LASALOCID - INDUCED NEUROPATHY

IN BROILER CHICKENS

By

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If - by ***Rudyard Kipling***

If you can keep your head when all about you
Are losing theirs and blaming it on you;
If you can trust yourself when all men doubt you,
But make allowance for their doubting too:
If you can wait and not be tired by waiting,
Or, being lied about, don't deal in lies,
Or being hated don't give way to hating,
And yet don't look too good, nor talk too wise;
If you can dream---and not make dreams your master;
If you can think---and not make thoughts your aim,

If you can meet with Triumph and Disaster
And treat those two impostors just the same:.
If you can bear to hear the truth you've spoken
Twisted by knaves to make a trap for fools,
Or watch the things you gave your life to, broken,
And stoop and build'em up with worn-out tools;
If you can make one heap of all your winnings
And risk it on one turn of pitch-and-toss,
And lose, and start again at your beginnings,
And never breathe a word about your loss:
If you can force your heart and nerve and sinew
To serve your turn long after they are gone,
And so hold on when there is nothing in you
Except the Will which says to them: "Hold on!"

If you can talk with crowds and keep your virtue,
Or walk with Kings---nor lose the common touch,
If neither foes nor loving friends can hurt you,
If all men count with you, but none too much:
If you can fill the unforgiving minute
With sixty seconds' worth of distance run,
Yours is the Earth and everything that's in it,
And---which is more---you'll be a Man, my son!

PREFACE

This is a study of the neurotoxic potential of lasalocid in broiler chickens. Lasalocid, monensin and salinomycin are commonly incorporated in broiler rations to prevent coccidiosis. There is currently little information concerning the neurotoxic effects of lasalocid. There are a few published reports and many empirical observations from poultry producers and veterinarians describing an ataxic syndrome labeled "duck-walking". The purpose of this study was to replicate a field syndrome, describe the syndrome, ensure that it was neurotoxic and not another expression of myotoxicity and begin to elucidate some possible mechanisms. The first phase of these studies (Chapter II) was a large-scale feeding trial with increasing doses of lasalocid, monensin and salinomycin in broiler rations. I used a clinical ataxia scoring system, serum electrolyte and enzymes, and ATPase activities in various organs to describe the neurotoxicity. These studies were an attempt to replicate the field observations with lasalocid and determine if monensin or salinomycin could cause ataxia. These studies also allowed for the determination of a toxic dietary dose (TD_{25}) that would cause ataxia in 25% of birds. The next phase of these studies (Chapter III) refined the dose-response relationship between lasalocid and ataxia using a per os dosing regime. These studies also examined the effects of vitamin E pretreatment and water deprivation on the incidence of lasalocid-induced neurotoxicity as well as recovery in the affected birds. In these studies, the effects of lasalocid on hepatic submitochondrial respiratory chain are examined by measuring the activities of NADH oxidase, succinate oxidase and

submitochondrial ATPase. Also found in Chapter III is information that describes the direct effects of lasalocid on the sciatic nerve *in vitro* .

The findings of these studies identify and characterize lasalocid-induced ataxia as a peripheral neuropathy in broiler chickens. Affected broilers develop dose-dependent ataxia with a reduced motor nerve conduction velocities, histologic and ultrastructural evidence of damage to the peripheral nervous system. Affected birds do not exhibit signs associated with myotoxicity: elevated serum enzymes (LDH, CPK, AST), electrolytes (K^+) or histopathology (myonecrosis). These studies show that there does not seem to be a mechanistic role for ATPase enzymes, specific serum cations (Na^+, Ca^{++}), oxidative stress or water deprivation in this syndrome.

This work is the result of my labors with the help of many individuals and the support of my department. I am most indebted to Dr. Sangiah, my major professor, for his guidance and encouragement during my thesis research. I was drawn to Sangiah because of his exciting classroom persona and thirst for knowledge. He is a solid, well-trained scientist but more importantly, he is caring, gentle man. He has cared for my family much like a father, during good and trying times. I would also like to thank the members of my committee, Dr. George Burrows, Dr. Chuck Qualls and Dr. Steven Wikel for their help, support and encouragement.

Much of the financial support for this study was provided by a research grant from Hoffam-LaRoche Fine Chemicals. I would like to thank Dr. Ken Powell for his help and advice in the early stages of these studies. I would like to thank the Department of Physiological Sciences for the support and the family-like atmosphere, especially when my own family needed the support. I would like to thank Dr. Larry Stein and Dr. Alastair Watson for affording me the opportunity to teach veterinary anatomy – an experience of a lifetime. I owe Dr. Ownby a special thanks for her help with microscopy and her open ear as Department Head. Dr. Stanley VanHooser was an excellent source of information and

helped me learn what little poultry histopathology I know. I would also like to thank Dr. James Breazile for his help with the nerve conduction studies and his support as a deacon when my family needed his strength.

I would also like to thank Dr. Chun Lin Chen for his help with the ATPase study and his friendship during many late nights. I owe a special thanks to a very dear friend, Dr. Eric Stair who lent a helping hand whenever asked. He reminded me to laugh and perhaps not take myself too seriously. We spent many hours studying for the ABVT – that we will pass in Louisville. I look forward to the day when we can give them all the "pitohoui".

My family deserves much of the credit for this work. I am fortunate that I come from a home where books, reading, learning and curiosity was encouraged. From my mother, Barbara, I learned to enjoy the process of learning, the desire to teach and the gift of gab. From my father, John, I learned healthy skepticism, intellectual curiosity and the honor of hard work. Kathy and Terry Stuck have been good friends, family and role models for my children. My three sons: Adam, Alexander and Andrew, are the joys of my life and always have a hug or kiss at just the right time. The greatest acknowledgment for this work and for who I am today belongs to my beautiful wife, Karen. She has made many sacrifices to allow me to pursue this dream and I will always remember her patience. She is truly the best friend I have ever had, a confidant, a cheerleader, a taskmaster and a constant, stable force in my hectic life. She has given me three beautiful boys and the best years of my life. I am either very intelligent or fortunate to have found such a wonderful woman as my companion for the journey of life. Thank you Karen!

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CHAPTER I

INTRODUCTION AND REVIEW OF THE LITERATURE

Introduction

Drug-induced neurotoxicity is an environmental, therapeutic or occupational hazard for humans and animals. The prevalence of human disease range from rare and sporadic (buckthorn neuropathy caused by *Karwinski humboldtii*, a poisonous plant) to endemic and common (Minamata Bay disaster caused by methylmercury from industrial effluents). Toxicants capable of damaging the nervous systems are ubiquitous in an industrialized society as illustrated by peripheral neuropathy caused by tri-ortho-cresyl phosphate (TOCP) contaminated alcoholic beverages, "Ginger-Jake" paralysis, and TOCP contaminated olive oil in Morocco (Anthony & Graham, 1991). Additionally, many chemotherapeutics can produce neurotoxicity as an undesired side-effect: streptomycin, doxorubicin, taxol, and isoniazid. Recently, some reverse transcriptase inhibitors used in AIDS therapy have been shown to cause a therapy-limiting peripheral neuropathy (Anderson et al.,1994; Feldman et al., 1992; Anderson et al.,1992).

The carboxylic acid ionophores are anticoccidial and growth promotant compounds extensively used in animal production in the United States. These

chemotherapeutics exhibit complex behaviors in biological systems, and their actions represent a dynamic interplay of drug, animal, nutritional and environmental factors. Recent evidence suggests that some of these drugs may cause neurotoxicity in animals. To understand the possible role of ionophore-induced neurotoxicity, it is necessary to review the literature of the polyether, ionophorus antibiotics (classification, mechanisms of action, pharmacology, toxicity) and cursorily examine the field of neurotoxicity

The Ionophores: Polyether Antibiotics

Classification

Pressman et al. (1967) coined the term ionophore, literally "ion-bearer ", as a descriptive and dynamic definition for antibiotics with the ability to bind and transport ions across biological membranes. A simplistic classification system is to place the ionophores into families with similar or shared properties; e.g., the valinomycin group, the nigericin group. A more precise method of classification is based on the mode of ion transport :neutral, channel-forming quasi- and carboxylic ionophores.

Neutral Ionophores. The neutral ionophores (e.g. valinomycin) mediate electrogenic transport of ions governed by membrane concentration gradients and membrane potential. At equilibrium, these ionophores will follow the Nernst equation if a single species is transported and the Goldman equation if several species of monovalent ions are being transported.

Channel-Forming Ionophores. The channel-forming quasi-ionophores (e.g. gramicidin) mediate a transport characterized by periodic bursts of current representing very rapid turnover. These ionophores work through existing membrane channels as well as forming new "channels" in membranes (Pressman, 1976).

Carboxylic Ionophores. The carboxylic ionophores (e.g. monensin, lasalocid, salinomycin) mediate concentration-dependent, pH-sensitive transport of ions independent of membrane potential. These compounds act as "exchange diffusion" carriers, transporting a cation during both stages of the exchange cycle (Pressman, 1976). The majority of the ionophores of veterinary importance are carboxylic acid ionophores and as such the remaining discussions will focus on their properties.

History of the Carboxylic Ionophores

In 1951, several antibiotics were isolated from *Streptomyces* that exhibited a wide antibacterial spectrum (Berger et al.,1951). This discovery was followed in 1970 by the determination of the chemical structure of X-537A (lasalocid) (Westley et al.,1970; Johnson et al.,1970) and in 1974 isolation of the fungus (*Streptomyces lasaliensis*) responsible for producing lasalocid (Westley et al.,1974).

Development of the ionophores into therapeutic agents required an additional 25-30 years of work. Monensin was approved for use as a coccidiostat in chickens (COBAN®) in 1971 and as a growth promoter for cattle (RUMENSIN®) in 1975. Lasalocid was approved for use as a coccidiostat in chickens (AVATEC®) in 1977 and as a growth promoter in cattle (BOVATEC®) in 1982. Salinomycin (BIOCOX ®) was approved for use as a coccidiostat in chickens in 1983 (Novilla, 1992; Ruff, 1982).

Chemistry of Carboxylic Acid Ionophores

The carboxylic ionophores (nigericin, grisyxin, monensin, dianemycin, salinomycin, lasalocid [X-537A] and A23187) are open-chained oxygenated heterocyclic rings with a single terminal carboxyl group. These compounds are of moderate molecular weight (200-2000), with the ability to form lipid-soluble transport complexes with polar cations (K^+, Na^+, Ca^{++} and Mg^{++}). Figures 1-3 show the molecular structures for lasalocid, monensin and salinomycin, respectively. Lasalocid is an asymmetrical compound with a tetrahydropyran, a tetrahydrofuran ring and an aromatic ring that exists as a ring by head-to-tail hydrogen binding in biological membranes (Pressman, 1976). The dimerization of two ionophore molecules forms a highly polar cation liganding site, the oxygen atoms, surrounded by a nonpolar carbon skeleton of the ionophore. The three dimensional structure of monensin and salinomycin resemble a "doughnut" with the "hole" as the seat for ion complexation. This confers a degree of cation selectivity to these ionophores. In contrast, lasaslocid assumes a "shell-shaped" configuration as a dimer. On the rim of the shell are the oxygen groups responsible for ion complexation. This open configuration may explain the ability of lasalocid to complex with a variety of mono- and divalent cations (Pressman, 1972,1976).

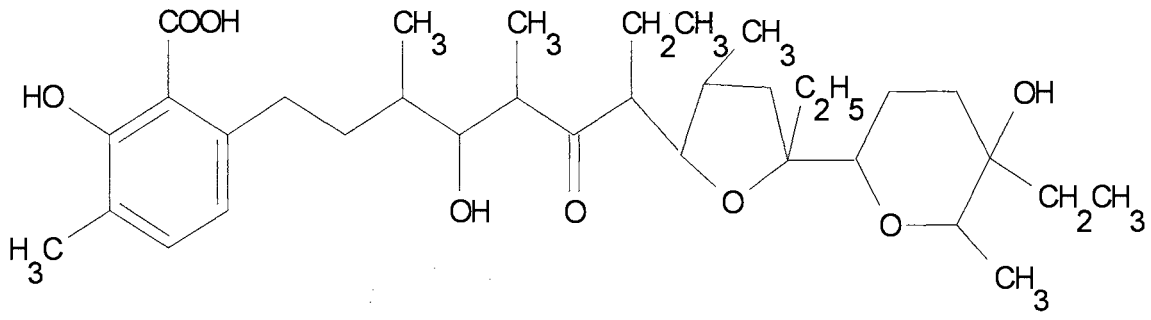

Figure 1. The Chemical Structure of Lasalocid

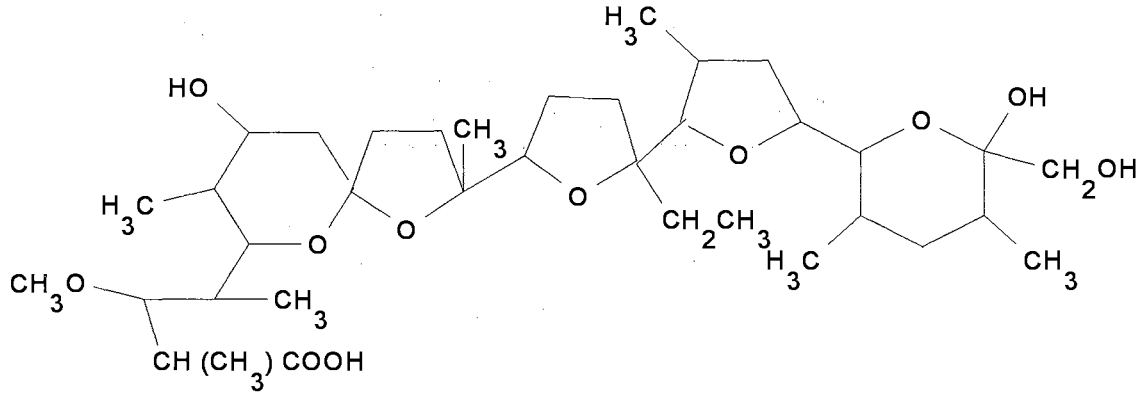

Figure 2. The Chemical Structure of Monensin

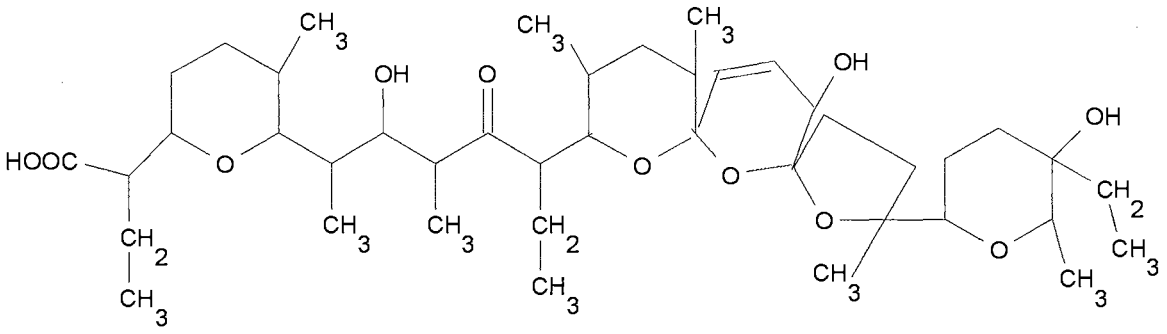

Figure 3. The Chemical Structure of Salinomycin

A common property of ionophores is complexation and transport of deprotonated, anionic cations. The transport of specific ions, Na^{+},K^{+} and Ca^{++}, across membranes accounts for the pharmacologic effects of the ionophores. The ensuing transport is electrically neutral involving a cation-for-proton exchange. The kinetics of ion transport by ionophores is rapid with turnover rates in biologic membranes achieving values of several thousand per second (Pressman, 1976). Each ionophore has a characteristic ion affinity and transport capacity described by *in vitro* complexation and transport studies (Pressman, 1976).

Biologic Effects Of Ionophores

Mitochondrial Effects. The fundamental mitochondrial function is aerobic production of ATP by electron transport linked to oxidative phosphorylation. The endergonic synthesis of ATP from ADP and Pi in mitochondria is catalyzed by ATP synthetase (ATPase). The chemiosmotic hypothesis postulates that the "power" for ATPase activity is generated by an electrochemical, proton gradient between the

mitochondrial matrix and the intermembrane space. This reaction requires an intact inner mitochondrial membrane impermeable to H^+, OH^-,K^+, and Cl^-, to prevent discharge electrochemical gradients.

One of the first noted properties of ionophores was the ability to alter mitochondrial respiration *in vitro* (Pressman, 1976). Recently, lasalocid-induced mitochondrial inhibition were shown to involve electroneutral K^+ for H^+ exchange across the inner mitochondrial membrane, altering pH and membrane potential in a Ca^{++}-dependent manner (Antonio, 1991). A23187 inhibits mitochondrial ATPase in sperm in a Ca^{++} dependent manner by releasing membrane-bound Mg^{++} and uncoupling oxidative phosphorylation (Reed & Lardy, 1972). Alternatively, lasalocid (0.05-0.01 μM) decreases mitochondrial glutamate oxidation by a Ca^{++}-independent mechanism at ionophore concentrations 2x lower than required for ion transport. The authors suggest that lasalocid complexes with membrane-associated Mg^{++} ions that are critical in mitochondrial energy production (Lin & Kun, 1973). A23187 stimulates mitochondrial respiration in some cells, while in other studies A23187, monensin, and lasalocid decrease intracellular ATP levels (Ruff, 1982).

The impact of ionophores on mitochondrial function may depend on the energy status of cells. The utilization of ATP to drive homeostatic pumps (Na^+, K^+-ATPase, Ca^{++}-ATPase) to maintain normal cation concentration gradients may be a homeostatic response to ionophore exposure.

Effects on Golgi Apparatus. Monensin and lasalocid inhibit the function of the trans face of the Golgi apparatus in the areas of secretory vesicle formation, endocytosis, and product sorting. Vacuolation and swelling of cisternae is a common finding for monensin- and lasalocid-incubated cells due to ion movement across cell

membranes with concurrent movement of water (Emery et al., 1991; Somlyo et al.,1975; Mollenhauer, et al, 1990).

Cardiac Function. Carboxylic ionophores increase myocardial contractility indirectly by Na^+ transport, direct Ca^{++} transport or by neurotransmitter release (Reed, 1982). A23187 and lasalocid increase cycle length in spontaneously beating rabbit sinoatrial node cells (Satoh & Uchida, 1993). In canine Purkinje fibers, A23187 decreased while lasalocid slightly increased contractile force and both ionophores markedly shortened action potential duration, delayed afterpotential and aftercontracton, possibly due to cellular Ca^{++} overload (Satoh et al.,1992).

Skeletal Muscle Effects. Carboxylic ionophores produce skeletal muscle contraction by Ca^{++}-mediated processes (Statham & Duncan, 1975; Statham et al., 1976; Cochrane & Douglas, 1975). Ionophores transport Ca^{++} into cytoplasm from extracellular stores (lasalocid, A23187) or transport of Na^+ into cells with subsequent exchange of Na^+ for Ca^{++} (lasalocid and monensin) (Ruff, 1982). The elevated Ca^{++} binds to troponin C causing a conformational change in the troponin I, releasing tropomysosin inhibition leading to actin-myosin interaction and muscle contraction. Lasalocid reversibly increased the frequency of excitatory post-synaptic potentials in locust extensor tibiae nerve-muscle preparations, while prolonged (> 60 minutes) exposure to lasalocid caused irreversible "giant" miniature potentials, synaptic vesicle depletion, mitochondrial damage, and disintegration of microtubules and neurofilaments in nerve terminals (Fahim, 1992).

Effects on Membrane Potential. The carboxylic acid ionophores dissipate ionic gradients across membranes modifying resting potential. Lasalocid and A23187

depolarize frog skeletal muscle fibers (Cochrane & Douglas, 1975). Valinomycin reduces erythrocyte membrane potential via Cl^- efflux, while lasalocid, monensin, and nigericin have no effect (Wittenkeller et al., 1992). Monensin induces Na^+-dependent hyperpolarization of neuroblastoma cells by activating Na^+, K^+-ATPase (Lichtshtein et al.,1979).

Veterinary Uses Of Ionophores

The ionophores of veterinary significance (monensin, lasalocid, salinomycin) are used to control and treat coccidiosis, especially *Eimeria sp.*, and as growth promotants, increasing the feed efficiency of ruminants.

Anticoccidial Effects in Broilers. Coccidiosis is an economically important disease in the poultry industry. The short feeding period and narrow profit magins for broilers demand coccidial prevention rather than treatment. Commonly, coccidial infections are subclinical with a moderate to high morbidity, low mortality causing reduced profitability. Clinical signs of avian coccidiosis include diarrhea, bloody feces, ruffled feathers, reduced weight gains and birds huddled together for warmth. Most birds produce a strong, species-specific protective immunity, after survival from initial infection.

The success of the ionophores as a class of anticoccidials can be attributed to efficacy, introduction at a time of resistance to previous anticoccidial agents and absence of true drug resistance against the ionophores by the coccidia (Ruff, 1982). The actions of ionophores on coccidia relate to the drug's ability to transport ions across biological membranes. Coccidia are intracellular parasites that depend upon the host cell for energy (ATP). Ionophores mediate cation influxes that require coccidial energy to maintain ionic

homeostasis. The ionophores alter ionic balance within coccidia that leads to cell dysfunction and death. *In vitro* incubation of coccidia with monensin causes ultrastructural changes including cytoplasmic vacuoles, bulging and separation of the plasma membrane and pycnotic nuclei (Augustine et al., 1992). These changes are associated with a significant time- and dose-dependent inhibition of cellular invasion. The ultrastructural effects of lasalocid on *Eimeria tenella* showed blistering of the outer membrane, large surface swellings and enlarged mitochondria in first- and second-generation merozoites (Daszak et al.,1991).

Efficacy studies for anticoccidial drugs usually are performed in three stages: battery cage studies, floor pen studies and field trials. Efficacy of an ionophores may differ drastically in these stages. Monensin performs poorly in battery trials but is highly efficacious in field trials (Ruff, 1982). A recent report showed that lasalocid easily controlled monensin- and narasin- resistant strains of *Eimeria*, but failed to control other susceptible strains in battery cage studies (Weppelman et al.,1977).

Recently, chicken embryos have been used to screen ionophorous anticoccidials against different stains of *Eimeria* (Mora et al, 1991; Xie et al.,1991). The *in vitro* determination of the minimal inhibitory concentration and minimal toxic concentration have been evaluated for many anticoccidials. The toxic effects of these compounds against the embryos and the widely divergent effective concentrations in embryos may limit the application of this technique as a screening tool (Xie et al.,1991).

A recent report of *in vivo* and *in vitro* resistance to a field isolate of *Eimeria tenella* may suggest that the coccidia are developing mechanisms to circumvent the actions of the ionophores. In this study, birds developed clinical coccidial infections despite treatment with 2x recommended levels of monensin, salinomycin, and lasalocid (Zhu &

McDougald, 1992). *In vitro* incubation of the isolate with ionophores did not alter coccidial invasion, development (sporozoites to schizonts), or ultrastructure. This type of data may encourage the use of ever-increasing concentrations of the ionophores in rations by the poultry producer.

Ionophore Uses in Ruminants. Monensin and lasalocid improve feed efficiency (10-20%) in feedlot and pastured cattle by altering the ruminal microflora (Bergen & Bates, 1984; Van Soest, 1982; Dennis et al, 1982; Corah, 1991; Quigley et al.,1992; Helaszek & White, 1991). Ionophores can also reduce the incidence of bloat, acidosis, and prevent tryptophan-induced atypical bovine pulmonary emphysema. Another indication for ionophore use in ruminants is the prevention or treatment of coccidial infections (Eicher-Pruiett et al.,1992; Heinrichs & Bush, 1991; Sinks et al., 1992). Lasalocid can also increase carcass quality (lean protein quantity, longisssimus area, muscle color) of cattle (Krelowska-Kulas et al., 1992).

Future Therapeutic Uses of Ionophores

In addition to their continued use as effective anticoccidial agents, ionophores may be used to treat other diseases in the future. Lasalocid may have a role in the treatment of life-threatening *Cryptosporidium parvum* infections in immunocompromised patients as evidenced by recent animal studies (Lemeteil et al.,1993; Brasseur et al., 1991; Kimata et al., 1991). Monensin may be used as an adjunct therapy for certain forms of cancer. Liposome delivered monensin increases the effects of immunotoxins (Madan & Ghosh, 1992a,b; Griffin et al.,1993; Candiani et al.,1992; Colombatti et al.,1990), decreases lag time (Madan & Ghosh, 1992a), potentiates ricin cytotoxicity *in vivo* (Vasandani et al., 1992) and *in vitro* for many tumor cells (Griffin et al.,1993).

Ionophore Intoxication

Overview

The ionophores currently used in livestock production are safe and efficacious at prescribed use levels in intended species. The majority of the literature concerning the toxic effects of ionophores describes monensin, due to the earlier market entry and greater market share for this compound. Ionophore toxicosis is well documented in most domestic species including horses, the most sensitive species (Osweiler et al.,1983; Hanson et al, 1981), cattle (Mathieson et al, 1990; Galitzer et al, 1986; VanVleet & Ferrans, 1983), goats (Dalvi & Sawant, 1990) ,pigs (VanVleet et al.,1987; Morgan et al.,1991) ,chickens (Horovitz et al.,1988) and quail (Sawant et al., 1990). Certain management situations increase the probability of intoxication due to overdosages (mixing errors or premix consumption), misuse in non-target species, or drug interactions (chloramphenicol, tiamulin) (Novilla, 1992). Diagnosis of ionophore toxicosis is initially tentative since clinical signs and lesions are not pathognomic. Any feed-related problem characterized clinically by anorexia, diarrhea, dyspnea, ataxia, depression, recumbency, acute mortality, and pathologically by focal degenerative cardiomyopathy, skeletal muscle necrosis, and congestive heart failure, warrants a presumptive diagnosis of ionophore toxicosis (Novilla, 1992). Confirmation requires consideration of differential diagnoses and laboratory assays to determine the specific ionophore involved. There are no specific antidotes for ionophore intoxication but removal of affected animals from medicated feed, administration of activated charcoal and saline cathartics may lessen absorption and reduce the toxic effects (Osweiler et al.,1983).

Differential Diagnosis

The differential diagnosis for ionophore toxicosis should include other causes of neuropathy and myopathy. In cattle, the differential should include vitamin E / selenium deficiency and poisonous plant intoxication: coffee senna (*Cassia occidentalis*), coyotillo (*Karwinskia humboldtiana*), or white snakeroot (*Eupatorium rugosum*) (Osweiler et al.,1983; Novilla, 1992). In poultry the differential includes nutritional myopathy, coffee senna toxicosis, botulism, NaCl (water deprivation) intoxication, mycotoxicoses (moniliformin cyclopiazonic acid), round-heart disease, and downer syndrome (viral arthritis) (Novilla, 1992). In horses, the differential should include colic, blister beetle ingestion (cantheridin intoxication) and azoturia (Osweiler et al.,1983).

Clinical Signs

The most common clinical signs of intoxication include anorexia, hypoactivity, leg weakness, ataxia, dyspnea, depression and death. Of these, anorexia is most consistently associated with consuming toxic levels of an ionophore (Novilla, 1992; Osweiler et al.,1983; Simon et al.,1991; Novilla et al., 1994; Todd et al., 1984; Galitzer et al., 1982; VanVleet et al.,1987). Decreased body weight is a common finding in poultry (VanderKop & MacNeil, 1990; Bartov, 1994; Todd et al.,1984).

Clinical Pathologic Changes

The clinical pathologic changes associated with ionophore-induced toxicosis are elevated enzyme levels of muscle origin: aspartate transaminase (AST), creatine phosphokinase (CPK), lactic dehydrogenase (LDH), with increased alkaline phosphatase (ALP), (Horovitz et al.,1988) serum urea nitrogen (SUN) and bilirubin

(Osweiler et al.,1983). Serum Ca^{++} and K^{+} levels may decline to life-threatening levels in monensin-intoxicated ponies or horses (Novilla, 1992; Osweiler et al.,1983).

Gross Pathologic Lesions

The gross lesions noted with ionophore overdose relate to the musculoskeletal and cardiovascular systems. Common findings are pale skeletal and cardiac muscle, flabby myocardial tissue, dilated ventricles, or yellow-white streaks of necrosis in the myocardium (Osweiler et al.,1983; Novilla, 1992). Animals that die acutely may exhibit no gross pathologic changes (Osweiler et al.,1983).

Histopathologic and Ultrastructural Changes

The histologic lesions found in ionophore toxicosis include focal degeneration of myocytes, vacuolation, swelling and eosinophilic staining of cardiomyocytes. Ultrastructurally, vacuolation is caused by swelling of mitochondria with disrupted cristae, dense matrical granules, cristolysis, swollen sarcoplasmic reticula and disruption of myofibrillar architecture (Anderson et al.,1984). After ionophore-mediated damage occurs to cardiomyocytes injured tissue is replaced by fibrosis (Novilla, 1992).

Ionophore-Induced Myopathy/Cardiomyopathy

The most common manifestation of ionophore-induced intoxication is skeletal myopathy or cardiomyopathy, depending upon the species involved. In horses, the primary organ affected is the heart while in dogs and pigs lesions are restricted to skeletal muscles and in poultry, cattle and rodents there is an equal distribution of cardiac and skeletal muscle lesions (Novilla, 1992).

Ionophore-induced myopathy is well described in poultry. Monensin-gavaged (≥1.93 mg/kg/day) turkeys present with ataxia, rear limb paresis and paralysis. Histologically, birds exhibit dose-dependent, necrotizing skeletal myopathy of the leg muscles with intrafiber edema and myocardial vacuolation (Cardona et al.,1993). Lasalocid fed to broiler breeders caused leg weakness, severe myonecrosis, ataxia and decreased production (eggs, fertility and hatchability) (Perelman et al., 1993).

