Abstract

Gowdy, Kymberly. Selenium Supplementation and Antioxidant Protection in Broiler Chickens. (Under the direction of Dr. F. W. Edens)

Selenium supplementation in poultry has long been associated with energy metabolism, increased feed efficiency, improved reproduction, and improved immune responses. Poultry are constantly exposed to environmental stressors that require the antioxidant protection of selenium supplementation. Improved performance results in an improved product for the industry to thrive on. However, the debate has now come up to examine the selenium dose and chemical form that is commonly fed to poultry amongst other livestock. The inorganic forms of selenium commonly used have proven to have prooxidant properties and can be toxic at high levels. Organic forms of selenium, such as Sel-Plex®, have been shown to be generally safer and better absorbed. These developments raise questions as to which form of Se is best for dietary supplementation.

Herein, studies were designed to investigate three main areas associated with the different forms of selenium supplementation. 1) Effect of various selenium supplements on antibody and nitrite production in commercial broilers, 2) Effects of high levels of selenium supplements on the developing immune system, 3) Effects of selenium supplementation on a new selenoprotein, thioredoxin reductase.

Selenium supplementation with selenite or Sel-Plex® did show varying effects on the humoral and innate immune response. Birds fed diets with selenium overall had

higher antibody titer that was maintained longer than birds not fed selenium supplemented diets. Birds fed selenite diets generally had significantly higher IgM titers, while birds fed Sel-Plex® diets had significantly higher IgG titers. When nitrite values were examined, birds fed selenium-supplemented diets had lower resting values and higher values when macrophages were stimulated with LPS. The control fed birds had higher resting values and lower values when stimulated with LPS. This could be due to an enhancement of the immune system with selenium in the diet. However, selenite fed birds had significantly higher nitrite values that indicate prooxidant properties potentially damaging to cells.

High levels of inorganic or organic forms of selenium showed different effects on the developing immune system. At levels as low as 1.2 ppm of selenite, immune organ weights were significantly decreased and inflammatory responses were increased. Up to 15 ppm no signs of toxicity were noted with the organic supplement Sel-Plex®. This indicates that at high levels of dietary inclusion, Sel-Plex® has no detrimental effects on the immune system.

Selenium is an integral part of a range of selenoproteins. Thioredoxin reductase (TR) is one of those proteins, which is a key player in the antioxidant cascade. TR has been purified and characterized in many organisms but not the chicken. Chickens are exposed to many stressors, so it is of great importance to study this enzyme in chickens. The distribution of TR protein expression was uniform throughout the chicken's body, regardless of selenium supplementation. However, varying isoforms did appear that were not seen in mammalian tissues. When activity of TR was examined, significant differences were seen among treatments, with the highest activities in the Sel-Plex® fed

birds. The subcellular distribution of this enzyme showed majority of the activity to be in the mitochondrial fractions, while there was little to no protein expression in these fractions. All of this taken together indicates that chicken TR may be very different than mammalian TR, but further studies are needed to draw these conclusions.

. Attempts to purify TR from chicken liver were also made. The enzyme seemed to be dependent on selenium supplementation, age of the bird and the addition of FAD. Ammonium sulfate saturation of the enzyme caused it to fall out of solution at 70% instead of 80% like the mammal. Reduction with 0.2 M DTT did not restore activity after being dialysed overnight. All of these results indicate that chicken TR is very sensitive to enzymatic conditions and is not very stable. Purifying this enzyme from chickens could be a great benefit to poultry science.

The emphasis of this thesis is on the different supplemental forms of Se and their effects on broiler health. Selenium is essential whether it be for immune responses or antioxidant protection. However, careful consideration needs to be made when choosing an optimum form. Organic forms have proven to be more beneficial to the poultry immune system through many different aspects.

Selenium Supplementation and Antioxidant Protection in Broiler Chickens

By

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DEDICATED TO MY PARENTS

AND GRANDMOTHER

Biography
Written by Wayne and Roxanne Gowdy

Our daughter, Kymberly Mae Gowdy was born on November 28th 1979 in Hartford Connecticut. We believed that from the minute we laid eyes on her that she would be doing great things with her life and she continues to live up to that belief. She has always been a happy person with an infectious smile and great sense of humor. She understands that life's opportunities should not be taken lightly and she has worked hard to make her dreams a reality.

In her early years Kym demonstrated her independence by amusing herself with the objects around her. At 11 months, while supposedly in her crib for an afternoon nap, she had an interest in find out what was behind the wallpaper next to her bed. She found the seam in the wallpaper and started picking it off. In just a short time we went in to check on her only to find her standing in her crib with pieces of wallpaper in her hand and the biggest smile on her face. Needless to say we took down the rest of the wallpaper and placed her crib in the middle of the room to avoid further investigation.

She also had a great love for stuffed animals and books that occupied her for hours at a time. "Toby the Rock Hound" was one of her favorite books as a toddler. You could see her dragging "mister blankie", a stuffed animal and that book from place to place saying "please read to me….again"

Her days in Elementary school were full of adventures. She loved school from the very start. She was anxious to learn from her teachers but thought that vocal socialization

was part of the curriculum. Eventually she soon found a balance to both the learning and the talking with her friends.

Throughout grade school, Kym would always volunteer to take care of the class pets and reluctantly let other children have their turn with the care and feeding. We believe that this is where her interest in animals and science got started. Her compassion for animals showed its true colors when she witnessed the birth of a calf at her uncle's farm. The tradition is that the youngest family member present gets to name the calf. She named the calf "Beaker" after the Muppets character. Unbeknownced to Kym this calf was a male and destined for the veal section of the meat case. When she found out that Beaker was someone's dinner she was saddened to think that someone could be so cruel to her calf and that becoming a vegetarian is the right answer. As her parents, we thought that this would just be one of those phases that every eight-year-old goes through. Well the vegetarian phase lasted until she was a junior in college….now that is conviction!

At the age of 14, Kym wanted to continue her quest to help out animals by volunteering at the local veterinary hospital doing kennel work. The after school activity grew more into a passion and at the age of 16 she was hired as a kennel aide. Her activities included holding animals during their examinations and even being the "go-for" during operations. She would bring home stories of all of the animals that they had helped each day and sad stories of the ones that they could not help. Regardless of the outcome she really enjoyed being with the animals and that this would be her life's work.

Kym never faltered with her studies and her interest in science and math was the focus while at East Catholic High School. Ms. Milas was her Biology teacher and challenged Kym to learn every detail about the physiology of the animals. Ms. Milas and

Mr. McConville (her Spanish teacher) encouraged Kym to sign up for the class trip to Costa Rica. This would give her a chance to see exotic animals in their wild habitat and practice her Spanish as well. The stories and pictures of the monkeys, wild horses, bird and sloth she brought back were well worth the trip.

Upon graduation from East Catholic in 1997, Kym set her sights towards Virginia Tech and a Bachelor of Science degree in Animal and Poultry Science. We can remember the day that we packed up the car and U-Haul trailer for that first of many 12-hour rides to Blacksburg. Upon arrival on campus Kym was looking forward to the challenge ahead and the excitement that comes from living away from home for the very first time.

While at Virginia Tech Kym was very fortunate to encounter Drs. Paul Siegel and Audrey McElroy who proved to be both wonderful role models and great advisors. At their direction the classes Kym got involved in would be interesting and prepare her well for future degrees. Kym tells the stories of the time she fell in sheep manure while trying to straddle a large sheep to draw blood and the time she spent all night at a pig barn waiting to help a sow give birth.

Her studies at Virginia Tech kept her quite busy however she always made time for Chemistry Fraternity activities and Poultry Science Club. Graduation in May 2001 with degrees in Chemistry and Animal and Poultry Science was a just end to four years of hard work. Months before graduation day Kym had already set her sights for the next opportunity…a masters program in Immunology and Poultry Science at North Carolina State University.

Her arrival at NC State challenged her to acclimate to new surroundings, make new friends and fulfill the requirements of a new course of studies. Under the direction of Drs. Edens and Qureshi, and support from Sue Mann, Kym began her Masters program. She was thrilled to begin her research with selenium supplements in poultry development. Within a short period of time she was making great progress in her lab trials and had heard that a doctor at the US EPA had done similar research with mammals. She initiated a meeting with Dr. David Thomas and he shared not only his selenium research but also his laboratory for the next two years to supplement her research at NC State. He and Karen Herbin-Davis were quite impressed with Kym's enthusiasm and attention to detail.

During one of our visits to North Carolina, Kym asked us to help her with some chicken dissections she was doing as apart of a trial. Even though we were novices at this type of work, seeing Kym take charge in the laboratory by outlining each procedure in great detail made it a memorable experience.

Having completed all of the research requirements of her Masters program, Kym was asked to consider a PhD program under the direction of Dr. Ian Gilmour from the US EPA. She accepted the PhD grant with the same vigor that she had for her previous two degrees and cannot wait to further her academic career.

As her parents, we cannot say enough about how proud we are of her and her accomplishments. She continues to work hard to make her dreams a reality and we are blessed to be part of her life.

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Literature Review

Introduction

Minerals are vital nutrients for maintenance of the homeostatic condition that exists in all living organisms. Many of the minerals are involved in essential metabolic and physiological processes, which are critical for human and animal health and animal food production. Most mineral requirements are met through consumption of nutrients from plant, soil, and animal by-products. In some cases these minerals can exist in chemical complexes that are not readily available. This can be advantageous because large amounts of some minerals can be toxic, but if too little is consumed, it can be harmful due to development of a deficiency. Advances in mineral nutrition have proven the importance of consuming adequate amounts of macro- (Ca, P, Na, Cl, Mg, K, and S) and microminerals (Co, Cu, F, I, Fe, Mn, Mo, Se, and Zn). The microminerals or trace elements function as parts of proteins, hormones, enzymes, or as cofactors that activate specific enzymes (Surai, 2002).

The lack of trace elements can impact significantly the health and well being of animals. Dietary inclusion of these trace elements, particularly selenium (Se), may induce physiological and metabolic changes that maintain the normal biochemical processes in the body. Among the micronutrients, Se may be the most difficult to understand. Discovered in 1817 by Jons Jakob Berzelius, it was known as a toxic mineral for about 100 years because it was associated with alkali disease in humans and livestock in the Dakota and Wyoming territories in the 1850's. In 1957, Klaus Schwartz reported that Se was an essential factor protecting rats against liver necrosis and degeneration. In 1974, the FDA permitted the use of Se as a feed supplement for pigs and poultry at the level of 0.1ppm and 0.2ppm for turkeys.

The primary function of Se in animal systems is as a component of the antioxidant enzymatic cascade responsible for protection of cells from potentially damaging lipid peroxides and oxygen and nitrogen free radicals. Selenium is involved in the regulation of energy metabolism, spermatozoa function, thyroid hormone activation, and immunity. It is recognized as having anticarcinogenic and antiviral properties that are essential in the face of stress. All of these factors emphasize the necessity of this trace mineral in the diets of all humans and animals.

Selenium exists in four valence states: -2 (hydrogen selenide, sodium selenide, dimethyl selenium, trimethyl selenium, and selenoamino acids), 0 (elemental selenium), +4 (selenium dioxide, selenious acid and sodium selenite), and +6 (selenic acid and sodium selenate). Not only does the valence state make Se difficult to understand, it can affect its water solubility and gastrointestinal absorption rate. Selenium can be toxic in valence states –2, +4 and +6, but in appropriate trace levels in feed or drinking water, Se in valence states –2, +4, +6 serves in essential roles in the maintenance of homeostasis.

Dietary Se is essential for optimum immune responsiveness, although the mechanism(s) are not fully understood it affects both the innate and adaptive immune functions. Selenium-deficient lymphocytes are less able to proliferate in response to an antigen. Humoral immunity is suppressed during Se-deficiency. In endothelial cells, Se-deficiency can result in the up-regulation of adhesion molecules, which results in greater infiltration by lymphocytes into sites of inflammation. There are many additional effects of Se on the immune system.

Selenium supplements used in diverse products such as human multivitamins to poultry feeds contain Se in different chemical forms. In most instances, either sodium

selenite or sodium selenate is used (Wolfram, 1999). Results of current studies have provided evidence that organic forms of Se are generally safer and better absorbed (Edens, 2001). These developments raise questions as to which form of Se is best for dietary supplementation.

Selenium yeast has been reported to be an excellent source of organic Se (Kelly and Powers, 1995). Selenium yeast is manufactured by growing a commercial Sarccharomyces yeast strain in a sulfur deficient/Se rich medium that forces the yeast to incorporate Se into their proteins in the form of selenomethionine.

Poultry and livestock diets were approved for supplementation with Se yeast at 0.3ppm (Federal Register, 2000, 2003). The use of Se yeast in poultry diets has resulted in some remarkable improvements in poultry production (Edens, 1996;1997;2001). Selenium yeast reduces drip loss, improves feathering, increases body weights, increases fertility, and decreases disease incidences (Edens, 1996;1997;2001). This is only the beginning of a development that will eventually result in the complete replacement of inorganic Se feed supplements with organic Se supplements

.

Forms of Selenium and Their Use in Animal Production

Selenium is an essential antioxidant nutrient that has been found to be toxic in high doses (Franke, 1934). Selenium occurs naturally in two chemical forms, inorganic and organic. Inorganic Se can be found, depending on the oxidation state as selenite, selenate, or selenide. Organic Se is found incorporated within the amino acids methionine and cysteine. Most plants and plant products contain the organic forms of selenium, mainly selenomethionine (SeMet) (Combs and Combs, 1986). Plants

synthesize selenoamino acids by absorbing Se from the soil in the form of selenite or selenate and incorporating it into their amino acid sequence (Olson and Palmer, 1976).

The amount of Se in the soil can vary depending on pH, aeration, concentrations of other minerals, and solubility (Reilly, 1996; Combs and Combs, 1986). Therefore, plant-based organic Se can be influenced by several environmental factors and can be lower than necessary to maintain the Se-dependent antioxidant systems in vertebrate animals that consume plants and their products. Dietary Se supplementation solves this problem. In the animal industries, dietary supplementation of Se is necessary to maintain optimum performance and health. The main dietary supplementation, for 30 years, has been the inorganic forms of Se. The inorganic Se supplements provide an inexpensive source of this element, but the inorganic forms can be toxic in mid- to high concentrations, interact and chelate other minerals, and are unable to be incorporated into by-products such as meat, eggs or milk in concentrations equivalent to the organic form of Se. Instead of being stored, selenite and selenate are excreted by the body if not utilized.

In recent years, the organic form of Se has become an alternative Se supplement. Sel-Plex® (SP) is an organic form of Se produced by Alltech, Inc. (Nicholasville, KY 40356). Sel-Plex® is a product of yeast cells grown in a sulfur-deficient medium. This forces the cells to incorporate Se into their amino acids. The yeast cells become enriched with selenomethionine (Kelly and Powers, 1995). This form of Se is to the same as the form found in grains.

Although inorganic and organic forms are supplements for Se, they differ extensively in chemical properties. These forms are absorbed and metabolized in

differently. Selenite is passively absorbed via the intestine with the highest concentrations of Se in the duodenum, liver, and kidneys (Apsite et al., 1994). In the chicken, the greatest absorption of $Na_2{}^{75}SeO_3$ was in the duodenum and anterior ileum (Pesti and Combs, 1976). Whanger et al. (1976) demonstrated that neither selenite nor SeMet is absorbed from the rat stomach. They also reported that selenite and SeMet are absorbed in all segments of the small intestine, with the duodenum absorption to be slightly higher than the ileum or jejunum. Anudi et al. (1984) showed that intestinal cells can concentrate Se and that reduced glutathionine in the gut lumen plays a large role in the absorption of selenite. Unlike selenite, L-SeMet is actively absorbed. In a study by McConnell and Cho (1965), L-selenomethionine was actively transported from the mucosa to the serosal area of the intestine in hamsters. However, in the presence of L-methionine the transport of L-selenomethionine was inhibited. Spencer and Blau (1962) showed the accumulation of L-SeMet on the serosal surface of everted hamster intestinal sacs, and through chromatography, they demonstrated that ^{75}Se-selenomethionine was not degraded by active transport. Reasbeck et al. (1981) used dogs with triple lumen gut perfusion to show that the amount of transferred SeMet was about four times the amount of transferred sodium selenite during a two-hour period. The amount of selenocystine absorbed was about half of the amount of SeMet. All of these studies prove that selenoaminoacids are absorbed through active transport mechanisms similar to the transport of the sulfur analogues of the amino acids.

Selenium status of the body has little to no affect on the absorption of either form of Se. Vendeland et al. (1992) studied the absorption of ^{75}Se from selenite, selenate, and SeMet from segments of the small intestine of rats fed a Se-deficient diet (0.009 ppm Se)

or a selenite-supplemented diet (0.20 ppm). Although the forms of Se were absorbed in different concentrations, Se-deficiency had no effect on the absorption of any form of Se supplementation in the intestine.

Other elements have been found to affect Se absorption. In chickens, high amounts of lead (Pb) in the diet inhibited the amount of ^{75}Se-selenite absorbed in the intestine over a period of three weeks (Mykkanen and Humaloja, 1984). High doses of vitamin A (Combs, 1976) or vitamin C (Combs and Pesti, 1976) increased the amount of Se absorbed in the intestine. Combs examined the addition of ethoxyquin to poultry diets (Combs, 1978). Ethoxyquin increased the amount of selenium-containing enzyme glutathionine peroxidase (GSH-px) in the plasma and alleviated signs of exudative diathesis (ED), which is commonly associated with Se-deficiency.

Metabolism of Selenium

Inorganic and organic Se are metabolized in different ways, but both must be converted to the common selenide form before the Se can be inserted specifically into a selenoprotein. When methionine is limited, SeMet is incorporated nonspecifically into body proteins in place of methionine. There are two pathways for catabolism of SeMet. SeMet can go through the transsulfuration pathway via selenocystathione to produce selenocysteine. It is then degraded by a decarboxylase into hydrogen selenide (Beilstein and Whanger, 1992). The other pathway involves transamination-decarboxylation (Mitchell and Benevenga, 1978).

Inorganic forms of Se, like selenate, are reduced to selenite and metabolized to hydrogen selenide via selenodiglutathione and glutathione selenopersulfide (Turner et al.,

1998). Hydrogen selenide is the precursor for supplying Se in an active form that can be used for the synthesis of selenoproteins (Sunde et al., 1997). Further metabolism of hydrogen selenide involves methylation by S-adenosylmethionine to methylselenol, dimethyl-selenide and a triethylselenomium ion (Foster et al., 1986).

Rats injected with selenide retain Se as selenocysteine (Beilstein and Whanger, 1988). Since the body has no pathway to synthesize SeMet from inorganic sources of Se, it relies on plant and microbial sources for this selenoamino acid. In the vertebrates, SeMet cannot be synthesized from selenocysteine.

Tissue Concentration of Selenium

Body retention of Se is dependent on many factors: the particular tissue, and the amount of available Se in the diet, the length of time the diet is fed, species, age, and the form of Se. Tissue Se status will plateau as the concentration of inorganic Se in the diet increases. The Se status of the dam affects the tissue concentration of Se in her offspring. Generally, the organic forms of Se are easily retained in tissues, whereas the inorganic forms are absorbed as a mineral and little is retained in tissues (Wolfram, 1999).

There are different concentrations of Se in different parts of the cell. The highest Se concentrations in the sub-cellular fractions of human liver cells were in the mitochondrial and nuclear fractions (Chen et al., 1999), who noted varying concentrations of selenoproteins in the sub-cellular fractions. Bovine kidney cells have been reported to hold the highest Se concentrations in the nuclear and cytosolic fractions (Jayawickreme and Chatt, 1987).

In a study in which ^{75}Se was administered to growing chickens, Se concentrations were found to be dependent on the amount of vitamin E available. The majority of the accumulation of ^{75}Se was in the feathers (Leibetseder and Leskova, 1973). Selenium concentrations were highest in the kidney and retina, followed by the spleen, liver, bone and plasma. Chickens deficient in vitamin E had a significant decrease in the amount of ^{75}Se retained in the feathers, blood, and cerebellum, but an increase in the concentration in the liver and breast muscle (Leibetseder and Leskova, 1973). Another study showed similar results when ^{75}Se was administered by injection and Se distribution was determined 15 minutes later. The ^{75}Se concentrations were in the following order: duodenum> liver> kidney> feathers> jejunum and ileum> spleen> pancreas> blood> breast muscle (Atlavin and Apsite, 1980).

Latshaw and Osman (1975) investigated the tissue concentrations of Se with chickens fed organic Se found in natural feedstuffs compared to the same level of Se provided as a sodium selenite feed supplement. The results showed an increased Se retention in the liver and muscle when the chickens were given natural feedstuffs. An increase in pancreas Se concentrations was found in chickens fed SeMet (Osman and Latshaw, 1976). Cantor et al. (1982) also showed an increase in Se concentrations of the pancreas, muscle, and gizzard when birds were fed SeMet compared to selenite.

Excretion of Selenium

Excretion of Se is dependent on the amount of available dietary Se and the form of Se. Excess Se is eliminated by three major routes respiration, urine, and feces. Selenium excretion was studied by injecting rats with $H_2{}^{75}SeO_3$. Urinary excretion was

proportional to the concentration of dietary Se. Fecal elimination of Se was about 10% of the total dietary Se. Respiratory elimination was marginal when related to total dietary intake of Se. Thus, rats adjust to the variation of dietary Se by increasing the Se excreted in urine (Burk et al., 1972).

Several studies have reported the influence of Se form and its effect on excretion. Thomson et al. (1975), reported that rats, dosed orally with ^{75}Se as either selenite, SeMet, or SeCys had higher excretion rates one week later when they were dosed with either selenite or SeCys and that the excretion rate of SeMet was only about half that of selenite or SeCys. Nahapetian et al. (1983) reported similar results when rats were orally dosed with low amounts of Se. When rats were given a dose of 1.5mg Se/kg body weight, the amount of Se excreted in the rats given selenite was reduced to the same percentage as that of the SeMet-dosed rats. SeCys-dosed rats remained the same no matter what amount they were given. It was concluded that the decrease in urinary excretion in the selenite-treated rats was due to an increase in respiratory excretion.

Bio-availability

Many studies have examined the bio-availability of Se as either selenite or SeMet. SeMet can be easily incorporated into body proteins because the body cannot distinguish between SeMet and methionine, but very little of the selenite Se is retained in the body. The SeMet form allows the body to retain more Se and increase its bio-availability (Henry et al., 1995).

Bio-availability of Se has been examined using the prevention of exudative diathesis (ED) as an endpoint. Cantor and Scott (1974) found bio-availability to be 100%

for sodium selenite, 74% for sodium selenate, and 7% for elemental Se. Cantor and Scott (1974) also found the bio-availability in plant feedstuffs to range from 210% for Lucerne meal to 60% for soybean meal. The bio-availability of Se from fishmeals and fish solubles ranged from 25% for herring meal to 8.5% for fish solubles. In a similar study, SeMet shown to be four times more available than selenite or selenocystine with the respect to the prevention of pancreatic degeneration (Cantor et al., 1975). Miller et al. (1972) observed that the retention of Se was 43% for Na_2SeO_3, and 31% for selenomethionine.

There is a close relationship between plasma GSH-px and protection against ED (Cantor and Scott, 1974). The availability of Se in feedstuffs has been evaluated by measuring the blood serum GSH-px activity in Se-depleted chicks. Hassan et al. (1987) found the bio-availability of Se in oats, meat meal and SeMet and their efficacy in preventing ED in Se depleted chicks, based on elevated blood GSH-px activity, was 33, 21 and 77%, respectively. SeMet is superior to selenite and meat meal in increasing Se concentration muscle and blood. Moksnes and Norheim (1986) showed greater tissue concentrations of Se and GSH-px activity in SeMet-fed chickens compared to selenite-fed chickens. Some investigators have conflicting observations. Cantor and Tarino (1982) showed that bio-availability of Se from selenite was greater than SeMet when plasma GSH-px activity was used as an endpoint. Thus, it appears that SeMet feed supplementation increases tissue Se concentrations as compared with inorganic forms of Se.

The use of the inorganic forms of Se has improved the performance of poultry and swine. Genetic selection, however, has changed these animals significantly since 1974.

As an example, the modern day broiler has a higher metabolic rate and different nutritional needs as compared to thirty years ago. Therefore, there is a need to reevaluate the nutritional requirements in current poultry diets. Among the problems associated with the current poultry diet are the minimal levels of Se retained in the meat and the pro-oxidant properties associated with the inorganic Se forms. A need to re-examine the efficacy of different forms of Se (inorganic *vs*. organic) for feed supplementation is apparent. Since Sel-Plex® provides a highly available form of organic Se, it was chosen as the organic form to be investigated along with sodium selenite.

Selenoproteins

Selenium has been found to participate in various physiological functions, most importantly as an integral part of a range of selenoproteins. There are over 20 eukaryotic selenoproteins (Kohrl et al., 2000) and at least 35 Se-containing proteins or protein subunits (Behne et al., 2000). The expression of these selenoprotiens is dependant on the amount of Se available, hormones, and environmental conditions (Kohrl et al., 2000). The majority of the selenoproteins contains a single selenocysteine residue per polypeptide chain (Tujebajeva et al., 2000). The synthesis of a selenoprotein requires that a selenocysteine be inserted by the UGA stop codon (Patching and Gardiner, 1999). The presence of selenocysteine at the active site of an enzyme can increase its activity 100-1000 fold (Burk, 2002). The most abundant selenoprotiens in mammals are glutathione peroxidase (GSH-px) and thioredoxin reductase (TR) (Gladyshev et al., 1998).

Selenoproteins are important players in many diverse systems such as redox signaling, regulation of apoptosis, immunomodulation, spermatogenesis, and embryonic development (Surai, 2002). Impairment of the thioredoxin and glutathione systems in the cell may explain the many effects of Se-deficiency. However, the majority of the selenoproteins still have unknown mechanisms of action. The study of the selenoproteins is still a very new and incompletely understood area of protein chemistry.

