Effects of oral lead on serum proteins and on the development of specific antibody response in young sheep

Lorraine Johnson Hoffman

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Effects of oral lead on serum proteins and on the development of specific antibody response in young sheep

by

Lorraine Johnson Hoffman

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>HISTORICAL</td>
<td>9</td>
</tr>
<tr>
<td>EXPERIMENTAL PROCEDURES</td>
<td>15</td>
</tr>
<tr>
<td>RESULTS</td>
<td>23</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>61</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>81</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>83</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>88</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>90</td>
</tr>
</tbody>
</table>
INTRODUCTION

The genius of the human mind and the resourcefulness of the human hand have endowed future generations with an infamous legacy—their environment, for which man is now more responsible than God.

Although the United States has achieved technological supremacy during the past several decades, it is evident that changes and advancements in technology only serve to hasten the appearance of new issues and paradoxes. Certainly one of the major problems evolving from industrialization and mechanization is that of inordinate quantities of potentially toxic materials being inappropriately utilized and indiscriminately disseminated into our natural environment. When one thinks in terms of drinking, eating, and/or inspiring a portion of this humanly adulterated product each day, there is no need to belabor the potential hazard which has been created and will continue to be created for man and other animals. It appears that scientists along with the other citizenry of our country must take immediate action for controlling and preventing environment-induced diseases.

Metallic elements are environmental contaminants which merit recognition as culprits. Goldwater and Clarkson (1972) have discussed numerous physical adversities including death as a result of chronic or acute mercury poisoning. Cadmium may also elicit toxic effects such as nausea and vomiting, respiratory insufficiencies, and altered urine biochemistry (Fassett, 1972). Smith (1972) has reported that cadmium, nickel, vanadium,
arsenic, and manganese may be potential environmental assaultants if their use and dissemination is not properly controlled.

Lead may be described as a useful, desirable, and necessary heavy metal which is found in great abundance throughout the world; however, this natural substance holds a significant claim to notoriety. Excessive quantities of lead produce clinical disease affecting primarily the nervous system, the hematopoietic system, and the kidneys. Lead poisoning, spoken of in the classical sense, has been present on this earth for centuries. A vast array of scientific documentation already exists regarding incidence, toxic manifestations, diagnosis, and treatment of symptomatic lead poisoning in man and animals. Zook et al. (1969) and Buck (1970) described it as one of the most significant inorganic poisonings in domestic animals. Plumbism in children is generally associated with lower socio-economic groups who live in dilapidated housing where lead-contaminated paint or putty is readily accessible for nibbling (Chisolm and Harrison, 1956, Griggs et al., 1964, Chisolm and Kaplan, 1968, and Chisolm, 1971). Although pica (habit of eating nonfood substances) and lead fumes are the normal cause for symptomatic lead poisoning in children, adult plumbism is often a result of poor hygienic practices in lead-oriented industries (Haley, 1971). Chisolm (1971) reported that imbibers of lead-contaminated whiskey and individuals who who eat or drink from improperly lead-glazed vessels may also be potential victims of lead toxicity.

There are several recent books and review articles that provide detailed information relative to environmental lead, including its
distribution, input and disposition in man and animals, toxicology and pharmacology, biologic or health effects, and nonbiologic effects (Tepper and Pfizer, 1970, DeBruin, 1971, Committee on Biologic Effects of Atmospheric Pollutants, 1972, Goyer and Chisolm, 1972, and Hicks, 1972). Figure 1 is a schematic flow-chart as presented by Goyer and Chisolm (1972) which summarizes sources of lead in the environment and the consequent effects on our population.

Aside from symptomatic or acute lead poisoning which is the result of a large accumulation of lead over a relatively short period of time, there is yet another concern relative to health and the presence of lead in our environment. It is essential that we learn whether or not current levels of lead absorption are resulting in subtle, subclinical, nonspecific effects on the health of our living population. Indisputably there has been increased usage and dissemination of this heavy metal during recent years, especially in the form of airborne emissions from autos and industry; therefore, the margin of safety existing between the normal daily intake of lead and that level of consumption which actually precipitates overt, recognizable symptoms must be determined.

Although Goyer and Chisolm (1972) recently stated that the majority of the population has not sustained physical or mental debilitation from current levels of lead absorption, a preceding report by Tepper and Pfizer (1970) expressed the consensus of several authorities presently studying the effects of lead. They concurred that at some point short of lead toxicity it is quite possible the body's absorption of
Figure 1. Ecodiagram of lead in the environment and effects on man (taken from Goyer and Chisolm, 1972).
Lead Ores

Sources

- Manufacturing and Industrial Processes
  - Airborne Emissions from Autos and Industry
    - Soil Wash by Rain
    - Edible Plant Life
      - Poultry and Meat
      - Oral Intake by Man
      - Inhalation
- Industrial Wastes
  - Rivers, Lakes, Oceans
  - Drinking Water
  - Aquatic Life
- Lead Objects
  - Paint
  - Plaster (Pica)
  - Putty
  - Toys
  - Pottery

Distribution

- G.I. Absorption
- Feces
- Red Blood Cells (non-diffusible)
- Plasma (Ligand Bound, diffusible)
- Bone (non-diffusible)

Pharmacodynamics

- CNS
- Reticulocytes
- Kidney
- Urinary Excretion

Health Effects

- Encephalopathy
- Peripheral Neuropathy
- Anemia
- Tubular Dysfunction
- Miscellaneous Effects

Reproductive
Endocrine
Cytogenic
lead reaches a level sufficient to create significant alterations in normal physiologic function. In order to determine the existence and subsequent significance of these subclinical or subtle perturbations, it is necessary that techniques be developed which are more sensitive and specific than the conventional devices now being employed for detection of lead effects. When this has been accomplished, we will be better equipped to determine the magnitude of threat which industrial utilization of lead poses to the health and welfare of that portion of our human and animal population which is exposed to appreciable quantities of lead each day.

All normal mammals are equipped with a highly-developed immune system which is essential for their well-being throughout life. Immunologic phenomena have been classically placed into two definite categories: (1) nonspecific or innate immunologic responses and (2) specific acquired immunologic responses. According to Bellanti (1971), the three primary functions performed by these immunologic processes are "defense"—protection of the individual against invasion by microorganisms, "homeostasis"—removal of effete and damaged cells, and "surveillance"—recognition and destruction of mutant cells. Although complex and variable owing to the many interacting natural and acquired immunologic factors (Figure 2), this physiologic system is tremendously efficient and well-organized. If subtoxic levels of lead impair, destroy, alter, or diminish one or more of the major defensive components, the implications could be highly significant with regard to our human and animal populations. First of all, if such a phenomenon does exist, it could yield individuals
I. Natural immunity (nonspecific)
   A. Physical barriers (skin and mucous membranes)
   B. Cellular barriers (phagocytic cells, reticuloendothelial system and inflammatory response)
   C. Humoral barriers (complement, properdin, lysozyme, skin secretions, natural antibodies, etc.)

II. Acquired immunity (specific)
   A. Antibody production
   B. Cell-mediated response (delayed hypersensitivity)
with enhanced susceptibility to certain bacterial and viral organisms thereby dramatically increasing the severity of infection and disease. Subsequently, unwarranted stress may be imparted to vital organs of the body due to repeated and progressively debilitating infections. Depending upon the focal point of the infectious agent, it is also quite possible that specific organs would sustain permanent damage.

Although the literature is not replete with information concerning the effects of lead and other pollutants on the immune system, there has been definite indication that subclinical levels of lead influence or interfere with one or more specific defense mechanisms. Through the expansion of immunologic research into diverse clinical and experimental areas, resistance and immunologic activities have been well-defined during the past several years; therefore, the immune response system could provide an extraordinarily sensitive approach to studying subclinical effects of lead. It is imperative at this point in time that definitive, sensitive assays be developed for detection of minimal alterations in normal physiological mechanisms.

This project was initiated in an effort to determine the effects of various levels of lead on the humoral immunologic system of young sheep. Two areas of experimentation were followed in this research endeavor. The first was designed to evaluate the development and alterations in serum proteins, especially in immunoglobulins, as a response to dietary lead. The second area of investigation involved studying specific antibody synthesis and activity in lead-treated animals.
HISTORICAL

Williams et al. (1954) were the first to indicate that lead may be an immunosuppressive agent. This relationship was expressed by pathological evidence obtained from an autopsy of a 23-month-old child with an extensive history of paint nibbling. Although the youngster's blood lead level had reached 0.348 mg per 100 ml at the time of death, the pathological abnormalities of the central nervous system were more indicative of a bacterial septicemia than of lead toxicity. After demonstrating that lead precipitates gamma globulins in vitro, the investigators concluded that the child's death may have been the result of lead-imposed inactivation of antibodies, thus allowing unrestricted growth of pathogenic agents and elaboration of toxic products.

Evidence in the literature strongly suggests that various environmental pollutants alter an animal's resistance to infection. Research by Rylander (1969 and 1970) indicated that sulfur dioxide, carbon dust, and cigarette smoke may interfere with an animal's defense against subsequent infectious agents.

Recent documentations have revealed that lead acetate administered simultaneously with endotoxins from various gram negative bacteria potentiates the toxic effect of the endotoxins in certain laboratory animals. Selye et al. (1966) discussed this effect in rats, a species which is normally resistant to infection enhancement by bacterial endotoxins. They observed a 100,000-fold increase in endotoxin sensitivity of the rats which were exposed to a nontoxic dosage of lead acetate concurrently...
with the injection of endotoxin. The researchers speculated that the lead acetate acted as a blocking agent for the reticuloendothelial system, which plays an integral role in protecting the body against endotoxin invasion. Truscott (1970) studied this phenomenon in young chicks, with similar results. The toxicity of *Escherichia coli* endotoxin was increased 1,000 times when given simultaneously with non-toxic levels of lead acetate.

Another illustration of potential immunologic effects of chemical pollutants at sublethal concentrations was presented by Friend and Tanner (1970). They found young mallard ducks which had experienced previous exposure to nontoxic levels of an organo-chlorine substance suffered higher mortality upon challenge with duck hepatitis virus than ducklings which had not encountered the hydrocarbon pollutant. This report suggested that polychlorinated biphenyls may reduce an animal's ability to resist some pathogenic agents.

After studying the effects of subclinical levels of lead nitrate on resistance of mice to *Salmonella typhimurium*, Hemphill et al. (1971) concluded that lead-treated animals were far less tolerant to bacterial challenge than the controls which received no lead. The investigators first determined how much lead a mouse can tolerate without manifesting clinical symptoms. Once this had been established, the effect of lead levels on resistance to infection was tested as follows: 75 mice were divided into three equal-sized groups including a control group and two exposed groups which received 100 μg and 250 μg of soluble lead nitrate, respectively. These amounts were injected via the intraperitoneal
route on a daily basis for four weeks. Following the period of lead exposure, five dilutions ($10^{-3.0}$, $10^{-3.7}$, $10^{-4.0}$, $10^{-4.7}$, and $10^{-5.0}$) of a selected strain of *Salmonella typhimurium*, previously shown to have an LD$_{50}$ of $10^{-3.7}$, were prepared for challenging the mice. Each bacterial dilution was injected into five mice from each of the three groups. There was 100% mortality in the high lead group three days post challenge. The mice which received 100 μg of lead nitrate per day showed 50% mortality within seven days after challenge. Ten days subsequent to challenge only 13% of the control group had succumbed to bacterial infection. It was concluded that continuous exposure of mice to subclinical levels of lead significantly increased their susceptibility to the particular strain of *Salmonella typhimurium* used for challenge.

The aforementioned studies are highly suggestive that various forms of lead (and possibly other chemical pollutants) administered at levels below the dosage causing clinical toxicity may alter or reduce the efficiency of immune responses. It is yet to be ascertained exactly what defense mechanism or mechanisms are affected by lead and how lead acts to encroach upon their efficacy.

There has been scanty indication in the literature that lead may interfere with natural or innate factors of the defense system. Kiryachko (1957) and Goreczky et al. (1957) have reported lowered complement titers in rabbits suffering from acute or chronic lead poisoning. *In vitro* inhibition of the hemolytic action of guinea pig complement has been shown to occur in the presence of lead chloride (Miyazaki, 1959). Hemphill (1973) demonstrated a complete absence or marked reduction of properdin activity in lead-treated mice. Workers in a lead
smelting plant, where high level exposure to lead occurred on a daily basis, manifested derangement in phagocytic and bactericidal activity of their blood, deficiency in salivary lysozyme levels, and changes in anti-microbial activity of the buccal mucosa (Rakhimova, 1968). Contrary to this trend of inhibition, Goreczky et al. (1957) noted a rise in the opsonic activity of rabbit serum after exposure to lead acetate. Alterations in ciliary function and efficiency of alveolar phagocytes in the lung after exposure to carbon dust and toxic gases have also been discussed (Green, 1970).

