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Intracellular Binding of Lead in the Kidney: The Partial Isolation and Characterization of Postmitochondrial Lead Binding Components

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Gel chromatography of kidney postmitochondrial fractions from control rats 2 hr after injection of ^{203}Pb or after *in vitro* incubation with ^{203}Pb disclosed the presence of two fractionated Pb-binding components plus binding in the void volume and total volume regions. The binding of Pb to the two components, with molecular weights of 11,500 and 63,000 daltons, was markedly decreased in Pb-pretreated rats. Sodium dodecyl sulfate-gel electrophoresis and autoradiography showed the presence of one major ^{203}Pb band with an estimated molecular weight of 60,000 daltons. The 11,500-dalton peak did not incorporate ^{14}C-leucine nor did concomitant administration of cycloheximide with the ^{203}Pb inhibit incorporation of ^{203}Pb activity, suggesting that the component is a preformed constituent of the kidney. *In vitro* incubation of brain, liver and lung postmitochondrial supernatants with ^{203}Pb disclosed that these two binding components were also present in brain but not in liver or lung, suggesting a target tissue-specific localization for these Pb-binding macromolecules.

INTRODUCTION

Previous isotope studies (1-3) in the rat and in the rabbit (4) have shown that a high proportion of total cellular Pb is consistently recovered in kidney cytosol of control animals. Data on the chemical nature of lead in this fraction and molecular aspects of the cytoplasmic binding of Pb are currently lacking and need to be investigated to give a better understanding of the essential steps leading to the biological effects of this element.

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Preliminary reports of some of these data were presented at the 20th Annual Meeting of the Society of Toxicology (Toxicologist 1:81, 1981).

Abbreviations used: Pb, lead; PMSF, phenylmethylsulfonylfluoride; Cd, cadmium; MT, metallothionein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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The present investigation was undertaken to characterize the initial *in vivo* binding of lead in the kidney under control conditions and conditions of prior lead exposure. In addition, characterization studies were conducted on two previously unreported Pb binding components in kidneys of control rats. Brain, liver and lung were also examined for these components to determine their organ distribution.

MATERIALS AND METHODS

Experimental Animals and Chemical Reagents

Male Sprague-Dawley rats (CD strain, obtained from Charles River Laboratories, Boston, MA) weighing about 300 g were used in all experiments. The animals were fed Purina rat chow and tap water *ad libitum*. Carrier-free ^{203}Pb and ^{14}C-leucine (L-[^{14}C(U)], sp. act. 342.0 mCi/mmol) were obtained from New England Nuclear (Boston, MA). Carrier-free ^{203}Pb was used in this study after it had been shown in initial experiments that the binding of carrier-free ^{203}Pb to tissue supernatants was similar to that of carrier-free ^{203}Pb combined with trace amounts of unlabeled Pb. Sephadex G-75, G-200 and DEAE A-25 Sephadex were purchased from Pharmacia Inc. (Piscataway, NJ); and cycloheximide from Sigma Chem. Co. (St. Louis, MO). Other chemicals used were of analytical or reagent grade and were purchased from regular commercial sources.

Treatment of Animals for Analysis of Postmitochondrial Supernatant Fraction Pb Binding Components

Control rats or rats injected with Pb acetate (50 mg Pb/kg) six days earlier were injected i.p. with 50 µCi of carrier-free ^{203}Pb acetate in distilled water. Rats in each group were killed after 2, 24, or 29 hr and the kidneys homogenized in 1 volume of 0.25 M sucrose and 0.01 M Tris/HCl (pH 8.6) buffer containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor. The postmitochondrial supernatant fraction obtained by centrifuging the samples at 13,000 xg for 15 min was immediately applied to either a Sephadex G-75 or a Sephadex G-200 column as described below.

The *in vitro* binding of ^{203}Pb to kidney, liver, lung, and brain postmitochondrial supernatant fraction was studied by incubation of 3 ml of supernatant fraction, prepared from control rats as described above, with 5 µCi of carrier-free ^{203}Pb acetate.