The best characterized selenoprotein and enzyme is the GSH-px family. GSH-px is a tetrameric protein with 4 identical subunits each containing a single SeCys residue at the active site (Sunde et al., 1993). GSH-px is found in all tissues where oxidative stress may cause damage (Kohrl et al., 2000). This selenoenzyme reduces hydrogen and organic peroxides into water and alcohol molecules and is important in protecting cells from free oxygen radicals that can react with and damage cell membranes. GSH-px is also important in maintaining cellular redox status. There are 5 different isotypes of GSH-px, which are (1) gastrointestinal GSH-px (iGSH-px), (2) phospholipid hydroperoxide GSH-px (phGSH-px), (3) plasma GSH-px (pGSH-px), (4) cytosolic GSH-px (cGSH-px), and (5) sperm nucleus GSH-px (spGSH-px). The cGSH-px is considered the "emergency enzyme" (Kohlre et al., 2000) because it is responsible for the prevention of the damaging effects of oxidative stress in the cellular cytoplasm. IGSH-px has the same enzymatic properties as cGSH-px. Activity for iGSH-px is found in the villi and crypts of the intestines of rat mucosal epithelium (Esworthy et al., 1998). PhGSH-px is another form of GSH-px discovered by Ursini et al. in 1985. PhGSH-px uses phosphatidyl choline hydroperoxide as a substrate. It is active in its monomeric form and has a different amino acid composition than cGSH-px (Sunde, 1993). PGSH-px has a

molecular weight of 92 kDa, with each subunit weighing approximately 23 kDa, containing four Se atoms per molecule (Cohen and Avissar, 1993). SnGSH-px is a selenoprotein with a molecular weight of about 34 kDa. It is found in spermatid nuclei and accounts for approximately 80% of the total Se in the spermatid. It has properties similar to phGSH-px (Pfeifer et al., 2001). However, snGSH-px differs from phGSH-px in its N-terminal sequence. In Se-deficient rats the concentration of snGSH-px was decreased to about one third of the normal amount, and the condensation of chromatin was disrupted (Pfeifer et al., 2001). It was concluded that this was a result of snGSH-px being a protamine thiol peroxidase that is responsible for disulfide linking by the reduction of free oxygen radicals.

The activity of all forms of GSH-px is dependent on the availability of dietary Se. In the face of a Se-deficiency, various forms of Se are differentially affected (Brigelius-Flohe, 1999). Some Se forms are retained in tissues for longer periods during a Se-deficiency and will compensate for selenoenzyme activities that decline. IGSH-px is retained longer followed by phGSH-px, and pGSH-px and cGSH-px, which had similar retention times (Brigelius-Flohe, 1999).

Thioredoxin Reductase

Selenoproteins have a major role in the maintenance of redox status in the cell (Morel and Barouki, 1999). The most studied thiol redox system has been the glutathione system, which consists of glutathione, glutathione reductase, glutaredoxin, and glutathione peroxidase (Holmgren, 1989). Recently, another thiol redox system, the thioredoxin system, has generated great interest. The thioredoxin system consists of

thioredoxin, thioredoxin peroxidase, and thioredoxin reductase (Holmgren, 2000). Together these two systems supply electrons for dexoyribonucleotide formation, antioxidant protection, and redox regulation of certain genes (Arner and Holmgren, 2000). Since thioredoxin reductase is a selenoprotein; the entire thiol system is dependent on dietary Se.

Thioredoxin reductase (TR) was first characterized from calf liver and thymus (Holmgren, 1977) and then from rat liver cytosol (Luthman and Holmgren, 1982). TR is a selenoenzyme found in a dimer formation with a molecular weight of 116 kDa. Each subunit weighs approximately 58 kDa. TR from mammalian cells contains a selenocysteine residue in the conserved C-terminus. TR contains a FAD prostethic group that is tightly bound to the enzyme and is very sensitive to inhibition by heavy metals such as arsenic (Tamura and Stadman, 1996). This selenoenzyme is a flavoprotein that requires NADPH as an electron donor to reduce free radicals (Ganther, 1999). TR also belongs to a family of oxidoreductases that share a sequence and mechanism that is very similar to glutathione reductase (GR; Gasdaska et al., 1995). It has been suggested that TR actually evolved in mammals from GR instead of its archaeic ancestor in bacteria. TR only differs from GR by its selenocysteine insertion; all other aspects are homologous (Mustacich and Powis, 2000).

There are three known forms of TR that are significant to the thioredoxin system. TR1 is predominantly a cytosolic selenoenzyme that was first purified from ^{75}Se-labeled human lung cancer cells (Tamura and Stadtman, 1996). TR1 is a dimer with two identical 56-kDa subunits. It was the first thioredoxin reductase to be identified as a mammalian selenocysteine-containing TR (Gasdaska et al., 1995). The second TR (TR2)

is primarily located in the mitochondria and is involved in protection against oxidative stress (Behne and Kyriakopoulos, 2001). TR2 was first described in 1999 when its cDNA was cloned from various sources, and it was purified from bovine adrenal cortex (Watabe et al., 1999). TR2 has a molecular weight of about 56 kDa for human and bovine proteins and about 53 kDa for rat proteins. The sequence for TR2 is about 56% homologous to TR1. TR2 differs from TR1 by an N-terminal mitochondrial leader sequence (Miranda-Vizuete et al., 1999). A third TR (TR3) was purified from ^{75}Se-labeled mouse testis where it is predominantly expressed (Sun et al., 1999). TR3 has about 70% homology to TR1 and has a molecular weight of about 65 kDa and a long N-terminal extension. These three isoforms suggest the possibility of other thioredoxin reductase species that may differ in tissue distribution (Behne and Kyriakopoulos, 2001).

Thioredoxin, the primary substrate for TR, is the major ubiquitous disulfide reductase responsible for maintaining cellular proteins in a reduced state. Thioredoxin is approximately 12 kDa in molecular weight. It was first described in E coli in 1964 and then again in 1967 from rat hepatoma cells (Powis et al., 2000). To this date only a single type of thioredoxin has been identified (Arne and Holmgren, 2000). Thioredoxin acts as a radical scavenger. Under oxidative conditions, reactive oxygen intermediates are generated intracellularly and thioredoxin is able to facilitate the regeneration of damaged proteins (Fernando et al., 1992; Schallreuter and Wood, 1986). It can also be secreted and taken up rapidly by many different cell types, which suggests that it has a role in intercellular signaling (Besse and Buchanan, 1997). Electrons from NADPH and TR reduce thioredoxin, which allows it to reduce oxidized cysteine groups on proteins (Powis et al., 2000).

Thioredoxin peroxidase (TPx) is part of a new family of proteins that are considered to be antioxidants that function as peroxidases associated with a sulfhydryl reducing system. TPx consists of two 25 kDa subunits that contain two cysteines. The two cysteines reduce hydrogen peroxides with electrons from thioredoxin (Zhang et al., 1997). There are three known forms of TPx; TPx-I, TPx-2, and TPx-3 (Mitsumoto et al., 2001). A novel human TPx named AOE372 was identified and characterized to have a prominent role in NFκB activation (Jin et al., 1997). TPx plays an important part in the thioredoxin system by conserving sensing mechanisms for redox conditions in eukaryotes and the detoxification of hydrogen peroxide (Ross et al., 2000). In mice, TPx was found in many different tissues but had high activity in cells that metabolized high affinity for oxygen molecules such as erythroid cells, renal tubular cells, cardiac and skeletal muscle cells, and certain neurons (Ichimiya et al., 1997).

The mechanism by which thioredoxin system protects cellular components from oxidative damage is very important for cell survival. Mammalian TR contains a selenocysteine residue in the conserved C-terminal sequence Gly-Cys-SeCys-Gly, which forms a selenylsulfide bond in the oxidized enzymes. NADPH reduces the selenylsulfide, which forms a selenolthiol. The selenolthiol serves as the active site in the reduction of thioredoxin (Sandalova et al., 2001). Thioredoxin reduces oxidized proteins through a thiol-disulfide exchange reaction. In addition to reducing oxidized proteins, TR can also catalyze the reduction of many other substrates such as lipid peroxides, TPx, glutathione, ascorbate, selenite, selenodiglutathione, and protein disulfide isomerase (Chae et al., 1994; Luthman and Holmgren, 1982). Certain compounds such as arsenic or gold compounds can inhibit these reactions (Smith et al., 1999). Inhibition of TR could

prevent the regeneration of reduced thioredoxin from thioredoxin disulfide and allow oxidized proteins inside the cell (Lin et al., 2001) leading to the toxic and carcinogenic properties of arsenic. The thioredoxin system, which maintains free sulfhydryls, and the GSH-GPx system, which is a primary antioxidant system, work together to regulate a low intracellular redox potential (Arner and Holmgren, 2000).

TR plays an essential role in oxidative stress responses in Archea, Prokarya, and Eukarya (Mustacich and Powis, 2000). The thioredoxin system is involved in regulating DNA synthesis, gene transcription, cell growth and apoptosis in all levels of organisms (Arne and Holmgren, 2000). However, there is a distinct difference between TR in higher organisms versus lower organisms. The TR types in higher order and lower order organisms are members of the flavoprotein family of pyridine nucleotide disulfide oxidoreductases and function as homodimers with FAD prosthetic group and a NADPH binding site (Williams et al., 2000). Those similarities are overshadowed by other major differences. The amino acid sequences and catalytic mechanisms of TR from the two types of organisms have distinct differences. One type of TR is a high molecular weight (58 kDa) found in humans, rats, and mice (Gladyshev et al., 1999). The second type of TR is a low molecular weight protein (35 kDa) identified in Archea, prokarayotic, and eukaryotic organisms (Becker et al., 2000). There is only about a 20% sequence homology between the two types of TR, while the high weight TR has a 35% homology to GR (Hirt et al., 2002). Furthermore, the high molecular weight TR has a C-terminal domain that is absent in the low molecular weight TR. Even though high molecular weight TR is homologous to GR, there is significant homology between the secondary and tertiary structures, and the NADPH- and FAD -binding domains of GR and low

molecular weight TR (Kuriyan et al., 1991). Although the low and high molecular weight TRs are likely to have originated from a common ancestor, the two types seem to have developed into different forms of TR. Data have suggested that TR from higher organisms evolved from GR and possibly replaced a pre-existing low molecular weight TR system (Hirt et al., 2002).

There is a Se requirement for the formation of functional TR. Radiolabeling of proteins by the incubation of the cDNA transfected cells with ^{75}Se in the form of sodium selenite proved that Se was incorporated into the expressed TR protein (Fujiwara et al., 1999). Se also can increase the activity of TR. Beggren et al. (1997) showed that Se increased the activity of TR in human cancer cell lines by increasing the amount of selenocysteine incorporated into proteins. This resulted in an increase in the specific activity of TR and an increase in TR protein levels. TR activity can be increased in humans with levels as low as 0.01 to 10 μM of Se in human serum (Kitaoka et al., 1994). Mammalian TR increased its activity with Se supplementation at nutritional and supranutritional levels. Berggren et al. (1999) showed that supranutrtional levels of Se increased activity in the kidney, lung, and liver of the rat. They also observed that increased TR activity did not indicate an increase in the actual TR protein concentration. The demonstration of an increased TR activity at supranutritional levels of Se could possibly provide evidence to support the hypothesis that TR is involved in the cancer preventive activity of Se.

TR has a possible role in chemoprevention. In a study by Gladyshev et al. (1998), three types of cancer models were examined and compared with normal cells for the expression of TR and cGSH-px. These antioxidant enzymes were measured by labeling

proteins with ^{75}Se. CGSH-px was decreased in the cancer cells, but TR was increased in all cancer models when compared to the control. The generation of reactive oxygen species in carcinoma cell lines could result in both the oxidation of the selenocysteine in TR and an increase in the expression of the enzyme. This could represent an anticancer activity of TR. Another study showed that human colon cancer cells supplemented with selenite had an increase in TR activity but not with cells from lymphoid origin (Gallegos et al., 1997). However, thioredoxin and TR have been found to be overexpressed in many cancer and tumor models, which could contribute to the growth and decreased apoptosis of these cells (Powis et al., 1998). Ganther (1999) hypothesized that supranutritional levels of Se may have an ability to form a diselenide bond with the selenocysteine residue on TR and inactivate the protein in tumor cells. This idea might explain why Se has such a strong correlation with cancer prevention. This proposed mechanism might be very evident in slow growing tumors such as in the colon and prostate. Although the mechanism of chemoprevention with the thioredoxin system is still unclear, TR still has many antioxidant properties that might influence the process of Se-mediated cancer prevention.

Iodothyronine Deiodinases

Selenoproteins play an important role in thyroid hormone metabolism. Iodothyronine deiodinases (ID) are selenoenzymes responsible for converting the inactive thyroxine (T_4) into an active form of 3,5,3′-triiodothyronine (T_3) (Arthur et al., 1990). This process occurs primarily in the liver and kidney (Beckett et al., 1992). There are three forms of ID. Type I 5′-deiodinase has a systemic action that regulates T_3

production and its degradation into rT_3 and sulfated iodothyronines (Kohrle, 2000). Type II 5′-deiodinase controls the local production of T_3 while Type III 5′-deiodinase regulates the inactivation of T_3 and T_4 (Arthur and Beckett, 1994). Activity of ID-I was found to be the highest in the liver and kidney, ID-II was the highest in the brain, brown adipose tissue, and pituitary, and ID- III was the highest in the brain, skin and placenta (Arthur and Beckett, 1994). However, it has been reported that the thyroid gland is a major source of circulating T_3 in rats (Chanoine et al., 1993). This is relevant because the activity of 5′-ID contributes to the maintenance of thyroid hormone homeostasis.

It has been shown that there is a strong correlation between the amount of Se in the diet and thyroid hormone synthesis. Kohrle et al. (1992) reported that Se-deficiency alters both the synthesis of T_3 from T_4 in the thyroid gland and the activity of 5′-ID activity in rat tissues. Hepatic 5′-ID activity declined ten-fold and plasma T_3 was significantly decreased in Se-deficient rats as compared to the Se-supplemented rats (Beckett et al., 1992). Other studies have reported an increase in 5′-ID activity in the thyroid of Se-deficient rats (Arthur and Beckett, 1994).

Thyroid hormones play an important role in growth and protein turnover (Hayashi et al., 1991, 1993). Impaired T_3 production could account for an impaired growth rate of animals with Se-deficiency. Jianhua et al. (2000) fed T_3 to Se-deficient broilers and found enhanced growth in those broilers fed a low level (0.1 ppm) of T_3. This study supported the concept that Se is necessary to drive the conversion of T_4 to T_3 via activity of the Se-dependent 5′- ID.

Other Selenoproteins

There are many other selenoproteins that have not been fully characterized regarding their functions and vital roles that may play in the maintenance of health.

Selenoprotein W (SeW) is found in the muscle, spleen, testis, and brain (Whanger, 2001). This protein contains one gram-atom of Se in the form of selenocysteine per g-mol of protein. There are four different forms of this protein with molecular masses ranging from 9.5 to 10 kDa that have been characterized from rat muscle (Allan et al., 1999). Expression of SeW and SeW mRNA are dependent on dietary Se supplementation (Whanger, 2000). One function that has been identified for this selenoprotein is its involvement in antioxidant defense, although the metabolic functions have yet to be examined (Whanger, 2000).

Selenoprotein P10 (SeP10) is a glycoprotein that contains about 10 selenocysteine residues per 43 kDa polypeptide chain (Allan et al., 1999). SeP10 is believed to represent approximately one third of total plasma Se in humans (Allan et al., 1999) to about 60-80% in rodents (Arthur and Beckett, 1994). SeP10 was originally believed to be the major form of transport of Se in plasma (Motsenbocker and Tappel, 1982). More recent studies have linked SeP10 to antioxidant actions in the plasma (Burk and Hill, 1993), degradation of peroxynitrite (Arteel et al., 1999) and the binding of heavy metals in vitro (Kohrle et al., 2000). This selenoprotein has multiple beneficial effects in the antioxidant cascade of protection.

Selenoprotein P12 (SeP12) is a recently identified selenoprotein that was found in bovine cerebellar cortex (Saijoh et al., 1995). The nucleotide sequence of SeP12 was found to have high homology with SeP12 found in rats and humans. However, SeP12

found in humans and rats had twelve rather than ten selenocysteines found in SeP10. SeP12 was found in all parts of the brain, most notably in the cerebellar cortex, hippocampus, and the olfactory bulb. Saijoh et al. (1995) concluded that SeP12 is the major Se carrier in the brain and has a role in nerve and ganglion cell response to stimulation.

Another relevant selenoprotein is selenophosphate synthetase-2 (SPS). SPS is involved in the biosynthesis of selenocysteine, and provides regulation of selenoprotein expression (Guimaraes et al., 1996). SPS begins to catalyze the reaction of synthesizing selenophosphate from selenide and ATP, which is then incorporated into selenoproteins (Low et al., 1995). Two forms of SPS have been identified mammals (Kim and Stadtman, 1995). The catalytic properties on SPS require further investigation.

In a study using ^{75}Se in rats, a novel 15 kDa Se compound was found. This is a selenocysteine-containing protein found in various tissues but is highly expressed in the epithelial cells of the prostate. The 15 kDa selenoprotein was also found to be abundant in human T cells. The mRNA of this selenoprotein has all of the features that are necessary to promote selenocysteine insertion into proteins, but the function of this selenoprotein is still not known (Gladyshev et al., 1998). Further studies of this novel protein are of great interest because of the high correlation between Se supplementation and decreased incidence of prostate cancer (Clark et al., 1998). A recent study showed that the gene Se115 is on one of the chromosomes that is often affected in cancer (Kumaraswamy et al., 2000).

An 18 kDa selenoprotein was found in various tissues in the rat (Behne et al., 1988). It was identified as a selenocysteine containing selenoprotein, mainly found in

mitochondrial membranes (Kyriakopoulos et al., 1996). This novel 18 kDa selenoprotein is preferentially expressed in the mitochondrial membranes suggesting that it must possess properties of biological importance (Behne and Kyriakopoulos, 2001).

Other selenoproteins that have been identified are selenoprotein R, selenoprotein T, selenoprotein X, and selenoprotein N (Kryukov et al., 1999). Functions of these selenoproteins are not yet elucidated.

Selenium Deficiency

Selenium was detected in normal human tissue samples as early as 1916. In 1957, Schwartz and Foltz found that liver necrosis induced in rats by feeding a purified vitamin E diet could be prevented by the addition of Se to the diet. Many other studies have shown that Se is essential for adequate growth and fertility, and that a Se-deficient diet or a combined low Se and vitamin E status may lead to various disorders (Combs and Combs, 1986).

A Se-deficiency has been known to cause a disorder in humans known as Keshan Disease. Keshan disease was named after an epidemic outbreak in 1935 in Keshan County, China. This disease occurred in the Se-deficient soil areas (Cheng and Qian, 1990). Keshan disease is an endemic cardiomyopathy that occurs with signs of congestive heart failure or stroke from diffuse cardiac thromboses (Aro et al., 1994). The myocarditic Coxsackievirus has been associated with the pathogenesis of this disease. The virus will become virulent in Se-deficient or vitamin E-deficient animals (Beck et al., 1994). A change in virulence has been found to be due to specific mutations in the virus

itself (Beck et al., 1995). Se-enriched salt supplementation of the diets has reduced significantly the incidence of Keshan disease in Se-deficient areas (Li et al., 2000).

Another disease found in humans as a result of Se-deficiency is Kashin-Beck disease. Like Keshan disease, Kashin-Beck is most prevalent in areas of the world with Se-deficient soils. A range of bone and joint deformations that develop during childhood and puberty characterizes the disease. The hands and feet are usually affected, and the bones of the wrist and ankles may be smaller in size or sometimes completely absent (Yang et al., 1993). Radiographs of these affected individuals have shown that the disease causes a deformation and fragmentation of the epiphyseal plate, uneven mineralization of the extracellular matrix, and an irregular bone surface. One diagnostic feature of Kashin-Beck disease is the broken, blocky or conical shape of the growth apparatus (Yang et al., 1993). Histological observations attribute these signs to the necrosis of chondrocytes during bone growth and necrosis of the growth plate. Although, the importance of Se for bone metabolism is still unknown, the consequences of the disease demonstrates the requirement for Se in bone metabolism (Moreno-Reyes et al., 2001).

Se-deficiency can actually be a consequence of some diseases. Many illnesses cause an altered nutritional status, poor nutrient absorption from the GI tract, and decreased immunity. Se-deficiency in has been associated with muscle weakness and tenderness, nail bed changes, and cardiomyopathy with decreased cardiac function (Marcus, 1993). Se-deficiency is also characteristic of patients with a reduced protein intake due to phenylketanuria (Darling et al., 1992), gastrointestinal disorders such as Crohn's disease (Abrams et al., 1992), and renal failure (Zima et al., 1999). Other

possible health effects that have been reported in numerous studies indicate that Se-deficient patients are more prone to seizures, rheumatoid disease, arteriosclerosis, miscarriages, neurological disorders, depression, and even cancer (Ramaekers et al., 1994; Rayman, 2000).

Humans are not the only species that can be affected by Se-deficiency. Se-deficiency in poultry, especially when combined with a vitamin E deficiency, can be responsible for a range of avian diseases including exudative diathesis (Bartholomew et al., 1998), nutritional encephalomalacia (Century and Hurwitt, 1964; Combs and Hady, 1991), and nutritional pancreatic atrophy (NPA) (Cantor et al., 1975; Thompson and Scott, 1969). NPA is the only disease that is caused by Se-deficiency alone (Combs, 1994). Se-deficiency in chickens is associated with impaired immunocompetence, reduced egg production, and increased embryonic mortality (Combs and Combs, 1984). In a study done by Combs and Scott (1974), birds were fed a low Se diet and peroxidized fat. This resulted in a significant decrease in the hatchability of eggs, which was restored by Se and vitamin E supplementation.

Se-deficiency in poultry affects fertility and hatchability. Lathshaw and Osman (1974) reported low fertility and hatchability when birds were fed a basal (low Se) diet, but this reproductive disorder could be corrected partly by vitamin E supplementation and completely by Se-supplementation. Eggs from hens fed a very low level of Se were more likely to be infertile (12.6%), more likely to have high embryo mortality (29%) and lower rate of hatchability of fertile eggs (15%). Mean values for the control group for infertility, embryo mortality, and hatchability were 4.1, 2.9, and 9.1%, respectively (Latshaw et al., 1977). Hatching weights of chicks from hens supplemented with 0.05

and 0.1 ppm of Se were significantly heavier than those of hens with no supplemental Se. Exudative diathesis (ED) in hatchlings from hens with no Se supplementation was also elevated (Hassan et al., 1990).

ED is a common problem in chickens deficient in Se and vitamin E. ED is attributed to increased capillary permeability due to endothelial cell failure in skeletal muscle (Combs and Scott, 1974) and reduced levels of blood proteins (Kristiansen, 1973). The signs of ED arises from leakage of body fluids through the capillaries and from small hemorrhages in the muscle tissues (Hassan et al., 1990) and can occur at any age but most frequently in young chicks or turkey poults (Whitehead and Portsmouth, 1989). Hassan et al. (1990) studied ED in chicks from laying hens fed a Se and vitamin E- deficient diets. ED was observed at hatching whereas signs of ED were not present in the chicks from Se and vitamin E supplemented hens indicating that signs of ED can develop during the development of embryos from Se- and vitamin E-deficient dams.

ED has been associated with low levels of muscle Se, liver GSH-px, and an increase in liver non-Se-dependent GSH-px (Hassan et al., 1990). Bartholomew et al. (1998) hypothesized that ED may be an inflammatory response associated with a Se/vitamin E deficiency. Pro-inflammatory cytokines, recruited because of a lack of antioxidant enzymes, might be responsible for the fluid accumulation and hemorrhaging.

Supplementing the diet with Se rather than vitamin E is more effective in preventing the occurrence of ED. Vitamin E supplementation at 15 ppm was not enough to prevent ED while levels as low as 0.15 ppm of Se was sufficient (Hassan et al., 1990). Therefore, ED is considered to be a Se-deficiency syndrome in chickens (Machlin et al., 1962).

Se-deficiency can lead to weakened membranous structures of spermatozoa that is characterized by a significant increase in midpiece abnormalities and decreased motility (Edens, 1996). Watanabe and Endo (1991) studied mice fed Se-supplemented or Se-deficient. In the Se-deficient group, the proportion of abnormal sperm was significantly higher than in the Se-supplemented group. The same results were shown in boars under the same conditions, and it was observed that fewer sperm were able to penetrate oocytes in the Se-deficient group (Marin-Guzman et al., 1997). In chickens, a low Se diet increased the amount of spermatozoa with bent midpieces to 18.7%, while diets supplemented with either sodium selenite or SeMet decreased the amount of deformed midpieces to 6.2% and 0.7%, respectively (Edens, 2002). These studies have shown that adequate Se is required to maintain sperm membrane integrity, motility, and fertilizing capability. The combination of both vitamin E and Se further improves semen quality.

Health problems in different species of animals might possibly be associated with impaired selenoprotein functions associated with antioxidant systems that are caused by frank and marginal Se-deficiency. All selenoproteins contain Se in the form of selenocysteine, which is encoded by a UGA triplet in the mRNA of the selenoprotein (reference needed). The amount of available Se limits the synthesis of selenocysteine for the synthesis of selenoproteins. There are actually two forms of tRNA[Ser]Sec, which are required for the synthesis of selenoproteins. Both tRNA forms are isoforms, but differ by the methylation of a ribose portion of the wobble nucleotide of the anticodon (reference needed). The methylation of $tRNA^{[Ser]Sec}$ has been thought to be relevant to the translation of glutathione peroxidase and possibly other selenoproteins (Moustafa et al., 1998). Se-deficiency might reduce the amount of methlylated $tRNA^{[Ser]Sec}$, which

would account for the decrease in selenoprotein mRNA concentrations. An example of this hypothesis was seen in a severely Se-deficient rat model in which almost all transcription of cGSH-px in the liver and heart were suppressed and Type I ID was decreased by 50% (Saedi et al., 1988).