Cardiotoxicity in monensin-intoxicated pigs presents as moderate to extensive myofibrillar necrosis with sarcoplasmic vacuolation and swollen mitochondria with disrupted cristae and dense matrical granule accumulation (VanVleet & Ferrans, 1984). Holstein steers given lasalocid or monensin *per os* had gross (flabby, dilated hearts with petecchia and ecchymosis) and histopathologic (multifocal myocyte necrosis with mild to marked cardiomyopathy, mononuclear cellular infiltrates and Purkinje degeneration) signs of cardiomyopathy (Galitzer et al., 1986). In another study, Holstein steers given a single oral dose of lasalocid (25 - 125 mg/kg) exhibited muscle tremors, increased heart and respiratory rates, followed by anorexia. Indications of cardiac muscle damage, elevated CPK and LDH, were noted in animals given 125 mg/kg (Galitzer et al., 1982). The ultrastructural damage associated with monensin myocardial toxicosis in calves details extensive sarcoplasmic vacuolation from mitochondrial swelling and lipid droplet accumulation. The initial damage was followed by myocardial necrosis with disrupted contractile proteins, pycnotic nuclei and macrophage infiltration (VanVleet & Ferrans, 1983). *In vitro,* lasalocid causes Golgi apparatus cistern swelling in rabbit sino-atrial node cell that led to dilation and vacuolization after the addition of Ca^{++} (Satoh & Uchida, 1993).

Drug Interactions With Ionophores

Many ionophores are incompatible with other therapeutic agents (Burch & Stipkovits, 1991; Umemura et al.,1984) or dietary components (Williams, 1992). Lasalocid is incompatible with concurrent administration of chloramphenicol (Broz & Frigg, 1987; Perelman et al.,1986) and T-2 fusariotoxin (Varga & Vanyi, 1992). Tiamulin enhances the toxicity of monensin and salinomycin in poultry (Umemura et al.,1984; Simon et al.,1991; Laczay et al.,1991; Mezes et al.,1992) and swine (Morgan et al.,1991). This interaction in pigs is minimized by concurrent administration of vitamin E and selenium (VanVleet et al.,1987). In contrast, field trials indicate that tiamulin and salinomycin are compatible in mycoplasma-infected birds and result in decreased mortality with improvement , lesion scores, and feed-efficiency (Stipkovits et al.,1992).

Ionophore - Induced Neurotoxicity

In certain situations, some ionophores may cause neurotoxicity. A recent report describes a paralytic syndrome in dogs due to lasalocid incorporation into a commercial dog food by a mixing-mill error. The disease was marked by generalized lower motor neuron deficits that were gradually reversible (Safran et al., 1993a). An experimentally-induced syndrome in broilers caused by elevated feed levels of lasalocid produced irreversible disinclination to stand, followed by hock sitting and a characteristic walking "on tip toe" after seven days of consumption which were more severe after the addition of chloramphenicol. Pathologic examination revealed vacuolization and demyelination of the spinal cord (Shlosberg et al.,1986). Neurophysiologic examination of broilers fed monensin and tiamulin showed reduced motor nerve conduction velocity and

lengthened relative refractory period in the sciatic nerves of 4 (Laczay et al.,1991) and 5 week-old male broilers (Simon et al.,1991).

In vitro evidence suggests that lasalocid causes swelling of perikarya, followed by neuronal death without affecting glial cells, fibroblasts or cultured rat astrocytes. Lasaslocid induced a Ca^{++} influx inhibited by MK-801 (a non-competitive NMDA receptor antagonist) but was not affected by voltage-sensitive Ca^{++} channel blockers (nimodipine, D-600) (Safran et al.,1993b). Nigericin and valinomycin can inhibit nerve growth factor (NGF)-induced neurite outgrowth in PC12 cells, while monensin and A23187 are without effects. This may reflect a K^{+}-sensitive signal transduction pathway in the NGF-induced neuronal differentiation PC12 cells (Harada et al., 1994). In another study, A23187 produced a dose- and time- dependent degeneration of the PC12 cells with recovery upon removal of the ionophore (Michel et al.,1994).

Ultrastructural evidence also supports the possibility of neurotoxicity induced by the ionophores. A23187 causes destruction of microtubules and microfilaments leading to myelin vesiculation in the desheathed sciatic nerve of the rat (Schlaepfer, 1977a,b). Time-course, ultrastructural studies show ionomycin-induced demyelinating in the central (Smith & Hall, 1994) and peripheral (Smith & Hall, 1988) nervous systems. Schwann cells and oligodendrocytes exhibit vesicular demyelination due to elevated intracellular Ca^{++} levels which may activate phospholipase A_2 (PLA_2) or other endogenous Ca^{++} -activated enzymes (Smith & Hall, 1988,1994). In transected nerves, ultrastructural changes are mediated by Na^{+} and Ca^{++} concentrations in the media. Sodium in the media of damaged dendrites causes dilation of the Golgi cisternae and increased mitochondrial electron opacity similar to uninjured neurons incubated with monensin (Emery et al., 1991).

The occurrence of ionophore-induced neurotoxicity may be masked by skeletal or cardiac muscle damage. In broilers, concurrent administration of lasalocid and chloramphenicol produce decreased body weight gains, "toe walking", reluctance to move and complete ataxia. This syndrome is reversible in mildly affected birds after the removal of the medicated feed. Histopathologic and ultrastructural evaluation revealed a correlation between incidence of disease and muscle fiber damage and inconsistent demyelination of the spinal cord and sciatic nerves (Perelman et al.,1986). Novilla recently reported that narasin (*per os* or by inhalation) can cause focal degeneration of intramuscular nerves of dogs. In acute inhalation studies, dogs exposed to narasin for 14 days had slight to moderate degeneration while in three-month studies the damage was described as mild to severe inflammatory or degenerative lesions of sciatic nerves . The author noted that similar lesions were induced by salinomycin (Novilla, 1994).

Neurotoxicity

Neurotoxins produce a wide array of clinical manifestations. Many toxicants differentially affect specific regions of the brain or peripheral nervous system. Information from mechanistic neurotoxicologic studies provides scientists with tools to study neurophysiology (tetrodotoxin, GABA). Understanding mechanism(s) of neurotoxins involves an appreciation of the anatomy, physiology and regenerative potential of the nervous system. The broad class of compounds classified as "neurotoxic" interact dynamically with cells of the central and peripheral nervous systems. The role of behavioral changes and clinical observation are still very important in screening and identification of neurtoxic compounds as many toxins can alter nervous system function without causing any morphologic changes.

Classification of Neurotoxic Disease

Morphologic classification of neurotoxic diseases is a simple way of grouping toxicants that can also incorporates mechanistic data. In this scheme there is a primary assumption, that each toxicant has a primary focus of cellular damage (Spencer & Schaumburg, 1980). This is not true for many compounds, especially metals, but it allows for categorization of neurotoxicants. Another difficulty with morphologic grouping of neurotoxicants is that not all cause alteration to cell structure. A toxin can be so exquisitely potent that mortality occurs before any morphologic changes. Further, due to nervous system complexity, alteration of neurotransmitter release or electrical excitability could be the mechanism of toxicosis without concurrent pathologic changes. Currently, there are exhaustive lists of compounds of neurotoxins, the cell(s) involved and for some, the site of action. Many compounds lack an exact mechanism(s) or sub-cellular site of toxic effects. As more mechanistic information for various toxins is gathered, a more refined classification system will evolve.

The morphologic classification system uses anatomic location of action (soma, axon, or myelin) to group neurotoxicants. Some systems further subdivide toxicants by subcellular site of action, if known. Spencer & Schaumburg (1980) describe a schema including neuronopathies, axonopathies, and myelinopathies. A similar neuropathologic system described by Gopinath (1987) focuses on individual cell types affected by the toxin(s) and categorizes neuronopathies, axonopathies, myelinopathies, gliopathies, vasculopathies, and chroid plexus vacuolation.

Neuronopathy involves insult to the nerve cell body (Thomas, 1980) and is usually widely distributed and irreversible (Anthony & Graham, 1991). Neuronopathies are further subclassified as distal and proximal axonopathies, depending on the portion of the axon

affected (Thomas, 1980; Spencer & Schaumburg, 1980). Neuronopathy can lead to a rapid secondary breakdown of axons and dendrites by a process called Wallerian degeneration, and further exposure to the toxicant leads to the collapse and removal of the myelin sheath limiting regenerative ability the nerve (Spencer & Schaumburg, 1980).

Myelinopathy or Demyelination

Myelinopathy affects oligodendrocytes in the CNS and Schwann cells in the PNS. Myelinopathy is characterized by rapid onset, with greatest susceptibility of the longest internode fibers. Remyelination can occur rapidly with full recovery of strength and sensation to the affected areas.

Primary demyelination involves segmental degeneration of the myelin sheath with proximal and distal nerve involvement, no correlation to axonal damage and a random distribution of demyelinated segments (Cammer, 1980). Secondary demyelination involves inflammation or trauma to the myelin sheath that is characterized by focal areas of myelin damage and localized distribution of damage.

Mechanisms of primary demyelination relate to effects of the toxicant on myelin producing cells (Schwann cells, oligodendrocytes). Primary demyelination produced by triethyltin, hexachlorophene, isoniazid and salicylanilides. Triethyltin, the prototype of this class, produces a severe, non-inflammatory edema and vacuolation of the myelin sheath of sciatic nerves without altering myelin composition (Cammer, 1980). Demyelination caused by direct damage to the myelinating cells has been described for lead, cuprizone®, ethidium bromide, diptheria toxin, tellurium, thiamin deficiency, and chronic cyanide or carbon monoxide intoxication (Cammer, 1980; Pleasure et al., 1973; Harry et al.,1989).

Damaged and demyelinated nerves exhibit delayed conduction velocities and reduced amplitude of the waves of depolarization. In the normal myelinated nerve, Schwann cells surround the axon and insulate the nerve membrane restricting depolarization to the Nodes of Ranvier. This specialization allows for a more rapid passage of the depolarization wave, salutatory conduction, down the nerve. The resting membrane potential is established by the selective transport of sodium and potassium by the Na^+, K^+-ATPase enzyme. At resting membrane potentials, Na^+ channels are gated shut, impermeable to Na^+. When the membrane is depolarized, Na^+ channels open allowing Na^+ influx down concentration gradients leading to further depolarization. Indications of demyelination include the inability to transmit trains of impulses and a lengthened refractory period. An increased refractory period may be a more sensitive indicator of incomplete or "patchy" demyelination than measurement of the conduction velocity (Rasminsky, 1980).

Possible Mechanisms of Ionophore-Induced Toxicosis.

Calcium-Mediated Cell Death

Calcium levels are tightly regulated within cells due to the role of calcium as an intracellular signaling mechanism. The role of Ca^{++} in cell death is well described (Schilder et al.,1994; McKeage et al.,1994; Wrogemann & Pena, 1976) and ionophore-induced cytotoxicity may involve transport, directly or indirectly, of this cation (Osweiler et al.,1983). Ionophore intoxication probably involves an influx of Na^+ and Ca^{++} ions into affected cells with a concurrent efflux of K^+ ions leading to excessive Ca^{++} uptake by mitochondria, mitochondrial damage, lack of cellular energy, elevated cytoplasmic Ca^{++} levels and ultimately muscle necrosis (Novilla, 1992).

In several papers, the mechanism(s) ionophore-mediated cell death suggest that A23187, monensin and lasalocid cause dysfunction through a multi-staged process, involving ionophores, Ca^{++}, and Na^{+} (DuBourdieu & Shier, 1992; Shier & DuBourdieu, 1992; Shier et al., 1991) Interestingly, the cells most sensitive to ionophore-induced cytotoxicity were excitable cell, cardiac muscles. Cytotoxicity, as measured by LDH and arachidonic acid release, occurred in cultured cardiac myocytes by two different Ca^{++}-mediated steps only if they follow a Na^{+}-dependent cell injury. The Na^{+}-dependent step requires extracellular $Na^{+} \geq 75mM$ (half the physiologic levels) (DuBourdieu & Shier, 1992). The first Ca^{++}-- mediated step required A23187, extracellular Ca^{++} concentrations in the micromolar range and is inhibited by Mn^{+} or Ni^{++}. The second Ca^{++}-mediated step requires extracellular Ca^{++} concentrations in the millimolar range is not ionophore-dependent and is not inhibited by Mn^{++}. Ionophore-induced cytotoxicity caused blebs in cell membranes, cellular swelling, increased permeability to trypan blue and holes in the plasma membrane. Monensin-induced Na^{+} influx allows toxic levels of extracellular Ca^{++} into the cytoplasm by Na^{+}/ Ca^{++} antiporter protein(s) (Shier & DuBourdieu, 1992).

In mice, the lethality of monensin is potentiated by Ca^{++} channel blockade (verapamil, diltiazem), Na^{+} channel blockade (lidocaine), α-adrenergic blockade (tolazoline) or centrally acting α_2 adrenergic antagonists (yohimbine) (Mitema, et al. 1988). Nimodipine protects against monensin- and ouabain-induced, calcium mediated cytotoxicity by maintaining lysozomal integrity (Danks et al.,1992). Valinomycin and beauvericin, K+ specific ionophores, induce apoptosis by increasing Ca^{++} from intracellular stores which activates endonucleases (Ojcius et al.,1991). Mehrotra et al recently reported that Ca^{++}

ionophores, calcimycin and lasalocid, potentiated $CaCl_2$ -mediated mitochondrial swelling *in vitro* (Mehrotra et al., 1993).

ATPase

The Na^+, K^+-ATPase enzyme is an ubiquitous regulatory protein involved in cell volume regulation, release of neurotransmitters, and maintenance of ion gradients required for action potentials. The Na^+, K^+-ATPase enzyme has two subunits,α and β. The α subunit spans the cell membrane, with molecular weight of 95,000 daltons, as well as binding sites for ATP and ouabain. The β subunit is a glycoprotein with a molecular weight of 40,000 daltons. The α and β subunits of Na^+, K^+-ATPase catalyze movement of 3 Na^+ ions out of and 2 K^+ ions into the cell (Sweadner & Goldin, 1980).

Decollogne et al. (1993) recently reviewed some ionic and hormonal factors influencing Na^+, K^+-ATPase, with specific reference their significance in health and disease. The Na^+, K^+-ATPase isozymes and their various subunits can be influenced by intracellular Ca^{++} and K^+ levels. In skeletal muscle, the α_2 subunit of Na^+, K^+-ATPase is half maximally inhibited by 160 nM Ca^{++} (the resting Ca^{++} level), while the α_1 subunit is altered by 600 μM Ca^{++} (McGeoch, 1990). Increased intracellular levels of Ca^{++} stimulate mRNA production for α1 and β1 subunits in outer medullary kidney tubular segments (Rayson, 1990). Decreased intracellular levels of K+ decrease the number of functional α2 subunits in rat skeletal muscle and brain tissue (Azuma et al.,1991).

Some neurotoxic compounds alter Na^+, K^+-ATPase function. Interference with ion transport can lead to increased levels of Na^+, Cl^-, and water in neural tissue causing edema and vacuolation. This is suggested in the pathogenesis of demyelination

induced by triethyltin, hexachlorophene, cuprizone® and isoniazid (Anthony & Graham, 1991).

Mitochondrial Oxidative Phosphorylation

Many compounds alter intracellular levels of ATP as part of their mechanism of toxic effects. This can occur at any level along the electron transport chain, at ATPase itself or at the outer mitochondrial membrane. Since oxidation and phosphorylation are tightly regulated, alteration in either will reduce the generation of ATP.

One mechanism for altering the intracellular generation of ATP is inhibition of electron transport. Toxicants in this group interrupt the flow of electrons along the respiratory chain and can be classified by the site of inhibition (Moreland, 1980). Similarly preventing proton transport through the stalk portion of ATPase, as exhibited by oligomycin, will also reduce ATP production. Intracellular ATP levels can also be lowered by uncoupling oxidation of substrate from the phosphorlyation of ADP without interfering with electron transport. Dinitrophenol acts in this manner by dissipating the trans-membrane proton gradient that powers ATP production via ATP synthetase. Toxicants can also disrupt regulatory substrate concentration within mitochondria thereby altering ATP production. Carboxyatractloside ,from cockleburs, acts as on an ADP-ATP translocator of the external mitochondrial membrane.

Some neurotoxicants produce primary demyelination by altering the oxidative phosphorylation capabilities of cells thereby reducing energy available for myelin synthesis and maintenance. Examples of compounds acting by this mechanism include triethyltin, lead, hexachlorophene, acetyl ethyl tetramethyl tetralin (AETT) and salicylanilides (Cammer, 1980; Anthony & Graham, 1991). Carbon disulfide causes

axonopathy by altering neuronal mitochondrial ATP generation capability (Anthony & Graham, 1991) uncoupling oxidative phosphorylation, decreasing the P:O ratio and lowering ATP-Pi exchange rate (Tarkowski & Sobczak, 1971).

Recently, the carboxylic ionophores have been shown to alter the energy status of cells resulting in cellular dysfunction. Monensin induced intoxication results in K+ influx into cell organelles, especially mitochondria which inhibits ATP production (Osweiler et al.,1983). Monensin increases acetaminophen-induced hepatotoxicity by depleting ATP and collapsing mitochondrial membrane potential (Harman et al.,1991) or uncoupling oxidative phosphorylation similar to valinomycin (Albertin et al.,1994). Gramicidin- and Br-A23187-induced cytotoxicity is due to uncoupling oxidative phosphorylation and decreased ATP generation in rat hepatocytes (Nieminen et al.,1990).

Statement of Dissertation Problem and Hypotheses

The polyether carboxylic ionophores are commonly used in poultry production for the control of coccidiosis. Ionophore-induced toxicosis has classically been described as myotoxicity involving cardiac and skeletal muscles; but, may also involve damage to the nervous system.

Recent, unpublished field observations report clinical signs of neurotoxicity in broiler chickens consuming a ration containing lasalocid, an anticoccidial ionophore. Producers and poultry veterinarians have labeled this syndrome "duck-walking", "knockdown" or simply "down birds". The incidence of the disease can reach 5% of a broiler flock. This represents a significant economic loss to the producer since affected birds do not eat or gain weight and are usually destroyed. Clinically, the syndrome includes birds that are: 1) prone and ataxic with the pelvic limbs stretched out caudally, 2) able to walk, but waddle like ducks, or 3) reluctant to walk. These represent a spectrum of

toxic effects in an affected bird or flock. Some epidemiological factors associated with increased incidence are : gender (male), rapidly growing birds and heat stress/water deprivation. The widespread use of lasalocid in broilers coupled with recent published reports of possible neurotoxicity (Shlosberg et al.,1986; Safran et al, 1993a; Gregory et al, 1995) warrant further study. Little is known concerning this syndrome, except that lasalocid is commonly associated with ataxic birds. This is a complex interaction of the drug, bird genetics, nutrition, environmental and management factors that combine to produce clinical disease.

The basic pharmacologic property of ionophores is to move ions down a concentration gradient across cell membranes. As a result, the ionophores can cause disruptions in the normal ionic homeostasis of many cells. Altered cation concentrations in cell of the nervous system may cause dysfunction and cytotoxicity leading to ataxia or "downer" broilers. The mechanism(s) of ionophore-mediated toxicosis may involve altering the metabolic capability of cells by reducing intracellular ATP, interfering with the "ionic pumps" of cells that maintain ionic gradients, or increased oxidative stress.

The purpose of the first part of this study is to examine the potential for ionophores (monensin, lasalocid, and salinomycin) to cause ataxia in dose-response studies. These experiments will examine the effects of feeding elevated ionophore levels to broiler chickens on a starter (3 weeks of age) and a grower ration (6 weeks of age). Lasalocid has been implicated in field cases of ataxia, but it not known that monensin or salinomycin can cause similar effects. Monensin and salinomycin are compared to lasalocid because of their documented ability for skeletal and myocardial necrosis. These studies will establish that lasalocid-induced ataxia does not involve overt myotoxicity. The determination of a toxic dose of lasalocid that will elicit a reliable percentage of ataxic birds will aid in future mechanistic studies. The rationale for dose-response studies is to determine if the

ionophore alone might cause this disease with all other variables constant. Additionally, interaction studies will examine the effect of changing ionophores from the starter to the grower ration on the incidence of disease. The hypothesis for this phase of the study is that lasalocid alone causes a neuropathy.

The second phase of this study will examine some of the possible mechanisms involved in ionophore-induced neurotoxicity. The effects of ionophore feeding on the activity of the Na^+, K^+-ATPase of several organs is one parameter to be examined. Dissipation of ionic gradients across cell membranes is a possible action of the ionophores *in vivo*. To maintain homeostasis under these conditions, the activities of ATPase enzymes should change. Additionally, ionophores may directly inhibit ATPase enzymes, leading to toxicosis. The effects of the ionophores on mitochondrial respiration and ATP production will also be examined. The hypothesis of this phase of the project is that alteration of energy production and ionic homeostasis of various tissues mediates ionophore-induced neurotoxicity.

The third phase of this study will identify a *per os* gavage dose of lasalocid that elicits ataxia. Using this dose, preliminary studies will be performed to examine possible compounds (vitamin E) or environmental situations (heat stress, water deprivation) that modulate the severity of toxicosis. The ability of affected birds to recover from lasalocid-induced ataxia will also be examined. The hypothesis of these studies is that environmental and nutritional factors interact with lasalocid to cause neurotoxicity.

The next phase will examine the direct, *in vitro* effects of lasalocid on isolated sciatic nerve segments. Incubation of the nerve segments with lasalocid will be followed by analysis of any ultrastructural changes. These studies will establish if damage to the sciatic

nerve is possible in the absence of inflammatory cells and other *in vivo* mediators. The hypothesis of these studies is that lasalocid causes direct damage to the sciatic nerve.

Neurotoxicity will be characterized using clinical signs, an ambulation scoring system, electrophysiology, light and electron microscopic changes. Additionally, several indicators (body weight, serum electrolytes, serum enzyme activities) of generalized toxic effects will further describe this syndrome. These criteria will provide information concerning the organs and cell types affected which will be useful for a more complete description and future mechanistic studies. As depicted in Figure 4, below, the expression of this disease syndrome is multifactorial.

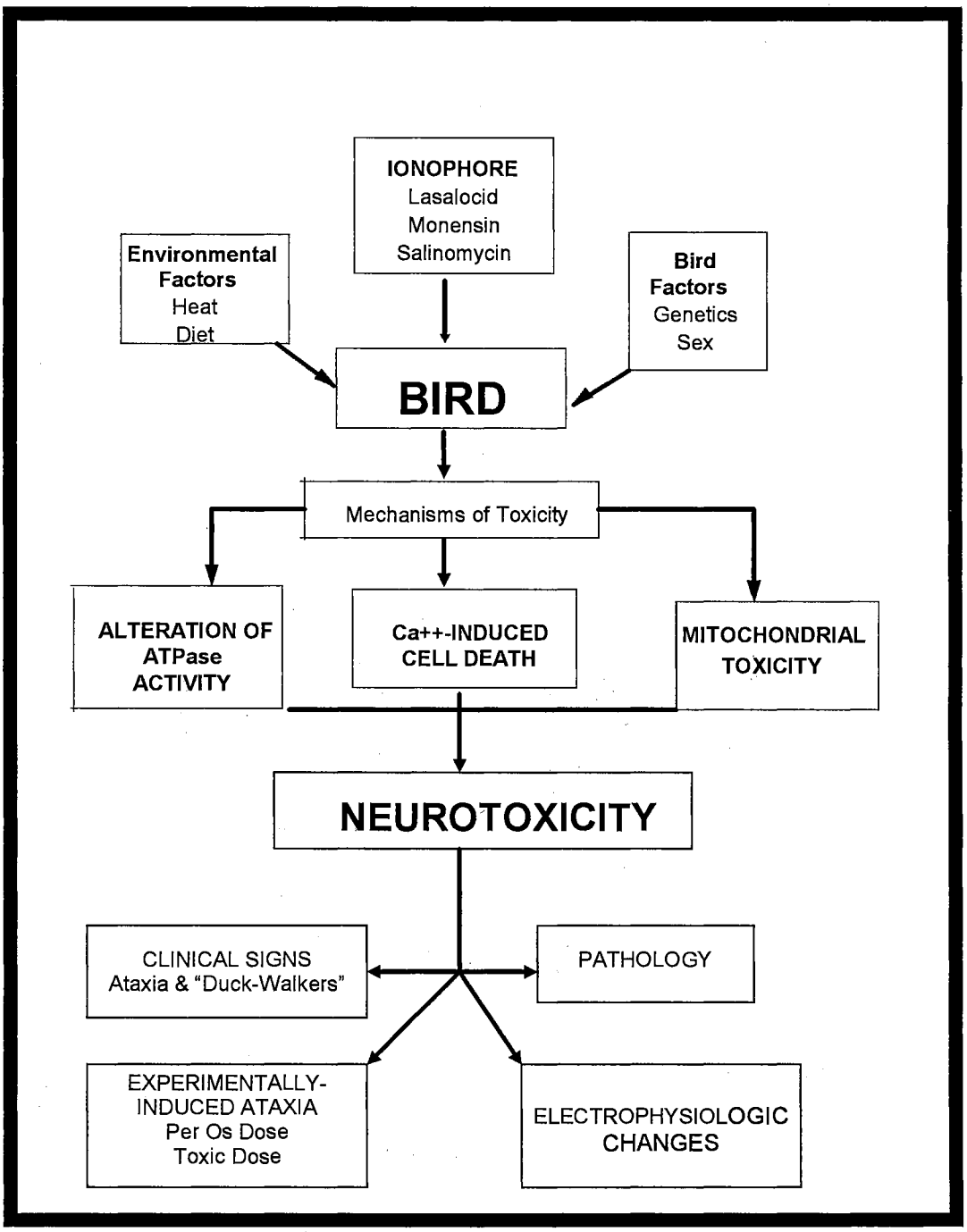

Figure 4. Proposed Pathogenesis of Ionophore-induced Neurotoxicity in Broilers

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CHAPTER II

THE INFLUENCE OF VARIOUS DIETARY LEVELS OF IONOPHORES ON NEUROPATHY IN BROILER CHICKENS

Abstract

Previous studies and field reports suggest that lasalocid may cause neurotoxicity in broiler chickens. It is currently unclear if the syndrome is due to the drug alone or in combination with other environmental, dietary or management factors. Lasalocid at ration concentrations greater than 90 ppm causes a dose-dependent ataxia that mimics the field syndrome in 3 and 6 week old, male, Cobb-cross broiler chickens. Two other ionophores, monensin and salinomycin, did not cause ataxia at the concentrations fed (up to 8x the recommended dose). Lasalocid-induced neurotoxicity primarily involves the sciatic nerve as described by clinical signs, neurologic examination, motor nerve conduction velocity, light and electron microscopy. Using a clinical scoring system the severity and incidence of lasalocid-induced ataxia were markedly increased at ration concentrations greater than 180 ppm, twice the recommended dose, and up to 360 ppm, the highest dose tested. The ration concentration of lasalocid that causes ataxia in 25% of the flock was determined to be 220 and 242 ppm for the 3 and 6 week old birds, respectively. Birds fed 270 ppm lasalocid for 10 days had significantly reduced superficial peroneal motor nerve conduction velocity (14.5 M/sec) as compared to birds fed no drug (35.5 M/sec). In vitro, lasalocid produced a dose-dependent inhibition of renal Na^+, K^+-ATPase with an IC_{50} of 46.58 - 50.96 μM. Light and electron

microscopic evaluation of the sciatic nerves indicate specific damage to the myelin (vacuoles and degeneration). Serum electrolyte changes did not correspond with the incidence of ataxia. There were no elevations in lactate dehydrogenase(LDH), creatinine phosphokinase (CPK), or aspartate transaminase (AST) activity indicating that ataxia was not related to myotoxicity. Elevated dietary levels of lasalocid (90 and 360 ppm) did not affect ATPase function of kidney, small and large intestinal mucosa in vivo.. These studies support the hypothesis that lasalocid, alone, can cause a peripheral neuropathy in broiler chickens.

Introduction

The ionophores (monensin, lasalocid and salinomycin) are carboxylic acid antibiotics produced by fungal fermentation (Novilla, 1992; Galitzer & Oehme, 1984; Pressman, 1976) with a wide range of antibacterial activities (Reed, 1982). In animal production systems, these compounds are safe and efficacious anticoccidial agents. However, there are numerous reports of acute ionophore toxicosis due to accidental ingestion by non-target species or overdose in target species (Novilla, 1992; VanVleet et al.,1987; Galitzer & Oehme, 1984; Shlosberg et al.,1992; Mezes et al.,1992). The toxic effects associated with these compounds is described as a cardiac or skeletal myopathy (Novilla, 1992). Recent reports suggest that neurotoxicity is a possible consequence of lasalocid toxicosis (Safran et al, 1993; Perelman et al, 1993; Shlosberg et al.,1986; Gregory et al, 1995). Unpublished field observations note an association with lasalocid in the ration and ataxia in broilers typically in male, rapidly growing birds within one week of changing to a ration containing the ionophore. The incidence and severity of disease is worse in the summer months. Poultry producers and field veterinarians have designated this syndrome "downer" or

"knockdown birds". It is currently unknown if this syndrome is associated with ionophore exposure or other environmental or management factors.

The clinical pathologic changes associated with ionophore intoxication relate to skeletal or cardiac muscle damage: elevated serum levels of aspartate aminotransaminase (AST), creatinine phosphokinase (CPK), lactate dehydrogenase (LDH). Additional indicators include elevated ALP, (Horovitz et al.,1988) SUN and bilirubin (Osweiler et al.,1983). Elevated serum enzyme activities can also occur at the recommended use levels. Elevate serum K^+ is commonly present with ionophore-induced myonecrosis due to release of intracellular stores from damaged muscle cells. Additionally, serum Ca^{++} and K^+ levels may decline to life-threatening levels in monensin-intoxicated ponies or horses (Novilla, 1992; Osweiler et al.,1983).

The most common clinical signs of ionophore intoxication are decreased feed intake and body weight gains, with anorexia most commonly associated in sublethal intoxications (Novilla, 1992; Osweiler et al.,1983; Simon et al.,1991;Novilla et al, 1994; Todd et al, 1984; Galitzer et al, 1982; VanVleet et al.,1987). This is consistent with the use of decreased body weight as a sensitive index of an adverse effect of a toxic substance (Gad & Weil, 1989b). Body weight gains are closely monitored by producers and can indicate possible ionophore intoxication (VanderKop & MacNeil, 1990; Bartov, 1994; Todd et al., 1984).

