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Simultaneous induction of Pb-metallothionein-like protein and Zn-thionein in the liver of rats given lead acetate

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Administration of a sublethal dose of lead acetate to rats induced the simultaneous synthesis of a Pb-metallothionein (Pb-MT)-like protein (Pb-BP) and Zn-thionein (Zn-BP) in the liver. The Pb-BP had an apparent molecule mass of 6900 Da and seemed to bind preferentially to lead in the liver cytosol. The Zn-BP was identified by comparison of the M_r, elution profiles from Sephadex G-75 and DEAE-Sephadex A-25 columns, and polyacrylamide-gel-electrophoretic mobility, with those of rat liver Zn-MT-II. The Pb-BP accumulated in the liver to a maximum 6 h after the intraperitoneal injection of lead acetate and accounted for about 60% of the lead in the liver cytosol at this stage. However, after that, it gradually decreased in the liver, until it was close to the basal amount 24 h after the induction. In contrast, the amount of Zn-MT increased gradually, reached a maximum 12 h after the administration of lead acetate and maintained a constant value until at least 24 h after the induction. Amino acid analysis of the Pb-BP indicated that it contained about 28% half-cysteine. These results strongly suggest that lead acetate induces the synthesis of Pb-MT as well as Zn-MT in rat liver.

INTRODUCTION

Heavy metals have become widely prevalent environmental pollutants, and an understanding of their effects on biological systems is important. However, information on the chemical nature of lead in the tissues of the animal body and on the molecular aspects of lead-binding proteins, which is necessary for a better understanding of the essential steps of the biological effects of this element, is currently lacking.

It has been proposed that metallothioneins, which are low-M_r, cysteine-rich, metal-binding proteins, are involved in heavy-metal detoxification (Nordberg *et al.*, 1972; Webb, 1972; Andersen *et al.*, 1978). Synthesis of metallothioneins is induced in the liver, kidney and other tissues when animals are treated with sublethal doses of metal salts (Shaikh & Lucis, 1971; Winge & Rajagopalan, 1972; Piotrowski *et al.*, 1974; Winge *et al.*, 1975; Sokolowski & Weser, 1975; Bremner & Davies, 1975; Bremner & Young, 1976; Cherian & Goyer, 1978; Zelazowski & Piotrowski, 1980). Administration of lead compounds to rats has been reported to cause the appearance in the liver cytosol of a Zn-binding protein which was co-eluted with Zn-thionein (Zn-MT) on size-exclusion chromatography (Suzuki & Yoshikawa, 1976; Arizono *et al.*, 1982). Ulmer & Vallee (1969) have indicated that proteins with large number of free thiol groups, e.g. thionein, bound lead firmly both *in vitro* and *in vivo*. However, the induction of synthesis of Pb-metallothionein (Pb-MT) in response to lead-salt administration has not been observed.

In the present paper, we deal with the inductive synthesis of a Pb-metallothionein-like protein (Pb-BP) as well as Zn-MT in the liver of rats given a sublethal dose of lead acetate.

MATERIALS AND METHODS

Animals and chemical reagents

Male Wistar rats (obtained from the Shizuoka Laboratory Animal Center, Shizuoka, Japan) weighing about 240 g were used in all experiments. The animals were housed in stainless-steel cages with food and water *ad libitum* under laboratory conditions. Zn-MT fraction was obtained by gel filtration on a Sephadex G-75 column of the liver cytosol of rats 24 h after the injection of zinc acetate, by the method of Cherian (1974). Purified Zn-MT-I and -II were isolated from the Zn-MT fraction by DEAE-Sephadex A-25 column chromatography (Cherian, 1974). $^{210}Pb(NO_3)_2$ (13 mCi/mmol) and [^{35}S]cysteine (1039 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). Sephadex G-75 and DEAE-Sephadex A-25 were purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Actinomycin D was from Makor Chem (Jerusalem, Israel). All other chemicals were of analytical grade and were purchased from regular commercial sources.

