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Effect of Lead on Globin mRNA *In Vivo* and *In Vitro*

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To the Graduate Council:

I am submitting herewith a thesis written by C. A. Aethranis entitled "Effect of Lead on Globin mRNA *In Vivo* and *In Vitro*." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

Walter R. Farkas, Major Professor

We have read this thesis and recommend its acceptance:

Daniel C. White, Charles C. Cogdon

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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EFFECT OF LEAD ON GLOBIN mRNA

IN VIVO AND *IN VITRO*

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

C. A. Aethranis

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ABSTRACT

The total amount of mRNA recovered from Pb^{2+}-treated rabbits was not significantly influenced by lead but dose-dependent loss of template activity of the poly A+ fraction was observed. These observations lead to the conclusion that the messenger RNA of rabbit reticulocytes exposed to Pb^{2+} in vivo was damaged and inactivated without extensive depolymerization of the mRNA.

In vitro experiments on the effect of Pb^{2+} on purified rabbit globin mRNA were also performed. After incubation with Pb^{2+} at micromolar concentrations, the 7-methyl guanosine cap was removed from the mRNA, while damage to the remainder of the molecule seems to be slight. These data indicate that Pb^{2+} causes the loss of the template activity of reticulocyte mRNA in vivo by causing a nucleolytic cleavage of the mRNA at or near to the 5' cap.

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1. INTRODUCTION

Some elements once thought to be nonessential for living cells have later been found to be essential. Selenium is an example. Considering the ubiquitous nature of lead, it is surprising that evolution has not found a use for this metal. However, to date no organisms has been found for which lead is essential or even useful. Man first started using lead in about 3500 BC (Waldron, 1973) and it has become virtually indispensable in the twentieth century. Lead is a contaminant of water, air and soil (Grandjean, 1975) and remains a danger to all organisms.

Lead Production and Pollution

In 1978, over 2.5 million tons of lead were taken from the earth (Harrison, et al., 1981). Smelting of lead in the 1960's was nearly 4 million tons (Grandjean, 1975). The USA and the USSR process about 15 and 17% respectively. About 14% of new lead products are made of recycled lead. Tetra-alkyl lead accounted for about 10% of the lead production in 1981, although this amount is constantly decreasing due to new regulations in the U.S.A. that have resulted in the removal of lead from gasoline. Most lead is used in the production of lead-acid storage batteries. Other uses of lead include

pigments for paints, components of electrical equipment, chemicals, pipes and solder (Harrison, et al., 1981).

In 1979, the following human activities resulted in the following emissions of lead into the atmosphere - gasoline consumption, 141 Kton; waste oil incineration, 5.5 Kton; copper smelting, 1.4 Kton; municipal incinerators, 1.3 Ktons (Harrison, et al., 1981). In 1974, particulate lead emissions from municipal incinerators was 10 to 100 mg of lead per kilogram of solid emissions (Kolbye, et al., 1974). This particulate lead is a serious problem because the lead is carried by rainwater into our water supplies (EPA-600, 1977). Sediments of fresh water lakes have been found to contain as much as 500 ppm Pb and ocean sediments have been recovered with up to 400 ppm Pb (Harrison, et al., 1981). Canned foods contain about 10 times more lead than raw foods (Kolbye, et al., 1974). This source accounts for a daily lead intake of 100 to 200 micrograms. As much as 18% of this lead appears in the blood (Rabinowitz, et al., 1976). Lead in canned food has decreased in recent years.

History of Man's Relationship with Lead

Since ancient times lead has been mined and smelted into useful products. Perhaps the oldest surviving reference to lead is in the book of Job, which was probably written about 1500 BC. Job said that he was going to inscribe his promises to God on tablets of lead, so that they would be incorruptible (Job 19:24). Ancient Assyria used lead as currency (Waldron,

1973). From the Greek physician Nicander in the second century BC comes the oldest existing documentation of lead poisoning (Eisenger, 1977). Rome developed an impressive lead technology which was used for the construction of elaborate water delivery systems. Romans also used lead vessels in cooking and the production of wine. Studies have demonstrated that wine and syrup made in leaden vessels contained enough lead to cause "Saturnine gout" (see Farkas' experiments, detailed below), impairment of mental capacities and death. At the time of Christ, many members of the Roman aristocracy exhibited behavior which could be attributed to chronic exposure to lead (Nriagu, 1983).

The Industrial Revolution saw a great increase in the manufacture of metal products, and concurrently, an increase in lead poisoning. It was at this time that the first simple chemical tests were devised to detect the presence of lead in wines and ciders. Certain foods, such as butter and milk, were thought to protect against lead poisoning. We now know that Ca^{2+} from milk products blocks absorption of lead. These efforts to solve the lead poisoning problems of the early industrial revolution was the birth of Occupational Medicine (Eisenger, 1977). Despite knowledge of the metal's toxicity, the lead problem is still with us. The metal has been used for centuries to make pigments for paints and many old buildings in the USA contain many layers of lead-based paint. Many children have been fatally poisoned by eating chips of this paint (Ross Laboratories, 1972). The paint on old metal structures, especially such

as bridges and towers, has caused lead poisoning in workers who repaint or repair these structures (Landrigan, et al., 1982). The mode of poisoning is usually inhalation of fumes generated during welding, or heating and scraping to remove old paint. Also, chips of old paint removed from the exteriors of these structures fall to the ground and become sources of poisoning for children playing in the area.

The major source of environmental lead until recently was "regular" gasoline. Lead alkyl additives have been added to gasoline for fifty years for three reasons. (First) They raise the octane rating of poorer grades of gasoline by making them burn more efficiently, (second) they serve as a top end lubricant and (third) lead helps make the engine run cooler. The absence of lead alkyls must be compensated for by making the fuel mixture richer. This requires more fuel. For the past fifty years lead salts have been spewed from the tail pipes of automobiles. This lead settles to the ground as microscopic particles. The lead is absorbed by plants and animals which may be food sources. The group at greatest risk are children when they play outdoors. The IQ's of American children have increased since the ban on the use of lead containing gasoline (Bellinger, et al, 1984). Needlemann (et al., 1974) presented statistical data to support a negative correlation between lead content of the deciduous teeth of school children and their I.Q.s. The data in this report is controversial.

Chromosome breaks have been found in lead workers. These aberrations rapidly disappear when workers leave the workplace. The mode of exposure in these intoxications is by inhalation of the fumes generated by smelting and of dust during grinding of the metal (Forni, et al., 1976). Industrial lead poisoning occurs less frequently nowadays because of safeguards in the lead industries.

It would be difficult to imagine life without this metal, especially in the U.S.A. where there is so much dependence on electronic products which contain lead solder, and our automobiles, which depend upon rechargeable lead-acid batteries. Total curtailment of the use of lead is unrealistic and naive. Instead, obtaining knowledge of the mechanisms of the toxicity seems the wiser approach to coping with the problem, for in this way we may learn how to live with lead.

Lead and Anemia

Many of the symptoms of lead poisoning are nonspecific and range from general malaise and gastroenteritis to encephalopathy and mental retardation. Among the most common symptoms of lead poisoning is anemia. Biologists have performed many sophisticated experiments to try to elucidate the mechanisms of lead toxicity. Pb^{2+} binds with high affinity to sulfhydryl groups of proteins, which leads to inhibition of enzymes containing these groups (Vallee, 1972). The enzyme ferrochelatase, is exquisitely sensitive to lead.

This enzyme inserts Fe^{2+} into protoporphyrin to form heme. In the presence of lead, zinc ions are inserted into the protoporphyrin molecule nonenzymatically to form zinc protoporphyrin. The presence of zinc protoporphyrin provides a very convenient assay for lead intoxication (Eisenger, 1979). Inhibition of the enzyme 8-aminolevulinic acid dehydratase by lead has been noted at blood lead levels as low as 10 to 20 lg/dl. Free erythrocyte protoporphyrin (FEP) has been shown to accumulate at blood lead levels as low as 17 to 20 lg/dl in children. The EPA has adopted FEP accumulation as the primary indicator of lead intoxication (EPA-600, 1977). Most enzymes thus far studied are inhibited by concentrations of Pb^{2+} at 10^{-4} to 10^{-3} M. Some enzymes are sensitive at much lower concentrations (Vallee, 1972).

Borsook (et al., 1959) has shown that lead is the most potent heavy metal inhibitor of hemoglobin synthesis in rabbit reticulocytes. In 1972, White and Harvey studied the effect of lead on hemoglobin syntheses in the reticulocytes of a pair of lead-poisoned three year old twins. More beta globin chains were made than alpha chains. The amount of mature hemoglobin was decreased by an amount proportional to the concentration of lead in the bloodstream. Our work offers a possible explanation for these observations.