A Se-deficiency affects the total antioxidant system because the mineral in the inorganic forms can be a pro-oxidant (Edens, 2002) and in the organic form (SeMet) it can be an anti-oxidant as well (Annika et al., 1998; Sies and Arteel, 2000). This lack of antioxidant protection can lead to an increase in oxidative stress and alterations in redox signaling (Gladyshev et al., 1999). Kayanoki et al. (1996) demonstrated that Se-deficient bovine renal epithelial cells were more susceptible to hydrogen peroxide induced apoptosis than the cells supplemented with Se. Several signaling pathways are also disrupted by reactive oxygen species (ROS). Se has been shown to have influence on signaling molecules such as NFκB and caspase-3 (Nomura et al., 1999). Both molecules are important in apoptosis signaling. Se has a direct effect on mitogen-activated protein (MAP) kinases, which are upstream molecules for the activation of NFκB (Adler et al., 1996). All of this information supports the hypothesis that Se-deficiency can be detrimental because of increased cellular damage and apoptosis.

Selenium Toxicity

Although Se is an essential trace mineral, it is also toxic at high levels of dietary supplementation (Franke, 1934). Se toxicity was observed in the Western United States in the 1930's range animals that consumed plants with high levels of accumulated Se showed signs of toxicity (Trelase and Beath, 1949; Spallholz, 1997). Selenites and

selenates from the soil accumulate in these plants as methylated Se compounds, which the plants convert in dimethyldiselenide and dimethylselenide. The consumption of high levels of Se resulted in selenosis, which involved hair loss, cracking of hooves, and interruption in coronary band development of the hoof (Miller and Shoening, 1938; Moxon, 1937).

Selenosis or Se toxicity in any species, can be classified in two categories depending on the amount of Se ingested and the duration of exposure (Kim and Mahan, 2001). Acute selenosis is a result of a high dietary intake (>20mg/kg) of Se for a short period of time (Miller and Williams, 1940). Signs of acute selenosis are respiratory distress, ataxia, diarrhea, and even death (Mahan and Moxon, 1984). Chronic selenosis is a result of a dietary intake of 5 to 20 mg/kg over a long period of time (Goehring et al., 1984). Chronic selenosis produces reduced feed intake and growth rate followed by hair loss, shedding of hooves, liver cirrohsis, and anemia (Ekermans and Schneider, 1982). Selenosis can inhibit production and depress health status of animals including humans.

There are several mechanisms through which Se can produce toxic effects. The most common and important mechanism is the production of the superoxide radical (Stohs and Bagchi, 1995). The superoxide radical has the ability to interact with thiols in mammals (Klaassen et al., 1985) and in birds (Hoffman et al., 1991). Reactions with thiols can alter the activity of many antioxidant sulfhydryl-containing enzymes as well as structural proteins in the body (Spallholz and Hoffman, 2002). When superoxides react with thiols they can generate destructive free radicals, which can cause irreversible cellular damage (Spallholz, 1994). Another way that Se can exert its toxic effects is through generation of excess hydrogen selenide as an intermediate in inorganic and

organic Se metabolism. Hydrogen selenide accumulates in animals receiving excess selenocysteine as a result of Se methylation metabolism being inhibited (Sayato et al., 1997). Excess hydrogen selenide can cause hepatoxicity and can result in other Se-related injuries (Spallholz and Hoffman, 2002). Since Se can substitute for sulfur in the formation of selenoproteins, Spallholz and Hoffman (2002) hypothesized that excess dietary SeMet might result massive incorporation into sulfur-containing enzymes and structural proteins that might cause embryo malformations and toxicity. However, Spallholz and Hoffman (2002) pointed out that pure SeMet injected into the embryo was far more toxic than Se incorporated into the embryo's proteins.

Toxicity of Se is not only related to the amount and duration of exposure but also the form that is ingested. Most toxicity studies have examined the effects of sodium selenite. In a study done by Lathshaw and Ort (1978), female chickens were fed graded levels of sodium selenite to determine at what level toxicity occurred. Toxic effects were observed at 5 ppm. In a separate study, adult male albino rats were fed 6 and 8 ppm of selenite for 6 and 9 weeks. Excess dietary selenite caused a reduction in body weights, reproductive organ weights and a significant increase in the amount of abnormal spermatozoa (Kaur and Kaur, 2000). Other studies have shown selenite toxicity to disrupt oestrous cycles, ovarian follicles, ovulation, and fetal development (Parshad, 1999).

Recent studies have compared the toxic effects of selenite to organic forms of Se. Since organic forms of Se do not generate superoxides it is hypothesized that they are less toxic than inorganic forms (Spallholz and Hoffman, 2002). In vitro studies with human lymphocytes have demonstrated toxic responses to the addition of sodium selenite

at 5μM/l but not with the addition of selenomethionine (Kelly and Powers, 1995). The same results were seen in an in vivo study in which male BALB/c mice were exposed to 0, 1, 3 or 9 ppm of Se as sodium selenite or seleno-L-methionine (SeMet) in the drinking water for 14 days. Thymus/body weight ratio was significantly reduced at 3ppm of sodium selenite. Selenite at 9 ppm also significantly increased the production of proinflammatory cytokines in LPS stimulated macrophages. Mice exposed to seleno-L-methionine (SeMet) did not express any signs of toxicity at any level. This indicated that inorganic Se is more toxic (Johnson et al., 2000). SeMet and selenocysteine at 2.5, 5, and 12.5 μM, have also been shown to have no effects on induced cytoxicity, oxidative DNA lesions, or apoptosis (Stewart et al., 1999). However, Lowry and Baker (1989) showed that pure SeMet at high dietary incorporation was toxic in avian species. In mallard ducks, pure L-selenomethionine was significantly more toxic than DL-selenomethionine (Heinz et al., 1996). All of these studies have led to the conclusion that high levels of Se in diets may induce negative health effects, but certain forms are more toxic than other forms.

Organic Se is becoming a more popular Se supplement in the world livestock sector. Organic Se as Sel-Plex® is the only source of organic Se that has been approved for use in poultry and livestock diets industry by the US Food and Drug Administration (Federal Register, 2000 and 2002). At the present time, there are no toxicity data available on the Se yeast products such as Sel-Plex®, suggesting that there is little if any negative effects with levels used in animal feeds.

Selenium and the Immune System

It has been evident since the 1970s that an adequate amount of dietary Se is necessary for optimum functioning of the immune system. Se has beneficial influences at several points in both the cell-mediated and antibody-mediated branches of the immune system. Not only does the immune system rely on the antioxidant properties of Se to control oxidative bursts by phagocytic cells it also has some endocrine effects that benefit antibody-mediated immunity.

Se at levels as low as 0.1 mg Se/kg diet can increase significantly the amount of plasma 3,5,3′-triiodothyronine (T_3) concentration (Jianhua et al., 2000). T_3 is converted from thyroxine (T_4) as a result of the action of the Se-dependent iodothyronine deiodinase selenoenzymes. In fact, Se-deficient chickens exhibit decreased plasma T_3 and increased plasma T_4 concentrations (Jianhua et al., 2000). Masaly et al. (1998) noted that there was a strong correlation between the increase in plasma T_3 and circulating antibody 2 days post injection with Brucella abortus (BA). They also showed that there was a decrease in plasma T_3 3 to 6 hours after BA injection, which was attributed to increased circulating corticosterone concentration (Decuypere et al., 1983). Corticosterone inhibits the antibody response to SRBC in mice (Johnson et al., 1982). These observations support the concept that there is a strong relationship between T_3 and the production of antibodies by plasma cells. Thus, in the face of a stressor, Se-supplementation appears to act as a suppressor of corticosterone secretion, increases the concentration of T_3, and increases antibody levels in circulation.

Se impacts the immune system through up-regulation of the interleukin-2 receptor α and β subunits on lymphocytes (Roy et al., 1994), which results in a greater affinity for the cytokine IL-2. IL-2 enhances proliferation of B-cells, T-cells, and natural killer cells (Kiremidjian-Schumacher et al., 1996). Kiremidjian-Schumacher et al. (1996) gave patients a 200μg/day sodium selenite supplement for a period of eight weeks. Se supplementation up-regulated the activity of cytotoxic T-cells (118%), natural killer cells (82%), and down-regulated suppressor T-cells. These results were likely a result of the up-regulation of IL-2 receptors on lymphocyte surfaces. However, the effect on the immune system has been shown to be dose-dependent when using sodium selenite. For example, Koller et al. (1986) gave in the drinking water 0.5 ppm, 2.0 ppm, and 5.0 ppm sodium selenite supplements to rats. The activity of natural killer cells was enhanced in rats given 0.5 and 2.0 ppm. However, the response in the group given 5.0 ppm was equivalent to the unsupplemented animals. Antibody production was not significantly increased but did decrease in the group supplemented with 5.0 ppm (Koller et al., 1986). A negative effect was also seen in natural killer cells and lymphocytes when the cells were cultured with 0.8 μg of sodium selenite/ml of medium. Lymphocyte and T-cell proliferation was inhibited by sodium selenite at 0.5-1.0μg/ml (Nair and Schwartz, 1990). These observations demonstrate the importance of the appropriate chemical form and dosage of Se in the immune system.

Up-regulation of adhesion molecules and pro-inflammatory cytokines is often observed during recruitment of inflammatory cells to traumatized tissues. Se has an inhibitory effect on adhesion molecules and pro-inflammatory cytokines. Horvatha et al. (1999) found that asthmatics have a significantly higher expression of P-selectin, vascular

adhesion molecule-1, E-selectin, and intercellular adhesion molecule-1 on endothelial cells. The asthmatic patients were treated with Se supplements for three months and were found to exhibit a significant decrease in vascular adhesion molecule-1 and E-selectin. D'Alessio et al. (1998) explained that Se caused down-regulation of these adhesion molecules. Additionally, D'Alessio et al. (1998) demonstrated that glutathione peroxidase prevented tumor necrosis factor-α-stimulated expression of P-selectin and E-selectin and the release of other pro-inflammatory cytokines.

Inflammatory responses can be induced by oxidative stress. Se has been found to bind to the activation sites for AP-1 and NF-κB on several pro-inflammatory cytokine gene promoters (Powis et al., 1997). Selenium status has plays an important role in the reduction of oxidative stress in the body. Se-deficiency can result in a significant decrease in the activity of selenoenzymes and an increase in the production of reactive-oxygen-species (ROS) (Allan et al., 1999). Se-deficiency is associated with an increase in reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite production (Prabhu et al., 2002). The RNS, similar to ROS, cause irreversible damage to cells by free radical damage to proteins, lipids and DNA. The addition of selenocysteine and especially, selenomethionine can protect plasmid DNA from peroxynitrite damage (Roussyn et al., 1996). It was later proven that this protection was seen only in the SeMet and selenocysteine groups, not the sodium selenite group (Briviba et al., 1996). The reason for this difference resides in the ability of SeMet residues in selenoproteins and in structural proteins to act in an antioxidant capacity. In conjunction with glutathione, even in micromolar concentrations, the selenoxide form of SeMet (MetSeO) that results from oxidative action of peroxynitrite is regenerated (Sies and Arteel, 2000; Assmann et al.,

1998). This is a very important redox mechanism that is dependent on Se in SeMet and to a limited extent selenocysteine. Selenocysteine is specifically incorporated into selenoproteins using the specific selenocysteine insertion element (Larsen and Berry, 1995), but SeMet is incorporated into protein random replacing the sulfur analogue of methionine (Schrauzer, 2003). The reactivity of peroxynitrite toward SeMet in proteins is much higher than for methionine as evidenced by a higher second order rate constant (Padmaja et al., 1996) and by more effective protection against oxidation and nitration reactions (Brivida et al., 1996; Sies and Arteel, 2000). These observations show that the organic selenium as SeMet, even in protein, can act as an antioxidant but the sulfur analogue cannot perform the same functions.

The role of Se in the immune system of chickens is of growing interest to many commercial production companies. Marsh et al. (1986) fed low levels of sodium selenite to chickens and observed an increase in the relative weights of lymphoid organs. The increase in lymphoid organ weights was an indication of a more proliferative immune system. Marsh et al. (1986) saw a degeneration of the bursal epithelium in Se-deficient birds. Chang et al. (1994) studied the influence of sodium selenite and vitamin E on T and B cell markers and lymphocyte proliferation. Lymphocyte proliferation was impaired with Se and vitamin E deficiency, and the amount of $CD4^{+}CD8^{+}$ T cells increased significantly when chickens were fed the Se-deficient diet. These results indicate that vitamin E and Se deficiencies affect the maturation of T cells and the proliferative responses of peripheral lymphocytes.

Leng et al. (2003) compared the influence of either sodium selenite or organic Se sources on the immune system of layer chickens. They determined that organic Se

sources increased $CD3^+$, $CD4^+$, and $CD8^+$ surface markers on T cells located in several lymphoid structures in young chickens. All markers are involved in associating the T cells with antigen presenting cells (APCs). Leng et al. (2003) concluded that organic Se supplements improved the status of the avian immune by increasing the ability of immunocompetent cells to respond to an antigen challenge.

The relationship between the immune system and modern-day poultry performance is complex. The general opinion is that modern poultry have been selected to have a less responsive immune system in order to provide energy for the increased growth rate and body weight in a confined environment. In fact, the decreased immune functions are due to energy demand and nutrient requirements that are no longer being met by the nutrition of the broiler chicken. Supplementation of the appropriate form of Se in modern-day poultry diets is one factor that can enhance the immune functions of poultry. Organic Se in Sel-Plex® has been shown to improve immunocompetence when compared with sodium selenite.

Selenium and Broiler Performance

In commercial poultry production, stress is a common factor that has a detrimental effect on performance and reproduction of birds. The effects of stress are often expressed as an increased oxidative process, which can be countered with antioxidant supplementation (Surai, 1999). Among the natural antioxidants in poultry diets, Se is of great importance. Even in normal conditions, Se supplementation appears to have a beneficial affect in poultry production.

Many studies have reported that Se is essential in poultry diets. Edens (2001) saw a decrease in heat shock proteins, improved feed conversion, decreased mortality, and an improved body weight in the face of an E coli challenge with birds that were supplemented with organic Se. Positive effects of Se supplementation were seen when immunostimulating properties were evaluated (Gowdy and Edens, 2003). In a study by Stanely et al. (1998) chickens were subjected to cold stress and fed aflatoxin-contaminated feed supplemented with 0.1 ppm of organic Se with 500mg/kg of vitamin E. The birds given the antioxidant supplements had a decreased mortality and incidence of pulmonary hypertension syndrome. Roch et al. (2000) saw similar results when broiler chickens were exposed to the same stressors and supplemented with varying levels of organic or inorganic Se and vitamin E. The birds fed 0.3ppm of organic Se in combination with 250 ppm of vitamin E performed the best in the face of the challenge.

In normal conditions, Se supplementation to broiler diets will result in beneficial effects. Body weights of broilers at 42 days of age was 2.38 kg in the control group, 2.43 kg with 0.2 ppm of selenite supplementation, and 2.45 kg with 0.2 ppm of Sel-Plex® supplementation (Edens, 2001). Naylor et al. (2000) fed inorganic and organic Se supplemented diets at 0.1 and 0.25 ppm and found that the higher level of dietary Se improved feed efficiency. Improved growth rate of broiler chickens fed an organic Se-supplemented diet may be a result of an increased concentration of T_3 or even expression of less stress (Edens, 2001). Organic Se supplementation has been associated with better feathering of chickens, particularly during cold stress conditions (Edens, 1996, 1997, 2001).

Dietary Se supplementation appears to have a positive effect on the cut-up yields of broilers. The inclusion of organic Se in the diet improved the eviscerated weight and breast yield of broilers (Naylor et al., 2000) confirming the observations made by Edens (1997). Edens (1997, 2001) evaluated the carcass weight, yield of viscera, feet, neck, and leg yields with broilers supplemented with organic Se and found a significant increase in total carcass yield and cut-up parts with the exception of the pectoralis major muscle.

A synergism between Se and vitamin E may enhance meat quality (Edens, 1997, 2001). GSH-px activity is elevated in chickens fed organic selenium suggesting an improved redox status (Mahmoud and Edens, 2003). Therefore, Se supplementation may allow increased antioxidant protection through elevated GSH-px activity in the living broiler that leads to improved cell membrane integrity resulting from less lipid peroxidation and free radical production in the post-mortem state. Broiler diets supplemented with 0.25 ppm of Se increased GSH-px activity in the breast (2.1- fold) and leg (4.1 fold). The elevated GSH-px activity resulted in a decrease in lipid peroxidation after 4 days of storage at 4ºC (DeVore et al., 1983). In a study by Surai and Dvorska (2002), spontaneous and iron stimulated peroxidation in the muscle tissue of birds fed vitamin E and organic Se were significantly lower than birds with no antioxidant supplementation.

Se supplementation has an effect on the maintenance of muscle and red blood cell membrane integrity of poultry. Drip loss of breasts was decreased with birds fed organic Se, but not sodium selenite (Edens, 1996). The membrane stability of red blood cells was also improved in birds fed organic Se (Edens, 2001). Further studies concluded that pale, soft, exudative meat problems could be controlled by increased antioxidant

supplementation. Organic Se specifically was more efficient than selenite in reducing the frequency of PSE breast meat in chickens (Edens, 2001). Similarly, pigs, fed either sodium selenite or organic Se, had poorer loin quality, greater drip loss, and a paler color (Mahan et al., 1999). These data suggest that a diet supplemented with organic Se improves meat quality during storage.

Objectives of work

Selenium is an essential trace mineral for all animals. Organic Se is more available and safer than inorganic selenium. Sodium selenite that has been used for many years has pro-oxidant properties, which causes cellular damage instead of preventing it. Organic Se has antioxidant properties that benefit many physiological and biochemical functions. Organic Se is easily integrated into body proteins whereas inorganic forms of Se are loosely associated with thiols through a labile bond. Selenomethionine in body proteins provide a reversible storage of Se in body tissues and organs. Se must function through specific selenoproteins of which 25 are known today and play essential roles in the protection of the body against stress.

Poultry similar to mammals express the essential selenoproteins, but the functions of the selenoproteins remain to be described in both mammals and birds. Poultry selenoproteins that have been studied are, GSH-px, iododeiodinases, and TR. Performance of these birds as indicated by body weight, feed conversion, meat yield, and disease resistance are influenced greatly by selenoproteins. Physiological functions such as immunity, resistance to stressors, reproduction, growth, development, endocrine functions, and digestive functions are all influenced by the Se status in poultry.

The emphasis of this thesis is on the different supplemental forms of Se and their effects on broiler health. The experimental process was divided into four main areas: 1) to evaluate different Se supplements and their effects on poultry immunity, more importantly T cell production, antibody production, and nitrite production of macrophages; 2) to evaluate the high levels of Se supplements and their effects on body/organ weights, delayed hypersensitivity response, feed conversion, antioxidant enzyme activity and T cell proliferation; 3) to evaluate the expression of TR from broilers fed different Se supplements; 4) to attempt to purify and characterize chicken TR.

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Chapter 1

The Effects of Selenium Supplements on Antibody and Nitrite Production in Broilers

Abstract. Diets supplemented with 0.3 ppm of sodium selenite, Sel-Plex®, or a combination of both were fed to male broiler chicks from the time of hatching until 3 weeks of age. The effects of these different forms of selenium on antibody production and sephadex elicited nitrite production were examined. A control group was also examined fed a basal diet with no supplemental selenium. The amount of antibodies produced against a 7% SRBC suspension increased numerically with selenium supplementation. The IgG response was significantly higher with birds fed Sel-Plex®, while the IgM response was higher with birds fed selenite. All selenium supplemented groups maintained higher titers longer than the control group. The nitrite production was higher at resting levels with the control group indicating the antioxidant properties of selenium. However, when stimulated with LPS, selenium supplemented groups had a higher nitrite production than the control with the selenite fed group being the highest. Both the combination and Sel-Plex® fed groups had a higher response than the control, but less than selenite. This indicates the prooxidant properties of selenite that other studies have previously noted. These results support the conclusion that supplementing with an organic form of selenium such as Sel-Plex® results in a heightened immune response that is more controlled than selenite fed groups. Selenium is essential to a healthy avian immune system.

Key Words: Poultry, Selenium, Immune system, Antibody, Nitrite, Antioxidant.

Introduction

Selenium (Se) is essential for the optimum functioning of the immune system. Selenium has beneficial effects on both the innate and adaptive (cell-mediated and antibody-mediated) immune responses (Brown and Arthur, 2001), and selenium influences the immune system at several mechanistic levels in these immunological responses. Not only does the immune system rely on the antioxidant properties of Se to control oxidative bursts by phagocytic cells, it also has some endocrine effects that benefit antibody-mediated immunity.

Dietary Se significantly increases the amount of plasma 3,5,3′-triiodothyronine (T_3) concentration at levels as low as 0.1 mg Se/kg diet (Jianhua et al., 2000). T_3 is part of a Se dependent pathway that relies on a major class of selenoproteins, iodothyronine deiodinase enzymes (IDs). The IDs catalyze the 5′-monodeiodonation of thyroxine (T_4) to the active thyroid hormone T_3 (Brown and Arthur, 2001). Selenium deficient birds have decreased plasma T_3 concentrations and an increased plasma T_4 concentrations (Jianhua et al., 2000). There is a strong correlation between the increase in plasma T_3 and circulating antibody 2 days post injection (Mashaly et al., 1998). Mashaly et al. (1998) demonstrated that there was a decrease in plasma T_3 3 to 6 hours after a *Brucella abortus* (BA) challenge. The decrease in plasma T_3 might be due to increased plasma coricosterone (primary adrenal glucocorticoid secreted in response to stress) concentrations (Edens and Siegel, 1975), which had been reported previously by Decuypere et al. (1983). Corticosterone inhibits the antibody response to SRBC in mice (Johnson et al., 1982) and in chickens (Glick, 1967; Elliot and Sinclair, 1968). These observations demonstrate a relationship between T_3 and the production of antibodies.

Thus, in the face of a stressor, selenium-supplementation appears to act as a suppressor of corticosterone secretion, increases the concentration of T_3, and increases the antibody levels in circulation.

Selenium status plays a vital role in the protection of the body against oxidative stress. Se-deficiency can result in a significant decrease in the activity of selenoenzymes and allow increased production of reactive oxygen species (ROS) (Allan et al., 1999). Se-deficiency has also been associated with an increase in reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite production (Prabhu et al., 2002). These reactive elements can cause irreversible damage to cells by free radical damage to proteins, lipids and DNA. Some studies have shown that the addition of selenomethionine can protect plasmid DNA from peroxynitrite damage (Briviba et al., 1996; Roussyn et al., 1996).

The role of Se in the immune system of chickens is of growing interest in the realm of commercial poultry production. Marsh et al. (1986) fed low levels of sodium selenite to chickens and observed an increase in the relative weights of lymphoid organs and noted degeneration of the bursal epithelium in Se-deficient chickens. Chang et al. (1994) studied the interaction of sodium selenite and vitamin E on T and B cell markers and lymphocyte proliferation. Lymphocyte proliferation was impaired with Se and vitamin E deficiency, and the expression of $CD4^+CD8^+$ T cells increased significantly when the Se-deficient diet was fed. These results indicate that vitamin E and Se deficiencies affect the maturation of T cells and the proliferative responses of peripheral lymphocytes.

Leng et al. (2003) compared the influence of either sodium selenite or organic Se sources on the immune system of layer chickens. They determined that organic Se sources increased $CD3^+$, $CD4^+$, and $CD8^+$ surface markers on T cells located in several lymphoid structures in young chickens. All markers are involved in associating the T cells with antigen presenting cells (APCs). Leng et al. (2003) concluded that organic Se supplements improved the status of the avian immune system by increasing the ability of immunocompetent cells to respond to an antigen challenge.

The relationship between the immune system and modern-day poultry performance is complex. The general opinion is that poultry have been selected to have a less responsive immune system in order to provide energy for the increased growth rate and body weight in a confined environment. In fact, the decreased immunological functions are due to energy demand and nutrient requirements that may no longer be met by the diets of the modern-day broiler chicken. Supplementation of the appropriate form of Se in modern-day poultry diets is one factor that can be addressed to enhance the immunological functions of today's broiler chicken. Organic Se in Sel-Plex® (Alltech Inc., Nicholasville, KY 40356) has been shown to improve immunocompetence when compared with sodium selenite (Leng et al., 2003).

This investigation examined immune responses of modern-day broilers given a diet with either no supplemental selenium (Control), supplemented with sodium selenite, supplemented with organic selenium as selenium enriched yeast, or supplemented with a combination of sodium selenite and organic selenium in equal levels. Parameters examined included antibody response to a primary and secondary SRBC injection and nitrite production of Sephadex stimulated macrophages.

Materials and Methods

Animals and diets

This project was approved and conducted under the supervision of the North Carolina State University Animal Care and Use Committee which has adopted Animal Care and Use Guidelines governing all animal use in experimental procedures.

Ross male broilers were fed the North Carolina Agricultural Research Service broiler starter feed (3154 kcal/kg ME, 21% CP) from 1d of age to 3 wk of age. Feed was supplemented with either 0.3 ppm of sodium selenite (SE), Sel-Plex® (SP; Alltech, Inc., Nicholasville, KY 40356), 0.15 ppm of both sodium selenite and Sel-Plex® (SS), or a control diet (C) that was diluted with sand to be equivalent to the experimental diets in terms of total nutrients available per unit-volume. The study was conducted as 2 battery trials with 3 replicates per treatment and 10 birds per replicate. Replicates were arranged in a completely randomized design with blocking for light and position within the battery. The broiler chickens were fed and provided water for ad libitum consumption.

Antibody Production by SRBC

Sheep red blood cells (SRBC) were used to elicit an antibody response. In both trials, six birds per treatment were injected intravenously with SRBC (1mL/chick of a 7% suspension in phosphate-buffered saline[1]) at 1 wk of age followed by a booster injection of SRBC at 10d post primary injection. Blood samples were collected at 0, 5, and 10d after the first injection and at 5 and 10d after the second injection. Serum from each sample was collected and frozen (-40°C) until analysis. Serum was heat inactivated at 56°C for 30 min and then analyzed for total, mercaptoethanol[1]-sensitive (MES) IgM and

[1] Fisher Scientific, Pittsburgh, PA 07410

mercaptoethanol-resistant IgG anti-SRBC antibodies as previously described (Delhanty and Solomon, 1966; Qureshi and Havenstein, 1994; Yamamoto and Glick, 1982).