Although results have been somewhat inconsistent, several reports can be found in the literature relative to serum protein levels in individuals exposed to abnormally high levels of lead. Kapetanovic et al. (1960) analyzed serum protein fractions from 36 persons with chronic lead poisoning. The major alterations appeared in the albumin fraction which decreased by an average of 20% and the gamma-globulin component which showed an average diminution of 50%. Contradictory to these findings, Soliman et al. (1970), in their study of 112 Egyptian lead workers, found 17% exhibited a significant reduction in alpha₁-globulin and alpha₂-globulin levels. The albumin, beta-globulin, and gamma-globulin levels were virtually unchanged. Observations made by Cervetti and Casucci (1960) on individuals suffering from severe lead poisoning yielded results somewhat divergent from those previously discussed. Their study revealed a decrease of total protein and albumin levels in several individuals while an enhancement beyond normal levels was shown in the alpha, beta, and gamma serum constituents. Hayashi et al. (1959)
induced lead poisoning in rabbits by various routes of exposure. Electrophophoretic analysis indicated elevated gamma-globulin in animals poisoned via the injectant or inhalant routes. The other major alteration occurred in the albumin fraction which decreased significantly after inhalation of lead compounds.

Minimal documentation is available concerning the effect of lead and other environmental irritants on specific immunity or the synthesis and mobilization of antibodies against antigenic stimuli. In 1955 Belli et al. published a series of papers which evaluated immunologic processes in rabbits exposed to high levels of tetraethyl lead. They found no increase in antibody globulins following injection of poisoned rabbits with *Streptococcus* antigen. Another group of rabbits was subjected to *Salmonella typhi* in order to determine specific agglutinin titers produced against that organism. Again, the antibody response was definitely impaired. To further verify these studies, Giuliani and Belli (1956a and 1956b) demonstrated reduced agglutinin titers in lead-treated rabbits challenged with *Brucella melitensis* and typhoid antigen. Hemphill (1973) recently noted a significant reduction of antibody titers against *Salmonella typhimurium* in lead-treated mice, as compared to control mice.

Control and carbon-dust exposed mice have been compared in their ability to respond to antigens administered via the intraperitoneal, intravenous, and inhalation routes (Zarkower and Morges, 1972). Although carbon manifested an overall immunosuppressive effect, the diminution in antibody-forming cells and serum agglutinin titers was most pronounced when the antigen was given as an aerosol by inhalation. After four days
pre-exposure to carbon dust, there occurred an increase in antibody-forming cells in the mediastinal lymph nodes. However, this was apparently a transient effect because it disappeared when carbon pre-exposure was continued for 15 days.
EXPERIMENTAL PROCEDURES

Lambs

Twenty-five normal, healthy, Columbia-Rambouillet crossbred lambs obtained from inresidence breeding stock maintained at the Behavioral Toxicology Laboratory, Iowa State University were used in this study. All of the lambs were born between June 6, 1972 and August 22, 1972. Their individual birthweights were in the range of 4 to 8 kg. At birth, each animal was assigned a number in sequential fashion (lamb 1-#51, lamb 2-#61, lamb 3-#71, lamb 4-#81, lamb 5-#91, lamb 6-#52, etc.) and was then placed by that number into one of five groups as follows: group 1, lambs #51-#55; group 2, lambs #61-#65; group 3, lambs #71-#75; group 4, lambs #81-#85; group 5, lambs #91-#95. Numbered ear tags and back markings were used to identify each of the 25 animals. After the lambs were weaned at approximately 6 weeks of age, they were maintained on a commercial lamb supplement and received water ad libitum.

Lead Compounds

Lead acetate crystals (reagent grade) were obtained from a commercial source. Two basic lead stock solutions were utilized for oral exposure of lambs. These solutions were prepared by dissolving lead acetate in distilled water at concentrations of 10 mg/ml and 20 mg/ml, respectively. Test lambs received a prescribed volume of one of these

1 B-P Lamb Starter Pellets, Protein Blenders, Inc., Iowa City, Iowa.
2 J. T. Baker Chemical Co., Phillipsburg, New Jersey.
standard lead solutions each day, as dictated by their weights and the exposure group to which they belonged. All lambs were force-fed using plastic tubing and a syringe.

**Lead Exposure**

Each lamb, except for those in the control group, received a daily (5 days/week) oral dosage of lead in the form of solubilized lead acetate according to the following paradigm: group 1 (control group), no lead; group 2, 2 mg lead/kg body weight; group 3, 4 mg lead/kg body weight; group 4, 8 mg lead/kg body weight; and group 5, 16 mg lead/kg body weight. Lead exposure for each lamb began at 5 days of age and continued for approximately 12 weeks (60 dosage days). The lambs were weighed each Monday throughout the period, in order to determine the amount of lead which they were to receive during the forthcoming week. Lead exposure was terminated after 12 weeks because 3 lambs in group 5 (16 mg/kg) succumbed to irreversible lead toxicosis, and our objective was to standardize the period of lead exposure across all groups.

**Blood Collection**

Venous blood samples were collected in heparinized lead-free Vacutainers\(^1\) from the lead-treated and control lambs. The initial heparinized blood sample (10 ml) was obtained from each lamb at 5 days of age. Subsequent samples were collected at intervals of 2, 4, 8, 12, 16, 20, 24, and 28 weeks. After each collection, these blood samples

\(^1\)Becton-Dickinson and Company, Rutherford, New Jersey.
were analyzed for lead by the method of atomic absorption spectrophotometry (Hessel, 1968).

Serum Collection and Storage

Blood samples (10 ml) collected in anticoagulant-free Vacutainers were allowed to clot at room temperature for approximately 1 hr. The clotted blood samples were refrigerated at 4 C overnight and then were centrifuged at 850 x g for 15 min to separate sera. Each of the serum samples was divided into two equal portions, one of which was stored under refrigeration at 4 C with merthiolate added to a final concentration of 0.0001 %, and the other portion was frozen and stored at -20 C. After obtaining the first serum sample from each animal at 5 days of age, serum collection continued on a weekly basis for a period of 6 months (28 weeks).

Serum Protein Evaluations

Total protein determinations

Total serum protein was measured with a refractometer. This was accomplished by holding the instrument in a horizontal plane and applying a drop of serum sample (0.05 ml) directly onto the glass window. By exposing the unit to an illuminating source, the value on the scale as seen through the eyepiece could be read. This reading represented the total quantity of serum protein in g/100 ml.

---

1 Becton-Dickenson and Company, Rutherford, New Jersey.
Cellulose acetate electrophoresis

Serum proteins were separated in a microzone chamber\(^1\) on cellulose acetate membranes\(^2\) using high resolution Tris-barbital buffer\(^3\), pH 8.8 and ionic strength of 0.05. Eight serum samples each of approximately 0.25-\(\mu\)l volume were applied to each membrane. As the microzone cell accommodated 3 membranes, it was possible to electrophoretically separate 24 serum samples simultaneously. Twenty-five minutes treatment at 250 V separated the serum proteins. Following electrophoretic migration, the proteins were stained with Ponceau S.\(^4\)

A photoelectric densitometer\(^5\) was utilized to scan the membranes and to print out an integrated density curve based on the amount of light absorbed by the stained protein bands. Optical density of the separated protein fractions was evaluated at 600 nm. For quantitative evaluation of the completed electrophoretic patterns, individual peaks were separated by drawing vertical lines through their lowest points. As the number of integrative pulse strokes between vertical lines is representative of the area under the curve, division of each component area count by total count yielded the relative percentage of each major

\(^1\) Brinkman Sartorius, Brinkman Instruments, Westbury, New York.
\(^2\) Beckman Instruments, Fullerton, California.
\(^3\) Gelman Instrument Company, Ann Arbor, Michigan.
\(^4\) Buchler Instruments, Ft. Lee, New Jersey.
\(^5\) Model R110, Beckman Instruments, Fullerton, California.
serum protein fraction contained in the sample. Relative concentrations were readily converted to absolute weights by multiplying total protein content by percentage composition.

Details of this procedure may be found in the Appendix.

**Immunoelectrophoresis**

A modification of Scheidegger's (1955) microslide immunoelectrophoresis technique was utilized in this study for separating and differentiating serum protein components, including the immunoglobulin subgroups. Immunoelectrophoresis was carried out in 1% Noble Agar\(^1\) and high resolution Tris-barbital buffer at pH 8.8 and 0.05 M ionic strength. After deposition of serum into the wells, the samples were electrophoresed in a chamber\(^2\) for 120 min at 4-5 ma (75 v) per frame. The troughs were then filled with approximately 0.15 ml of rabbit antisheep serum\(^3\), and the slides were allowed to incubate in a humidified atmosphere for 16-20 hr at room temperature. Following complete development of the precipitin lines, the immunoelectrophoretic patterns were photographed using a Nikon Photomic FT\(_N\) (automated reflex) camera.\(^4\)

Details of this procedure may be found in the Appendix.

---

\(^1\)Difco Laboratories, Inc., Detroit I, Michigan.

\(^2\)Gelman Deluxe Electrophoresis Chamber, Gelman Instrument Company, Ann Arbor, Michigan.

\(^3\)Schwarz/Mann, Division of Becton-Dickinson and Company, Orangeburg, New York.

\(^4\)Nippon Kogaku K. K., Japan.
Development of Specific Immune Response

Antigen

A heat-killed bacterin of *Serratia marcescens* was used in this study. The vaccine was prepared by seeding the surface of layered nutrient agar contained in a Kolle flask with 10 ml of a 19-hr *Serratia marcescens* (obtained from culture collection, Department of Bacteriology, Iowa State University, Ames, Iowa) broth culture. After incubation at 25 °C for 24 hr, the cells were collected into a sterile centrifuge tube by washing the agar several times with sterile physiological saline. The bacterial suspension was boiled for 2 hr, then it was collected in a RC2-B refrigerated centrifuge\(^1\) for 1/2 hr at 1400 x g using the SS-34 angle head rotor. This step was followed by resuspension in sterile saline to a concentration of 10% (v/v). Sterility was checked by appropriate bacteriologic tests; the suspension was then placed in a sterile vaccine bottle, and it was stored in the refrigerator at 4 °C. For the purpose of immunizing lambs, it was diluted with saline to a density of Tube 4 on the McFarland nephelometer scale (approximately \(10^9\) bacteria/ml) (Campbell et al., 1970).

Immunization schedule

Upon reaching 6 weeks of age, each of the 25 lambs was inoculated intravenously (jugular vein) with 6 injections of heat-killed *Serratia marcescens*, according to the paradigm presented in Table 1.

\(^1\)Ivan Sorvall, Inc., Norwalk, Connecticut.
Table 1. Injection schedule for 6 week old lambs

<table>
<thead>
<tr>
<th>Day</th>
<th>Ml</th>
<th>No. of cells</th>
<th>Route</th>
</tr>
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<tr>
<td>1</td>
<td>0.5</td>
<td>$1.5 \times 10^9$</td>
<td>Intravenous</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>$1.5 \times 10^9$</td>
<td>Intravenous</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>$1.5 \times 10^9$</td>
<td>Intravenous</td>
</tr>
<tr>
<td>7</td>
<td>1.0</td>
<td>$3.0 \times 10^9$</td>
<td>Intravenous</td>
</tr>
<tr>
<td>14</td>
<td>1.0</td>
<td>$3.0 \times 10^9$</td>
<td>Intravenous</td>
</tr>
<tr>
<td>21</td>
<td>1.0</td>
<td>$3.0 \times 10^9$</td>
<td>Intravenous</td>
</tr>
</tbody>
</table>

Approximately 60 days after the final injection of the initial series, each animal received a secondary injection of *Serratia marcescens* (1 ml, $3.0 \times 10^9$ cells) intravenously or subcutaneously. This later exposure was given for the purpose of evaluating the secondary or anamnestic response.

**Antibody titration**

Modifications of combined passive hemagglutination techniques of Neter et al. (1956) and Field et al. (1970) were used to determine antibody titers. The Microtiter System\(^1\) was employed in this study.

*Serratia marcescens* endotoxin\(^2\) prepared at a concentration of 10 mg/ml was used to sensitize sheep erythrocytes at a ratio of 1 part

\(^1\) Cooke Engineering Company, Alexandria, Virginia.

\(^2\) Difco Laboratories, Inc., Detroit 1, Michigan.
red blood cells to 4 parts endotoxin. This red cell-lipopolysaccharide mixture was incubated for 1/2 hr at 37 C. The treated cells were then washed 3 times by serial centrifugation, using 0.85% NaCl as suspending medium. After washing was completed, the modified cells were resuspended to a concentration of 2.5% (v/v) with P04-buffered saline, and were again diluted to a concentration of 0.5% for use in the passive hemagglutination test.

The procedure for the hemagglutination test was as follows: employing a Microtiter pipette, 0.025 ml of 1% normal rabbit serum was placed in each well of a Microtiter U plate. After adding 0.025 ml of test serum (diluted 1:5) to the first well, two-fold serial dilutions were made. Next, 0.025 ml of the endotoxin-sensitized sheep red blood cells was added to each well. Plates were incubated for 1 hr at 37 C, and then were stored overnight in the refrigerator. Serum antibody titers were expressed as the reciprocal of the highest dilution of serum that produced complete agglutination of the lipopolysaccharide-coated sheep red blood cells.