Treatment of rats with lead acetate and the preparation of liver cytosol

Rats were intraperitoneally injected with 24 μmol of lead acetate/100 g body wt., and killed by cervical dislocation at the time indicated. The liver was immediately excised and homogenized in 4 vol. of ice-cold 0.25 M-sucrose in 10 mM-Tris/acetate (pH 8.2) with a glass/Teflon motor-driven Potter–Elvehjem-type homogenizer. The homogenate was centrifuged at 15000 *g* for 20 min, and the supernatant was again centrifuged at 105000 *g* for 60 min at 4 °C with a Hitachi ultracentrifuge model 70P-72. The supernatant solution was applied

Abbreviations used: MT, metallothionein.

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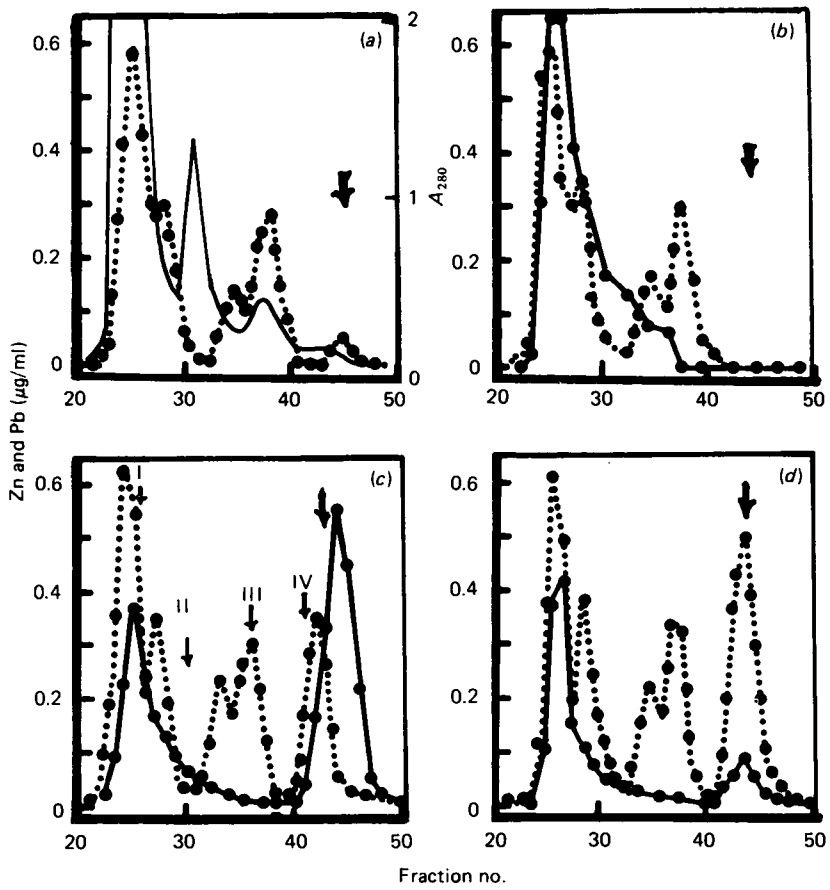

Fig. 1. Sephadex G-75 column chromatography of liver cytosol from rats intraperitoneally injected with 24 μmol of lead acetate/100 g body wt.

Liver cytosol was prepared at (*a*) 0, (*b*) 2, (*c*) 6 or (*d*) 24 h after the administration of lead acetate. Untreated rats were used for the preparation of the cytosol at 0 h. Cytosol (4 ml) was applied to a Sephadex G-75 column (45 cm × 2.5 cm) equilibrated with 5 mM-Tris/acetate (pH 8.2) containing 0.02% NaN_3. Arrows indicate the location of the authentic Zn-MT-II eluted from the same column. Flow rate, 10 ml/h; fraction volume, 3.5 ml. M_r standards are designated as: I, bovine serum albumin (67000); II, ovalbumin (46000); III, soya-bean trypsin inhibitor (24000); IV, cytochrome *c* (12500). ——, A_{280}; —●—, Pb; ··●··, Zn.

to a Sephadex G-75 column as described below. The supernatant could be stored at −20 °C for at least 1 month without changing the chromatographic pattern.

When experiments were carried out with radioactive materials, $^{210}Pb(NO_3)_2$ (2 μCi/100 g body wt.) or [^{35}S]cysteine (17 μCi/100 g body wt.) was intravenously injected into rats 2 h before excision of the liver, and the liver cytosol was prepared as described above. The radioactivity of ^{210}Pb in polyacrylamide gels was measured as described previously (Ikebuchi & Kametani, 1977).

