(γ-Glu-Cys)Gly,[6] and others.[7] In this chapter we describe the induction and isolation of metal–cadystin complexes, properties of the metal complexes, and the chemical structure of cadystins.

Induction by Administration of Cadmium Ions

Preparation of Cadmium–Peptide Complexes

The fission yeast *Schizosaccharomyces pombe* L972 h$^-$ is grown to the early stationary phase of culture by shaking at 30° in YPD medium [1% (w/v) yeast extract, 2% (w/v) polypeptone and 2% (w/v) dextrose (Wako Pure Chem., Osaka, Japan)], and this culture is used as the preculture. The main culture of 500 ml is started in a 3-liter flask by addition of preculture to 0.3 or 1.2×10^7 cells/ml; $CdCl_2$ is added at various concentrations (0.1–1 m*M*). After an appropriate incubation time (2–24 hr), cells are harvested by centrifugation, washed once with 0.14 *M* NaCl and twice with distilled water, and stored at −30° until use. Frozen cells are ground in a chilled mortar and pestle with acid-washed quartz sand (three times the weight of the cells), and extracted with 5 vol of 50 m*M* Tris-HCl (pH 7.5)–0.1 *M* KCl. The extract is centrifuged at 17,000 *g* for 20 min at 4°,

[1] A. Murasugi, C. Wada, and Y. Hayashi, *J. Biochem. (Tokyo)* **90**, 1561 (1981).
[2] N. Kondo, M. Isobe, K. Imai, T. Goto, A. Murasugi, and Y. Hayashi, *Tetrahedron Lett.* **24**, 925 (1983).
[3] N. Kondo, K. Imai, M. Isobe, T. Goto, A. Murasugi, C. W. Nakagawa, and Y. Hayashi, *Tetrahedron Lett.* **25**, 3869 (1984).
[4] E. Grill, E.-L. Winnacker, and M. H. Zenk, *Science* **230**, 674 (1985).
[5] R. N. Reese, R. K. Mehra, E. B. Tarbet, and D. R. Winge, *J. Biol. Chem.* **263**, 4186 (1988).
[6] P. J. Jackson, C. J. Unkefer, J. A. Doolen, K. Watt, and N. J. Robinson, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6619 (1987).
[7] D. W. Weber, C. F. Shaw III, and D. H. Petering, *J. Biol. Chem.* **262**, 6962 (1987).

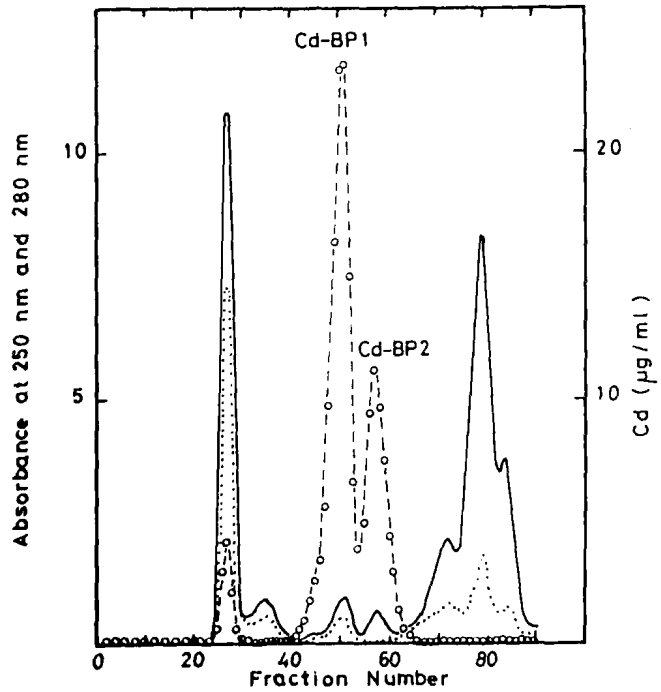

FIG. 1. Sephadex gel-filtration analysis of induced cadmium-cadystin complexes (Cd-BP1 and Cd-BP2). The overnight culture of the fission yeast is diluted with the fresh YPD medium containing 0.5 m*M* $CdCl_2$ (1.2×10^7 cells/ml) and incubated for 17 hr with vigorous shaking at 30°. Cell extract is prepared as described in the text and about one-third volume of the extract is applied to the Sephadex G-50 SF column. Fractions (1.5 ml) are collected and absorbances at 250 nm (—) and 280 nm (····) and cadmium content (O—O) in each fraction are determined.

the supernatant is applied to Sephadex G-50 Superfine (Pharmacia, Piscataway, NJ) column (1.6×65 cm), and eluted with the extraction buffer. Two peaks of cadmium-binding peptides are detected in the low-molecular-weight region (Fig. 1). The time course study of Cd-BP formation indicates that the amount of Cd-BP per cell reaches saturation after 10 hr of 1 m*M* $CdCl_2$ administration,[1] although cell growth continues and the total amount of Cd-BP per culture still increases (Fig. 2). This is consistent with the time needed for maximum cadmium uptake per cell, implying that cellular cadmium uptake occurs as long as Cd-BP synthesis continues.

Subspecies of Cadmium-Binding Peptides

Cd-BP1 and Cd-BP2 from 0.1 m*M* $CdCl_2$-induced cells are rechromatographed in a Sephadex G-50 SF column, and the purified Cd-BP1 or

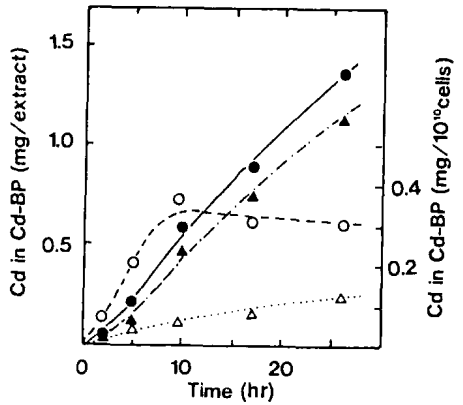

FIG. 2. Increases of Cd-BPs with time in extracts from 500 ml of cultured cells. Amounts of Cd-BPs are expressed as their cadmium amounts. (●), Cd-BPs in the total cell extract; (○), Cd-BPs/10^{10} cells; (▲), Cd-BP1 in the total cell extract; (△), Cd-BP2 in the total cell extract.

Cd-BP2 is diluted and applied to a DEAE-Toyopearl (Tosoh, Tokyo, Japan) 650S column (0.9 × 10 cm) equilibrated with 25 m*M* Tris-HCl (pH 7.5)–50 m*M* KCl–10 μM $CdCl_2$. The subspecies of Cd-BP1 and Cd-BP2 are isolated by KCl gradient elution (Fig. 3). Each subspecies is rechromatographed on the DEAE-Toyopearl column and some properties of the cadmium–cadystin complex are analyzed.

Determination of Heavy Metals, Sulfhydryl Groups, and Acid-Labile Sulfides

The content of heavy metals, e.g., cadmium, zinc, and copper, in a solution containing metal–peptide complexes is determined with a Perkin-Elmer (Norwalk, CT) atomic absorption spectrophotometer or Hitachi (Tokyo, Japan) Z-7000 polarized Zeeman atomic absorption spectrophotometer.

Sulfhydryl group is determined by Ellman's method, using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).[8]

The acid-labile sulfide content is determined according to King and Morris,[9] except for the addition of an appropriate amount of $CdCl_2$ (5–50 mg/ml) to the standard Na_2S reaction mixture. This modification is necessary because the coupling reaction is affected by heavy metal ions,[9] and cadmium–peptide complexes contain cadmium ions. With the use of an Na_2S solution standardized iodometrically, the effect of cadmium ions is

[8] G. L. Ellman, *Arch. Biochem. Biophys.* **82**, 70 (1959).
[9] T. King and O. Morris, this series, Vol. 10, p. 634.