Nitrite Production

Nitrite production of macrophages was determined in both trial 1 and 2. Six birds per treatment were injected with a 3% suspension of Sephadex G-50[1] at a concentration of 1mL/100g BW. Birds were injected intra-abdominally at 21 d of age. At 24 h after injection, birds were killed by CO_2 asphyxiation and the abdominal cavity was flushed with sterile heparinized saline (0.85%). Abdominal exudates were collected in siliconized tubes and spun at 1500 rpm for 10 minutes. Cell pellets were resuspended in RPMI-1640[2] with 5% heat inactivated fetal calf serum and 1% antibiotics. Macrophages were then counted in a hemocytometer and then diluted to make a $1x10^6$ cells/1mL suspension.

Nitrite production of macrophages from the abdominal exudates in response to LPS[2] stimulation was analyzed by Greiss reagent method (Green et al., 1982). Macrophages from 24 birds were pooled into four groups based on treatment, cultured in a 24-well plate ($1x10^6$ cells per well), and stimulated with LPS from E. coli (1μg/5μl/well) for 24 h. Supernatants were then collected and analyzed for nitrite production by the Greiss Reagent method (Green et al., 1982). A 100μL volume of macrophage supernatant and Greiss Reagent were added into each well of a 96-well flat-bottomed plate[3]. After 10 minutes of incubation at room temperature, the plates were read at 540 nm on an ELISA plate reader[3]. For each treatment, an average of 13 readings was used for an accurate calculation of nitrite production. Using dilutions of 10mM

[2] Sigma, St.Louis, MO 63103
[3] Bio-Rad, Richmond, CA 94547

stock solution of sodium nitrite in RPMI-1640[2] medium, a standard curve was generated for each assay. Nitrite levels in the supernatants were calculated by using optical density against the standard curve.

Statistical Analysis

Data were analyzed for main effects and their interactions using the general linear model procedure of SAS (SAS Institute, 1995). Means were examined for significance by Least Square Means test at the significance level of $P<0.05$. Data from each trial were analyzed separately due to responses that differed between trials.

Results

The antibody response data for trials 1 and 2 are presented in Tables 1 and 2, respectively. Antibody titers were measured at 0, 5, and 10d post-primary injection, and at 5 and 10d post-secondary injection in both trials 1 and 2. Day 0 serum was measured for the presence of maternal and nonspecific antibody titers. In both trials, d 0 resulted in no agglutination of SRBC. In trial 1, there were no significant differences among treatments for primary antibody titer. However, numerically at 10d, the birds fed SP had a higher titer. At 5d post secondary injection SE fed birds had a numerically higher titer, and a significantly higher IgM response ($P=0.0288$). The IgG titer at 5d was numerically higher in birds fed SP and SS when compared to the control and SE groups. There was a significant difference among treatments at 10d post-secondary injection (20d). All groups fed Se had a higher total antibody titer than the control group. The SE treatment had the highest total and IgM titer at 20d. In trial 2, there were significant differences among treatments for the primary antibody response. At 5d, all of the Se treatments were

significantly higher than the control group (P= 0.0298). SE and SP total and IgM titers were significantly higher than control or SS on d5. The secondary antibody response on day 15 was not significantly different among treatments, but all of the Se treatments were numerically higher than the control. At 15d, there was a tendency for higher IgG titers in the SP group and higher IgM titers in the SE group. The 20d IgG titers were significantly higher for the SE group (P=0.0118). The nitrite levels differed significantly among treatments for both trial 1 and 2 (Tables 3 and 4, respectively). Unstimulated macrophages from birds fed SP and SS produced comparable nitrite levels (SP= 0.29μM, SS= 0.12μM Trial 1), but unstimulated macrophages from the control fed group produced a negative value (C= -0.17μM Trial 1). The negative value for the unstimulated macrophages from the control-fed group represents the possibility of mechanical trauma and increased cell death at the time of collection. Nitrite levels of unstimulated macrophages from SE-fed birds were significantly higher (SE= 1.42μM Trial 1) than levels from other nonstimulated macrophages (Table 1) suggesting that sodium selenite had pro-oxidant potential.

Unstimulated macrophages from Trial 2 (Table 4) produced nitrite levels that were significantly higher than the levels found in trial 1 (Table 3). In trial 2, nitrite levels from the control group were significantly higher than the levels of nitrite produced by unstimulated macrophages from the selenium fed groups. Among the selenium-fed groups, unstimulated macrophages from the SS group had the highest nitrite production level followed by SP and SE groups (SP= 1.84μM, SE= 1.25 μM, and SS= 2.71μM). These results indicate that Se through the glutathione-glutathione peroxidase system is

reducing the number of reactive nitrogen species (RNS) being produced by the macrophages at resting levels.

Stimulated macrophages from trial 1 and 2 yielded similar results (Tables 3 and 4, respectively). Nitrite levels from control-fed LPS-stimulated macrophages were significantly lower ($P<0.05$) in trial 1 compared with trial 2 (C= 1.51μM Trial 1; C= 5.52μM Trial 2). The LPS-stimulated macrophage nitrite levels from Se-fed birds were significantly higher than nitrite levels from LPS-stimulated control-fed birds in both trials 1 and 2 (SP= 6.42μM, SE= 7.88μM, SS= 6.45μM, Trial 1; SP= 7.06μM, SE= 9.24μM, SS= 7.10μM Trial 2). In both trials, LPS-stimulated macrophages from SE-fed birds produced the highest nitrite levels (Tables 3 and 4). Nitrite levels produced by LPS-stimulated macrophages from SP- and SS-fed groups were similar and lower than the nitrite levels produced by LPS-stimulated macrophages from SE-fed birds (Tables 3 and 4). The data show that Se supplementation affected nitrite production of macrophages at rest, and in the organic form, Se enhanced nitrite production when macrophages were stimulated but to a lower level than the results from macrophages from SE-fed birds.

Discussion

Previous studies have demonstrated that adequate levels of Se are crucial for the proper development of the immune system. A dietary deficiency of both Se and vitamin E has been shown to cause a decreased response to mitogens such as phytohemagglutinin antigen (PHA-P) or Concanavalin A (ConA) and bacterial lipopolysaccharide (LPS) (Sheffy and Schultz, 1979). Most of the earlier studies only compared the immune response of animals supplemented with sodium selenite (Chang et al., 1994; Marsh et al.,

1986), but there has been no examination of immune responses in birds supplemented with organic Se supplemented as the selenium yeast product Sel-Plex®. Our previous work demonstrated that signs of toxicity in lower immune organ weights such as the thymus and bursa of Fabricius and in lower T cell numbers in the modern high-yielding broiler chicken began to develop when sodium selenite was fed at a level as low as 1.2 ppm (Gowdy and Edens, 2003). Birds supplemented with Sel-Plex® up to 15 ppm had normal lymphoid organ weights and T cell numbers. Thus, sodium selenite, while having a strong history showing stimulated growth and improved feed conversion in broilers, appears to have some negative influences on the development of the immune system in broilers. With the exception of the control-fed unstimulated macrophage response in trial 1, nitrite levels in trials 1 and 2 followed similar trends. Even though the control-fed nitrite levels in trial 1 were lower than the nitrite levels in trial 2, attributed to mechanical trauma and increased cell death, similar selenium related responses of stimulated macrophages were found in both trials. Prabhu et al. (2002) noted that there is an inverse relationship between cellular Se status and nitrite production in stimulated macrophages. Selenium supplementation provides antioxidant protection at resting levels and an enhancement of macrophage nitrite production when there is stimulation by mitogens. However, there is a selenium form influence on nitrite production. The inorganic sodium selenite allows for greater nitrite production by stimulated macrophages and sets the stage for the possibility of increased peroxynitrite production, which can cause damage to cell membranes and the nucleic acids within the cell (Masumoto and Sies, 1996; Sies et al., 1997; Assmann et al., 1998; Sies and Arteel, 2000). The apparently more highly controlled nitrite production by stimulated macrophages from organic selenium fed birds

possibly was due to the organic selenium influence on higher activities of glutathione peroxidase (GSH-px) and thioredoxin reductases (Masumoto and Sies, 1996; Sies et al., 1997; Assmann et al., 1998; Sies and Arteel, 2000). These selenoproteins are involved in many biological processes such as the protection against oxidative stress from both reactive nitrogen (RNS) and oxygen species (ROS) (Holben and Smith, 1999). Se-deficiency results in a decrease in the activity of these enzymes and an increase in the amount of RNS and ROS, which have been associated with many diseases such as cardiomyopathy (Azoicai et al., 1997), rheumatoid arthritis (Knekt et al., 2000), cancer (Combs, 1999), and multiple sclerosis (Liu et al., 2001).

The majority of previous macrophage research dealing with Se was done *in vitro*. The present study is indicative of *in vivo* reactivity of the macrophage that has been stimulated. The intra-abdominal Sephadex injection elicits an inflammatory response, but the SP and SS groups had a controlled response that demonstrated the importance of organic selenium in the antioxidant process. In our previous studies, with birds fed either selenite or Sel-Plex® at 0.3, 0.6, or 1.2 ppm, there was an obviously improved health status for broilers fed organic selenium as compared with the feeding of sodium selenite (Gowdy and Edens, 2003; Mahmoud and Edens, 2003). Intradermal wingweb injections of PHA-P induced a delayed type cutaneous basophilic hypersensitivity (CBH) response that was measured 24 h after injection (Gowdy and Edens, 2003). Birds fed Sel-Plex® had a significantly lower CBH than the sodium selenite-fed birds at 0.6 and 1.2 ppm. Both selenium-supplemented groups had a greater CBH than the control group that had no supplemental selenium, and birds fed sodium selenite had a greater CBH than did Sel-Plex®-fed birds. The lessened CBH response associated with Sel-Plex® feeding is

associated with increased glutathione peroxidase activity in lymphocytes and granulocytes that migrate to the site of antigen stimulation (Brown et al., 2000). An increase in the production of ROS following sodium selenite supplementation was observed by Johnson et al. (2000) who attributed the increased ROS production of proinflammatory cytokines such as TNF α and IL-1β. Johnson et al. (2000) found the highest production of ROS in the sodium selenite group compared with the selenomethionine group. Although measurements of neither ROS, RNS, nor GSH-px were made in the current study, redox studies done in this laboratory support this as a mechanism for the control of nitrite production in stimulated macrophages (Mahmoud and Edens, 2003).

In a study similar to the current investigation, mice were exposed to 0, 1, 3, or 9 ppm of sodium selenite or seleno-L-methionine in the drinking water for two weeks (Johnson et al., 2000). Primary immune organ weights, cytokine production, and PHA-P induce lymphocyte proliferation were measured. Results indicated that sodium selenite at greater than 3 ppm enhancement inflammatory responses that were not seen in the seleno-L-methionine supplemented mice (Johnson et al., 2000). Those results are consistent with our present study where it was apparent that nitrite production was significantly higher in the SE-fed birds than in the SP- or SS-fed groups. The data from this current investigation shows a strong correlation between pro-oxidant responses of LPS-stimulated macrophages and SE-feeding.

The effect of Se on antibody production has also been described in other studies. Larsen et al. (1988) demonstrated a significant increase in serum IgG concentrations of lambs fed selenomethionine compared to the groups fed sodium selenite. However, the

group fed sodium selenite at 1.0 ppm had the lower antibody titer to parainfluenza virus challenge when compared with the control. The results from Larsen et al. (1988) suggest an inhibitory effect of sodium selenite on antibody-mediated response to a viral challenge. Spallholz et al. (1990) made a similar observation when mice fed sodium selenite at 1.25 ppm showed a decrease in the number of spleen lymphocytes producing antibodies to SRBC. Although the current study did not produce an inhibitory effect on antibody production with sodium selenite, all antibody titers of selenium-supplemented birds were significantly higher than the control. Similar results were noted by Berenstein et al. (1972) in rabbits given selenium supplements with or without vitamin E.

The use of selenomethionine (SeMet) as a feed additive increases whole blood Se concentrations and total GSH-px values more efficiently than selenite (Larsen et al., 1988). Intestinal absorption is also greater with organic forms of Se during investigations of the functions of the immune system (Young et al., 1982). Thus, more absorbed selenium should result in an increase in selenoprotein activity, most importantly GSH-px, iodothyronine deiodinases, and thioredoxin reductase.

Selenium has an impact on the immune system through the up-regulation of the interleukin (IL)-2 receptor α and β subunits on lymphocytes (Roy et al., 1994) that causes a greater affinity for the cytokine IL-2, which enhances proliferation of B cells, T cells, and natural killer (NK) cells (Kiremidjian-Schumacher et al., 1996). A negative effect was seen on NK cells and lymphocytes when cells were cultured with 0.8 μg of sodium selenite/mL of medium. Lymphocyte and T cell proliferation was inhibited by 0.5-1.0μg/mL (Nair and Schwartz, 1990) supporting the importance of the appropriate chemical form and dosage of selenium supplementation on the immune system.

The current investigation suggests that selenium supplementation has a beneficial effect on antibody production and that the organic form of selenium (SeMet in yeast protein) aids in the production and removal of nitrite through the glutathione-glutathione peroxidase system (Masumoto and Sies, 1996; Sies et al., 1997; Assmann et al., 1998; Sies and Arteel, 2000). The chemical forms of selenium and selenium in the diet each have important influences on immune responses to various types of bacterial and viral challenges. The observations made in the current study are consistent with immunological responses of mammals and avian species investigated earlier. It appears that a selenium deficiency or inorganic selenium supplement can result in either impaired innate immune responses or an impaired inflammatory response. In the current investigation with poultry, organic selenium, provided as SeMet in the yeast product- Sel-Plex®, has been shown to be superior to inorganic forms in aiding immunological responses to antigenic challenge or macrophage stimulation.

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Table 1: The effect of different selenium supplements on the antibody response to SRBC in 3 weeks old Ross male broilers. At 1 week of age, 6 birds/group are immunized with 1mL of 7% SRBC suspension to examine the antibody-mediated immune response. Serum is collected at day 0, 5, and 10 after primary injection, and day 5 and 10 after secondary injection. The values are reported in $\log_2$ (2 log reduction in titers). Trial 1.

	DAYS POST SRBC INJECTION				
	0	5	10	15	20
		Total Anti-SRBC Antibody Response			
Control	0	1.167	0.67	6	1.167[c]
SE 0.3ppm	0	1.67	0.833	7	2.5[a]
SP 0.3ppm	0	1.67	1.167	6.167	1.83[b]
SS 0.3ppm	0	1.0	0.67	6	1.83[b]
Pooled SEM	0	0.512	0.431	0.768	0.428
P value	0	.2637	.7875	.3684	.0396
		IgG Anti-SRBC Antibody Response			
Control	0	0	0	0.833	0.33
SE 0.3ppm	0	0	0	1.33	1.167
SP 0.3ppm	0	0.167	0	1.5	1.167
SS 0.3ppm	0	0.167	0	2.17	1.167
Pooled SEM	0	0.1178	0	0.4518	0.3395
P value	0	1	0	.4431	.098
		IgM Anti-SRBC Antibody Response			
Control	0	1.67	0.67	5.167[ab]	0.833
SE 0.3ppm	0	1.67	0.833	5.67[a]	1.33
SP 0.3ppm	0	0.833	1.167	4.67[ab]	0.67
SS 0.3ppm	0	1.0	0.67	3.83[b]	0.67
Pooled SEM	0	0.512	0.431	0.55	0.271
P value	0	.1195	.1379	.0288	.1395

[a,b,c] Means with unlike superscripts differ significantly ($P<0.05$).

Table 2: The effect of different selenium supplements on the antibody response to SRBC in 3 week old male broilers. At 1 week of age, 6 birds/group are immunized with 1mL of 7% SRBC suspension to examine the antibody-mediated immune response. Serum is collected at day 0, 5, and 10 after primary injection, and day 5 and 10 after secondary injection. The values are reported in $\log_2$ (2 log reduction in titers). Trial 2.

	DAYS POST SRBC INJECTION				
	0	5	10	15	20
		Total Anti-SRBC Antibody Response			
Control	0	1.5^{a}	0.5	5.0	4.0
SE 0.3ppm	0	3.5^{b}	0.167	7.0	3.83
SP 0.3ppm	0	3.83^{b}	0.5	6.67	3.67
SS 0.3ppm	0	2.5^{ab}	0.167	6.5	2.83
Pooled SEM	0	0.6412	0.268	0.578	0.456
P value	0	.0298	.07	.0556	.7989
		IgG Anti-SRBC Antibody Response			
Control	0	0	0	2.0	1.5^{ab}
SE 0.3ppm	0	0.167	0	1.3	2.5^{b}
SP 0.3ppm	0	0	0	2.67	1.5^{ab}
SS 0.3ppm	0	0	0	2.0	0.67^{a}
Pooled SEM	0	0.083	0	0.649	0.541
P value	0	1	0	.1623	.0118
		IgM Anti-SRBC Antibody Response			
Control	0	1.5^{a}	0.5	3.0	2.5
SE 0.3ppm	0	3.3^{a}	0.167	5.67	1.33
SP 0.3ppm	0	3.83^{b}	0.5	2.83	1.83
SS 0.3ppm	0	2.0^{a}	0.167	4.5	2.17
Pooled SEM	0	0.653	0.269	0.6169	0.6164
P value	0	.0201	.5421	.7064	.2267

^{a,b,c} Means with unlike superscripts differ significantly ($P<0.05$).

Table 3: The effect of different selenium supplements on nitrite response of abdominal exudates macrophages from 3 week old male broilers. Macrophage cultures (from 6 birds/treatment) were stimulated to LPS from Escherichia coli (1μg/1 x 10^6 macrophages) for 24 h at 3 wk of age. The culture supernatant was tested for nitrite levels by the Greiss reagent method. Trial 1.

Treatment	Nitrite (μM) with LPS	P value	Nitrite (μM) without LPS	P value
Control	1.51		-0.17	
SP 0.3ppm	6.42	<.0001	0.29	0.0183
SE 0.3ppm	7.88	.0026	1.42	<.0001
SS 0.3ppm	6.45	.0035	0.12	0.035

[a,b,c] Means with unlike superscripts differ significantly (P<0.05).

Table 4: The effect of different selenium supplements on nitrite response of abdominal exudates macrophages from 3 week old male broilers. Macrophage cultures (from 6 birds/treatment) were stimulated to LPS from Escherichia coli (1μg/1 x 10^6 macrophages) for 24 h at 3 wk of age. The culture supernatant was tested for nitrite levels by the Greiss reagent method. Trial 2.

Treatment	Nitrite (μM) with LPS	P value	Nitrite (μM) without LPS	P value
Control	5.52		3.53	
SP 0.3ppm	7.06	<.0001	1.84	<.0001
SE 0.3ppm	9.24	<.0001	1.25	<.0001
SS 0.3ppm	7.10	<.0001	2.71	<.0001

[a,b,c] Means with unlike superscripts differ significantly (P<0.05).

Chapter 2

Determination of Potential Toxic Influences of Organic Selenium on Growth and Immunologic Endpoints in Young Chickens

Abstract. Selenium is an essential trace mineral that is also toxic at high levels of dietary incorporation. This study evaluated the effect of varying levels of Se using organic and inorganic Se on fast growing, high yielding broilers. Sodium selenite and Sel-Plex® was added to broiler starter diets at 0.3, 0.6, 1.2, 5, 10, and 15 ppm. Basal diets were also examined. Body weights and relative immune organ weights were monitored at day 7, 14, and 21. Wing web PHA-P measurements were taken at day 21 as an indication of the cell mediated inflammatory response. With experiment 1, a natural Salmonella type B infection broke out. This gave an example of how chickens immune systems with supplemental selenium react to a pathogen. Early signs of toxicity were noted at levels of 1.2 ppm of selenite with suppressed body and organ weights. With the salmonella infection the Sel-Plex® fed birds performed significantly better than the basal or selenite fed birds. With levels of 5, 10, and 15 ppm the selenite fed birds had a significant decrease in body and organ weights. At levels of 10 and 15 ppm of selenite, there was no thymic tissue present. CD4/CD8 T cell numbers were also significantly suppressed in the selenite fed birds. Feed conversion was also abnormally high in the selenite group. However, Sel-Plex® fed birds preformed normally when compared to the control group at all levels, indicating no signs of toxicity. Antioxidant enzyme levels (GPx and TR) were significantly higher with birds fed Sel-Plex® and selenite, proving that these enzymes are dependent on dietary selenium for activity. Experiment 3

addressed the issue that palatability was not a problem with the groups fed high levels of selenite. Overall, selenium toxicity was found in birds fed 1.2 ppm of selenite and not found up to 15 ppm with birds fed Sel-Plex®. These results suggest that dietary selenium is essential for a healthy immune response, but careful consideration should be taken with level and chemical form.

Keywords: Poultry, Selenium, Toxicity, Immunity, Antioxidant, T cell, Thioredoxin Reductase, and Glutathione Peroxidase.

Introduction

Selenium is an essential trace mineral that is also toxic at high levels of dietary incorporation (Franke, 1934). In nature, Se is found in the elemental form, the inorganic form, or incorporated into an organic form. Animals are able to receive adequate amounts of Se in the organic form as the selenoamino acid, selenomethionine, from many feedstuffs (Combs and Combs, 1986).

The relationship of selenium and the antioxidant system has been studied extensively (Combs and Combs, 1986) and continues to receive intensive study (Surai, 2002). The Se-dependent antioxidant system utilizes several selenoproteins along with vitamin E and C. In mammals there are at least 18 known (Hatfield and Gladyshev, 2002) and possibly more genes that encode selenoproteins (Behne et al., 1996). Recent evidence strongly suggests that there are at least 25 selenoproteins in the mammalian selenoproteome (Kryukov et al., 2003), and this would suggest more genes that encode selenoproteins. The expression of these selenoproteins is dependant on the amount of Se available, hormones, and environmental conditions (Kohrl et al., 2000). The majority of

the selenoproteins contain a single selenocysteine residue per polypeptide chain (Tujebajeva et al., 2000; Patching and Gardiner, 1999).

Selenoproteins have important and diverse roles in functions such as redox signaling, regulation of apoptosis, immunomodulation, spermatogenesis, and embryonic development (Surai, 2002). Recent studies have suggested impaired functions of the thioredoxin and glutathione systems in the cell as an explanation for the many effects attributed to Se-deficiency. However, a majority of these selenoproteins still have undefined mechanisms of action. The study of selenoproteins is still a new and an incompletely understood area of protein chemistry.

Although Se is essential many biochemical and physiological functions in the body, high dietary levels cause toxicity. There are several mechanisms that allow development of Se toxicity. The most common and important mechanism is the production of the superoxide radical (Stohs and Bagchi, 1995). The superoxide radical has the ability to interact with thiols in mammals (Klaassen et al., 1985) and in birds (Hoffman et al., 1991). Reaction of superoxide radicals with thiols can alter the activity of many antioxidant sulfhydryl-containing enzymes as well as structural proteins in the body (Spallholz and Hoffman, 2002). When superoxides react with thiols they can generate additional destructive free radicals that can cause irreversible cellular damage (Spallholz, 1994).

Toxicity of Se is related to the concentration of the element, duration of exposure, and the form that is ingested. Most toxicity studies have examined the effects of sodium selenite. In a study done by Latshaw and Ort (1978), female chickens were fed graded levels of sodium selenite to determine the dietary selenium at which toxicity occurred.

Toxic effects were observed at 5 ppm. In another study, adult male albino rats were fed 6 and 8 ppm of selenite for 6 and 9 weeks. These high levels of dietary sodium selenite caused a reduction in body weights, reproductive organ weights and a significant increase in the amount of abnormal spermatozoa (Kaur and Kaur, 2000). Additionally, sodium selenite toxicity has a severely detrimental effect on oestrous cycles, ovarian follicles, ovulation, and fetal development (Parshad, 1999).

Recent studies have compared the toxic effects of selenite to organic forms of Se. Organic Se does not generate superoxides, and it is hypothesized that organic selenium is less toxic than inorganic forms (Spallholz and Hoffman, 2002). *In vitro* studies with human lymphocytes have established a toxicity level of sodium selenite at 5μM/l but not with the addition of selenomethionine (Kelly and Powers, 1995). Similar results were seen in an *in vivo* study in which male BALB/c mice were given 0, 1, 3 or 9 ppm of Se as sodium selenite or seleno-L-methionine in the drinking water for 14 days. The thymus/body weight ratio was significantly reduced at 3 ppm of sodium selenite. Selenite at 9 ppm also significantly increased the production of proinflammatory cytokines in LPS stimulated macrophages. Mice exposed to seleno-L-methionine did not express any signs of toxicity at any level, indicating that inorganic Se is more toxic (Johnson et al., 2000). Selenomethionine and selenocysteine at 2.5, 5, and 12.5 μM/L, did not induced cytoxicity, oxidative DNA lesions, or apoptosis (Stewart et al., 1999). However, Lowry and Baker (1989) showed that pure selenomethionine at high dietary incorporation was toxic in avian species. In mallard ducks, pure L-selenomethionine was significantly more toxic than DL-selenomethionine (Heinz et al., 1996). All of these

studies have led to the conclusion that high levels of Se in diets may be detrimental to health, but certain forms are more toxic than others are.

Adequate amounts of Se are essential for a healthy avian immune system. Marsh et al. (1986) fed low levels of sodium selenite to chickens and observed an increase in the relative weights of lymphoid organs and observed a degeneration of the bursal epithelium in Se-deficient birds. Chang et al. (1994) studied the influence of sodium selenite and vitamin E on T and B cell markers and lymphocyte proliferation. Lymphocyte proliferation was impaired with Se and vitamin E deficiency, and the number of $CD4^+$ and $CD8^+$ T cells increased significantly with the feeding of the Se-deficient diet. These results indicate that vitamin E and Se deficiencies affect the maturation of T cells and the proliferative responses of peripheral lymphocytes.

Organic Se is becoming a more popular Se supplementation in the commercial poultry and livestock sectors of the world. Organic Se as the selenium yeast product (Sel-Plex®) has been approved by the US Food and Drug Administration for use in poultry and livestock diets (Federal Register, 2000, 2003). Currently, there are no toxicity data available on Sel-Plex® in poultry species, suggesting that there might not be a cause for concern about toxicity. Dietary organic Se as selenomethionine from grain has been reported to be less toxic than the inorganic forms of Se (Levander, 1987). It is also known that the avian immune system has an affinity for Se (Larsen, 1988). Because no studies have compared organic vs. inorganic sources of Se on the development of the avian immune system, the objective of this study was to use known toxic doses of sodium selenite with comparable levels of Sel-Plex® (organic selenium incorporated in yeast cells) in broiler diets and assess their influence on body weight and weight of organs that

are associated with the development of the avian immune system. Our specific goal, however, was to focus on the thymus response because Se has been reported to influence its development (Marsh et al., 1986).