Gel filtration and ion-exchange chromatography

Gel filtration was carried out on a Sephadex G-75 column equilibrated with 10 mM-Tris/acetate (pH 8.2) at 4 °C. The column was eluted with the same buffer. Ion-exchange column chromatography was carried out with a DEAE-Sephadex A-25 column, equilibrated with 5 mM-Tris/acetate (pH 8.2), eluted by the method of Bremner & Davies (1975) with a linear gradient of the buffer concentration.

Lead and zinc in the fractions were measured by flame atomic absorption with a Hitachi model 180–40 spectrometer.

Polyacrylamide-gel electrophoresis

This was performed by the method of Davis (1964), with 7.5%-acrylamide gels. If necessary, gels were stained with Coomassie Brilliant Blue R-250.

Amino acid analysis of the Pb-BP

Amino acid analysis of the Pb-BP and Zn-MT was performed as follows. Proteins in the Pb-BP fraction were separated by slab-gel electrophoresis as described above. After the gel was stained with Coomassie Brilliant Blue R-250, protein bands corresponding to the Pb-BP and Zn-MT were cut out and eluted electrophoretically from the gel as described previously (Suzuki *et al.*, 1985). The eluted proteins were oxidized with performic acid (Moore, 1963) and hydrolysed with 6 M-HCl for 24 h at 110 °C. The amino acid analysis of the hydrolysate was performed with an amino acid analyser (Hitachi 835).

Estimation of M_r of the Pb-BP

The M_r of the Pb-BP was determined as described by Andrews (1965), on a Sephadex G-75 column (80 cm × 5 cm) eluted with 10 mM-Tris/acetate (pH 8.2). The following proteins and peptides were used as M_r standards; bovine serum albumin (67000), ovalbumin

(46000), soya-bean trypsin inhibitor (24000), cytochrome *c* (12500) and bacitracin (1420).

RESULTS

Induction of synthesis of Pb-binding macromolecules in the liver of rats given lead acetate

To determine whether or not an intraperitoneal injection of lead acetate into male rats induces the synthesis of Pb-binding components, especially proteins with M_r similar to that of metallothioneins, the liver cytosols from rats treated with lead acetate were analysed by gel filtration on a Sephadex G-75 column. Typical chromatographic patterns are shown in Fig. 1. As seen in Fig. 1(*a*), the liver cytosol from rats not treated with lead acetate contained no detectable lead, and almost all the zinc in the cytosol was present in a form bound to macromolecules. The chromatographic pattern of the liver cytosol 2 h after lead treatment of the rats was essentially the same as that of the cytosol from the untreated rats (Fig. 1*b*) when A_{280} and zinc concentration were measured. However, when the fractions were analysed for lead, a Pb-containing peak was detected in the high-M_r region (more than 25000; Fig. 1*b*). At 6 h after the treatment, new peaks appeared between fractions 41 and 47 (Fig. 1*c*). These peaks were barely detectable by their A_{280} and were located only by measurement of zinc and lead by atomic absorption analysis. Since free Zn^{2+} and Pb^{2+} were eluted from this column between fractions 60 and 80, it is evident that the zinc and lead present in these peaks were complexed to some biological macromolecules. However, the Pb-bound component disappeared from the cytosol 24 h after the treatment, and only the Zn-bound component was clearly detected (Fig. 1*d*).

An apparent M_r of the Zn-bound component (Zn-BP) was about 10000 and that of the Pb-bound component (Pb-BP) was 6900, calculated from the elution positions of the standard proteins run on the same column. It is likely that these two components behave somewhat abnormally on gel filtration and give slightly larger M_r values. Therefore their exact M_r values are not known at present.

To confirm that the newly synthesized components are protein in nature, the Pb- and Zn-bound fractions were examined for incorporation of [^{35}S]cysteine. As shown in Fig. 2(*b*), the Pb-BP and Zn-BP were labelled with [^{35}S]cysteine. In contrast, only a small amount of [^{35}S]cysteine was incorporated in this region in the liver of rats not treated with lead acetate (Fig. 2*a*). These results indicate that the Pb-BP and Zn-BP components are proteins and that their synthesis was induced by the administration of lead acetate to rats.