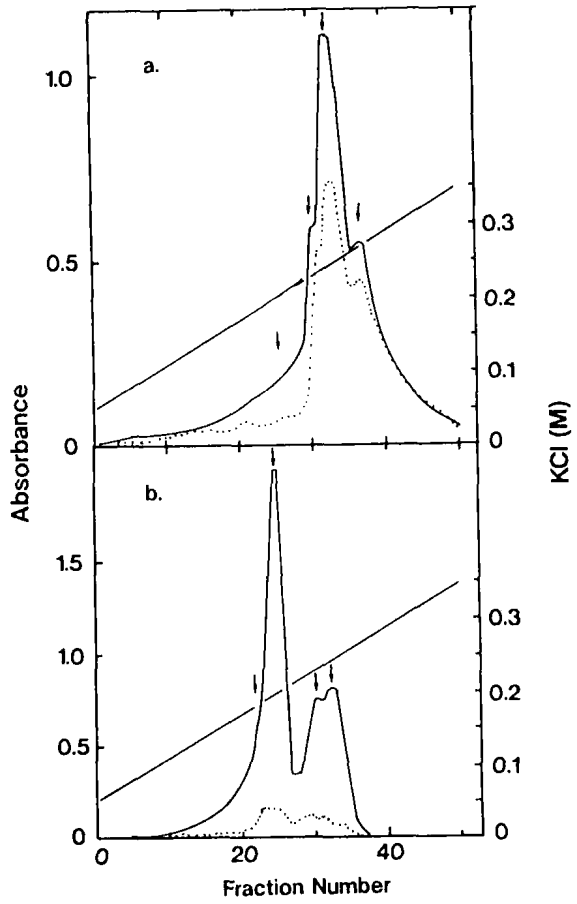

FIG. 3. Separation of cadmium–cadystin complex subspecies by ion-exchange chromatography. The Cd-BP1 or Cd-BP2 fraction from the Sephadex G-50 SF column is chromatographed on a DEAE-Toyopearl 650S column (Tosoh). Absorbances are read at 250 nm (—) and at 280 nm (····). (a) Cd-BP1 subspecies separation. The arrows indicate 0.19, 0.22, 0.25, and 0.27 *M* subspecies, respectively. (b) Cd-BP2 subspecies separation. The arrows indicate 0.18, 0.20, 0.24, and 0.26 *M* subspecies.

checked; the presence of 20 μg/ml Cd^{2+} in the sample decreased the efficiency of the coupling reaction to 81%.[10]

Peptide content in metal–peptide complexes can be determined by one of the following methods: (1) nitrogen content determined by the Kjeldahl–Nessler method, (2) quantitative amino acid analysis, or (3)

[10] A. Murasugi, C. Wada, and Y. Hayashi, *J. Biochem. (Tokyo)* **93**, 661 (1983).

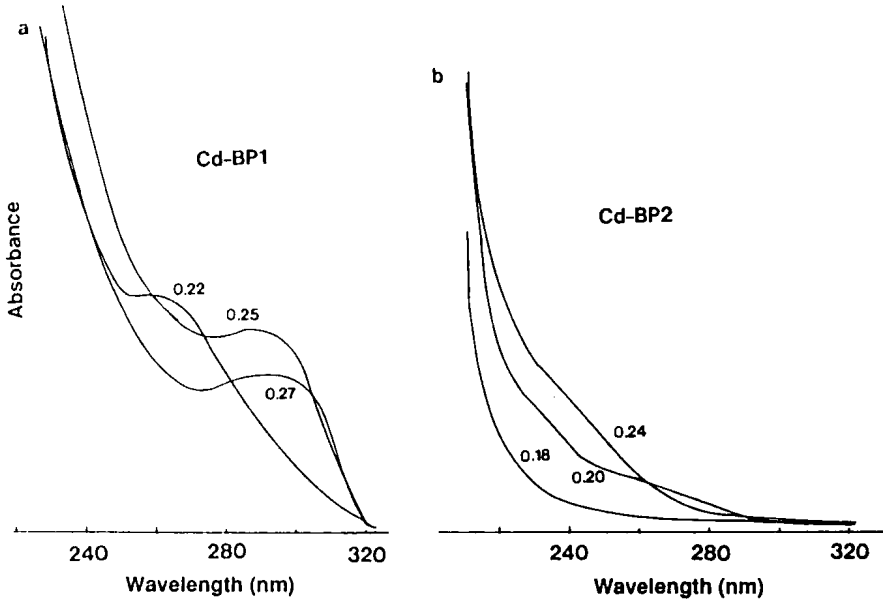

FIG. 4. Ultraviolet absorption spectra of Cd-BP subspecies. Spectra are shown for the eluted fractions from the DEAE-Toyopearl column. The numbers by the curves indicate the salt concentration of the elution fractions normalized to contain cadmium (2 μg/ml) and 25 mM Tris-HCl (pH 7.5)–50 mM KCl. (a) Cd-BP1 subspecies. (b) Cd-BP2 subspecies.

sulfhydryl groups or the absorption units of peptides fractionated by high-performance liquid chromatography (HPLC).

Ultraviolet Spectra of Cadmium–Cadystin Complexes

Cd-BPs purified by rechromatography are dialyzed against 25 mM Tris-HCl (pH 7.5)–50 mM KCl–1 μM $CdCl_2$, diluted to the same Cd^{2+} concentration (2 μg Cd/ml) with the same buffer, and ultraviolet (UV) spectra are determined.

Among Cd-BP1 subspecies, the higher the eluting salt concentration from the anion-exchange column, the longer the wavelength of the optical transition characteristic for cadmium–sulfide in Cd-BP1 (Fig. 4a). The physical basis of the optical properties relate to the quantum nature of Cd–S crystallite that is formed in these Cd-BP1 species.[11]

The UV spectra of Cd-BP2 subspecies reveal that these are cadmium complexes with characteristic cadmium–thiolate charge-transfer transi-

[11] C. T. Dameron, R. N. Reese, R. K. Mehra, A. R. Kortan, P. J. Carroll, M. L. Steigerwald, L. E. Brus, and D. R. Winge, *Nature (London)* **338**, 596 (1989).

tions (Fig. 4b). The Cd-BP2 subspecies are devoid of the labile sulfide and do not show absorbances between 260 and 320 nm due to CdS.

The effects of salt concentration on the UV spectra of these cadmium complex subspecies are determined by increasing the KCl concentration in a sample having the same cadmium–cadystin concentration. With Cd-BP1 subspecies, very little absorbance shift is observed over the entire UV

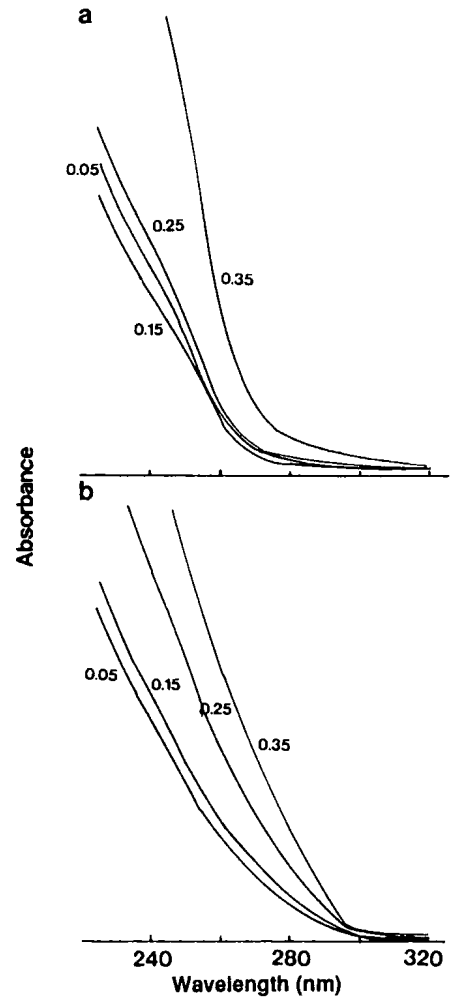

FIG. 5. Effect of the salt concentration on UV spectra of the cadmium–cadystin complexes. Absorbance shifts are observed in (a) 0.20 M and (b) 0.24 M subspecies of Cd-BP2 (cadmium, 1 μg/ml) with increasing concentration of KCl in 25 mM Tris-HCl (pH 7.5) as indicated by the number by the respective curve.