Incorporation of ^{14}C-leucine into the 11,500-dalton Pb binding component was studied in rats injected i.v. with 3.5 mg Pb/kg (as Pb acetate). Immediately after and again 30 min later, the rats were injected i.p. with 15 µCi ^{14}C-leucine. Two hours after the Pb injection, the rats were killed and the kidney postmitochondrial supernatant fraction chromatographed on Sephadex G-75.

Effects of cycloheximide treatment on Pb binding to the 11,500-dalton Pb binding component were studied in rats injected i.v. with 100 µCi ^{203}Pb plus 135 µg Pb (as Pb acetate). One group of rats was injected i.v. with cycloheximide (20 mg/kg body weight) in saline and controls received saline alone. Both groups were killed after 2 hr and samples of the kidney postmitochondrial supernatant fraction applied to a Sephadex G-75 column as described below.

Binding of ^{203}Pb to the cadmium-metallothionein (Cd-MT) peak was studied in rats injected s.c. with 0.35 mg/kg Cd as $CdCl_2$ daily for five days. On the eighth day the rats were given a final injection of Cd and 2 hr later an i.p. injection of 50 µCi carrier-free ^{203}Pb. Control rats were given the same dose of ^{203}Pb. Twenty-four hours after the Pb injection, the rats were killed and the kidney postmitochondrial supernatant fraction applied to a Sephadex G-75 column. Cadmium in the fractions was measured by flame atomic absorption (Perkin Elmer Model 305B).

Gel Filtration and Ion-Exchange Chromatography

Three-milliliter samples of cytosol were applied to a Sephadex G-75 column (61.5 x 2.6 cm) equilibrated at 4°C with 0.01 M Tris/HCl (pH 8.6) buffer. Fractions (5.3 ml) were monitored for ^{203}Pb radioactivity using a Beckman Biogamma II gamma counter and for absorption at 280 nm. Where applicable ^{14}C-radioactivity was determined by liquid scintillation counting using Hydrofluor scintillation fluid (National Diagnostics, Somerville, NJ) in a Beckman LS 9000 liquid scintillation counter.

For Sephadex G-200 chromatography, 2-ml samples of kidney postmitochondrial supernatant fraction were applied to a 26.5 x 2.6 cm column equilibrated with 0.01 M Tris/HCl (pH 8.6). Fractions of 2.7 ml were collected and assayed as described above. For lung, liver and brain tissue, 3 ml of postmitochondrial supernatant were applied to a 60.5 x 2.6 cm column. Fractions of 5.3 ml were collected.

The 11,500-dalton fractions from Sephadex G-75 chromatography and the 63,000-dalton fractions from Sephadex G-200 chromatography were applied to a DEAE Sephadex A-25 ion-exchange column (8.5 x 1.4 cm) equilibrated with 0.01 M Tris/acetate buffer (pH 7.4). The column was eluted in a stepwise fashion with 50 ml each of 0.01 M, 0.05 M, 0.1 M and 0.2 M Na acetate or Tris/acetate (pH 7.4). Fractions of 2.7 ml were collected and measured for radioactivity and absorption at 280 nm following elution from the columns and radioactivity content of the gel was also measured.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Autoradiography

Pooled fractions of either the void volume peak or the 11,500-dalton peak from Sephadex G-75 chromatography of kidney postmitochondrial supernatant cytosol were concentrated approximately 10-fold using an Amicon cell with a UM2 filter. The concentrates were lyophilized and then dissolved in distilled water. After centrifugation in an Eppendorf model 5412 centrifuge operated at top speed for 5 min, the samples were heat-treated with SDS and mercaptoethanol and applied in duplicate to a 6.5% SDS-polyacrylamide slab gel of 1.5 mm thickness according to the method of O'Farrell (5). A mixture of molecular weight standards, consisting of myosin (480,000), β-galactosidase (135,000), phosphorylase b (94,000), bovine serum albumin (67,000), and ovalbumin (43,000), was run on the same gel. After electrophoresis the gel slab was cut in half with equivalent samples on each half. One part of the gel was stained in 0.25% Coomassie blue. The other half was immediately treated with 2% glycerol in water and dried on filter paper using a BioRad Model 224 slab gel dryer. The dried gel was exposed to an X-ray film (Kodak X-Omat R) for four days and then developed. The stained gel was also dried after destaining and treatment with glycerol. The molecular weight of the ^{203}Pb bands in the autoradiogram was estimated from a standard curve of the logarithm of the molecular weights of the protein standards vs. their R_f values.