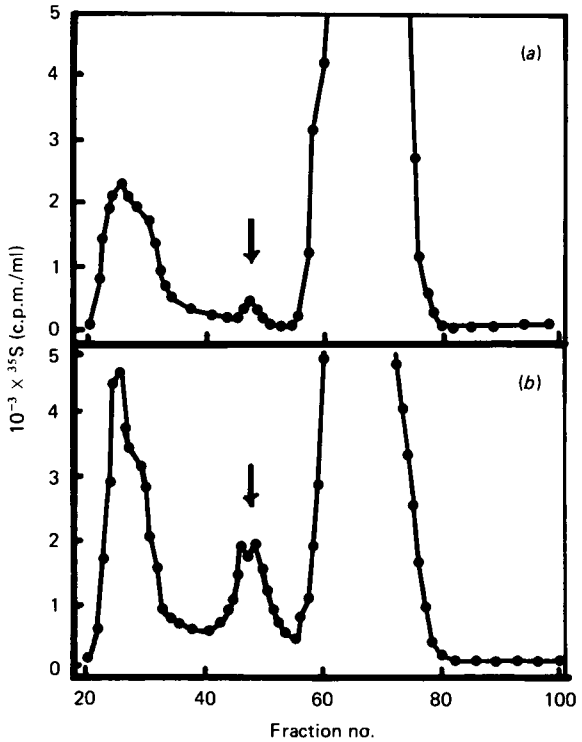

Fig. 2. Sephadex G-75 column chromatography of liver cytosol from lead acetate-treated (*b*) and untreated (*a*) rats intravenously injected with [^{35}S]cysteine 2 h before death

Liver cytosol was prepared at 6 h after the administration of lead acetate. The cytosol (4 ml) was applied to a Sephadex G-75 column (45 cm × 2.5 cm) equilibrated with 5 mM-Tris/acetate (pH 8.2) containing 0.02% NaN_3. Arrows indicate the location of the authentic Zn-MT-II from the same column. Flow rate, 10 ml/h; fraction volume, 3.5 ml.

Effect of actinomycin D on the synthesis of Pb-BP and Zn-BP

To provide evidence that these two proteins are produced by mRNA synthesis *de novo*, we investigated the effect of actinomycin D on the inductive synthesis of these proteins. Actinomycin D (0.8 mg/kg) was intraperitoneally injected 4 h before the administration of lead acetate (24 μmol/100 g body wt., intraperitoneally). At 6 h after lead acetate administration, liver cytosol was prepared as described in the Materials and methods section and the Pb-BP and Zn-BP were analysed by gel filtration. As shown in Fig. 3(*b*), actinomycin D treatment completely abolished the formation of both proteins.

Since the amount of actinomycin D used in our experiment is known to block the synthesis of mRNA in rats effectively (Drysdale & Munro, 1966), the present results suggest that the induction of formation of the Pb-BP and Zn-BP was due to the synthesis of the proteins *de novo* after the induction of mRNA synthesis by lead acetate.

Time-dependent changes in the amounts of Pb-BP and Zn-BP in the liver

It has been reported that the synthesis of hepatic metallothionein mRNA starts within 1 h of subcutaneous injection of cadmium and that the mRNA synthesis is accompanied by a high rate of synthesis of hepatic metallothioneins (Andersen & Weser, 1978; Durnam & Palmiter, 1981).

We therefore measured the time-dependent changes in the amounts of Pb-BP and Zn-BP after a single dose of lead acetate. The results are shown in Fig. 4. Synthesis of the Pb-BP started 2 h after the intraperitoneal administration of lead acetate, and the amount reached a maximum after 6 h and then decreased to close to the basal value by 24 h. In contrast, the amount of Zn-BP