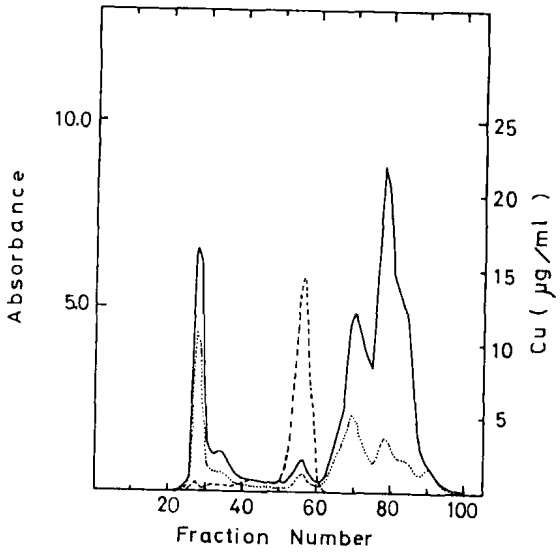

FIG. 6. Induction of the copper–cadystin complex. $CuCl_2$ is added to the YPD medium at 2.5 m*M* and culture continued for 17 hr. The cell extract from a 500-ml culture was applied to a Sephadex G-50- SF column as described in the text. Absorbances at 250 nm (—) and at 280 nm (· · · ·) and copper concentration (– – –) in each fraction are determined.

range. On the other hand, some Cd-BP2 subspecies show significant absorbance shifts with increasing salt concentration (Fig. 5).

Cadystin Induction by Other Heavy Metals and Oligosaccharides

Induction

Cadystin synthesis can be induced by other heavy metals in the fission yeast, although the synthesized amounts of cadystin are less than 10% of that by Cd^{2+}. With 2.5 m*M* $CuCl_2$ in YPD medium, copper–cadystin complex in the cell extract is observed (Fig. 6) by analysis of Sephadex gel filtration at the region around M_r 3000, which is an intermediate molecular weight between the values observed for the two cadmium–cadystin complexes (Cd-BP1 and Cd-BP2).[1]

With 2 m*M* $ZnCl_2$ in the medium, only a small amount of cadystin synthesis is detected after 10 hr of culture. However, with the simultaneous addition of oligosaccharides, such as hydrolysates of pectin, mannan, chitin, and chitosan, at 50 μg/ml the induction of cadystin synthesis is significant (about 20–40% of that induced by 0.1 m*M* cadmium) and a zinc–cadystin complex peak is observed at around M_r 1200.[12] The induction by

[12] Y. Hayashi, S. Morikawa, N. Mutoh, M. Kawabata, and Y. Hotta, submitted.

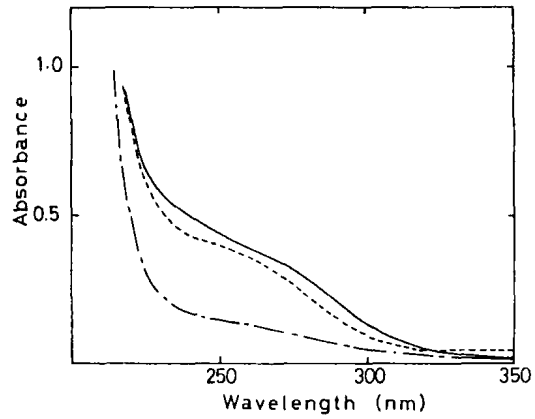

FIG. 7. Ultraviolet absorption spectra of copper–cadystin complexes. UV spectra are recorded for the native copper–cadystin complex (– – –), Cu(I)-replaced complex (—), and Cu(II)-replaced complex (– · – ·). Copper-replaced complexes are prepared by incubation of Cd-BP1 with an excess amount of Cu(I) or Cu(II) ion, and the replaced complexes are isolated by the use of a BioGel P-2 column or Sephadex G-10 column. Cadystin amounts in complexes are about 100 μg/ml in 5 m*M* Tris-HCl (pH 7.5)–10 m*M* KCl.

oligosaccharides is observed even without addition of $ZnCl_2$, and the zinc–cadystin complex is also detected at the same region in Sephadex G-50 SF column fractions. Oligosaccharides of fungal and plant origin have been identified that can act as chemical signals to activate a broad spectrum of genes for the defense mechanism in plants.[13] The induction of cadystin synthesis by oligosaccharides could be one of the defense reactions of the fission yeast. However, because the induction of cadystin synthesis in the fission yeast by oligosaccharides is accompanied by the appearance of a significant peak of zinc–cadystin complex even without special addition of zinc to YPD medium, the defense signal of oligosaccharides not only induces the cadystin synthesis but may increase the influx of Zn^{2+} from the usual YPD medium (Zn^{2+} concentration is 0.3–0.4 m*M*).

Ultraviolet Absorption Spectra of Copper–Cadystin and Zinc–Cadystin Complexes

When the UV absorption spectrum of the native copper–cadystin complex is compared with the spectra of the reconstituted Cu(I)–cadystin and Cu(II)–cadystin complex, the spectrum of the native copper–cadystin corresponds to that of Cu(I)–cadystin complex (Fig. 7). This indicates that

[13] C. A. Ryan, *Biochemistry* 27, 8879 (1988).

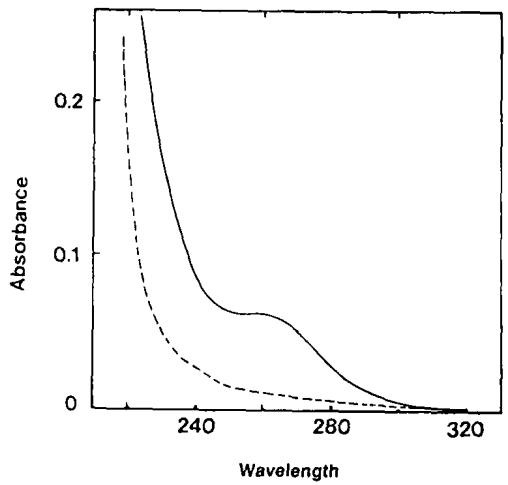

FIG. 8. Ultraviolet absorption spectra of zinc–cadystin complexes. The UV spectra are recorded for the native zinc–cadystin complex (—) and reconstituted zinc–cadystin complex (---) from free cadystin and excess zinc ions followed by BioGel P-2 isolation. The amount of complex is normalized as zinc (1 μg/ml) in 25 m*M* Tris-HCl (pH 7.5)–50 m*M* KCl.

the ionic state of copper in the copper–cadystin complex in the cell is Cu(I), like copper–thionein in *Neurospora* and in mammals. In the spectrum of the native copper–cadystin complex, the optical transition at 265 nm is also observed as in Cd-BP1 species (Fig. 7). The UV spectrum of the native Zn^{2+}–cadystin complex induced by the oligosaccharides and zinc also shows the optical transition at 265 nm, and this transition cannot be observed in the reconstituted zinc–cadystin complex (Fig. 8).

Chemical Structure of Cadystins

Purification of Cadystins

The metal–cadystin complexes from the Sephadex G-50 SF column fraction are purified by rechromatography on the same gel-filtration column and by an anion-exchange column [DE-52 (Whatman, Clifton, NJ) or DEAE-Toyopearl 650M]. The purified metal–cadystin complex is concentrated by ultrafiltration using SPECTRA/POR membrane (Spectrum, Los Angeles, CA) (molecular weight cutoff, 1000). The metal complex is acidified by the addition of trifluoroacetic acid (TFA) to 5%, centrifuged at 15,000 *g*, filtered through Chromatodisc 13A (Kurabou), and applied to an HPLC column [TSK- ODS80 (Tosoh, Tokyo, Japan), 0.46 × 25 cm or Beckman Ultrasphere ODS (San Ramon, CA), 1.0 × 25 cm with TSK-ODS80 precolumn, 0.46 × 1.5 cm]. Cadystin species are eluted from the column by a linear gradient of 0–20% acetonitrile in 0.05% TFA in 40 min (flow rate: 1 ml/min for 0.46-cm diameter column or 2 ml/min for 1.0-cm diameter column). Depending on the column size 1- or 2-ml fractions are collected, and the contents of SH groups are determined in an aliquot of each fraction to ascertain the position of cadystin species and to determine the amounts of cadystins synthesized.