Lead and Nucleic Acids

Nucleic acids can be depolymerized by some metal ions and lead has been shown to be a more potent catalyst for the depolymerization of RNA than

any other metal. In series of experiments, both Eichhorn (et al., 1965-1967) and Farkas, (1968) showed that single stranded synthetic polynucleotides are much more sensitive to depolymerization by metal ions than are double stranded polynucleotides. Waxman and Rabinowitz (1966) showed that lead caused the disaggregation of rabbit reticulocyte polyribosomes in vitro. Farkas and colleagues (see below) have shown that lead caused the depolymerization of many types of natural RNAs, including the globin messenger of rabbit reticulocytes.

Over a century ago, Archibald Garrod described a form of gout due to lead poisoning. Farkas' laboratory has shown the molecular basis for this gout which is known as Saturnine gout. Lead inhibits guanine aminohydrolase and causes the accumulation of guanine which precipitates in the joints (Farkas, et al., 1978). The Farkas laboratory has also investigated the effect of lead on transfer RNA. The tRNA's were found to be susceptible to cleavage by Pb^{2+} to a degree intermediate between that of single and double stranded polynucleotides. This data indicates that lead probably attacks the tRNA's in areas of little secondary structure, e.g. the loops. This laboratory has also investigated the effect of Pb^{2+} on the aminoacylation of the tRNA's. Interestingly, tRNA's for different amino acids were differentially susceptible to attack by lead indicating that different tRNA's possess different degrees of secondary structure. The effect of lead on the ability of tRNAs to bind to ribosomes was also investigated. The lead-treated tRNA's were severely

impaired in their ability to participate in protein syntheses (Farkas, et al., 1972). Brown (et al., 1983) also has presented evidence to support Pb^{2+} catalyzed cleavage of primary stucture of yeast tRNA.

Farkas also investigated the effect of Pb^{2+} on messenger RNA's in vitro. Brome mosaic virus mRNA, rabbit globin mRNA, and polyuridylic acid were exposed to Pb^{2+} and the loss of their ability to act as templates during protein synthesis in cell-free extracts of wheat germ was examined. An E. coli cell-free system was used for translation of poly U. Both the BMV and globin mRNA's were rendered untranslatable by incubation with 1mM lead acetate in only a minute. The polyuridylic acid retained about 55% of its polyphenylalaine synthesis-directing ability in the E. coli system under similar conditions. These studies demonstrated that Pb^{2+} acts as a true catalyst in depolymerizing messenger RNAs (Farkas, 1975). This laboratory has also studied the effect of Pb^{2+} on the 2', 5'-oligoadenylates that are synthesized by cells after exposure to interferon. Lead at millimolar concentrations resulted in 66% depolymerization of the 2', 5' oligoadenylate molecule (George, 1985).

Effect of Lead on Messenger RNA In Vivo

Whereas the effect of metals on RNA has been studied extensively in vitro, it is not known if Pb^{2+} depolymerizes RNA in vivo. The work in this thesis investigates the effect of lead on rabbit reticulocyte messenger RNA in vivo. Lead crosses the reticulocyte membrane and impairs the ability of the

mRNA to program protein synthesis--even though the absolute amount of mRNA recovered from reticulocytes treated at different dosages of lead does not decrease significantly with increased lead levels.

Effect of Lead on Messenger RNA *In Vitro*

This thesis also presents *in vitro* experiments which elucidate the effect of plumbous ion on purified globin messenger RNA. From these studies we can conclude that the attack of Pb^{2+} on globin mRNA removes the 7-methylguanosine cap from the 5′ end of the mRNA. The importance of the presence of the 7-methylguanosine cap as a recognition factor for translation in eukaryotes is well established. The works by Shatkin, Furiuchi, Muthukrishnan and others (see references) have shown that the cap is necessary for translation.

Synopses of Experiments

To investigate the effect of plumbous ion on the messenger RNA of rabbit reticulocytes *in vivo*, our experimental design was to induce reticulocytosis in rabbits, and then to transfuse these reticulocytes into recipient rabbits that had been dosed with lead by intravenous injection. About 20 hours later the blood was collected from each rabbit and the mRNA recovered. The amount of poly A+ RNA was determined spectrophotometically and assayed for template activity by measuring its ability to stimulate uptake of radio-labelled

amino acids into proteins in an mRNA-dependent reticulocyte lysate. The total mRNA per ml of packed reticulocytes and the activity per microgram of the mRNA from the lead-treated rabbits and control rabbits were compared. Figure 1[1] shows the scheme of these experiments. The effect of lead on polyribosome formation was also investigated by sucrose density gradient ultracentrifugation of reticulocyte lysates from normal and lead treated rabbits.

The effect of Pb^{2+} on rabbit globin mRNA was ascertained by incubating purified reticulocyte 9S RNA with varying lead concentrations and analyzing for the presence of free 5′ hydroxyl groups using a technique that incorporates ^{32}P into the 5′ hydroxyl group.

The first series of experiments involved incubation of the lead-treated RNA with T4 polynucleotide kinase, an enzyme that catalyzes the incorporation of ^{32}P from ATP into free 5-'hydroxyl groups of ribonucleotides. The labelled product was analyzed in three ways: by electrophoresis on agarose gels and subsequent autoradiography, by liquid scintillation counting of acid insoluble radioactivity, and by thin layer chromatography on polyethyleneimine.

From these studies it was evident that the 5′ end of the molecule was susceptible to attack by Pb^{2+}, that polynucleotides slightly smaller than the native messenger RNA were formed and that the attack by Pb^{2+} was truly catalytic. At higher Pb^{2+} concentrations or incubation times, all four of the ribonucleotides were liberated.

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*All figures and tables may be found in the Appendix.

2. MATERIALS AND METHODS

Preparation of Reticulocytes

Reticulocytosis was induced in New Zealand White rabbits (2 - 3 Kg) by four daily subcutaneous injections of aqueous phenylhydrazine - HCl (DeBellis, et al., 1964). In early experiments, the dose of phenylhydrazine was 3.2 mg per kg. In later experiments, the dose was increased to 7 mg/kg. On the sixth day, the animals were exsanguinated by cardiac puncture. All animals were anesthetized with rompun and ketamine. The rompun dose was 4.7 mg/kg, and the ketamine dose was 10 mg/kg. The blood was collected into heparinized vessels in an ice bath. Reticulocyte counts and hematocrit determinations were performed to monitor reticulocytosis.

Transfusion of Reticulocytes and Injection of Lead

The reticulocytes from the donor rabbit were washed 3 times by resuspending them in 0.9% saline and centrifuging them at 1000 xg for 20 min at 4#. The cells were resuspended at a hematocrit of 40 to 50% in 0.3 M glucose. Forty to 60 ml of the cell suspension was slowly infused into each recipient rabbit through the lateral ear vein. The volume of the cell suspension transfused into each rabbit was always the same in a single experiment. Infusion rate was 2 ml/min. The lead acetate was dissolved in 0.3 M glucose at 1 mg/ml and a single dose was injected i.v. into each animal immediately

after the cell infusion. Lead acetate doses were 0, 2, 3, 4, and 8 mg/kg. After 20 hours the rabbits were exsanguinated by cardiac puncture and the blood collected in heparinized vessels kept at 0#. Reticulocyte counts and hematocrits were determined and the RNA extracted.

Extraction of RNA

The cells were washed 3 times in 0.9% saline. Centrifugation was at 1000 xg for 20 min at 4#. The cells were lysed by gentle shaking for 5 min with 2 volumes of ice cold distilled water. The stroma was removed by centrifugation at 17,000 xg for 20 min at 4#. An equal volume of water-saturated phenol containing 1 mM EDTA was added to the lysate. The mixture was shaken vigorously for 10 min on a wrist action shaker in an ice bath. To this mixture, an equal volume of chloroform-isoamyl alcohol (24:1) was added and the mixture was again shaken on ice for 10 min. This mixture was then allowed to stand in the ice bath for 30 min to allow the phases to separate. The organic phase was aspirated from beneath the aqueous phase and an equal volume of fresh chloroform - isoamyl alcohol (24:1) was added to the aqueous phase and the mixture reshaken for 10 min. The suspension was centrifuged at 500 xg for 20 min at 4# to separate the phases.

The aqueous phase was collected and brought to 0.4 M with NaCl, and 2.5 volumes of 95% ethanol were added. The suspension was stored overnight at -20# to allow precipitation of the RNA. The RNA was collected by

centrifugation at 12,000 xg at -20# for 20 min. The pellets were stored under vacuum.

Purification of mRNA by Oligo dT Chromatography

The RNA was redissolved in loading buffer, 20 mM Tris HCl (pH 7.6), 0.5 M NaCl, 1 mM EDTA, and 0.1% sodium dodecyl sulfate (SDS) and loaded onto an oligo dT cellulose column (Aviv and Leder, 1972). The oligo dT column was poured in the loading buffer. Before each use, the column was washed with 0.1 M NaOH containing 5 mM EDTA and washed with distilled water until the pH was neutral. The column was then equilibrated with several volumes of the loading buffer. The columns were stored in 0.02% sodium azide at 4#C and were stable for more than two years.