RESULTS AND DISCUSSION

Cytosolic ^{203}Pb Binding Components

The kidney postmitochondrial supernatant fraction from control rats was found to contain ^{203}Pb binding components with estimated molecular weights of 11,500 and 63,000 daltons as well as ^{203}Pb in the void and total volumes as determined by Sephadex G-75 and G-200 gel chromatography (Fig. 1 and Fig. 2). The 11,500 component was present in samples incubated with ^{203}Pb *in vitro* (Table 1) and in samples obtained 2 hr after *in vivo* injection of ^{203}Pb

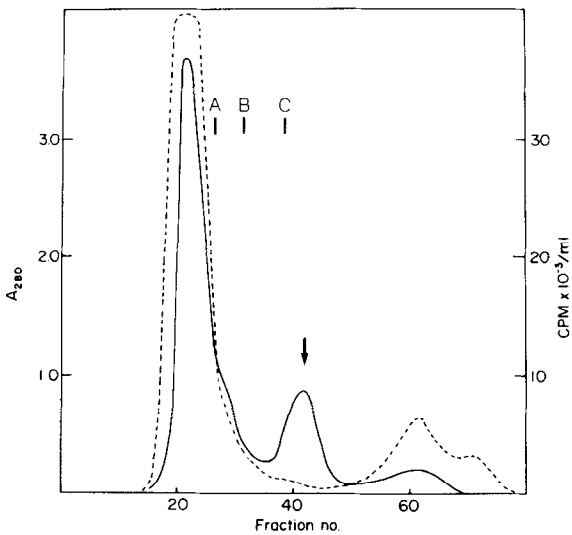

Figure 1. Sephadex G-75 column chromatography of kidney cytosol from rats injected i.p. 2 hr previously with 50 µCi ^{203}Pb showing a distinct 11,500-dalton molecular weight ^{203}Pb peak (arrow). -----A_{280}; ——— ^{203}Pb cpm. Molecular weight standards are designated as (A) ovalbumin (45,000); (B) chymotrypsinogen (25,000); and (C) ribonuclease (13,700).

(Fig. 2a and Table 1), but after 29 hr the amount of ^{203}Pb present in the peak was greatly diminished. The 63,000-dalton Pb binding component was detectable