Amino Acid Analyses of Cadystins

Cadystin species (HPLC purified) are lyophilized and oxidized by performic acid, and digested in 5.7 *N* HCl–0.02% (v/v) phenol for 24 hr at 120°. Samples are lyophilized again to remove HCl completely, and coupled with phenyl isothiocyanate.[14] Phenylthiocarbamyl-amino acids are analyzed in HPLC (TSK-ODS80, 0.46 × 25 cm with a precolumn) by a linear gradient of A solution [0.14 *M* sodium acetate (pH 6.3)–0.05% triethylamine/acetonitrile (940:60)] and B solution [acetonitrile/H_2O (60:40)] in 50 min from 100% A to 100% B. The results of quantitative amino acid analyses give the equimolar amounts of glutamic acid and cysteinic acid, and the respective amount of glycine according to cadystin species.

Chemical Structure of Cadystins

Cadmium–cadystin complex is desulfurized with Raney nickel W-2 at 50° for 12 hr in 50 m*M* Tris-HCl, pH 7.6.[2] The solvent is evaporated *in vacuo* and the catalyst is dissolved in a small volume of 6 *N* HCl below 30° and then the mixture is lyophilized. The residue is desalted in a BioGel P-2 column with 3 *M* acetic acid. Dethiocadystin thus obtained is separated by HPLC (ODS column) as previously described.[2,3] Amino acid analysis of dethiocadystins shows equimolar amounts of glutamate and alanine, and the respective amount of glycine, depending on the cadystin species. Then the chemical structures of cadystins are determined by carboxypeptidase P digestion of dethiocadystins and chemical analyses of the resulting dipeptide as follows.[15] The smallest dethiocadystin is digested with carboxypeptidase P for 96 hr at 35° in 0.1 *M* sodium acetate (pH 5.2). It yields glycine and two molar equivalents of dipeptides containing glutamate and alanine in 1:1 ratio. The dried dipeptide is esterified in 10 μl of a mixture of methanol, trimethyl orthophosphate, and thionyl chloride (80:20:5 w/w) for 4 hr at 40°, and dried *in vacuo*. The residue is reduced in 10 μl of 0.3 *M* $LiBH_4$ in tetrahydrofolic acid (THF) for 12 hr at 80° in a sealed tube. After

[14] R. L. Heinrikson and S. C. Meredith, *Anal. Biochem.* **136,** 65 (1984).
[15] N. Kondo, M. Isobe, K. Imai, and T. Goto, *Agric. Biol. Chem.* **49,** 71 (1985).

evaporation to dryness, the residue is hydrolyzed with 50 μl of 6 *N* HCl for 12 hr at 105°. The hydrolysate gives 4-amino-5 hydroxyvaleric acid and 2-aminopropanol in nearly quantitative yield without formation of alanine or 2-amino-5-hydroxyvaleric acid. Thus, the sequence of the dipeptide should be γ-Glu-Ala. The deduced general structures for cadystins are $(\gamma\text{-Glu-Cys})_n\text{Gly}$ ($n = 2,3,4, \ldots$).[3] These structures for cadystins are confirmed by comparing the behavior in HPLC (ODS column), CD spectra, and ^{1}HNMR spectra of natural cadystins vs chemically synthesized cadystins.[3,15]

Possible Functions of Cadystins in Cells

Cadystins induced on exposure to heavy metals work in detoxification of excess heavy metals. However, cadystin induction caused by the administration of oligosaccharides may be different from heavy metal detoxification, as follows. (1) The influx of Zn^{2+} increases. The chemical signals of the oligosaccharides presumably increase the influx of Zn^{2+} to the cell. The excess Zn^{2+} resulting from the increased influx is stored as the complex form with cadystin and can be used for the activation of zinc enzymes, which may be newly synthesized as the result of the defense reaction. (2) Active radicals are detoxified. Like the biological function of metallothionein, cadystins may have a role in the elimination of harmful oxygen radicals or hydroxy radicals because of their strong reduction activities. The reducing activities of cadystins stronger than glutathione indicate that the role of cadystins in the damaged cell could be mainly detoxification of reactive radicals.

[42] Isolation of Metallothionein from Ovine and Bovine Tissues

By P. D. WHANGER

Although metallothionein (MT) was first isolated and characterized from the kidney of a large animal, the equine species,[1] most of the work on its metabolism and involvement in metal metabolism has been performed with small animals, mainly the rat.[2] There are some major differences in the number of MT species between various animals.[2] Thus, a study of MT

[1] J. H. R. Kägi and B. L. Vallee, *J. Biol. Chem.* **235**, 3460 (1960).
[2] J. H. R. Kägi and Y. Kojima, eds., *Experientia, Suppl.* **52** (1987).

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metabolism in large animals was necessary to obtain information applicable to these animals.

Supplies and Reagents

A Potter–Elvehjem homogenizer, centrifuge, chromatography columns, freeze drier, fraction collector, an amino acid analyzer, equipment for electrophoresis on acrylamide gels, an absorption spectrophotometer, and an atomic absorption spectrophotometer are the necessary equipment for the isolation and characterization of MT. Sephadex G-75, DEAE-Sephacel, BioGel P-10, Tris-HCl, isotonic saline, and dialysis tubing are the necessary reagents for these procedures.

Procedures

Methods should be employed to minimize metal contamination, as was discussed in a previous volume of this series.[3] Since dietary zinc was shown to accumulate in tissue MT,[4,5] animals were fed elevated amounts of this metal in the diet to increase these levels in tissues. Dietary levels of zinc to 2000 mg/kg feed can be fed for 2 months without any affect on the performances of sheep and cattle. Excess zinc was shown to accumulate in MT in liver, kidney, pancreas, and small intestinal epithelia, but not in heart, testis, or epithelia of abomasum and rumen of cattle and sheep.[5]

About 50 g of tissue is homogenized in 2 vol of cold isotonic saline with a Potter–Elvehjem homogenizer. Homogenates and successive supernatant fractions are centrifuged (4°) at 37,000 *g* for 30 min and at 160,000 *g* for 90 min to obtain the tissue cytosols. The tissue cytosols are eluted through a Sephadex G-75 column (5.0 × 100 cm) with 0.05 *M* Tris-HCl buffer, pH 8.4. Fractions of 15 ml are collected at a rate of 120 ml/hr. The eluted fractions are monitored for zinc by atomic absorption spectrophotometry. The MT fractions are then combined, dialyzed against 0.003 *M* Tris-HCl, pH 8.4, for 12 hr. This is then chromatographed on a DEAE-Sephacel (Sigma Chemical Co., St. Louis, MO) column (2.0 × 30 cm) with a gradient of 0.003 *M* (350 ml) to 0.3 *M* (350 ml) Tris-HCl buffer, pH 8.4, at a flow rate of 20 ml/hr. About 4 ml/fraction is collected and the zinc monitored by atomic absorption spectrophotometry. A typical elution pattern is shown in Fig. 1. This pattern is very similar for both ovine and

[3] J. F. Riordan and B. L. Vallee, eds., this series, Vol. 158.
[4] R. L. Kincaid, W. J. Miller, P. R. Fowler, R. P. Gentry, D. L. Hampton, and M. W. Neathery, *J. Dairy Sci.* **59**, 1580 (1976).
[5] P. D. Whanger, S.-H. Oh, and J. T. Deagen, *J. Nutr.* **111**, 1196 (1981).