The RNA solutions were heated to 65#C for 5 min, cooled to room temperature in a water bath, and applied to the column. The effluent was collected, reheated, cooled, and reapplied to the column. This cycle was repeated 3 times for all samples. The column was then washed with the loading buffer until the absorbance at 260 nm was negligible. The poly (A+) RNA was eluted with 10 mM Tris HCl (pH 7.5), 1 mM EDTA and 0.5% SDS. Fractions of 1 ml were collected and the absorbance at 260 nm monitored. The peak fractions were pooled and brought to 0.4 M NaCl and combined with 2.5 volume volumes of 95% ethanol. This suspension was stored overnight at -20#C. The precipitate was collected by centrifugation at -20# for 20 min at

17,000 xg. The pellets, often invisible at this point were dried and stored under vacuum. These poly A+ samples were analyzed for mRNA activity by the protein synthesis assay described below.

Template Activity of Poly A+ Fraction

The poly A+ RNA was reconstituted to 0.5 ug/ul in ultrapure water for analysis in an mRNA-dependent rabbit reticulocyte lysate (Pelham and Jackson, 1976). Analysis of an equal amount of brome mosaic virus RNA was always included as a positive control. An incubation containing no exogenous messenger was included as a negative control. Zero time counts were subtracted from the counts in the incubated samples.

To assay for template activity of the poly A+ fractions, 1, 2 and 3 micrograms of the poly A+ RNA's recovered from the control and lead-treated rabbits were added to the lysate. The lysate was 20 IM in each of the amino acids, except methionine, which was labelled with ^{35}S and added at (5 ICi) 1 IM per incubation. All tubes were run in duplicate. The tubes were incubated at 30#C. At zero time and at 1 hr, a 5 Il aliquot from each tube was pipetted into 1 ml of ice cold 10% TCA. The suspensions were brought to 90# for 20 min (to hydrolyze aminoacyl tRNA), and then cooled in an ice bath. The TCA precipitates were collected on Whatman fiberglass discs. The tubes were washed 3 times with 3 ml ice cold 5% TCA and the washes were also filtered. The precipitate was washed with 3 ml ice cold 95% ethanol. The discs were

dried under an infrared lamp and counted in 10 ml of a toluene based fluor in a Packard TriCarb 1500 liquid scintillation counter.

Agarose Gel Electrophoresis of Poly A+ RNA

The products from oligo dT chromatography were also analyzed by electrophoresis on a 2% agarose submerged gel. The RNA samples were prepared for electrophoresis in 50 mM boric acid, 5 mM sodium borate, and 10 mM sodium sulfate (which was also the running buffer) which was made 10% in glycerol, 12.5% methylmercuric hydroxide, and 0.02% bromophenol blue. The gel was poured in the running buffer (Maniatis, et al., 1985). Electrophoresis was carried out at 65 volts until the bromophenol blue front had migrated about 5 cm. The gels were then stained with ethidium bromide (1 lg/ml) in 0.5 M ammonium acetate (which removes the methylmercuric ion) and observed under UV light or photographed at 254 nm. Some gels were stained with 0.04% methylene blue in 0.5 M sodium acetate and destained with running tap water.

Assay for Lead in RNA

Since exposure to lead caused inhibition of translation, the question as to whether the RNA isolated from lead-treated rabbits contained residual lead had to be addressed. To test this possibility, the effect of direct addition of lead on the translation assay was determined. The Poly (A+) RNA of Brome

Mosaic Virus was assayed in the presence of 1.0 lM to 1.0 mM lead acetate. We also mixed the mRNA from a rabbit that received 8 mg/kg lead acetate with BMV RNA. The globin mRNA was also assayed separately. The amount of ^{35}S methionine incorporated was the same as if the BMV RNA and the globin mRNA had been assayed separately indicating that the globin mRNA was not contaminated with Pb^{2+}.

Time Course for the Clearance of Lead

Lead acetate in 0.3 M glucose was infused i.v. into a rabbit. The dose was 2 mg/kg body weight. Aliquots of 1 ml were taken prior to the lead infusion, immediately afterwards and then at the times indicated in Fig 4. Aliquots of blood were collected via catheter into "vacutainer" vessels. In order to make the experiment more like our infusion study, 50 ml of whole blood was infused just before the lead injection. A 100 ll aliquot taken at each time point was digested with concentrated nitric acid as follows. The 100 ll sample of whole blood was added to 5 ml of water. 200 ll of 12 N nitric acid (J.T. Baker "Ultrex") was added and the mixture vigorously mixed on a vortex mixer. This mixture was centrifuged for 5 min in a clinical bench top centrifuge at 1000 xg. The resulting supernatant was analyzed by atomic absorption spectrometry for lead content.

Effect of In Vivo Pb^{2+} Treatment on Polyribosomes

To examine the effect of Pb^{2+} in vivo on the formation of polyribosomes, reticulocytosis was induced in animals as in all other experiments and transufed into recipient animals that received 0 and 2 mg/kg lead acetate. After allowing the animals to carry the transfusions overnight, the cells were collected and the lysate prepared as for extraction of RNA. A volume of 100 ll of each lysate was layered onto sucrose density gradients of 15 to 30% prepared in a buffer of 50 mM Tris-Hcl (pH 7.4), 25 mM KCl, and 5 mM $MgCl_2$. The tubes were ultracentrifuged at 150,000 xg for 90 min in an SW 41 Ti rotor. Centrifugation was continued for 90 min. The tubes were then punctured and fractions of 10 drops were diluted with 0.5 ml of buffer and the absorbance of each read at 260 nm.

Incubations with Plumbous Ion

In order to ascertain the effect of Pb^{2+} on rabbit globin messenger RNA (hereafter 9S RNA), varying amounts of 9S RNA were incubated with lead acetate at the indicated concentrations and incubation times in 10 mM Tris HCl (pH 7.4), 10 mM magnesium acetate at 37#C. The reaction was stopped by immersing the tubes in an ice bath, and adding a five fold excess of dithiothreitol (DTT).

Radiolabelling of the Lead-Treated RNA with ^{32}P

To radiolabel the residues at the 5′ end of the lead-treated 9S RNA, 150 ICi of gamma ^{32}P ATP (from ICN) 10 units of E. coli T4 polynucleotide kinase (from New England BioLabs), and a buffer containing 50 mM Tris HCl, pH 7.6, 10 mM magnesium chloride, 0.1 mM spermidine, 5 mM dithiothreitol, 0.1 mM EDTA was added to the products from above. (The T4 enzyme catalyzes the transfer of the gamma phosphate of ATP to the free 5′ hydroxyls of nucleotides and polynucleotides. See Maniatis, 1982.)

Analysis of Radio-labelled Products of Pb^{2+}-treated 9S RNA by Agarose Gel Electrophoresis

Some of the ^{32}P labelled products were analyzed by electrophoresis on 2% agarose gels containing methylmercuric hydroxide (from Serva Biochemicals) followed by autoradiography. After electrophoresis, the gels were removed from the buffer and blotted by placing Whatman #1 filter paper directly on them. Several layers of paper towels were placed behind the filter paper. After blotting, the gels were wrapped in plastic, placed in Kodak brand, 8 by 10 inch intensifying "X-Omat" cassettes directly against Kodak X-Omat X-ray film and developed for various times. The developing time was estimated from activity as estimated with a Geiger counter. The films were then removed and developed in a Kodak X-Omatic automated developer. Some of the ^{32}P-labelled product was acid precipitated by pipetting an aliquot from the T4

incubation into 1 ml of ice cold 10% TCA. The acid-insoluble precipitate was collected on Whatman fiberglass discs. The tubes were washed 3 times with ice cold 5% TCA and the wash added to the filters. The filters were washed with 3 ml ice cold 95% ethanol, dried for 20 min under an infrared lamp, and counted in 10 ml of a toluene based fluor. The number of free 5′ hydroxyl groups increased when 9S RNA was treated with Pb^{2+}.

Elution of [^{32}P]-Containing Species from the Agarose Gels

Some of the bands containing ^{32}P were recovered from the 2% agarose gels by cutting out the bands from the gels and placing the gel slices in 0.5 M ammonium acetate, and incubating at 37#C for 1 hour. After centrifugation the supernatant was collected and the RNA fragments precipitated by the addition of 2.5 volumes of 95% ethanol followed by storage overnight at -20#C and the precipitate collected by centrifugation.