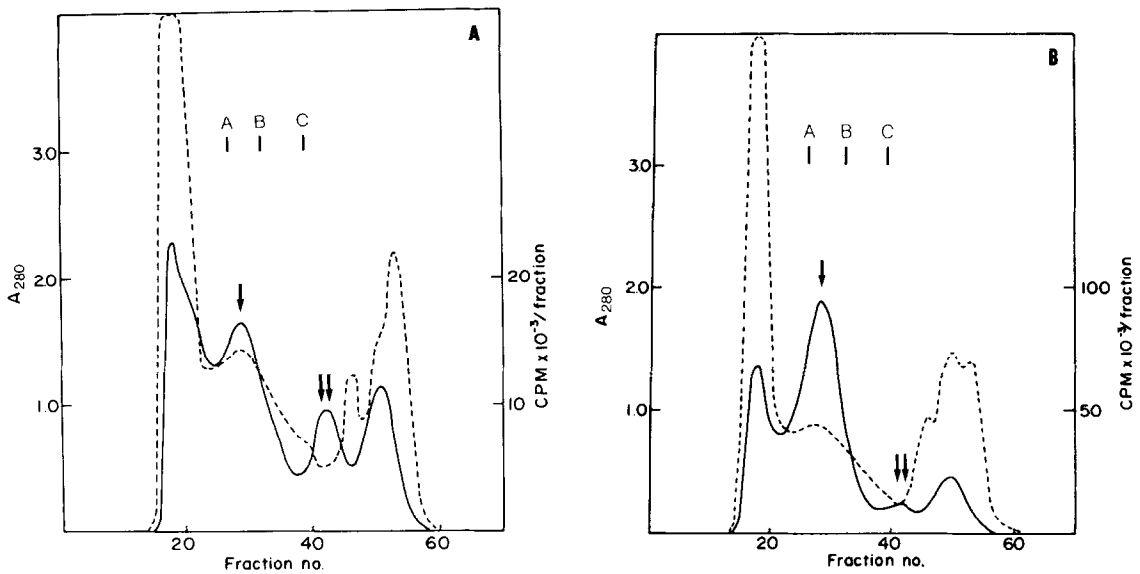

Figure 2. Sephadex G-200 column chromatography of kidney cytosol from control rats 2 hr (A) and 29 hr (B) following an i.p. injection of 50 µCi or 75 µCi of ^{203}Pb, respectively, showing the presence of both a 63,000 molecular weight peak (arrow) and also the low molecular weight peak at 11,500 daltons (double arrow). -----A_{280}; ——— ^{203}Pb cpm. The elution volumes of molecular weight standards are designated as: (A) albumin (67,000); (B) ovalbumin (45,000); and (C) chymotrypsinogen (25,000).

TABLE 1

Effect of experimental treatment and survival time on kidney cytosolic Pb binding

Treatment/Survival Time[a]	Proportion of total ^{203}Pb radioactivity eluted in fraction (%)	
	11,500 dalton[b]	63,000 dalton[c]
Control, *in vitro* incubation	10	15
Control, *in vivo*, 2 hr	15	30
Control, *in vivo*, 24 hr	3[d]	
Control, *in vivo*, 29 hr	5[d]	50
Pb-pretreated, *in vivo*, 2 hr	5[d]	
Pb-pretreated, *in vivo*, 24 hr	4[d]	20
Control, *in vivo*, 24 hr	3[d]	
Cd-pretreated, *in vivo*, 24 hr	4[d]	

[a]See "Materials and Methods" section for complete description.

[b]11,500-dalton peak isolated by Sephadex G-75 chromatography.

[c]63,000-dalton peak isolated by Sephadex G-200 chromatography.

[d]Not present as distinct peaks but as diffuse shoulders of the high molecular weight peak.

in control rat kidney following *in vitro* incubation (Table 1) and was also present after *in vivo* injection with ^{203}Pb at both 2 and 29 hr (Fig. 2a and b). The ^{203}Pb binding capacity of the 11,500- and 63,000-dalton binding components accounted for 15 and 30% of the total postmitochondrial radioactivity, respectively, at 2 hr. At 29 hr the amount of ^{203}Pb bound to the 63,000-dalton binding component was increased to 50% of the radioactivity, and there was a corresponding reduction in the void volume peak and 11,500-dalton peak (Fig. 2b). In kidneys of Pb-pretreated rats, the ^{203}Pb binding to the 11,500-dalton peak was greatly reduced. Binding to the 63,000-dalton component represented only 20% of the total radioactivity (Table 1) and most of the ^{203}Pb was eluted with the void volume peak. The 11,500-dalton component peak showed no detectable incorporation of ^{14}C-leucine when the labeled amino acid was injected together with lead (data not shown). The ^{203}Pb binding capacity of the component was also

not inhibited by cycloheximide administration. These data suggest that the 11,500-dalton component is a preformed constituent of the kidney and is subject to saturation with Pb and/or polymerization following prolonged lead exposure.

Although the 11,500-dalton component eluted from Sephadex G-75 columns at the same volume as Cd-MT, the binding of Pb to this component was not influenced by increased tissue levels of MT in rats treated with Cd prior to injection of ^{203}Pb. The chromatographic elution pattern of Pb from Sephadex G-75 for Cd-pretreated vs. control rat kidney cytosol was identical, indicating that induction of MT did not alter ^{203}Pb binding to the 11,500-dalton peak (Table 1). DEAE Sephadex A-25 anion-exchange chromatography disclosed that for both components, about 80% of the radioactivity applied to the column exhibited firm binding to the ion-exchange gel. About 10-15% of the bound radioactivity from the 63,000-dalton component could be released in a distinct peak by 0.20 M Na acetate. The 11,500-dalton component however, did not elute from the column in a discrete peak using the Tris/acetate gradient normally employed for the isolation of MT (6) but was released from the column in a continuous pattern. Ulmer and Vallee (7) have described the isolation of lead-MT by DEAE chromatography; however, the low molecular weight Pb binding component studied here does not behave in a similar manner. The 11,500-dalton component isolated by Sephadex G-75 chromatography hence does not appear to be MT by this parameter.

