The Proteins of Lead-induced Intranuclear Inclusion Bodies*  

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Characteristic intranuclear inclusion bodies have been isolated from the kidney cortex of lead-intoxicated rats. A protein has been identified which appears to be unique to the inclusion bodies. It was not detected in kidney or liver from normal rats, suggesting that it is induced by lead. However, sequestration of an extant protein by lead remains a possibility. The protein has an apparent molecular weight of 32,000 and an isoelectric point of 6.3. Although traces of related proteins were identified by 125I-tryptic peptide maps, these may be artifacts because they do not occur in some preparations. Identification of this protein provides an opportunity to characterize the molecular interactions between lead and the inclusion bodies. It has been suggested that the inclusion bodies have a protective effect in lead-intoxicated animals. The induction or sequestration of a unique protein lends support to this proposal.

Intranuclear inclusion bodies are found in kidney proximal tubular cells of lead-intoxicated animals (1, 2). They have been found in many mammals and in chickens (3). It is unknown whether or not these inclusion bodies reflect a physiological response to lead or a nonselective aggregation of nuclear protein. Goyer (4) has suggested that the inclusion bodies' function is to bind lead.

Goyer et al. (5, 6) have partially characterized isolated inclusion bodies and found them to be composed largely of acidic proteins and to be enriched in lead relative to other nuclear and cellular fractions. These findings are consistent with the proposal that inclusion body proteins are important in sequestering lead. This proposal would be strengthened if it could be shown that unique proteins occur in the inclusion bodies. We have isolated an inclusion body-enriched fraction and report the partial characterization of a protein which appears to be unique to this fraction.

**MATERIALS AND METHODS**

The methods of producing and isolating inclusion bodies generally followed those of Goyer et al. (5, 6) but were modified in some instances. Adult, male Sprague-Dawley rats were fed Ralston Purina Rat Chow and water ad libitum. Two methods of inducing inclusion bodies were utilized. In one, pulverized rat chow was made 1% in pulverized lead acetate. In the other, the drinking water was made 0.75% lead as lead acetate. Both methods induced inclusion bodies.

**Preparation of Kidney Nuclei**—The cortex portion of decapsulated kidneys was minced and then homogenized in a Potter-Elvehjem homogenizer in 2 volumes of 250 mM sucrose, 20 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 0.1 mM phenylmethylsulfonyl fluoride and 20 mM NaN3. The homogenate was filtered through four layers of cheesecloth and then mixed with 2 volumes of 2.3 mM sucrose, 50 mM Tris-HCl (pH 7.5), 25 mM KCl, 5 mM MgCl2, 0.1 mM phenylmethylsulfonyl fluoride and 20 mM NaN3. The diluted homogenate (27 ml) was layered over a two-step gradient consisting of 4.5 ml each of 61 and 64% sucrose in a 36-ml ultracentrifuge tube. Centrifugation was at 58,000 × g for 90 min in a SW 27 rotor. The clean nuclei were collected by aspiration from the 61/64% sucrose interface (Method 1).

Another procedure was developed and utilized in which the diluted homogenate was centrifuged over a step gradient of 58, 59, 60, 61, 64, and 69% sucrose (each contained 50 mM Tris-HCl (pH 7.5), 25 mM KCl and 5 mM MgCl2). The nuclei from lead-treated rats, which concentrated in the 58-60% sucrose, were sometimes purified on this multistep gradient and harvested by aspiration of the 58/59 to 61/64% sucrose zone (Method 2).

**Sonication of Nuclei**—The harvested nuclei were diluted with 250 mM sucrose, 20 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 0.1 mM phenylmethylsulfonyl fluoride and 20 mM NaN3, and collected by centrifugation at 650 × g for 5 min in an HB-4 rotor. The nuclear pellet from five rats was resuspended in 10-12 ml of 340 mM sucrose, 10 mM Tris-HCl (pH 7.5), 0.05 mM MgCl2, 0.1 mM phenylmethylsulfonyl fluoride and 20 mM NaN3 (7). A Heat Systems-Ultrasonics, Inc. Sonifier Cell Disruptor W-140 equipped with a 1/4″ horn (Method 1) was used to disrupt the nuclei. The nuclear suspension was placed in a rosette cooling cell which was immersed in an alcohol-ice bath maintained at -4°C. Sonication was performed in three 15-s bursts at maximal output with 15-s cooling periods between bursts. In some experiments, five bursts were used. In Method 2, a standard tapered microtip 419 was substituted for the 1/4″ horn.