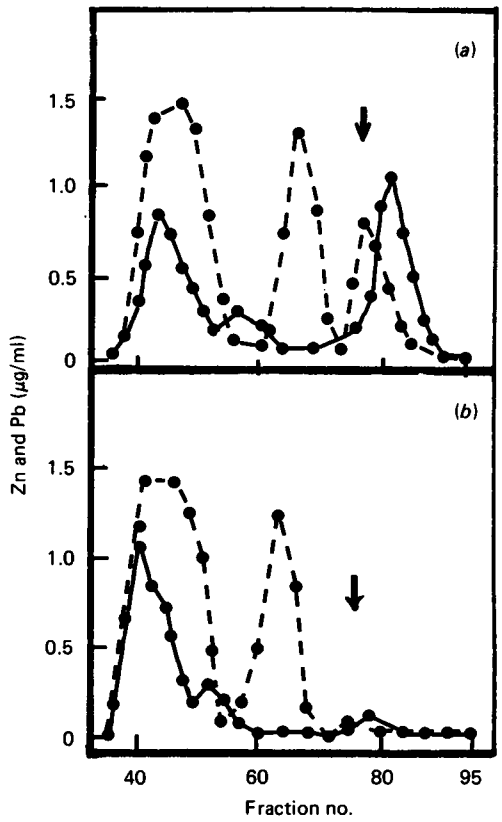

Fig. 3. Influence of the administration of actinomycin D on the synthesis of the Pb-BP and Zn-BP

Actinomycin D (0.8 mg/kg, intraperitoneally) was administered 4 h before the injection of lead acetate. Rats were killed 6 h after the administration of lead acetate. Chromatographic conditions were the same as those in Fig. 1. (*a*) Liver cytosol from rats not treated with actinomycin D; (*b*) liver cytosol from rats treated with actinomycin D. Arrows indicate the location of the authentic Zn-MT-II eluted from the same column. —●—, Pb; -●-, Zn

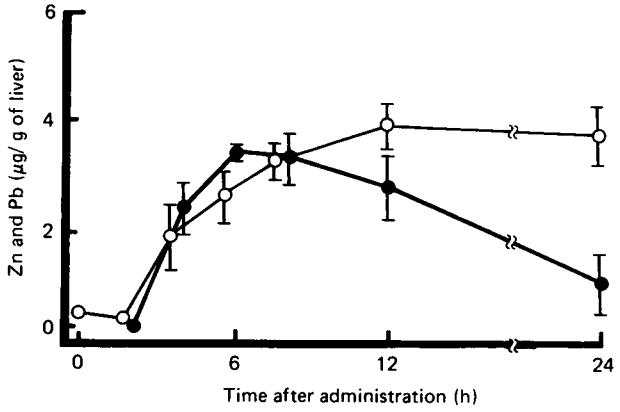

Fig. 4. Time-dependent changes in the amount of Pb-BP and Zn-BP in the liver cytosol from rats injected with lead acetate

The amounts of Pb-BP and Zn-BP in the cytosol were measured after their separation on a Sephadex G-75 column as described in Fig. 1. Each point represents the mean of five rats. ●, Pb; ○, Zn

increased gradually up to 12 h and maintained a constant value until at least 24 h after the injection. Thus it seems clear that the synthesis and degradation of these proteins occur at different rates in the rat liver.

Behaviour of the Pb-BP and Zn-BP on a DEAE-Sephadex A-25 column

For confirmation of the identity of the Zn-BP with Zn-MT and the development of a procedure for separating the Pb-BP and Zn-BP, the behaviour of the two proteins on a DEAE-Sephadex A-25 column was investigated. A typical chromatographic pattern is shown in Fig. 5(*a*). The Pb-BP was less tightly bound to the column and was easily separated from the Zn-BP.

The elution pattern of the Zn-BP coincided with that of the authentic Zn-MT-II from the same column (Fig. 5*b*). This suggests that the Zn-BP is identical with Zn-MT-II. Although a small Zn peak that was eluted between fractions 8 and 18 was not identified, this might be a component of Zn-MT.