SDS-PAGE of the void volume peak and the 11,500 ^{203}Pb peak isolated from kidney by Sephadex G-75 column chromatography demonstrated that both fractions contained many protein bands (Fig. 3a). Autoradiography of these gels (Fig. 3b) showed that most of the ^{203}Pb in the 11,500 peak migrated with the front to the gel bottom with a "tail" extending behind the front band. However, a discrete, major ^{203}Pb band with an estimated molecular weight of 60,000 daltons and faint bands (73,000 and 80,000 daltons) were observed in the Sephadex G-75 void volume peak. These data indicate that the major ^{203}Pb band with an estimated molecular weight close to that of the 63,000-dalton peak identified by Sephadex G-200 chromatography has a high affinity for Pb.

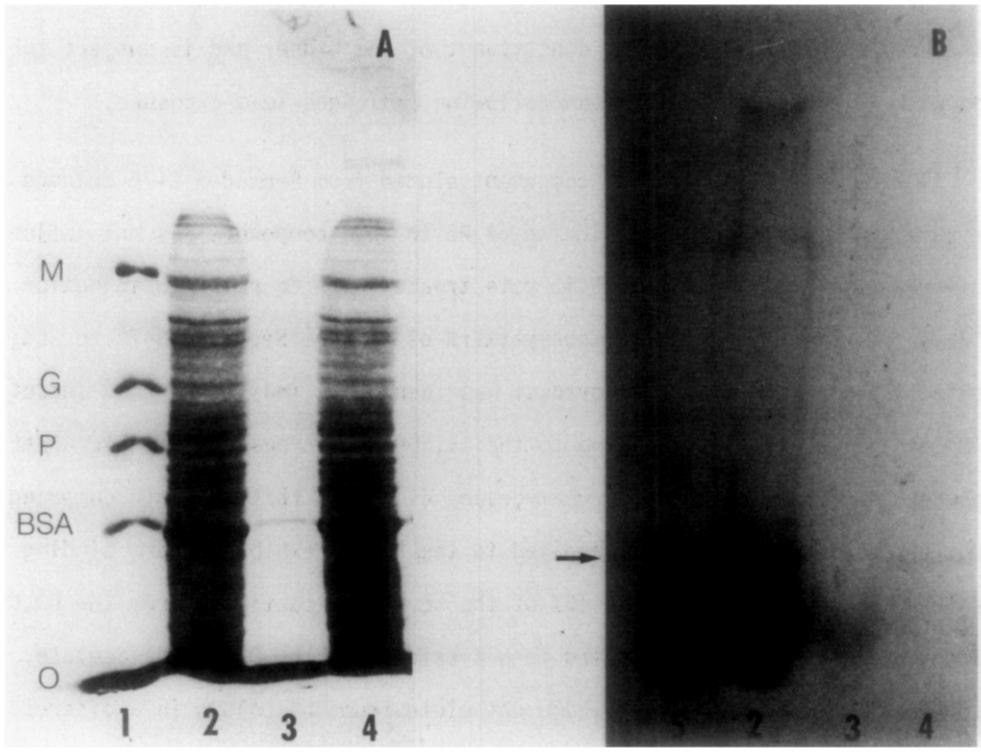

Figure 3. Coomassie blue stained 6.5% SDS gel (A) and corresponding autoradiogram (B). Lane: (1) molecular weight standards: myosin (M); β-galactosidase (G), phosphorylase (P), bovine serum albumin (BSA), and ovalbumin (O); (2) void volume peak from Sephadex G-75 chromatography of kidney cytosol incubated with ^{203}Pb *in vitro*; (3) 11,500-dalton fraction from the same gel chromatography as lane 2; (4) void volume peak from Sephadex G-75 chromatography of kidney cytosol 2 hr after *in vivo* Pb treatment (Fig. 1) and (5) 11,500-dalton fraction from Fig. 1. In the autoradiogram the arrow indicates the 60,000-dalton major lead-binding protein.