**Preparation of Inclusion Bodies**—Sonication disrupted the nuclei but the presence of residual chromatin necessitated enzyme and salt treatments. The sonication mixture was diluted with 1 volume of 58, 59, 60, 61, 64, and 69% sucrose and centrifuged at 650 × g for 5 min in an HB-4 rotor. The nuclear pellet from five rats was resuspended in 10-12 ml of 340 mM sucrose, 10 mM Tris-HCl (pH 7.5), 0.05 mM MgCl2, 0.1 mM phenylmethylsulfonyl fluoride and 20 mM NaN3. The diluted homogenate (27 ml) was layered over a two-step gradient consisting of 4.5 ml each of 61 and 64% sucrose in a 36-ml ultracentrifuge tube. Centrifugation was at 58,000 × g for 90 min in a SW 27 rotor. The clean nuclei were collected by aspiration from the 61/64% sucrose interface (Method 1).

**Analysis of the Inclusion Body Fraction Proteins by Two-dimensional Gel Electrophoresis**—The pellet fraction was dissolved in 10 mM sodium phosphate (pH 7.2), 5% mercaptoethanol, 4% sodium dodecyl sulfate, boiled for 3 min in a water bath, and stored at -20°C. Two-dimensional electrophoresis was performed as previously described (8). The two-dimensional polyacrylamide system of O'Farrell (9) was used for separating proteins in all fractions. Modifications were as follows. Ampholytes (Servalytes-AG 3-10) were obtained from Serva, Feinbiochemica, Heidelberg, Germany. The cylindrical gels were not prefocused and sample was applied at the anode. The gradient slab did not contain glycerol. The proteins in the first dimension were focused at 500 V, constant voltage for 3 h with cooling at a constant temperature of 15°C. The slab gels were electrophoresed at 30 V, constant voltage, overnight (14 to 16 h) and cooled at a constant temperature of 10°C.

Two systems were used for staining protein in the gels. For Coomassie brilliant blue staining, slabs were soaked for 6 h in 0.025% Coomassie brilliant blue R and destained at 45°C in 10% ethanol, 5%
acetic acid. Gel slabs were placed between two sections of plastic foam during destaining.

The second method of staining relied on the use of a commercial silver stain, GELCODE, obtained from The Upjohn Co. In addition to increased sensitivity, many protein spots exhibit characteristic colors which are an aid in comparing complex gel patterns.

$^{35}$S Tryptic Peptide Mapping of Proteins Obtained from Coomassie Blue-stained Spots—The procedure of Elder et al. (10) for analysis of stained polypeptides in gels was applied to inclusion body fraction proteins after separation of the proteins by two-dimensional electrophoresis. After the stain was washed out, the proteins were labeled with $^{35}$S and digested overnight with trypsin, and the $^{35}$S-peptides were recovered. The peptides, in 1 to 2% of 88% formic acid, were spotted on thin layer chromatography sheets and separated by electrophoresis and chromatography. Peptides were located by autoradiography.

RESULTS

Nuclei were purified in initial studies by centrifugation through 61% sucrose. Isolated inclusion bodies were very similar to those observed in thin sections of kidney tissue (compare Fig. 1, a and b). Inclusion bodies consisting of dense masses and dense masses surrounded by fibrous networks could be observed before and after isolation. Material other than inclusion bodies was also obtained in the inclusion body fraction. Fig. 19 was selected to present the most abundant contaminants. These include residual mitochondria, nuclear pore complex-fibrous lamina, collagen fibers, and a finely divided homogeneous material.

The protein constituents of the inclusion body fraction were characterized by two-dimensional gel electrophoresis. In Fig. 2, the proteins of the inclusion body fraction from control (Fig. 2a) and lead-treated (Fig. 2b) rats are presented and compared. Isoelectric focusing gels containing the fractions, separated in the first dimension, were positioned top-to-top on a single slab gel and the proteins were separated in the second dimension by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Only one of the proteins was unique to the lead-treated fraction (arrow, Fig. 2b). The isoelectric point of this protein was determined to be 6.3 (bovine carbonic anhydrase has a pI of 6.4 in our system). The molecular weight was 32,000 as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis migration. The protein is referred to hereafter as p32/6.3.