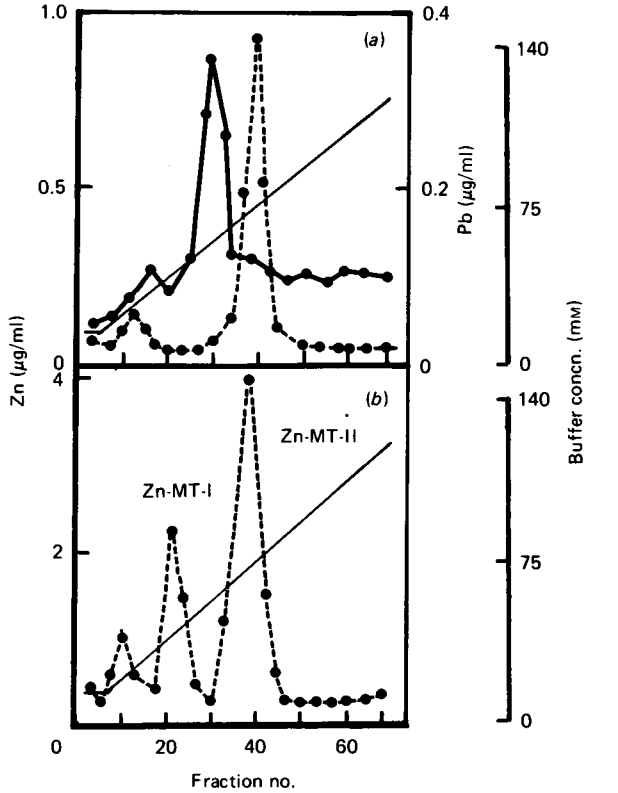

Fig. 5. Separation of the Pb-BP and Zn-BP on DEAE-Sephadex A-25

Liver cytosol was prepared at 6 h after the administration of lead acetate and applied to a Sephadex G-75 column as described in Fig. 1. (*a*) Fractions 40–50 in Fig. 1(*c*) were pooled and applied to a DEAE-Sephadex A-25 column (10 cm × 1 cm) equilibrated with 5 mM-Tris/acetate (pH 8.2). Elution was performed with a linear gradient (——) of 5–140 mM-Tris/acetate (pH 8.2). Flow rate, 10 ml/h; fraction volume, 5 ml. Total volume of the gradient was 400 ml. (*b*) Zn-thionein induced in 24 h in the liver of rats injected with zinc acetate was first fractionated as described in Fig. 1. Zn-thionein fractions were pooled and column chromatography was carried out under exactly the same conditions as in (*a*). —●—, Pb; -●-, Zn.

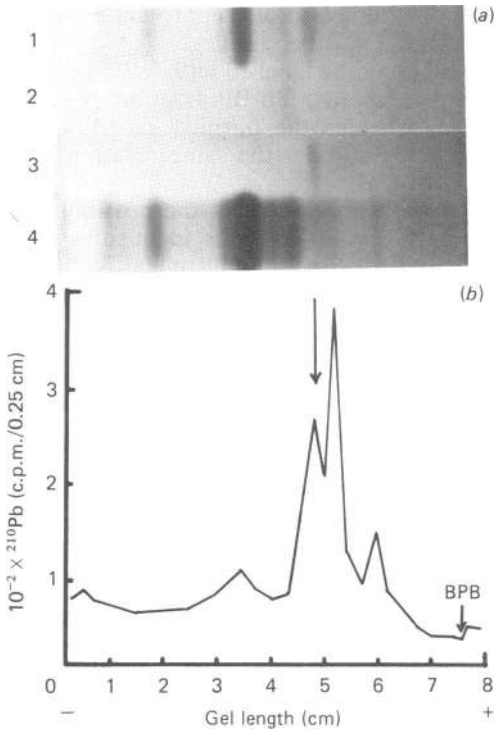

Fig. 6. Polyacrylamide-gel electrophoresis of the Pb-BP and Zn-BP

(*a*) Coomassie Brilliant Blue stain patterns. Lane 1, Zn-thionein fraction; lane 2, Zn-MT-I; lane 3, Zn-MT-II; lane 4, fractions 41–47 from Fig. 1(*c*). (*b*) The Pb-BP was detected by measurement of ^{210}Pb. The arrow indicates the location of the authentic Zn-MT-II run in the same gel. Abbreviation: BPB, Bromophenol Blue.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was carried out to study the behaviour of the Pb-BP and Zn-BP in gels. In this experiment, the Pb-BP was labelled with ^{210}Pb *in vivo* by intraperitoneal injection of $^{210}Pb(NO_3)_2$ 2 h after the induction as described in the Materials and methods section. After the run, the gel was cut into small pieces, 2.5 mm in width, and the radioactivity was measured. As shown in Fig. 6(*b*), three radioactive bands were detected. The major band (R_m 0.68) ran just ahead of the authentic Zn-MT-II. This finding is consistent with the results suggesting that the Pb-bound protein is a different molecular species from rat Zn-MT (Fig. 4). The second large peak (R_m 0.62) had the same mobility as that of rat Zn-MT-II. Therefore it is probably contaminating Zn-BP carrying a small amount of ^{210}Pb. The third band (R_m 0.81) was unidentified.