Materials and Methods

Experiment 1

Animals and diets

This project was approved and conducted under the supervision of the North Carolina State University Animal Care and Use Committee which has adopted Animal Care and Use Guidelines governing all animal use in experimental procedures.

Ross male broilers were fed the North Carolina Agricultural Research Service broiler starter feed (3154 kcal/kg ME, 21% CP) from 1d of age to 3 wk of age. Feed was supplemented with either 0.3, 0.6, or 1.2 ppm of sodium selenite (SE), Sel-Plex® (SP; Alltech, Inc., Nicholasville, KY 40356), or a control diet (Con) with no supplemental selenium. The study was conducted in 3 battery trials with 5 replicates per treatment and 10 birds per replicate. Replicates were arranged in a completely randomized design with blocking for light and position within the battery. The broiler chickens were fed and provided water for *ad libitum* consumption. Trial 1, by design had no bacterial challenge, but trials 2 and 3 were exposed, unintentionally, to a Salmonella type B infection.

Tissue collection

Live body weights were taken on 7, 14, and 21 d of age. Birds were then killed by CO_2 asphyxiation and the bursa of Fabricius, thymus, spleen, liver and heart were collected. All organs were blotted, cleaned of adherent tissues, and weighed. To adjust

for differences in body weights between treatment groups, all organ weights were expressed relative to live body weight ([grams organ weight/grams body weight] X 100).

Mitogen-Induced Lymphocyte Proliferation

Birds, 19 d of age, had feathers removed from right wingweb 24 hours before intradermal challenge with a mitogen. Phytohemagglutinin[1] (PHA-P) was dissolved in distilled water in the concentration of 1 mg/mL. At 24 h following the feather removal, the thickness of the wingweb was determined with micrometer calipers, and the PHA-P was injected intradermally in a volume of 100 μL that delivered 100 μg of the mitogen into the measured wingweb site in five birds per treatment. At 24 h after the intradermal injection, measurements were taken with the micrometer calipers to determine the increased thickness of the injected wingweb site. An index of stimulation was calculated ({[24 h post-injection wingweb thickness in microns - pre-injection thickness in microns]/ pre-injection thickness in microns}) X 100 = Index of Stimulation) to assess the influence of the selenium sources and dietary concentrations on the recruitment of leukocytes into the site of PHA-P intradermal injection.

Experiment 2

Animals and Diets

Ross male broilers were fed the North Carolina Agricultural Research Service broiler starter feed (3154 kcal/kg ME, 21% CP) from 1d of age to 3 wk of age. Feed was supplemented either with 5, 10, or 15 ppm of sodium selenite (SE), Sel-Plex® (SP), or a control diet (C) with no supplemental selenium. The control diet (C) was diluted with sand to account for volume displacement caused by the volumes of either sodium selenite

or Sel-Plex® that were added to diets. The study was conducted in 3 battery trials with 5 replicates per treatment and 10 birds per replicate. Replicates were arranged in a completely randomized design with blocking for light and position within the battery. The broiler chickens were fed and provided water for *ad libitum* consumption.

Tissue collection

Live body weights were taken on 7, 14, and 21 d of age. Birds were then killed by CO_2 asphyxiation and the bursa of Fabricius, thymus, spleen, liver and heart were collected. All organs were blotted, cleaned of adherent tissues, and weighed. To adjust for differences in body weights between treatment groups, all organ weights were expressed relative to live body weight.

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Quantification of $CD4^+$ and $CD8^+$ T cells by flow cytometry

Blood from 21 d old birds was drawn into each heparizined (100μL sterile phosphate buffered saline [PBS; pH 7.00] containing 25μg sodium heparin) syringes. Blood was diluted in a 1:1 ratio with sterile PBS. A volume of 2 mL of blood/PBS mixture was layered on 3 mL of ficolpaque in glass 15 mL tubes. Tubes were spun at 500 x g (Beckman L5-504) for 20 min. The interface was transferred into 5 mL of fluorescence activated cell sorter (FACS) buffer (1% bovine serum albumin [BSA] in sterile PBS). This mixture was centrifuged for 400 x g for 10 min. The supernatent was discarded and the pellet was resuspended in 5 mL of FACS buffer. The tubes were then spun again for 10 min at 400 x g. The supernatent was again discarded and the pellet was resuspended in 500μL of FACS buffer. A 25 μL volume of the resuspended pellet was transferred to FACS tubes 1, and 75 μL of FACS buffer was added. Normalized mouse serum[5] was added and tubes were incubated for an additional 3min. The CD4/CD8 cocktail was made of mouse anti-chicken CD8α-FITC[6] and mouse anti-chicken CD4α-PE[7] in FACS buffer. Each tube had 100 μL of antibody cocktail added, and was chilled for 30 min on ice. After chilling, 300 μL of FACS buffer was added to each tube, and tubes were spun at 400 x g for 8 min to wash the cells. Cells were washed two more times, and resuspended in 200 μL of FACS buffer. Cells were analyzed by flow cytometry using a FACScan flow cytometer[8]. For each sample, 15000 events were recorded.

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[4] Beckman Instuments, Palo Alto, CA 92634-3100
[5] Zymed Laboratories Inc, San Francisco, CA, 01-6501
[6] Southern Biotech Associates, Inc, Birmingham, AL, 35209
[7] Southern Biotech Associates, Inc, Birmingham, AL, 35209
[8] Becton-Dickinson Immunocytometry, San Jose, Ca 95131-1807

Thioredoxin Reductase Activity

Liver samples (0.5 to 1.0 gm) from birds at 3 wk of age were taken and homogenized with a polytron homogenizer[9] in cold 50mM TrisHCl 1mM EDTA pH 7.5 in a 1:3 ratio. The homogenates were centrifuged (Beckman L5-50) at 5,000 x g for 30 min. Supernatants were removed and analyzed for activity using the DTNB assay previously described by Luthman and Holmgren (1982). Working buffer (100mM Na phosphate, 10mM Na_4EDTA, 0.2 mM NADPH, 0.2 mg BSA/mL, 1% ethanol, 5 mM DTNB) and 0.5 mM FAD (4.2 mg of FAD in 500mM Tris, pH 7.4) were added to 96 well plate before adding sample. Absorbency was read at 412 ηm for 3 min. Samples were run in triplicate and statistical outliers were removed. Results were calculated based on the yield of 2 moles of 2-nitro-5-thiobenzoate per mole of NADPH consumed.

Glutathione Peroxidase Activity

GSH-px activity was measured as previously described (St. Clair and Chow, 1996). Liver samples were prepared by homogenizing[9] at least 0.5g of tissue in 2 mL of PO_4-EDTA buffer (50 mM PO_4, 5 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF), pH 7.4). Samples were centrifuged at 2500 x g for 30 min at 4°C. The supernatents were then centrifuged[10] for 30 min at 10000 x g at 4°C. The enzyme activity was measured by monitoring NADPH oxidation at 340 ηm by coupling reactions with excess GR using H_2O_2 as a substrate.

Cytosolic Protein Determination

[9] Heat System Ultrasonics, Plainview, N.Y. 11803
[10] Micromax R.F, Needham Heights, MA 02194

Total protein content was measured using a Protein Assay Kit[11] using BSA as a standard. The standard curve was linear ($r^2 > 0.98$) up to 80μg/mL of BSA. Coefficients of variation, among replicates of the same sample, were maintained less than 5%.

Experiment 3

Animals and Diets

In trial 1, a total of 64 Ross male broilers were orally gavaged daily with three levels of sodium selenite[12] (SE5, 10, and 15 ppm) dissolved in distilled water to provide a normal daily selenium intake equivalent to the calculated average of selenite consumed on a daily basis when consuming amounts of feed considered to be normal for a 0-3 week old Ross male broiler (data supplied by Ross breeders, Roslin- Midlothian, Scotland). Control birds were gavaged with distilled water only. The dosage of selenite gavaged was constant throughout the trial. Birds were gavaged once daily and were fed control feed on an *ad libitum* basis. Body weights, mortality, and feed conversion were recorded to 3 wk of age.

In trial 2, 64 Ross male broilers were orally gavaged daily with three levels of sodium selenite[9] (SE 5, 10, and 15ppm) dissolved in distilled water to provide a selenite intake equivalent to the amount of selenite consumed by these birds in these treatments as deduced from feed consumption data from experiment 2. To determine the amount of selenite consumed by birds fed selenite-supplemented diets at 5, 10, and 15 ppm, the total amount of feed consumed in 3 weeks for each SE level was divided by the number birds in each treatment. The selenite present in the amount of feed consumed in 3 weeks per bird per treatment was dosed in such a way to deliver this amount on an increasing $\log_2$

[11] Bio-Rad, Hercules, CA 94547
[12] Sigma Chemical Co., St. Louis, MO. 63103

scale throughout the trial. No selenite was gavaged on Day 0. Control birds were gavaged with distilled water only. Birds were gavaged once daily and were fed control feed with no supplemental Se on an *ad libitum* basis. Body weights, mortality, and feed conversion was recorded to 2 wk of age.

In trial 3, 90 Ross male broilers were orally gavaged daily with three levels of sodium selenite (SE5, 10 and 15 ppm) dissolved in distilled water to provide a daily selenite intake equivalent to the amount of selenite consumed by birds in these treatments as deduced from feed consumption data from experiment 2. Consumption of selenium per bird per treatment was calculated as in trial 2, but for this trial a weekly selenium consumption per bird per treatment was also calculated. In an attempt to finally explicate palatability or toxicity as the basis for decreased feed intake and consequential mortality as found in experiment 2, selenite was gavaged at two different feed consumption levels. Treatments, called SE in experiment 2, were SE5ppm(SE), SE10ppm(SE), and SE15ppm(SE) were gavaged with selenite at levels consistent with the amount of feed consumed weekly (0-3 wk) by birds fed SE5, 10, and 15 ppm in experiment 2, respectively. Treatments, called C in experiment 2, were SE5ppm(C), SE10ppm(C), and SE15ppm(C) were gavaged with selenite at levels consistent with the amount of feed consumed weekly (0-3 wk) by the control group in experiment 2. The daily dosage of sodium selenite remained constant instead of increasing by $\log_2$ scale daily, unless the birds exhibited signs of toxicity, at which time the amount of selenite delivered on a daily basis was decreased to the selenite level delivered on day 1 in trial 2. Birds were gavaged once daily and were fed control feed with no supplemental Se on an *ad libitum* basis. Body weights, mortality, and feed conversions were recorded to 5 days of age.

Statistical Analysis

Data from all completely randomized design experiments were analyzed using the ANOVA procedure of the Statistical Analysis System (SAS Institute, 1995). Differences among treatment means and interactions between treatments were separated by the Student Newman Kuehls test at $p \leq 0.05$.

Results

At 3 wk of age, Sel-Plex® fed at 0.3, 0.6, and 1.2 ppm exerted positive effects on BW (Figure 1). Selenite fed birds also had an increased BW at 0.3 and 0.6 ppm, but when fed 1.2 ppm, they had a significant decrease in BW, which strongly suggested early signs of toxicity in the SE group. In the face of a Salmonella infection, at 0.3 ppm Sel-Plex®-fed birds had a significantly greater 3 wk BW than birds fed sodium selenite (Figure 1). All levels of Se supplements to the diet resulted in numerically higher BW than in the control with no supplemental selenium. Selenium at 0.3, 0.6, and 1.2 ppm had no significant effect on the 3 wk relative weight of the bursa of Fabricius, spleen, or liver (Figures 2, 3, 4). Although there were no significant selenium source treatment effects, Sel-Plex®-feeding resulted in an increasing trend for lymphoid organ relative weights even with the unavoidable Salmonella infection (Figures 2, 3, 4). Thymus relative weights were significantly greater in Se-supplemented birds than the control birds (Figure 5). At 0.3 and 0.6 ppm, selenite feeding resulted in significantly greater thymus relative weights, but at 1.2 ppm thymus relative weights were less than the thymus relative weights in Sel-Plex®-fed birds. Sel-Plex®-fed birds had about the same thymus relative weight at all levels of intake, indicating a constant development and no signs of toxicity.

With the Salmonella infection, Sel-Plex® fed birds had significantly greater thymus relative weights than both the control and all levels of selenite intake (Figure 5). There wer no significant differences amongst treatments with the relative heart weights (data not shown).

When birds were examined for resistance to subcutaneous basophil hypersensitivity to intradermal PHA-P injection, the control- and selenite-fed birds had a significantly greater index of stimulation indicated by a greater inflammatory response, more swelling and tissue damage than did the Sel-Plex®-fed groups (Figure 6). When groups were challenged with both PHA-P and Salmonella infection, both Se-supplemented groups mounted a greater response than did the control group (Figure 7). The groups fed selenite at 1.2 ppm mounted the greatest response, but all of the Sel-Plex®-fed birds mounted a remarkably consistent response across all levels (Figure 7).

To assess the influence of high levels of dietary selenium on thymus development, known toxic levels of selenium as sodium selenite (5, 10, and 15 ppm) were supplemented in chicken starter feed. Comparable levels of Sel-Plex® (5, 10, and 15 ppm) were also supplemented. Figure 8 represents the 3 wk BW data from experiment 2. The BW of the selenite fed birds were significantly lower than the BW of Sel-Plex®-fed and control-fed birds, indicating the potential for selenium toxicity related to sodium selenite but not organic selenium from Sel-Plex®-feeding (Figure 8). In sodium selenite-fed birds, there was a sharp decrease in BW observed with selenite feeding at 10 and 15 ppm but no similar signs when Sel-Plex® was fed.

It was observed that the relative bursal weight was decreased in groups fed selenite at 10 and 15 ppm (Figure 9). The decrease in relative bursal weights suggests a

decrease in the number of bursal follicles and B cells production. Once again no toxic effects were observed with any of the groups fed Sel-Plex®.

There were no significant differences among selenium source treatments for spleen relative weights (Figure 10), or relative heart weights (data not shown).

Relative liver weights were significantly greater in selenite-fed birds (Figure 11). The increased relative weight of the liver was attributed to slow body weight accretion. The livers of the selenite birds appeared to be hard and bright yellow in color- almost cirrhotic in appearance and texture. The Sel-Plex®-fed birds had no significant differences in relative liver weights in comparison to the control birds suggesting that even at 15 ppm in the diet, Sel-Plex®-feeding was not toxic to young chickens. Thymic relative weights were sharply decreased in birds fed 5, 10, and 15 ppm of selenite (Figure 12). The feeding of sodium selenite 10 and 15 ppm caused total atrophy of the thymic tissue by 2 weeks of age. Control- and Sel-Plex®-fed treatments had normal thymic relative weights.

Non-specific mitogen stimulation of the cutaneous basophilic hypersensitivity reaction via intradermal PHA-P wingweb injection did not result in significant differences among selenium source treatments (Figure 13). PHA-P stimulation has a B and T cell component, which suggests that the selenite birds were still able to mount a response despite the cellular atrophy of the thymus. However, when the presence of $CD4^+$ and $CD8^+$ T cells were examined, birds fed all levels of sodium selenite had a sharp decrease in the number of T cells (Table 1). The Sel-Plex®-fed birds had a normal numbers of $CD4^+$ and $CD8^+$T cells compared with the control.

Feed conversion was abnormally elevated in the selenite-fed birds, indicating that they simply were not gaining weight no matter how much feed they consumed (Table 2). The BW of the selenite-fed birds were decreased significantly ($p \leq 0.05$). All of the Sel-Plex®-fed treatments had normal feed conversion and BW indicating a normal rate of gain even at the highest level of supplementation (15 ppm).

The influence of feeding high levels of sodium selenite and Sel-Plex® on the activity of thioredoxin reductase (TR) was also examined (Table 3). Birds with no supplemental Se had the lowest liver TR activity. Feeding either sodium selenite or Sel-Plex® from 0.3 ppm to 15 ppm Se induced higher activities of TR, but Sel-Plex®-fed birds at 15 ppm had the highest activity of TR (Table 3).

The GSH-px activity was significantly increased by Se supplementation in both trial 1 and trial 2 of experiment 2 (Tables 4 and 5). However, inconsistent results between trials were found. The GSH-px activity for selenite and Sel-Plex®-fed groups was elevated compared with controls. Birds fed Sel-Plex® at 5 ppm had significantly higher GSH-px activity than birds fed selenite at 5 ppm. In trial 1, Sel-Plex® at 10 ppm had a higher GSH-px activity than birds fed selenite at 10 ppm. However, the opposite was seen in trial 2. In trial 1, Sel-Plex® fed at 15 ppm resulted in significantly lower GSH-px activity than selenite feeding at 15 ppm. In trial 2, the reverse was seen in which Sel-Plex feeding at 15 ppm gave a higher GSH-px activity than did the selenite feeding at 15 ppm.

Because there was such a sharp decrease in BW and livability among chickens fed toxic levels of sodium selenite, questions arose concerning the issue of palatability of diets with high levels of sodium selenite. The birds that were fed diets with sodium

selenite at 5, 10, and 15 ppm had a significantly lower growth rate and feed intake. Thus, the question of reduced feed intake due to unpalatable diets had to be addressed. To investigate this question, birds were gavaged with 5, 10, and 15 ppm of sodium selenite. Body weights and feed conversion were recorded up to 2 wk of age. The first trial of experiment 3 resulted in total mortality among gavaged chickens (data not shown). In the second trial where birds were gavaged daily with a $\log_2$ increase of sodium selenite (Day 1, SE5=0.0129mg/bird, SE10=0.0161mg/bird, SE15= 0.0206mg/bird) resulted in birds performing and feeding normally (Table 6) which raised the question about the potential for insufficient amounts of selenium being given to the birds at a time when the birds might consume more selenite per gram of body weight before showing signs of morbidity. In the toxicity trials of experiment 2, it had been noted that all birds fed high levels of sodium selenite had normal growth and feed intake during the first week of each trial. Therefore, a third trial was conducted in which birds were gavaged with an average daily intake of sodium selenite based on daily feed intake data from experiment 2. The birds gavaged with selenite at the selenite (SE) feed consumption levels (Week 1 SE5(SE)=0.0875mg/bird, SE10(SE)=0.170mg/bird, SE15(SE)=0.25mg/bird) from experiment 2 performed slightly better than the birds gavaged with the Control (C) level feed consumption (Week 1 SE5(C)=0.095mg/bird, SE10(C)=0.20mg/bird, SE15(C)=0.35mg/bird) (Table 6). Both groups exhibited signs of toxicity, with the SE group being less severe than the C group. The results from trial 3 of experiment 3 conclusively show that sodium selenite in high concentrations is toxic and has detrimental effects on poultry growth and health. Palatability was not an issue in these toxicity trials.

Discussion

The results from this investigation proved that the chemical form of Se in high levels had diverse effects on the health of chickens and that in the face of a bacterial infection, organic selenium was better than sodium selenite for the overall health and well-being of chickens. Marsh et al. (1986) showed an increase in body weights and relative bursa, spleen, and thymus weights when birds were supplemented with 0.1 ppm of sodium selenite. In this experiment, levels of sodium selenite to 0.6 ppm resulted in data that were similar to those reported by Marsh et al. (1986), but when sodium selenite was elevated to 1.2 ppm in the diet of chickens, BW, and organ weights decreased significantly. All groups fed the organic form of selenium in Sel-Plex® had increased BW and organ relative weights compared to the control- and selenite-fed groups. The thymus relative weight data showed dramatic differences between the two Se supplements in the face of a bacterial challenge. The divergent responses of sodium selenite and Sel-Plex®-fed chicken thymus relative weights showed that the thymus and T cells have a high affinity for Se but high levels of the inorganic selenite can have severe negative effects on its development (Spallholz et al., 1990).

Marsh et al. (1986) demonstrated that low levels (0.1 ppm) of sodium selenite were beneficial to the immune system, but in modern high-yielding poultry with significantly higher metabolic rates and different nutritional needs, 0.3 ppm is now the desired standard supplement level. Inorganic Se dietary supplementation has some problems. Among these problems are the minimal levels of Se in meat proteins and the potential for toxicity if too high a dietary level of inorganic Se is supplemented in the diet (Edens and Gowdy, 2003). Ort and Latshaw (1978) showed that signs of toxicity are

seen at 5 ppm in laying hens. However, with modern day broilers we were able to see early signs of toxicity with sodium selenite at 1.2 ppm.

Few studies have compared the toxicity of sodium selenite versus organic selenium. Kim and Mahan (2001) examined the two forms of Se at high levels in growing-finishing swine. They noted a decrease in body weights, hair loss, and cracked hooves with inorganic forms of Se at greater than 5 ppm. Hair loss and cracked hooves occurred with organic Se level of 20 ppm. Plasma GSH-px activity increased with high levels of either Se source. Our results with broilers showed decreased body weights, feed efficiency, and relative organ weights with sodium selenite at levels of 1.2 ppm or higher. Sel-Plex® levels as high as 15 ppm showed no signs of toxicity in the current investigation. It was noted that GSH-px levels increased with high levels of both Se source, but Sel-Plex®-fed birds generally had significantly higher GSH-px activities than did the selenite fed birds. These observations were consistent with those from swine data reported by Kim and Mahan (2001).

Broilers fed sodium selenite at 10 and 15 ppm had severe thymic atrophy by 2 wk of age. Severe thymic atrophy in response to stressful stimuli is not surprising because in avian species this primary lymphoid tissue is the first to show signs of toxicity. Thymic atrophy is characterized by decreased weight and decreased medullary/ cortex ratio when chickens are exposed to toxic substances or even pathogenic organisms (Kuper et al., 2000). A similar atrophic thymic response was observed in both experiments 1 and 2 when dietary levels of sodium selenite were higher than 1.2 ppm. No thymic atrophy was observed with any level of dietary Sel-Plex® in this study.

Chang et al. (1994) studied the influence of sodium selenite and vitamin E on T and B cell markers and lymphocyte proliferation. Proliferation was impaired with Se and vitamin E deficiency, but $CD4^+$and $CD8^+$ T cell populations increased slightly with vitamin E and Se supplementation. Leng et al. (2003) also compared the influence of sodium selenite and organic Se sources on immunity in layer chickens. They reported an increase in the number of T cell surface markers with organic selenium. In experiment 2, T cell numbers and T cell surface markers decreased significantly with high dietary levels of sodium selenite. However, T cell numbers and T cell surface markers in the control group, with no supplemental selenium, and in birds fed high dietary levels of Sel-Plex® were significantly greater than the numbers in the selenite-fed birds. Using this as a basis for assessing the general immunological status of chickens, one comes to a conclusion that selenium is required for an efficient response to an antigenic challenge and that birds fed organic selenium have an advantage over birds fed sodium selenite. Organic selenium enhances T cell function and surface markers, and at high dietary levels, no adverse effects are observed, and this was exactly the opposite from the observations with sodium selenite.

Birds fed Sel-Plex® had a significantly lower cutaneous basophilic hypersensitivity (CBH) response than did sodium selenite fed chickens given dietary levels of either 0.6 or 1.2 ppm. Selenium-supplemented groups had a greater CBH response than did the control group, with no supplemental selenium, when faced with a bacterial challenge. Presumably, the increased CBH response associated with Se supplementation was associated with increased glutathione peroxidase activity in lymphocytes and granulocytes that migrate to the site of antigen stimulation (Brown et

al., 2000). An increase in the production of reactive oxygen species (ROS) following sodium selenite supplementation but not selenomethionine was observed in a study by Johnson et al. (2000). They attributed increased ROS production in sodium selenite (>3.0 ppm) fed animals to increased production of proinflammatory cytokines such as TNF α and IL-1β.

Those results are consistent with our present study where it was clear that the PHA-P response was significantly higher in the control and selenite fed birds than in the Sel-Plex® group. The results of this current investigation suggest a potential additive effect between pro-oxidant activity of the immunocompetent cells recruited to a site of inflammation and sodium selenite when the chicken responds to a mitogen challenge.

The analysis of the selenoprotein, thioredoxin reductase (TR), and the effect of high dietary selenium levels on the activity of TR were remarkable. Liver TR activity in 3 wk old birds was lowest in the control-fed group. The feeding of either sodium selenite or Sel-Plex® from 0.3 ppm to 15 ppm induced higher activities of TR, but Sel-Plex® at 15 ppm induced the greatest hepatic TR activity. Induction of higher TR activity in the chicken liver was similar to an observation of selenite induction of TR activity in the rat liver (Berggren et al., 1999). Thus, chickens and rats respond comparably with selenium-mediated induction of TR. The ability of selenium to induce TR activity is an important event in growing birds because there is a constant need to cope with oxidative stress.

Broiler performance is improved with the use of Se in the diet. Edens (1996 and 2001) pointed out that organic Se improves BW, reduced drip loss, and improved feed efficiency in growing birds. High levels of dietary sodium selenite had significant

negative effects on feed efficiency, but feed efficiency in birds given high levels of dietary organic selenium provided by Sel-Plex® were comparable with feed efficiencies of birds given no supplemental selenium. It is important to understand that even though no supplemental selenium was provided in the control feed, there was organic selenium in that feed that was provided via the feed grains. The selenite fed groups had no significant BW gain regardless of the quantity of feed consumed. The issue of feed palatability with high levels of dietary selenite feeding arose. However, palatability was not the problem as shown in trial 3 of experiment 3. The sodium selenite supplement at high levels caused significantly decreased BW gain and organ relative weights due to toxicity but not as a result of decreased feed intake.

The relationship between the immune system and modern day poultry performance is complex. The general opinion is that poultry have a decreased immune system functions due to an increase in selection pressures for increased growth characteristics in a confined environment. In fact, the decreased immune system functions are probably due to nutrient requirements that are no longer being met by the modern day diet. A proper source of Se supplementation in current poultry diets may be one factor that can enhance the immune system of poultry. This concept has been demonstrated in many animal studies in which organic Se in Sel-Plex® has been compared with inorganic sodium selenite. In the current study, organic selenium in Sel-Plex® was shown to enhance the immune system while there was a negative impact of sodium selenite. Additionally, Sel-Plex® has been shown to be safe at levels as high as 15 ppm whereas sodium selenite fed birds showed signs of toxicity as low as 1.2 ppm.