Thin Layer Chromatography on PEI Cellulose

The 5′ termini of the lead-nicked ^{32}P labelled 9S RNA were identified by two dimensional thin layer chromatography on polyethyleneimine cellulose (PEI cellulose) plastic backed sheets from Machery-Nagel. We converted the ^{32}P-labelled polynucleotides to mononucleotides by incubation with micrococcal nuclease from Staphylococcus aureus (0.2 lg/ll) and calf spleen nuclease (0.2 lg/ll) in a buffer of 2.5 mM Tris-HCl, pH 8.8, and 2.5 mM $CaCl_2$. The incubation

was continued for 2 hrs at 37#. Then, nuclease P1 from Penicilium citrinum was added (0.2 ug/ul) and the incubation continued for one more hour at 37#. These nucleases degrade RNA to 5'-mononucleotides (Wilson, et al., 1985). The product was spotted directly onto PEI cellulose thin layer plates for two dimensional chromatography in 1 M acetic acid, pH adjusted to 3.5 with ammonium hydroxide (solvent "Aa") for the first dimension, and saturated ammonium sulfate, pH adjusted to 3.5 with ammonium bisulfate (solvent "Sb") for the second dimension. The chromatograms were washed with methanol for 20 min after development in the first dimension to remove solvent and dried with a heated air stream. The four canonical RNA 5'-monophosphate ribonucleotides were also chromatographed as markers. These methods are essentially those of Bochner and Ames (1982).

The chromatograms containing the authentic 5'-monophosphate ribonucleotides were viewed at 254 nm. The chromatograms containing the ^{32}P labelled 5'-monophosphate ribonucleotides were placed in Kodak brand cassettes directly against Kodak X-Omat X-ray film and developed for various times according to the activity as estimated with a Geiger counter. The films were then removed and developed in Kodak X-Omatic automated developer.

3. RESULTS

Recoveries of Poly A+ RNA

Table 1 shows the recoveries of the poly A+ RNA per ml of packed reticulocytes from each experiment. The data indicates that there was no significant difference in the recoveries of poly A+ RNA from control and lead-treated rabbits at any lead dosage.

Template Activities of Poly A+ RNA

Table 2 and figure 2 show the results of the assays for the ability of the poly (A+) RNA to support proteins synthesis in the mRNA-dependent system. These data indicate that there was substantially less template activity in the poly (A+) RNA from the reticulocytes recovered from lead-treated rabbits. Furthermore, the loss of template activity was dose-dependent.

Effect of Lead on Translation of BMV RNA

Table 3 shows the effect of lead acetate on the translation of BMV mRNA and Table 4 shows the results of the experiment where we mixed the lead-treated and control RNA or lead-treated RNA plus BMV RNA. There was actually an increase in the net cpm in both the BMV and the globin mRNA after addition of RNA from lead-treated rabbits. This is to be expected since lead

treated RNA does have some template activity. These data show that the reduced activity in the mRNA recovered from lead-treated rabbits was not due to the mRNA being contaminated with Pb^{2+}.

Effect of <u>In Vivo</u> Pb^{2+} Treatment on Polyribosomes

Figure 3 shows the results of the ultracentrifugation of the reticulocyte lysates from normal and 2 mg/kg Pb^{2+} treated rabbits. (These cells are the result of transfusions.) The population of monoribosomes is clearly increased and the polyribosomes decreased in the lead treatment product lysate.

Time Course for Lead Clearance From Rabbit Blood

Figure 4 shows the amount of lead in the bloodstream of a rabbit at different times after injection. The lead concentration was soon reduced to a level which remained fairly constant for at least 24 hours.

Agarose Gel Electrophoresis of the Poly A+ Products

In UV photographs of ethidium bromide stained agarose gels of poly A+ RNA isolated from control and lead-treated rabbits, a 9S RNA band is apparent in both control and lead treatment products. (Data not presented.)

Agarose Gel Electrophoresis of ^{32}P Labelled RNA

Two ll of purified 9S mRNA was incubated with 1 lm Pb^{2+} for 5 min. This material was end-labeled with ^{32}P as described above and analyzed on a 2% agaose gel (Fig. 5). The figure shows that the attack by Pb^{2+} resulted in the creation of a population of molecules containing free 5′ hydroxyl groups which serve as substrates for T4 polynucleotide kinase. The largest of these polynucleotides is only slightly smaller than the parent molecule. (The band at the front is ATP, which was run as a standard.) This data indicates that the 5′ end of the mRNA is removed during short term incubations with Pb^{2+} at micromolar concentrations.

Time Course of Depolymerization of mRNA by Lead

The time course of the formation of free 5′ ends was determined by exposing 9S mRNA to Pb^{2+}. At the indicated times, aliquots were removed, labeled with polynucleotide kinase and precipated with TCA. The precipitates were counted and the results are summarized in Fig 6.

Dependence of Depolymerization of mRNA on Pb^{2+} Concentration

Figure 7 shows the effect of varying the lead concentration on the depolymerization of 9S RNA. The incubation times with the Pb^{2+} was 5 min, and the incubation with the T4 enzyme was 1 hour for all samples. The samples were precipitated with TCA and counted in a scintillation counter as

above. These data indicate that the phenomenon is complex. Multple sites become susceptible to attack by lead at higher concentrations.

Assay for Free 5′ Ends on the Poly A^+ RNA extracted from Pb^{2+}-Treated Rabbits

In light of the information obtained with the in vitro experiments, we decided to determine if the mRNA exposed to Pb^{2+} in vivo could be labeled by the T4, ^{32}P labeling system. The assay was performed as described in materials and methods, except that 3 ug of RNA was used instead of 1. The lead dose that the animal had received was 2 mg/kg. The control in one experiment was commercial 9S RNA. The results in Table 5 indicate that the poly A+ RNA extracted from Pb^{2+} treated rabbit reticulocytes has many more free 5′-hydroxyl groups than does the control poly A+ RNA.

The experiment was repeated with control poly (A+) RNA that was recovered from reticulocytes that had been incubated in rabbits that had not been dosed with Pb^{2+}. Table 6 shows the results of this experiment. The poly (A+) RNA from the rabbit that had been treated with lead had many more 5′ hydroxyl groups than the control RNA. The control RNA was labeled to only 1% of that of the poly A+ RNA from the Pb^{2+} treated rabbit. From these data we conclude that lead attacks the reticulocyte messenger in vivo in a manner very similar to that which we observed in vitro, and that the 7 methyl guanosine cap is removed in vivo as well as in vitro.

Thin Layer Chromatography

Autoradiographs of the ribonucleotides formed when mRNA end-labeled with ^{32}P as described above was digested to 5′ mononucleotides with a mixture of enzymes consisting of Staphylococcus aureus nuclease and calf spleen nuclease show that all four of the canonical ribonucleotides were labelled. This data indicates that Pb^{2+} at high concentrations attacks rabbit globin mRNA in vitro, at many phosphodiester bonds (Data not presented).

4. DISCUSSION

The fact that the amount of poly (A+) RNA per ml of packed reticulocytes was not less in the lead-treated rabbits was unexpected. Also, gel electrophoresis of the poly (A+) material from normal and lead-treated rabbits showed no difference in their mobilities. However, a loss of the template activity was apparent and related to the lead dose that had been administered to the rabbits. These data indicate that the damage conferred to the messenger by the plumbous ion resulted in loss of biological activity, but not in the amount of recoverable poly (A+) RNA. The poly (A+) RNA recovered from the lead-treated animals apparently has the same mobility, and therefore is about the same size as the native 9S RNA.

These data lead us to conclude that the mRNA has lost its biological activity due to the loss of only a small segment from the polynucleotide. However, the lost part of the RNA was required for interaction of the mRNA with the components of the translation system. Our working hypothesis was that some recognition factor or factors that were integral parts of the mRNA were removed by the action of the Pb^{2+}. A likely explanation was that removal of the 7-methyl guanosine cap on the 5' end of the mRNA had occurred. The cap is necessary for efficient translation of the messenger RNA of all eucaryotic species (Kozak, et al., 1979).

Our in vivo experiments indicate that Pb^{2+} at blood concentrations of 50 lg per dl (about 25 micromolar) can cross the reticulocyte membrane without lysing of the membrane and cause significant damage to the mRNA. These results may explain the observations of Borsook (et al., 1957) in which Pb^{2+} (as lead acetate) was observed to be the most potent inhibitor of protein synthesis in rabbit reticulocytes. Farkas (1975) elaborated upon these experiments and demonstrated that synthesis of the alpha chain of hemoglobin was affected to a greater degree than beta globin. These observations agree with those of White and Harvey (1972) who reported a disorder in the synthesis of hemoglobin in a pair of lead poisoned three-year-old twins. These authors reported surplus beta globin synthesis by their reticulocytes. Explanations of these observations may be that of damage of the protein synthesizing systems or that of lead-catalyzed depolymerization of nucleic acids. It is known that in the hemoglobin synthesizing system that the alpha mRNA rather than the beta mRNA is more readily recognized by the ribosomes. However, the relative concentrations of the alpha and beta mRNA are also known to compensate for this binding affinity differential. There is no evidence to suggest that two protein synthesizing systems exist for alpha and beta hemoglobin -- one of which may be more greatly susceptible to the toxicity of lead. Likewise, discrimination in the insertion of the heme into beta over alpha-hemoglobin is also difficult to imagine in the synthesis of mature hemoglobin. Moreover, White and Harvey's experiments demonstrated the effect to be independent of

the presence of additional heme (op cit.). A more likely explanation is that the messengers are differentially susceptible to the attack of Pb^{2+}, most probably due to different structural conformations of alpha and beta globin messengers. Thus, the explanation of the relative abundance of the beta chain of hemoglobin in Harvey and White's experiments could be explained by our data.