On the other hand, the Zn-BP had the same mobility as the authentic rat Zn-MT-II (Fig. 6*a*). This finding indicates that the Zn-BP is identical with rat Zn-MT-II.

Amino acid composition of the Pb-BP

The most typical characteristic of metallothioneins is their extremely high content of cysteine (about 30%). The amino acid composition of the Pb-BP is shown in the left column of Table 1 and that of the Zn-BP isolated from the same slab gel is indicated in the middle column. The results indicate that the Pb-BP is a metallothionein-type protein, with a characteristic cysteine content of about 28%. Another characteristic of metallothioneins is that they are lacking in aromatic amino acids. However, the Zn-BP isolated from the slab gel contained a small amount of phenylalanine. This indicates that our Zn-BP sample might have been contaminated with a small amount of other proteins and that phenylalanine was derived from these contaminants. Amino acid analysis of the Pb-BP also showed the presence of a small amount of phenylalanine. It is unclear at present whether or not phenylalanine is a component of the Pb-BP. However, one possibility is that we had not been able to obtain sufficiently pure Pb-BP by slab-gel electrophoresis and that the protein was, like Zn-BP, also contaminated with a small amount of phenylalanine-containing proteins. Since we first oxidized the protein samples with performic acid, methionine in the proteins should have been converted into methionine sulphone. Unfortunately, methionine sulphone was not completely separable from threonine under our analytical conditions, and the amount of methionine is not indicated in Table 1.

Table 1. Amino acid composition of the Pb-BP and Zn-BP

Experimental details are described in the Materials and methods section. The values are expressed as percentages of the total number of residues in the molecule. Values were obtained from duplicate 16 h hydroysates. Numbers in parentheses indicate the composition of Zn-MT-II reported by Bremner & Davies (1975).

Amino acid	Pb-BP	Zn-BP	
Lysine	13.4	10.9	(12.5)
Aspartate	11.2	9.7	(6.7)
Threonine + methionine	4.9	4.8	(3.9+1.8)
Glutamate	7.0	8.2	(6.3)
Proline	3.7	2.8	(4.9)
Glycine	5.2	8.4	(7.3)
Alanine	6.5	6.7	(8.1)
Half cystine*	28.1	29.2	(27.2)
Valine	1.8	1.6	(2.4)
Isoleucine	3.1	2.8	(1.7)
Leucine	4.2	2.4	(1.0)
Tyrosine	0.0	0.0	(0.0)
Phenylalanine	1.5	1.7	(0.5)
Serine	9.2	10.7	(14.1)

* Determined as cysteic acid.

DISCUSSION

Metallothioneins are a group of low-M_r cytoplasmic metalloproteins with high affinity for cadmium, zinc and other metals. The protein moieties of these metallothioneins are similar in amino acid composition and have the common characteristics that they contain about 30% cysteine and are completely devoid of aromatic amino acids, as so far determined (Cherian & Goyer, 1978).

In the present paper, we reported evidence indicating the simultaneous induction of Zn-thionein and a Pb-metallothionein-like protein (Zn-BP and Pb-BP) in rats injected intraperitoneally with lead acetate. Since the amounts of zinc and cadmium contaminating the lead acetate used in our present experiments were less than 0.05%, we concluded that lead itself can induce the synthesis of the Pb-BP and Zn-BP.

In this study, we used a rather large amount of lead acetate to induce metallothionein synthesis, owing to the low sensitivity of lead in atomic absorption analysis. However, intravenous injection of 0.3 μmol of [^{210}Pb]lead acetate/100 g body wt. could also induce synthesis of the Pb-BP (results not shown).