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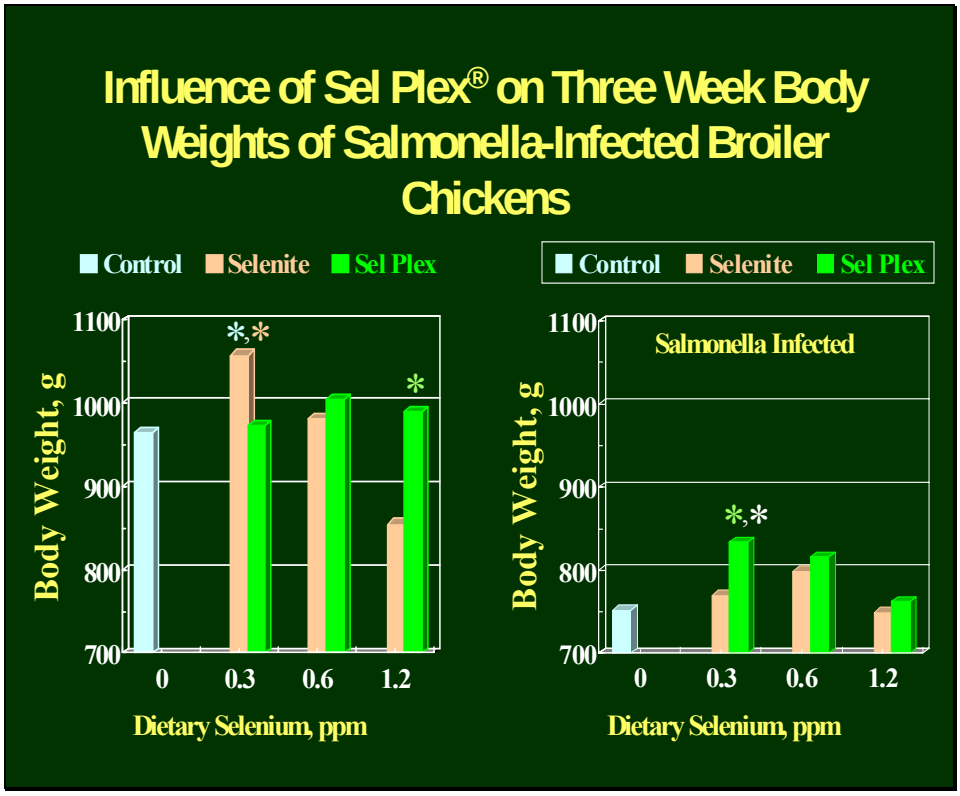
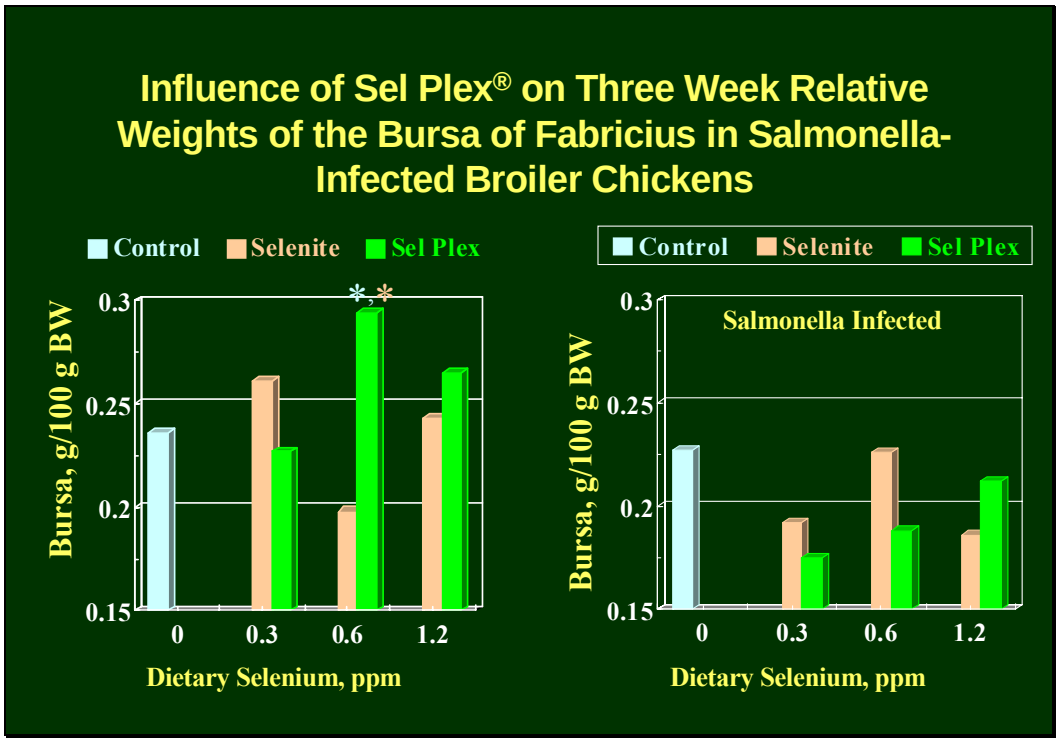


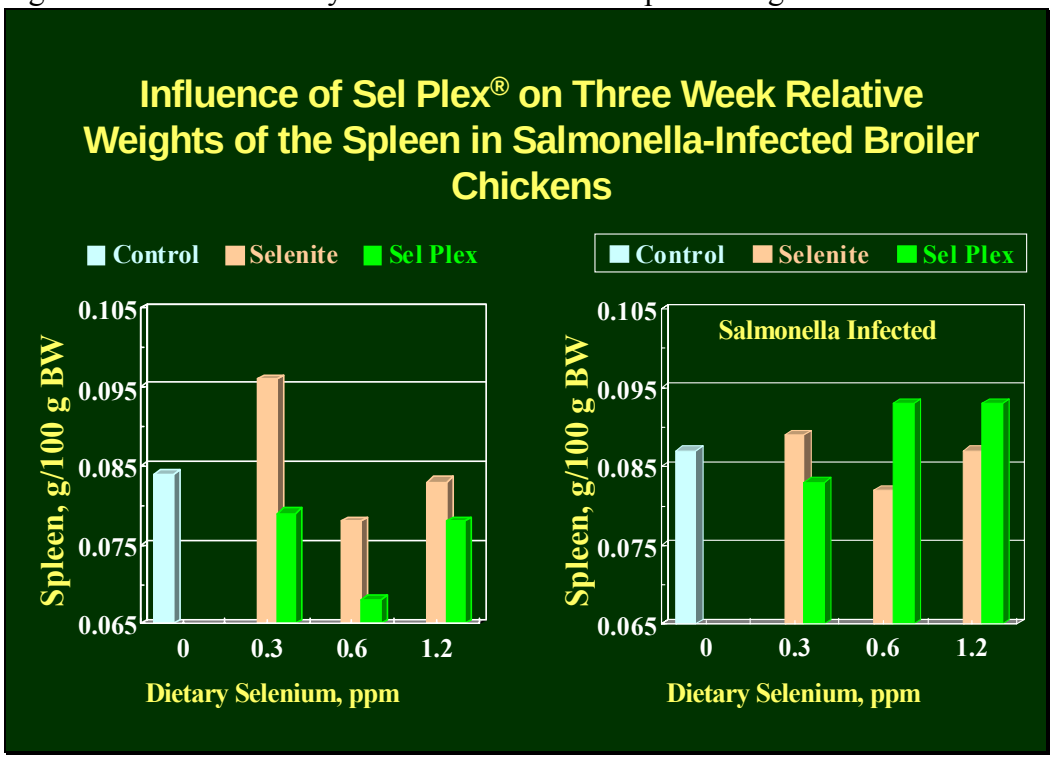
Figure 1. Body weights are expressed in grams. Birds were supplemented with either sodium selenite or Sel-Plex® at 0.3, 0.6, or 1.2 ppm as described in Materials and Methods. Control diets were not diluted with sand. Five birds per treatment were sampled per time period. Significant differences ($P<0.05$) are indicated by the asterisk.

Figure 2. Effect of dietary treatments on 3 week bursa weights



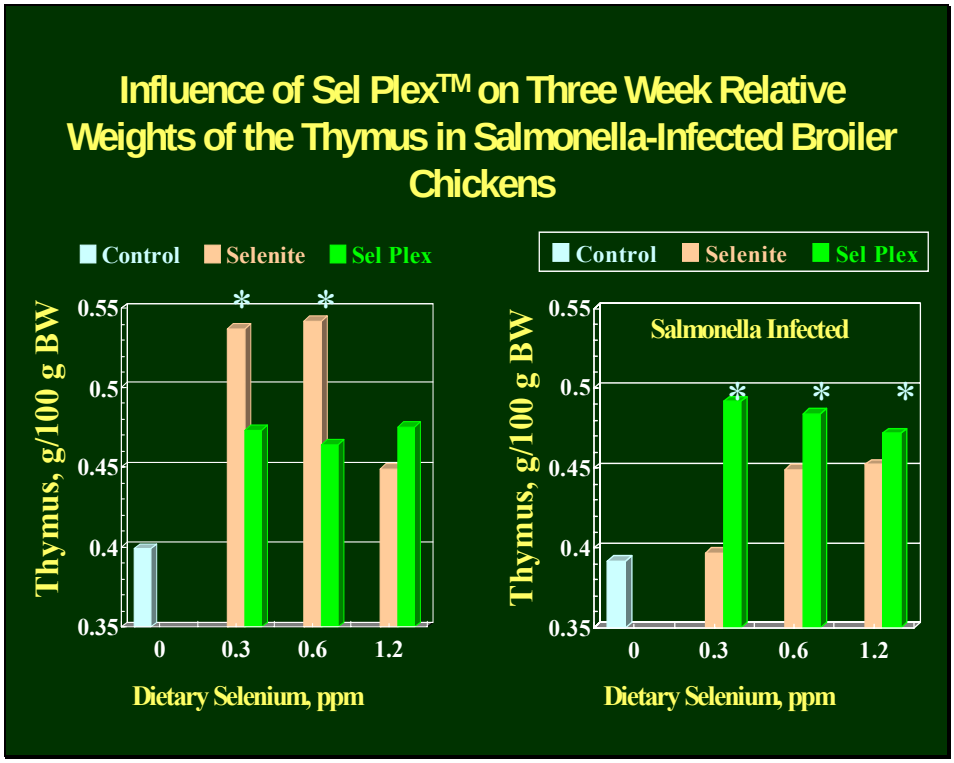
Bursa weights are expressed in grams/100 grams of body weight. Birds were supplemented with either sodium selenite or Sel-Plex® at 0.3, 0.6, or 1.2 ppm as described in Materials and Methods. Control diets were not diluted with sand. Five birds per treatment were sampled per time period. Significant differences ($P<0.05$) are indicated by the asterisk.

Figure 3. Effect of dietary treatments on 3 week spleen weights



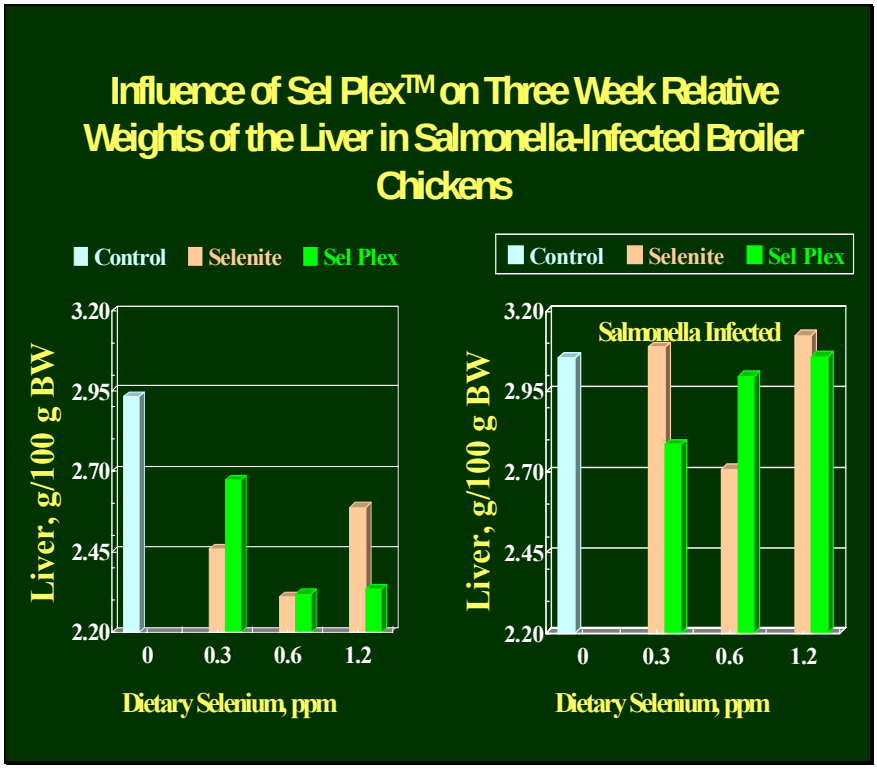
Spleen weights are expressed in grams/100 grams of body weight.
Birds were supplemented with either sodium selenite or Sel-Plex® to provide selenium at 0.3, 0.6, or 1.2 ppm. Control diets were not diluted with sand to account for dilution effects associated with the addition of large quantities of selenium sources to the experimental diets.
N= 5 per treatment per time point.
*Significant differences ($P<0.05$) are indicated by the asterisk.

Figure 4. Effect of dietary treatments on 3 week thymus weights.



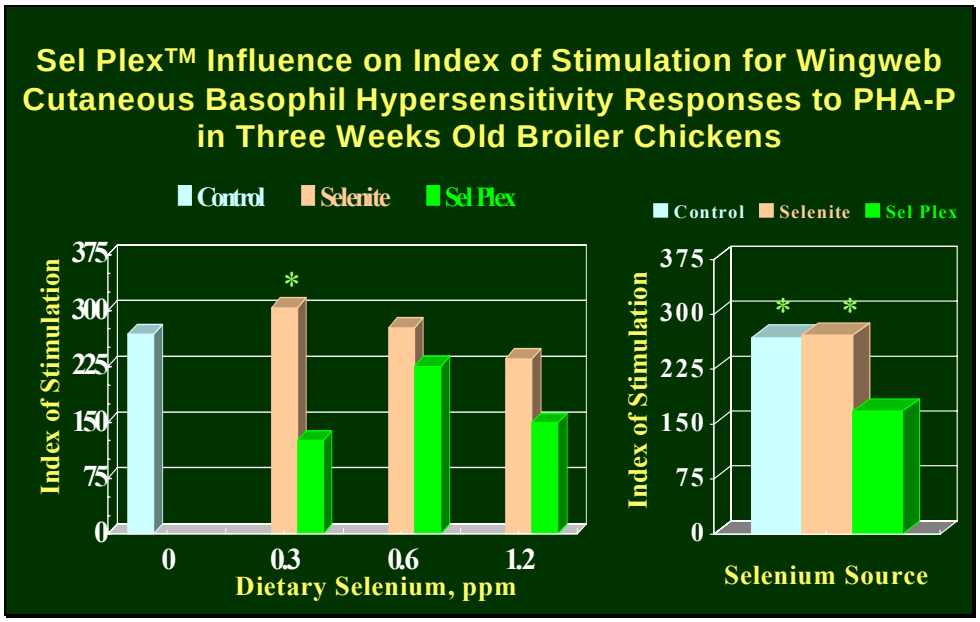
Thymus weights are expressed in grams/100 grams of body weight. Birds were supplemented with either sodium selenite or Sel-Plex® at 0.3, 0.6, or 1.2 ppm as described in Materials and Methods. Control diets were not diluted with sand. Five birds per treatment were sampled per time period. Significant differences ($P<0.05$) are indicated by the asterisk.

Figure 5. Effect of dietary treatments on 3 week liver weights.



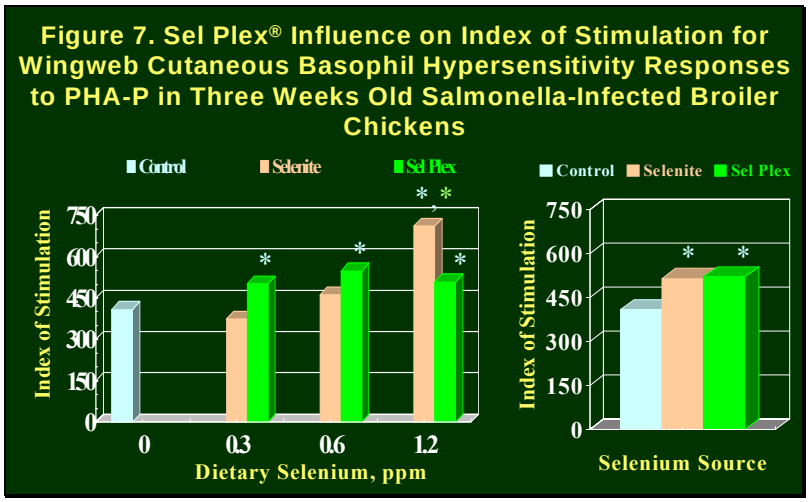
Liver weights are expressed in grams/100 grams of body weight. Birds were supplemented with either sodium selenite or Sel-Plex® at 0.3, 0.6, or 1.2 ppm as described in Materials and Methods. Control diets were not diluted with sand. Five birds per treatment were sampled per time period. Significant differences ($P<0.05$) are indicated by the asterisk.

Figure 6. Effect of dietary treatments on PHA-P stimulation in 3 week old broilers.



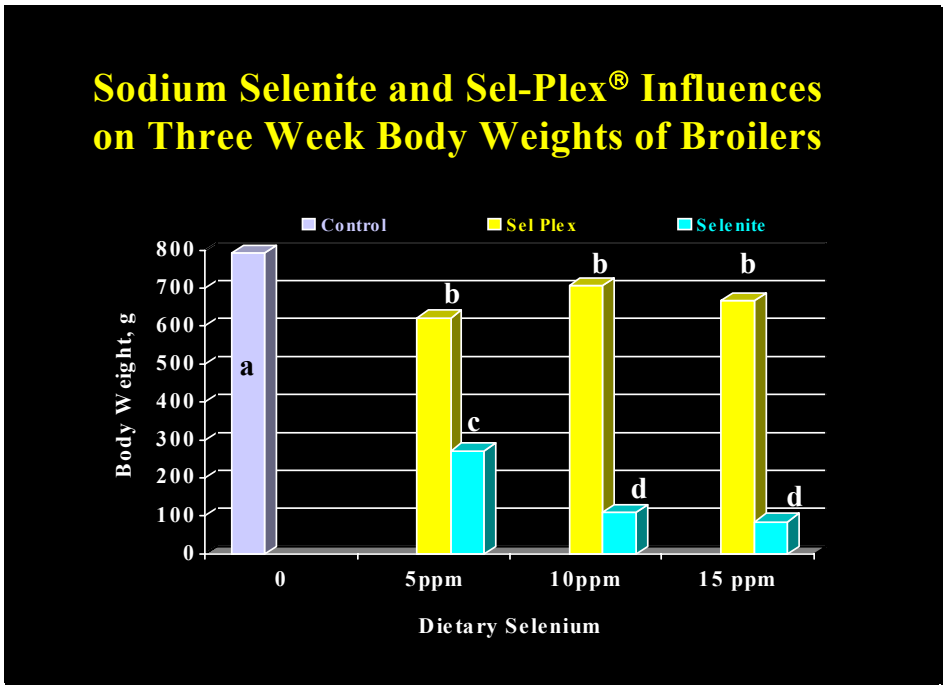
Index of stimulation was measured at 0 and 24 hours post injection. Birds were supplemented with either sodium selenite or Sel-Plex® at 0.3, 0.6, or 1.2 ppm as described in Materials and Methods. Control diets were not diluted with sand. Five birds per treatment were sampled per time period. Graph on right represents a pooled sample of all levels of dietary treatments. Significant differences ($P<0.05$) are indicated by the asterisk.

Figure 7. Effect of dietary treatments on PHA-P stimulation in 3 week old Salmonella infected broilers.



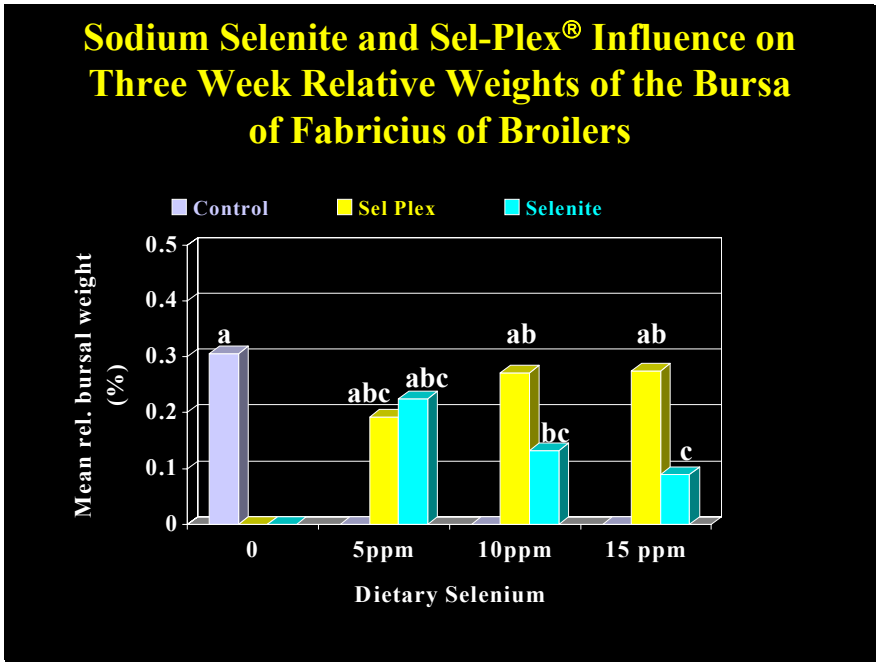
Index of stimulation was measured at 0 and 24 hours post injection. Birds were supplemented with either sodium selenite or Sel-Plex® at 0.3, 0.6, or 1.2 ppm as described in Materials and Methods. Control diets were not diluted with sand. The graphs represent results with birds Salmonella Type B infection. Five birds per treatment were sampled per time period. Graph on right represents a pooled sample of all levels of dietary treatments. Significant differences ($P<0.05$) are indicated by the asterisk.

Figure 8. Sodium selenite and Sel-Plex influences on three week body weights of broiler chickens.



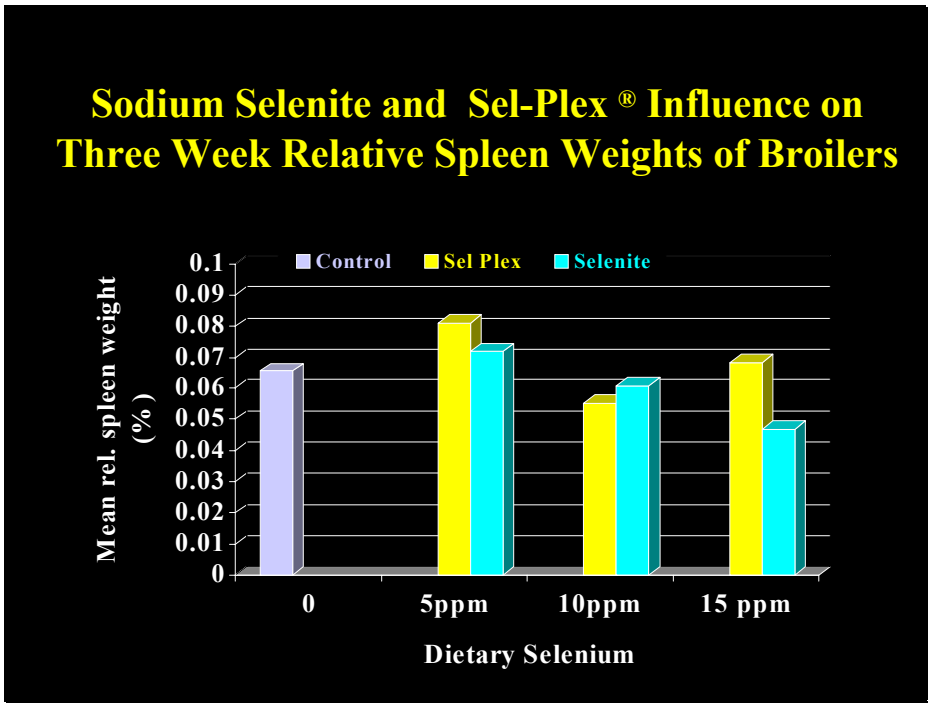
Body weights are expressed in grams. Birds were supplemented with either sodium selenite or Sel-Plex® to provide selenium in the diet at 5, 10, or 15 ppm. Control diets were diluted with sand to account for dilution effects from the addition of large quantities of selenium sources to the diets. Significant differences ($p<0.05$) among treatments are indicated by the lower case letters associated with the bars of the histogram.

Figure 9. Effect of high levels of selenium supplementation on 3 week body weights.