Treatment of the serum samples with 2-mercaptoethanol (2-ME), a sulfhydryl reducing agent which inactivates 19S gamma globulin, served as a crude indication of 7S and 19S antibody. Duplicate samples of sera were incubated for 1 hr at 37 C with an equal volume of 0.2 M 2-ME before dilution. The sera were then treated as previously described (Field et al., 1970).

Details of this procedure may be found in the Appendix.
RESULTS

Blood Lead Determinations of Control and Lead-Treated Lambs

The mean blood lead level for the control lambs (group 1) was 11.7 μg/100 ml; groups 2, 3, 4 and 5 which individually received 2, 4, 8 and 16 mg lead/kg body weight displayed mean blood lead levels of 37.2, 56.8, 67.6 and 99.8 μg/100 ml, respectively. Group means for blood lead residues obtained at specific intervals are displayed in Table 2.

After subjecting these data to an analysis of variance, the LSD values (Steel and Torrie, 1960) were utilized to determine whether the overall mean blood lead levels of the five groups were significantly different from one another. A summarization of the statistical comparisons between the experimental groups of lambs appears in Table 3. A statistically significant difference at the 1% level existed between all groups, except for groups 3 and 4. Although analysis of overall mean values reflected at least three distinct levels of lead feeding, group means for blood lead were not consistently distinct throughout the experimental period, especially after termination of lead exposure.

Serum Protein Analysis of Control and Lead-Treated Lambs

Total serum protein values, as measured with a refractometer, and serum protein patterns obtained by electrophoresis on cellulose acetate membranes, were employed to derive quantitative data for individual lambs at weekly intervals following initiation of lead treatments at
Table 2. Blood lead levels in control and lead-treated lambs

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Group 1 Control</th>
<th>Group 2 2 mg/kg</th>
<th>Group 3 4 mg/kg</th>
<th>Group 4 8 mg/kg</th>
<th>Group 5 16 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>11.4</td>
<td>12.0</td>
<td>15.6</td>
<td>14.2</td>
<td>15.2</td>
</tr>
<tr>
<td>14</td>
<td>10.8</td>
<td>47.6</td>
<td>66.8</td>
<td>66.6</td>
<td>108.4</td>
</tr>
<tr>
<td>28</td>
<td>12.6</td>
<td>64.6</td>
<td>106.8</td>
<td>112.5</td>
<td>163.8</td>
</tr>
<tr>
<td>56</td>
<td>16.2</td>
<td>55.0</td>
<td>77.0</td>
<td>125.0</td>
<td>160.0</td>
</tr>
<tr>
<td>84</td>
<td>14.6</td>
<td>39.5</td>
<td>57.0</td>
<td>85.6</td>
<td>99.3</td>
</tr>
<tr>
<td>112</td>
<td>6.0</td>
<td>18.0</td>
<td>36.5</td>
<td>45.6</td>
<td>48.0</td>
</tr>
<tr>
<td>140</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>168</td>
<td>8.6</td>
<td>20.5</td>
<td>28.0</td>
<td>32.6</td>
<td>31.5</td>
</tr>
</tbody>
</table>

*Groups 1, 2, 3, 4 and 5 ingested 0, 2, 4, 8 and 16 mg lead/kg body weight on a 5 day/week regimen for approximately 12 weeks (60 dosage days) beginning at 5 days of age.

*b Four animals/group - adjusted group means were obtained if unequal cell frequencies occurred.

*c Lead exposure was terminated prior to 84th day.

*d Two animals/group - adjusted group means were obtained if unequal cell frequencies occurred.

*e No data due to laboratory error.
Table 3. Statistical comparisons of blood lead levels in control and lead-exposed lambs

<table>
<thead>
<tr>
<th>Group comparisons</th>
<th>Probability values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 vs 2</td>
<td>(P&lt;0.01)</td>
</tr>
<tr>
<td>1 vs 3</td>
<td>(P&lt;0.01)</td>
</tr>
<tr>
<td>1 vs 4</td>
<td>(P&lt;0.01)</td>
</tr>
<tr>
<td>1 vs 5</td>
<td>(P&lt;0.01)</td>
</tr>
<tr>
<td>2 vs 3</td>
<td>(P&lt;0.01)</td>
</tr>
<tr>
<td>2 vs 4</td>
<td>(P&lt;0.01)</td>
</tr>
<tr>
<td>2 vs 5</td>
<td>(P&lt;0.01)</td>
</tr>
<tr>
<td>3 vs 4</td>
<td>No significant difference</td>
</tr>
<tr>
<td>3 vs 5</td>
<td>(P&lt;0.01)</td>
</tr>
<tr>
<td>4 vs 5</td>
<td>(P&lt;0.01)</td>
</tr>
</tbody>
</table>
5 days of age. All of the electrophoretic patterns showed excellent resolution into five component peaks composed of the following proteins: albumin, \( \alpha_1 \) (\( \alpha_2 \))-globulin, \( \alpha_2 \) (\( \alpha_2 \))-globulin, \( \beta \)-(\( \beta \))-globulin, and \( \gamma \)-(\( \gamma \))-globulin.

It was considered highly redundant to engage in the cumbersome task of presenting, evaluating, and analyzing weekly data from each of the test animals; therefore, nine sampling dates were selected to provide a representative set of data for determining the existence of alterations or significant trends which might be occurring in the serum protein profiles of the lambs. All individual lamb data, and other data compilations obtained throughout this experimental undertaking are on file at the Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa.

Tables 4, 5, 6, 7, 8 and 9 summarize mean values of the calculated serum protein concentrations in grams/100 ml for each group of lambs at 5, 14, 28, 42, 56, 84, 112, 140 and 168 days of age, respectively. Range values, along with overall group-and time-means, also appear in the tables.

All serum-protein data were subjected to an analysis of variance to test for significant effects of lead exposure on each parameter measured. The analysis of mean total protein levels (Table 4) indicated that no significant group differences were present. Although one of the lead-treated groups manifested a sharp decrement in total protein at 84 days, there is no indication that this change resulted directly
Table 4. Total protein values in control and lead-treated lambs

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Group 1 Control</th>
<th>Group 2 2 mg/kg</th>
<th>Group 3 4 mg/kg</th>
<th>Group 4 8 mg/kg</th>
<th>Group 5 16 mg/kg</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5.55 (5.30-5.90)</td>
<td>5.06 (4.10-6.40)</td>
<td>5.90 (5.30-6.80)</td>
<td>5.52 (4.50-6.60)</td>
<td>5.62 (5.00-6.30)</td>
<td>5.53</td>
</tr>
<tr>
<td>14</td>
<td>5.44 (5.30-6.00)</td>
<td>5.28 (4.70-5.90)</td>
<td>5.24 (4.00-6.30)</td>
<td>5.76 (4.80-6.70)</td>
<td>5.54 (4.50-6.20)</td>
<td>5.45</td>
</tr>
<tr>
<td>28</td>
<td>5.26 (4.70-6.60)</td>
<td>5.33 (4.95-6.40)</td>
<td>5.34 (4.90-5.80)</td>
<td>5.30 (5.20-6.30)</td>
<td>5.50 (4.80-6.20)</td>
<td>5.35</td>
</tr>
<tr>
<td>42</td>
<td>5.50 (4.70-6.60)</td>
<td>5.43 (4.70-6.50)</td>
<td>5.05 (4.10-5.80)</td>
<td>5.14 (4.80-5.00)</td>
<td>5.24 (4.80-5.00)</td>
<td>5.27</td>
</tr>
<tr>
<td>56</td>
<td>5.63 (4.20-6.70)</td>
<td>5.19c (4.50-5.80)</td>
<td>5.92 (4.80-8.80)</td>
<td>5.82 (5.00-6.70)</td>
<td>5.62 (4.60-7.20)</td>
<td>5.71</td>
</tr>
<tr>
<td>84</td>
<td>6.50 (5.60-7.50)</td>
<td>5.10c (4.60-6.50)</td>
<td>4.97c (4.40-8.40)</td>
<td>5.70 (5.10-6.00)</td>
<td>5.77c (4.90-6.30)</td>
<td>5.64</td>
</tr>
<tr>
<td>112</td>
<td>6.65 (5.70-7.20)</td>
<td>6.24c (5.70-7.30)</td>
<td>6.22c (5.50-6.55)</td>
<td>6.17 (5.40-7.20)</td>
<td>5.96d (5.40-5.80)</td>
<td>6.25</td>
</tr>
<tr>
<td>140</td>
<td>6.61 (6.00-7.50)</td>
<td>6.63c (5.85-8.35)</td>
<td>6.29c (5.80-6.85)</td>
<td>6.63 (5.40-7.40)</td>
<td>6.26d (5.60-6.40)</td>
<td>6.43</td>
</tr>
<tr>
<td>168</td>
<td>6.72 (6.30-7.30)</td>
<td>6.34c (5.80-7.40)</td>
<td>6.30c (5.80-6.60)</td>
<td>6.63 (5.60-7.30)</td>
<td>6.41d (5.60-6.30)</td>
<td>6.41</td>
</tr>
<tr>
<td>Mean</td>
<td>5.98</td>
<td>5.65</td>
<td>5.64</td>
<td>5.79</td>
<td>5.59</td>
<td></td>
</tr>
</tbody>
</table>

a Groups 1, 2, 3, 4 and 5 ingested 0, 2, 4, 8 and 16 mg lead/kg body weight on a 5 day/week regimen for approximately 12 weeks (60 dosage days) beginning at 5 days of age.

b Mean and range of values observed from each group.

c Four animals/group - adjusted group means were obtained if unequal cell frequencies occurred.

d Two animals/group - adjusted group means were obtained if unequal cell frequencies occurred.
from lead encounter. A general trend of increasing total protein concentration with increasing age was observed in all groups.

The analysis of the albumin data (Table 5) indicated a highly significant effect (P<.0001) of time, but no difference among the groups. All of the lead-treated groups displayed a drop in albumin on day 84, while the control group showed an increase at this point. The mean values did not differ markedly throughout the experiment; however, there was a trend of increasing albumin in all groups.

A significant difference among groups (P<.046) was detected for the $\alpha_1$-globulin component (Table 6). In order to determine where the differences occurred, LSD values were utilized for comparing group means (LSD value for comparing any two group means at the 5% level=.0593 and at the 1% level=.0889). The two highest lead treatment groups (4 and 5) differed significantly from the control group, as well as from group 2 at the 5% level. Although the validity of comparing overall mean values may be questioned when there is substantial variation in normal 5-day levels across all groups, in this particular instance, groups 4 and 5 were consistently higher than the other groups.

Analysis of variance indicated a significant group effect (P<.046) for mean $\alpha_2$-globulin levels, which are displayed in Table 7. Overall group means were then compared using LSD values (LSD value for comparing any two group means at the 5% level=.1711 and at the 1% level=.2333); groups 3, 4 and 5 were found to be significantly different from the control group. The trend exhibited in this parameter may well be given
<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Group 1 Control</th>
<th>Group 2 2 mg/kg</th>
<th>Group 3 4 mg/kg</th>
<th>Group 4 8 mg/kg</th>
<th>Group 5 16 mg/kg</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2.05 (1.86-2.26)</td>
<td>2.00 (1.67-2.40)</td>
<td>2.19 (1.74-2.71)</td>
<td>1.92 (1.57-2.24)</td>
<td>1.98 (1.93-2.12)</td>
<td>2.03</td>
</tr>
<tr>
<td>14</td>
<td>1.99 (1.64-2.44)</td>
<td>2.16 (1.93-2.39)</td>
<td>1.90 (1.60-2.53)</td>
<td>2.19 (1.78-2.91)</td>
<td>2.14 (1.61-2.51)</td>
<td>2.08</td>
</tr>
<tr>
<td>28</td>
<td>2.17 (1.90-2.56)</td>
<td>2.28 (1.80-2.55)</td>
<td>2.08 (1.96-2.16)</td>
<td>2.19 (1.75-2.63)</td>
<td>2.27 (1.75-2.84)</td>
<td>2.20</td>
</tr>
<tr>
<td>42</td>
<td>2.41 (1.96-3.00)</td>
<td>2.29 (1.97-2.48)</td>
<td>2.28 (1.78-2.42)</td>
<td>2.10 (1.46-2.76)</td>
<td>2.25 (1.79-2.44)</td>
<td>2.25</td>
</tr>
<tr>
<td>56</td>
<td>2.29 (1.80-2.93)</td>
<td>1.98 (1.73-2.26)</td>
<td>2.33 (1.77-3.46)</td>
<td>2.41 (2.19-2.76)</td>
<td>2.30 (1.87-3.57)</td>
<td>2.28</td>
</tr>
<tr>
<td>84</td>
<td>2.50 (2.03-3.04)</td>
<td>1.88 (1.60-2.15)</td>
<td>1.94 (1.61-2.06)</td>
<td>2.13 (1.89-2.25)</td>
<td>2.07 (1.91-2.30)</td>
<td>2.12</td>
</tr>
<tr>
<td>112</td>
<td>2.52 (2.38-2.76)</td>
<td>2.33 (1.98-2.95)</td>
<td>2.40 (1.86-2.82)</td>
<td>2.42 (1.71-3.23)</td>
<td>2.15 (2.04-2.60)</td>
<td>2.38</td>
</tr>
<tr>
<td>140</td>
<td>2.52 (2.37-2.67)</td>
<td>2.43 (2.14-3.07)</td>
<td>2.52 (1.98-2.79)</td>
<td>2.40 (1.91-2.94)</td>
<td>2.69 (2.34-2.84)</td>
<td>2.47</td>
</tr>
<tr>
<td>168</td>
<td>2.55 (1.98-2.88)</td>
<td>2.37 (2.06-2.71)</td>
<td>2.50 (2.03-2.51)</td>
<td>2.53 (2.40-2.63)</td>
<td>2.45 (2.17-2.18)</td>
<td>2.45</td>
</tr>
<tr>
<td>Mean</td>
<td>2.33 (2.33-2.20)</td>
<td>2.20 (2.20-2.20)</td>
<td>2.26 (2.26-2.26)</td>
<td>2.16 (2.16-2.16)</td>
<td>2.16 (2.16-2.16)</td>
<td>2.16</td>
</tr>
</tbody>
</table>

aGroups 1, 2, 3, 4 and 5 ingested 0, 2, 4, 8 and 16 mg lead/kg body weight on a 5 day/week regimen for approximately 12 weeks (60 dosage days) beginning at 5 days of age.