The relative amounts of poly (A+) RNA recovered by oligo dT chromatography from control and lead-treated animals did not vary significantly. When this RNA was tested for its ability to stimulate protein synthesis in an mRNA-dependent cell free system, translational activity was found to be impaired. Also, the 5', ^{32}P labelled mRNA moved slightly faster than intact 9S RNA on electrophoresis in a denaturing gel. This data, combined with the oligo dT chromatography data, led us to hypothesize that the RNA must be damaged by a mechanism involving cleavage near the 7-methyl guanosine cap at the 5' end. The cap is required for recognition of mRNA by eukaryote initiation factors. (For an excellent series of articles, see Shatkin, Furuichi, Kozak, and Muthukrishnan in refs.) Since oligo dT chromatography demonstrates that the poly (A+) tail is present, the explanation for these observations may be the loss of a small piece from the 5' region of the mRNA. Our in vitro data below supports this explanation.

Our second series of experiments, performed in vitro, were designed to probe the effect of Pb^{2+} on the purified 9S messenger RNA from rabbit reticulocytes. Our in vitro experiments showed that even at micromolar

concentrations, Pb^{2+} depolymerized RNA releasing large oligonucleotides with all four of the ribonucleotides present at their 5′ ends. This, of course, means that the 7-methyl guanosine cap is lost from these molecules.

Our experiments also indicate that in lead-treated reticulocytes there are fewer polyribosomes than in untreated reticulocytes indicating that the mRNA in the Pb^{2+}-treated cells was defective. It is well known that the 7-methyl guanosine cap is a recognition factor for the binding of the ribosomal subunits. The absence of polyribosomes may be explained by the absence of the cap in the mRNA of Pb^{2+}-dosed rabbits. This data also indicates that the mRNA packaging proteins may leave a region of the messinger RNA unprotected and these are the regions susceptible to attack by Pb^{2+}.

These studies have revealed that at micromolar concentrations of Pb^{2+}, damage to the messenger RNA is extensive in vivo as well as in vitro and that the inactivation of enzymes by Pb^{2+} does not completely explain the detrimental effects of lead on organisms since only very few enzymes have been shown to be effected by Pb^{2+} at micromolar concentrations. These data suggest that the 7-methyl guanosine cap structure and the adjacent region of the mRNA may not be well protected by the RNA packaging proteins since it is apparently very susceptible to the attack by lead. The lead associated anemia in humans may in part be due to depolymerization of this region of this mRNA.

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APPENDIX

TABLE 1. Recovery of mRNA from control and Pb^{2+}-treated Rabbits

Exp.	Pb (mg/kg)	ug mRNA/ml packed retics
1	0	3.3
	2	3.2
	4	3.5
2	0	13.2
	2	15.5
3	0	19.4
	2	15.6

TABLE 2. Template activities of Reticulocyte mRNA.

Exp.	Pb mg/kg	% of control
1	0	100
	2	35
	3	13
2	0	100
	2	66
	3	54
3	0	100
	2	43
4	0	100
	8	16
5	0	100
8	45	
6	0	100
	8	12

Table 3. Effect of Lead on mRNA Dependent Translation by Reticulocyte Lysate

	cpm per 5 ul	% Inhibition
BMV mRNA	318,737	-
" + 10^{-3}MPb	123,613	62
" + 10^{-4}MPb	246,817	23
" + 10^{-5}MPb	321,149	0
" + 10^{-6}MPb	297,345	3

Table 4. Effect of Globin mRNA Isolated from Lead-Treated Rabbits* on the Translation of BMV mRNA and on Globin mRNA

	net cpm per 5 ul
BMV alone	349,070
" + Pb mRNA	360,211
GlobinRNA alone	84,005
"RNA + Pb mRNA	115,331
Pb mRNA alone	1435
no messenger	0

*Lead dose was 8 mg/kg at the time of the reticulocyte transfusion.

Table 5. Assay for Free 5′ Hydroxyls with T4 Polynucleotide Kinase on the poly (A+) RNA of a Lead-treated Rabbit*

commercial 9S mRNA preparation	2664 cpm
Poly (A+) RNA from lead treated rabbits	254,550 cpm

*Rabbit was dosed with lead acetate (2 mg/Kg).

Table 6. Assay for free 5′ Hydroxyls with T4 polynucleotide kinase on the poly (A+) RNA of Lead-treated and Non-Lead treated rabbits*

control poly (A+) RNA	95,204 cpm
poly (A+) RNA from lead treated rabbits	1,163,936 cpm

*Rabbit was dosed with lead acetate (2 mg/kg).

Legends to Figures

Figure 1. Scheme of Reticulocyte Transfusions. Reticulocytosis was induced in Juvenile New Zealand white rabbits by four daily injections of phenylhydrazine. Doses of phenylhydrazine were 3.2 mg/kg in early experiments and were increased to 7 mg/kg in later experiments. On the sixth day, the animals were rompun (4.7 mg/kg) and ketamine (10 mg/kg) anesthetized and exsanguinated by cardiac puncture. These cells were washed 3x in normal saline, reconstituted to 40 to 50% cells and infused into naive recipients who received either a mock injection containing only 0.3 M glucose or doses of lead acetate in 0.3 M glucose. These animals carried the cells and lead doses overnight, were exsanguinated as above, and the cells lysed and used for RNA extraction or for ultracentrifugation.

Figure 2. In vivo effect of Pb^{2+} on mRNA Template Activity. 1, 2 and 3 micrograms of the poly(A+) RNA for control and lead treated rabbits of various doses (8 mg/kg shown) were incubated in an exogenous mRNA dependent reticulocytelysate to which ^{35}S methionine was added. Total activity was taken as the slope of control product line. Slope of lead treatment poly A+ RNA product was expressed in percent of control slope. The results of several experiments are summarized by this method in Table 2.

Figure 3. There are fewer polyribosomes in Lead-Treated Rabbits (2 mg/kg). Reticulocytes were raised in phenylhydrazine treated rabbits and transfused into animals that received 0 and 2 mg/kg lead acetate doses. After carrying the transfusions and lead overnight, the

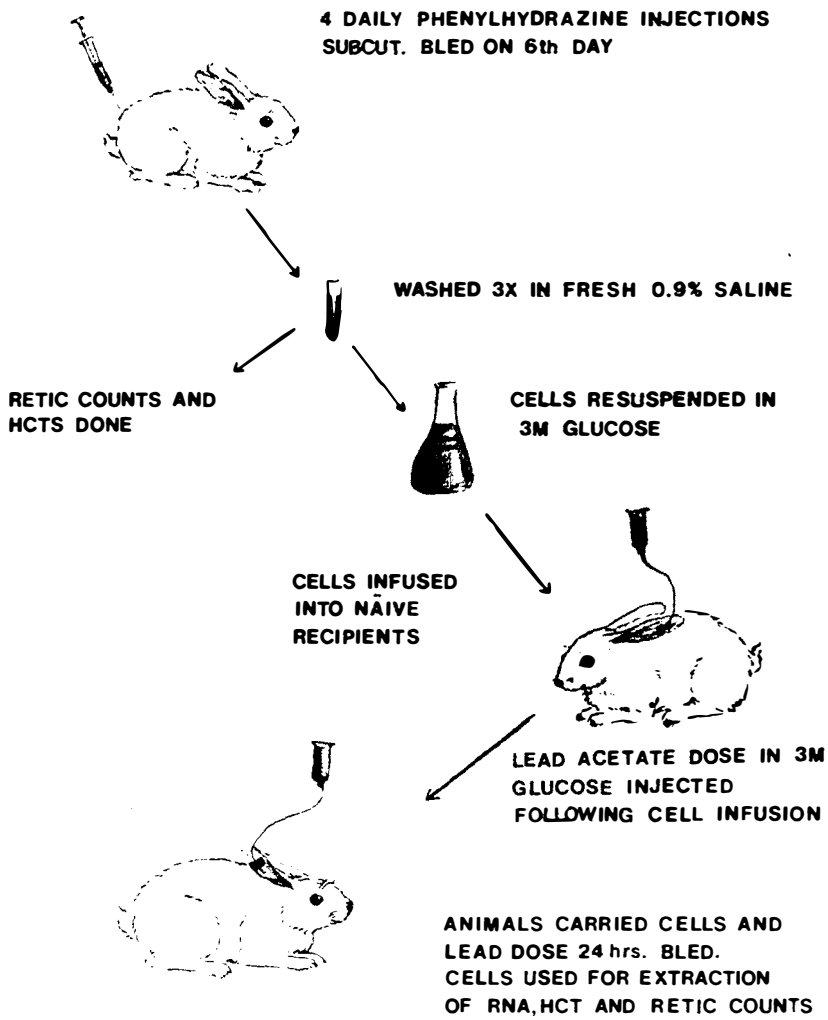
cells were collected and a lysate prepared. Aliquots of the lysates were applied to a 15-30% sucrose density gradient and subjected to ultracentrifugation. The tubes were punctured and fractions collected and analyzed for absorbance at 260 nm.