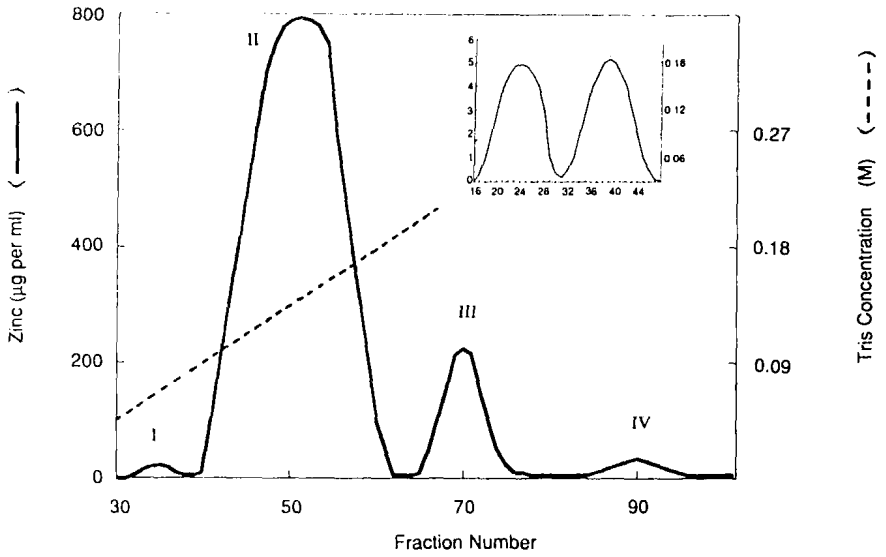

FIG. 1. Chromatography of the bovine or ovine hepatic metallothionein preparation from the gel-filtration step on DEAE-Sephacel. The inset is the pattern from a rat preparation.

bovine preparations. There is one predominant MT species, with up to three minor ones. For comparison, the pattern for rat liver is shown in the inset (Fig. 1), indicating a major difference between these two species of animals. The number of MT species in tissues of various animals has been discussed.[2,6]

The individual MT fractions are combined, dialyzed, and rechromatographed on DEAE-Sephacel columns (1.5 × 20 cm) with a salt gradient of 0.003 *M* (160 ml) to 0.3 *M* (160 ml) Tris-HCl buffer, pH 8.4, at a flow rate of 26 ml/hr, collecting 5 ml/fraction. The individual fractions are combined, dialyzed against distilled water, and freeze dried. The freeze-dried samples are dissolved in 0.05 *M* Tris-HCl, pH 8.4, and then chromatographed in this same buffer on BioGel P-10 (Bio-Rad Laboratories, Richmond, CA) columns (2.0 × 60 cm) at a flow rate of 11 ml/hr with collection of 3.5-ml fractions. All these chromatographic procedures are conducted at 4°. The resulting purified MT preparations can then be used for metal and amino acid analyses. In addition to zinc, the absorbance at 240 nm can also be monitored to follow the purification of MT.

Each of these preparations yields one band on disc gel electrophoresis.

[6] P. D. Whanger, S.-H. Oh, and J. T. Deagen, *J. Nutr.* **111**, 1207 (1981).

The amino acid analyses on the hydrolyzed MT preparations with an amino acid analyzer give the amino acid content expected for MT.[6] The cysteine content ranges from 20.4 to 35.7% for the various MTs in bovine tissues and from 27.3 to 35.6% for the various MTs in ovine tissues. The lower cysteine content is found in the minor MT species (I, III, and IV; Fig. 1). The minor MT species appear to have a low content of aromatic amino acids.

A procedure was developed in the author's laboratory using hydroxylapatite after the first DEAE-Sephacel step to obtain pure MT from rat tissues.[7] However, this method could not be used for the minor MT species (I, II, and IV; Fig. 1) from bovine or ovine tissues, and it was suggested that their amino acid content may account for this behavior.[6] This hydroxylapatite step, however, could be used for the major MT species (II; Fig. 1) from both ovine and bovine tissues.

The gram atoms of zinc per mole MT ranged from 0.5 to 6.4 for the various preparations from bovine tissues and from 2.0 to 6.3 for the various preparations from ovine tissues. Again, the lower zinc content was present in the minor MT species (I, II, and IV; Fig. 1).

Metals in Metallothioneins

When animals are fed zinc in the diet, this is essentially the only metal present in MT. There are traces of copper but never on a molar basis to MT.

When cadmium is included in the diet, this metal is also incorporated into MT, but not at the exclusion of zinc. The accumulation of cadmium in MT will also cause zinc to accumulate in MT.[8] Thus, it is impossible to biologically incorporate only cadmium into tissue MT. Of course, this can be done easily by *in vitro* methods.[3]

It is impossible to incorporate only copper biologically into MT unless there is an inducer for MT. This is apparently because copper is a poor inducer of MT, at least in the ovine and bovine species. One way to incorporate copper into MT has been to elevate the zinc status of cattle,[9] as demonstrated in Fig. 2. When only elevated copper was included in the diet, this metal accumulated with high-molecular-weight proteins (void volume of Sephadex G-75) in tissues. However, when elevated zinc was also included in the diet, copper instead accumulated with MT. This pattern was also demonstrated for the kidneys and pancreas. The molar

[7] R. W. Chen and P. D. Whanger, *Biochem. Med.* **24**, 71 (1980).
[8] J. T. Deagen, S.-H. Oh, and P. D. Whanger, *Biol. Trace Elem. Res.* **2**, 65 (1980).
[9] P. D. Whanger and J. T. Deagen, *Biol. Trace Elem. Res.* **28**, 69 (1990).

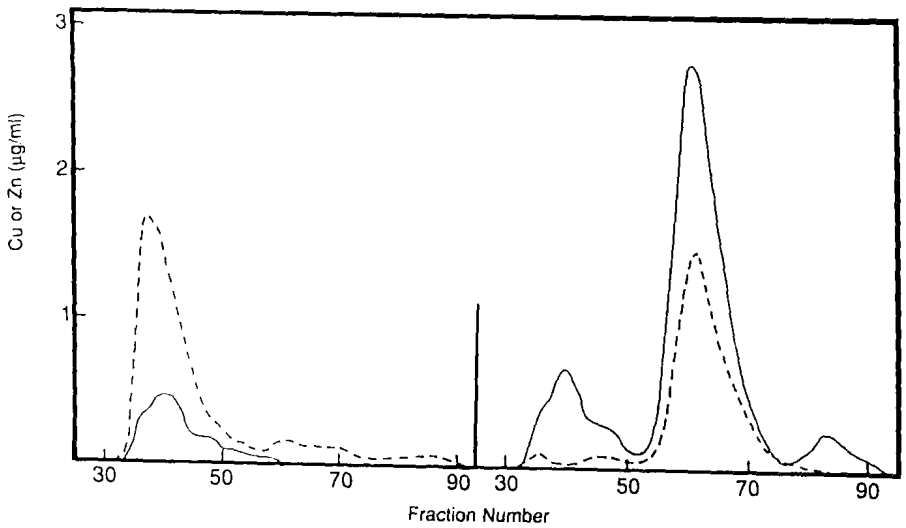

FIG. 2. Gel filtration of hepatic cytosols from cattle fed elevated copper (left) in the diet or copper plus zinc (right). (—), Zinc; (---), copper.

ratios of copper and zinc in MT vary with the relative amounts of these metals in the diet. However, it is impossible to biologically obtain MT with only copper because zinc will also be present. In order to obtain MT with only copper, this has to be done *in vitro* by metal substitution similar to that used for cadmium.

Extra precaution must be taken in the purification of MTs containing copper because the cysteine in copper-MT is susceptible to oxidation.[10] Measures must be taken to avoid this problem. Also, the gels should be treated to reduce the interaction with copper during purification of the copper-MT. Treatment with sodium borohydride appears to be the simplest method that has been used.[11] Although this was not done in the above procedures for purification of MT from animals fed elevated zinc in the diet, it is probably prudent to use borohydride-treated gels for this isolation.

Half-Life of Metallothionein

The degree of saturation of MT with metals influences its turnover.[12] The more metal-saturated MTs have a longer half-life than the less saturated ones. Also, the type of metal influences this half-life. Cadmium-MT has a longer half-life than when zinc is the predominant metal bound to it.[12] The half-life of MT in bovine and ovine tissues is markedly longer than that in tissues of rats or chicks.[13] The half-life of MT in ovine and bovine livers was found to be 22 to 24 days, whereas in rats and chickens it ranged from 10 to 65 hr, depending on the zinc status of the animals.[13] The half-life was determined in sheep and cattle MT from the zinc turnover, and is assumed to be valid because the turnover of zinc parallels that of the protein moiety in adults animals.[14] Adult sheep and cattle were used in these studies.