Tissue Distribution Studies of Cytosol ^{203}PB Binding Components

The results of Sephadex G-200 column chromatography, conducted with post-mitochondrial supernatant fractions from liver, lung, and brain tissue from control rats following *in vitro* ^{203}Pb incubations (Fig. 4), show the presence of both the 11,500- and 63,000-dalton components in brain tissue but not liver or lung tissue. In brain the 63,000-dalton component accounted for about 70% of the total radioactivity. In the liver and lung chromatograms, there is an intermediate ^{203}Pb and 280 nm peak which elutes at a molecular weight of approximately 35,000 daltons. The peak is clearly separate from the 63,000-dalton Pb peak observed in brain and kidney and probably represents Pb bound to hemoglobin. Hemoglobin

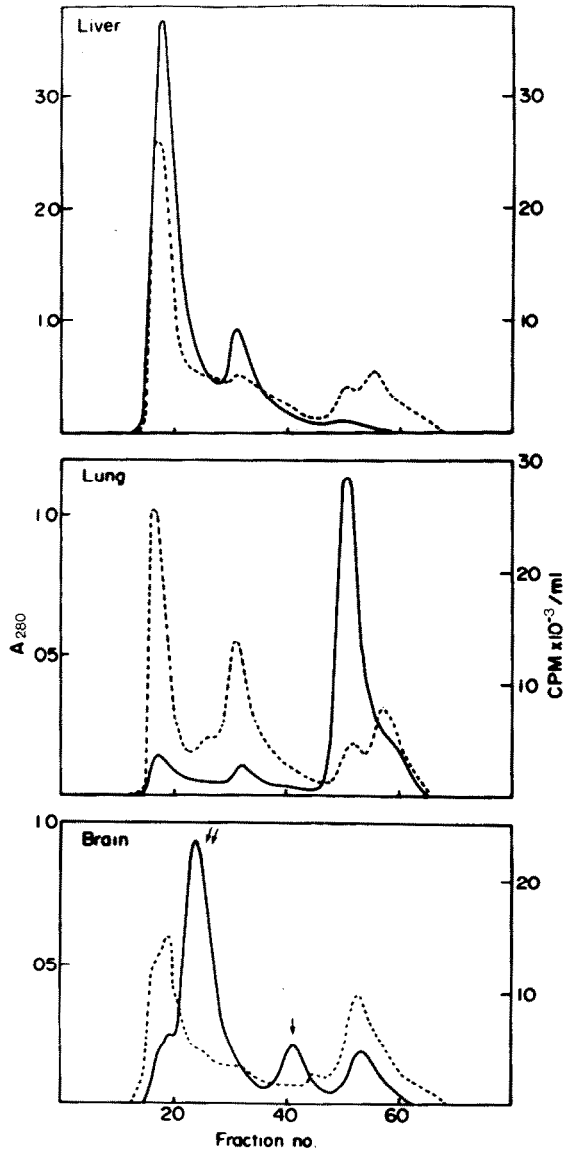

Figure 4. Sephadex G-200 column chromatography of cytosol fractions from liver, lungs and brain of control rats incubated with 5 μCi ^{203}Pb *in vitro*. Both the 11,500- (single arrow) and the 63,000-dalton (double arrow) ^{203}Pb binding peaks are present in brain but not liver or lung. -----A_{280}; ——— ^{203}Pb cpm.

(molecular weight, 64,000-daltons) dissociates into subunits in dilute solutions on Sephadex columns and migrates with a lower apparent molecular weight (8). The apparent tissue specificity of both the 11,500- and 63,000-dalton ^{203}Pb binding components in kidney and brain, but not liver or lung, following *in vitro* addition of Pb is of great potential interest since they appear to be present only in

target tissues (9). This may suggest that target tissue metal-specific ligands play a role in determining the sensitivity of an organ or cell type to lead toxicity.

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