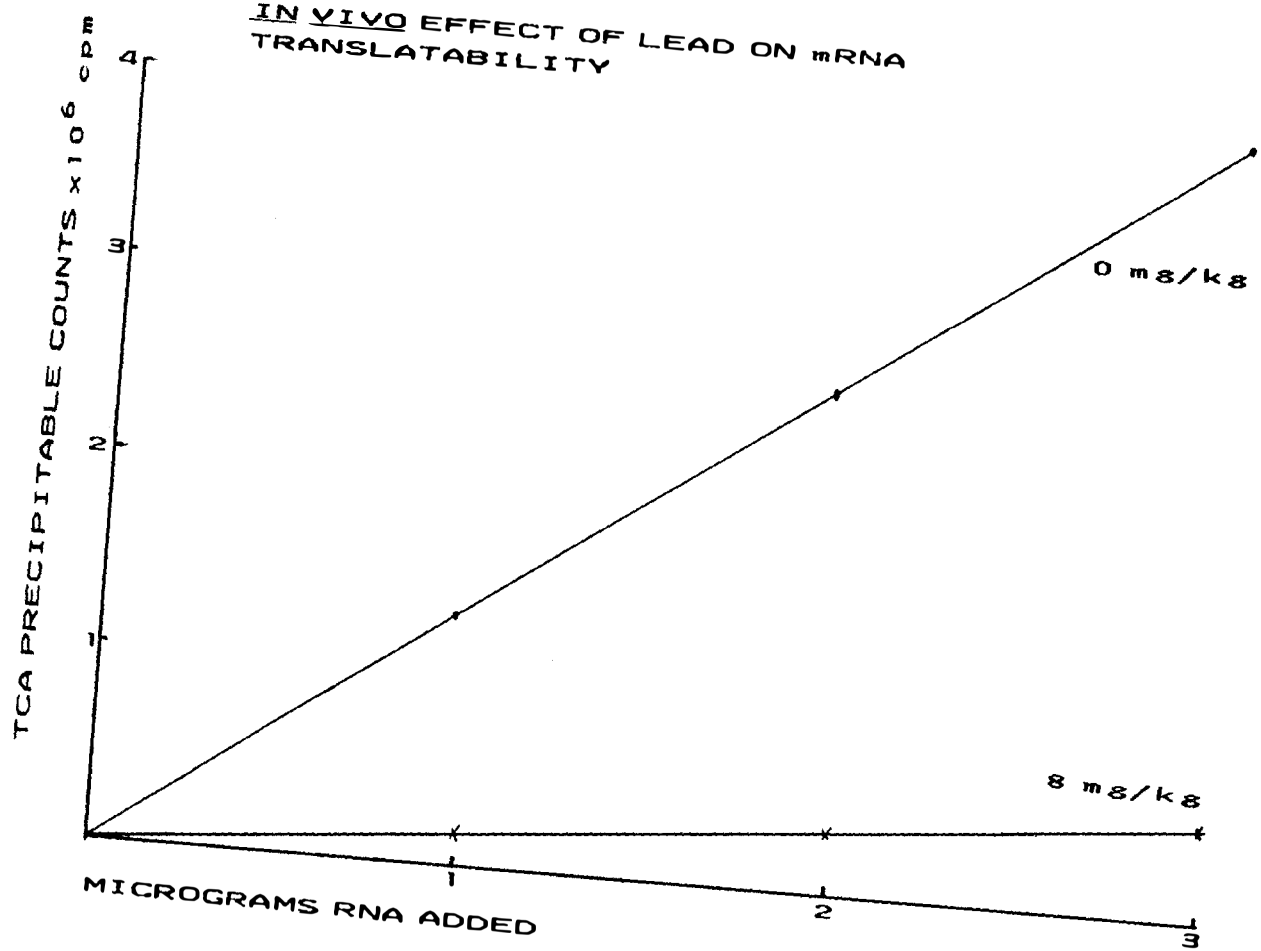
Figure 4. Time Course for Clearance of Lead from Blood of Rabbit. Lead dose was 2 mg/kg. At times indicated, 1 ml samples of blood were collected of which small aliquots were analyzed for Pb^{2+} by atomic absorption spectrophotometry.

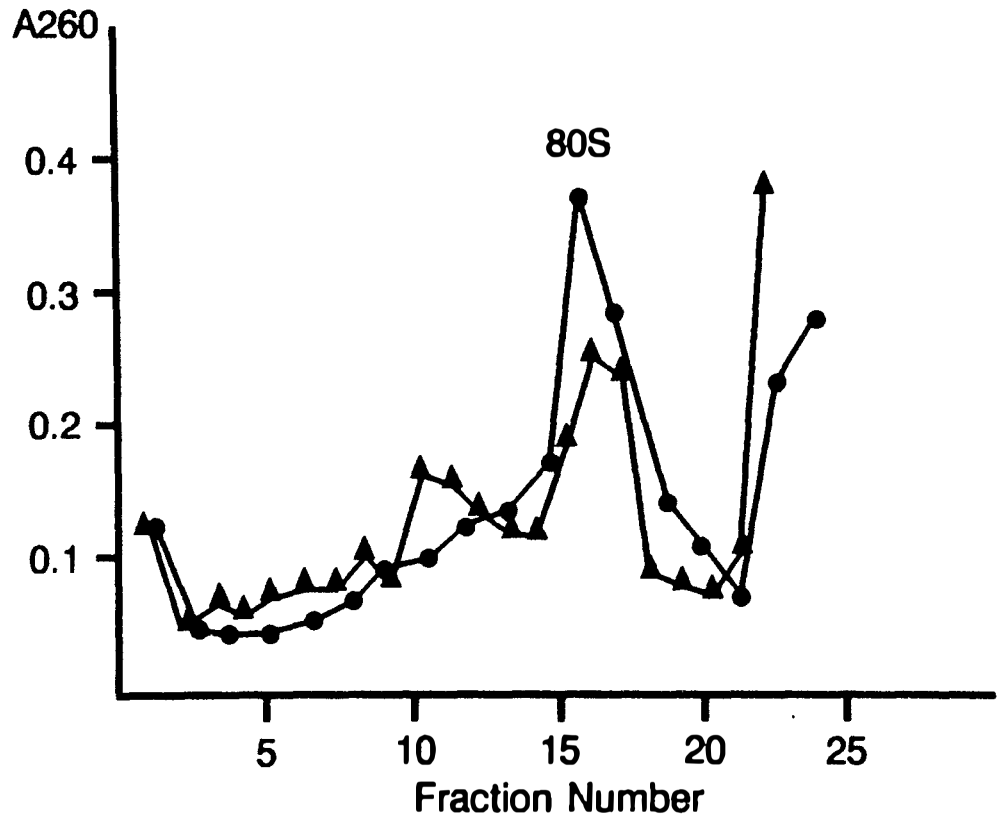
Figure 5. Autoradiogram of a 2% Agarose Gel ^{32}P Containing Fragments Produced by the Action of Lead. Commercial 9S RNA was incubated with lead at the concentrations indicated for 5 min, treated with DTT to stop the reaction and electrophoresed on a 2% agarose gel. The gel was blotted, wrapped in plastic wrap, exposed to X-ray film in a Kodak X-omat cassette, and developed with a Kodak automated developer. Lanes: A, B and C are markers. A is total E. coli RNA, B is 9S rabbit reticulocyte RNA, C is total S. cerevisiae RNA, D is the product of 5 min incubation of 9S at 1 mM Pb^{2+}, E at 0.1 mM Pb^{2+}, F at 0.001 mM Pb^{2+}, G is 9S incubation product without Pb^{2+} and H is no 9S RNA plus 1 mM Pb^{2+}