Clinical observation and the use of ataxia scoring systems have been used by other researchers to evaluate the toxic effects of the organophosphates that produce delayed neuropathy (OPIDN)(Abou-Donia & Graham, 1978; Abou-Donia, 1977; Abou-Donia et al.,1980; Dyer et al.,1992; el-Fawal et al, 1990a,b) . Establishing a dose-response relationship with an ionophore and the incidence of neurotoxicity would support the causal relationship of lasalocid to the syndrome.

The fundamental hypothesis of these studies is that carboxylic ionophores (especially lasalocid) cause a dose-dependent neurotoxicity in broiler chickens. By maintaining other factors (environmental, dietary) constant, the capability of these compounds to cause this syndrome will be determined. These dose-response studies will use increasing dietary concentrations of ionophores in an attempt to reproduce the clinical syndrome previously described and seen in the field. The parameters to be measured include the incidence of neurotoxicity (ataxia), body weight, mortality, histologic examination, ultrastructural changes and serum electrolytes or enzyme activities will be used to evaluate the hypothesis that lasalocid can cause a peripheral neuropathy. The effects of elevated dietary ionophore concentration on the activity of the Na^+, K^+-ATPase in several vital organs and *in vitro* will be examined as a possible mechanism for ionophore-induced neurotoxicity. Another objective is to determine a specific dose (a toxic dose) that will cause ataxia in 25 % of a flock of birds.

Materials and Methods

Animals

Three thousand-three hundred, male, Cobb-cross broilers were used in these studies. One-day-old chicks were purchased from a commercial source (Tyson Hatchery, Springdale, AR). Birds in the starter studies (1-21 days of age) were housed in battery cages at a density of 10 animals to a cage. Temperature was maintained between 24° - 27° C with constant tungsten filament lighting. Birds used in grower studies (1-42 days of age) were housed in floor pens, bedded on fresh rice hulls. Supplemental heat was provided by 2-3 lamps per pen, to maintain the temperature at floor level near 30° C, during the first 2 weeks of the study. The birds were also exposed to constant tungsten filament

lighting. Birds in the grower studies were fed a basal ration (no ionophore) for days 1-21. Feed and water were provided *ad libitum*.

Diet and Drugs

The birds were fed a corn-soy basal ration as outlined in Appendix A. Ionophores were purchased as feed grade additives: lasalocid (AVATEC®), monensin (RUMENSIN®), and salinomycin (BIOCOX®). Lasalocid was added to the diet at 1x, 2x, 3x and 4x the recommended levels (90 ppm, 180 ppm, 270 ppm, and 360 ppm). Monensin and salinomycin were added to the ration at 1x, 2x, 4x, and 8x the recommended levels (100 ppm, 200 ppm, 400 ppm, and 800 ppm for monensin and 60 ppm, 120 ppm, 240 ppm, and 480 ppm for salinomycin). Ionophores were weighed and incorporated into 100 pounds of feed and mixed completely in a hopper-type mixer. The medicated feed was then added to 900 pounds of the basal diet in a large mixer and mixed for 35-45 minutes. This allowed for uniform distribution of the ionophore which is a common practice to reduce the incidence of "hot spots" where high concentrations of medicated feed are found in the diet.

Experimental Protocols: Starter, Grower and Interaction Studies

Starter and Grower Studies. 100 birds were randomly assigned to each treatment groups at 1 day (starter study) or 21 days (grower study) of age. The dietary treatment groups used in these studies are outlined in the previous section. Twice each day, birds were observed for mortality and clinical signs of neurotoxicity using an ataxia scoring system. The ataxia scoring system was used initially on the birds at rest, and

then following a brief (2 min) exercise period as described below. At the end of the trial birds were euthanitized by cervical dislocation or CO_2 overdose.

Interaction Study. The interaction study examined the effects of previous administration of other ionophores on the incidence of lasalocid neurotoxicity in the grower ration. Birds were fed a corn-soybean meal ration (Appendix A) with the addition of ionophores as described in Table I. Assessment of ataxia, mortality, sample collection and methods of euthanasia were as previously described.

TABLE I

TREATMENT GROUPS FOR AN IONOPHORE INTERACTION STUDY

Treatment Group	Diet from Days 1 - 20	Diet from Days 21 - 42	Number of Birds
1. controls	no drug	no drug	100
2.	121 ppm monensin	no drug	100
3.	66 ppm salinomycin	no drug	100
4.	121 ppm monensin	121 ppm lasalocid	100
5.	121 ppm monensin	220 ppm lasalocid	100
6.	66 ppm salinomycin	121 ppm lasalocid	100
7.	66 ppm salinomycin	220 ppm lasalocid	100
8.	no drug	220 ppm lasalocid	100

Clinical Assessment Of Neurotoxicity

All birds were assessed twice each day for clinical signs of ataxia. Initial observation of undisturbed birds allowed for identification of paralyzed or severely ataxic birds. All birds were forced to walk around the battery cage or floor pen for several minutes during each examination period. Any bird showing signs of ataxia or reluctance to walk was categorized by the clinical ataxia scoring system (Table II). Forced ambulation allowed detection of slightly ataxic birds and to determine if ataxia worsened after exercise. This scoring system evaluates the gait characteristics and the attitude of the bird, with scores ranging from normal birds to those that are paralyzed and depressed.

Further evaluation of toxic effects in lesioned birds was accomplished by a clinical neurologic examination using cranial nerve function and spinal reflexes (Appendix B). This checklist permits the localization of lesion(s) to the central or peripheral nervous system and region of the body affected. Each lesioned bird was evaluated at two different time periods to accurately measure any alteration in reflexes.

TABLE II

CLINICAL SCORING SYSTEM FOR IONOPHORE-INDUCED ATAXIA

Score	Description
0	Normal bird
1	Slight Ataxia after exercise
2	Slight Ataxia, Ataxia worsens after exercise
3	"Duck Walkers", Ataxic, Waddling-type gait
4	Non-ambulatory (down), alert, aware of surroundings
5	Non-ambulatory (down), not alert, depressed

Determination of Toxic Dose

The dose of lasalocid that produced ataxia in 25% of a group of birds (Toxic Dose 25 or TD_{25}) was determined for the starter (1 - 21 days) and grower (21 - 42 days) studies. The raw data (percent neurotoxicity vs. concentration of ionophore) were transformed into a probit vs. log concentration. The percent response data was expressed by a probit scale while the concentration of ionophore in the ration was expressed by a logarithmic scale using the method previously described by Gad and Weil (1989a). The log-probit transformed data was analyzed by linear regression to determine the specific TD_{25} for starter and grower studies

Serum Electrolytes and Chemistries

Blood was drawn via cardiac puncture from 10 birds in each treatment group and analyzed for serum electrolytes (Na^+, K^+, Mg^{++}, Ca^{++}, and Cl^-) and enzymes (LDH, AST, CPK) using a Cobas Mira wet chemistry analyzer (Roche Diagnostic Systems Inc., Montclair, NJ 07042-5199). Na^+ and K^+ were measured with a selective electrode module of the Cobas Mira system (No. 44498). Other electrolytes and enzyme activities were determined using Roche kits (Hoffman-LaRoche, Nutley, NJ 07042) for Cl^- (No. 44029), Mg^{++} (No. 44169), Ca^{++} (No. 44903), LDH (No. 43623), AST (No. 44645). CPK was assayed using Sigma kit (No. 520)

Body Weights and Mortality

In each treatment group, 25 randomly selected birds were weighed on day 20 (starter trial) and day 40 (grower and interaction trial). During the daily examination

periods, the number of dead birds were counted and recorded. Total mortality for each treatment group were determined end of the feeding trial.

Microscopic and Ultrastructural Examination

Light Microscopy. At necropsy, sciatic nerves (from an area caudal to the crest of the tibia to the lateral condyle of the femur) and portions of liver, skeletal and cardiac muscle were removed from 10 birds in each ionophore treatment group. Tissues were fixed in a phosphate buffered, 10% formaldehyde solution and kept cool until processing. Following fixation, the tissues were processed in a series of alcohol dehydration steps and embedded in paraffin. Tissues in paraffin blocks were sectioned into 4 - 6μm slices, placed on glass slides and stained with hematoxylin and eosin in a Fisher's automatic stainer. Sciatic nerve tissue was sectioned longitudinally and transversely for microscopic examination. All tissues were evaluated by a veterinary pathologist blinded to the specific treatment of each slide.

Electron Microscopic Evaluation. At necropsy, the sciatic nerves from normally gaited birds fed no ionophore (control) and 2 ataxic birds (clinical score 2) fed 270 ppm lasalocid were removed and fixed in buffered gluteraldehyde-sodium cacodylate solution for 2 hours. Post-fixation, the tissues were placed in a 1:1 solution of 2% osmium and 0.27 M cacodylate buffer for 2 hours. Segments of sciatic nerve were washed in a 1 M sodium cacodylate solution. After rinsing, tissues were dehydrated with a series of ethanol and propylene oxide rinses. The tissues were embedded in Polybed® and placed in an oven.

Blocks of resin containing sciatic nerve segments were thick sectioned with a glass knife to approximately 0.550 nm. Areas of each block containing nerve tissue were then chosen for thin sectioning, mounted on 200 mesh copper grids and stained with uranyl acetate and lead citrate. Grids were examined using a JEOL 100 CX transmission electron microscope at 80 KV. Areas of normal and abnormal appearing tissue were photographed for more detailed analysis.

Motor Nerve Conduction Velocity Studies

The motor nerve conduction velocities (MNCV) of normal and ataxic birds was determined using clinical electrophysiology. A total of seven birds, 40 days old, were evaluated for the MNCV of the superficial peroneal nerve. Three control (no drug) and four ataxic birds (clinical score 3) were fed 270 ppm lasalocid for 10 days. Birds were anesthetized with a combination of xylazine (5 mg/kg) and ketamine (15 mg/kg), i.m., and placed in lateral recumbency. The peroneal nerve was identified by digital palpation and instrumented with transcutaneous needle-type electrodes at a the level of the lateral condyle on the femur (stimulating electrode) and distolateral to the tarsometatarsal joint (recording electrode). The nerve was stimulated with supramaximal, square-wave pulses of 1.0 millisecond duration at a rate of 1/second. The evoked action potential was recorded from the lateral digital extensor. The right and left pelvic limb of each birds was instrumented and evaluated.

ATPase Activity

Experimental protocol. In the ATPase studies, two different experiments were performed. First the activity of these enzymes was assayed from various organs from

birds fed different levels of ionophore. Additional tissues (kidney) were taken from control birds to examine the inhibitory effects of lasalocid incubation on ATPase activity, *in vitro.* The procedures for enzyme isolation are as follows.

Enzyme Isolation. ATPase enzymes from kidney, small and large intestinal mucosa were prepared following the method of Chen (Chen et al.,1994), a modification of an earlier procedure (Akera et al., 1969). Tissues were rapidly removed after sacrificing the bird and washed several times with ice-cooled isotonic solution containing 0.25 *M* sucrose, 5.0 *mM* L-histidine, 5.0 m*M* ethylene diamine tetraacetate (EDTA×Na_2) and 0.15% sodium deoxycholate, pH 6.8 with Trizma® base. Approximately 5 g of kidney, small intestinal mucosa and all of the large intestinal mucosa (usually about 2-3 grams) was minced and homogenized in a polytron homogenizer (Polytron® Model #PT 10/35, Brinkman Instruments, Switzerland) twice for 45 seconds duration in 6 volumes of above ice-cooled isotonic sucrose solution. The homogenate was centrifuged for 30 minutes at 12,000 x g. Following centrifugation of the supernatant for 60 minutes at 100,000 x g, the sediment was suspended in 30 ml of a suspension solution containing 0.25 *M* sucrose, 5.0 m*M* L-Histidine, and 1 m*M* ethylene diamine tetraacetic acid, with pH 7.0 with Trizma® base. The suspension was centrifuged again at the same speed for the same time. The pellet was resuspended in 20 ml of the above suspension solution. The same volume of LiBr solution (2.0 *M*) was added, and the new solution was stirred gently for 1 hour. The mixture was centrifuged for 60 minutes at 100,000 x g. The sediment was resuspended in the same suspension solution and recentrifuged. A final suspension was filtered through four-layers of gauze. All above procedures were carried out at 2 °C.

Determination of Enzyme Activity. ATPase activity was determined by using a slight modification of previously reported procedures (Broekhuysen et al., 1972; (Akera & Brody, 1971). A final volume of 1ml reaction medium containing 50 m*M* Tris HCl buffer (pH 7.5) with 5 m*M* $MgCl_2$, 15 m*M* KCl, 100 m*M* NaCl, and 0.1 ml of enzyme preparation was used in all experiments. The reaction medium was preincubated at 37°C for 5 minutes. The reaction was started by adding 2 m*M* of Tris ×ATP (Sigma Chemical Co., St. Louis) and continued for 20 min. at 37 °C and was terminated with 1 ml of ice-cooled 15% trichloroacetic acid. After centrifugation, the inorganic phosphate liberated in a 1.0 ml aliquot of supernatant was measured with 3 ml of 6 *N* H_2SO_4:water:2.5% ammonium molybdate:10% L-ascorbic acid (1:2:1:1) by a modified method of Fisk and SubbaRow (Fisk & SubbaRow, 1925). Absorbance was measured at 660 nm after 20 minutes at 37 °C. The Mg^{2+}-dependent ATPase activity was assessed in the absence of K^+ and Na^+. The Na^+,K^+-ATPase activity was calculated by subtracting Mg^{2+}-activated ATPase activity from total ATPase activity. Protein content of enzyme preparations was determined by bicinchoninic acid protein assay (Smith et al.,1985) with bovine serum albumin as the standard.

Data Analysis

All data were initially examined by analysis of variance (ANOVA) with a general linear model (GLM) using SAS (SAS Institute, Carey, NC). Mean ± standard deviation of serum electrolytes and enzyme activity for each group were calculated and compared to appropriate controls using Dunnett's test. Ataxia, as evaluated by the clinical scoring system, was transformed by a probit (response) by logarithmic dose scale. Birds were grouped in the ataxic group if they had a clinical score of 3,4 or 5.

The toxic dose 25% (TD25) , the concentration of lasalocid in the ration causing ataxia in 25% of the birds was determined by linear regression of transformed data.

Results

Dose-Dependent Ataxia in Starter and Grower studies

There is a dose-response relationship between lasalocid and neurotoxicity. As the levels of lasalocid increased in the ration, a greater percentage of birds were ataxic. In the starter study, 1 - 21 days of age, the incidence of ataxia noted with the corresponding concentration of lasalocid was 1% at 90 ppm, 9% at 180 ppm , 36% at 270 ppm and 69 % at 360 ppm. In the grower study, day 21 - 42 of age, the incidence of ataxia noted with the corresponding concentration of lasalocid was 1% at 90 ppm, 13.4% at 180 ppm , 27.6% at 270 ppm and 50.6% at 360 ppm. At concentrations greater than 180 ppm in the ration, lasalocid-induced ataxia increased exponentially (Figure 1). In these studies, no monensin or salinomycin treatment group had an incidence of ataxia greater than 5% (data not shown).

The onset and progression of lasalocid-induced ataxia is similar to field case reports. The onset of overt ataxia (clinical score 3- 5) in the 90 and 180 ppm groups began 5 - 7 days after introduction of the ionophore. The 270 and 360 ppm lasalocid treatment groups had a more rapid onset of ataxia, usually occurring within 3-5 days. In all lasalocid groups, there is a progression of ataxia severity with time. An individual bird may exhibit a clinical ataxia score of 3 on day 7 of the feeding trial and progress to a clinical score of 4 or 5 by day 12 or 14.

The affected birds present with a flaccid, floppy paresis/paralysis of the pelvic limbs with upper motor neuron signs with a generalized reduction in muscle mass of affected birds. Extensor muscle function was preferentially affected that leads to collapse of the stifle and tibiotarsal joints when bearing weight in the later stages of the syndrome. Birds with an ataxia score of 2 have a clinical presentation that mimics myasthenia gravis. Following a brief period of exercise, birds collapse and after 30 - 60 seconds of rest they stand again. The "duck walking" birds with an ataxia score of 3, ambulate primarily by abducting and adducting the hip without extending the stifle or tibiotarsal-tarsometatarsal joint. This gives the chicken a "waddling" type of gait that somewhat resembles a duck. Birds with an ataxia score of 4 or 5 are non-ambulatory, even with vigorous encouragement; differing in their attitude and desire to eat and drink.

Evaluation of the thoracic limbs (wings) did not provide consistent, reproducible findings indicative of altered function. Subjectively, birds with clinical ataxia scores of 1 or 2 could flapped their wings stronger than non-ambulatory birds.

Birds with ataxia scores of 2-4 had normal cranial nerve function, as measured by the visual menace, pupillary light reflex, mandibular tone, palpebral reflex, hearing, vestibular reflexes, and gag reflex (swallowing). Lesioned birds with an ataxia score of 5 were too depressed to assess most cranial nerve function. These birds did maintain the ability to swallow until approximately 12 hours before death.

The spinal reflexes of the birds with ataxia scores of 2 were essentially the same as control, no ionophore in diet, birds. They were able to right themselves when placed in dorsal recumbency and had superficial and deep pain responses and proprioreceptive placing responses. Birds with an ataxia score of 3 were able to right themselves with greater difficulty and had normal pain responses. These "duck-

walking" birds had diminished proprioreceptive responses. Most birds with a score of 4 were unable to right themselves, but still attempted, while birds with a score of 5 would make feeble attempts. Birds with a score of 4 had varied pain responses, while birds with a score of 5 usually did not respond to painful stimuli.

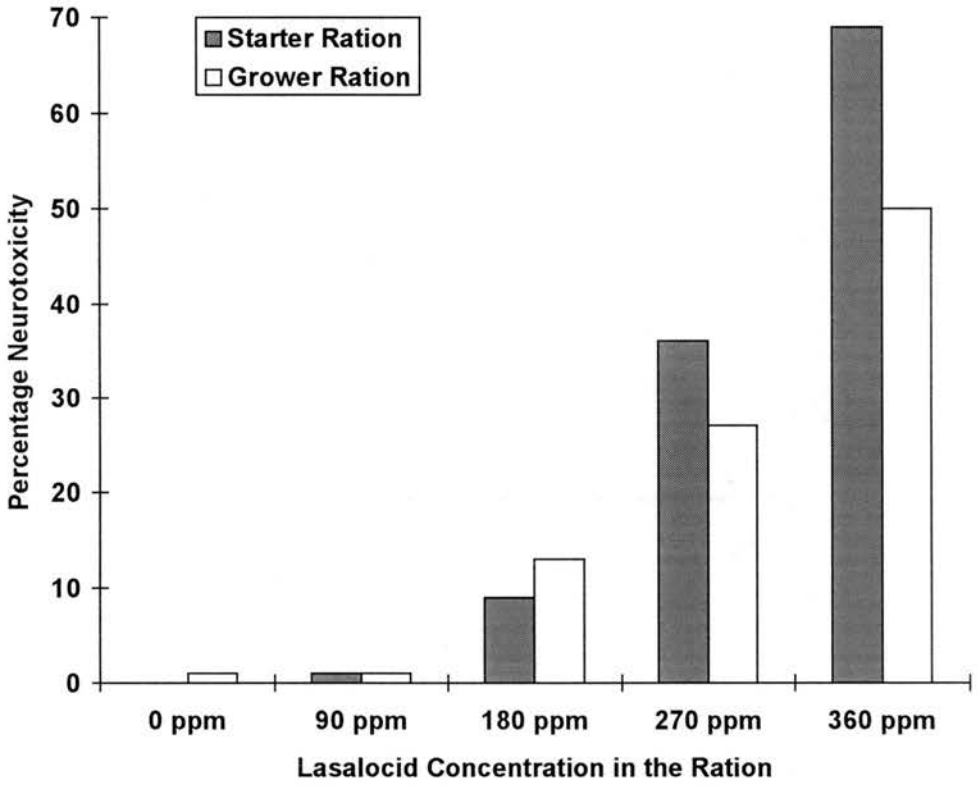


Figure 1. Dose Dependent Ataxia in Cobb-cross Broiler Chickens (20 or 40 days of age) fed Increasing Dietary Concentrations of Lasalocid .

Toxic Dose of Lasalocid Causing 25% Ataxia (TD_{25})

Transformation of the percentage ataxia verses linear dose data to a probit response versus log dose produced a linear, dose-response curve. These lines allow for more precise predictions using linear regression analysis. The ration dose of lasalocid eliciting 25% ataxia in these birds is 220 ppm and 242 ppm for the starter and grower ration, respectively (Figure 2 & 3).

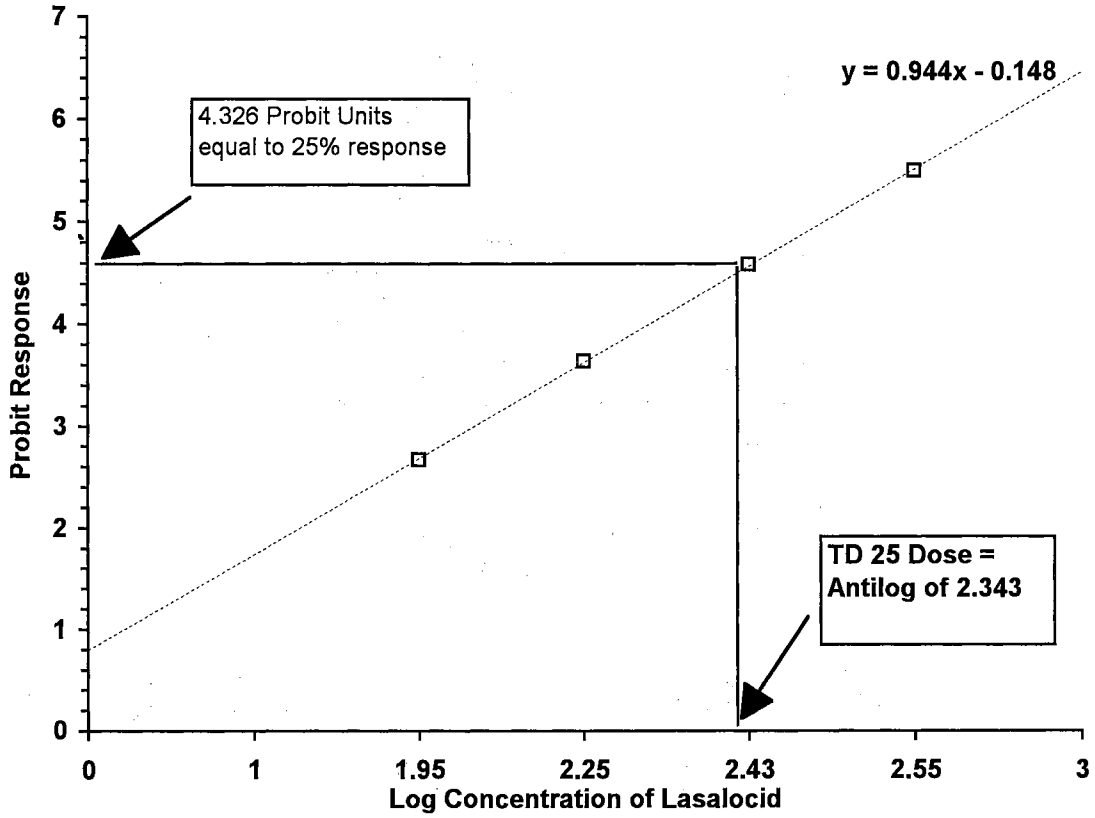


Figure 2. Toxic Dose 25% as Determined by Linear Regression of a Log-Probit Transformation in Cobb-cross Broiler Chickens (20 days old) fed Increasing Dietary Levels of Lasalocid.

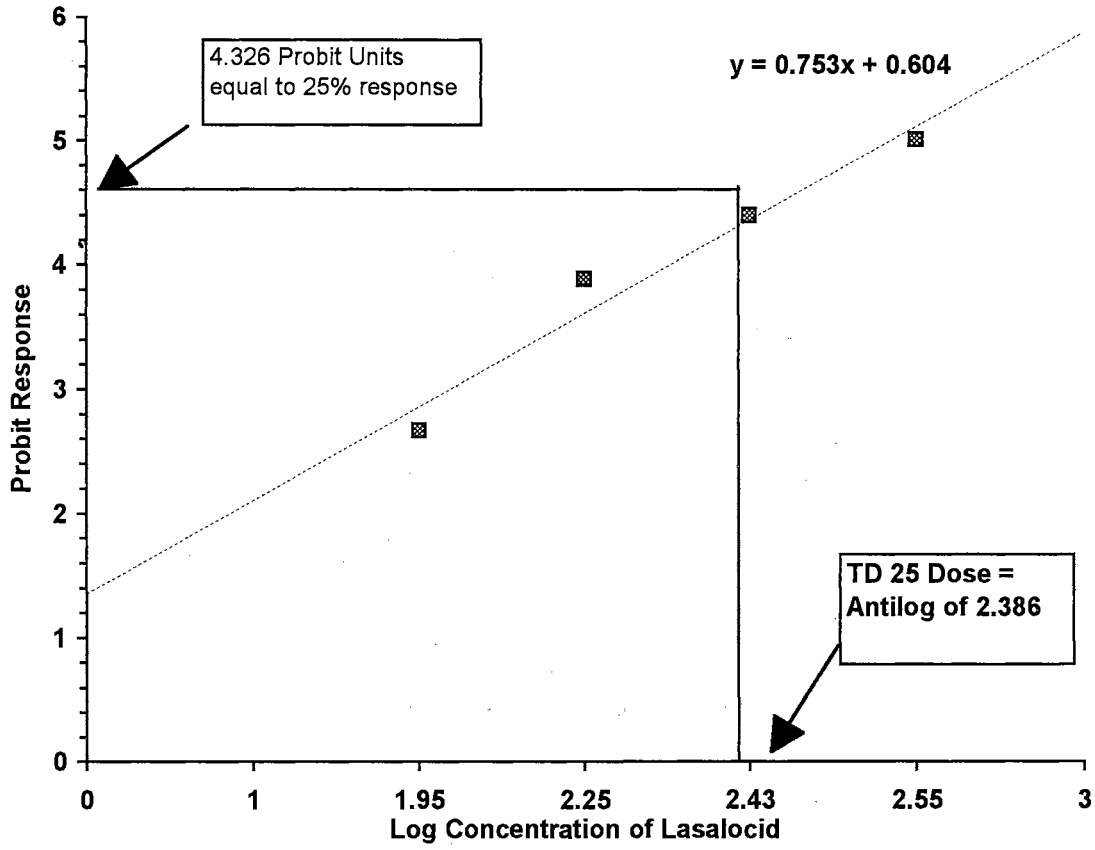


Figure 3. Toxic Dose 25% as Determined by Linear Regression of a Log-Probit Transformation in Cobb-cross Broiler Chickens (40 days old) fed Increasing Dietary Levels of Lasalocid.

Ataxia in the Interaction study

The interaction study was conducted using the toxic dose of lasalocid that would produce ataxia in 25% of the birds (220 ppm). These results show no significant increase in the incidence of ataxia for birds fed monensin or salinomycin at

recommended levels and then switched to 121 ppm lasalocid (highest recommended dose). The increase in incidence of ataxia is most notable in the birds fed 220 ppm lasalocid in the grower ration. All three groups have an incidence statistically different from the negative control (no drug) group but not different from the positive control group (no drug in the starter ration and 220 ppm lasalocid in the grower ration) (Figure 4).

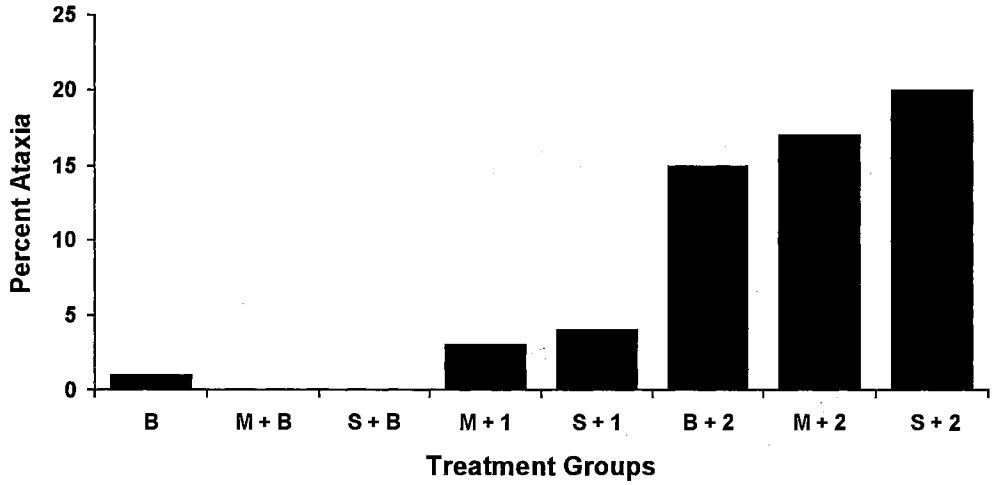
The time to onset and severity of lasalocid-induced ataxia was similar to the results observed in the grower study. The birds fed 220 ppm lasalocid in the interaction trial began to exhibit clinical signs (scores 3,4 or 5) of ataxia within 5 - 7 days. Some of these birds progressed in severity from a clinical score of 3 to a clinical score of 5.

Serum Electrolytes and Chemistries

Serum sodium concentrations are reduced in all lasalocid dietary treatments, statistically significant at 90, 180 and 270 ppm treatments in the starter study. Conversely, in the monensin and salinomycin treatments, the general trend was for an elevated serum sodium. There were no statistically significant changes in serum calcium for any of the tested ionophores at any dietary level. Serum potassium was elevated in birds consuming greater than recommended concentrations of monensin and salinomycin. Lasalocid-treated birds had serum potassium levels lower than the controls, especially at concentrations greater than approved levels (Table II & III). Reduced serum potassium was the most significant cation trend noted in the interaction studies. In the positive control group (no drug starter: 220 ppm lasalocid in grower) the serum potassium was significantly reduced (Table IV). Serum magnesium

concentrations are reduced in all lasalocid treatments in starter and grower rations, although not statistically significant. In contrast, birds fed diets containing monensin or salinomycin had elevated serum magnesium concentrations. Reduced serum magnesium is seen in the positive control group (no drug starter: 220 ppm lasalocid in grower). Serum chloride was elevated in all monensin treatments in the starter study. Broilers fed lasalocid had lowered serum concentrations chloride.