bMean and range of values observed from each group.

cFour animals/group - adjusted group means were obtained if unequal cell frequencies occurred.

dTwo animals/group - adjusted group means were obtained if unequal cell frequencies occurred.
Table 6. Alpha_1-globulin values for control and lead-exposed lambs^d

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Group 1 Control</th>
<th>Group 2 2 mg/kg</th>
<th>Group 3 4 mg/kg</th>
<th>Group 4 8 mg/kg</th>
<th>Group 5 16 mg/kg</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.38 (0.34-0.42)</td>
<td>0.32 (0.14-0.47)</td>
<td>0.46 (0.41-0.51)</td>
<td>0.37 (0.23-0.50)</td>
<td>0.41 (0.23-0.55)</td>
<td>0.39</td>
</tr>
<tr>
<td>14</td>
<td>0.21 (0.00-0.37)</td>
<td>0.26 (0.17-0.43)</td>
<td>0.23 (0.00-0.43)</td>
<td>0.38 (0.26-0.56)</td>
<td>0.36 (0.19-0.44)</td>
<td>0.29</td>
</tr>
<tr>
<td>28</td>
<td>0.34 (0.20-0.40)</td>
<td>0.20 (0.17-0.23)</td>
<td>0.23 (0.20-0.44)</td>
<td>0.42 (0.32-0.50)</td>
<td>0.47 (0.26-0.76)</td>
<td>0.34</td>
</tr>
<tr>
<td>42</td>
<td>0.30 (0.14-0.51)</td>
<td>0.23 (0.17-0.27)</td>
<td>0.27 (0.18-0.40)</td>
<td>0.46 (0.30-0.69)</td>
<td>0.37 (0.21-0.56)</td>
<td>0.33</td>
</tr>
<tr>
<td>56</td>
<td>0.39 (0.33-0.46)</td>
<td>0.27^c (0.20-0.44)</td>
<td>0.42 (0.23-0.63)</td>
<td>0.33 (0.23-0.39)</td>
<td>0.35 (0.20-0.51)</td>
<td>0.36</td>
</tr>
<tr>
<td>84</td>
<td>0.18 (0.00-0.36)</td>
<td>0.36^c (0.16-0.50)</td>
<td>0.31^c (0.27-0.35)</td>
<td>0.31 (0.18-0.37)</td>
<td>0.35^c (0.27-0.45)</td>
<td>0.30</td>
</tr>
<tr>
<td>112</td>
<td>0.25 (0.00-0.45)</td>
<td>0.35^c (0.25-0.47)</td>
<td>0.41^c (0.35-0.50)</td>
<td>0.37 (0.28-0.46)</td>
<td>0.41^d (0.40-0.41)</td>
<td>0.35</td>
</tr>
<tr>
<td>140</td>
<td>0.44 (0.28-0.57)</td>
<td>0.44^c (0.27-0.80)</td>
<td>0.47^c (0.45-0.51)</td>
<td>0.45 (0.28-0.59)</td>
<td>0.33^d (0.22-0.42)</td>
<td>0.44</td>
</tr>
<tr>
<td>168</td>
<td>0.43 (0.17-0.50)</td>
<td>0.25^c (0.16-0.47)</td>
<td>0.44^c (0.39-0.54)</td>
<td>0.39 (0.00-0.58)</td>
<td>0.40^d (0.32-0.47)</td>
<td>0.38</td>
</tr>
<tr>
<td>Mean</td>
<td>0.32 (0.17-0.50)</td>
<td>0.29 (0.16-0.47)</td>
<td>0.36 (0.39-0.54)</td>
<td>0.39 (0.00-0.58)</td>
<td>0.39 (0.32-0.47)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

^a Groups 1, 2, 3, 4 and 5 ingested 0, 2, 4, 8 and 16 mg lead/kg body weight on a 5 day/week regimen for approximately 12 weeks (60 dosage days) beginning at 5 days of age.

^b Mean and range of values observed from each group.

^c Four animals/group - adjusted group means were obtained if unequal cell frequencies occurred.

^d Two animals/group - adjusted group means were obtained if unequal cell frequencies occurred.
<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Group 1 Control</th>
<th>Group 2 2 mg/kg</th>
<th>Group 3 4 mg/kg</th>
<th>Group 4 8 mg/kg</th>
<th>Group 5 16 mg/kg</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.17 (0.87-1.13)</td>
<td>1.08 (0.75-1.40)</td>
<td>1.26 (0.92-2.00)</td>
<td>1.12 (0.74-1.46)</td>
<td>1.02 (0.84-1.14)</td>
<td>1.13</td>
</tr>
<tr>
<td>14</td>
<td>1.48 (1.07-1.80)</td>
<td>1.19 (0.96-1.40)</td>
<td>1.26 (1.07-1.42)</td>
<td>1.22 (0.86-1.59)</td>
<td>1.12 (0.92-1.37)</td>
<td>1.26</td>
</tr>
<tr>
<td>28</td>
<td>1.18 (1.05-1.34)</td>
<td>1.28 (1.13-1.57)</td>
<td>1.21 (0.76-1.88)</td>
<td>1.09 (0.80-1.51)</td>
<td>1.09 (0.78-1.31)</td>
<td>1.17</td>
</tr>
<tr>
<td>42</td>
<td>1.32 (1.22-1.55)</td>
<td>1.32 (1.04-1.72)</td>
<td>0.96 (0.79-1.14)</td>
<td>0.95 (0.70-1.21)</td>
<td>0.95 (0.80-1.26)</td>
<td>1.07</td>
</tr>
<tr>
<td>56</td>
<td>1.11 (0.94-1.23)</td>
<td>1.17 ^c^ (0.89-1.56)</td>
<td>1.17 (0.93-1.84)</td>
<td>1.21 (0.89-1.38)</td>
<td>1.21 (0.97-1.14)</td>
<td>1.15</td>
</tr>
<tr>
<td>84</td>
<td>1.53 (1.22-2.02)</td>
<td>0.96 ^c^ (0.87-1.13)</td>
<td>0.84 ^c^ (0.72-0.83)</td>
<td>1.15 (0.95-1.50)</td>
<td>1.15 (0.82-1.24)</td>
<td>1.13</td>
</tr>
<tr>
<td>112</td>
<td>1.45 (1.11-1.81)</td>
<td>1.23 ^c^ (0.93-1.60)</td>
<td>0.96 ^c^ (0.81-1.14)</td>
<td>1.20 (0.88-2.09)</td>
<td>1.20 (0.85-0.97)</td>
<td>1.20</td>
</tr>
<tr>
<td>140</td>
<td>1.11 (0.96-1.25)</td>
<td>1.26 ^c^ (0.89-1.63)</td>
<td>0.92 ^c^ (0.69-1.25)</td>
<td>1.04 (0.88-1.25)</td>
<td>1.04 (0.76-0.91)</td>
<td>1.06</td>
</tr>
<tr>
<td>168</td>
<td>1.11 (0.99-1.25)</td>
<td>1.23 ^c^ (1.12-1.36)</td>
<td>0.91 ^c^ (0.85-0.91)</td>
<td>0.86 (0.57-1.04)</td>
<td>0.86 (0.92-0.96)</td>
<td>1.02</td>
</tr>
<tr>
<td>Mean</td>
<td>1.27</td>
<td>1.22</td>
<td>1.06</td>
<td>1.09</td>
<td>1.04</td>
<td></td>
</tr>
</tbody>
</table>

a Groups 1, 2, 3, 4 and 5 ingested 0, 2, 4, 8 and 16 mg lead/kg body weight on a 5 day/week regimen for approximately 12 weeks (60 dosage days) beginning at 5 days of age.

b Mean and range of values observed from each group.

c Four animals/group - adjusted group means were obtained if unequal cell frequencies occurred.

d Two animals/group - adjusted group means were obtained if unequal cell frequencies occurred.
consideration as a legitimate effect of lead, even though some disparity did exist in normal mean levels at 5 days of age.

An analysis of the β-globulin data (Table 8) indicated a significant effect of time (P<.0001), but the changes due to time were not linear over the course of the experiment. Although there was no significant group effect, the analysis indicated a significant group-by-time interaction (P<.002), but the measured values for each group of animals were so variable from one sampling date to the next that it was difficult to attach a meaningful interpretation to these findings.

Mean γ-globulin levels (Table 9) increased significantly (P<.01) during the course of this experimentation, but no group differences were indicated. The general trend of decreasing γ-globulin concentrations from day 5 to day 42, followed by a constant increase throughout the remaining weeks, was observed in all groups.

In an effort to exclude the unpredictable effect which an antigenic stimulus might impose upon the distribution of various protein components (possibly masking the effect of lead as a single known variable), an analysis of variance was performed on those data collected on days 5, 14, 28 and 42 (prior to antigenic exposure). Only in the case of the α₁-globulin fraction was there an indication of a significant group (treatment) effect. However, these differences were thought to be due to chance and were not considered to be reflective of change induced by lead exposure.

There was often a marked disparity in normal mean serum protein values among groups of lambs at 5 days of age; therefore, the
<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Group 1 Control</th>
<th>Group 2 2 mg/kg</th>
<th>Group 3 4 mg/kg</th>
<th>Group 4 8 mg/kg</th>
<th>Group 5 16 mg/kg</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.44 (0.30-0.67)</td>
<td>0.29 (0.16-0.39)</td>
<td>0.58 (0.31-1.00)</td>
<td>0.23 (0.00-0.42)</td>
<td>0.38 (0.30-0.53)</td>
<td>0.39</td>
</tr>
<tr>
<td>14</td>
<td>0.55 (0.51-0.59)</td>
<td>0.42 (0.40-0.45)</td>
<td>0.49 (0.42-0.56)</td>
<td>0.45 (0.36-0.59)</td>
<td>0.49 (0.40-0.59)</td>
<td>0.48</td>
</tr>
<tr>
<td>28</td>
<td>0.52 (0.47-0.58)</td>
<td>0.53 (0.31-0.68)</td>
<td>0.54 (0.48-0.57)</td>
<td>0.35 (0.00-0.57)</td>
<td>0.47 (0.00-0.57)</td>
<td>0.48</td>
</tr>
<tr>
<td>42</td>
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<td>0.40 (0.40-0.78)</td>
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<td>0.46 (0.36-0.54)</td>
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</tr>
<tr>
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<td>0.49 (0.40-0.61)</td>
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</tr>
<tr>
<td>84</td>
<td>0.55 (0.40-0.66)</td>
<td>0.36 (0.32-0.50)</td>
<td>0.41 (0.30-0.58)</td>
<td>0.44 (0.34-0.51)</td>
<td>0.43 (0.28-0.56)</td>
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</tr>
<tr>
<td>112</td>
<td>0.43 (0.35-0.48)</td>
<td>0.44 (0.44-0.48)</td>
<td>0.38 (0.32-0.43)</td>
<td>0.48 (0.38-0.63)</td>
<td>0.41 (0.35-0.42)</td>
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</tr>
<tr>
<td>140</td>
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<td>0.59 (0.47-0.73)</td>
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<td>0.51 (0.44-0.62)</td>
<td>0.34 (0.29-0.33)</td>
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</tr>
<tr>
<td>168</td>
<td>0.59 (0.47-0.76)</td>
<td>0.57 (0.44-0.96)</td>
<td>0.45 (0.37-0.54)</td>
<td>0.57 (0.44-0.84)</td>
<td>0.46 (0.31-0.56)</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Mean 0.53 0.48 0.49 0.45 0.44

*a* Groups 1, 2, 3, 4 and 5 ingested 0, 2, 4, 8 and 16 mg lead/kg body weight on a 5 day/week regimen for approximately 12 weeks (60 dosage days) beginning at 5 days of age.