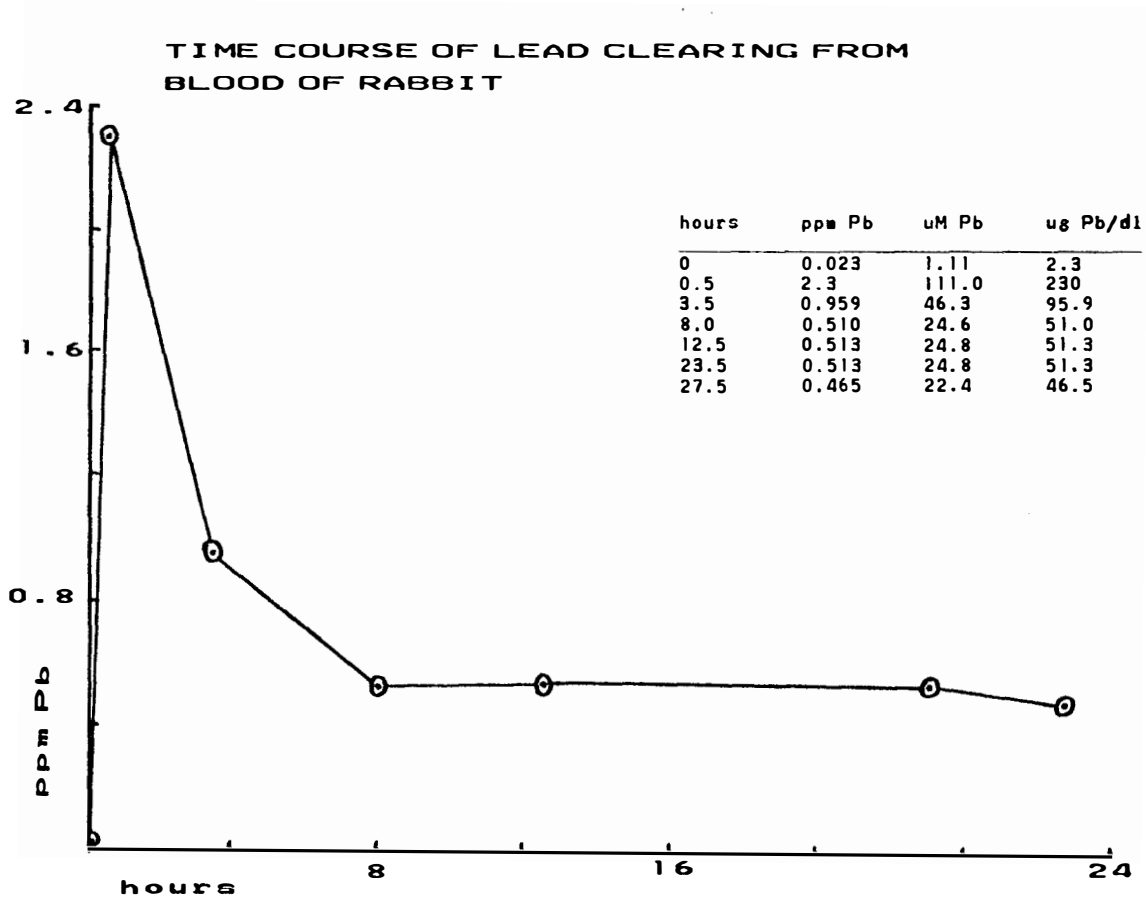
Figure 6. Time course for degradation of mRNA by lead. Commercial 9S reticulocyte RNA was incubated with Pb^{2+} at 10 mM. At the times indicated, aliquots were removed and the Pb^{2+} removed by treatment with DTT, end labeled with ^{32}P, precipated with TCA, end labeled with ^{32}P filtered onto fiberglass discs and liquid scintillation analyzed in toluene fluor.

Figure 7. Effect of Lead Concentration on Degradation of mRNA. Commercial 9S RNA was incubated with lead at the concentrations indicated for 5 min, treated with DTT to stop the reaction, precipated with TCA, filtered onto fiberglass discs and liquid scintillation analyzed in toluene fluor.

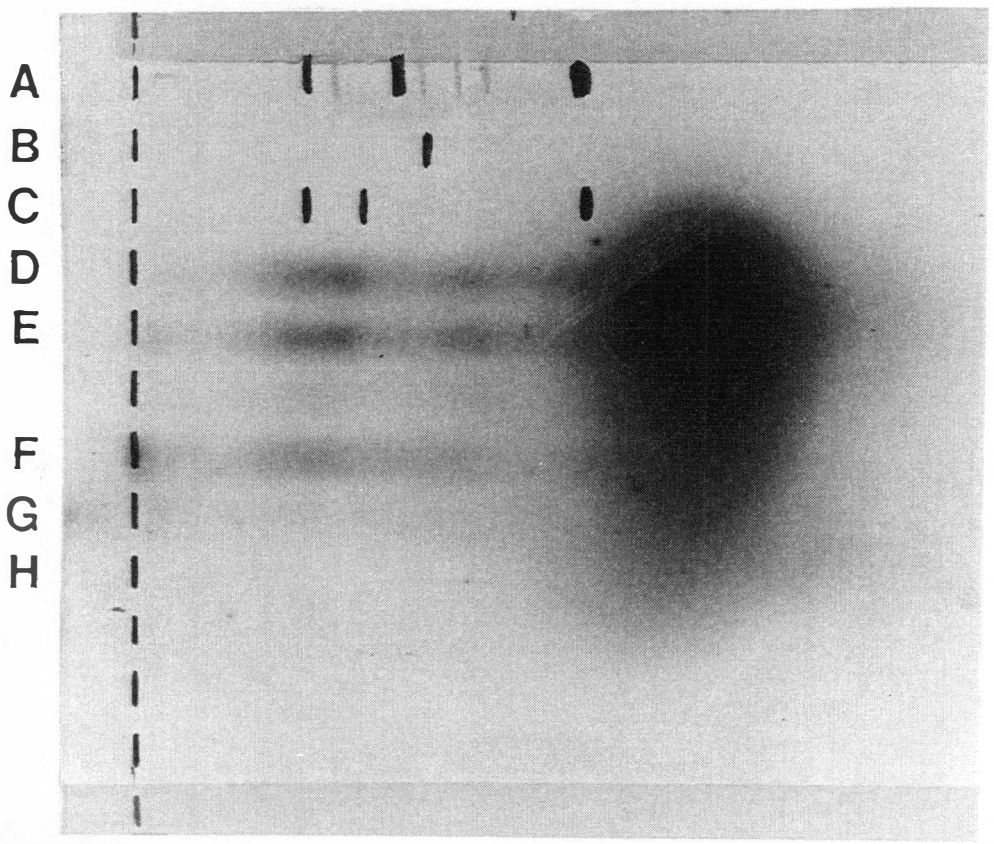

4 DAILY PHENYLHYDRAZINE INJECTIONS
SUBCUT. BLED ON 6th DAY
WASHED 3X IN FRESH 0.9% SALINE
RETIC COUNTS AND
HCTS DONE
CELLS RESUSPENDED IN
3M GLUCOSE
CELLS INFUSED
INTO NAIVE
RECIPIENTS
LEAD ACETATE DOSE IN 3M
GLUCOSE INJECTED
FOLLOWING CELL INFUSION
ANIMALS CARRIED CELLS AND
LEAD DOSE 24 hrs. BLED.
CELLS USED FOR EXTRACTION
OF RNA, HCT AND RETIC COUNTS