In lasalocid-treated birds serum AST, LDH or CPK activity were similar to controls. Monensin- or salinomycin- treated birds had markedly elevated serum activities of these enzymes, especially CPK. These increases are most notable at greater than 400 ppm monensin and greater than 120 ppm salinomycin (Figures 9 - 11). In the interaction study, AST was the only serum enzyme in the positive control group statistically different from the control (data not shown).



Legend for Treatments in Interaction Trial:

B = (No drug in starter or grower rations)

M + B = 121 ppm Monensin in Starter: No drug in Grower

S + B = 66 ppm Salinomycin in Starter: No drug in Grower

M + 1 = 121 ppm Monensin in Starter: 121 Lasalocid in Grower

S + 1 = 66 ppm Salinomycin in Starter: 121 Lasalocid in Grower

B + 2 = (Positive Control) No drug starter: 220 ppm Lasalocid in Grower

M + 2 = 121 ppm Monensin in Starter: 220 ppm Lasalocid in Grower

S + 2 = 66 ppm Salinomycin in Starter: 220 ppm Lasalocid in Grower

Figure 4. Percent Ataxia (as Measured by a Clinical Ataxia Scoring System) for Broilers (40 days of age) in an Ionophore Interaction Study.

TABLE II.

ELECTROLYTE CONCENTRATIONS (MEAN ± S.D.) OF SERUM FROM COBB-CROSS BROILER CHICKENS (20 DAYS OLD) FED INCREASING DOSES OF IONOPHORES. * = DIFFERENT FROM CONTROL (P<0.05)

Treatment Group	Na^+ (mEq/L)	Ca^{++} (mEq/dl)	K^+ (mEq/L)	Mg^{++} (mEq/L)	Cl^- (mEq/L)
Control (no drug)	157.70 ± 5.11	7.16 ± 0.41	5.54 ± 0.35	1.90 ± 0.09	114.56 ± 2.39
90 ppm Lasalocid	136.50 ± 4.13 *	7.54 ± 0.28	4.98 ± 0.35	1.55 ± 0.04	110.70 ± 3.46
180 ppm Lasalocid	143.00 ± 1.27 *	7.08 ± 0.25	4.73 ± 0.30	1.65 ± 0.05	114.60 ± 0.94
270 ppm Lasalocid	140.50 ± 2.94 *	6.91 ± 0.27	5.32 ± 0.26	1.73 ± 0.06	114.10 ± 1.97
360 ppm Lasalocid	146.60 ± 2.14	7.39 ± 0.30	5.37 ± 0.26	1.77 ± 0.04	117.40 ± 1.74
100 ppm Monensin	191.88 ± 2.42 *	9.41 ± 0.79 *	6.76 ± 0.33	3.04 ± 0.08 *	154.88 ± 2.84 *
200 ppm Monensin	156.60 ± 2.69	7.31 ± 0.69	6.84 ± 0.36 *	2.33 ± 0.15 *	140.60 ± 4.22 *
400 ppm Monensin	177.70 ± 2.20*	4.77 ± 0.43*	5.87 ± 0.26	1.98 ± 0.13	124.60 ± 1.48 *
800 ppm Monensin	163.80 ± 5.27	6.76 ± 0.27	6.24 ± 0.30	1.88 ± 0.11	133.40 ± 5.88*
60 ppm Salinomycin	166.88 ± 1.75	5.72 ± 0.45	6.32 ± 0.28	2.38 ± 0.20 *	119.38 ± 1.71
120 ppm Salinomycin	172.70 ± 5.91*	8.27 ± 0.41	6.91 ± 0.47*	2.37 ± 0.08*	152.20 ± 3.54*
240 ppm Salinomycin	161.80 ± 0.09	5.96 ± 0.27	4.04 ± 0.23*	1.87 ± 0.06	116.90 ± 1.72
480 ppm Salinomycin	173.10 ± 2.55*	6.44 ± 0.55	4.85 ± 0.31	1.82 ± 0.10	128.80 ± 1.16

TABLE III.

ELECTROLYTE CONCENTRATIONS (MEAN ± S.D.) OF SERUM FROM COBB-CROSS BROILER CHICKENS (40 DAYS OLD) FED INCREASING DOSES OF IONOPHORES. * = DIFFERENT FROM CONTROL (P<0.05)

Treatment Group	Na^+ (mEq/L)	Ca^{++} (mEq/dl)	K^+ (mEq/L)	Mg^{++} (mEq/L)	Cl^- (mEq/L)
Control (no drug)	153.33 ± 4.50	8.73 ± 0.60	6.55 ± 0.27	2.05 ± 0.13	116.61 ± 15.56
90 ppm Lasalocid	141.60 ± 2.95	7.77 ± 0.20	5.59 ± 0.21	1.61 ± 0.04*	103.70 ± 6.67
180 ppm Lasalocid	150.60 ± 5.48	7.99 ± 0.35	5.14 ± 0.38*	1.82 ± 0.10	112.10 ± 13.44
270 ppm Lasalocid	147.60 ± 6.27	8.44 ± 0.38	5.16 ± 0.33*	1.84 ± 0.11	111.50 ± 15.70
360 ppm Lasalocid	147.44 ± 5.11	7.68 ± 0.35	4.38 ± 0.22*	1.70 ± 0.08	109.00 ± 11.48
100 ppm Monensin	165.44 ± 1.46	7.03 ± 0.58	6.48 ± 0.26	2.48 ± 0.12	128.89 ± 5.84
200 ppm Monensin	164.67 ± 2.04	9.92 ± 0.64	6.15 ± 0.33	2.71 ± 0.22*	115.00 ± 18.69
400 ppm Monensin	162.40 ± 1.18	7.59 ± 0.62	6.18 ± 0.31	2.09 ± 0.04	123.90 ± 4.59
800 ppm Monensin	154.28 ± 1.71	10.40 ± 0.97	4.92 ± 0.24*	2.28 ± 0.09	127.40 ± 5.01
60 ppm Salinomycin	159.28 ± 1.71	6.20 ± 0.63	5.53 ± 0.42	2.03 ± 0.11	123.00 ± 5.89
120 ppm Salinomycin	160.22 ± 1.42	8.39 ± 0.58	4.89 ± 0.39*	2.51 ± 0.11*	122.22 ± 5.26
240 ppm Salinomycin	157.10 ± 1.06	10.19 ± 0.84	4.65 ± 0.25*	2.54 ± 0.11*	131.50 ± 3.95
480 ppm Salinomycin	162.88 ± 2.05	10.41 ± 0.74	5.05 ± 0.29*	2.29 ± 0.17	126.50 ± 6.85

TABLE IV.

ELECTROLYTE CONCENTRATIONS (MEAN ± S.D.) OF SERUM FROM COBB-CROSS BROILER CHICKENS (40 DAYS OLD) FED OF IONOPHORES.IN AN INTERACTION STUDY * = DIFFERENT FROM CONTROL (P<0.05)

Treatment Group	Na^{+} (mEq/L)	Ca^{++} (mEq/dl)	K^{+} (mEq/L)	Mg^{++} (mEq/L)	Cl^{-} (mEq/L)
Control (no drug)	153.33 ± 4.50	8.73 ± 0.60	6.55 ± 0.27	2.05 ± 0.13	116.61 ± 3.67
M1	151.18 ± 3.90	7.93 ± 0.36	4.76 ± 0.16*	1.73 ± 0.07	113.09 ± 3.13
M2	136.10 ± 7.93	6.50 ± 0.41*	5.11 ± 0.37*	1.55 ± 0.10*	100.60 ± 5.89
S1	133.60 ± 7.76*	6.74 ± 0.40*	4.59 ± 0.30*	1.53 ± 0.11*	99.00 ± 5.79*
S2	152.30 ± 6.35	7.92 ± 0.35	6.39 ± 0.42	1.74 ± 0.09	112.40 ± 5.05
PC	142.50 ± 8.46	7.27 ± 0.55	5.01 ± 0.34*	1.55 ± 0.12*	103.80 ± 6.45

Legend for Treatments in Interaction Trial:

Control = No drug in starter or grower rations

M1 = 121 ppm Monensin in Starter: 121 Lasalocid in Grower

M2 = 121 ppm Monensin in Starter: 220 ppm Lasalocid in Grower

S1 = 66 ppm Salinomycin in Starter: 121 Lasalocid in Grower

S2 = 66 ppm Salinomycin in Starter: 220 ppm Lasalocid in Grower

PC = (Positive Control) No drug starter: 220 ppm Lasalocid in Grower

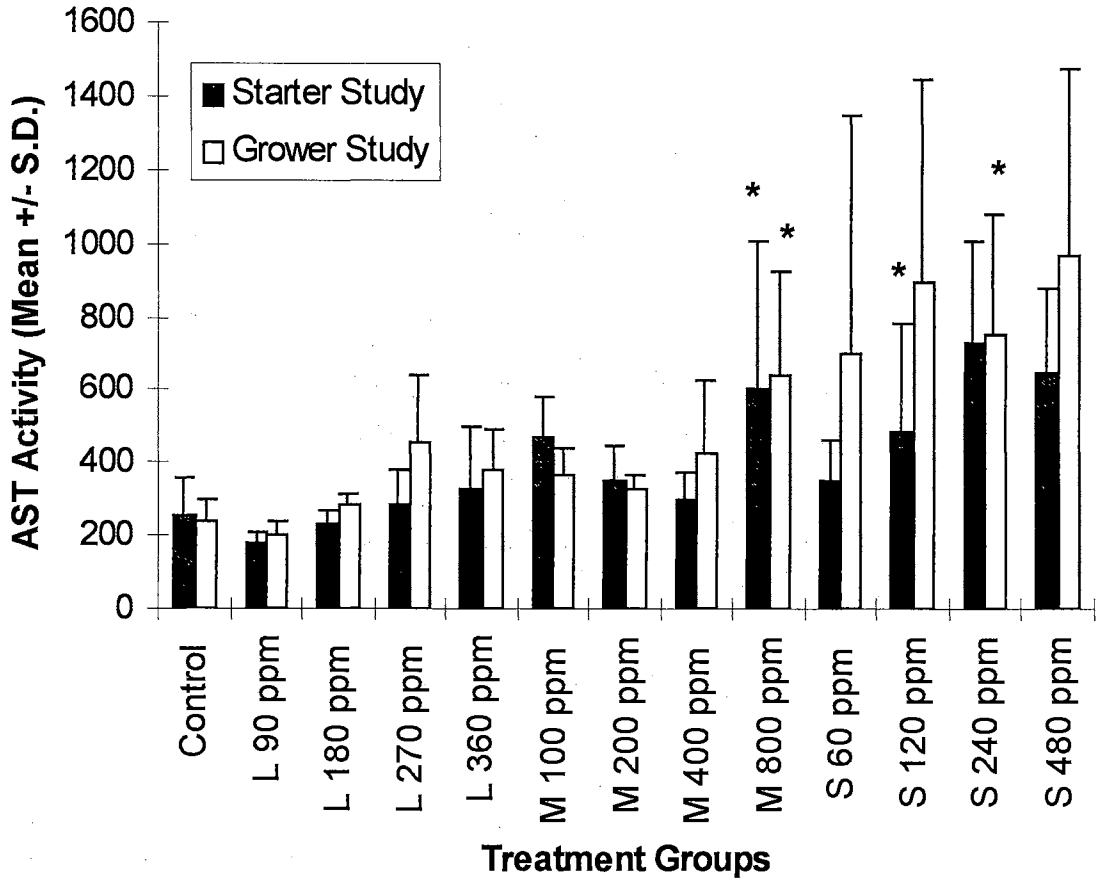


Figure 5. Serum Aspartate Transaminase (AST) activity of Cobb-cross broilers (20 or 40 days of age) in a Starter or Grower Study fed Various Levels of Ionophores (L = Lasalocid, M = Monensin, S= Salinomycin). (* = Statistically Significant, $P < 0.05$).

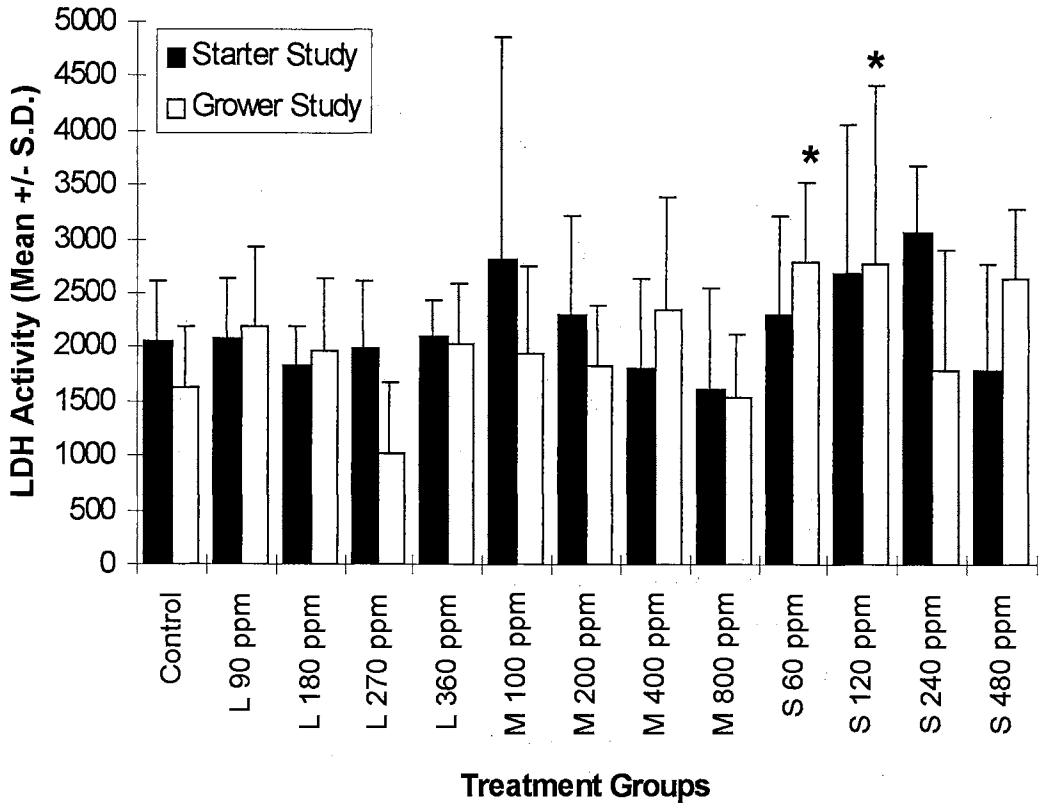


Figure 6. Serum Lactate Dehydrogenase (LDH) Activity of Cobb-cross Broilers in a Starter or Grower Study fed Various Levels of Ionophores (L = Lasalocid, M = Monensin, S= Salinomycin). (* = Statistically Significant, $P < 0.05$).

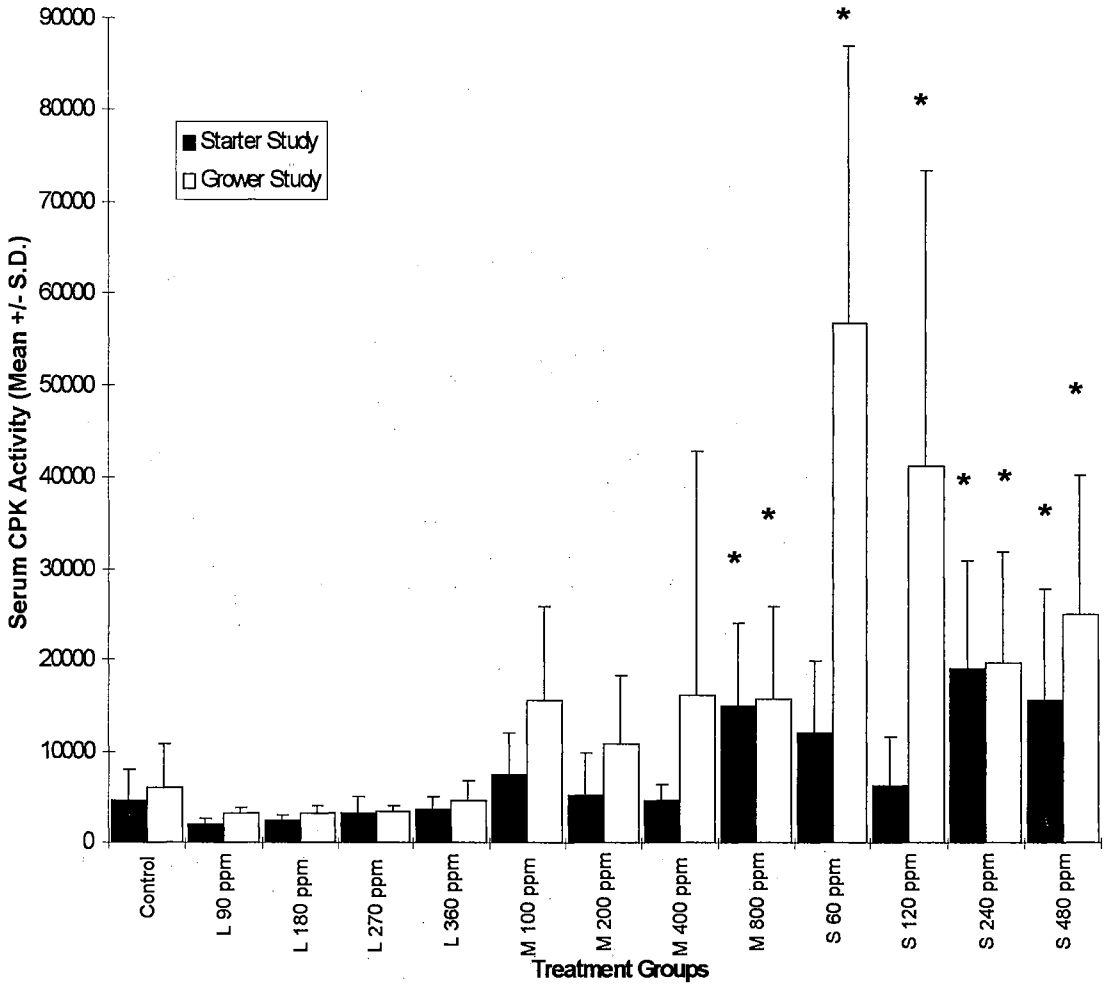


Figure 7. Serum Creatinine Phosphokinase (CPK) Activity of Cobb-cross Broilers in a Starter or Grower Study fed Various Levels of Ionophores (L = Lasalocid, M = Monensin, S= Salinomycin). (* = Statistically Significant, $P < 0.05$).

Body Weights

Increased dietary concentrations of ionophores caused a dose-dependent decrease in body weight. The body weights of broilers (3 or 6 weeks) fed 270 and 360 ppm lasalocid are significantly ($p<0.05$) reduced from controls. The birds (3 or 6 weeks old) fed 800 ppm monensin, 240 or 480 ppm salinomycin were significantly lower than controls (Figure 8). In the interaction study, the body weight was reduced in the groups fed the higher levels of lasalocid, 220 ppm. The groups of birds fed 60 ppm salinomycin in the starter ration and switched 220 ppm lasalocid had significantly reduced body weights as compared to controls (Figure 8).

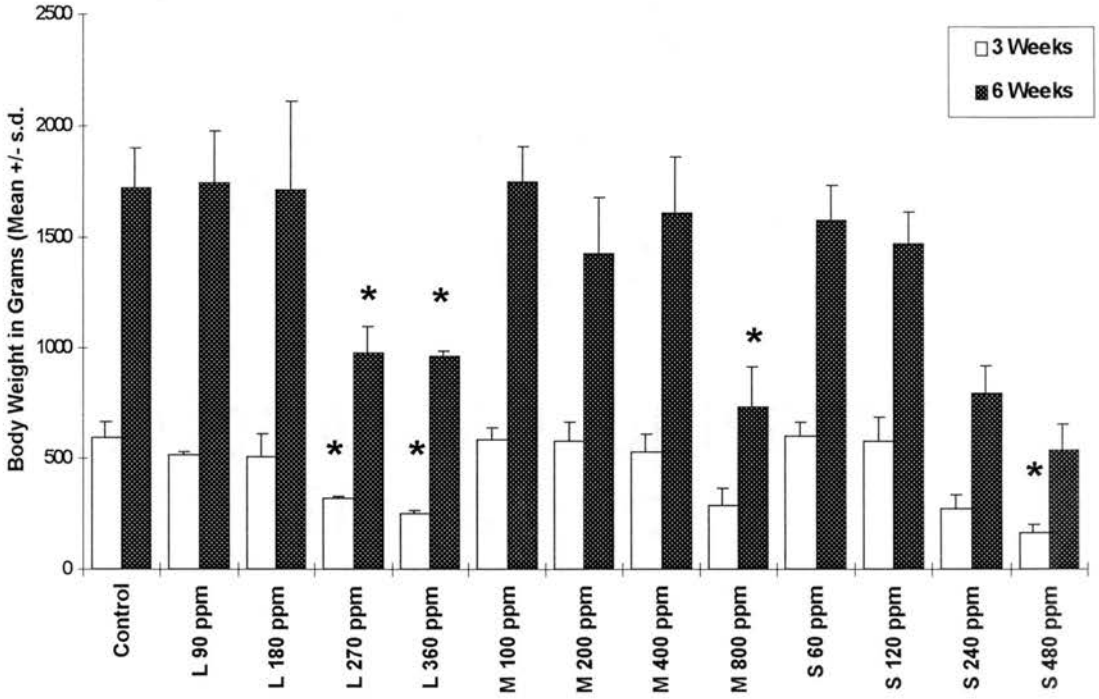
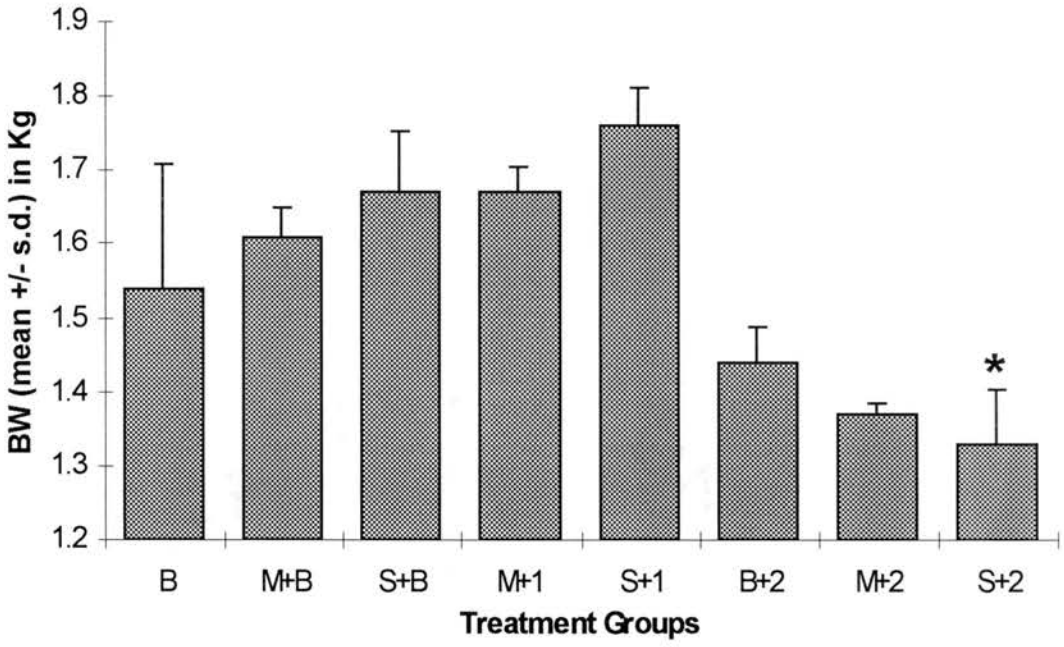


Figure 8. Body Weights of Cobb-cross Broiler Chickens (3 or 6 Weeks of age) Fed Increasing Dietary Concentrations of Ionophores (L = lasalocid, M = monensin, S = salinomycin). (* = $P < 0.05$).



Legend for Treatments in Interaction Trial:

B = (No drug in starter or grower rations)

M + B = 121 ppm Monensin in Starter: No drug in Grower

S + B = 66 ppm Salinomycin in Starter: No drug in Grower

M + 1 = 121 ppm Monensin in Starter: 121 Lasalocid in Grower

S + 1 = 66 ppm Salinomycin in Starter: 121 Lasalocid in Grower

M + 2 = 121 ppm Monensin in Starter: 220 ppm Lasalocid in Grower

B + 2 = (Positive Control) No drug starter: 220 ppm Lasalocid in Grower

S + 2 = 66 ppm Salinomycin in Starter: 220 ppm Lasalocid in Grower

Figure 9. Body Weights of Cobb-cross Broiler Chickens (40 Days of age) Fed Increasing Dietary Concentrations of Ionophores in an Interaction Study. (* = $P < 0.05$).

Mortality in the Starter and Grower Rations

There was minimal mortality (< 6%) in the groups fed higher concentrations of monensin or salinomycin. This may have been due to feed refusal exhibited by these

birds (data not presented). The mortality data for birds (3 or 6 weeks old) fed lasalocid showed low mortality at ration concentrations below 270 ppm. Broilers (3 or 6 weeks of age) fed 360 ppm lasalocid had a significantly higher rate of mortality than did the controls (Figure 10).

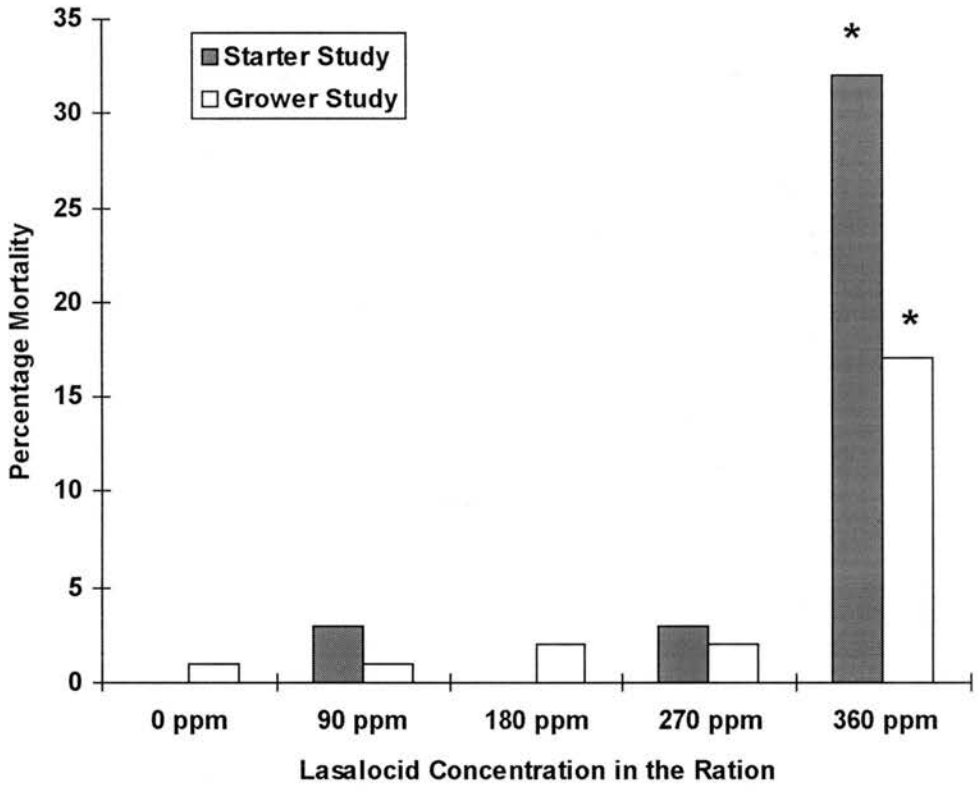


Figure 10. Mortality in Cobb-cross Broiler Chickens (20 or 40 Days Old) Fed Increasing Dietary Concentrations Of Lasalocid in a Starter (day 1-21) or Grower (day 21-42) Study. (* = $P < 0.05$).

Light Microscopic Evaluation of Sciatic Nerves

The results of light microscopic evaluation of tissues from lasalocid - fed birds, at all dose levels, failed to detect any significant skeletal or cardiac muscle necrosis. The only lesions consistently noted in these birds were degenerative changes in the sciatic nerve. These changes were noted in ataxic birds fed any level of lasalocid or more commonly in birds fed greater than 180 ppm lasalocid. Areas of myelin disruption, foamy myelin, axonal swelling and rupture were common findings (Figure 11). No lesions were detected by light microscopy in the spinal cord or brains of lasalocid-fed broilers. Monensin or salinomycin produced dose-dependent cardiac and skeletal muscle necrosis with no lesions in the sciatic nerve.

Electron Microscopic Evaluation of Sciatic Nerves

Ultrastructural changes observed in lasalocid fed, ataxic broilers were localized in the sciatic nerve. In these tissues, edema and separation of myelin lamella, vacuolation and degeneration of myelin and vacuoles within the cytoplasm of the Schwann cells were common findings (Figures 12 & 13). In most of the sections examined, the neurofilaments and microtubules of the axonal cytoplasm were intact and homogenous. Often the axon was compressed and deformed, but this was primarily due to the disruption and degredation of the myelin.