*b* Mean and range of values observed from each group.

*c* Four animals/group - adjusted group means were obtained if unequal cell frequencies occurred.

*d* Two animals/group - adjusted group means were obtained if unequal cell frequencies occurred.
Table 9. Gamma-globulin values for control and lead-exposed lambs

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
<th>Mean</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>2 mg/kg</td>
<td>4 mg/kg</td>
<td>8 mg/kg</td>
<td>16 mg/kg</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.50</td>
<td>1.37</td>
<td>1.41</td>
<td>1.88</td>
<td>1.83</td>
<td>1.60</td>
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<tr>
<td></td>
<td>(1.26-2.14)</td>
<td>(0.89-2.25)</td>
<td>(0.57-1.97)</td>
<td>(1.36-2.61)</td>
<td>(1.29-2.06)</td>
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</tr>
<tr>
<td>14</td>
<td>1.21</td>
<td>1.24</td>
<td>3.77</td>
<td>1.52</td>
<td>1.43</td>
<td>1.35</td>
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<tr>
<td></td>
<td>(1.02-1.54)</td>
<td>(0.75-1.70)</td>
<td>(0.56-1.89)</td>
<td>(1.40-1.73)</td>
<td>(0.96-1.74)</td>
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</tr>
<tr>
<td>28</td>
<td>1.06</td>
<td>1.05</td>
<td>2.22</td>
<td>1.25</td>
<td>1.20</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>(0.97-1.11)</td>
<td>(0.64-1.45)</td>
<td>(0.90-1.55)</td>
<td>(0.61-1.80)</td>
<td>(1.02-1.56)</td>
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<tr>
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<td>1.02</td>
<td>1.06</td>
<td>1.01</td>
<td>1.24</td>
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<td>(0.68-1.27)</td>
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<td>56</td>
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<td>1.56</td>
<td>1.29</td>
<td>1.43</td>
<td>1.37</td>
</tr>
<tr>
<td></td>
<td>(0.99-1.42)</td>
<td>(0.93-1.70)</td>
<td>(1.16-2.37)</td>
<td>(0.85-1.68)</td>
<td>(0.99-1.64)</td>
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<tr>
<td>84</td>
<td>1.74</td>
<td>1.54</td>
<td>1.47</td>
<td>1.68</td>
<td>1.85</td>
<td>1.65</td>
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<tr>
<td></td>
<td>(1.32-2.05)</td>
<td>(1.19-2.25)</td>
<td>(1.04-1.84)</td>
<td>(1.17-2.30)</td>
<td>(1.49-2.11)</td>
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</tr>
<tr>
<td>112</td>
<td>1.99</td>
<td>1.89</td>
<td>2.07</td>
<td>1.69</td>
<td>2.04</td>
<td>1.89</td>
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<td>(1.36-2.38)</td>
<td>(1.77-2.07)</td>
<td>(1.68-2.17)</td>
<td>(0.90-2.18)</td>
<td>(1.73-1.97)</td>
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<td>2.00</td>
<td>2.02</td>
<td>2.29</td>
<td>2.01</td>
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<td>(1.61-2.60)</td>
<td>(1.56-2.23)</td>
<td>(1.56-2.79)</td>
<td>(1.89-2.10)</td>
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</tr>
<tr>
<td>Mean</td>
<td>1.53</td>
<td>1.45</td>
<td>1.52</td>
<td>1.59</td>
<td>1.56</td>
<td></td>
</tr>
</tbody>
</table>

*aGroups 1, 2, 3, 4 and 5 ingested 0, 2, 4, 8 and 16 mg lead/kg body weight on a 5 day/week regimen for approximately 12 weeks (60 dosage days) beginning at 5 days of age.

*bMean and range of values observed from each group.

*cFour animals/group - adjusted group means were obtained if unequal cell frequencies occurred.

*dTwo animals/group - adjusted group means were obtained if unequal cell frequencies occurred.
establishment of a uniform or constant baseline for comparison was virtually impossible. It was decided to refine the two previous analyses by statistically comparing the mean differences of the control group from day 5 to day 42 and from day 5 to day 168 with the mean differences of each treatment group during those time periods.

An analysis of mean differences for total protein levels revealed no significant differences between the control group and any of the four treatment groups over the time periods evaluated. Examination of albumin levels in the same manner yielded similar results.

Changes in $\alpha_1$-globulin from day 5 to day 168 were consistent in all groups, but the F-test, from the analysis of variance of the mean differences between the control group and group 3 ($P<.065$) and between the control group and group 4 ($P<.023$) from day 5 to day 42, indicated significant results. However, group 3 exhibited a decrease in $\alpha_1$-globulin level, while group 4 showed an elevation. A lack of consistency in the pattern of response indicates that the alterations could have been due to chance or random error.

A similar analysis of $\alpha_2$-globulin levels revealed the following; from day 5 to day 168, group 2 ($P<.04$) and group 3 ($P<.07$) each differed significantly from the control group, but again group 2 levels increased, while group 3 levels decreased. However, $\alpha_2$-globulin levels in groups 4 and 5 also decreased, which may indicate a trend attributable to lead. The same pattern developed when mean differences from day 5 to day 42 were compared.
When comparing mean differences in β-globulin levels, group 2 (P<.02), group 3 (P<.0004), and group 4 (P<.008) differed significantly from the control group. Again, the pattern was inconsistent with respect to levels of lead exposure, because β-globulin levels in groups 2 and 4 increased, and in group 3 decreased over time. The β-globulin in group 5 increased at the same rate as it did in the control group. Similar inconsistencies occurred in the analysis of data covering the period from day 5 to day 42.

No significant differences were detected when comparing alterations in γ-globulin levels from day 5 to day 168, as this protein increased at a comparable rate in all groups. Each of the groups showed a decrement in γ-globulin level from day 5 to day 42.

Compilation of results from several statistical analyses indicate that, in most instances, protein levels did not increase or decrease in a consistent pattern that might reflect the influence of varying levels of lead. Results of protein level analyses were in many cases erratic and irregular, over the course of the experiment, and the observed variations in protein concentrations could be attributed more to chance, or uncontrolled variation than to the direct effect of different levels of lead exposure.

Normal immunoelectrophoretic patterns (employing adult sheep serum) were established to serve as a basis of comparison for immunoelectrophoregrams developed from lamb test sera collected during the course of this experimentation. Rabbit anti-whole sheep serum, when allowed to react with electrophoresed normal sheep serum yielded
numerous arcs of precipitation (Figure 3a). These included an albumin band, several $\alpha_1$- and $\alpha_2$-globulin arcs, many $\beta$-globulin bands, and 2 $\gamma$-globulin arcs.

Serums collected from each individual lamb at 5, 14, 28, 42, 56, 84, 112, 140 and 168 days of age were analyzed immunoelectrophoretically using rabbit anti-whole sheep serum and/or rabbit anti-sheep $\gamma$-globulin. Of primary concern was the development of IgG and IgM bands since these proteins are the ones which possess major immunologic significance. The immunoelectrophoresis slides seen in Figures 3b and 3c were utilized to establish the exact location and conformation of the IgG and IgM bands of precipitation (purified IgM was kindly supplied by Dr. Ralph Heimer, Department of Biochemistry, Jefferson Medical College, Philadelphia, Pennsylvania).

Although sometimes weak and indistinct (almost imperceptible on photographs), the IgM arc was observed at 5 days of age in nearly every lamb utilized in this experiment; the IgG band was sometimes short and weak in the younger lambs. However, as the animals advanced in age, the IgG arc increased in length and intensity, eventuating in a pattern paralleling that of the normal adult pattern.

Evaluative comparisons (Figures 4, 5 and 6) of immunoglobulin development in lead-exposed and in control lambs, from 5 days of age to 168 days of age, indicated no noticeable lead-based impairment, alteration, or enhancement in the development of IgG and IgM as assessed by inspection of immunoelectrophorograms. There were no significant qualitative or semi-quantitative changes noted in the other serum
Figure 3. Pictures of immunoelectrophoretic slides of electrophoresed normal sheep serum or fractions. Arrows indicate IgG arc; straight lines indicate IgM arc.

a. Well, normal sheep serum; trough, rabbit anti-whole sheep serum.

b. Well, normal sheep serum; trough, rabbit anti-sheep \( \gamma \)-globulin.

c. Well, purified IgM; trough, rabbit anti-sheep \( \gamma \)-globulin.
Figure 4. Pictures of immunoelectrophoretic slides of electrophoresed serum from a control lamb. Upper troughs contain rabbit anti-whole sheep serum; lower troughs contain rabbit anti-sheep γ-globulin. Arrows indicate IgG arc; straight lines indicate IgM arc.

a. Upper well, serum from 5-day-old lamb; middle well, serum from 14-day-old lamb; lower well, serum from 28-day-old lamb.

b. Upper well, serum from 42-day-old lamb; middle well, serum from 56-day-old lamb; lower well, serum from 84-day-old lamb.

c. Upper well, serum from 112-day-old lamb; middle well, serum from 140-day-old lamb; lower well, serum from 168-day-old lamb.
Figure 5. Pictures of immunoelectrophoretic slides of electrophoresed serum from a lead-treated lamb (4 mg lead/kg body weight). Upper troughs contain rabbit anti-whole sheep serum; lower troughs contain rabbit and anti-sheep $\gamma$-globulin. Arrows indicate IgG arc; straight lines indicate IgM arc.

a. Upper well, serum from 5-day-old lamb; middle well, serum from 14-day-old lamb; lower well, serum from 28-day-old lamb.

b. Upper well, serum from 42-day-old lamb; middle well, serum from 56-day-old lamb; lower well, serum from 84-day-old lamb.

c. Upper well, serum from 112-day-old lamb; middle well, serum from 140-day-old lamb; lower well, serum from 168-day-old lamb.
Figure 6. Pictures of immunoelectrophoretic slides of electrophoresed serum from a lead-treated lamb (16 mg lead/kg body weight). Upper troughs contain rabbit anti-whole sheep serum; lower troughs contain rabbit anti-sheep γ-globulin. Arrows indicate IgG arc; straight lines indicate IgM arc.

a. Upper well, serum from 5-day-old lamb; middle well, serum from 14-day-old lamb; lower well, serum from 28-day-old lamb.

b. Upper well, serum from 42-day-old lamb; middle well, serum from 56-day-old lamb; lower well, serum from 84-day-old lamb.

c. Upper well, serum from 112-day-old lamb; middle well, serum from 140-day-old lamb; lower well, serum from 168-day-old lamb.
proteins. Figures 4, 5 and 6 are representative of all slides observed and photographed.

Development of Antibody Response in Control and Lead-Treated Lambs

Control and lead-treated lambs were assessed for their ability to produce antibodies, after several intravenous injections of a *Serratia marcescens* heat-killed bacterin. None of the 25 lambs displayed pre-inoculation titers against this organism, but the bacterin did evoke a significant response from each animal. Lamb #63, receiving lead at a dosage level of 2 mg/kg, died within three hours after receiving its initial bacterin injection. Post-mortem examination revealed that death resulted from a rapid hypersensitivity reaction.

Weekly serum antibody titers were obtained from each lamb for 12 weeks following the initial injection at 6 weeks of age. The weekly mean passive hemagglutination titers for each group of lambs are presented in Table 10, along with overall group- and time-means, plus range values. Weekly mean titers are also displayed graphically in Figure 7. From examination of the mean values, it is apparent the levels of lead employed in this experiment did not impose an immunosuppressive effect upon the immune response to *Serratia marcescens*. In fact, the antibody levels on days 7, 14 and 21 were generally higher in lead-exposed groups than in the control group.

Hemagglutination titers began to stabilize and exhibit uniformity across all groups at day 35; therefore, only those data obtained from sera collected on days 7, 14, 21 and 28 were subjected to statistical
Table 10. Passive hemagglutination titers for control and lead-exposed lambs at weekly intervals following the initial injection of heat-killed *Serratia marcescens*. Injections occurred on the 1st, 3rd, 5th, 7th, 14th and 21st days.

<table>
<thead>
<tr>
<th>Days after Initial Injection</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>2 mg/kg</td>
<td>4 mg/kg</td>
<td>8 mg/kg</td>
<td>16 mg/kg</td>
<td></td>
</tr>
<tr>
<td>7</td>
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<td>1064</td>
<td>432</td>
<td>192</td>
<td>478</td>
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<tr>
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<td>80-640</td>
<td>80-1280</td>
<td>40-1230</td>
<td>80-640</td>
<td>160-320</td>
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</tr>
<tr>
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<td>1027</td>
</tr>
<tr>
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<td>160-2560</td>
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</tr>
<tr>
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<td>800</td>
<td>480</td>
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<td>827</td>
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</tr>
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<td>10-80</td>
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<td>60&lt;sup&gt;c&lt;/sup&gt;</td>
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</tr>
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<td>411</td>
<td>244</td>
<td>529</td>
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</table>

<sup>a</sup>Mean and range of titers obtained from each group.