[10] U. Weser, H.-J. Hartmann, A. Fretzdorff, and G.-J. Strobel, *Biochim. Biophys. Acta* **493**, 464 (1977).
[11] B. Lonnerdal and T. Laas, *Anal. Biochem.* **72**, 527 (1976).
[12] P. D. Whanger, J. W. Ridlington, and C. L. Holcomb, *Ann. N.Y. Acad. Sci.* **355**, 336 (1980).
[13] P. D. Whanger and J. W. Ridlington, *in* "Biological Roles of Metallothionein" (E. C. Foulkes, ed.), p. 263. Elsevier/North-Holland, New York, 1982.
[14] S.-H. Oh and P. D. Whanger, *Am. J. Physiol.* **237**, E18 (1979).

[43] Metallothioneins and Other Zinc-Binding Proteins in Brain

By M. EBADI

Introduction

With the exception of calcium and magnesium, zinc is the most abundant cation in the brain. In addition, the distribution of zinc in the brain is nonuniform and its concentration is highest in the cerebellum, hippocampus, retina, and the pineal gland. Furthermore, the mammalian hippocampi not only contain high concentrations of zinc, but also exhibit subregional variation in this essential element, with concentration being highest in the hilar region and lowest in the fimbria.

In addition to protein-bound zinc designated as "structural pool of zinc" and to enzyme-bound zinc designated as "metabolic pool of zinc," the brain contains a unique synaptosomal pool of zinc designated as "vesicular pool of zinc." This latter and restricted pool of zinc is found in zinc-containing neurons of the limbic, cerebrocortical, and corticifugal systems.[1-3]

[1] C. J. Frederickson and G. Danscher, *in* "Nutritional Modulation of Brain Function" (J. E. Morley, M. B. Sterman, and J. H. Walsh, eds.), p. 289. Academic Press, San Diego, 1988.
[2] J. Perez-Clausell, *J. Comp. Neurol.* **267**, 153 (1988).
[3] C. J. Frederickson, *Int. Rev. Neurobiol.* **31**, 145 (1989).

The concentration of zinc in the gray matter is 0.15–0.2 m*M*, which is significantly higher than the concentration of acetylcholine, a classical transmitter, or than the concentrations of enkephalin or cholecystokinin, two peptide transmitters. Therefore, in addition to its established roles with zinc metalloenzymes,[4] it has been proposed that zinc participates in neurosecretion, neuromodulation, and neurotransmission. For example, zinc participates in the synthesis of γ-aminobutyric acid and in the action of glutamate receptor, two widely utilized inhibitory and excitatory transmitters in the brain.[5]

All living organisms have developed efficient mechanisms to utilize essential trace elements such as zinc and copper in expressing their biological functions, and to minimize the cytotoxicity of nonessential posttransition metal ions such as cadmium and barium. The ability to bind excess metals and to limit concentration of "free" ions is achieved mostly by cysteine-rich polypeptides such as γ-glutamyl peptide (phytochelatin) found in plants, and metallothioneins found in animals.[6] The brain regions contain metallothioneins and other low-molecular-weight zinc-binding proteins of unknown nature. The biochemical properties of neuronal metallothioneins seem to be similar to those of metallothioneins found in nonneuronal tissues. However, the regulation of the synthesis and most probably the functions of neuronal metallothioneins appear to be different from those found in the peripheral tissues. In view of the fact that neither essential elements such as zinc and copper nor nonessential elements such as cadmium and barium traverse the brain readily and rapidly, it is very doubtful that the brain metallothioneins play major roles in acute metal detoxification—a task that is carried out efficiently by the hepatic and the renal metallothioneins. On the other hand, a complex structure such as brain, possessing an estimated 30,000 mRNAs and having one-third of the mammalian genome exclusively dedicated to its function,[7] may have developed unique processes for transporting, compartmenting, releasing, and utilizing zinc, copper, calcium, and other essential elements, commensurate with the diversified and vital functions endowed and invested in its various regions.

[4] B. L. Vallee and A. Galdes, *Adv. Enzymol.* **56,** 284 (1984).
[5] M. Ebadi, *in* "Selected Topics from Neurochemistry" (N. N. Osborne, ed.), p. 341. Pergamon, London, 1985.
[6] J. H. R. Kägi and Y. Kojima, *Experientia, Suppl.* **52,** 25 (1987).
[7] J. G. Sutcliffe, *Annu. Rev. Neurosci.* **11,** 157 (1988).

Classification of Zinc-Binding Proteins in Brain

Zinc-binding proteins in mammalian brains may be divided into three major categories of (1) zinc-metallothioneins, (2) zinc-metalloenzymes, and (3) zinc-containing structural proteins other than metalloenzymes or metallothioneins.

Neuronal Metallothioneins

Metallothioneins have been identified first in rat brain,[8-10] and later characterized further in monkey brain,[11] in bovine pineal gland,[12] retina,[13] cerebellum,[14] and hippocampus.[14,15] In addition, hippocampal neurons in primary culture,[16] retinoblastoma Y79 cell line,[17] and neuroblastoma IMR-32 cell line,[18] are all able to synthesize and express metallothionein isoforms.

Events Leading to Discovery of Metallothionein-Like Protein in Rat Brain

The discovery of a low-molecular-weight zinc-binding and zinc-inducible protein in the rat brain in our laboratory[8] and its designation as metallothionein-like protein[19] was a fortuitous observation that took place while attempting to comprehend the mechanism of convulsive seizures associated with Pick's disease, a rare neurological disorder, which allegedly results in higher than normal accumulation of zinc in the hippocampus, causing inhibition of γ-aminobutyric acid (GABA) transmission. In a study, Itoh and Ebadi[20] showed that the intracerebroventricular (icv) administration of zinc sulfate (0.3 μmol/10 μl) caused seizures that were

[8] M. Itoh, M. Ebadi, and S. Swanson, *J. Neurochem.* **44,** 823 (1983).
[9] M. Ebadi, *Biol. Trace Elem. Res.* **11,** 101 (1986).
[10] M. Ebadi, V. K. Paliwal, T. Takahashi, and P. L. Iversen, *in* "Metal Ion Homeostasis: Molecular Biology and Chemistry" (D. H. Hamer and D. R. Winge, eds.), p. 257. Alan R. Liss, New York, 1989.
[11] S. Gulati, V. K. Paliwal, M. Sharma, K. D. Gill, and R. Nath, *Toxicology* **45,** 53 (1987).
[12] A. Awad, P. Govitrapong, Y. Hama, M. Hegazy, and M. Ebadi, *J. Neural Transm.* **76,** 129 (1989).
[13] T. Takahashi, V. K. Paliwal, and M. Ebadi, *Neurochem. Int.* **13,** 525 (1988).
[14] S. M. Sato, J. M. Frazier, and A. M. Goldberg, *J. Neurosci.* **4,** 1662 and 1671 (1984).
[15] V. K. Paliwal and M. Ebadi, *Exp. Brain Res.* **75,** 477 (1989).
[16] P. Thakran, M. P. Leuschen, and M. Ebadi, *In Vivo* **3,** 191 (1989).
[17] C.-Z. Ou, T. E. Donnelly, and M. Ebadi, *Pharmacologist* **30,** 141 (1988).
[18] M. Ebadi, T. Takahashi, and P. Timmins, *Biol. Trace Elem. Res.* **22,** 233 (1989).
[19] M. Ebadi, *Fed. Proc.* **43,** 3317 (1984).
[20] M. Itoh and M. Ebadi, *Neurochem. Res.* **7,** 1287 (1982).

prevented by GABA, but not by other putative transmitters. During the course of our investigation some interesting observations were made. The administration of substantially larger doses of zinc sulfate (up to 100 mg/kg), either intravenously or intraperitoneally, did not cause convulsive seizures in rats. Since the convulsive dose of 0.3 μmol/10 μl of zinc sulfate was considerably lower than the concentrations of zinc found in 14 regions of rat brain,[21] we postulated that most of the zinc in the brain was bound and did not exist in "free" form. Based on these observations and conclusions, we searched for and identified a metallothionein-like protein in the rat brain.[8,10,19]

Induction of Rat Brain Metallothionein by Using Surgically Implanted Minipump

The intraperitoneal administration of zinc sulfate (7.5 mg/kg) given either in a single dose or daily for 10 days does not stimulate the synthesis of brain metallothionein.[9] On the other hand, the intracerebroventricular administration of zinc sulfate (0.20 μmol/μl/hr for 48 hr) not only induces the synthesis of brain metallothionein, but also induces the synthesis of hepatic metallothionein. The results of this study add further credence to a hypothesis that the steady state concentration of zinc in the brain is firmly regulated and is not disturbed readily. Indeed, unbound and "free" zinc in higher than physiological concentration is an extremely neurotoxic substance[22] with an inherent ability to inhibit an extensive number of sulfhydryl-containing enzymes (e.g., glutamic acid decarboxylase) and of sulfhydryl-containing receptor sites (e.g., glutamate and aspartate receptors).[23-25] Therefore, it is believed that the excess and unbound zinc most probably flows out from the brain via cerebrospinal fluid and reenters into plasma by transport through the choroid plexus, which lies outside of the blood–brain barrier.