All the results in this paper support the concept that the Zn-BP is identical with rat Zn-MT-II. The Pb-BP is also a low-M_r cysteine-rich protein which is inducible with lead acetate. It has affinity for lead, and 6 h after the intraperitoneal injection of lead acetate into rats, lead bound to the Pb-BP accounted for about 60% of the lead in the liver cytosol. In this study, one-third of the lead in the liver was located in the cytosol, and the remaining two-thirds was present in a particulate-bound form. Lead contents (μg/g liver wt.) in the rat liver at 0, 2, 6, 12 and 24 h after the administration of lead acetate (24 μmol/100 g body wt.) were 0.22 ± 0.03, 32.4 ± 4.7, 26.1 ± 5.1, 17.6 ± 4.2 and 13.6 ± 3.0 respectively (4 rats for each measurement). From these results, we tentatively conclude that the Pb-BP is also a kind of metallothionein or a metallothionein-like protein. This concept is also supported by our preliminary experiments showing that the Pb-BP and Zn-BP are cross-reactive with rabbit anti-(rat Zn-MT-II) serum (H. Ikebuchi, R. Teshima, K. Suzuki, J. Sawada, T. Terao & Y. Yamane, unpublished work). However, owing to the instability of the Pb-BP and gradual release of Pb from the protein moiety during the isolation, we could not calculate the exact amount of Pb per mole of the protein.

Rat Zn-metallothioneins are separable on a DEAE-Sephadex A-25 column into Zn-MT-I and -II (Failla & Cousins, 1978). On examination of fractions 41–47 from Fig. 1(*c*) on a DEAE-Sephadex A-25 column, as shown in Fig. 5(*a*), only a single Zn-containing peak, corresponding to Zn-MT-II, was observed. Since the chromatographic conditions used in this experiment could separate Zn-MT-I and -II on this column (Fig. 5*b*), the results shown in Figs. 5(*a*) and 5(*b*) indicate that mainly Zn-MT-II is inducible in the liver by lead treatment. Rat liver metallothionein has been demonstrated to exist in at least two different forms separable by DEAE-cellulose column chromatography and polyacrylamide-gel electrophoresis (Winge *et al.*, 1975). Therefore, the fact that the Zn-BP mainly consists of Zn-MT-II might reflect a different influence of Pb on the expression of Zn-MT genes, although the molecular mechanism of regulation of Zn-MT-I and -MT-II gene expression is not known.

Zinc usually exists as one of the constituent metals of rat liver metallothionein regardless of inducing metals (Suzuki & Yoshikawa, 1976). The results shown in Fig. 5(*a*), however, indicated that the Pb-BP did not contain an appreciable amount of Zn. The reason for this unusual behaviour of the Pb-BP is not known.

Induction of a Zn-thionein-like protein in the rat liver by various metals, including lead, has been reported (Suzuki & Yoshikawa, 1976; Arizono *et al.*, 1982). Time-dependent changes in the amounts of the Pb-BP and Zn-BP revealed that the turnover rates of the two proteins were different. The Pb-BP was decomposed and/or removed faster than the Zn-BP and, 24 h after the administration of lead acetate, most of the Pb-BP had disappeared from the liver (Fig. 1*d*). This might be the reason why the induction of this protein by lead acetate was not observed in other studies.

The Pb-BP could be labelled with ^{210}Pb by incubation *in vitro* of the isolated Pb-BP fraction (Fig. 1*c*) with $^{210}Pb(NO_3)_2$, whereas the purified Zn-MT-II was not labelled with ^{210}Pb under the same reaction conditions (results not shown).

Although we cannot rule out the possibility that the protein moiety of the Pb-BP is identical with that of Zn-thionein and only the bound metal is different, the results shown in Fig. 4 strongly suggest that they are different protein species.

The kidney is the other main organ in which metallothioneins are produced (Pulido *et al.*, 1966; Piotrowski *et al.*, 1974). Therefore it would be interesting to know whether or not the Pb-BP is also induced in the kidney of Pb-treated rats. Our preliminary results show that the same metallothionein was also induced in rats intravenously injected with lead acetate (H. Ikebuchi, R. Teshima, T. Terao & Y. Yamane, unpublished work).

The biological and toxicological significance of the Pb-BP should be studied in relation to lead poisoning.

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