<sup>b</sup>Four animals/group - adjusted group means were obtained if unequal cell frequencies occurred.

<sup>c</sup>Two animals/group - adjusted group means were obtained if unequal cell frequencies occurred.
Figure 7. The comparative long-term antibody response of the control group and the lead-treated groups to six injections of *Serratia marcescens* received on days 1, 3, 5, 7, 14 and 21. Small arrows indicate 0.5 ml injection; large arrows indicate 1.0 ml injection. Each point represents the mean passive hemagglutination titer of groups of five lambs.
analysis. In order to stabilize the variation which is inherently present in titer data because of the wide range between individual values, log transformation of the data was deemed necessary before an analysis of variance could be conducted. The analysis revealed no significant difference in antibody response among the groups.

To test for the presence of 7S and 19S immunoglobulins in control and lead-exposed lambs during and after exposure to *Serratia marcescens*, sera from several individual animals were exposed to 2-ME. Serum titers remaining after such treatment were considered to be due to 7S antibody, or IgG. Figures 8 and 9 are representative of results obtained by this treatment of sera with 2-ME. Relatively high levels of 19S antibody (IgM) were consistently observed throughout the 84-day period following initial injection of the bacterin. This was accompanied by appreciable levels of 2-ME-resistant antibody (IgG) until titers began to decline, at which time little or no 7S antibody remained.

Approximately 60 days post-challenge, each animal received a secondary injection of *Serratia marcescens*. Because lambs #51, #61, #71, #81, #52 and #62 exhibited shock-like symptoms (or endotoxin reaction) shortly after receiving an intravenous secondary challenge, it was decided to expose the remaining lambs by the subcutaneous route to allow for slower diffusion and absorption of antigen. Individual secondary titers are shown in Figure 10 for those animals challenged via the intravenous route and in Figure 11 for those injected
Figure 8. The effect of 2-mercaptoethanol on serum antibody developed in a control lamb injected with *Serratia marcescens* on days 1, 3, 5, 7, 14 and 21. Small arrows indicate 0.5 ml injection; large arrows indicate 1.0 ml injection.
Passive Hemagglutination Titer

Time in Days

Whole Serum

2-ME Treated Serum

Injected

0 10 20 40 80 160 320 640 1280 2560 5120

10240

14 21 28 35 42 49 56 63 70 77 84
Figure 9. The effect of 2-mercaptoethanol on serum antibody developed in a lead-treated lamb (16 mg lead/kg body weight) injected with *Serratia marcescens* on days 1, 3, 5, 7, 14 and 21. Small arrows indicate 0.5 ml injection; large arrows indicate 1.0 ml injection.
Passive Hemagglutination Titer

Time in Days

Whole Serum
O--O 2-ME Treated Serum
→ Injected
Figure 10. Secondary antibody response of six individual lambs to an intravenous injection of *Serratia marcescens* approximately 60 days after the primary series of injections was completed.
Animals

Passive Hemagglutination Titer

- Titer before secondary injection
- Titer after secondary injection

Titer before secondary injection:
- 51
- 52
- 61
- 71
- 81

Titer after secondary injection:
- 62

Values represent the titer levels for different animals before and after a secondary injection.
Figure 11. Secondary antibody response of fourteen individual lambs to a subcutaneous injection of *Serratia marcescens* approximately 60 days after the primary series of injections was completed.
Ani Titer before secondary injection

Titer after secondary injection
subcutaneously. All lambs exposed intravenously responded with an
increase in antibody titer regardless of group. Although the rise
in antibody level was not as marked in those lambs challenged by the
subcutaneous route, all but two responded to the secondary injection.
The lambs were not being treated with lead at the time of secondary
injection, but those animals in the exposed groups were all manifesting
residual blood lead levels above the normal range.
DISCUSSION

A recent study clearly demonstrated that mice exposed to nonlethal levels of lead for 30 days were more susceptible to a pathogenic strain of *Salmonella typhimurium* than were their normal counterparts (Hemphill *et al.*, 1971). Although there have been no subsequent reports of a similar phenomenon occurring in other mammalian species, numerous individuals have speculated on the effects which lead might impose on the various mechanisms of the immune response. Thus, these ideas and hypotheses are generally based on analysis of the literature relative to heavy metals and their overall effect upon biological systems, rather than on original research.

It is quite possible that lead may interfere with specific immunity (synthesis and/or mobilization of antibodies) by (1) causing cytopathic effects in lymphoid tissues and in other cells which are requisite to antibody formation; (2) binding to and/or hydrolyzing nucleic acids which may be essential to induction of antibody synthesis; or (3) depressing immune mobilization through complexing (binding) antibodies already in the circulation. Lead may also impose adverse influences upon innate or natural immune factors. For example, since it is known that certain enzymes are very sensitive to lead exposure (Tepper and Pfitzer, 1970), it is possible that lead could interfere with the enzyme systems necessary for immunologic activities of complement and also with those enzymes which come into play during phagocytosis of harmful microorganisms.
As previously mentioned, there are multitudinous reports relative to the pathological and other biochemical effects of lead, but studies dealing with immunological changes associated with lead exposure have been sparsely documented. The variability and complexity of interactions involved in the immune system negated the possibility of testing and evaluating the effect of lead on every known factor involved in the development of immunity in young sheep. Therefore, this investigation was launched with two specific goals in mind; first, to conduct an exhaustive survey and comparison of serum protein levels in normal and lead-treated lambs from 5 days to 6 months of age, with special interest directed toward the development of major immunoglobulin subgroups, and secondly, to study the development of specific immunity in control and lead-treated lambs after they had been exposed to a known antigenic stimulus.

Levels of oral lead exposure were established with the aid of a recent investigation by Sharma (1971) who reported overt toxicosis in 12 ewes which had ingested 11.8 mg lead/kg body weight for approximately 45 days. It was initially projected that the force-feeding of several graded doses of lead to the lambs would be continued for a period of 6 months, in an effort to produce varying rates of lead absorption. In other words, it would have been desirable to develop graded pathological states, e.g. (1) several lambs with increased blood lead levels, but no metabolic or functional symptoms; (2) others exhibiting altered metabolites in the blood and urine, but no obvious functional injury;
(3) a group of animals which displayed functional anomalies of the kidneys, hemopoietic system, and central nervous system; and (4) a group manifesting acute toxicity.

However, after 10-11 weeks of lead encounter, several lambs began to express motor clumsiness, lassitude, anorexia, and gastrointestinal irregularities. Before the syndrome could be properly treated, three lambs in the highest exposure group succumbed to irreversible lead poisoning. In order to standardize the period of exposure across groups and to reduce variability when evaluating experimental data, all lambs were removed from the lead regimen at approximately 12 weeks of age. Each of the exposed lambs continued to manifest elevated blood lead levels throughout the 6 months of observation; and since it has been established (Chisolm, 1971) that many residual effects persist even after blood lead levels return to normal, collection of serum samples was continued on a weekly basis throughout the entire period.

Serum proteins in vertebrates are indeed a diverse group manifesting a wide variety of functions, including maintenance of plasma pH, colloid pressure, and viscosity; transportation of various substances of exogenous and endogenous origin; and regulation of cellular activity and function. Along with the immunoglobulins, which possess the primary function of endowing the body with a defense against infection and injury, many other serum proteins wield a role of prominence as overall effectors and mediators of immunity. For example, it is conjectured that transferrin, the principal β-globulin of serum, may serve as a compensatory
defense mechanism in some instances because of its capacity to assert a suppressive effect on the growth of certain microorganisms (Turner and Hulme, 1971).

Complement, a complex group of enzymes, which is present in the \( \alpha^- \), \( \beta^- \), and \( \gamma^- \)-globulin fractions, also merits mention in this discussion. It functions harmoniously with antibodies and plays a highly significant role as a mediator of immune and allergic reactions (Mayer, 1973). Various components of this effector system serve numerous biologic functions which include potentiating phagocytosis, mediating immune adherence, increasing vascular permeability, augmenting neutrophilic chemotaxis, and destroying bacteria. Gewurz (1971) has discussed virus neutralization, mediation of graft rejection, destruction of neoplastic cells, and participation in immediate and delayed hypersensitivity as other immunologic phenomena which might be affected by complement; worldwide research is underway to completely explore these areas. Investigators have also discovered that complement mediates a definite interaction between clotting mechanisms and host defense responses (Ratnoff, 1971). The revelation of this relationship has been helpful in studying idiosyncratic reactions to drugs, mechanisms of endotoxic shock, and rejection of organ transplants. Based upon this discussion, it becomes immediately clear that if lead incites modification or instability of consequence in these physiologic components, it is quite possible that defense mechanisms may be significantly disrupted.
In comparing control and lead-treated lambs, this study revealed some statistically significant alterations in concentrations of albumin, \( \alpha_1 \)-globulin, \( \alpha_2 \)-globulin, \( \beta \)-globulin, and \( \gamma \)-globulin. However, there was seldom a pattern of consistency developing in response to different levels of lead. There were some trends in the data which may or may not have signified a direct effect of lead. Perhaps if greater numbers of animals had been employed in this study, the data would have been less confusing and the trends would have been more readily understood.

Total protein levels tended to increase in all groups from day 5 to day 168. This observation was not considered abnormal because in many species total protein levels manifest an appreciable gain with increasing age. The marked variability across all groups from day 42 to day 84 may be attributable to numerous factors, e.g. weaning of lambs, exposure of lambs to antigenic stimulus, or termination of lead exposure. The only report in the literature contradictory to the findings of this investigation was that of Cervetti and Casucci (1960) who indicated a significant decrease in total serum protein in several humans suffering from severe lead poisoning.

This research endeavor disclosed relatively little change in albumin levels among control lambs and lambs exposed to various levels of lead. Rather, there was a trend of slight increase in albumin with increasing age, in all groups. These findings do not appear consistent with those of Kapetanovic et al. (1960) who studied persons suffering from chronic lead poisoning, or with those of Hayashi et al. (1959) who
reported on lead poisoning in rabbits, as they both reported a significant diminution in the albumin fraction of their subjects.

Soliman et al. (1970) reported a decrease of α-globulins in several Egyptian lead workers, while Cervetti and Casucci (1960) documented an enhancement in these fractions. The animals surveyed in this project did not manifest meaningful deviation in α₁-globulin values throughout the experimental period; the changes which did occur could not be associated with the presence or absence of lead. A trend which merits consideration occurred in the α₂-globulin concentrations. Groups 3 and 4, which received 4 mg lead/kg body weight and 8 mg lead/kg body weight, respectively, when compared to the control group manifested a rather marked decrease in the α₂-globulin component. However, the mean values for the group exposed to the highest level of lead did not display such a dramatic decrease, and those animals which received 2 mg lead/kg body weight generally showed an enhancement of α₂-globulin over the 6 months time period. Although these results may be interpreted to mean that lead was asserting a suppressive effect of some nature on the α₂-globulin component, the assumption would not be a firm one.

A general increase in the β-globulin component was demonstrated in the control group and in all treatment groups except group 3 (4 mg lead/kg body weight), which showed a slight decrease. Cervetti and Casucci (1960) reported this fraction to be enhanced beyond the normal range in lead-poisoned humans.

Each group of lambs exhibited a depression in mean γ-globulin levels from day 5 to day 42, with a marked decrease noted in group 4
(8 mg lead/kg body weight) from day 5 to day 42. A possible explanation for this phenomenon would be a reduction in maternal antibodies (obtained in the colostrum) as the lambs progressed in their development and their periods of nursing became shorter and less frequent. There appeared an increase in the antibody globulin at day 56, which continued throughout the remaining period of evaluation. This is probably reflective of the artificial antigenic stimulation which began at approximately 42 days of age. Also, as an animal increases in age, there is naturally more exposure to exogenous stimuli accompanied by a tendency to develop subclinical infection, which would probably result in an enhancement of γ-globulin.

It has been documented that after an animal is weaned, it will manifest lower concentrations of serum proteins than does its adult counterpart. The protein components, then, usually increase in concentration as the animal becomes older (Dimopoullos, 1963). Some of the observations made during the course of this study are probably a reflection of this normal pattern of events.

Several heavy metals possess the potentiality to interact with proteins, nucleic acids, or integrated systems such as organelles. Williams et al. (1954) reported the in vitro precipitation of γ-globulin, and subsequently speculated on the likelihood of a similar phenomenon occurring in a living physiological system. Albumin is particularly susceptible to metal binding. Bryan and Bright (1973) reported a depression in several serum proteins after short periods of exposure to nonfatal levels of cobalt. Mercury, on the other hand, was found...
to produce an immediate enhancement of several globulin components. The same investigators have suggested a form of protein induction-repression mechanism to be responsible for metal-induced variations in serum protein patterns. In other words, an interaction between metals and specific enzymes might influence the rate of accumulation and/or removal of the protein components.