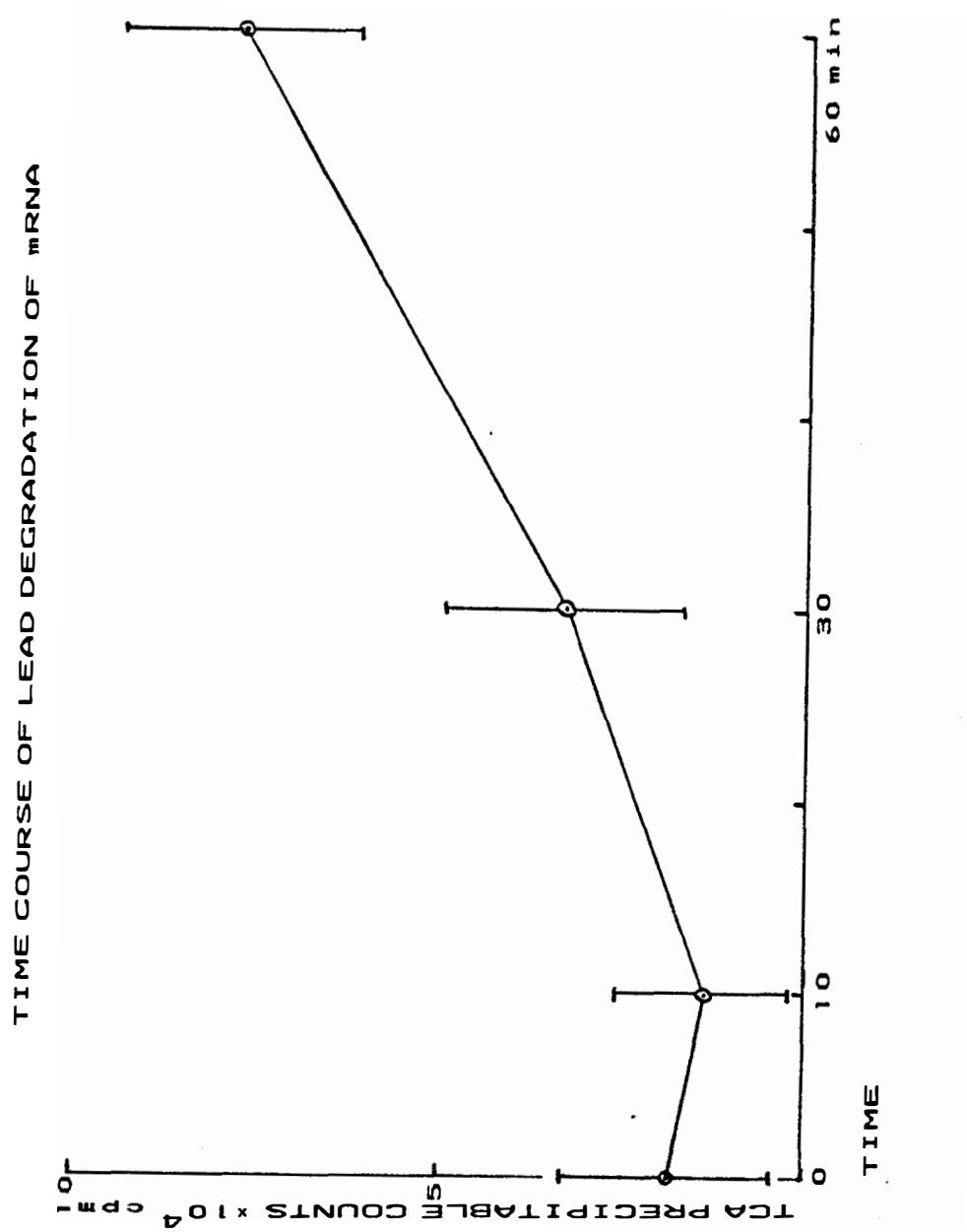

IN VIVO EFFECT OF LEAD ON mRNA TRANSLATABILITY
TCA PRECIPITABLE COUNTS x 10⁶ cpm
4
3
2
1
0 mg/kg
8 mg/kg
1
2
3
MICROGRAMS RNA ADDED

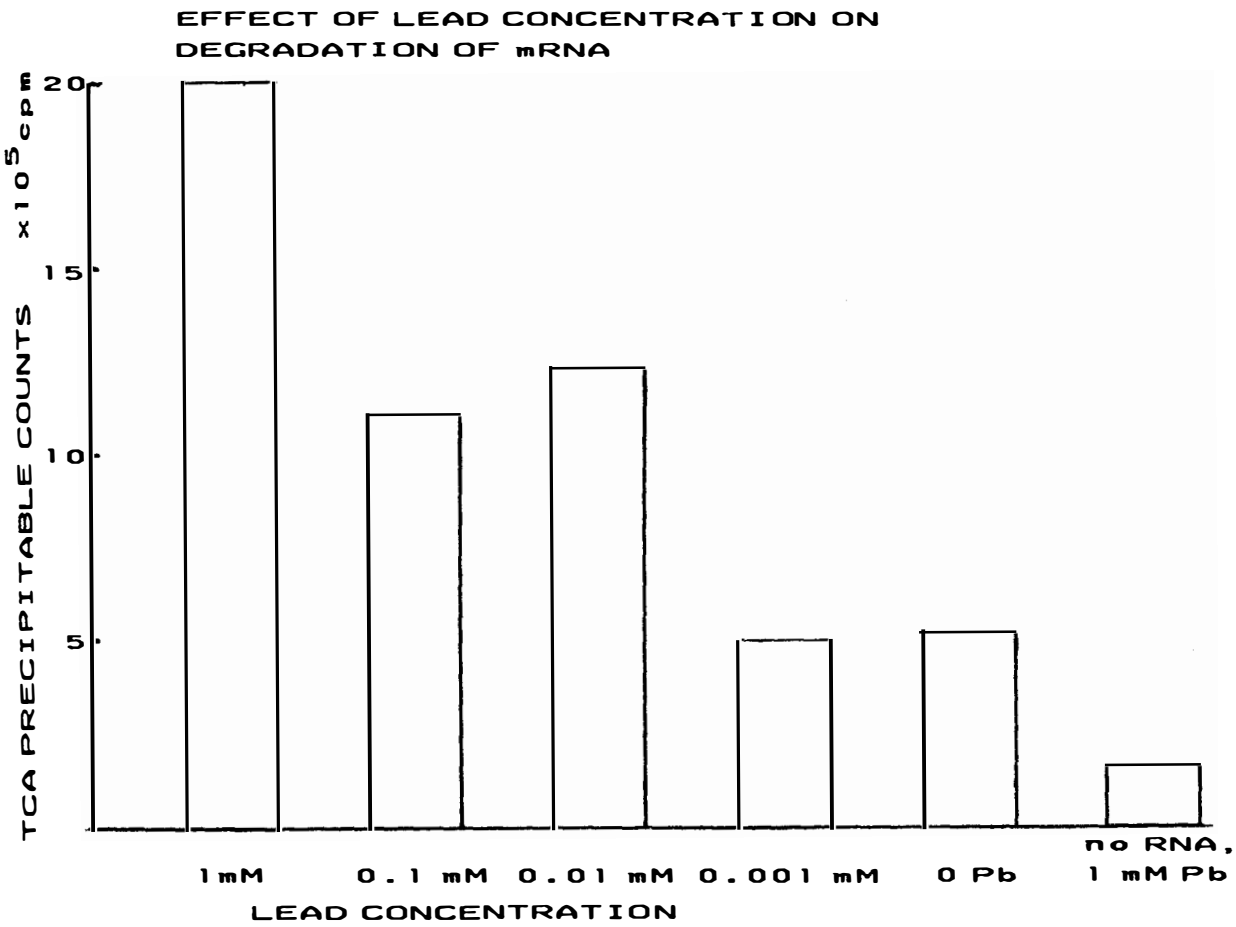

A260
0.4
0.3
0.2
0.1
80S
5
10
15
20
25
Fraction Number



hours	ppm Pb	uM Pb	ug Pb/dl
0	0.023	1.11	2.3
0.5	2.3	111.0	230
3.5	0.959	46.3	95.9
8.0	0.510	24.6	51.0
12.5	0.513	24.8	51.3
23.5	0.513	24.8	51.3
27.5	0.465	22.4	46.5


A
B
C
D
E
F
G
H


TIME COURSE OF LEAD DEGRADATION OF mRNA
TCA PRECIPITABLE COUNTS x 10⁴ cpm
10
5
0
10
30
60 min
TIME


EFFECT OF LEAD CONCENTRATION ON
DEGRADATION OF mRNA
TCA PRECIPITABLE COUNTS x10^5 cpm
20
15
10
5
1mM
0.1 mM
0.01 mM
0.001 mM
0 Pb
no RNA, 1 mM Pb
LEAD CONCENTRATION

VITA

This degree is being awarded post-humously.

Anthony Aethranis was killed in an accident at a construction site. Tony was a skilled carpenter and drew pleasure from the fact that his work benefitted others. Although he could have made a comfortable living in the construction industry, Tony felt it essential that he obtain a higher education. He was awarded a Bachelor's Degree in Science from The University of Tennessee in Knoxville. In 1986 he was accepted as a graduate student in The Graduate Program in Environmental Toxicology at The University of Tennessee. In 1980 he had completed the laboratory work that is the content of this thesis. At this time, he realized that he needed more than a career in science to fulfill his lifework. Tony was a very religious person and he enrolled in The Southern Baptist Seminary in Louisville, Kentucky which is the reason that this thesis is being submitted after the laboratory work was completed. He graduated from the seminary in May, 1993 and would have presented this thesis in completion of the requirements for an M.S. degree to the University of Tennessee during the summer of 1993. His untimely death prevented that.

There is an increasing awareness on the impact of toxic substances on the lives of our population and the environment. The knowledge of the public

about toxicology is often marginal and there giving rise to distrust in the scientists and industrialist who try to describe the benefits and risks of industry and its potentially harmful products. A person with a knowledge of the science of toxicology and training in tending to peoples spiritual needs would have been of great value to us. He will be missed.