Since the intraperitoneal administration of zinc sulfate does not stimulate the synthesis of brain metallothionein, this protein is induced by administering zinc sulfate intracerebroventricularly using a surgically implanted minipump (Alzet model 2M14; Alza Corp., Palo Alto, CA). In short, rats are anesthetized with sodium pentobarbital (35 mg/kg body

[21] M. Ebadi, M. Itoh, J. Bifano, K. Wendt, and A. Earle, *Int. J. Biochem.* **13,** 1107 (1981).
[22] M. Yokoyama, H. Koh, and D. W. Choi, *Neurosci. Lett.* **71,** 351 (1986).
[23] M. Ebadi, R. J. White, and S. Swanson, *Neurol. Neurobiol.* **11A,** 39 (1984).
[24] M. Ebadi and R. Pfeiffer, *Neurol. Neurobiol.* **11B,** 307 (1984).
[25] M. Ebadi and Y. Hama, *Adv. Exp. Med. Biol.* **203,** 557 (1986).

weight). The dorsal surface of the head is shaved, and a midline incision is made through the skin and underlying periosteum of the skull. The skin and periosteum are reflected and held in place with hemostats. A small hole is drilled with a dental drill approximately 2 mm posterior to the coronal suture and 3 mm lateral to the midline. The hole should be slightly larger than a 21-gauge hypodermic needle. A second hole of the same size is drilled into the dorsal surface of the skull 3 to 4 mm posterior to the first hole and 3 mm lateral to the midline. A small stainless steel machine screw is threaded into the second hole and screwed into the skull so that the head of the screw is 1.5 mm above the surface of the skull.

A 21-gauge hypodermic needle is gently squeezed in a pair of wire cutters to prepare a 15-mm length of hypodermic tubing. The tip of the needle is bent, allowing it to break off without crimping the needle. A second area of the needle is scored in a similar manner 15 mm below the broken end so that the length of the hypodermic tubing is 15 mm. This hypodermic tubing is then held in the flame of a gas burner, and the temper of the steel is changed so that the softened metal can be bent without cracking. The tubing is placed in a vise and a right angle bend made in it. One end of the angle will be 2.5 mm in length while the other will be 12.5 mm.

A short piece of plastic tubing is fitted over the long end of the hypodermic tubing, and the plastic tubing and hypodermic tubing are filled with saline (control group) or zinc sulfate (test group) by means of a syringe. The 2.5-mm end of the hypodermic tubing is placed in the remaining hole in the dorsal surface of the skull of the rat. As mentioned, this hole is 2 mm posterior to the coronal suture and 2 mm lateral to the midline. If properly placed, the end of the hypodermic tubing should rest in the lateral ventricle.[26] The long end of the hypodermic tubing is placed underneath the projecting head of the machine screw and secured in place with Durelon carboxylate cement (Espe GmbH, Seefeld/Oberbayern, Germany). The dental cement is allowed to dry thoroughly, and a subcutaneous tunnel is made between the scapulae of the rat with a blunt forceps. The minipump is then inserted into the subcutaneous pocket that had been made between the scapulae, and the wound is closed with silk sutures.

In addition to implanting a single pump for delivery of zinc sulfate (test) or saline (control), a dual pump system may be implanted, wherein one pump may deliver zinc sulfate and the second pump may deliver [^{35}S]cysteine; or one pump may deliver zinc sulfate and the second pump may deliver a protein synthesis inhibitor such as actinomycin D.

[26] E. P. Nobel, R. J. Wurtman, and J. Axelrod, *Life Sci.* **6,** 281 (1967).

Synthesis of Metallothionein in Hippocampal Neurons in Primary Culture

The metallothionein synthesis has been studied in newborn (7–8 days old) rat hippocampal neurons in primary culture.[16]

Tissue Preparation and Cell Culture Conditions. The animals are killed by decapitation and the brains quickly and aseptically removed into sterile Petri dishes containing Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (GIBCO, Grand Island, NY) with 100 U/ml penicillin and 100 μg/ml streptomycin. The hippocampi are dissected free of meningeal vessels and cut into explants of 1-mm diameter. Hippocampal explants from rat pups are plated in 60-mm Permanox Lux dishes (NUNC, Inc., Naperville, IL) that have been coated previously with a solution containing carbodiimide (130 μg/ml) and collagen (500 μg/ml) (Vitrogen-Flow) according to the protocol of Macklis *et al.*[27] Explants from adult rats are plated in similar dishes treated with poly (L-lysine) (Sigma, St. Louis, MO) according to Pettman *et al.*[28] Cultures are fed twice a day during the first week with 9 drops (330 μl) of Iscove's modification of Dulbecco's MEM (IMDM), containing 40.3 ml/liter $NaHCO_3$, 30 m*M* D-glucose, 293.2 mg/L-glutamine, 24.5 m*M* KCl, 100 mU/liter insulin, 7 μM *p*-aminobenzoic acid, 100 μg/ml transferrin, 10 mg/ml bovine serum albumin (BSA), 10^{-12} *M* β-estradiol, 100 g/ml gentamicin, and 3 μg/ml Fungizone, completely removing the spent medium from the dishes before feeding.[29] Thereafter, cultures are fed every other day with 770 μl of fresh medium, following the same procedure. Cultures are equilibrated with 90% O_2–10% CO_2 (v/v) in a humid incubator at 37° and monitored daily for survival and growth using a Leitz phase-contrast microscope (American Optical Corp., Buffalo, NY).

Staining Procedure. Neurons and their processes are identified by direct histochemical staining for cholinesterase according to the method of Karnovsky and Roots.[30] Briefly, the presence of cholinesterase is detected by its ability to hydrolyze the thiocholine ester used as substrate. The thiocholine thus liberated reduces ferricyanide to ferrocyanide. The latter compound combines with Cu^{2+} to form the insoluble copper ferrocyanide (Hatchett's Brown). On the tenth day *in vitro,* cultures derived from rat hippocampi originally plated on poly(L-lysine) are exposed to various concentrations of zinc and incubated with [^{35}S]cysteine in order to ascertain its incorporation into metallothionein. Spent medium is aspirated off

[27] J. D. Macklis, R. L. Sidman, and H. D. Shine, *In Vitro* **21,** 189 (1985).
[28] B. Pettman, T. C. Lewis, and M. Sensenbrenner, *Nature (London)* **281,** 378 (1979).
[29] V. Silani, G. Pezzoli, E. Motti, A. Falini, A. Pizzuti, C. Ferrante, A. Zecchinelli, F. Marossero, and G. Scarlato, *Appl. Neurophysiol.* **51,** 10 (1988).
[30] M. J. Karnovsky and L. Roots, *J. Histochem. Cytochem.* **12,** 219 (1964).

and fresh, unsupplemented medium containing a different concentration of zinc plus 10 μCi [^{35}S]cysteine/ml is added to the dishes. Control dishes do not receive zinc. In preliminary experiments, incubation was carried out for 72 hr, but subsequently the medium was aspirated off after 48 hr and the cultures were rinsed three times with 1 ml of Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). The cells are harvested with a rubber policeman and washed twice with PBS at 4°. Cell extracts are prepared by freezing and thawing followed by sonication. An aliquot is taken for protein determination and the remaining suspension is centrifuged at 13,600 *g* for 3 min in a Fisher (Pittsburgh, PA) microcentrifuge (model 235B).