Based upon the results of serum protein quantitation in lead-exposed sheep, lead apparently did not exert an effect of the nature just described (unless possibly in the case of the $\alpha_2$-globulin component). However, there is the possibility that the sheep developed a rapid tolerance to nonlethal quantities of lead through some adaptive process.

Although reporting results that were often contradictory, a few previous investigators have indicated "significant" alterations in serum protein levels for various species exposed to lead compounds; therefore, it seemed somewhat surprising that such phenomena could not be more readily detected. The weakness of many reports is that even when changes did occur in serum proteins, lead could not be positively incriminated as the responsible agent. This investigator feels that there is a tenable explanation for the inconsistencies which appear in the literature relative to the effect of lead on serum protein components. Interpretation of data obtained from electrophoregrams must be undertaken with extreme caution because serum protein profiles may be altered by numerous pathological, physiological, and experimentally-induced stress factors. It is important to look for abrupt alterations or gradual trends which occur with consistency in animals within a given treatment
group or groups, if using this as a means to determine the effects of a toxic substance. Of course, the appearance of new components, or the absence of normal fractions, may indeed be diagnostic, if related to a specific treatment.

Also, it appears as though many investigators, when interpreting quantitative serum protein data, fail to accept the fact that there is a wide range of values which are considered normal in a given species. Thus, in order to attain more precise and accurate evaluations of such parameters, biological variation must be drastically reduced, e.g. a homogeneous group of well-defined animals (sex, genotype, weight, and age) should be obtained, and the experimentation should be well-controlled in regard to environment, husbandry, nutrition, and exposure to pathogenic microorganisms. Lack of sufficient numbers of subjects is another inherent defect in most research endeavors of this kind. Discrepancies and inconsistencies in results would probably be reduced if it were economically feasible to utilize more animals.

Serum samples from control and lead-exposed lambs were further analyzed by the technique of immunoelectrophoresis, using commercial antisera prepared against normal sheep serum and against normal γ-globulin. Although I was interested in all antigenic components, of which there are 23 in sheep serum, as reported by Chordi and Kagan (1964), this was primarily an attempt to follow and compare the development of immune globulins among the various groups of lambs, noting time and sequence of appearance and intensity of precipitin bands.
Immunoglobulins of all mammalian species studied have been divided into several classes on the basis of molecular weight, electrophoretic mobility, and chemical and immunological properties of their heavy chains. Five immunoglobulin subgroups have been defined thus far in sheep. There are two antigenically related 7S immunoglobulins designated as IgG₁ (fast component) and IgG₂ (slow component), which usually present a "gull-wing" shape on immunoelectrophoretic slides, and there is a component analogous to human IgM (Aalund et al., 1965, Heimer et al., 1969, and Sullivan et al., 1969). Lascelles and McDowell (1970) isolated IgA from mucous secretions of ewes, but there is only circumstantial evidence that IgA exists in sheep serum. A component designated as IgG₁ₐ has also been described (Curtain, 1969), but this subgroup is definitely lacking in serum of some sheep. Only the IgG₁, IgG₂, and IgM components are resolvable and easily identified on immunoelectrophoresis slides.

Although the ovine animal does not receive immunoglobulins from the maternal circulation via the placenta, it has been established by Silverstein et al. (1963a) that the fetal lamb is not agammaglobulinemic. Their study demonstrated that, in utero, the unstimulated lamb does engage in the production of IgM, along with small amounts of IgG. Therefore, it seemed highly probable that the IgM and IgG subgroups would be detectable in the lambs at day 5, and therefore should be observable throughout the entire experimental period unless lead was affecting both components, or possibly interfering with one immune fraction preferentially over the other fraction.
These studies indicated no existence of significant qualitative or semi-quantitative differences in the immuneelectrophoretic patterns of immunoglobulins (IgG and IgM) obtained from control and lead-exposed lambs for a 6 months' period. It appears that lead, at the concentrations employed, neither removed the serum immunoglobulins from the circulation, nor impaired their development.

Although Belli et al. (1955a, 1955b, and 1955c) found that rabbits that had been poisoned with tetraethyl lead via the subcutaneous route were less capable of producing antibodies against bacterial antigens than were normal rabbits, and Hemphill (1973) reported a similar phenomenon in mice exposed to intraperitoneal lead nitrate, the results of this present study were not in agreement with those findings. In fact, upon exposure to heat-killed Serratia marcescens, the lambs which had ingested lead for several weeks, in many instances, responded immunologically more promptly and efficiently than did their normal counterparts.

However, when comparing contradictory results such as these, many factors must be taken into consideration, such as species of animals utilized, and their relative susceptibilities to lead. Even animals of the same speciation may individually differ in their tolerance to this heavy metal, such has been observed in the porcine animal (Lassen, 1973). Also, the route of administration and dosage level of lead used are important. For example, Hemphill (1973) demonstrated a

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Lassen, E. D., Department of Pathology, College of Veterinary Medicine, Iowa State University, Ames, Iowa. Information concerning biochemical effects of lead in swine. Personal communication, 1973.
reduction in antibody response, but his animals were receiving either 5 ppm or 12 ppm of lead injected into the peritoneal cavity, whereas the lambs which I used received 2 ppm, 4 ppm, 8 ppm and 16 ppm via the oral route; the percentage amount of lead absorbed through the intestinal tract after oral ingestion remains undefined. Finally, blood lead levels, per se, may not be highly correlated with the toxic effects of lead; it is really more significant to know the amount of lead which is in the interstitial tissues. For example, those animals which retain high concentrations of lead attached to their red blood cells may manifest high blood lead values, but other individuals which bind less lead in their blood cells and release soluble lead into the soft tissues of the body may be more debilitated. Conversely, Sharma (1971) has shown a high correlation between blood lead levels and toxic signs in adult sheep.

In this study, the lambs were allowed to reach 6 weeks of age before being exposed to a test antigen. Therefore, those lambs in the lead-treatment groups had received lead for several weeks prior to initiation of their immunization series. *Serratia marcescens* was used as the standard antigenic stimulus because it is a rather innocuous organism, and is not normally considered part of the normal microflora of sheep. It was assumed that there would be no pre-existing antibody titers against this antigen to interfere with our immunological evaluation, as was later proven to be the case. Also, elicitation of a febrile response or any other clinical symptoms would have been undesirable in this study; therefore, sheep pathogens were purposely avoided in the selection of a test antigen.
A rather protracted series of antigen injections was imposed, because antigenic stimulants are often more effective when given in several divided doses. There was a concerted effort made to reach maximum antibody titers so that additional injections would have little or no effect on peak antibody level. However, it was conjectured that hyperimmunization might have masked part of the true toxicity picture by production of excess immunoglobulins which combined through sulfhydryl groups with heavy metals, thereby overcoming subtle effects of lead.

Potential immunocompetence of the 6-week-old lamb was given particular consideration before beginning the series of injections. It was reported by Silverstein et al. (1963b) that stimulation of the immature lamb in utero by a variety of antigens induces, in the fetus, an active antibody response. Another research endeavor clearly demonstrated that both the fetal, and the newborn lamb, presumably insulated from antigenic stimuli, are actively engaged in the production of at least one of the ovine gamma globulins customarily associated with immunologic function (Silverstein et al., 1963a). Therefore, it was assumed that these 6-week-old animals would be completely immunocompetent and able to respond to an artificial exogenous stimulus, not depending on passively conferred maternal antibodies for immunity.

As the graph displaying mean antibody titers (Figure 7) indicates, a latent, or lag, period in immune response was virtually nonexistent for all groups. This apparent irregularity may be explained by the fact that the animals had already received three injections before the
initial serum samples were obtained; generally a secondary immune response can be anticipated within two days after intravenous injection of a particulate antigen, such as the one employed in this study. Apparent duration of the latent period also depends upon the sensitivity of the method used to detect it. Passive hemagglutination is an extremely sensitive assay procedure (e.g., 1,000 times more sensitive than precipitin reactions).

The mean hemagglutination titers are grouped closely together on the graph (Figure 7), indicating that there was no great disparity in the response of the various groups to this particular antigenic stimulus. It is interesting to note that these results, with the control group consistently displaying the lowest mean titer on days 7, 14 and 21, were in accordance with data obtained from a previous investigation in this laboratory. In that study, two groups of ewes receiving, respectively, daily oral dosage of approximately 4.5 and 2.3 mg lead/kg body weight for several weeks, manifested consistently higher titers to *Serratia marcescens* than did control ewes (Hoffman, 1972).¹

It should be pointed out that, on day 28, while the treatment groups had previously reached their maximum titers and were beginning to decline even after an additional injection, the mean response of the control group manifested a marked increase. This enhancement of response carries little significance because there was one individual lamb whose

¹Hoffman, L. J., Department of Bacteriology, Iowa State University, Ames, Iowa. Information concerning effects of low level lead ingestion in ewes. Personal communication, 1972.
hemagglutination titer catapulted to over 10,000 after the final injection, thus altering the mean drastically. The decline which was detected in the mean titers of the lead-treated groups, even after an additional injection of antigen, may have been caused by immune exhaustion, a phenomenon in which memory cells, or immunocytes, are unable to continue responding, or by feedback inhibition, where pre-existing specific antibody molecules themselves interfere with initiation of synthesis of this specific antibody.

It is important to comment on the individual response of individual lambs to the test antigen. For example, the control group harbored one lamb which was an excellent responder, but the others in this group were immunologically sluggish, with sera of two animals not manifesting hemagglutination titers beyond 160. However, none of the sera of lead-treated animals were below a maximum titer of 320, only four out of 19 were below 640, and sera of 10 of the 19 lead-treated lambs reached a titer of 1280, or above. Could it be possible that substances, which are lethal or toxic at certain concentrations in the body, may actually be stimulatory, at lesser concentrations, to various physiologic factors or functions? In the case of lead exposure, perhaps there was an increase in cellularity of tissues responsible for antibody formation. In support of this explanation, Zarkower and Morges (1972) demonstrated an enhancement of antibody-forming cells in the mediastinal lymph nodes of mice after four days' exposure to carbon dust.

Under the conditions of this particular experimental study, lead apparently did not interfere with the functions of the reticuloendothelial
system (RES); therefore, the capacity of RES to direct antibody induction, synthesis, and mobilization was not impaired. Also, since there were no marked depressions in antibody response among the lead-treated groups, there is good indication that immunoglobulin molecules retained their integrity (no binding or separation of polypeptide chains) after reaching the circulatory system.

In order to determine whether lead influenced either the appearance or disappearance of high and/or low, molecular weight immunoglobulins formed in response to the *Serratia marcescens* bacterin, sera from the control lambs and from several of the treated lambs were exposed to 2-ME. Lead apparently did not interfere with the production of either 7S or 19S antibodies nor did it preferentially affect the serum levels of these immunoglobulins, because the results of this test were essentially the same for all lambs evaluated.

Although Field et al. (1970) demonstrated no significant amounts of 2-ME-resistant antibody (IgG) appearing in sera of mice injected with *Serratia marcescens*, the present study revealed the presence of both 7S and 19S antibody. However, at no time did IgG appear to be the predominant immunoglobulin, as is most often the case in responses to bacterial stimuli; IgM usually predominates during the early stages of an immune response, only to be outclassed by IgG after a short time interval. This observation may be explained by the fact that gram negative organisms often elicit predominantly 19S antibody. Also, 19S antibody is a more efficient mediator of passive hemagglutination; thus,
perhaps more 7S antibody was present, but was not detected with our assay procedure.

There is no documentation in the scientific literature relative to the effect of lead on the secondary or "anamnestic" response to an antigenic stimulus. This phenomenon refers to the antibody production obtained with a subsequent injection of the same antigen, at a considerable interval after the preceding injection or injections, usually as titers are decreasing to just above detectable levels. The secondary response is characterized by the following: (1) rapidly rising titer, (2) peak titer attained which is usually greater than that obtained in the primary response, and (3) an antibody titer attained which persists for a longer period of time than the primary titer.

The true scientific value of this phase of the experiment is subject to question. First of all, the treated lambs had received no lead for several weeks prior to secondary injection; therefore, it was impossible to reach positive conclusions concerning the response of lambs to a subsequent stimulus while concomitantly receiving lead, which had been the case during the primary injection period. Also, there was variability introduced by using two different routes of exposure.

There were only two animals, both of which were exposed by the subcutaneous route, which did not manifest an elevation in antibody titer after secondary stimulation. Much as expected, the intravenous injections elicited a more marked response; each lamb exposed through the jugular vein matched or surpassed its previous high titer.
Evidence that residual lead affected the lambs' response to secondary antigenic exposure did not exist. Although the intensity of response did vary among the animals receiving an injection via a common route, the within group variations were often greater than the differences between the groups.