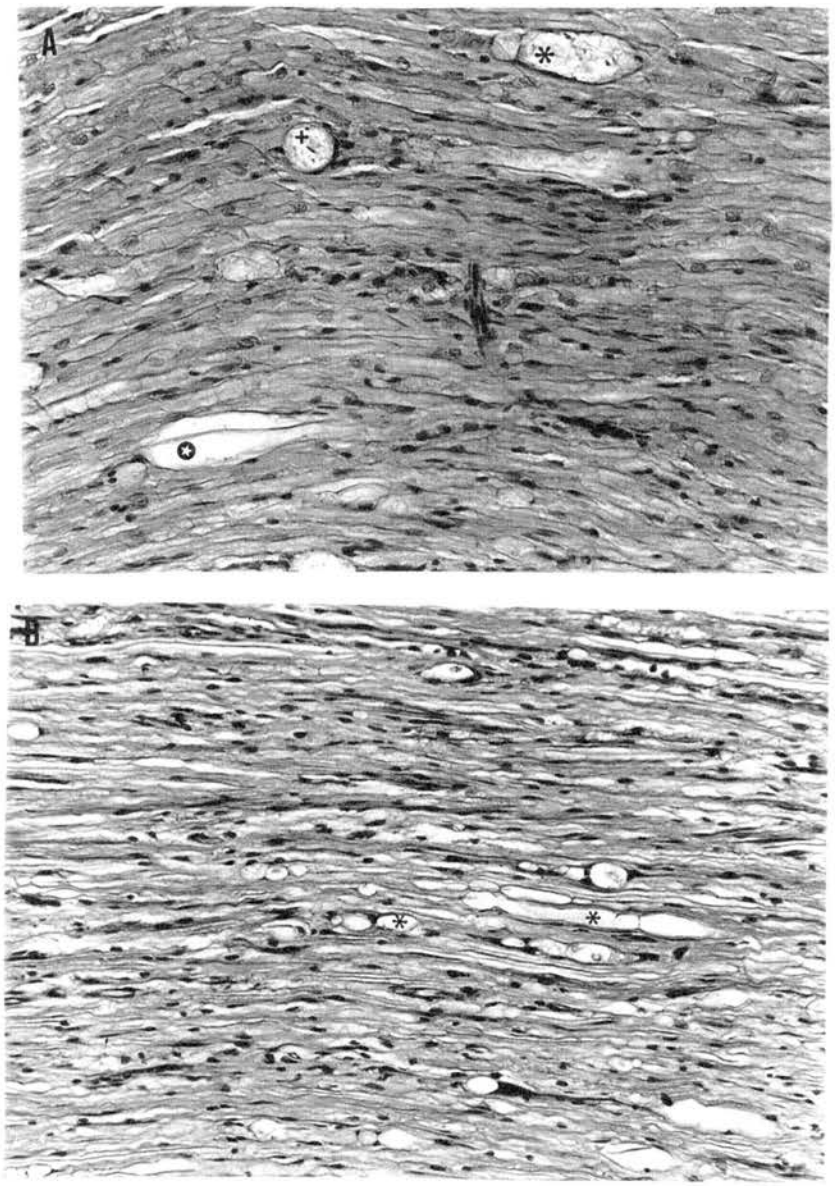


Figure 11. Light Micrograph of a Sciatic Nerve from an Ataxic, Male, Cobb-Cross Broiler Chicken fed 220 ppm Lasalocid. A) * Foamy Myelin; + Degrading Myelin With Axonal Fragment; ✪ Swollen Axon. B) * Multiple Areas of Myelin Vacuolation.

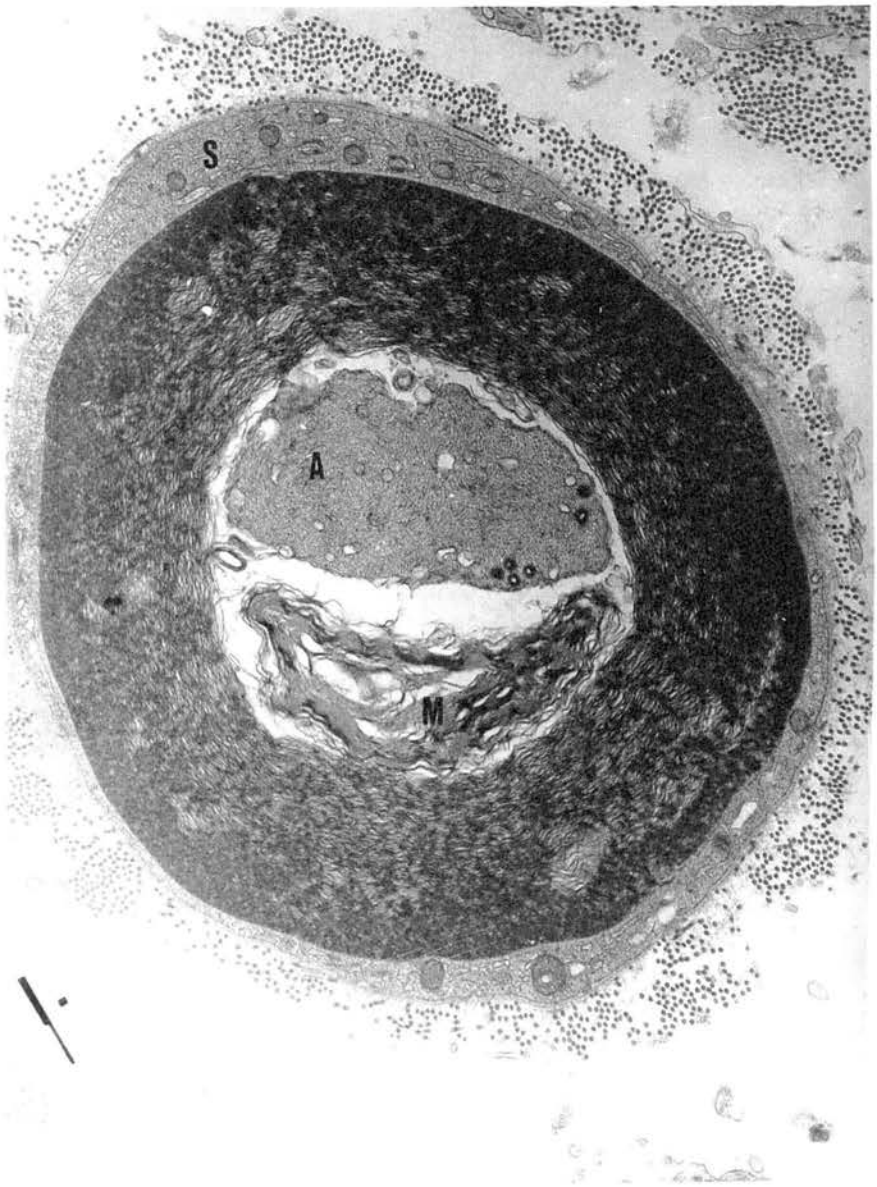


Figure 12. Electron Micrograph of a Sciatic Nerve from a Cobb-cross Broiler Chicken (40 days old) fed 90 ppm Lasalocid. Note the Concentric Lamella of Myelin and Intact Axon (a) Schwann cell (s), (m) Myelin Debris. (Magnification 7200)

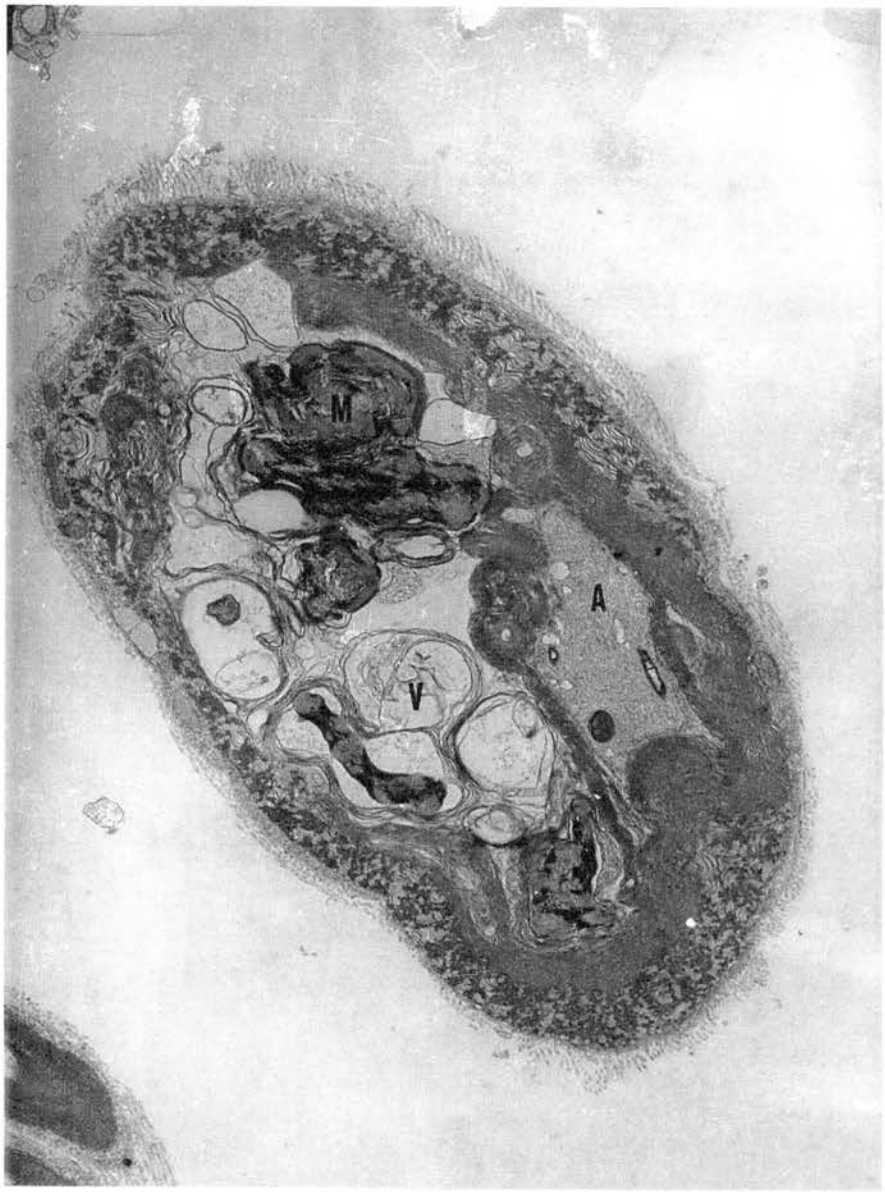


Figure 13. Electron Micrograph of a Sciatic Nerve from an Ataxic, Male Cobb-cross Broiler Chicken fed 220 ppm Lasalocid for 10 days. Note Intramyelinic Edema, Myelin Fragments (m), Extensive Vacuolation (v) of the Myelin and Compression of the Axon (a). (Magnification 4800)

Motor Nerve Conduction Velocity

Control birds (no drug) had an average MNCV of 35 ± 2 and 34 ± 1.5 M/sec for the right and left peroneal nerves, respectively. Ataxic birds with a clinical score of 3 (fed 270 ppm lasalocid) had a significantly reduced average MNCV of 15.2 ± 5.3 and 13.9 ± 4.6 M/sec for the right and left superficial peroneal nerves, respectively. The average percent reduction in the MNCV for the ataxic birds was 47 ± 15 and 59 ± 13 for the right and left nerves, respectively (Table IV)

TABLE IV.

MOTOR NERVE CONDUCTION VELOCITIES (M/SEC) OF THE SUPERFICIAL PERONEAL NERVES FROM MALE, COBB -ROSS BROILER CHICKENS (40 DAYS OF AGE) FED NO DRUG (CONTROL) OR 270 PPM LASALOCID (ATAXIC) FOR 10 DAYS. (* = $P < 0.05$)

Treatment Group	Velocity of Left Nerve (M/s)	% Reduction	Velocity of Right Nerve (M/s)	% Reduction
Control (n=3)	35 ± 2	--	34 ± 1.5	---
Ataxic (n = 4)	15.3 ± 5.3*	47 ± 15	13.9 ± 4.6*	59 ± 13

ATPase Activity

The results for ATPase activity from selected organs of broilers fed increased levels lasalocid showed no significant differences from control (no drug) in the starter and grower studies (Tables VI and VII, respectively). The specific ATPase activity from selected organs of broilers fed increasing levels of monensin and salinomycin similarly show no significant differences from control (no drug) in the starter and grower studies

(Table VIII & IX, respectively). *In vitro* incubation of ATPase with lasalocid , 10 - 100 μM, produced a dose-dependent inhibition of activity. The Na^+, K^+ - ATPase IC_{50} of lasalocid was 46.6 μM for the Sigma (95% purity) product and 51 μM for the biomass product (88% purity). The inhibitory effects of lasalocid on Mg^{++}-ATPase was less than 50 % with the concentrations examined (Figures 14 & 15). There is little difference between the biomass product (Roche, 88% purity) and the more purified preparation (Sigma Chemical Co., 95 % purity).

TABLE VI

SPECIFIC ACTIVITY OF ATPASE ENZYMES FROM ORGANS OF BROIILER CHICKENS FED LASALOCID IN A STARTER RATION (DAY 1 - 21). (* = P < 0.05)

Organ	Dose (Lasalocid)	Na+, K+-ATPase	Mg++-ATPase	Total ATPase
Brain	0 ppm	5748.16 ± 1195.84	906.91 ± 232.53	6655.07 ± 1323.45
Heart	0 ppm	1332.68 ± 484.00	517.74 ± 179.49	1850.42 ± 428.41
Kidney	0 ppm	4470.84 ± 2026.99	1398.64 ± 601.11	5869.47 ± 1746.16
LI Mucosa	0 ppm	1656.13 ± 676.45	2683.48 ± 489.29	4339.61 ± 787.92
SI Mucosa	0 ppm	4355.71 ± 584.18	3614.35 ± 1917.21	7970.06 ± 2275.64
Brain	90 ppm	4841.26 ± 2107.87	901.90 ± 393.77	5743.16 ± 2440.88
Heart	90 ppm	1009.99 ± 383.56	472.24 ± 151.86	1482.24 ± 513.86
Kidney	90 ppm	4571.84 ± 785.61	1090.14 ± 160.70	5661.98 ± 920.82
LI Mucosa	90 ppm	1079.64 ± 148.88	2617.43 ± 1249.44	3697.07 ± 1390.76
SI Mucosa	90 ppm	2498.94 ± 2175.61 *	3404.11 ± 1178.88	5903.05 ± 3107.79
Brain	360 ppm	5391.12 ± 1306.47	932.12 ± 195.72	6323.24 ± 1448.83
Heart	360 ppm	1379.00 ± 607.23	487.63 ± 214.57	1866.64 ± 732.67
Kidney	360 ppm	5146.67 ± 1089.02	1073.86 ± 184.04	6220.53 ± 1212.61
LI Mucosa	360 ppm	1635.65 ± 664.70	1872.60 ± 404.39 *	3508.25 ± 457.06*
SI Mucosa	360 ppm	2992.47 ± 1992.85	2346.86 ± 1483.28	5339.33 ± 3446.64

TABLE VII

SPECIFIC ACTIVITY OF ATPASE ENZYMES FROM ORGANS OF BROILERS FED LASALOCID IN A GROWER RATION (DAY 21 - 42 OF AGE)

Organ	Dose (Lasalocid)	Na+, K+- ATPase	Mg++ - ATPase	Total - ATPase
Kidney	0 ppm	2470.64 ± 600.91	702.96 ± 41.89	3173.59 ± 630.62
SI Mucosa	0 ppm	1489.93 ± 165.46	1430.96 ± 412.81	2920.88 ± 524.34
LI Mucosa	0 ppm	884.53 ± 79.49	1869.05 ± 309.29	2753.58 ± 347.14
Kidney	360 ppm	3001.12 ± 1179.97	758.96 ± 208.15	3760.08 ± 1329.45
SI Mucosa	360 ppm	1445.15 ± 144.57	1329.54 ± 482.00	2774.69 ± 600.71
LI Mucosa	360 ppm	1113.05 ± 478.34	1750.08 ± 1261.90	2863.13 ± 1567.15

TABLE VIII

SPECIFIC ATPASE ACTIVITY FROM ORGANS OF BROILER CHICKENS FED MONENSIN (M) OR SALINOMYCIN (S) IN STARTER RATION (DAY 1-21 OF AGE)

Organ	Dose (Ionophore)	Na+, K+, ATPase	Mg++ ATPase	Total ATPase
Kidney	0 ppm	1156.35 ± 512.44	724.67 ± 86.64	1881.02 ± 578.49
LI Mucosa	0 ppm	1348.37 ± 586.17	1029.95 ± 326.94	2378.32 ± 596.40
SI Mucosa	0 ppm	1673.57 ± 571.22	1234.53 ± 266.27	2908.10 ± 600.78
Kidney	M 100 ppm	1452.63 ± 861.16	320.69 ± 178.87	1773.32 ± 990.48
LI Mucosa	M 100 ppm	1849.55 ± 584.39	1039.35 ± 241.98	2888.90 ± 1973.33
SI Mucosa	M 100 ppm	1938.51 ± 1107.62	920.16 ± 315.51	2858.67 ± 1046.46
Kidney	M 400 ppm	675.44 ± 392.85	1210.86 ± 1011.51	1886.30 ± 1144.29
LI Mucosa	M 400 ppm	1697.65 ± 1754.35	756.69 ± 644.67	2454.34 ± 2376.89
SI Mucosa	M 400 ppm	939.39 ± 650.75	1201.01 ± 758.14	2140.40 ± 177.06
Kidney	S 60 ppm	1324.67 ± 686.61	850.35 ± 472.00	2175.02 ± 1096.33
LI Mucosa	S 60 ppm	1150.14 ± 547.29	872.30 ± 450.80	2022.44 ± 715.75
SI Mucosa	S 60 ppm	2397.66 ± 1357.89	1530.56 ± 811.62	3928.22 ± 2098.54
Kidney	S 240 ppm	1718.63 ± 930.51	777.85 ± 761.76	2496.47 ± 943.35
LI Mucosa	S 240 ppm	1193.12 ± 492.88	543.42 ± 226.54	1736.54 ± 418.72
SI Mucosa	S 240 ppm	2425.01 ± 1858.60	959.36 ± 429.60	3384.37 ± 1650.75

TABLE IX.

SPECIFIC ATPASE ACTIVITY FROM ORGANS OF BROILER CHICKENS FED MONENSIN (M) OR SALINOMYCIN (S) IN A GROWER RATION (DAY 21- 42 OF AGE)

Organ	Dose (Ionophore)	Na+, K+, ATPase	Mg++ ATPase	Total ATPase
Kidney	0 ppm	2390.78 ± 987.43	353.12 ± 113.08	2743.90 ± 1009.76
LI Mucosa	0 ppm	1835.13 ±306.10	866.38 ± 259.87	2701.50 ± 412.19
SI Mucosa	0 ppm	2355.33 ± 1067.22	530.97 ± 265.63	2886.30 ± 1312.99
Kidney	M 100 ppm	2134.94 ± 516.42	357.14 ± 131.62	2492.08 ± 558.09
LI Mucosa	M 100 ppm	2641.14 ± 1178.51	588.64 ± 139.82	3229.78 ± 1300.70
SI Mucosa	M 100 ppm	2945.00 ± 947.09	776.50 ± 364.87	3721.50 ± 1230.40
Kidney	M 400 ppm	1645.69 ± 505.14	246.17 ± 89.29	1891.87 ± 565.42
LI Mucosa	M 400 ppm	2186.38 ± 1027.47	420.90 ± 233.72	2607.27 ± 1249.67
SI Mucosa	M 400 ppm	2841.76 ± 1939.35	709.50 ± 511.05	3551.27 ± 2445.57
Kidney	S 60 ppm	2053.57 ± 1185.88	374.34 ± 166.24	2427.91 ± 1334.88
LI Mucosa	S 60 ppm	2760.95 ± 1447.49	727.19 ± 191.13	3488.13 ± 1519.73
SI Mucosa	S 60 ppm	2611.57 ± 1028.09	742.76 ± 292.43	3354.33 ± 1311.26
Kidney	S 240 ppm	2186.56 ± 1475.86	549.71 ± 271.72	2736.27 ± 1742.27
LI Mucosa	S 240 ppm	2437.67 ± 1048.99	704.80 ± 268.80	3142.47 ± 1298.31
SI Mucosa	S 240 ppm	2849.91 ± 1234.06	646.56 ± 199.15	3496.46 ± 1291.68

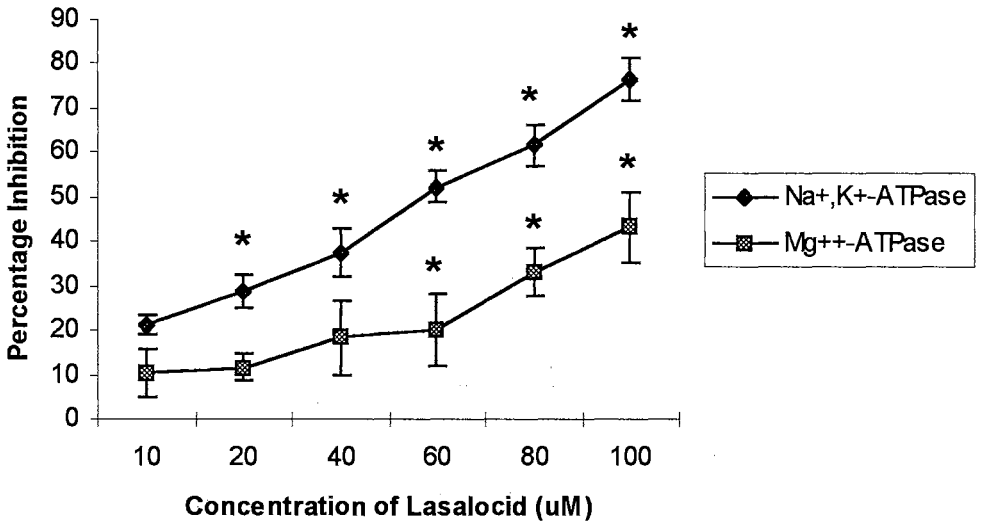


Figure 14. *In Vitro* Dose-Dependent ATPase Inhibition from Cobb-cross Broiler Chicken Kidneys by Lasalocid (Sigma Co. 95 % Purity). (* = P < 0.05)

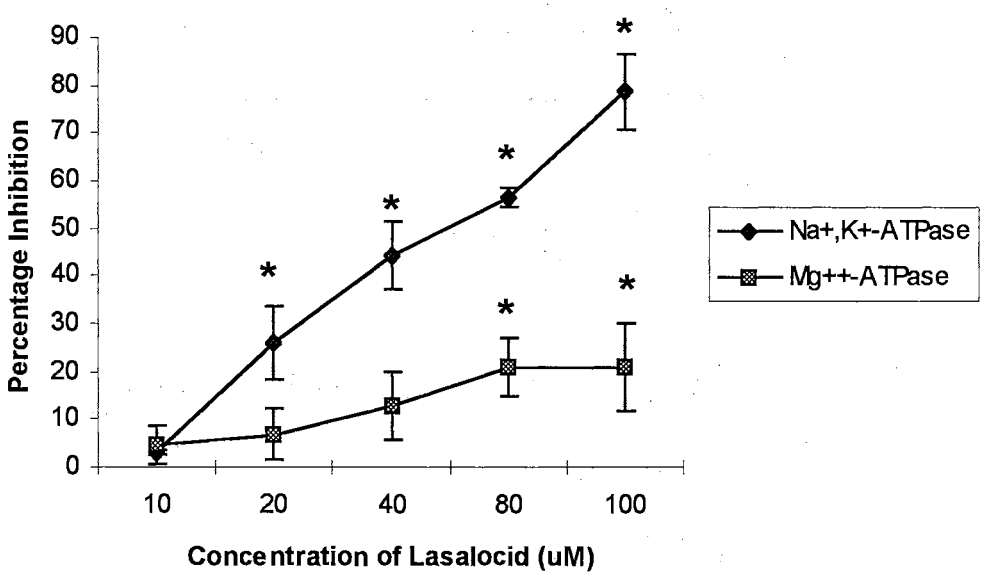


Figure 15. *In Vitro* Dose-Dependent ATPase Inhibition from Cobb-cross Broiler Chicken Kidneys by Lasalocid (Roche 88% Purity). (* = P < 0.05)

Discussion

The data presented provide evidence that lasalocid can cause peripheral neuropathy in Cobb-cross broiler chickens. This dose-dependent, experimentally-induced toxicosis is described by a clinical ataxia scoring system and mimics field cases in description, time of onset and severity. Increased incidence of ataxia is noted in 3 and 6 week old Cobb-cross broilers with increased dietary levels of lasalocid. A dietary dose that cause 25% ataxia in a group of birds was determined to be 220 ppm and 242 ppm for 3 and 6 week old broilers, respectively. The neurotoxic effects of lasalocid are characterized by a markedly reduced superficial peroneal MNCV and pathologic findings in the sciatic nerve that included: myelin vacuoles, intramyelinic edema and secondary axonal degeneration.

The ataxia scoring system used in these studies is similar to the procedures used by others (Abou-Donia & Graham,1978; Abou-Donia,1977; Abou-Donia et al.,1980; Dyer et al.,1992; el-Fawal et al, 1990a,b). Using this clinical scoring system, lasalocid produced a dose-dependent ataxia in 3 and 6 week old broiler chickens. Ataxia dramatically increased at dietary concentration greater than 180 ppm. These levels could occur in a production system by an accidental overdose of the ionophore. In contrast, rations containing monensin or salinomycin produced sporadic, less than 2%, ataxia.

Affected birds in the earlier stages (Clinical Scores 1 or 2) are alert, able to eat and drink until the severity increases to 3 or 4 when birds become increasingly ataxic and are unable to walk to feed and water. In the last stage (Clinical Severity Score 5), the birds are depressed and lethargic, refuse to eat and frequently die. The onset of

ataxia is usually 5 - 7 days after introduction of lasalocid-containing feed, which is similar to field reports.

Assessment of neurotoxic effects with neurologic examinations suggest that the syndrome is due to peripheral nervous system damage (Anon., 1986). Central nervous system function of the vestibular system, vision, hearing and the limbic systems are normal in affected birds.

Incidence of ataxia was linear following a logarithmic-probit transformation. Linear regression analysis of the transformed data demonstrated similar TD_{25} for the 3 week (220 ppm) and 6 week (242 ppm) broilers suggesting that the neuropathy is not age-dependent up to 6 weeks. These toxic doses are approximately twice the recommended use levels of lasalocid and could be used to increase the number of ataxic birds for future studies. Increased numbers of affected birds would be beneficial in examining potential neurotoxic mechanisms or to evaluate the efficacy of possible therapies for lasalocid-induced neuropathy.

The motor nerve conduction velocities in the control, no drug, birds are similar to previous reports in broiler chickens (Konegay et al, 1983; Robertson et al, 1986). The marked reduction in conduction velocity in ataxic broilers fed 270 ppm lasalocid for 10 days indicate specific damage to the peripheral nervous system. This is similar to the reduced nerve conduction velocities caused by phenyl saligenin phosphate, an organophosphate capable of producing OPIDN, in chickens (Lidsky et al, 1990) and ddC, a reverse transcriptase inhibitor, in rabbits (Anderson et al, 1990). The marked decrease of the MNCV in lasalocid-fed birds was associated with gait abnormalities and an ataxia score of 3.

The light and electron microscopic findings in lasalocid-induced ataxia are specific to the peripheral nervous system. The more prominent findings include

degenerative changes in the sciatic nerve with separation of myelin lamella, myelin vacuolation, myelin degeneration, intramyelinic edema, axonal swelling and rupture. These results are similar to a previous report (Gregory et al, 1995;). Myelin pathology may be the primary change with lasalocid-induced ataxia with subsequent compression on the axon. This is demonstrated by the numerous fibers with severe intramyelinic edema without concurrent axonal damage. Ultrastructurally, axons with damaged myelin had normal appearing neurofilaments and microtubules of the axonal cytoplasm. No lesions were detected by light microscopy in the spinal cord or brains of lasalocid-fed broilers. Broilers fed monensin and salinomycin did not exhibit any lesions in sciatic nerves. The specificity of lasalocid-induced damage to peripheral nervous system is not known. A possible explanation could relate to the different tissues levels of lasalocid in the PNS as compared to the CNS. Another possible reason for the differential response may be different responses of central and peripheral myelin to lasalocid.

Reduced body weights of birds fed increased concentrations of all three ionophore indicate generalized toxic effects. This agrees with a previous reports (Keshavarz & McDougald, 1982; Horovitz et al., 1988; Owawoye & Krueger, 1986; Bartov, 1994) of feeding elevated levels of ionophore. In the current studies, lasalocid-induced depression of body weight and greater variability in weights may be due to a lack of ability to obtain feed, generalized malaise or cachexia of disease. In previous reports, hens affected with OPIDN had reduced body weights that was most prominent after the birds became ataxic, lost their appetites and exhibited poor eating habits (Abou-Donia et al.,1980; Abou-Donia & Graham, 1978). This effect was more pronounced in the more severely ataxic birds with respiratory and swallowing disorders. In the current study, the greater variability in body weights could indicate to producers

that there is increased risk for lasalocid-induced ataxia. This would most likely occur at lasalocid concentrations in excess of 220 ppm in the ration, as shown by the current studies and would be very marked at 270 and 360 ppm.

The serum electrolyte and chemistry results for 3 & 6 week controls are similar to previously reported values (Ross et al., 1978). Serum sodium concentrations are significantly reduced in the 90, 180 and 270 ppm lasalocid treatments of the starter study. The reduced serum sodium in lasalocid-fed broilers may provide a clue to the role of this cation in the syndrome. As noted earlier, ataxia is more prevalent during the summer and on farms that have water with a high salinity. In these situations, elevated serum sodium may be shuttled into the cell by lasalocid and cause cell dysfunction and death as previously described (Shier & DuBourdieu, 1992; Shier et al, 1991).

Monensin and salinomycin at greater than recommended levels increased serum concentrations of potassium and AST, LDH and CPK indicating myotoxicity as previously reported (Horovitz et al.,1988; Galitzer et al, 1982; Duncan & Prasse, 1986). Of these enzymes, CPK is the most specific and sensitive indicator of muscle damage. Monensin and salinomycin cause myonecrosis with a resulting hyperkalemia due to the higher concentration of intracellular potassium in the myocytes (Duncan & Prasse, 1986). Lasalocid-treated birds from any age group did not have elevated enzyme activities.