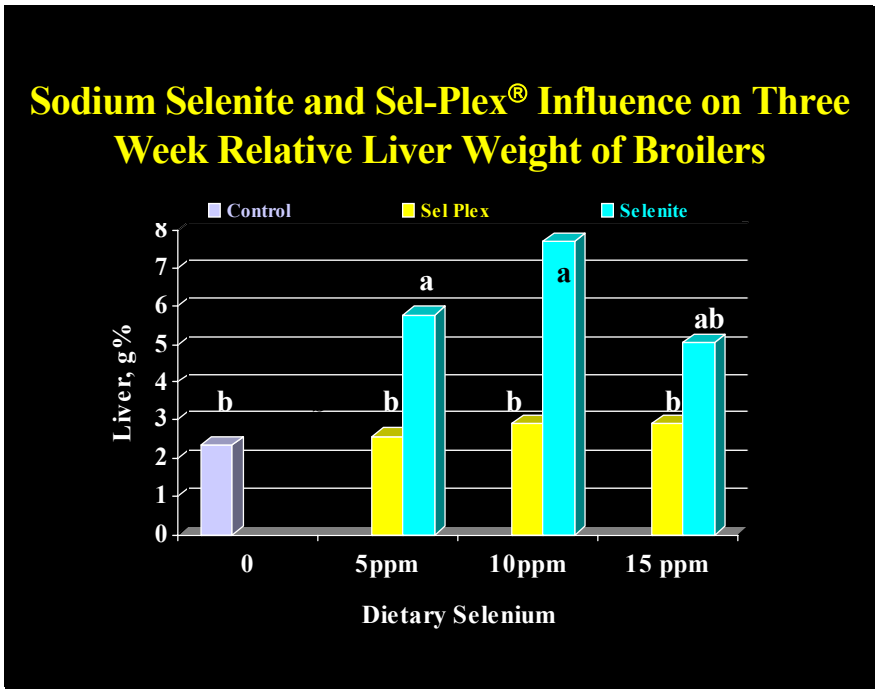
Bursa weights are expressed in grams/ 100 grams of body weight. Birds were supplemented with either sodium selenite or Sel-Plex® at 5, 10, or 15 ppm as described in Materials and Methods. Control diets were diluted with sand. Five birds per treatment were sampled per time period. Significant differences ($P<0.05$) are indicated by the lower case letters.

Figure 10. Effect of high levels of selenium supplementation on 3 week spleen weights.



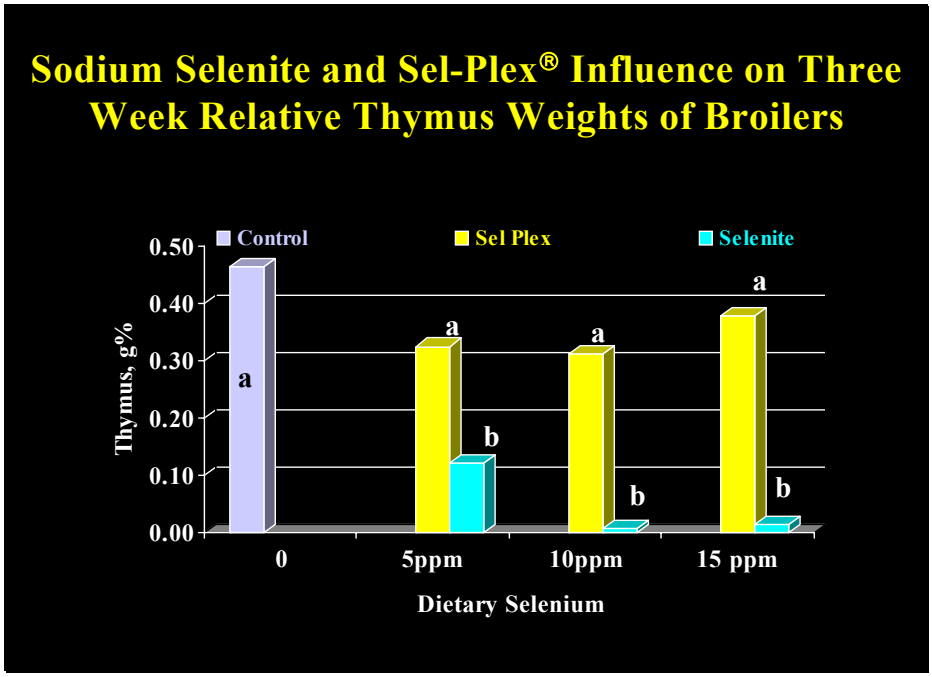
Spleen weights are expressed in grams/ 100 grams of body weight. Birds were supplemented with either sodium selenite or Sel-Plex® at 5, 10, or 15 ppm as described in Materials and Methods. Control diets were diluted with sand. Five birds per treatment were sampled per time period. Significant differences ($P<0.05$) are indicated by the lower case letters.

Figure 11. Effect of high levels of selenium supplementation on 3 week liver weights



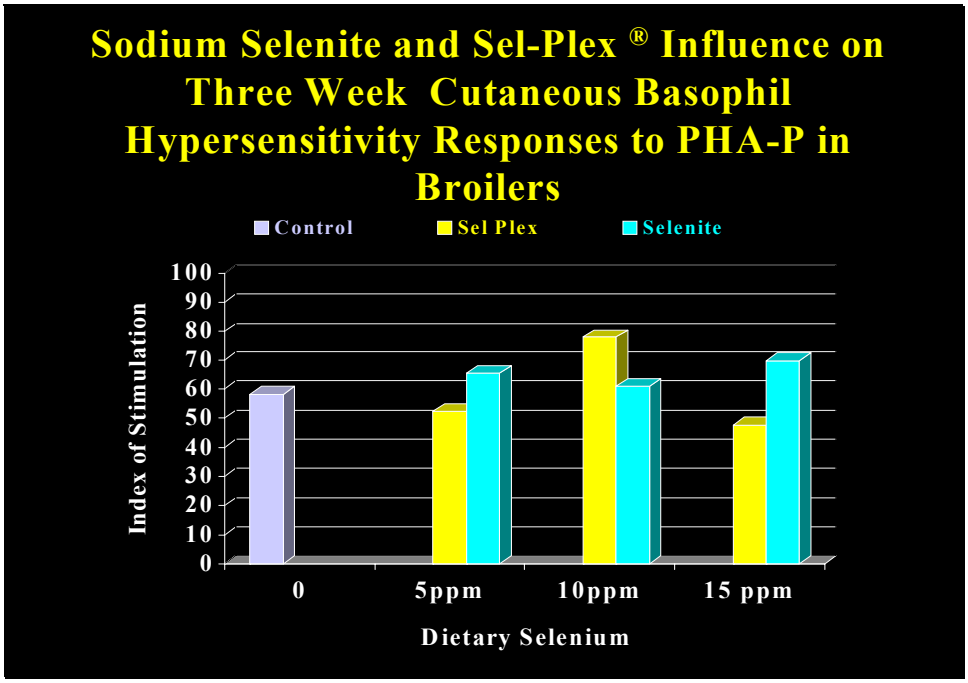
Liver weights are expressed in grams/ 100 grams of body weight. Birds were supplemented with either sodium selenite or Sel-Plex® at 5, 10, or 15 ppm as described in Materials and Methods. Control diets were diluted with sand. Five birds per treatment were sampled per time period. Significant differences ($P<0.05$) are indicated by the lower case letters.

Figure 12. Effect of high levels of selenium supplementation on 3 week thymus weights.



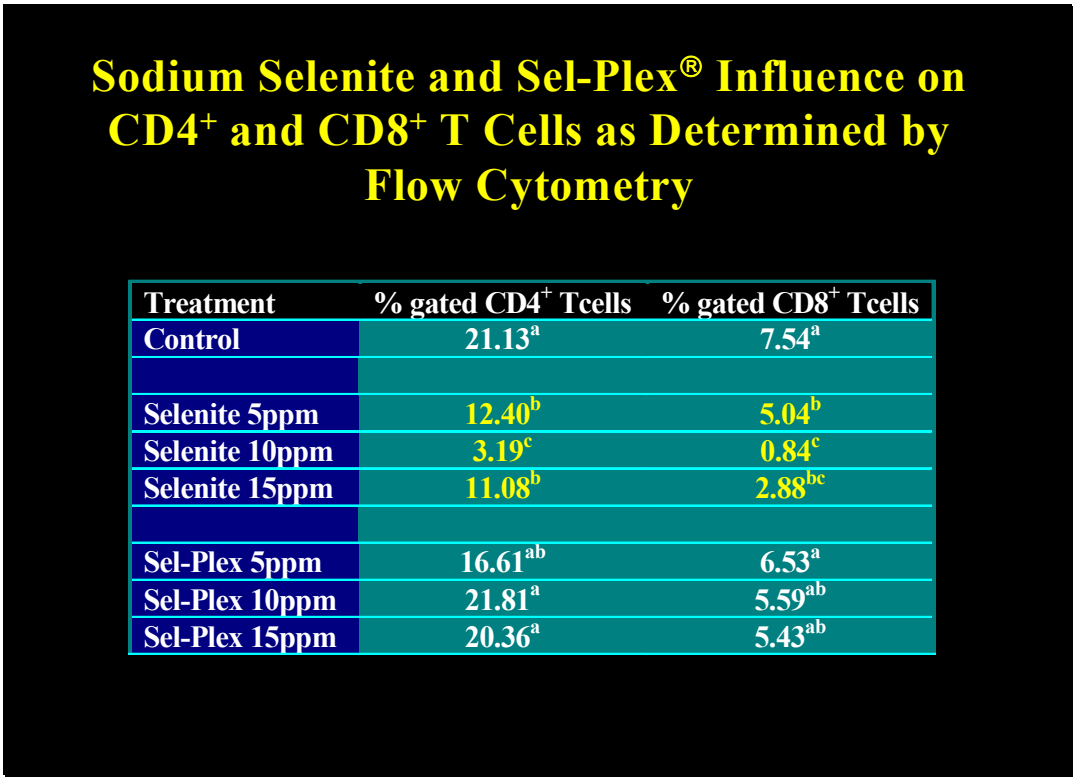
Thymus weights are expressed in grams/ 100 grams of body weight. Birds were supplemented with either sodium selenite or Sel-Plex® at 5, 10, or 15 ppm as described in Materials and Methods. Control diets were diluted with sand. Five birds per treatment were sampled per time period. Significant differences ($P<0.05$) are indicated by the lower case letters.

Figure 13. Effect of high levels of selenium supplementation on PHA-P stimulation in 3 week old broilers.



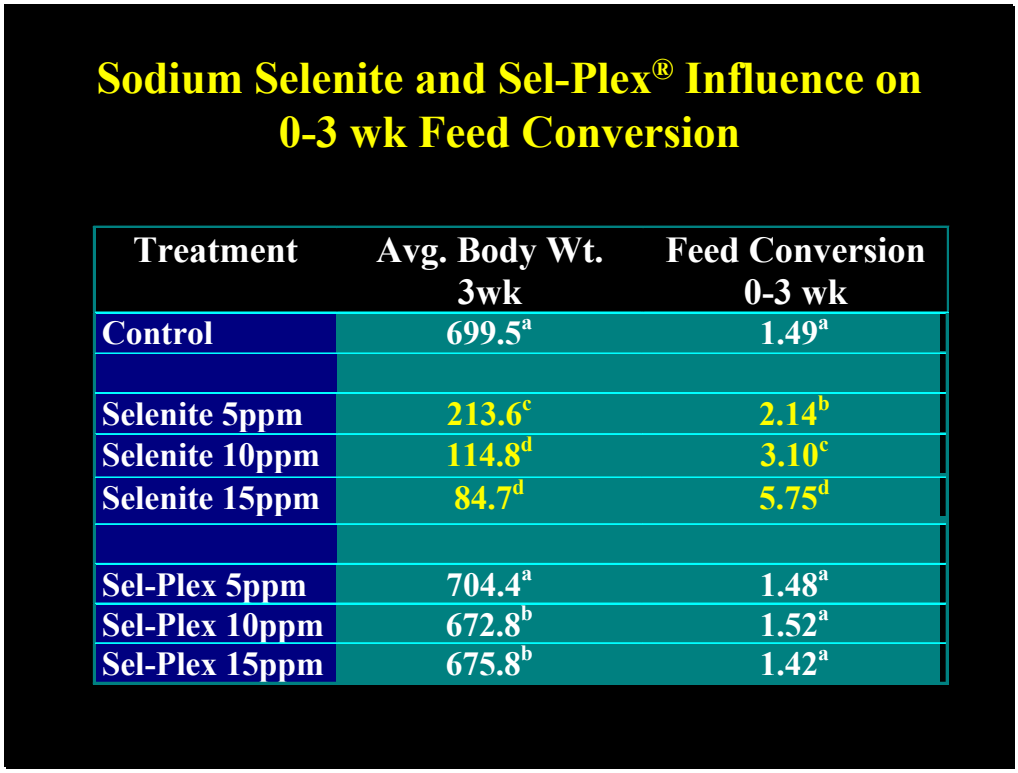
Index of stimulation was measured at 0 and 24 hours post injection. Birds were supplemented with either sodium selenite or Sel-Plex® at 5, 10, or 15 ppm as described in Materials and Methods. Control diets were not diluted with sand. Five birds per treatment were sampled per time period. Significant differences ($P<0.05$) are indicated by the lower case letters.

Table 1. Effect of high levels of selenium supplementation on CD4 and CD8 T cell populations.



Effect of dietary treatments on T cell surface marker expression. Birds were supplemented with either sodium selenite or Sel-Plex® at 5, 10, or 15 ppm as described in Materials and Methods. Control diets were diluted with sand. Five birds per treatment were sampled on day 21. Significant differences ($P<0.05$) are indicated by the lower case letters.

Table 2. Effect of high levels of selenium supplementation on feed efficiency in 3 week old broilers.



Effect of dietary treatments on 0-3 wk feed conversion. Birds were supplemented with either sodium selenite or Sel-Plex® at 5, 10, or 15 ppm as described in Materials and Methods. Control diets were diluted with sand. Significant differences ($P<0.05$) are indicated by the lower case letters.

Table 3. Thioredoxin Reductase activity (mmol BNADPH/min/mg total protein) from chicken liver homogenates from 3 week old male broilers fed high levels of selenite or Sel-Plex®.

Treatment	nmol/min/mg protein
Control (0 ppm Se added)	42.00[c]
Selenite 0.3 ppm Se	84.00[bc]
Sel-Plex® 0.3 ppm Se	71.00[bc]
Selenite 5 ppm Se	80.53[b]
Sel-Plex® 5 ppm Se	98.12[ab]
Selenite 10 ppm Se	90.25[ab]
Sel-Plex® 10 ppm Se	95.77[ab]
Selenite 15 ppm Se	102.37[ab]
Sel-Plex® 15 ppm Se	145.81[a]

TR activity was monitored using the DTNB assay that measures the NADPH-dependent reduction of the disulfide bond in 5,5'-dithiobis(2-nitrobenzoic acid) at 412nm. Calculated resulted are based on yield of 2 moles of 2-nitro-5-thiobenzoate per mol of NADPH consumed. Results are given as the μmol of NADPH oxidized.

[a,b,c] Means are significantly different at $P \leq 0.05$

Table 4. Glutathione Peroxidase activity (mU/mg total protein) from chicken livers homogenates from 3 week old male broilers (Trial 1) fed high levels of selenite or Sel-Plex®.

Treatment	mU/mg protein
Control (0 ppm Se added)	20.69^{cd}
Selenite 5 ppm Se	29.23^{bcd}
Sel-Plex® 5 ppm Se	38.397^{ab}
Selenite 10 ppm Se	59.015^{d}
Sel-Plex® 10 ppm Se	43.37^{abc}
Selenite 15 ppm Se	68.412^{bcd}
Sel-Plex® 15 ppm Se	20.43^{a}

GSH-px activity was monitored using excess GR and H_2O_2 as a substrate.
^{a,b,c,d} Means are significantly different at $P \leq 0.05$

Table 5. Glutathione Peroxidase activity (mU/mg total protein) from chicken livers homogenates from 3 week old male broilers (Trial 2) fed high levels of selenite or Sel-Plex®.

Treatment	mU/mg protein
Control (0 ppm Se added)	23.808^{ab}
Selenite 5 ppm Se	34.47^{bcd}
Sel-Plex® 5 ppm Se	57.02^{d}
Selenite 10 ppm Se	12.93^{a}
Sel-Plex® 10 ppm Se	46.819^{cd}
Selenite 15 ppm Se	50.60^{cd}
Sel-Plex® 15 ppm Se	63.64^{d}

GSH-px activity was monitored using excess GR and H_2O_2 as a substrate.
^{a,b,c,d} Means are significantly different at $P \leq 0.05$

TABLE 6. Feed conversion and 2 week body weights of male broilers orally gavaged with increasing amounts of sodium selenite. Trial 2.

TREATMENT	AVG 2 WK BW (g)	FC 0-1WK	FC 0-2WK
CONTROL	322.85	0.775	2.41
SELENITE 5PPM	304.3	0.82	2.48
SELENITE 10PPM	327.2	0.81	2.27
SELENITE 15PPM	333.8	0.78	2.40

Birds were orally gavaged with increasing log doses of sodium selenite dissolved in distilled water.

[a,b,c] Means are significantly different at $P \leq 0.05$

TABLE 7. Feed conversion, 5-day body weights, and mortality of male broilers orally gavaged with sodium selenite.

TREATMENT	AVG 5 DAY BW (g)	FC 0-DAY 5	% MORTALITY
SE 5PPM (SE)	105.9	0.965	0
SE 10PPM (SE)	91.2	1.05	0
SE 15PPM (SE)	80.7	1.33	56
SE 5PPM (C)	96.1	0.975	12.5
SE 10PPM (C)	62.1	2.19	50
SE 15PPM (C)	34.0	-0.26	100

Birds were orally gavaged with sodium selenite based on control (C) and selenite (SE) feed intake, dissolved in distilled water.

[a,b,c] Means are significantly different at $P \leq 0.05$.

Chapter 3

Comparative Effects of Various Forms of Selenium on Thioredoxin Reductase Activity in Broiler Chickens.

Abstract. Thioredoxin reductase (TR) is an essential antioxidant enzyme that catalyzes the reduction of thioredoxin by NADPH. This enzyme was shown to catalyze an NADPH-dependent reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in a simple rapid assay. The importance of TR is attributed to its ability to protect against oxidative stress. There has been little work done to characterize this enzyme in the chicken, an animal that encounters many stressors such as crowding, disease, and 24-hour photoperiods. This study examined TR activity and protein distribution in organs and cellular fractions in young chicken. Birds were raised on a (1) basal diet (no supplemental selenium) or diets providing either 0.3 ppm of (2) sodium selenite, (3) Sel-Plex®, or (4) a combination of both selenium sources. At 3 weeks of age, organ samples were taken and processed for Western blotting and DTNB activity that is indicative of TR activity. The highest TR activity was seen primarily with birds fed 0.3 ppm of Sel-Plex®. However, TR protein distribution did not differ among treatments. In the subcellular fractions, the majority of the TR activity was in the mitochondrial lysate and post mitochondria supernatant. The TR protein distribution within the cell was primarily in the post mitochondrial supernatant. These results suggest that dietary selenium is essential for TR activity, but not TR protein expression. It also indicates that the majority of the TR activity within the cell lies in the mitochondria. These results show that in the chicken the optimum TR activity is selenium-dependent.

Keywords: Chickens, Selenium, Thioredoxin Reductase.

Introduction

Selenium has been found to participate in various physiological functions, with its most notable roles as a member of the selenoprotein family. There are over 20 eukaryotic selenoproteins (Kohrl et al., 2000) and at least 35 Se containing proteins or protein subunits (Behne et al., 2000). The most abundant selenoproteins in mammals are glutathione peroxidase (GSH-px) and thioredoxin reductase (TR) (Gladyshev et al., 1998). The expression of these selenoproteins is dependant on the amount of available Se, hormones, and environmental conditions (Kohrle et al., 2000). The majority of the selenoproteins contain a single selenocysteine residue per polypeptide chain inserted by the UGA stop codon during protein synthesis (Tujebajeva et al., 2000; Patching and Gardiner, 1999). The presence of a selenocysteine at the active site of an enzyme can increase its activity 100-1000 fold (Burk, 2002).

Selenoproteins are important in many diverse systems such as redox signaling, regulation of apoptosis, immunomodulation, spermatogenesis, and embryonic development (Surai, 2002). Recent studies have attributed impaired functions of the cellular thioredoxin and glutathione systems as just one of the myriad of the many effects of Se deficiency. However, a majority of these selenoproteins still have undefined functions. This lack of knowledge and the clear multifunctional role of selenoproteins serve as the impetus for increasing research focus in the area of protein chemistry.

Thioredoxin reductase (TR) was first isolated and characterized from calf liver and thymus (Holmgren, 1977) and then from rat liver cytosol (Luthman and Holmgren, 1982). TR is a selenoenzyme found as a dimer with a molecular weight of 116 kDa with

each subunit weighing approximately 58 kDa. TR from mammalian cells contains a selenocysteine residue in the conserved C-terminus and a FAD prostethic group that is tightly bound to the enzyme and very sensitive to inhibition by heavy metals such as arsenic (Tamura and Stadman, 1996). It appears that TR is a flavoprotein that requires NADPH as an electron donor to reduce free radicals (Ganther, 1999). TR belongs to a family of oxidoreductases that share a sequence and mechanism of action very similar to glutathione reductase (GR) (Gasdaska et al., 1995). TR only differs from GR by its selenocysteine insertion and all other aspects of the enzyme are homologous with GR (Mustacich and Powis, 2000).

There are three known forms of TR that contribute to the thioredoxin system. TR1 is predominantly a cytosolic selenoenzyme that was first purified from ^{75}Se-labeled human lung cancer cells (Tamura and Stadtman, 1996). TR1 is a dimer with two identical 56-kDa subunits. The second TR (TR2) is located primarily in the mitochondria and is involved in protection against oxidative stress (Behne and Kyriakopoulos, 2001). The sequence for TR2 is about 56% homologous with TR1. TR2 differs from TR1 at the N-terminal mitochondrial leader sequence (Miranda-Vizuete et al., 1999). A third TR (TR3) was purified from ^{75}Se- labeled mouse testis where it is found predominantly (Sun et al., 1999). TR3 has 70% homology with TR1. These isoforms suggest the possibility of other thioredoxin reductase species, which may differ on the basis of their tissue distribution (Behne and Kyriakopoulos, 2001).

The mechanism by which the thioredoxin-TR system protects cellular components from oxidative damage is very important for cell survival. Mammalian TR contains a selenocysteine residue in the conserved C-terminal sequence (-Gly-Cys-

SeCys-Gly), which forms a selenylsulfide bond in the oxidized enzymes. NADPH reduces the selenylsulfide, which forms a selenolthiol. The selenolthiol serves as the active site in the reduction of thioredoxin (Sandalova et al., 2001). Thioredoxin, in turn, reduces oxidized proteins through a thiol-disulfide exchange reaction. In addition to reducing oxidized proteins, TR catalyzes the reduction of many other substrates such as, lipid peroxides, thyroid peroxidase (T-px), glutathione, ascorbate, selenite, selenodiglutathione, and protein disulfide isomerase (Chae et al., 1994; Luthman and Holmgren, 1982). Compounds containing arsenic or gold can inhibit these functions (Smith et al., 1999). Inhibition of TR prevents the regeneration of reduced thioredoxin from thioredoxin disulfide and allows for the accumulation of oxidized proteins inside the cell (Lin et al., 2001). The inhibition of these pathways has been proposed as the means by which arsenic mediates its toxic and carcinogenic effects.

Selenium is requirement for the synthesis of functional TR. Radiolabeling of proteins by incubation of cDNA transfected cells with ^{75}Se in the form of sodium selenite demonstrated that Se is incorporated into the expressed TR protein (Fujiwara et al., 1999). Se supplementation can increase the activity of TR. Berggren et al. (1997) showed that Se increased the activity of TR in human cancer cell lines by increasing the amount of selenocysteine incorporated into proteins resulting in an increase in the specific activity of TR and TR protein levels. In humans, TR activity increases when serum Se levels from 0.01 to 10 μM (Kitaoka et al., 1994). Mammalian TR activity is induced with dietary Se supplementation in nutritional and supranutrtional ranges. Berggren et al. (1999) showed that supranutrtional levels of Se increased activity in the kidney, lung, and liver of the rat. Berggren et al. (1999) also demonstrated that an

increase in TR activity was not necessarily indicative of an increase in the actual TR protein levels. The demonstration of increased TR activity at supranutritional supplementation of Se supports the hypothesis that TR plays a role in suppression of cancer.

An extensive examination of TR function in chickens has not been conducted to date. Recent evidence and preliminary observations from this laboratory suggest that chicken TR may be different from mammalian TR (Gowdy et al., unpublished). It is important to examine TR in chickens because the poultry industry has increasing problems associated with oxidative stress resulting from higher rates of metabolism and oxidation of feed ingredients such as dietary fat. Thus, it is important to identify potential interventions that would alleviate the problems associated with oxidative stress in poultry. An easily amenable problem of oxidative stress could be stopped with the proper dietary Se supplementation. Given that the poultry industry is a multibillion dollar a year business in the United States alone, the increasing problems associated with oxidative stress and tumorigenesis, and the role that TR has played in these functions in other organisms, it was important to ascertain the role that Se may play in TR pathways in chickens. The objective of this study was to use different chemical forms of selenium in broiler diets and assess their influence on cellular and organ distribution of TR activity and protein expression in the chicken.

Materials and Methods

Animals and diets

This project was approved and conducted under the supervision of the North Carolina State University Animal Care and Use Committee which has adopted Animal Care and Use Guidelines governing all animal use in experimental procedures.

Ross male broilers were fed the North Carolina Agricultural Research Service broiler starter feed (3154 kcal/kg ME, 21% CP) from 1d of age to 3 wk of age. A basal feed (Control) was supplemented either with 0.3 ppm of sodium selenite (SE), Sel-Plex® (SP), or 0.15 ppm of both sodium selenite and Sel-Plex® (SS). The control diet (C) was diluted with sand to adjust for dilution effects associated with addition of large quantities of either SE or SP to the test diets. The study was conducted in 2 battery trials with 3 replicates per treatment using 10 birds per replicate. Replicates were arranged in a completely randomized design with blocking for light and position within the brooder battery. Birds were given feed and water for *ad libitum* consumption.

Subcellular Preparation

Livers from five 3 wk old male Ross broilers fed 0.3 ppm of Sel-Plex® (SP) were collected fresh, minced and washed immediately in ice cold 0.9% saline. The minced liver was then diluted to a 1:5 (w:v) mixture with an ice cold 250 mM sucrose, 5 mM Tris HCl pH 7.5 buffer. Tissue was ground with a 40 mL Dounce homogenizer with a glass pestle. The homogenate was spun at 1500 x g for 30 min at 4°C followed by removal of the post-nuclear supernatant and isolation of the nuclear pellets that were pooled. The post-nuclear supernatant was centrifuged at 12000 x g for 30 minutes at 4°C, which yielded the mitochondrial pellet and post-mitochondrial supernatant. The mitochondrial

pellets were pooled and resuspended in 5 mM Tris HCl pH 7.5 and homogenized with the Dounce homogenizer. The pooled pellets were then centrifuged at 12000 x g for 30 min at 4°C, which yielded the mitochondrial lysate and mitochondrial membranes. A 2 mL aliquot of each fraction was taken and analyzed for protein and TR activity.