In conclusion, this study indicated that various selected levels of lead ingestion (generally "subclinical" in nature) caused no major qualitative or quantitative alterations in serum proteins of treated animals, including the immunoglobulins, and no significant difference in antibody response was noted between control and lead-exposed lambs. Under certain circumstances, of course, lead is a highly toxic metal; however, there were no outstanding upsets in the humoral defense system of the normal, healthy lambs which were evaluated here. It must be remembered that this response pattern may not be true under another set of experimental conditions, or for other species. For example, the immune competence of disadvantaged children suffering from vitamin and mineral deficiencies may be altered significantly after accidental exposure to relatively low quantities of lead.

These results obtained with sheep do not necessarily concur with all previous reports on the effects of exposure to subclinical dosage of lead, but may be explicable in a number of ways; however, one can only speculate on why immune responses of lambs were not impaired, altered, or enhanced by exposure to lead. Perhaps the lead molecules did not progress to a site or sites which would be critical for induction of immunological mechanisms; therefore, adverse changes were so
inconsequential that they went undetected. Also, it is possible that temporary abnormalities were produced, but were easily repaired by normal bodily mechanisms, with no resultant lasting effects. Finally, it is conceivable that these lambs may have possessed the capacity to detoxify (tolerate) the prescribed dosages through physiological mechanism inherent in sheep.

This study is extremely significant from a practical point of view, because a random heterogeneous population of animals was used, which is representative of sheep in commercial production. Also, results of lead exposure obtained in this study seem to be more extrapolatable to human populations than are those results obtained in several earlier studies because (1) blood lead levels were in a range which is not unusual for children exposed to excessive quantities of lead, and (2) a normal route of exposure was employed, in contrast to the abnormal process of subcutaneous, or intravenous, injection.

This research project definitely points out the need for more information in the area of heavy metal effects on immune response mechanisms. First of all, consideration must be given to developing more realistic and sensitive models for natural exposure to this substance. Since one of our major concerns is how atmospheric lead affects the health of living populations, it would seem logical to study the long-term effects of inhaled lead by simulated exhaust fumes, etc. in experimental inhalation chambers. Perhaps the antigen should also be introduced by the aerosol route to determine exact effects of lead on the respiratory defense system. It is imperative that more quantitative
information be obtained relative to how lead affects antibody-forming cells, lymphoid organs, and various classes of immunoglobulins. A long-term study to evaluate the possible differences between genotypes in relative susceptibility to lead should be given consideration in the future.

Finally, it is most important that we remain mindful of the complexity which is inherent in biological systems. In our attempt to make research more manageable, we often tend to isolate, or oversimplify, components of physiology. Nothing biological operates with a single variable, and the immune response system is no exception. Resistance to bodily stress encompasses a number of defense mechanisms, whose responses are mobilized by an intermeshing of numerous physiological systems.
SUMMARY

Twenty-five lambs of Columbia-Rambouillet cross were divided into five equal groups which received 0, 2, 4, 8 and 16 mg lead/kg body weight, respectively, via the oral route. Ingestion of lead, in the form of solubilized lead acetate, was permitted on a daily basis (5 days/week) for 12 weeks. This period of ingestion resulted in mean blood lead levels of 11.7, 37.2, 56.8, 67.6 and 99 μg/100 ml, respectively, over the 6-months' evaluation period. Nearly every lamb in each of the treated groups continued to manifest significantly high blood lead residues (as compared to those lambs in the control group) after lead exposure was terminated.

Cellulose acetate electrophoresis and agar gel immunoelectrophoresis were used to examine serum protein patterns at regular intervals, starting when the lambs were 5 days of age and continuing until they were 6 months old. Although statistical analyses of absolute concentrations of total protein, albumin, α₁-globulin, α₂-globulin, β-globulin and γ-globulin detected significant differences among groups in certain instances, there was little basis for concluding that these alterations were an effect of increasing levels of lead and not due to uncontrolled variations, or random error in the experimentation. However, certain trends seemed evident (from time to time) which perhaps would be more pronounced if the number of subjects in each treatment group had been larger. There were no detectable differences noted among groups in the development and maintenance of immunoglobulin subgroups, IgG and IgM, as evaluated by electrophoresis precipitin patterns.
The various treatment groups were also compared in their responses to injections of heat-killed *Serratia marcescens*. There were no significant differences noted in passive hemagglutination titers among treatment groups, but during the actual period of immunization, the lead-treated groups consistently displayed higher serum titers than did the control group.

Of the immunologic parameters investigated in this study, none were consistently affected by the selected levels of oral lead exposure. Under these research conditions, one could conclude that lead does not impose a state of immunologic deficiency in the ovine species by altering antibody synthesis or mobilization of preformed antibody, nor by interfering with the production or maintenance of serum proteins.
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Deepest appreciation is extended to my parents for their sincere and enduring support of my academic career. They have given me an education in honesty, respectfulness, and dedication to life's tasks---this has truly been an integral part of my success.

Finally, I am profoundly grateful to my husband, Peter, who has supplied the primary inspiration and motivation for much of this academic accomplishment. He has never failed to provide me with the true gifts of life, those of mind, spirit, and heart.

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APPENDIX

Serum Protein Electrophoresis

A. Reagents

1. Buffer: Tris-barbital (Gelman Catalog #51104)
   Dissolve 1 packet of High Resolution Buffer in distilled water and dilute to 1000 ml, pH 8.8, ionic strength 0.05.

2. Stain: Ponceau S (Dye II, Buchler Catalog #3-1133)

3. Decolorized: Acetic acid
   Prepare 300 ml (3 trays, 100 ml each) of 5% aqueous acetic acid (v/v).

B. Procedure

1. Preparation of electrophoresis chamber.
   Place chamber on a level, firm surface and fill the chamber with Tris-barbital buffer. Attempt to obtain equal levels in all compartments.

2. Preparation of cellulose acetate strips (Beckman cellulose acetate manufactured by Millipore Filter Corporation, #324330, Size 57 x 145 mm).
   a. Label the strips at one end using nondiffusible ink.
   b. Float the strips on the buffer surface until they are completely wetted and then immerse them.
   c. After a thorough wetting has been effected, remove the membrane from the buffer and blot dry between two pieces of filter paper until all excess moisture is removed.
d. Each strip is then stretched across a plastic bracket which is in turn positioned in the chamber with the ends of the strip submerged directly in the buffer.

3. Application of samples.
   a. Using the microzone sample applicator pick up the serum sample, making sure that the sample is in a continuous film across the length of the applicator wire.
   b. On the cathode side of the chamber, place the applicator over the strip in the position and press the applicator button down to apply the sample. Clean and rinse applicator wire between each sample.

4. Electrophoresis of samples.
   a. Place cover on chamber.
   b. Connect tank to power supply and set it at 250 v.
   c. Turn on power supply and allow separation to continue for 25 min.
   d. Turn off power supply and disconnect the chamber.

5. Staining of strips.
   a. With blunt-tipped forceps, remove the cellulose-acetate strips from the chamber.
   b. One at a time, float the strips in Ponceau S solution. When the strip is completely wet, immerse it, and stain for 5-10 min.
c. Decolorize the stained strips by rinsing successively in 3 baths of 5% acetic acid, until background is white.

d. After blotting between paper toweling, the membrane should be pressed flat until completely dry.


Integrated electrophoretic density plots were obtained with a scanning densitometer.

Immunoelectrophoresis

A. Reagents

1. Buffer: Tris-barbital (Gelman Catalog #51104)
   
   Dissolve 1 packet of High Resolution Buffer in distilled water and dilute to 1800 ml, pH 8.8, ionic strength 0.05 M.

2. Agar: Noble Agar (Difco Catalog #0142-01-8)
   
   Weigh out 1 1/2 g of Noble Agar and suspend it in 50 ml distilled water plus 50 ml of Tris-barbital buffer. Add several drops of merthiolate preservative (1:10,000=final concentration). Heat in a water bath until solution is completely clear.

B. Procedure

1. Preparation of agar-covered slides.
   
   a. Load each immuno frame (Gelman Catalog #51447) with 6 25 mm x 75 mm glass slides.
b. Place the spirit level within the engraved circle in the center of the leveling table, (Gelman Catalog #51433) and adjust the table until the bubble is in the proper position.

c. Secure 3 loaded immuno frames between the clamping bars on the leveling table.

d. Fill the small depressions at either end of the immuno frames with warm agar. Proceed to cover the slides until each half of each frame contains 12 ml of the warm agar in a uniform layer. Place the leveling table cover over the coated plates and allow to set for 30 min.

e. Prepare a humidity chamber by placing wet paper toweling in the bottom of a rinsing tray.

f. After the agar has set, place the frames into the immuno frame holder (Gelman Catalog #51448) and put into the humidity chamber. Allow the slides to remain in the chamber overnight.

2. Pattern cutting and application of serum samples.

a. After removing the frames from the humid atmosphere chamber, the gel punch (Gelman Catalog #51449) is positioned over the frame. By moving the punch along the frame and applying pressure at the appropriate times, a pattern consisting of troughs and wells is thus punched out in the gel layer of each slide.
b. Suck out the plugs of agar remaining in the wells with a fine-tipped micropipette connected to an aspirator.

c. Place the serum samples to be analyzed in the wells with a fine-tipped micropipette.

3. Electrophoresis of samples.

a. Fill the chamber (Gelman Catalog #51211) with buffer solution.

b. Place the frames across the chamber.

c. Saturate 4 strips of filter paper per frame in buffer solution; place wicks in chamber, one end on agar and opposite end in the buffer solution.

d. Place cover on chamber, and connect chamber to power supply.

e. Electrophorese for 120 min at 3-5 ma per frame (12 ma; 200 v when running 3 frames simultaneously).


a. Remove gel from troughs with the gel knife (Gelman Catalog #51480).

b. Place approximately 0.15 ml of the appropriate antisera into the trough and spread evenly.

c. Incubate for 16-20 hr in a humidity chamber or until the lines of precipitation have completely developed.

d. Rinse the slides for 2 days in 2 different solutions of 2% NaCl.
e. Rinse for a short time in distilled water to remove all residual unreacted serum and antiserum.

5. Photographing precipitin patterns.
Precipitin patterns are photographed using a special viewer box which employs indirect lighting. Converging rays of light are allowed to strike the slide which is then photographed against a dark background.

Passive Hemagglutination Test

A. Reagents

1. Alsever's Solution
   a. Solution A: dextrose, 20.5 g; sodium citrate, 12 g; sodium chloride, 4.2 g; triple distilled water, q. s. to 100 ml.
   b. Solution B: citric acid, 1 g; triple distilled water, 10 ml. Adjust pH of Solution A to 6.1 using the citric acid solution. Multiply the amount of citric acid required to attain the above pH x 49; add that amount of citric acid to Solution A.
   c. Filter sterilize using 0.2 μm millipore filter.
   d. Store in the refrigerator as a 10x solution. Add 20 ml of stock solution to 180 ml of sterile distilled water to obtain the final concentration (working solution).
2. Endotoxin (Dykstra, personal communication)
   a. Suspend *Serratia marcescens* lipopolysaccaride W
      (Difco Catalog #3130-25) in 0.85% NaCl to a concentra-
      tion of 10 mg/ml.
   b. Add 4.5 ml of 0.25 N NaOH to this suspension of endo-
      toxin (1 ml), and incubate in a 37 C water bath for
      3 hr.
   c. Neutralization of the endotoxin solution is accomplished
      by adding 4.5 ml of 0.25 N HCl after the 3 hr incubation
      period.
   d. Divide the final solution into 2 ml aliquots and store
      in vials at -20 C until needed.

B. Procedure

1. Preparation of sheep red blood cells.
   a. Collect sheep RBC in Alsever's Solution (equal volumes
      of each).
   b. Wash and centrifuge RBC 3 times in 0.85% NaCl at 600 x g
      for 10 min.
   c. Add 0.25 ml RBC to 1 ml *Serratia marcescens* endotoxin
      solution, and incubate this mixture in a 37 C water
      bath for 30 min.
   d. Wash and centrifuge treated cells to remove excess
      endotoxin. Repeat washing step 3 times.
   e. Resuspend cells in PO₄-buffered saline, pH 7.2, to
      yield a final concentration of 0.5% (v/v).
2. Preparation of antiserum.
   a. Thaw serum in 37°C water bath; remove 1 ml aliquots and inactivate by incubating in water bath at 56°C for 30 min.
   b. Dilute serum 1:5 with 0.85% NaCl.

3. Preparation of 1% normal rabbit serum.
   a. Thaw serum and inactivate as in part 2.
   b. Absorb serum with equal volume of packed normal sheep RBC by adding 1 ml normal rabbit serum to 1 ml packed RBC suspended in 4 ml 0.85% NaCl. Allow to stand for 30 minutes at room temperature.
   c. Centrifuge this suspension at 600 x g for 10 min.
   d. Further dilute this 1:5 concentration of rabbit serum by mixing 1 ml with 19 ml of 0.85% NaCl, to give a 1% solution.


   The complete test is presented in Protocol 1.
### Protocol 1

**Passive Hemagglutination Test Using Endotoxin - Sensitized Cells**

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**Negative Controls**

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**Serial Two-fold Dilutions**

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98