The results of the ATPase studies indicate that *in vivo* there is not significant alteration in the activity of these enzymes. The results of *in vitro* incubation of lasalocid with ATPase enzymes demonstrated a dose-dependent inhibition. These results might be explained by different enzyme isoforms or upregulation of the proteins. There are Na+,K+-ATPase isoform differences between these cell types with α_2 more prevalent in

glial cells and α_3 more commonly found in neurons. Additionally, there are species (dog, rat, human) differences in the abundance and importance of these isoforms of Na+,K+-ATPase (Decollogne et al.,1993). The current studies did not measure the activities of various isoforms of Na+,K+-ATPase. The ionophores may impact the function of one or more different isoform, *in vivo* leading to alterations in ionic homeostasis. Another possible explanation for these results involve the complex intracellular regulation of ATPase activity *in vivo*. Inside the cell, Na+,K+-ATPase is subject to complex, highly regulated control mechanisms. Ionic regulation, *in vivo*, exerts a powerful control over these enzymes (McGeoch, 1990; Azuma et al.,1991; Rayson, 1990) . Increased intracellular levels of calcium drastically increase the function of the Na+,K+-ATPase. Elevated calcium increases ATPase mRNA, enzyme synthetic rates and releases enzyme inhibition (Decollogne et al.,1993). This would help offset the ionophore-induced degradation of the ionic gradients, to maintain homeostasis in the long term. This may also explain differential effects on the ATPase enzyme *in vivo* and *in vitro*. Alternatively, lasalocid may have only an indirect effect on the function of the activity of the Na+,K+-ATPase. The ionophore may alter critical membrane-bound cations (Mg^{++}) that regulate the structure or activity of the enzyme (Antonio et al, 1991).

In summary, lasalocid incorporated in the feed produced a dose-dependent ataxia in male, Cobb-cross broilers similar to field cases. The incidence and severity of ataxia is described by a clinical scoring system. Lasalocid neuropathy is characterized by a marked reduction of superficial peroneal MNCV and pathologic findings in the sciatic nerve that include: myelin vacuoles, intramyelinic edema and secondary axonal degeneration. This is the first report of the dose-dependent nature of lasalocid-induced

neuropathy. The use of 220 - 242 ppm lasalocid in the ration of broiler chickens could provide a non-mammalian, acute model of chemically-induced peripheral neuropathy.

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CHAPTER III

LASALOCID-INDUCED ATAXIA IN BROILERS: ELUCIDATION OF POSSIBLE MECHANISMS

Abstract

Previous studies suggest that increased dietary concentrations of lasalocid are associated with a peripheral neuropathy in broiler chickens that manifests clinically as ataxia. Determination of a precise oral dose capable of causing ataxia similar to field cases is essential for mechanistic and therapeutic studies. This study characterized the oral doses of lasalocid necessary to cause ataxia within 48 hours. Lasalocid caused a dose-dependent neurotoxicty in 4-6 week old, male, Cobb-cross broiler chickens at concentrations greater than 11.25 mg/kg, t.i.d. given orally. As measured by a clinical ataxia scoring system, the severity and incidence of neurotoxicity are dose related. Recovery from lasalocid-induced ataxia occurs 10 days after removal of the drug. Vitamin E (100 IU) pretreatment and 12 hour water deprivation do not appear to play a major role in the pathogenesis of this syndrome. Lasalocid administration (33.75 mg/kg, t.i.d.) did not influence sciatic nerve ATPase activity from ataxic broilers. In vitro, 1 - 8 μM lasalocid inhibited submitochondrial NADH oxidase activity in a dose-dependent manner. Additionally, submitochondrial ATPase activity was inhibited by 25 - 200 μM lasalocid. Sciatic nerves

exposed to lasalocid (100 μM), *in vitro*, demonstrate time- and calcium-dependent ultrastructural changes (intramyelinic edema and myelin vacuolation). The results of these studies suggest that lasalocid may elicit peripheral neuropathy in broilers indirectly by reducing intracellular ATP levels causing deregulation of ionic homeostasis or directly by activating calcium-mediated catabolic enzymes. The oral doses of lasalocid used in these studies provide a useful model of chemically-induced, acute peripheral neuropathy.

Introduction

The ionophores (monensin, lasalocid and salinomycin) are carboxylic acid antibiotics produced by fungal (Streptomyces) fermentation (Novilla, 1992; Galitzer & Oehme, 1984; Pressman, 1976), with a wide range of antibacterial activities (Reed, 1982). The ionophores are commonly used anticoccidial agents added to poultry diets. There are numerous reports of acute toxicoses in domestic livestock species due to ingestion (accidental or overdose) of these compounds (Novilla, 1992; VanVleet et al.,1987; Galitzer & Oehme, 1984; Shlosberg et al.,1992; Mezes et al.,1992). The toxic effects due to these compounds have traditionally been described as a skeletal or cardiac myopathy (Novilla, 1992). Recently, intoxication with lasalocid produced neurologic clinical signs (Safran et al, 1993; Perelman et al, 1993; Shlosberg et al.,1986; Gregory et al, 1995). Unpublished field observations indicate an association with lasalocid in the ration and ataxic broilers. Ataxia is most commonly seen in male, rapidly growing broilers one week after changing to a ration containing lasalocid. The incidence and severity of this syndrome is worse in the summer months. Poultry producers and field veterinarians have designated this syndrome "downer" or "knockdown birds". It is currently unknown if this effect is directly related to lasalocid exposure or lasalocid and a combination of environmental or management factors.

We have recently described a feed concentration of lasalocid (220 - 242 ppm) that produced ataxia in broiler chickens (Roder et al, 1996). This dose-dependent neuropathy is specific for the peripheral nervous system as evidenced by light and electron microscopic findings. The onset and severity of the neurotoxicity is similar to field cases with clinical signs beginning 7 days after introduction of lasalocid.

The dose-response relationship is fundamental to toxicology and understanding the mechanisms of toxic agents (Klaassen & Eaton, 1991). Accurate measurement of a toxin-induced response depends on a quantifiable, repeatable method of measurement (Klaassen & Eaton, 1991). Clinical observation and the use of ataxia scoring systems have been used by other researchers to evaluate organophosphate-induced delayed neuropathy (OPIDN) (Abou-Donia & Graham, 1978; Abou-Donia, 1977; Abou-Donia et al.,1980; Dyer et al.,1992; el-Fawal et al, 1990a,b). Refinement of a precise oral dose that reliably produces ataxic birds will allow for mechanistic and therapeutic studies.

Some possible pathophysiologic mechanisms involved in lasalocid-induced peripheral neuropathy may include: water deprivation/heat stress (as noted in the field), depletion of intracellular antioxidant systems, alteration of neuronal ATPase activity, alteration of oxidative phosphorylation or direct damage to peripheral nerves via a calcium-mediated process.

The increased incidence of lasalocid-induced ataxia in the summer months and field observations of increased severity on farms with increased water salinity suggest a role for hypernatremia and the incidence of lasalocid-induced ataxia. The combination of water deprivation/heat stress would increase the osmolality of serum possibly potentiating toxicosis. Antioxidants, especially vitamin E, protect neurons and myocytes from damage and cytotoxicity caused by different agents including ionophores (Horvath et al, 1992;

Schubert et al, 1992; Behl et al.,1992; VanVleet et al.,1987). Oxidative stress and the resulting cellular damage may play a role in the neurotoxicity caused by lasalocid.

Inhibition or reduction of peripheral nerve ATPase activity has been associated with diabetes-associated neuronopathy (LoPachin et al.,1993; Brismar & Sima, 1981; Llewelyn & Thomas, 1987; Greene et al, 1987). The diminished activity of this constitutive enzyme would alter the ionic homeostasis of the cells and reduce the electrical excitability of the nerve. It is possible that lasalocid produces ataxia due in part to inhibition of sciatic nerve ATPases.

Many compounds can alter the function of the mitochondrial respiratory chain and reduce the production of ATP. Without this source of energy, the membrane bound "pumps" , Na+-K+-ATPase and Ca++ ATPase, can not adequately regulate the intracellular concentrations of sodium and calcium. This can lead to increases in intracellular calcium and cell death.

Calcium-mediated cell death is well established *in vitro* and *in vivo* (Trump & Berezesky, 1995; Nicotera et al, 1992; Iacopino et al.,1992; Pollardet al.,1994). Increased intracellular calcium can activate a myriad of processes, calcium-dependent endonucleases, phospholipases, destabilization of the cytoskeleton (microtubules), condensation of mitochondria, expression of immediate-early genes (c-fos, c-jun, c-myc) and apoptosis (Trump & Berezesky, 1995; Nicotera et al, 1992), that lead to dysfunction and death. Some degenerative changes in axons and neuronal cell lines have been shown to calcium-dependent (Fano et al.,1993) as have the degenerative changes of transected neurites (Schlaepfer, 1974; Schlaepfer & Bunge, 1973), which can be prevented by chelation of extracellular calcium.

Carboxylic ionophores can increase the intracellular concentrations of calcium either directly (lasalocid, A23187) or indirectly (monensin, salinomycin) and serve as a model for studying the process of cell death (DuBourdieu & Shier, 1992; Shier & DuBourdieu, 1992; Shier et al, 1991). Ionophores can also increase calcium flux across neuronal cell membranes causing ultrastructural damage *in vitro* (Schlaepfer, 1977b,a) and *in vivo* (Smith & Hall, 1988). It is possible that lasalocid causes direct neuronal cell damage in vitro though a calcium-mediated process.

The objectives for the in vivo studies are: identification of oral dose(s) of lasalocid that would reliably produce dose-dependent ataxia in broilers, determine if affected birds can recover from lasalocid-induced ataxia and to examine the roles of Vitamin E (oxidative stress), ATPase (ionic homeostasis) and water deprivation/mild heat stress on the pathogenesis of this syndrome. The objectives for the in vitro studies are to examine the effect of lasalocid on mitochondrial respiratory chain function, explore the ultrastructural changes associated with direct exposure to lasalocid and to determine the role of calcium in these ultrastructural changes.

Materials and Methods

Animals

Male, Cobb broilers were used in these studies. One-day-old chicks were purchased from a commercial source (Tyson Hatchery, Springdale, AR) and were housed in floor pens, bedded on fresh rice hulls. Supplemental heat was provided by 2-3 lamps per pen, to maintain the temperature at floor level near 30° C, during the first 2 weeks of the study. Birds were exposed to constant tungsten filament lighting. At 4 weeks of age, the birds were randomly assigned to treatment groups and housed in individual stainless steel cages

in an environmental chamber (1 bird per cage). The broilers were fed a basal ration (no drugs) throughout the study. The composition of the corn-soy basal ration is detailed in Appendix A. Feed and water, from automatic waterers, were provided ad libiditum.

Drugs

Lasalocid as a semi-purified (88%), biomass product was provided as a gift from Roche Fine Chemicals. The drug was weighed and placed into gelatin capsules (UPJOHN®). An injectable Vitamin E product (VITAL E™-300 ; Schering -Plough Animal Health, Kenilworth, NJ) was purchased for use. All other chemicals used in the oxidative-phosphorylation, ATPase studies and sciatic nerve exposure, in vitro (EGTA, Tris HCl, Trizma® base, $MgCl_2$, KCl, CaCl2, NaCl, Tris-ATP, TCA, lasalocid, DMSO, phenol red,) were purchased from Sigma Chemical Company (St. Louis, MO).

Experiment One: Characterization of Oral Dose Response

In these studies, 4-6 week old, male, Cobb-cross broiler chickens were acclimatized for 3 days and randomly assigned to 6 treatment groups. The treatment groups were: control, no lasalocid, (n =10), 11.25 mg/kg lasalocid, t.i.d. (n =10); 22.5 mg/kg lasalocid, t.i.d. (n = 10); 30 mg/kg lasalocid, t.i.d. (n = 10); 33.75 mg/kg lasalocid, t.i.d. (n = 10) ; 50 mg/kg lasalocid, t.i.d. (n = 7). Lasalocid, biomass product, was weighed and placed into gelatin capsules. Gelatin capsules were given orally to each bird in the treatment group. Ataxia was scored 48 hours after the initial dose using a clinical ataxia scoring system.

All birds were assessed twice each day for clinical signs of ataxia. Initial visual observation examined the character and attitude of undisturbed animals. Any

paralyzed or severely ataxic birds were noted by this unobtrusive observation. All birds were then forced to walk around their cages for several minutes. Birds were categorized by the clinical ataxia scoring system presented in Table I. Forcing ambulation allows detection of slightly ataxic birds or observe if ataxia worsens after exercise. This scoring system evaluates the gait characteristics and the attitude of the bird and ranges from normal to paralyzed and depressed birds.

Further evaluation of intoxication in lesioned birds was accomplished by a clinical neurologic examination of cranial nerve function and spinal reflexes, Appendix B. This permits localization of lesion(s) to the central or peripheral nervous system and region of the body affected. Each lesioned bird was evaluated at two different time periods to accurately measure any alteration in the reflexes.

TABLE I

CLINICAL SCORING SYSTEM FOR LASALOCID-INDUCED ATAXIA

Score	Description
0	Normal bird
1	Slight Ataxia after exercise
2	Slight Ataxia, Ataxia worsens after exercise
3	"Duck Walkers", Ataxic, Waddling-type gait
4	Non-ambulatory (down), alert, aware of surroundings
5	Non-ambulatory (down), not alert, depressed

Experiment Two: Evaluation of Recovery From Lasalocid-induced Ataxia

In the recovery studies, 4 - 6 week old, male, Cobb-cross broilers were randomly assigned to three treatment groups with 10 birds each. The lasalocid doses used were 11.25 mg/kg, t.i.d., 22.5 mg/kg, t.i.d., or 33.75 mg/kg, t.i.d. Birds were given lasalocid, biomass product, in gelatin capsules orally for 2 days after which they were evaluated and scored using a clinical ataxia scoring system. Birds with clinically observable ataxia (scores 2,3 or 4) were selected for use in the recovery portion of these studies. The number of ataxic birds used for each treatment group : 11.25 mg/kg (n=2), 22.5 mg/kg (n=6), 33.75 mg/kg (n=7). Ataxic birds were maintained on a drug-free diet for 10 days. Ataxia was re-evaluated 3, 5 and 10 days following the last dose of lasalocid, using a clinical ataxia scoring system as described in experiment one.

Experiment Three: Determination of the Influence of Water Deprivation

This experiment was designed to examine the role of water restriction and mild heat stress on the incidence of lasalocid-induced ataxia. In these studies, 5 week-old, male Cobb-cross broilers were divided into three groups of 5 birds each. The treatment groups were: no drug (control); 33.75 mg/kg lasalocid, t.i.d, + ad lib water; 33.75 mg/kg lasalocid, t.i.d. + 12 hour water restriction. Birds were housed in an environmental chamber where the ambient temperature cycled daily from a low of $25 \pm 1\ ^{0}C$ (0700 hrs) to a high of $33.5 \pm 1\ ^{0}C$ (1800 hrs) with a relative humidity maintained at 55 ± 5 %. Water was restricted for 12 hours (0800 - 2000) during the times the birds were exposed to elevated environmental temperatures. These environmental conditions mimic a mild, non-lethal heat stress typical

of a summer day in poultry producing areas of the country. After 2 days, the severity of ataxia was evaluated using the clinical ataxia scoring system.

Experiment Four: The Role of Vitamin E on Lasalocid-induced Ataxia.

Five week-old, male Cobb-cross broilers were divided into three groups of 5 birds each. The treatment groups were: no drug (control); 33.75 mg/kg lasalocid, t.i.d; and 33.75 mg/kg lasalocid, t.i.d. with Vitamin E (100 IU/kg). The Vitamin E was administered 45 - 60 minutes prior to dosing the bird with lasalocid. Ataxia was evaluated and severity was determined after 2 days. After neurotoxic assessment, birds were euthanitized and the sciatic nerves were removed to examine ATPase activity.

ATPase Isolation ATPase enzymes from sciatic nerves of broilers at the completion of the study prepared following the method of Hermenegildo (Hermenegildo et al.,1992). The sciatic nerves were rapidly removed after sacrificing the bird and washed several times with ice-cooled isotonic solution containing 0.25 *M* sucrose, 1.25 mM EGTA, 10 mM Tris hydrochloride, pH 7.5 with Trizma® base. A portion of the nerve was homogenized in 20 volumes of ice-cooled solution in a polytron homogenizer (Polytron® Model#PT 10/35, Brinkman Instruments, Switzerland) twice for 45 seconds. Aliquots of the homogenates were used immediately while replicates were frozen (- 80 °C) for later use. All above procedures were carried out at 2 °C.

Measurement of ATPase Activity ATPase activity was determined using the method of Chen (Chen et al, 1995) which is a slight modification of previous methods (Broekhuysen et al., 1972; Akera & Brody, 1971). The activity was measured in

triplicate for each bird in each treatment group. After the ATPase assay was stopped with TCA, the liberated inorganic phosphate in the supernatant was measured by adding 3 ml of 6 *N* H_2SO_4:water:2.5% ammonium molybdate:10% L-ascorbic acid (1:2:1:1) by the modified method of Fiske and SubbaRow (Fisk & SubbaRow, 1925). Absorbance was measured at 660 nm after 20 minutes at 37 °C. The Mg^{2+}-dependent ATPase activity was assessed in the absence of K^+ and Na^+. The Na^+,K^+-ATPase activity was calculated by subtracting Mg^{2+}-activated ATPase activity from total ATPase activity. Protein content of the enzyme was determined by bicinchoninic acid protein assay (Smith et al.,1985) in microplate with bovine serum albumin as the standard.

Experiment Five: The Effects of Lasalocid on Mitochondrial Respiration In Vitro

Liver mitochondria and submitochondria were isolated from fasted, 4-5 week old, male, Cobb-cross broilers following a previous method (Chen et al., 1988). The birds were decapitated by guillotine and the livers were immediately removed. The tissue was washed with ice-cooled 0.25 M sucrose- I mM Tris-I mM EDTA, pH 7.4. The livers were minced with scissors, and homogenized in 4 volumes of the above solution using a polytron homogenizer (Model PT 10/35, Brinkmann Instruments, Switzerland) for 30 seconds. The homogenate was centrifuged at 1,000 x g for 10 minutes. The supernatant was centrifuged at 15,000 x g for 15 minutes. Then the pellets were suspended in 0.25 *M* sucrose-5 mM Tris (pH 7.4) and centrifuged at 15,000 x g, for 15 minutes. The pellets were resuspended in the above solution and subjected to ultrasonification for 3 minutes (treated for 1 minute followed by a 2 minute pause for cooling down). The solution was centrifuged at 15,000 x g for 15 minutes to remove

unbroken mitochondria. The supernatant was centrifuged at 105,000 x g for I hr to obtain submitochondrial particles. The submitochondrial particles are stored at -60 °C until analysis. All procedures were carried out at below 4 °C.

Following isolation, the submitochondria were exposed to lasalocid and the activities of NADH oxidase, succinate oxidase and ATPase enzymes were determined in vitro. Lasalocid was dissolved and mixed in ethanol at concentrations of 1 - 400 μM. Control reactions, that contained the same volume of ethanol, were performed concurrently experimental groups.

Protein content of the submitochondria was determined by bicinchoninic acid protein assay (Smith et al.,1985) with bovine serum albumin as the standard.

NADP Oxidase Activity. The activity of NADH oxidase was measured via a polarographic technique with a Clark electrode using the following reaction medium (1.0ml total volume): 50 mM KH_2PO_4-NaOH buffer (pH 7.4); 100 μg cytochrome c; and 0.1-0.3 mg submitochondrial particles. The doses of lasalocid used in these studies were 0, 1, 2, 4, 6, 8 μM. The medium was incubated at room temperature (25± 1°C) for 5 minutes. Then the reaction was started by the addition of 1 mM NADH.

Succinate Oxidase Activity. The activity of succinate oxidase was measured via a polarographic technique with a Clark electrode using the following reaction medium (1.0 ml total volume): 50 mM KH_2PO_4-NaOH buffer (pH 7.4); 200 μg cytochrome c; and 0.1-0.3 mg submitochondrial particles. The doses of lasalocid used in these studies were 0, 50, 100, 200, 300, and 400 μM. The reaction was started by the addition of 30 mM sodium succinate.

Submitochondrial ATPase Activity. ATPase activity was measured using the following medium (1ml total volume): 10 mM Tris-HCl (pH 7.8); 2 mM $MgCl_2$; and 50 - 100 μg submitochondrial protein. The doses of lasalocid used in these studies were 0, 25, 50, 100, 150 200 μM. After incubation at 37 °C for 5 minutes, the reaction was started with the addition of 3mM ATP. The reaction proceeded for 10 minutes at 37 °C and terminated with 1 ml 15% trichloroacetic acid. After centrifugation, the liberated inorganic phosphate was measured using a previous method (Chen et al, 1992).

Experiment Six: Direct Effects of Lasalocid on the Sciatic Nerve In Vitro

Two, 5 week-old, male, Cobb-cross broilers were euthanitized by decapitation after a 1 week acclimatization period. The right and left sciatic nerves were rapidly removed from 2-5 mm distal to the greater trochanter of the femur to 2-5 mm proximal to the stifle joint. The nerves were cut into 3 section, approximately 15 mm long, 2 mm wide and 1 mm thick. The segments were randomly assigned to an incubation treatment group.

The nerve segments were incubated at varying time periods (30, 60 or 120 min.) at 37° C in an oxygenated (95% O_2: 5% CO_2), complete ringer's solution with or without the addition of 100 μM lasalocid. Additional treatment groups used a calcium-free ringer's solution with EGTA (to chelate Ca^{++}) with or without the ionophore. Lasalocid was dissolved and mixed in 100% DMSO to make a 2mM stock solution. This stock solution was added to the complete or calcium-free ringer's solution immediately before the incubation to give a final concentration of 100 μM lasalocid. Any solution that appeared milky or cloudy was discarded. The greatest volume of DMSO that was added to any ringer's solution was 5%. All control incubations contained 5% DMSO to exclude a DMSO effect. The complete

ringer's solution contained: NaCl (860 mg/100 ml), KCl (30 mg/100 ml) and $CaCl_2$ (33 mg/100 ml). The calcium-free ringer's contained the same concentrations of NaCl and KCl without any $CaCl_2$, additionally, 1 mM EGTA was added to chelate any residual calcium. All incubation solutions contained phenol red (1:2000) to assess any pH changes that might occur during the incubation period. Any solution that turned yellow (indicating an acidic pH) during the incubation period was discarded.

There were 12 different treatment groups: 1) complete ringer's, 30 min., 2) complete ringer's + lasalocid, 30 min., 3) calcium-free ringer's, 30 min., 4) calcium-free ringer's + lasalocid, 30 min., 5) complete ringer's, 60 min., 6) complete ringer's + lasalocid, 60 min., 7) calcium-free ringer's, 60 min., 8) calcium-free ringer's + lasalocid, 60 min., 9) complete ringer's, 120 min., 10) complete ringer's + lasalocid, 120 min., 11) calcium-free ringer's, 120 min., 12) calcium-free ringer's + lasalocid, 120 min. After incubation for the prescribed times, the nerve segments were fixed and processed for electron microscopy.

Electron Microscopy Following incubation, nerve segments were immediately removed and fixed in buffered gluteraldehyde-sodium cacodylate solution for 2 hours. Post-fixation, the tissues were placed in a 1:1 solution of 2% osmium and 0.27 M cacodylate buffer for 2 hours. Segments of sciatic nerve were washed in a 1 M sodium cacodylate solution. After rinsing, tissues were dehydrated with a series of ethanol and propylene oxide rinses. The tissues were embedded in Polybed® and placed in an oven.

Blocks of resin containing sciatic nerve segments were thick sectioned with a glass knife to approximately 550 microns. Areas of each block containing nerve tissue were then chosen for thin sectioning. Thin sections were mounted on 200 mesh copper grids and stained with uranyl acetate and lead citrate. Grids were examined

using a JEOL 100 CX transmission electron microscope at 80 KV. Areas of normal and abnormal appearing tissue were photographed for more detailed analysis.

Statistical Analysis of the Data

All data was initially examined by analysis of variance (ANOVA) with a general linear model (GLM) using SASS. Mean ± standard deviation for ATPase activity were calculated for each group and compared to controls using a Dunnett's test. Using a clinical scoring system to describe ataxia gives ordinal discrete data. These data are nonparametric and as such can be described by frequency distributions, median or rank transformations. Parametric test (mean +/- s.d.) are not appropriate for this data. Median ataxia score are presented for these studies with rank tests (Kruskal-Wallis or median test) used to measure differences between groups at the $P < 0.05$ level. For the frequency graphs, birds were classified as overtly ataxic if they had a clinical score of 3,4 or 5.

Results

Oral Dose-Response Characteristics

Preliminary studies using 2.5 to 10 mg/kg lasalocid, p.o., t.i.d. failed to produce any consistent, dose-dependent ataxia (data not shown). Dose-dependent neurotoxic effects were consistently noted with doses ranging from 11.25 to 50 mg/kg produced ataxia. The incidence of overt ataxia, with a clinical ataxia score greater than 3, was 10% at 11.25 mg/kg, 30 % at 22.5 mg/kg, 73% at 33.75 mg/kg and 100% at 50 mg/kg (Figure 1). The severity of ataxia was also dose related as the median ataxia score

increased with higher doses of lasalocid. The median ataxia score was 0 for 11.25 mg/kg, 1.0 for 22.5 mg/kg, 3.0 for 33.75 mg/kg and 4.0 for 50 mg/kg (Figure 3).

The neurotoxic effects of lasalocid noted in these studies are ataxia, flaccid or floppy paresis/paralysis of the pelvic limbs. Extensor function is more severely affected as the joints of the pelvic limbs collapse as the syndrome worsens. The "duck walking" birds, clinical score 3, ambulate by abducting and adducting the hip without extending the stifle or tibiotarsal-tarsometatarsal joint giving the broiler a "waddling" gait. Birds with a clinical score of 4 or 5 are non-ambulatory, even with vigorous encouragement.

The onset of overt ataxia (clinical score 3- 5) in birds dosed with greater than 22.5 mg/kg lasalocid, t.i.d. occurs within 48 hours. This is more rapid than field reports and feeding trials (Roder et al, 1996), but these doses exceed the daily intake for a bird consuming a ration containing 220 ppm lasalocid.

The clinical neurologic examination results of these studies mirror the findings of the feeding trial (Roder et al, 1996), indicating a lesion in the peripheral nervous system. Birds with a clinical ataxia score of 2-4 had normal visual menace, pupillary light reflex, mandibular tone, palpebral reflex, hearing, vestibular reflexes, and gag reflex (swallowing). Lesioned birds with an ataxia score of 5 were too depressed to assess function of most cranial nerves, yet the birds maintained the ability to swallow until approximately 12 hours before death. The spinal reflexes (righting reflex, proprioreceptive placing, superficial and deep pain) of birds with ataxia scores of 1 or 2 were the identical to controls (no drug). At a clinical score of 3, the birds were still able to right themselves, but with greater difficulty and still had normal responses to deep and superficial pain. These "duck-walking" birds also had diminished proprioreceptive responses. When the birds reached a clinical score of 4, most were unable to right themselves, but still attempted, while the birds with a score of 5 would make only feeble

attempts. Birds with a clinical score of 4 had varied responses to deep and superficial pain, while the birds with a score of 5 usually did not respond to painful stimuli.

There was no mortality in birds receiving 33.75 mg/kg or less, t.i.d. for the 2 day experiments. Birds dosed with 50 mg/kg lasalocid, t.i.d. had a significantly higher (30%) mortality during the 2 day dosing period (data not presented). This may represent the upper level of the range of doses that can elicit neurotoxicity in this acute exposure model.

Recovery Studies

The recovery studies show that over a period of 10 days, ataxic birds given 11.25, 22.5 or 33.75 mg/kg exhibit clinical recovery. Birds dosed with 11.25 mg/kg had an initial median ataxia score of 2.5 while birds given 33.75 mg/kg had a median score of 3.0. Seven days after the last dose of lasalocid, the median ataxia score was the same (2.5) in the 11.25 mg/kg group and lower (1.0) for the 22.5 and 33.75 mg/kg groups. After 10 days the median ataxia scores were 1.5 (11.25 mg/kg) and 1.0 (22.5 and 33.75 mg/kg) indicating recovery (Figure 4).

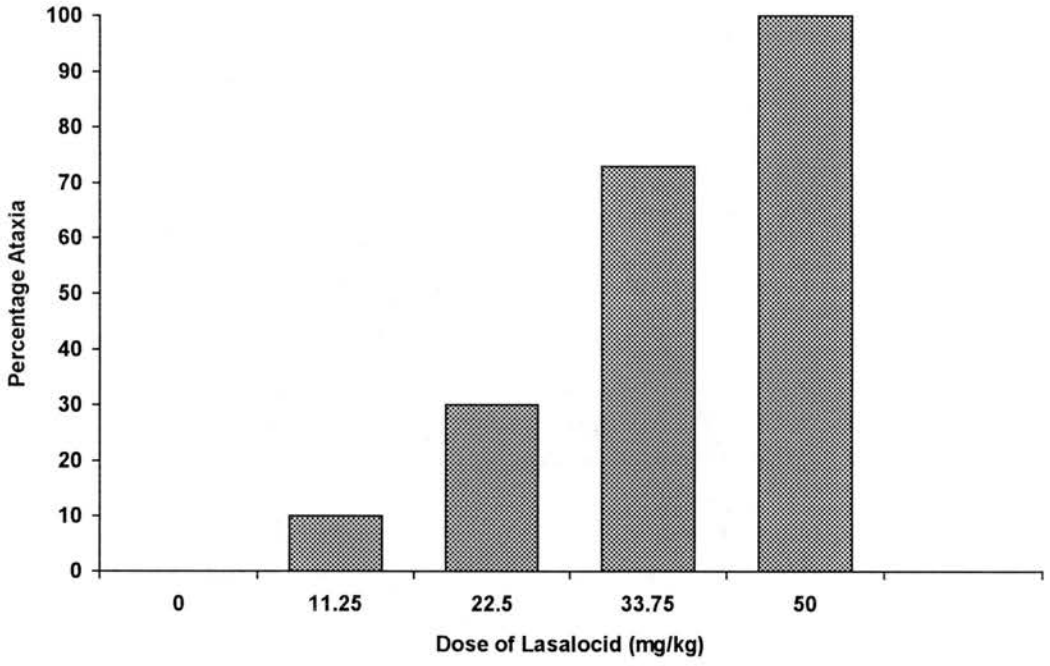

Figure 1. Dose-Dependent Ataxia in 5 Week, Male, Cobb-cross Broiler Chickens Given Various Oral Doses of Lasalocid, t.i.d. for 2 Days.