Aliquots of the resulting supernatant are chromatographed on a Sephadex G-75 column (56 × 0.9 cm) using 10 m*M* Tris-HCl, pH 7.5, containing 1 m*M* dithiothreitol as the eluent.[9] The radioactivity of each fraction is determined in a Packard (Downers Grove, IL) liquid scintillation counter (model Tri-Carb 4530). In order to verify the authenticity of the metallothionein peaks isolated from the hippocampal primary cultures by gel-filtration chromatography, zinc-induced hepatic metallothionein is used as a marker protein according to earlier studies conducted in our laboratory.[8,9]

Zinc sulfate at a concentration of 1×10^{-9} to 1×10^{-6} *M* stimulated the incorporation of [^{35}S]cysteine and the synthesis of metallothionein in a time-dependent fashion and the maximum effects were seen 48 hr after incubation with zinc.[16]

Synthesis of Metallothionein in Neuroblastoma IMR-32 Cell Line in Culture: Lack of Induction by Dexamethasone

Although neuroblastoma cells are of tumor origin, they possess several features in common with neurons,[31-33] thus making them a useful model to study the effects of substances on the nervous system. The Chang liver cells may be used as control.

Human neuroblastoma IMR-32 cells are cultured in Eagle's minimum essential medium with nonessential amino acids containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37° in a humid atmosphere of 5% (v/v) CO_2 in air. Semiconfluent plates (2×10^6 cells/4 ml of culture medium/60 mm in diameter) of neuroblastoma or Chang cells are used in all experiments.

After aspiration of the culture media of the semiconfluent plates, fresh culture medium plus 5 μCi [^{35}S]cysteine/ml, 1 μM zinc or cadmium plus 5 μCi [^{35}S]cysteine/ml, or 2.5–100 μM dexamethasone plus 5 μCi [^{35}S]cys-

[31] G. Augusti-Tocco and G. Sato, *Proc. Natl. Acad. Sci. U.S.A.* **64,** 311 (1969).
[32] K. N. Prasad, *Biol. Rev.* **50,** 129 (1975).
[33] S. C. Haffke and N. W. Seeds, *Life Sci.* **16,** 1649 (1976).

teine/ml, is added to each semiconfluent plate. The control plates do not receive cadmium, zinc, or dexamethasone, but otherwise receive identical treatments. After 24 hr of incubation, the medium is aspirated and the cells are rinsed with 1 ml phosphate buffer solution without Ca^{2+} and Mg^{2+} [PBS(−)], followed by treatment with 1 ml 0.05% (w/v) trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA, v/v) solution. After addition of

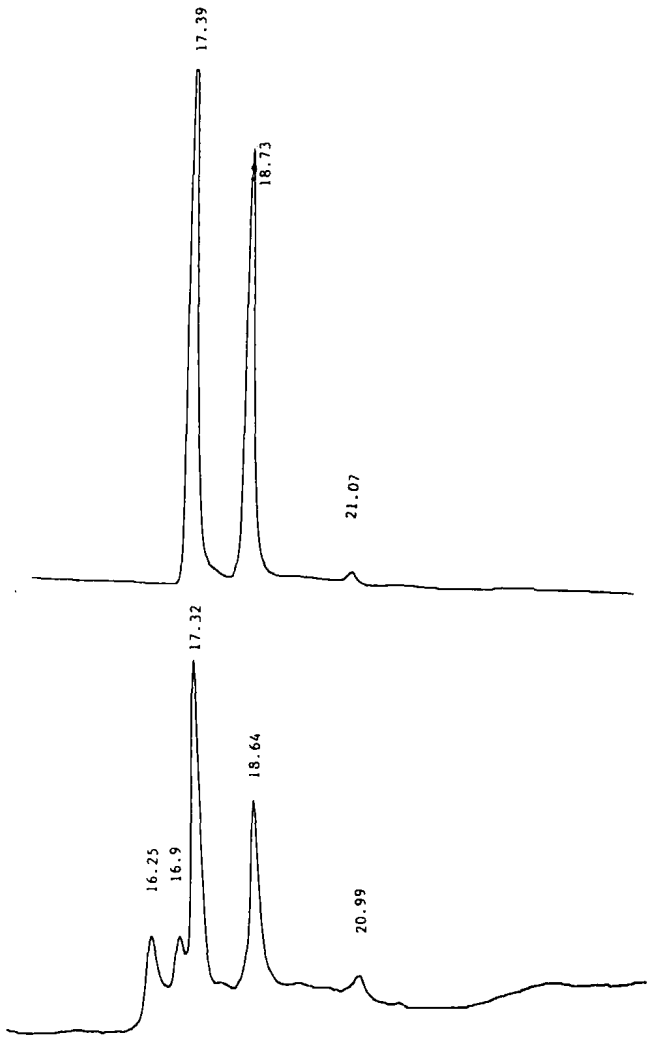

FIG. 1. Comparative high-performance liquid chromatography (HPLC) profiles of hepatic metallothionein (top) and brain metallothionein-like protein (bottom). One group of rats was administered intraperitoneally with 7.5 mg/kg of $ZnSO_4$ and killed 18 hr thereafter. The

1 ml of ice-cold PBS(−), the cells are harvested by pipetting, and the collected cells are washed with ice-cold PBS(−) twice and resuspended in 1 ml of 10 m*M* Tris-HCl, pH 7.5, containing 1 m*M* dithiothreitol. The cell extracts are prepared by freezing and thawing, followed by sonication. The extracts are centrifuged at 13,600 *g* for 3 min in a Fisher microcentrifuge (model 235B).

Aliquots of the resulting supernatant are chromatographed on a Sephadex G-75 column (0.9 × 56 cm) using 10 m*M* Tris-HCl, pH 7.5, containing 1 m*M* dithiothreitol as the eluant, as described by Ebadi.[9] The radioactivity of each fraction is determined in a Packard liquid scintillation counter (model TRI-CARB 4530).

The control Chang cells or the human neuroblastoma IMR-32 cells, untreated with either metals or glucocorticoid hormone, are able to incorporate [^{35}S]cysteine slightly (250–900 cpm × 10^{-3}). The incubation of Chang liver cells with 100 μM zinc for 24 hr stimulated 6.7-fold over the control value the incorporation of [^{35}S]cysteine into metallothionein over the control group, whereas the incubation of neuroblastoma cells with an identical amount of zinc, under identical conditions, stimulated the synthesis of metallothionein only 2.3-fold over the control value. The incubation of Chang liver cells with 1 μM cadmium for 24 hr stimulated 5.0-fold over the control value the incorporation of [^{35}S]cysteine into metallothionein; whereas the incubation of neuroblastoma cells with an identical amount of cadmium, under identical conditions, stimulated the synthesis of metallothionein only 2.25-fold over the control value. As expected, 10 μM dexamethasone induced the synthesis of metallothionein in the Chang cells, whereas dexamethasone in concentrations ranging from 2.5 to 100 μM had no significant effects on the same parameter in the neuroblastoma cells. The minimum tested concentration of cadmium stimulating the synthesis of metallothionein in the Chang liver cells was 1 μM, and 7.5 μM caused cell death. The minimum tested concentration of zinc stimulating the synthesis of metallothionein was 100 μM in the neuroblas-

second group of rats was administered $ZnSO_4$ intracerebroventricularly with 0.22 μmol/μl/hr/48 hr by Alzet minipumps and then decapitated. The zinc-binding proteins were determined by HPLC using the technique of Klauser *et al.*,[34] with only one modification of using 10 m*M* Tris-HCl buffer instead of 50 m*M* Tris, as recommended by Suzuki *et al.*[35] These studies have shown the adult rat brain contains three zinc-binding proteins. The intracerebroventricular (icv) administration of zinc stimulates the synthesis of only one of these proteins, producing metallothionein-like protein isoforms 1 and 2, with retention times of 17.32 and 18.64 min, respectively. The synthesis of the other two zinc-binding proteins, with retention times of approximately 16.25 and 16.9 min, are not stimulated by intracerebroventricular administration of zinc. (Data from Ebadi.[9])

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