The purified inclusion body fraction contained two to three major proteins in addition to p32/6.3 (Fig. 2). Surprisingly, the relatively insoluble nuclear envelope lamins (8) are not prominent components. Thus, the traces of nuclear pore complex-lamina detected in the preparations by ultramicroscopic examination (Fig. 1) must contribute a minor fraction of the total protein. Lamins can be detected in the fraction by the more sensitive silver staining (see Fig. 6). Similarly, collagen is not a prominent constituent although collagen fibrils could be detected in the preparation (Fig. 1). Collagen appears to be a minor and variable contaminant as judged by the detection of large molecular weight proteins which are stained pink (11) by Coomassie blue (data not presented). The large amount of collagen recovered in the inclusion body fraction by Moore et al. (6) is probably due to differences in the sucrose gradients used. These observations confirmed that structural components which might be significant contaminants in the inclusion body fraction have been largely removed.

![Fig. 1. Ultramicroscopic comparison of inclusion bodies in the isolated inclusion body fraction and in intact kidney. The inclusion body fraction was prepared as described under “Materials and Methods.” Samples were fixed in 2.5% glutaraldehyde in 100 mM sodium phosphate (pH 7.2), rinsed in 100 mM sodium phosphate (pH 7.2), and then treated in 2% osmium tetroxide in 100 mM sodium phosphate (pH 7.2). After dehydration in alcohol, the samples were embedded in Durcupan for sectioning. Samples were stained with 5% uranyl acetate and Reynolds’s lead citrate and examined on a Hitachi HU-12 electron microscope. a, a thin section from a rat fed a diet including 1% lead acetate for 13 weeks reveals a large inclusion body. This inclusion body consists of two adjacent dark masses centrally located in the nucleus. One of these is surrounded by a more open, fibrous cortex. Tapering fibers extend from each mass into the surrounding chromatin. b, ultrastructure of material in the inclusion body fraction. A thin section reveals three inclusion bodies, the largest one surrounded by a fibrous cortex. This view was selected to illustrate other material which co-isolated with the inclusion bodies. These include structures reminiscent of the nuclear pore complex-lamina (L), mitochondria (M), collagen fibers (C), and a homogeneous material which appears in the shape of circles and ribbons (H). The bars represent 1 μm.](image-url)
The comparison of lead-treated and control fractions suggested that p32/6.3 was uniquely associated with inclusion bodies. In order to determine whether p32/6.3 is a normal constituent of other cellular fractions, cellular (12) and nuclear (13) fractions were examined by two-dimensional electrophoresis. The fractionation procedures are summarized in Fig. 3. Carbonic anhydrase migrates slightly below and slightly to the basic side of p32/6.3 (Fig. 4a). However, it was possible to demonstrate by 125I-tryptic peptide mapping (see below) that these proteins were not related to p32/6.3 (data not presented). Thus, p32/6.3 has only been detected in the inclusion body fraction from lead-treated rats.

In the experiments presented thus far, all nuclei were purified by centrifugation through a layer of 61% sucrose. Microscopic examination of the preparative step gradient indicated that many nuclei from lead-treated rats did not penetrate the 61% barrier. This observation was confirmed by centrifugation of nuclei from control and lead-treated rats on extended step gradients (Fig. 5). Drop fractions were collected and examined microscopically for nuclei. DNA analyses were performed on 5% trichloroacetic acid precipitates (14). The DNA results

![Image of Fig. 4](image-url)

**Fig. 4. Electropherogram of soluble rat liver nuclear protein.** This fraction was obtained as described in Fig. 3. *a*, carbonic anhydrase (→) migrates near p32/6.3 (▲) and was included in other fractions as a marker. *b*, soluble rat liver nuclear protein with carbonic anhydrase (▲). Two proteins migrate just over the carbonic anhydrase.

![Image of Fig. 5](image-url)

**Fig. 5. Altered sedimentation of kidney nuclei from rats fed a diet of 1% lead acetate.** Sucrose step gradients (58, 59, 60, 61, 64, and 66% sucrose) were prepared in 36-ml tubes. Rat kidney cortex (approximately 1.5 g) was homogenized as described under "Materials and Methods" and applied to the gradients. After centrifugation at 58,000 × g for 90 min, 40-drop fractions were collected and the percentage of sucrose in each fraction was determined by refractometry. DNA was assayed in cold trichloroacetic acid precipitates by the diphenylamine method (14). The DNA results expressed relative to the A∞ of the most concentrated fraction. *a*, nuclei from control rats. *b*, nuclei from rats fed a diet of 1% lead acetate for 13 weeks.

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**Fig. 3. Fractionation of normal tissue and nuclei for protein analysis by two-dimensional electrophoresis.** *a*, fractionation of rat liver and kidney. *b*, fractionation of rat liver and kidney nuclei. Protein was precipitated from extracts with acetone:NH₄OH (5.3:0.3) and redissolved in 9 M urea, 5% Nonidet P-40, 1% 2-mercaptoethanol. Pellets were dissolved in the same final solvent.
presented in Fig. 5 were in accord with the microscopic examination. While control nuclei largely passed through the 61% sucrose barrier, nuclei from lead-treated rats were found predominantly in 58% sucrose with only a small proportion penetrating 61% sucrose. In subsequent studies, nuclei were sedimented on the gradients described in Fig. 5 to increase the yield of nuclei and therefore of p32/6.3.