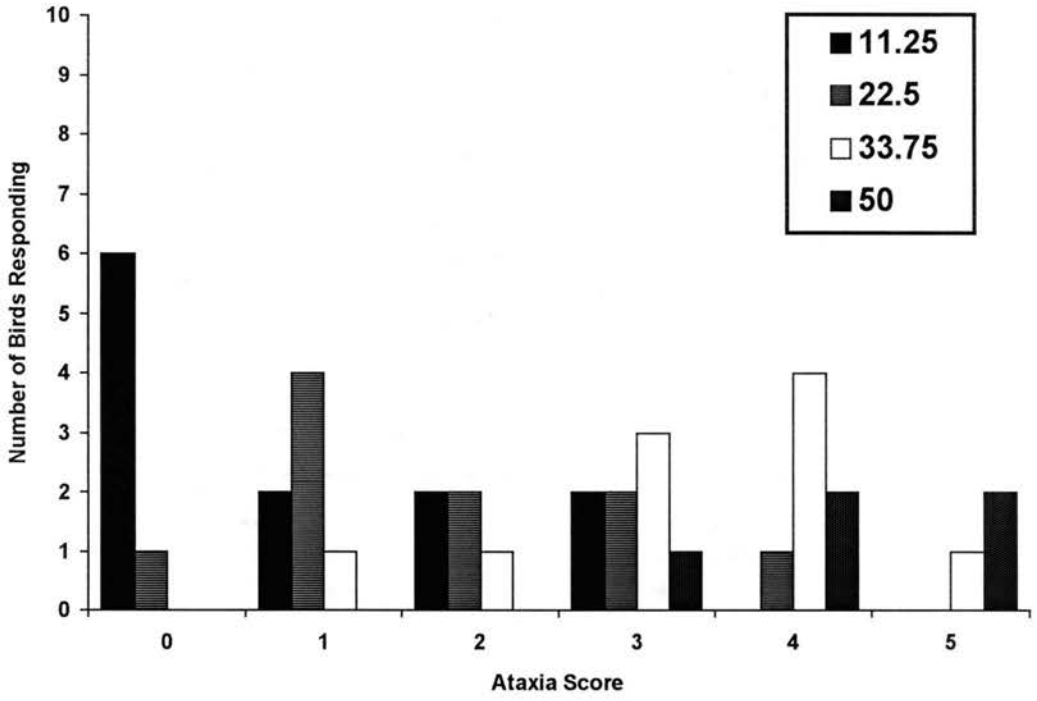

Figure 2. Frequency Distribution of Birds Exhibiting Ataxia after Oral Dosing with Various Doses of Lasalocid, t.i.d., for 2 days.

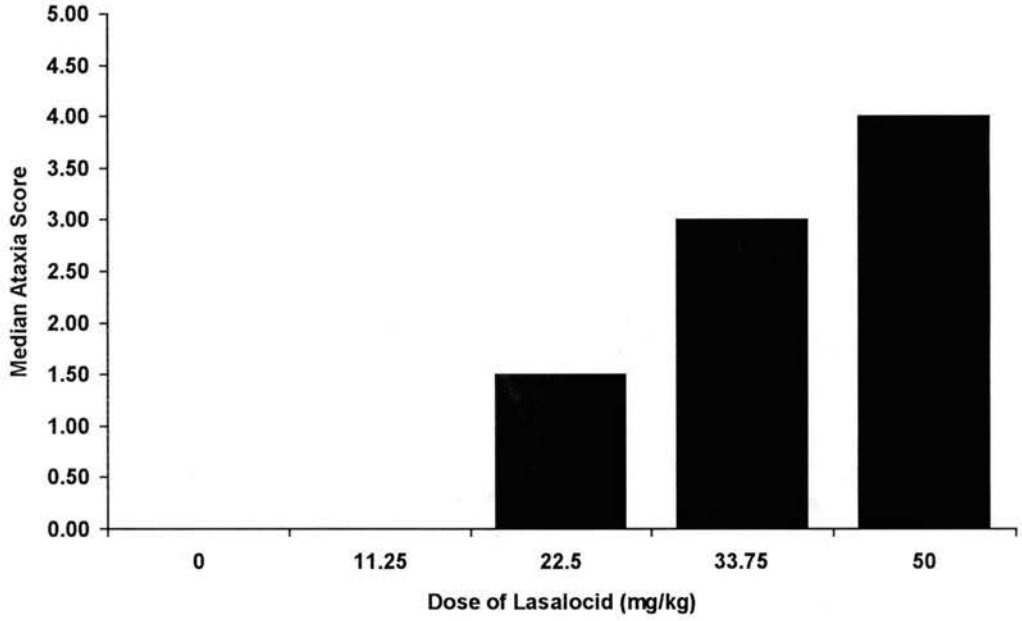

Figure 3. Median Ataxia Score for 5 Week, male, Cobb-cross Broilers Given Various Oral Doses of Lasalocid, t.i.d. for 2 days.

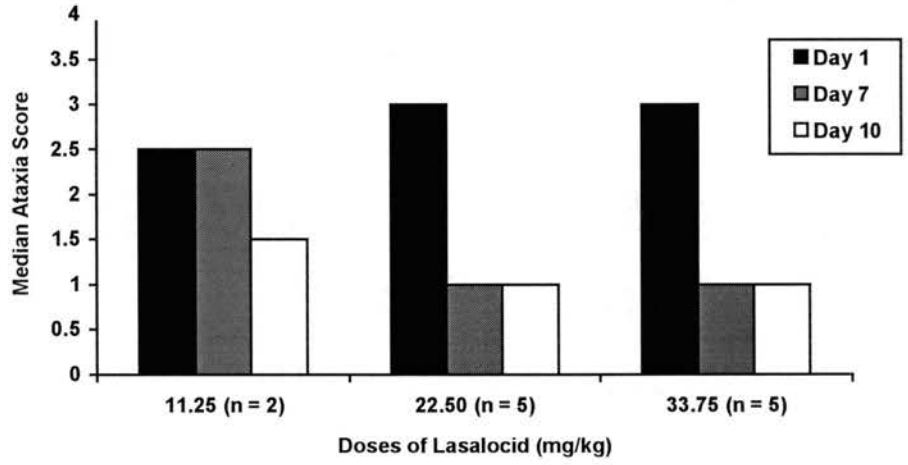

Figure 4. Median Ataxia Score for 5 Week, Male, Cobb-cross Broilers Given Various Oral Doses of Lasalocid, t.i.d. for 2 days and Allowed to Recover (fed Drug Free Ration) for 10 Days.

Effects of Water Deprivation on Lasalocid-induced Ataxia

Birds given 33.75 mg/kg lasalocid, t.i.d., for 2 days had a median ataxia score of 2.5. A 12 hour/day water deprivation + lasalocid 33.75 mg/kg, p.o., t.i.d. resulted in a median ataxia score of 2.5 which is not statistically different from birds with ad lib exposure to water (Figure 5). The most notable difference between the treatment groups in this experiment was onset of clinical signs . Birds deprived of water attained their final ataxia scores by 24 hours while birds with ad lib exposure water attained their final ataxia score between 40 and 48 hours.

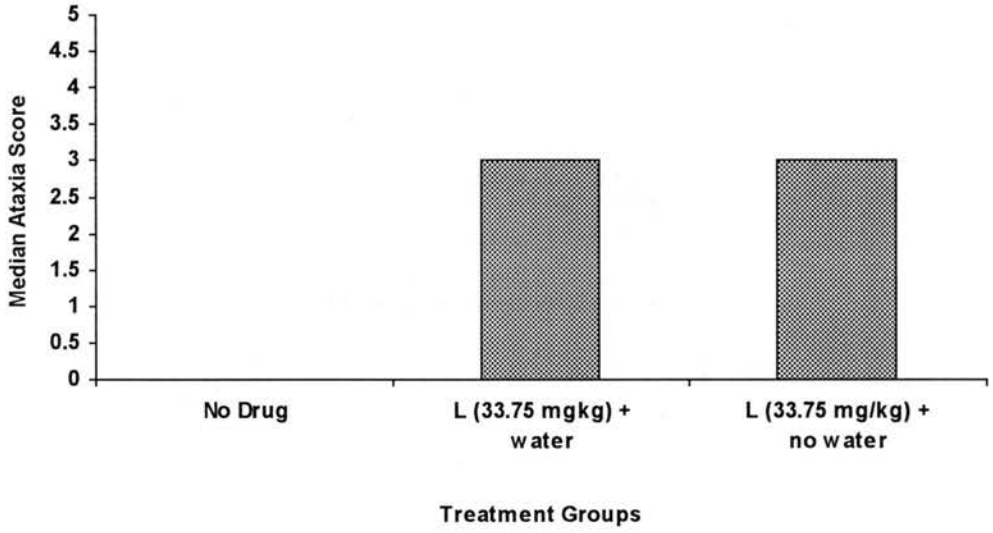

Figure 5. Median Ataxia Score for 5 Week, Male, Cobb-cross Broilers Given: no Drug, Lasalocid (33.75 mg/kg, t.i.d.) + Water, or Lasalocid (33.75 mg/kg, t.i.d.) + 12 Hour Water Deprivation. Score is Median of 5 birds

Effects of Vitamin E Pretreatment on Lasalocid-Induced Ataxia

Pretreatment with 100 IU Vitamin E had no effect on the median ataxia score of lasalocid dosed broilers. Birds given 33.75 mg/kg lasalocid orally had a median ataxia

score of 3.0 as did broilers given 100 IU Vitamin E /kg + 33.75 mg/kg lasalocid (Figure 6). The ATPase activity of sciatic nerve homogenates showed a similar trend. The specific Na^{+}, K^{+}-ATPase activity (mean ± s.d.) for nerve homogenates was 61.4 ± 35.2 (no drug), 40.9 ± 27.8 (33.75 mg/kg lasalocid), 84.3 ± 23.7 (33.75 mg/kg lasalocid + Vitamin E). The specific Mg^{++}-ATPase activity (mean ± s.d.) for nerve homogenates was 339.1 ± 115.7 (no drug), 271.1 ± 58.1 (33.75 mg/kg lasalocid), 282.9 ± 14.7 (33.75 mg/kg lasalocid + Vitamin E). The specific Total -ATPase activity (mean ± s.d.) for nerve homogenates was 280.2 ± 62.2 (no drug), 282.5 ± 15.9 (33.75 mg/kg lasalocid), 313.3 ± 81.3 (33.75 mg/kg lasalocid + Vitamin E) (Table II). Replication of the measurement of ATPase activity using frozen aliquots of sciatic nerve homogenates gave similar results (data not shown).

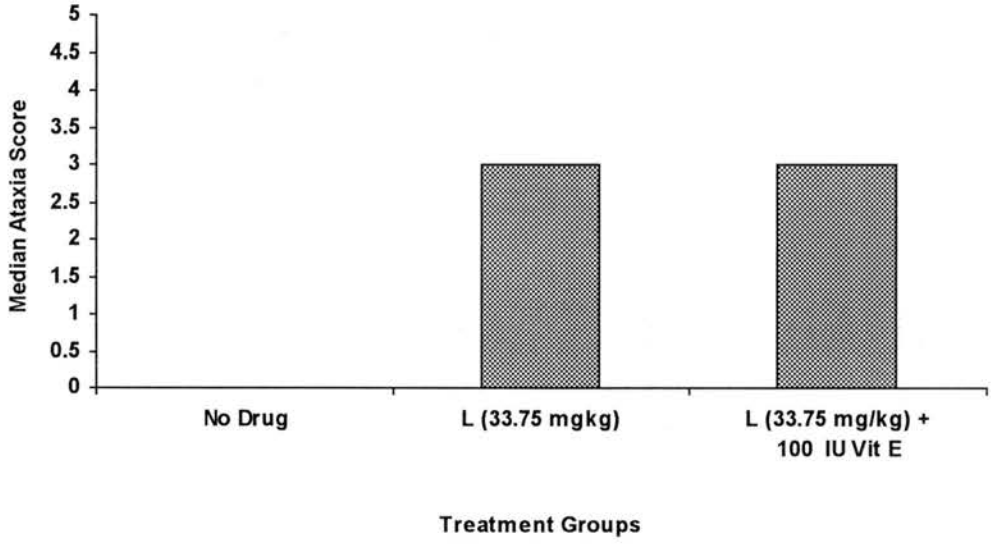

Figure 6. Median Ataxia Score for 5 Week, Male, Cobb-cross Broilers Given: no Drug , Lasalocid (33.75 mg/kg, t.i.d.), or Lasalocid (33.75 mg/kg, t.i.d.) + Vitamin E (100 IU/kg). Score is the Median of 5 Birds.

TABLE II

SCIATIC NERVE HOMOGENATE ATPASE ACTIVITY FROM 5 WEEK, MALE COBB-CROSS BROILER CHICKENS GIVEN NO DRUG, LASALOCID (33.75 MG/KG, T.I.D.) OR LASALOCID + VITAMIN E (100 IU/KG) PRETREATMENT.

Specific ATPase Activity (mM Pi/mg protein/min) Mean +/- s.d.			
Treatment	Na+,K+-ATPase	Mg++-ATPase	Total ATPase
Control (no drugs)	61.4 (35.2)	339.1 (115.7)	280.2 (62.2)
Lasalocid	40.9 (27.8)	271.1 (58.1)	282.5 (15.9)
Lasalocid + Vitamin E	84.3 (23.7)	282.9 (14.7)	313.3 (81.3)

Effect of Lasalocid on Submitochondrial Enzymes

Lasalocid shows a dose-dependent inhibition of hepatic submitochondrial NADH Oxidase specific activity from 5 week old broilers. The IC_{50} for lasalocid on NADH oxidase activity in vitro was 2.90 μM. The doses of lasalocid used with the resulting percent inhibition (mean ± s.d.) were: 1μM (9 ± 12 %); 2 μM (43 ± 3 %); 4μM (53 ± 12 %); 6 μM (57 ± 9 %); and 8 μM (58 ± 12 %) (Figure 7).

Lasalocid exhibited a dose-related inhibition of submitochondrial ATPase activity in vitro. The concentration of lasalocid and the percentage inhibition of ATPase were : 25 μM:7.1 ± 12.6%; 50 μM: 14.2 ± 8.1; 100 μM: 12.0 ± 16.9 %; 150 μM: 28.4 ± 6.15; 200 μM: 26.5 ± 0.64 (Figure 8).

Lasalocid did not show any dose-related inhibition of hepatic submitochondrial succinate oxidase activity. At 50 μM, lasalocid stimulated (10%) enzyme activity, while

100 μM, 200 μM and 300 μM were essentially the same as control (no drug). At a concentration of 400 μM succinate oxidase activity was inhibited 33% (Table III).

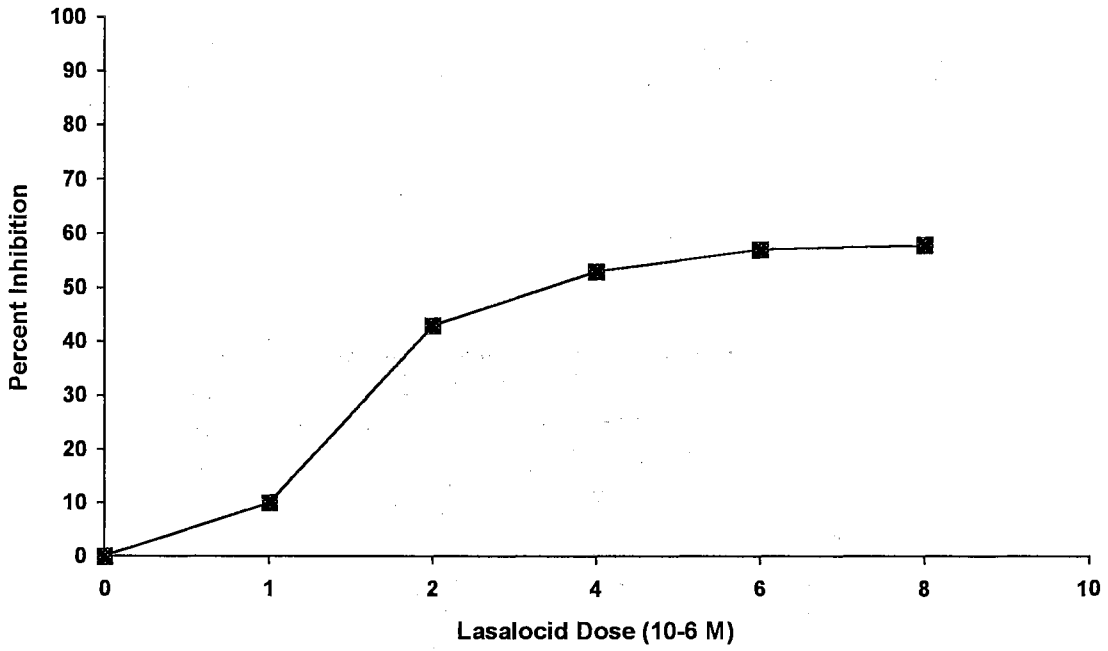

Figure 7. Effect of Lasalocid (μM) on Submitochondrial NADH Oxidase Activity of Livers from Cobb-cross Broiler Chickens

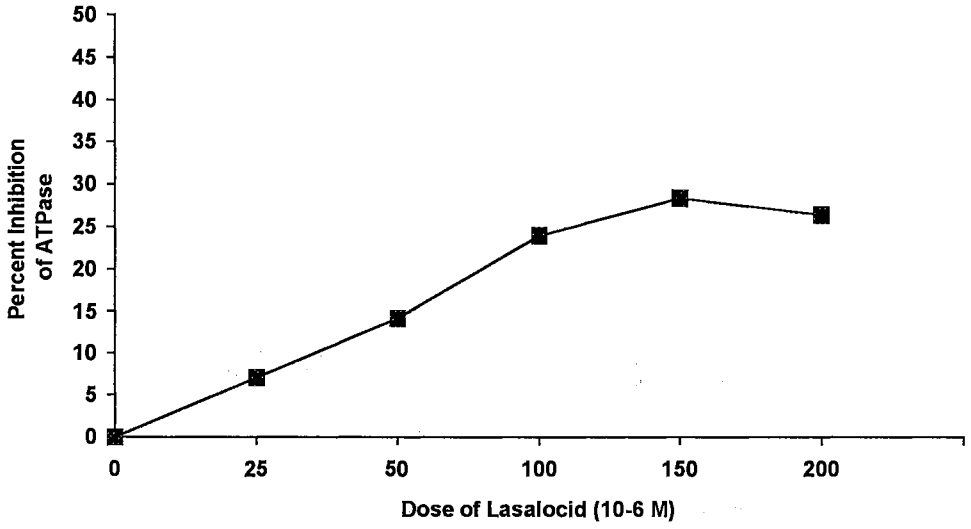

Figure 8. Effect of Lasalocid (μM) on Submitochondrial ATPase Activity from Livers of Cobb-cross Broiler Chickens

TABLE III

EFFECTS OF LASALOCID (μM) ON HEPATIC SUBMITOCHONDRIAL SUCCINATE OXIDASE ACTIVITY

Dose of Lasalocid (μM)	Percent Inhibition
0	--
50	-10
100	3
200	0
300	3
400	32

Effects of Direct Lasalocid Exposure on the Sciatic Nerve In Vitro

Examination of the control nerve fragments that were incubated in complete Ringer's solution without ionophore at the earliest time period (30 minutes) showed little or no

damage to the ultrastructure of the axon, myelin or Schwann cell (Figure 9). The neurofilaments and microtubules in the axon were homogeneous with a granular appearance. In most of the sections observed the axolemma was adhered to the Schwann cell membranes. The Schwann cells had normal ultrastructure and the lamella of myelin were concentric and compact. Segments incubated in complete media with lasalocid for 30 minutes were very similar to controls (Figure 10).

Nerve segments exposed to 100μM lasalocid and calcium-containing Ringer's for 60 minutes had more extensive damage to the myelin and Schwann cells. Many axons had early and reversible, indications of cell damage. Media with calcium and lasalocid had more pronounced changes, swelling of Schwann cells, some areas intramyelinic edema with resulting compression of the axolemma (Figures 11 & 12). Membrane-lined vacuoles are noted within Schwann cells.

Nerve segments incubated for 120 minutes in calcium-containing Ringer's solution without the addition of lasalocid had only slight ultrastructural changes. There is some mild intramyelinic edema and vacuoles in the Schwann cell cytoplasm (Figure 13). In sections of nerve incubated with lasalocid (100 μM) and calcium for 120 minutes there was marked ultrastructural changes, especially to the Schwann cell cytoplasm and myelin. These sections exhibit severe disruptions of the myelin, intramyelinic edema resulting in compression of axonal cytoplasm (Figure 14). The neurofilaments of some axons were dispersed and unorganized in the ionophore and calcium -treated nerve segments, while others appeared normal.

An interesting observation was made during tissue processing. The electron microscopist, blinded to treatments, noted that segments incubated for 120 minutes with lasalocid and calcium were very difficult to section. The ultrastructure of the nerves from these blocks was difficult to interpret , especially the longitudinal sections. Transverse sections of these tissues had many artifacts, tears and rips in the Polybed®.

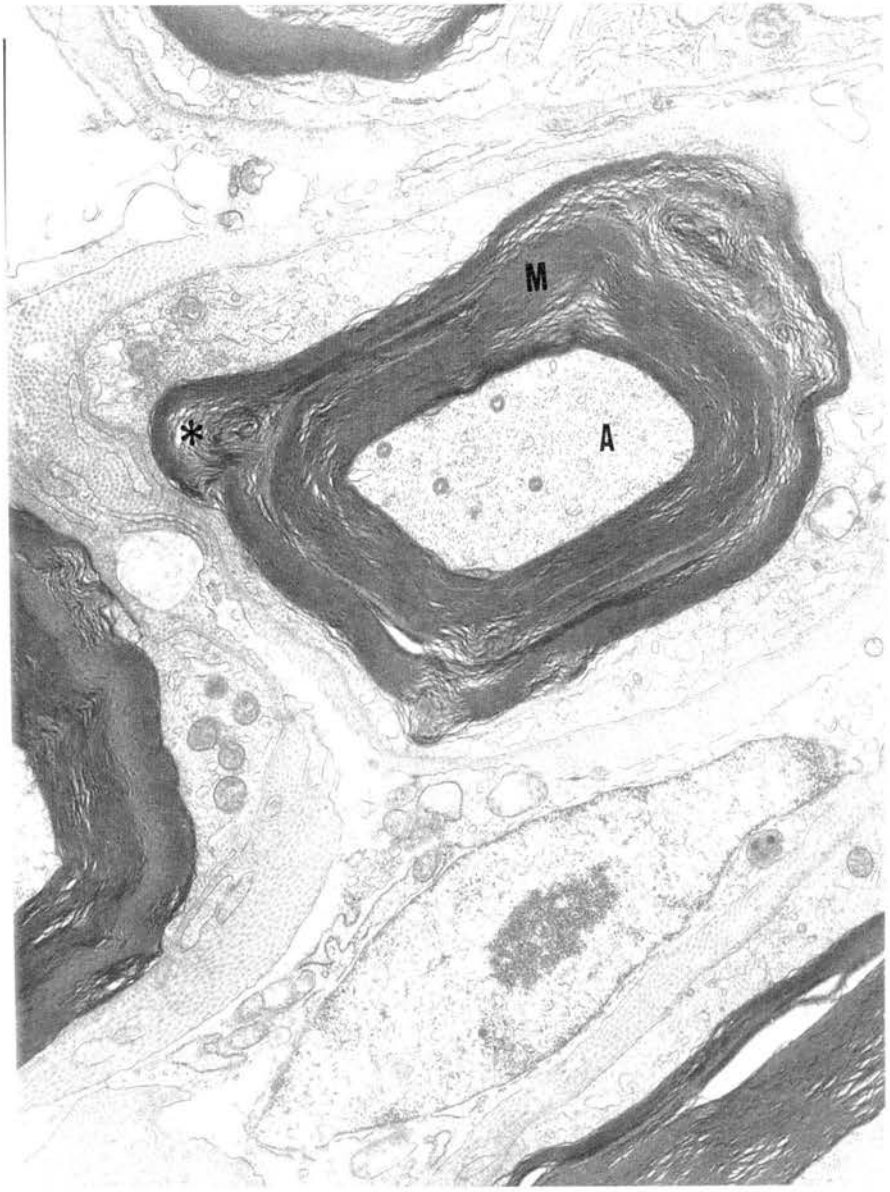

Figure 9. Electron Micrograph of a Sciatic Nerve Incubated *In vitro* in Complete Ringer's Solution for 30 Minutes. Note the Intact Myelin Sheath (M) with Concentric and Compressed Laminae with a Normal Appearing Axon (A). There is an Artifact (*) in the Myelin. (Magnification 7200)

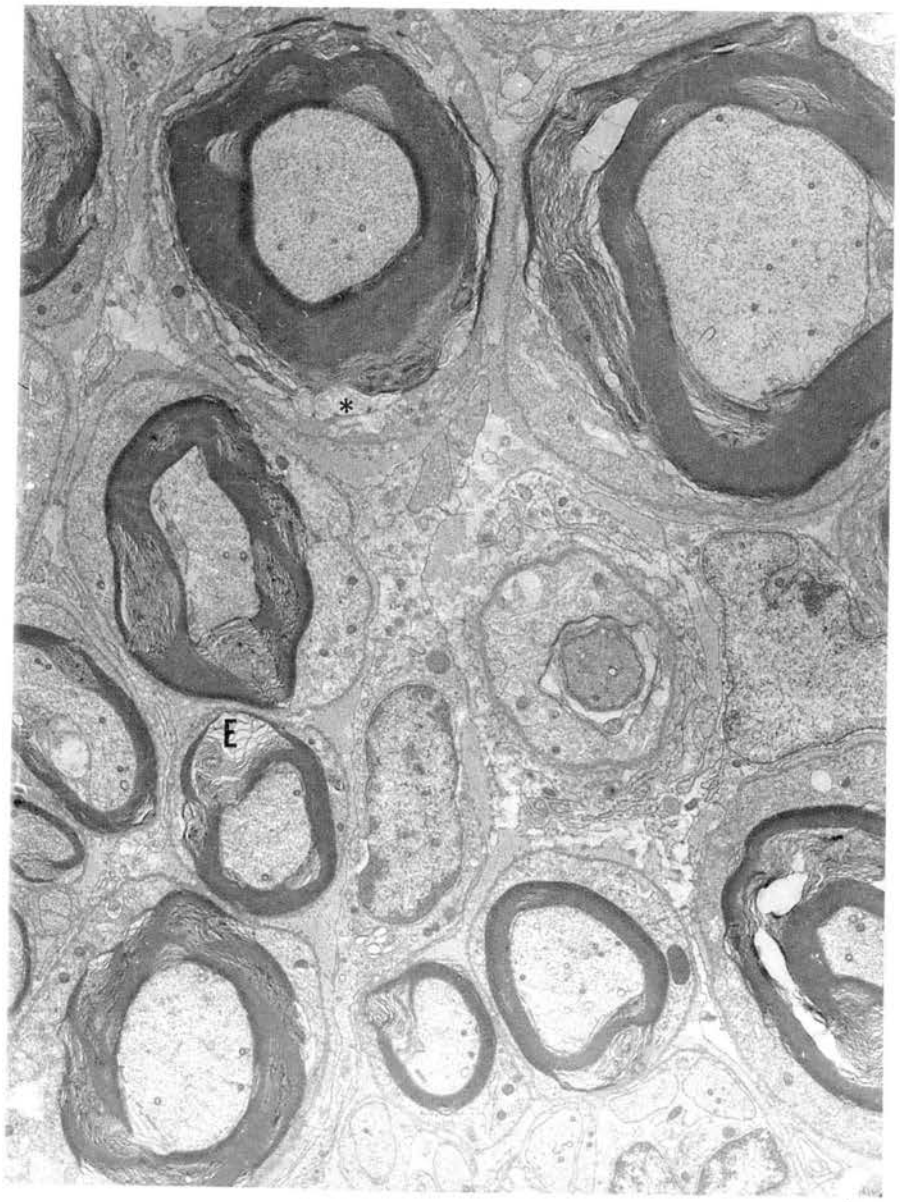

Figure 10. Electron Micrograph of a Sciatic Nerve Incubated *In vitro* in Complete Ringer's Solution with Lasalocid (100 μM) for 30 Minutes. Note the Early Damage to the Myelin as Evidenced by Intramyelinic Edema (E) and Vesiculation at the Myelin - Schwann Cell Interface (*). The Axons Appear Normal. (Magnification 3600)