The possibility that low levels of p32/6.3 occur in the inclusion body fraction from control rats was investigated. In these studies, a silver stain was used to increase sensitivity. A commercially available kit, GELCODE, from The Upjohn Co. was employed. The variety of colors for different proteins yielded by this procedure assists in comparing spots in complex gel patterns. In Fig. 6, the inclusion body fraction proteins from control (Fig. 6a), and lead-treated rats (Fig. 6b) are compared. With this stain, p32/6.3 yields a yellowish color at the center of a concentrated spot and a grayish color at the periphery of the spot. Thus, it appears in black and white (Fig. 6b) as a dark ring with a white center. No p32/6.3 could be detected in the control protein, even when the applied protein was increased from 50 to 100 μg (data not shown). In contrast, p32/6.3 could be detected in the lead-treated fraction when as little as 0.5 μg of total protein was applied. Thus, p32/6.3 is at least 200-fold more abundant in the fraction from lead-treated rats.

The silver stain revealed several other proteins which migrated near p32/6.3 and which had similar staining properties. Some of these are indicated in the enlargement of the p32/6.3 gel region presented in Fig. 7. In order to determine if these constitute a related family of proteins, the labeled spots were sectioned out of Coomassie blue-stained gels and the proteins were characterized by 125I-tryptic peptide mapping. The spots at A, B, and C contained related proteins as evidenced by their similar maps (Fig. 8,a, b, and c) but the proteins at D and E were unrelated to p32/6.3 (maps not shown).

In order to further confirm the relationship between intranuclear inclusion bodies and p32/6.3, an alternative method of lead administration was utilized. Mice (15) and rats (16) produce ultramicroscopically detectable inclusion bodies rapidly following an intracardiac injection of lead acetate. Rats were injected by this route, thin sections were prepared for ultrastructural examination, and the inclusion body fraction was prepared for gel electrophoresis. The appearance of inclusion bodies was slower than previously reported (15, 16). The reasons for this difference are unknown. Approximately 1% of the nuclei in thin sections exhibited inclusion bodies after 11 days and 4–12% after 14 days (200 nuclear sections were examined for each count). The inclusion bodies were small; one of the largest is presented in Fig. 9. Light microscopic examination also revealed relatively few inclusion bodies in these nuclear preparations as compared to 17-week preparations in which large inclusion bodies are found in nearly half of the nuclei. When the inclusion body fraction proteins were examined by two-dimensional electrophoresis with silver staining, p32/6.3 was questionable at days 2 and 4, but was more positive by day 7. A 14-day preparation is presented in Fig. 10. p32/6.3 is present as a gray spot. These observations again relate p32/6.3 to the appearance of intranuclear inclusion bodies. Although quantitative correlations have not been attempted, a trend of increased p32/6.3 with an increase in inclusion bodies is apparent when the two methods of induction are considered.

**DISCUSSION**

Lead adversely affects several organelles and enzymes. It is especially harmful to nervous tissue, the hematopoietic system, and kidneys (see Refs. 2 and 17 for reviews). Intracellular
Electrophoretic Analysis of Inclusion Body Proteins

Fig. 8. Comparison of the 125I-tryptic peptides of three proteins from the inclusion body fraction. The proteins were obtained in Coomassie-stained gel fragments. They were iodinated in the gel by the chloramine-T procedure, digested with trypsin, and maps of the labeled peptides were prepared by high voltage electrophoresis and thin layer chromatography. Autoradiograms are presented. a, b, and c, peptide maps of proteins identified as A, B, and C in Fig. 7. The protein in a is p32/6.3. d, this is a tracing of the spots that can be detected in a. C and E indicate the direction of chromatography and electrophoresis. The autoradiograms have been cropped to eliminate empty areas and spots or streaks which arise primarily from background (such a streak is present in the upper left corner of b). These proteins appear to be closely related by sequence. Proteins identified as D and E in Fig. 7 were unrelated and their fingerprints are not presented.

Fig. 9. Ultrastructure of an inclusion body formed 14 days after an intracardiac injection of lead acetate (30 µg/g body weight). The central dark area is very small but a typical loose cortex with extensions into the chromatin is apparent.

lead concentrates in the nucleus of kidney cells (5). It might be predicted that lead would have a profound effect in the nucleus because of the ability of metals to alter nucleic acid conformations. Further, lead is the most active metal in the degradation of RNA (18), increases misincorporation in DNA synthesis (19), and stimulates chain initiation by Mg2+-activated RNA polymerase (20). Lead affects the proliferation of cells in vivo (21). There have also been reports of chromosomal abnormalities (22) and renal tumors (see Ref. 17) associated with lead poisoning. The specificity of lead for these in vivo effects has been questioned, however, because of the complexity of the reported studies (17).

The formation of intranuclear inclusion bodies appears to be a consistent feature in lead-intoxicated animals. Goyer (4) has proposed that the intranuclear inclusion bodies function to bind lead and to minimize its effects. Several observations support this proposal. The appearance of the inclusion bodies, following lead exposure, appears to depend on protein synthesis (15). This observation argues against the trivial precipitation of pre-existing nuclear components. The inclusion bodies are enriched in lead in comparison with other nuclear fractions (5). When lead-intoxicated animals are treated with EDTA to remove lead, the inclusion bodies are disaggregated and exit the nucleus (23). Thus, there appears to be a direct relationship between lead and the inclusion bodies. However, elucidation of the molecular constituents and mechanisms involved has been prevented by the insolubility of the proteins and the structural complexity of the nucleus. It has not been possible.
to determine whether the lead-inclusion body relationship has the specificity of the zinc and cadmium interactions with metallothionein, a cytoplasmic, soluble protein (24).

An essential step in characterizing the molecular interactions between the inclusion bodies and lead is to isolate and characterize the inclusion body proteins. The resistance of the intranuclear inclusion bodies to dissolution by a variety of chemical and physical treatments permits their isolation in a residual nuclear fraction (5). This fraction of residual proteins has proven amenable to two-dimensional electrophoretic procedures used to characterize the insoluble nuclear envelope fraction (25).

Of the three to four major proteins in the inclusion body fraction, only one protein, p32/6.3, was uniquely present when inclusion bodies were present. The others were all found in control preparations. Thus, p32/6.3 may be the only protein component of the inclusion bodies. Alternatively, the other proteins might occur in the inclusion bodies as well as in other materials. These questions are difficult to resolve by isolation of essentially insoluble components. For instance, the examples of collagen and the lamins described under "Results" indicate that material stained in thin sections can be difficult to detect as stained proteins in electrophorograms. Further, it is often assumed that experimental treatments do not alter the parameters by which cellular structures are isolated and this is an implicit assumption in the comparison of control and lead-treated fractions (see Fig. 2). However, lead treatment alters the sedimentation characteristics of the bulk of the kidney nuclei (Fig. 5). Other materials recovered in these fractions might be affected also. Despite these assignment problems, which are especially difficult for shared proteins, additional observations support the localization of p32/6.3 to the inclusion bodies.

Altered sedimentation of the lead-treated nuclei required that nuclei be collected from less dense regions of the gradient when material was limiting. As might be expected, both Coomassie blue-stained (results not presented) and silver-stained electrophorograms (Fig. 6) of these preparations revealed more spots than are present in Fig. 2. However, p32/6.3 was again a major component, particularly when the electrophoretic sample was restricted to 0.5 μg.

The assignment of p32/6.3 to the lead-induced inclusion bodies is of particular interest because it does not appear to be a constituent of liver or kidney cells in normal rats. The possibility exists, of course, that it is a very minor constituent of normal tissue but if this is true, then its recruitment into the inclusion bodies by lead is highly selective. Alternatively, p32/6.3 may be induced by lead. This possibility would be consistent with the study reported by Choie et al. (15) in which inhibition of protein synthesis prevented inclusion body formation. In the present study, the rate of inclusion body formation observed upon intracardiac injection of lead did not lend itself to the use of metabolic inhibitors to test this hypothesis.

Numerous proteins are now known to occur as closely related families. The detection of other proteins of similar molecular weight, isoelectric point, and silver staining properties suggested that there might be a family of p32/6.3-like proteins. However, it appears more likely that the two proteins found to have related 125I-tryptic peptide maps are formed artifically from p32/6.3. One occurs as a streak on the acid side of p32/6.3; the other is of slightly lower molecular weight and could be a cleavage product. Neither is present in some preparations.

The available evidence strongly suggests that p32/6.3 is a component of lead-induced intranuclear inclusion bodies. The evidence is also consistent with the induction of p32/6.3 by lead. Further study of this protein and its metabolism in lead intoxicated animals may explain the role of the inclusion bodies.

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