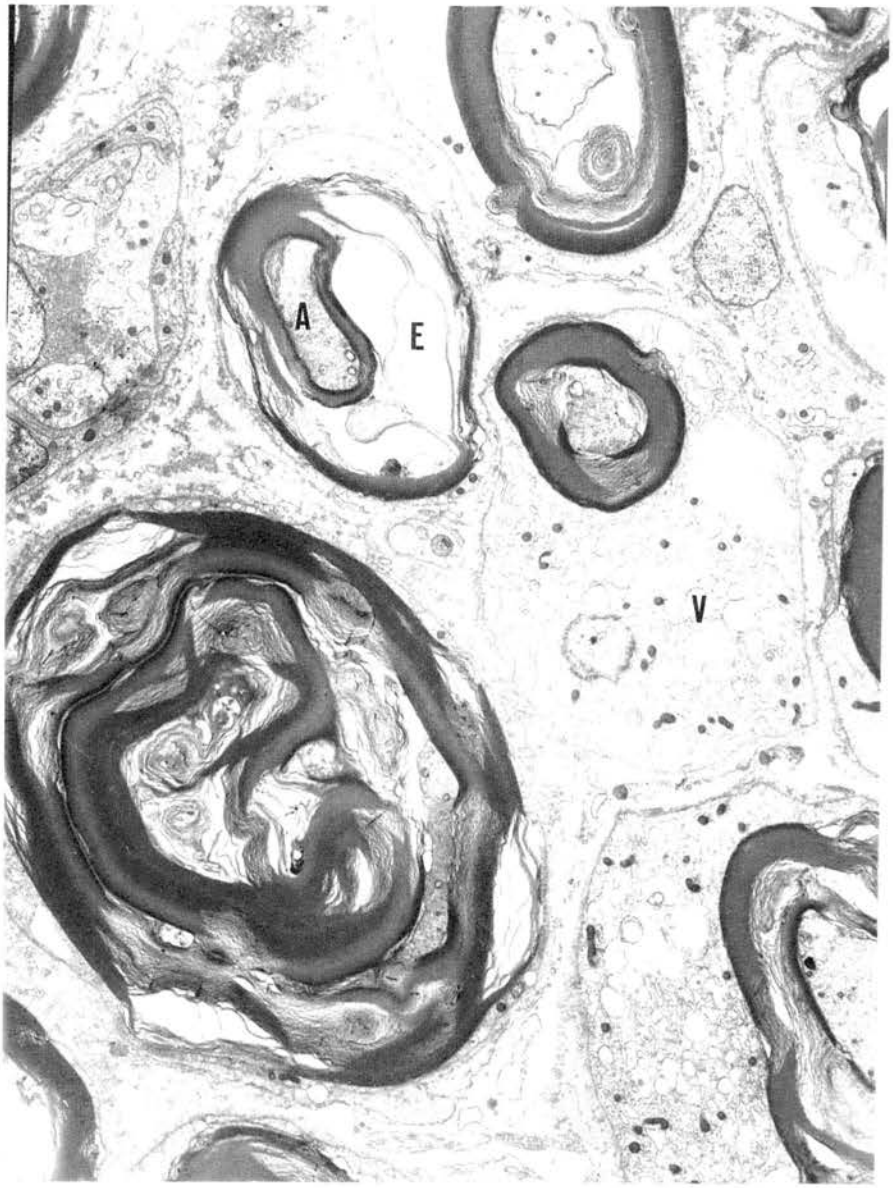

Figure 11. Electron Micrograph of a Sciatic Nerve Incubated *In vitro* in Complete Ringer's Solution with Lasalocid (100 μM) for 60 Minutes. Note the Severe Damage to the Myelin, Intramyelinic Edema (E), Vacuolation (V) of the Schwann Cell Cytoplasm, Degredation of the Myelin and Compression of Axons. (Magnification 2900)

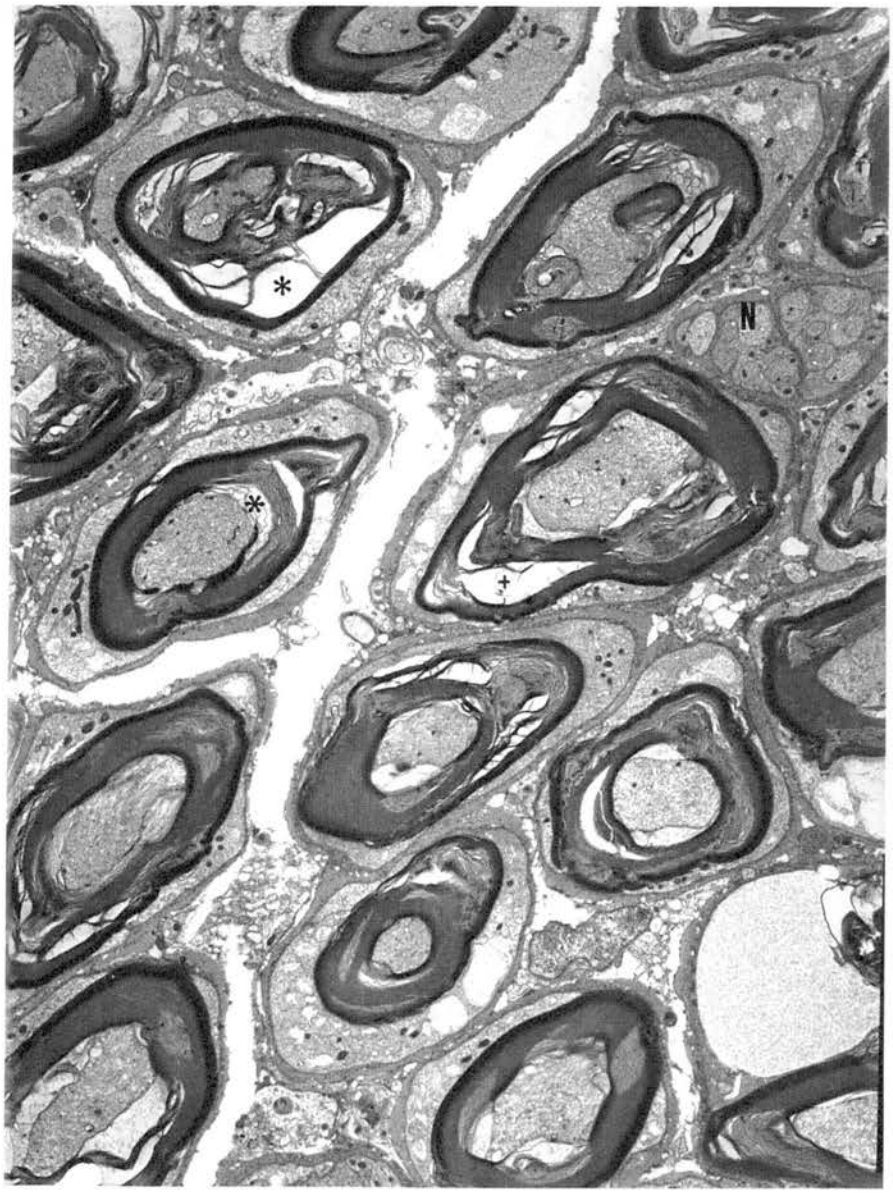

Figure 12. Electron Micrograph of a Sciatic Nerve Incubated *In vitro* in Complete Ringer's Solution with Lasalocid (100 μM) for 60 Minutes. Note the Severe Damage to the Myelin, Intramyelinic Edema (*), and Vacuolation of the Schwann cell Cytoplasm. Note the Collection of Non-myelinated Nerves (N) not Affected by Treatment. (+) is a tear in the Polybed. (Magnification 1900)

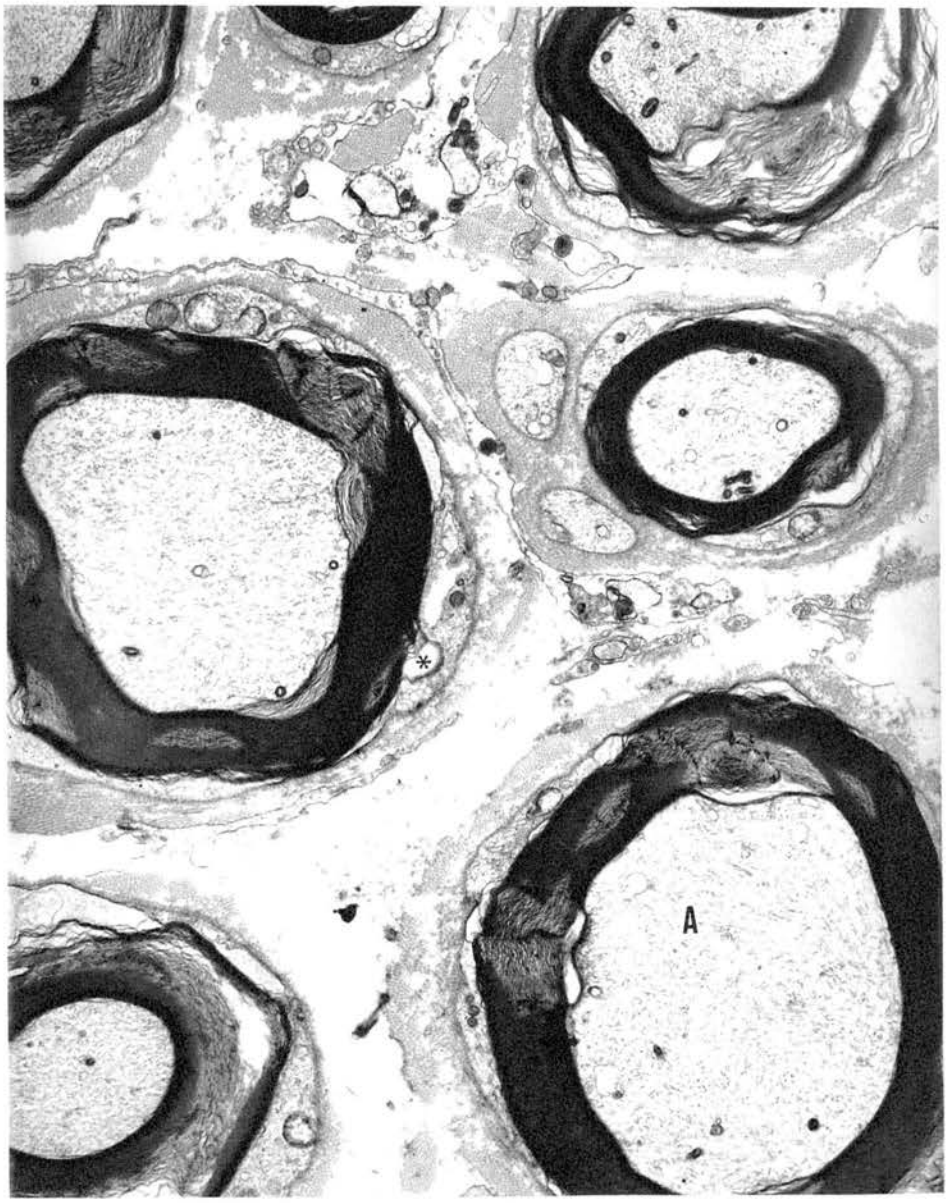

Figure 13. Electron Micrograph of a Sciatic Nerve Incubated *In vitro* in Complete Ringer's Solution without Lasalocid for 120 Minutes. Note the Mild Changes in the Myelin with Some Early Edema, Vacuoles and Vesicles (*) and Swollen Mitochondria in the Cytoplasm of Schwann Cells. The Axons (A) are Not Compressed. (Magnification 2900)

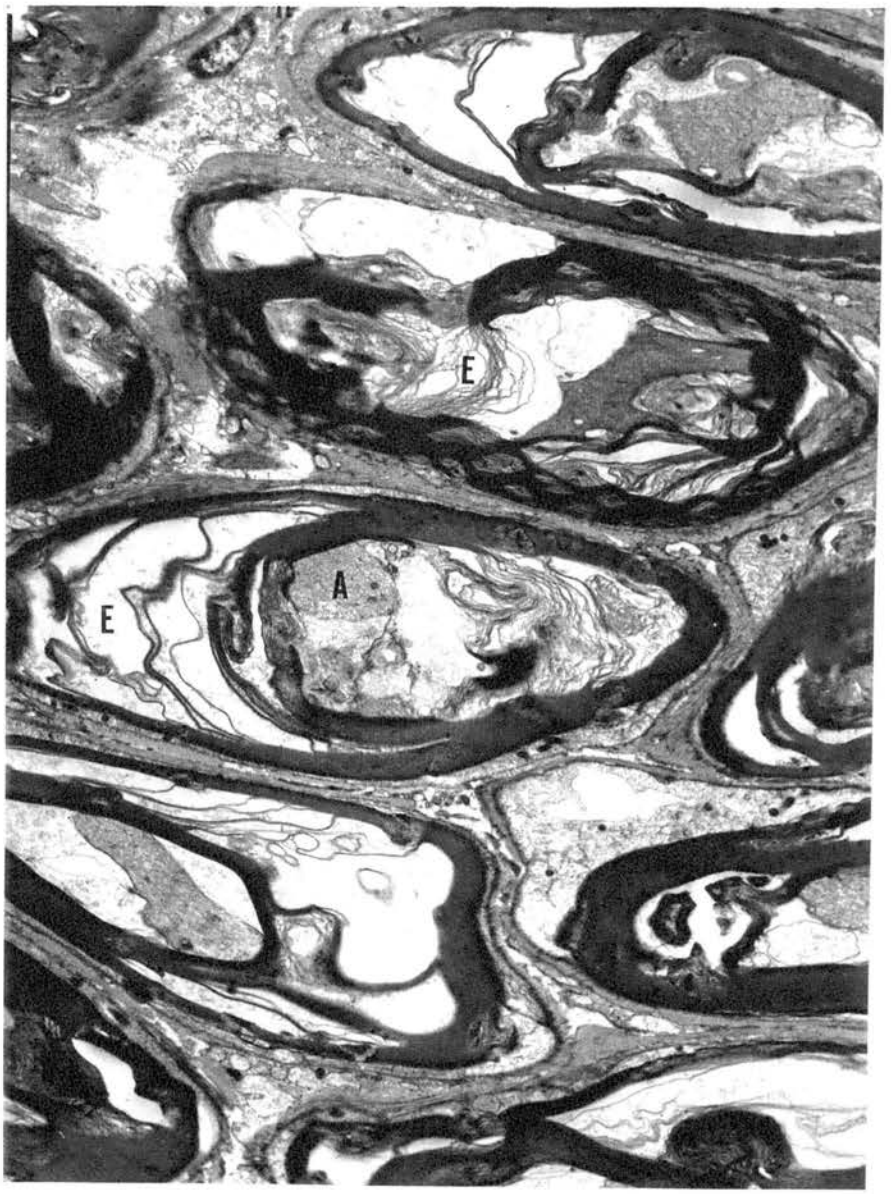

Figure 14. Electron Micrograph of a Sciatic Nerve Incubated *In vitro* in Complete Ringer's Solution with Lasalocid (100 μM) for 120Minutes. Note Severe Damage to the Myelin, Extensive Intramyelinic Edema (E), Vacuolation and Degredation of the Myelin with Compression of Axons (A). (Magnification 2900)

Discussion

Oral administration of lasalocid at doses ranging from 11.25 - 50 mg/kg, t.i.d. cause a dose-dependent neurotoxicity in broiler chickens. The neurotoxicity primarily affects the peripheral nervous system and is adequately assessed by means of a clinical ataxia scoring system. The experimentally-induced toxicity in these studies mimics the field case reports and the previous feeding trials (Roder et al., 1996) in clinical description, time of onset and severity. These studies show that providing lasalocid *per os* by means of gelatin capsules can serve as a reliable model of chemically-induced peripheral neuropathy. The short treatment period (2 days), high incidence (>95 % at doses exceeding 30 mg/kg) and low mortality (<5%) provide sufficient number of affected animals for use in studies examining the possible pathophysiologic mechanisms. This experimental paradigm is much easier to execute, more cost-effective and less laborious than feeding trials.

The ataxia scoring system used in these studies is similar to other investigators (Abou-Donia et al., 1978; Abou-Donia, 1977; Abou-Donia et al.,1980; Dyer et al.,1992; el-Fawal et al, 1990a,b) and effectively describes lasalocid-induced neurotoxicity by *per os* dosing. The scoring system is sensitive enough to follow the progression and recovery of an affected bird. Similar to the scoring system used by Abou-Donia et al for OPIDN, this system forces the birds to walk to increase the probability of detecting gait abnormalities. This is essential in detecting the birds that have less severe lesions that are more prominent upon exercise with many affected birds appearing normal at rest.

The assessment of neurotoxicity by means of clinical signs and neurologic examinations suggests damage to the peripheral nervous system. The central nervous system functions seem to remain intact in the lesioned birds. Affected birds proceed through a progression of clinical signs that preclude the "duck-walking" and the "downer" birds.

Affected birds in the earlier stages (Clinical Severity Scores 1 or 2) are alert, able to eat and drink. As the neurotoxicity progresses and the clinical severity score increases to 3 or 4 the birds become increasingly ataxic and are unable to walk to feed and water. In the last stage of intoxication (Clinical Severity Score 5), birds are depressed and lethargic, refuse to eat and frequently die

The effects of withholding water for 12 hours on the incidence and severity of lasalocid-induced neurotoxicity were subtle. There is no statistically significant difference in the median severity score of ad lib and water withheld birds at the 48 hour ataxia assessment period. Empirical observations suggest that the onset of ataxia may be more rapid in the birds deprived of water for 12 hours/day during the treatment period. The birds in this group reached their final ataxia score by 24 hours, while those that had access to water did not develop ataxia until 36-48 hours into the treatment period. Birds exposed to water deprivation and heat stress usually have hypernatremia. These birds may exhibit signs of ataxia more rapidly than broilers under more ideal management conditions.

These experiments suggest that oxidative stress and protection by Vitamin E is not involved in the pathogenesis of lasalocid-induced ataxia. This is different from the studies by VanVleet that describe a protective role for Vitamin E in the pathogenesis of monensin-induced myopathy in pigs (VanVleet et al, 1987). The addition of Vitamin E (100 IU/kg/day) also had no effect on sciatic homogenate ATPase activity .

Alternatively, the dose of Vitamin E (100 IU) or the frequency of administration may not be adequate to protect against lasalocid-mediated damage.

Inhibition or reduction of sciatic nerve ATPase does not appear to be involved in the pathogenesis of lasalocid-induced ataxia. These results agree with findings in streptozocin-treated diabetic Sprague-Dawley rats (LoPachin et al.,1993) and diabetic mutant C57Bl/Ks mice (Bianchi et al.,1987). In these studies peripheral neuropathy and axonal ionic changes (LoPachin et al.,1993) were not explained by changes in ATPase function. In contrast, the influence of lasalocid on sciatic nerve ATPase differs with previous reports of diabetes-induced peripheral neuropathy (Brismar & Sima, 1981; Llewelyn & Thomas, 1987; Greene et al, 1987; Hermenegildo et al.,1992). The findings of the current study might be explained by the different cell types affected in these syndromes. Diabetic neuropathy primarily affects the axon while lasalocid seems to first affect the myelin. There are Na+,K+-ATPase isoform differences between these cell types with α_2 more prevalent in glial cells and α_3 more commonly found in neurons. Additionally, there are species (dog, rat, human) differences in the abundance and importance of these isoforms of Na+,K+-ATPase (Decollogne et al.,1993). Another possible explanation for these results surround the complexity of intracellular regulation of ATPase activity *in vivo*. *In vitro* , lasalocid causes a dose-dependent inhibition of ATPase activity (Roder et al, 1996). In the intracellular environment Na+,K+-ATPase is subject to complex, highly regulated control mechanisms. Ionic regulation, *in vivo*, exerts a powerful control over these enzymes (McGeoch, 1990; Azuma et al.,1991; Rayson, 1990) . Increased intracellular levels of calcium drastically increase the function of the Na+,K+-ATPase, an expected result of introducing lasalocid into the biologic system. The elevated calcium increases ATPase

mRNA, enzyme synthetic rates and releases enzyme inhibition (Decollogne et al.,1993). This would help offset the ionophore-induced degradation of the ionic gradients, to maintain homeostasis. This may also explain differential effects on the ATPase enzyme *in vivo* and *in vitro*. Alternatively, lasalocid may have only an indirect effect on the function of the activity of the Na+,K+-ATPase. The ionophore may alter critical membrane-bound cations (Mg^{++}) that regulate the structure or activity of the enzyme (Antonio et al, 1991).

The inhibition of NADH oxidase and submitochondrial ATPase activity by lasalocid in chicken liver mitochondria suggest that the ionophore may alter intracellular energetics that may play a role in ataxia. Previous studies have shown lasalocid can inhibit mitochondrial energy coupled processes, even below the concentration necessary for cation transport (Lin et al, 1973). Lasalocid may depress the mitochondrial respiratory chain leading to decreased ATP production. The lowered intracellular ATP concentration would affect the higher metabolic tissues (nervous system) and diminish a myriad of intracellular functions. Previously, cisplatin nephrotoxicity has been shown to mediated by an early mitochondrial injury, causing depressed ATP, which precedes inhibition of Na+,K(+)-ATPase activity (Brady et, 1993). A similar process may be occurring in lasalocid-induced neurotoxicity in broilers.

Direct lasalocid exposure can produce time- and calcium- dependent ultrastructural damage similar to that found in ataxic birds fed the ionophore (Roder et al, 1996, Gregory et al, 1995). The electron micrographs indicate that these changes primarily affect the myelin and secondarily the axon. The changes noted in these studies are similar to those reported earlier with lasalocid (Gregory et al, 1995) , A23187 (Schlaepfer, 1977b,a), ionomycin (Smith & Hall, 1988) and the results of ataxic birds fed 270 ppm

lasalocid (Roder et al, 1996). The ability of lasalocid to complex with and transport calcium ions across cell membranes is the most plausible mechanism for these changes. The elevated intracellular concentrations of calcium probably interact with a calcium-reactive site within the myelin or activate some calcium-dependent enzymes within the Schwann cells. The lack of direct axonal effects with lasalocid incubation with a calcium containing is an interesting observation to this study. This may relate to tissue composition and the selective affinity for the ionophore. The hydrophobic nature of lasalocid may cause it to be found at greater concentrations in lipid-rich tissues such as myelinated nerves.

In summary, the results of these studies demonstrate the ability of lasalocid to directly cause a dose-dependent neurotoxicity with specific ultrastructural changes in the sciatic nerve. This experimental model mimics field cases of "duck walking" broilers. The determination of a range of per os doses that can reliably reproduce this ataxia is a useful tool for further mechanistic studies. Recovery from lasalocid-induced ataxia is possible by removing the drug and providing good supportive care. Water deprivation and Vitamin E do not appear to have a significant role in the pathogenesis of this syndrome. Alteration of mitochondrial respiratory chain function, especially NADH oxidase and ATPase, may be an important subcellular site of action for lasalocid. Calcium-mediated ultrastructural changes may involve activation a calcium-dependent enzyme within the myelin sheath that leads to breakdown or a calcium mediated alterations of cellular energetics. Additionally, lasalocid may bypass the normal, intracellular regulatory mechanisms and activate calcium-mediated cell death in the sciatic nerve.

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CHAPTER IV

SUMMARY AND CONCLUSIONS

The results of all of the previous studies indicate that lasalocid causes a peripheral neurotoxicity in broiler chickens. In the feeding trials (Chapter II), the incidence of ataxia was low at normal use levels (90 ppm) but increased exponentially at greater than 2x normal use levels (180 ppm). In these studies, lasalocid exhibited a dose-dependent ataxia in broilers of 3 weeks and 6 weeks of age. Transforming the percentage neurotoxicty data with a log dose by probit response produced a straight line that could be further analyzed by linear regression. The toxic ration concentration of lasalocid that would cause ataxia in 25% of the flock, was approximately 220 ppm for both age groups.

The feeding trials also established the ability of the clinical ataxia scoring system to screen large numbers of birds for evidence of neurotoxicity. The scoring system can also be used on an individual, following the animal through the different stages of disease. The scoring system is organized such that the classifications indicating overt neurotoxicty (Scores 3,4,5) would be detected by untrained individuals. The clinical score 3 also represents a threshold of gross observable ataxia. The simplicity of the tool allows it to be used by several different individuals concurrently.

Clinical neurologic evaluation of lesioned birds indicate that the peripheral nervous system is primarily involved. The significantly reduced nerve conduction velocities of ataxic

birds support the neurotoxicity of lasalocid. Serum electrolytes, enzymes (CPK, AST, LDH) and gross necropsy findings confirm that lasalocid does not cause any significant muscle damage which is further evidence that lasalocid-induced ataxia is not due to myonecrosis

Histologically and ultrastructurally, the myelin of the sciatic nerve is most commonly affected. Damage to the myelin include: myelin splitting, intramyelinic edema, myelin vacuolation and Schwann cell degeneration and appear to precede any axonal damage. In many sections, the myelin would have significant damage and the microtubules and neurofilaments looked normal.

These studies also showed that monensin and salinomycin do not elicit ataxia at concentrations up to 8x the recommended use levels. Gross pathologic lesions, histopathology, serum electrolytes and enzymes (CP, LDH, AST) reconfirmed the myotoxic nature of salinomycin and monensin.

The *per os* dosing studies (Chapter III) further refined the dose and time interval necessary to cause ataxia in broiler chickens. A dose between 33.75 and 50 mg/kg, t.i.d., will produce greater than 90 % ataxic birds with less than 5 % mortality. These per os doses (30 and 50 mg/kg, t.i.d.) correspond to ration levels of 990 ppm and 1500 ppm, respectively. These calculations assume that the birds consume 10% of their body weight in feed per day. The clinical ataxia scoring system is a useful, reliable toxicologic endpoint that can screen large numbers of animals. The scoring system also has merit for examining time to onset of ataxia or recovery from the syndrome. The results of the clinical ataxia scoring system were validated by histopathology, electrophysiology and serum chemistries.

The *per os* dose is reliable and should prove useful in prophylactic, therapeutic or mechanistic studies. The results of the water deprivation (12 hours/day) study did not show a difference in the severity of ataxia after 48 hours of lasalocid (33.75 mg/kg) treatment.

The water deprived birds exhibited a more rapid onset of ataxia as compared to birds provided water ad lib. Although in the current experiments, vitamin E did not provide any protection at 100 IU/kg, there is potential for performing small scale preliminary studies to examine several possible therapies before attempting larger scale studies. To accomplish similar results using the recommended dose of lasalocid in a feeding trial would require between 300 - 500 birds.

The effects of lasalocid on the mitochondrial function are also presented in Chapter III. The NADH oxidase inhibition indicate that lasalocid can reduce the amount of ATP generated in hepatic mitochondria. This can result in a decrease in the function of the various cation "pumps" in cells responsible for ionic homeostasis. This may be a subcellular site of action for lasalocid-induced ataxia.

The direct effect of lasalocid (100μM) on the sciatic nerve of broilers in vitro was described in Chapter III. There are similar ultrastructural changes (primarily to the myelin) in vitro as compared to the feeding trials. The severity of these changes seem to be related to calcium levels in the incubation media. Nerves incubated for 120 minutes with lasalocid and calcium had serve intramyelinic edema with resulting compression of the axolemma. Sciatic nerve segments incubated in calcium-containing media for 120 minutes had little ultrastructural change. These results suggest that some calcium-sensitive mechanisms, perhaps a calcium-sensitive Phospholipase A_2, are involved in the pathogenesis of lasalocid-induced neurotoxicity.

These studies failed to find a relationship between alteration of ATPase activity and neurotoxicity The ATPase activity from various organs of birds in the feeding trials was not significant different from controls while in vitro, lasalocid caused a dose-dependent reduction in ATPase activity. The in vitro results are probably due to the ionophore's actions

on a magnesium- or calcium- sensitive of the enzyme. Lasalocid administration had no detrimental effects on sciatic nerve ATPase activity in the per os gavage studies (Chapter III). Sciatic nerve ATPase activity has been implicated in the pathogenesis of diabetes-induced neuropathy. The differences between the in vitro studies and the whole animal studies may be due the fact that in vivo, there are adaptations (upregulation, production of new enzyme, removal of inhibitory factors) by cells to increase overall ATPase function and ensure ionic homeostasis.

Lasalocid causes a dose-dependent peripheral neurotoxicity in broiler chickens. These studies show that the ataxia caused by lasalocid is not due to muscle necrosis , as evaluated by serum chemistries, gross pathology and histopathology. The use of a clinical ataxia scoring system is a useful tool in measuring the incidence and severity of the neurotoxicity. The sciatic nerve myelin is the primary tissue damaged in this disease as shown by reduced MNCV, rapid recovery, histologic and ultrastructural damage. Lasalocid can also directly damage the sciatic nerve after in vitro exposure and evaluated by electron microscopy.

Lasalocid-induced neurotoxicty may provide a useful, non-mammalian , acute model of drug-induced peripheral neuropathy. Additional mechanistic data must be determined before this model will be of practical use.

APPENDIXES

APPENDIX A

RATION COMPOSITION FOR FEEDING TRIALS

Ingredients	Starter Diet (%)	Grower Diet (%)
Corn	47.18	52.74
Soybean meal	42.00	36.50
Fat	6.30	7.00
Dicalcium phosphate	2.35	1.61
Calcium carbonate	1.20	1.23
Salt	0.40	0.40
Vitamin mix[1]	0.20	0.20
Trace elements[2]	0.10	0.10
DL-methionine	0.25	0.20
Calculated Analysis:		
ME Kcal/Kg	3165	3282
Crude Protein (%)	24.79	22.56
Calcium (%)	1.00	0.92
Phosphorus (% ave)	0.53	0.45

1 Mix supplies the following per kilogram of diet. Vitamin A (14,109 I.U.), Vitamin D_3 (5,291 I.U.), vitamin E (47.63 I.U.), vitamin B_{12} (0.014 mg), Niacin (26.5 mg), riboflavin (3.6 mg), Choline (705.5 mg), Menadione (1.16 mg), Folic Acid (1.76 mg), Pyridoxine (3.52 mg), Thiamine (3.52 mg), d-Biotin (0.167 mg).

2 Mix supplies the following per kilogram of diet. Manganese (120 mg), zinc (80 mg), copper (10 mg), iodine (1 mg), calcium (180 mg), selenium (0.15 mg), iron (75 mg).

APPENDIX B

CLINICAL NEUROLOGIC EVALUATION CHECKLIST

Area Evaluated	Reflexes Examined	Specific Nerve(s) Tested
Head		
	Visual menace test	CN II (optic),CN VII (Facial)
	Pupillary light reflex	CN II, CN III (Oculomotor)
	Mandibular tone	CN V (Trigeminal)
	Palpebral reflex	CN V, CN VII
	Hearing , Vestibular reflexes	CN VIII (Vestibulocochlear)
	Gag reflex (Swallowing)	CN IX (Glossopharyngeal), CN X (Vagus), CN XII (Hypoglossal)
Neck and Forelimbs		
	Righting Reflex	
	Pain (Superficial and Deep)	
Pelvic Limbs		
	Righting Reflex	
	Pain (Superficial and Deep)	
	Proprioceptive positioning	

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VITA

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