Thioredoxin Reductase activity

Liver samples (0.5-1.0g) from birds at 3 wk of age were taken and homogenized with a polytron homogenizer[13] in cold 50mM TrisHCl 1mM EDTA pH 7.5 in a 1:3 ratio. The homogenates were centrifuged (Beckman L5-50) at 5,000 x g for 30 min. Supernatants were removed and analyzed for activity using the DTNB assay described by Luthman and Holmgren (1982). Working buffer (100mM sodium phosphate, 10mM Na_4EDTA, 0.2mM NADPH, 0.2mg bovine serum albumin (BSA)/mL, 1% ethanol, 5 mM DTNB), and 0.5mM FAD (4.2mg of FAD in 500mM Tris, pH 7.4) were added to a 96 well plate before adding sample. Absorbency was read at 412 ηM for 3 minutes. Samples were run in triplicate and statistical outliers were removed. Results were calculated based on the yield of 2 moles of 2-nitro-5-thiobenzoate per mol of NADPH consumed.

Cytosolic Protein Determination

Total protein content was measured using a Protein Assay Kit[14] with BSA as a standard. The standard curve was linear ($r^2 \geq 0.98$) up to 80μg/mL of BSA. Coefficients of variation, between replicates of the same sample, were maintained less than 5%.

[13] Heat System Ultrasonics, Plainview, N.Y. 11803
[14] Bio-Rad, Hercules, CA 94547

Western Blot Analysis

A sample of 15 μg of cytosolic protein from each tissue or blood sample was loaded on a 10% Tris HCl Criterion gel[2]. A recombinant rat thioredoxin reductase[15] was included in each gel as a positive control. The proteins were separated on the Tris Gel at constant current (250 mV) for 1 hour. The separated proteins were then electro-transferred onto a nitrocellulose membrane after 1 hour at constant voltage (100 volts) using a transfer buffer (25mM Tris, 192mM Glycine, 20% Methanol, 0.1% SDS). The membranes were blocked for 1 hour with 5% nonfat milk powder in TPBS (PBS, 0.05%Tween-20) with continuous shaking. The membranes were then washed 3times for 10 min/wash in TPBS. The primary antibody was a gift from Dr. J. R. Arthur from the Rowett Research Institute (Aberdeen, Scotland). The antibody was raised in rabbits against the whole rat protein. The membranes were treated with primary antibody diluted 1:5000 of antiserum in 3% nonfat milk powder in TPBS with continuous shaking overnight to obtain maximum binding. The membranes were again washed 3 times for 10 min/wash in TPBS with continuous shaking. The membranes were then treated with secondary antibody- anti-rabbit HRP[2] diluted 1/3000 in 3% nonfat milk powder in TPBS for 1 hour with continuous shaking. The membranes were again washed 3 times for 10 min/wash in TPBS and 1 time for 10 min in PBS. The blots were developed using the Immun-Star HRP substrate Kit[2] with horseradish peroxidase for antibody detection, followed by exposure to x-ray film. The signals were captured using an Alpha Imager 2000[16] for quantitative analysis. A kaleidoscope prestained protein standard[2] was used to determine molecular weights of separated proteins.

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[15] American Diagnostica Inc, Stamford, Ct 06902
[16] Alpha Innotech Corporation, 400859.

Statistical Analysis

Data from all experiments were analyzed using the ANOVA procedure of the Statistical Analysis System (SAS Institute, 1995). Differences among means were determined with the Student Newman Kuehls test. The level of significance was set at $p \leq 0.05$.

Results

The TR activities in various chicken tissues differ in response to the chemical forms of dietary supplemental selenium. The Se-deficient chickens (Control) had the lowest TR activity in the majority of the organs (Table 1). With few exceptions, TR activity generally was highest in the Sel-Plex®-fed birds as compared to the sodium selenite-fed or combination (selenite + Sel-Plex®)-fed birds. The organs with the highest activities were the thymus, brain, heart, bursa of Fabricius, and lung. TR activity in the liver increased significantly when the birds were fed selenium supplemented diets (C=0.02, SE= 0.08, SP= 0.07, SS= 0.05). The TR activity in the lung responded similarly to selenium supplementation (C=0.05, SE= 0.08, SP=0.09, SS=0.03). The TR activity in the heart showed the greatest increase in the groups fed SP (C=0.02, SE=0.06, SP=0.11, SS=0.06). The brain showed high TR activity when compared to the other tissues, but selenium supplementation appeared to cause a decrease in TR activity in the brain (C=0.32, SE= 0.08, SP=0.15, SS=0.13). An explanation for the divergent response to selenium supplementation in TR activity in the brain is not readily apparent at this time. It is not known if the brain TR activity results were due to a variation n the assay procedure or whether the brain is an organ in which TR activity is maintained at high levels at all times. Selenium will accumulate in high concentrations in endocrine glands

in decreasing order in chicken pituitary, pineal, adrenals, kidneys, pancreas, brain and ovary and testes (Vohra *et al*., 1973). Dietary supplementation of SP resulted in the highest TR activity in breast muscle (C=0.03, SE=0.07, SP=0.08, SS=0.07), bursa of Fabricius (C= 0.03, SE =0.07, SP= 0.10, SS=0.06), thymus (C=0.04, SE=0.09, SP=0.14, SS=0.08), and spleen (C=0.03, SE=0.04, SP=0.07, SS=0.08). The RBC (C=0.03, SE= 0.07, SP=0.04, SS=0.02) and plasma (C=0.03, SE=0.03, SP=0.02, SS=0.04) had the lowest TR activity among the various tissues sampled.

Within a tissue, no significant differences were seen when integrated density values (IDV) were used to assess the influence of different selenium supplements on expression of the of the 58 kDa TR protein (Figure 2). The IDV for TR bands in the breast muscle were very low for SE, SP and SS fed birds and were not seen for control fed birds. There were no IDVs generated for the rat RBC fraction regardless of the selenium treatment. Plasma IDVs were the highest of all tissues and were more than two times the IDVs of all other tissues. A reason for the extremely high IDVs for plasma was not readily available, but antiserum binding in plasma could be nonspecific because plasma had a very low TR activity (Table 1). Therefore, the extremely high plasma TR IDV did not represent TR activity. Nevertheless, when Western blots of the antibody bound-TR were analyzed, all organs and tissues expressed a TR band that had a molecular weight slightly higher than the ≈58 kDa rat standard (Figure 1). Some organs such as the heart and kidney had multiple bands indicating multiple weights or isoforms of TR (Figure 1).

The subcellular distribution of TR in chicken hepatic cells is presented in Table 2. The high TR activity of the liver homogenate is primarily cytoplasmic. When different

cellular fractions were analyzed, the TR activities in the nuclear pellet (0.1289) and the mitochondrial lysate (0.1333) were the highest followed by post-mitochondrial supernatant (0.1066), mitochondrial membranes (0.0930), post-nuclear supernatant (0.0865), and the mitochondrial pellet (0.065). All of these TR activities were higher than the organ distribution activities. A possibility exists that the differences may be due to the ages of the tissues used for organ distribution and for subcellular distribution.

The Western blot analysis of the subcellular TR activity provided conflicting results (Figure 3). The majority of the TR bands were the same molecular weight as the rat standard, but there was also a second lower molecular weight band in all of the subcellular fractions. The heaviest bands were in the post-mitochondrial supernatant, and no bands were seen in the mitochondrial lysate or mitochondrial membranes, but spectrophotmetric analysis of the TR activity showed the mitochondrial lysate to have the highest activity among the subcellullar fractions (Table 2). The antibody used in this investigation was developed for rat cytosolic TR, and these results, therefore, might indicate a different TR isoform in the mitochondria. The post-mitochondrial supernatant was also the only fraction without hemoglobin in it. The TR distribution assessment by Western blot showed that the rat cytosolic antibody would not bind to either the RBC, the mitochondrial lysate, or the membranes with associated hemoglobin. These observations suggest that the avian RBC TR is different from that found in mammals.

Discussion

The fact that chicken TR is selenium-dependent is not surprising. Breggen et al. (1999) observed similar results with rats fed sodium selenite at 0.1 and 0.01 ppm and

compared their TR activity to that of rats fed a Se-deficient diet. Breggen et al. (1999) found that TR activity was enhanced by dietary selenium supplementation and that the TR activity was more stable. Initial results with chicken TR reflect a similar condition in which chickens fed supplemental selenium had higher and more stable TR activity (Chapter 2).

In comparison to mammals (Smith et al., 2001), chickens, as found in this research, have extremely low TR activities. The low TR activities in chickens might indicate low TR protein expression or might indicate that chicken TR(s) differ significantly from mammalian TRs (Liu and Stadtman, 1997). These conclusions are drawn from Western blot observations in this experiment that showed TR protein expression at relatively low levels. All organs expressed a band that was slightly higher in molecular weight (≈70 kDa) than the rat standard (≈56-58 kDa). Perhaps, this increase in molecular weight could be attributed to glycosylation of chicken TR, but even if this were to be the case, the active sight containing the selenocysteine should have been conserved even in the chicken as it is in other species (Gromer et al., 2003).

In the various tissues, it was not uncommon to find multiple TR bands on the Western blots. Some of the TR bands were quite heavy and others very light (Figures 1 and 2). Based on spectrophotometric assays (Tables 1 and 2), TR activity in the different tissues was variable and apparently selenium form-dependent because TR activity was generally greater in tissues from birds that had been fed SP-supplemented diets. A similar observation was made *in vitro* when it was noted that TR protein expression and activity in cultured cells was dependent on the availability of selenium in the media (Gallegos et al., 1997). However, it is surprising that there were very diverse responses

within the RBC. The TR activity in RBC was relatively high with the DTNB assay, but no bands were present with the Western blot. These observations clearly indicate a difference between mammalian TR and avian TR because the rat cytoplasmic anti-TR antiserum simply did not bind to the RBC.

Rigobello et al. (1998) documented subcellular TR distribution in rat hepatic cells, and the results from the current subcellular study with chicken hepatic cells was similar. Rigobello et al. (1998) found higher activity in the matrix of the mitochondria than the mitochondrial pellet. The post-nuclear and post-mitochondrial supernatants were primarily cytosolic fractions with comparable activity to that of the cytosol, and those results were comparable with subcellular results made in the rat (Rozell et al., 1985; Rozell et al., 1988) and in human tissue (Chen et al., 2002; Ejima et al., 1999). Similar subcellular results for the chicken hepatacyte are documented herein.

The distribution of thioredoxin and TR in subcellular structures such as the endoplasmic reticulum, secretory granules, plasma membranes, and at the subplasma membrane were strategically placed indicating that TR has a role to play in protein processing, secretion, and formation of protein disulfides (Rozell et al., 1985 and 1988). Chen et al. (2002) found high concentrations of TR in the mitochondria, lysosome, microsome and cytosol in the human liver. In a similar study by Ejima et al. (1999), high levels of TR were found in the cytosol (90%) and the mitochondria (10%) of placental cells. All of these results point to the fact that TR plays an important role in not only the cytosol of the cell, but also in the organelles as well. The role of TR in the nucleus should be apparent when one understands the importance of thioredoxin function in the maintenance of DNA and in the transcription of DNA (Mustacich and Powis, 2000;

Powis and Montfort, 2001). Without such functions the genome would rapidly deteriorate or it would become disorganized leading to the production of tumorigenic cells.

The DTNB assay has been widely used for the determination of TR activity because it is economical, easy, and yields instant results. The insulin assay for determining TR activity is more costly than the DTNB assay, although both give comparable results. However, enzymatic methods for TR activity have been questioned (Nicol et al., 2001). Therefore, it seemed prudent to explore the use of Western blotting as a means to determine TR protein expression. Unfortunately, little is known about chicken TR, and reagents specific for chicken TR do not exist. However, it was reasoned that if the active site in many of the selenoproteins has a highly conserved sequence and that significant homology has been recognized among species, it might be possible to use a well defined anti-rat cytosolic TR antiserum to bind to chicken TR. Nevertheless, the issue of specificity was considered a limiting factor in this approach. Binding of the rat cytosolic anti-TR antiserum to chicken proteins did occur, but it quickly became apparent that chicken TR is clearly different from the rat TR in molecular weight, and that there were multiple isoforms in some organs and subcellular fractions. Nicol et al. (2001) reported two isoforms when using a Western blot on rat liver preparations. In the current study, up to five different isoforms were found in some organs, and the TR molecular weights ranged from 100 kDa to 30 kDa. Whether the different isoforms, revealed via Western blotting, represented true isoforms or conjugates of oxidized TR protein was not determined in this study.

The spectrophotometric TR activity results and the TR protein expression documented with Western blotting were not consistent. Breggen et al. (1999) showed that activity of TR was significantly increased with dietary selenium supplementation, but protein expression was not altered by selenium supplementation. A significant increase in TR activity, especially with organic selenium supplementation was evident, but protein expression was not significantly altered with this intervention. Thus, a selenium-dependent increase in TR activity is not because of a greater amount of protein, but rather an increase in the specific activity of the enzyme, which should result in better antioxidant protection (Allan et al., 1999).

The increased TR activity was also associated with the amount of selenium incorporated into TR (Gladyshev et al., 1996). TR isolated from human placental cells had a higher rate of selenocysteine incorporation when more selenium was added to the cell culture media (Gladyshev et al., 1996). Mahmoud and Edens (2003) have confirmed that GSH-px activity was increased to a higher rate organic selenium dietary supplementation than with sodium selenite dietary supplementation, and Edens (2002) has reviewed research reports that show organic selenium to be more readily available for selenoprotein synthesis than sodium selenite. Thus, it was no surprise to find TR activity to be somewhat higher in organs and tissues from chickens fed the organic selenium supplements in this study

There were no significant differences among organs for TR activities. The only organ that seemed to differ was breast muscle in which selenium supplemented birds had Western blots for TR that was not evident in control birds. The reason for this difference might be that selenium retention in skeletal muscle is relatively poor and that in the Se-

deficient birds inadequate selenium was available to meet the needs for all selenoprotein production. Jurado et al. (2003) examined the expression of TR mRNA in different organs of the mouse and observed elevated TR mRNA in the testis, spleen, and kidney. However, the presence of TR mRNA does not instantly signal that there will be increasing levels of TR protein.

This study represents the first in depth investigation on chicken TR. These preliminary results indicate that chicken TR differs from mammalian TR in molecular weight, form, and activity, but chicken TR is sensitive to selenium supplementation. Future studies must incorporate analysis of chicken TR mRNA and its tissue distribution. Furthermore, there is a need for chicken/avian-specific reagents that can be used in the study of TR *in vivo* and *in vitro*.

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Table 1. Thioredoxin reductase activity (nmol NADPH/ min/ mg total protein) from chicken tissues from 3-week-old male broilers fed supplemental levels of selenite (SE) at 0.3 ppm, Sel-Plex® (SP) at 0.3 ppm, 0.15 ppm selenite + 0.15 ppm Sel-Plex® (SS) Or a Control diet with no supplemental selenium. TR activity was monitored using the DTNB assay that measures the NADPH-dependent reduction of the disulfide bond in 5,5'-dithiobis(2-nitrobenzoic acid) at 412nm. Calculated resulted are based on yield of 2 moles of 2-nitro-5-thiobenzoate per mol of NADPH consumed. Results are given as the μmol of NADPH oxidized per minute per mg of protein.

	Treatments			
	Control	**SE 0.3 ppm**	**SP 0.3 ppm**	**SS 0.3 ppm**
Liver	$20.0^{b} \pm 10$	$80.0^{a} \pm 40$	$70.0^{a} \pm 50$	$50.0^{ab} \pm 20$
Lung	50.0 ± 50	80.0 ± 30	90.0 ± 70	30.0 ± 10
Heart	$20.0^{b} \pm 10$	$60.0^{b} \pm 30$	$110.0^{a} \pm 40$	$60.0^{b} \pm 20$
Kidney	60.0 ± 70	90.0 ± 10	80.0 ± 30	50.0 ± 30
Brain	$320.0^{a} \pm 280$	$80.0^{a} \pm 30$	$150.0^{a} \pm 100$	$130.0^{a} \pm 100$
Breast Muscle	$30.0^{b} \pm 10$	$70.0^{ab} \pm 20$	$80.0^{a} \pm 40$	$70.0^{ab} \pm 30$
Bursa	$30.0^{b} \pm 10$	$70.0^{ab} \pm 20$	$100.0^{a} \pm 50$	$60.0^{ab} \pm 30$
Thymus	$40.0^{c} \pm 20$	$90.0^{b} \pm 30$	$140.0^{a} \pm 40$	$80.0^{b} \pm 30$
Spleen	$30.0^{b} \pm 10$	$40.0^{ab} \pm 10$	$70.0^{a} \pm 40$	$80.0^{a} \pm 20$
RBC	30.0 ± 30	70.0 ± 60	40.0 ± 20	20.0 ± 10
Plasma	30.0 ± 10	30.0 ± 10	20 ± 35	40.0 ± 20

[a,b,c] In a row, means with unlike supescripts differ significantly ($P \leq 0.05$).

Figure 1. Western blot analysis shows the protein expression of thioredoxin reductase. Protein (15 μg) was loaded into each well, and samples were run in duplicate. This example is from a 3-week-old male broiler fed 0.3 ppm of Sel-Plex®. A rat standard (8 μg) reference was run in lane 1.

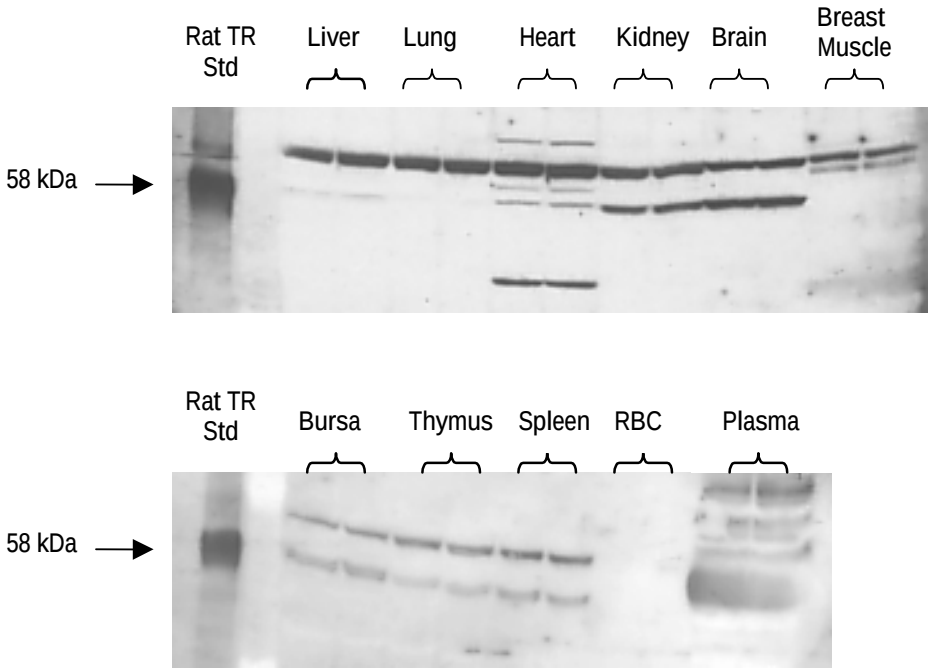


Figure 2. Integrated Density Values (IDV) of 58 kDa TR band on various chicken organs. The IDV was based on the mean of two TR protein bands assessed via Western Blot. Six birds per treatment were examined. Within tissues, there were no significant differences attributed to selenium treatment

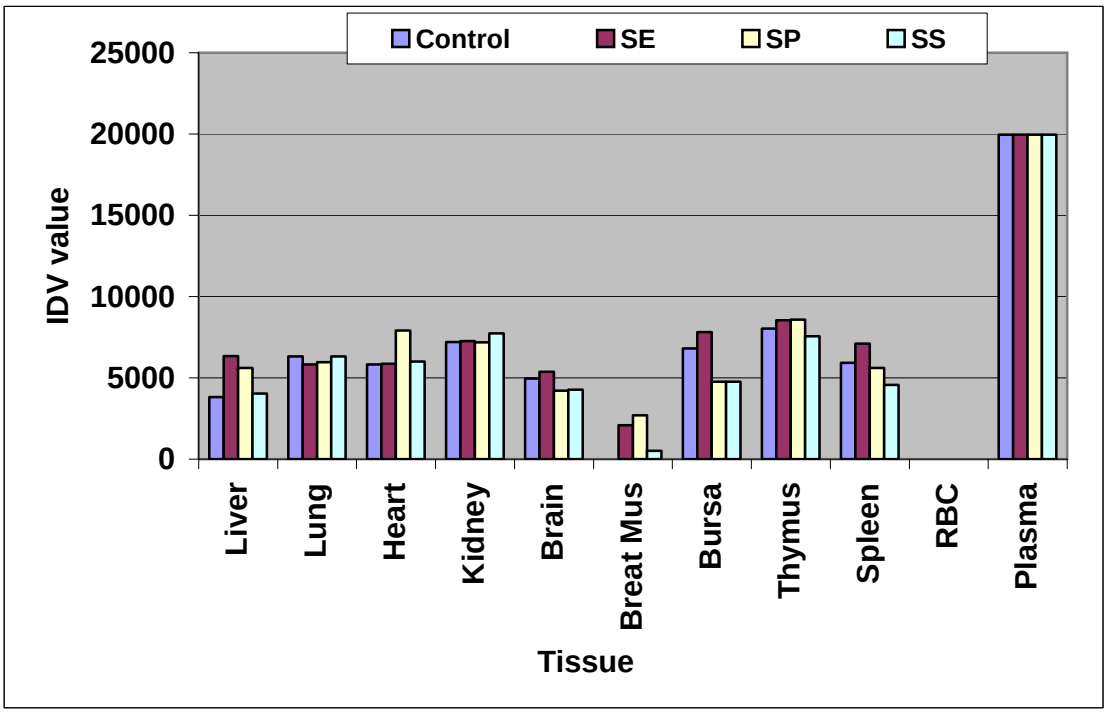
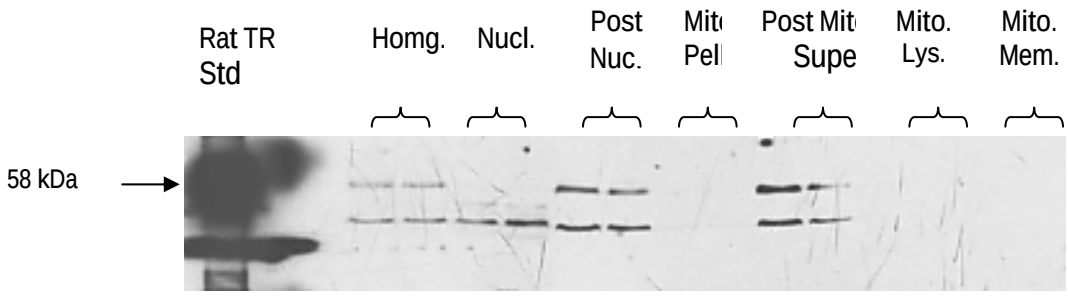


Table 2. Subcellular distribution of chicken thioredoxin reductase (TR) activity (nmol/min/mg total protein) in liver cells. TR activity was monitored using the DTNB assay that measures the NADPH-dependent reduction of the disulfide bond in 5,5'-dithiobis(2-nitrobenzoic acid) at 412nm. Calculated resulted are based on yield of 2 moles of 2-nitro-5-thiobenzoate per mol of NADPH consumed. Results are given as the μmol of NADPH oxidized per minute per mg of protein.

Cellular Distribution	μmol/min/mg protein
Liver Homogenate	**107.2**
Nuclear Pellet	**128.9**
Post Nuclear Supernatant	**86.5**
Mitochondria Pellet	**65.0**
Post Mitochondria Supernatant	**106.6**
Mitochondria Lysate	**133.3**
Mitochondria Membranes	**93.0**

[a, b]In a column, means with unlike superscripts differ significantly ($P \leq 0.05$).

Figure 3. Western blot analysis shows the subcellular protein expression of thioredoxin reductase. Protein (15 μg) was loaded into each well, and samples were run in duplicate. This example is from a 3-week-old male broiler fed 0.3 ppm of Sel-Plex®. A rat standard (8 μg) reference was run in lane 1.



Chapter 4

Attempts to Purify and Characterize Thioredoxin Reductase from Chicken Liver

Abstract. Thioredoxin reductase (TR) is an essential antioxidant enzyme that catalyzes the reduction of thioredoxin. TR has been shown to catalyze an NADPH-dependent reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in a simple, rapid assay. TR provides protection against oxidative stress. Until this time, no work has been conducted to characterize TR in the chicken. This report describes an attempt to purify and characterize TR from chicken liver. Preliminary work has shown that chicken TR activity is responsive to dietary selenium supplementation, activity was age dependent, and required the addition of FAD in the incubation media to stabilize enzyme activity. Ammonium sulfate saturation of the enzyme caused it to fall out of solution at 70% instead of 80% like the mammal. Reduction with 0.2 M DTT did not restore activity after overnight dialysis. These results suggest that chicken TR is very sensitive (labile) under conditions that are optimized for mammalian TR. This study describes attempts to redefine pre-column preparatory events for chicken TR. Isolation and characterization of chicken TR is important for the worldwide poultry industry and would provide additional evidence concerning the evolution of the enzyme in different vertebrates. Furthermore, development of reagents necessary to adequately study avian TR goes beyond the poultry world and extends into wild bird populations around the world that are being threatened by environmental pollutants that could endanger many species.

Keywords: Poultry, Selenoprotein, Protein Purification, Thioredoxin Reductase

Introduction

Selenium is involved in various physiological and biochemical functions in all vertebrates. Its most notable role is in the selenoprotein family. There are over 20 eukaryotic selenoproteins (Kohrl et al., 2000) and at least 35 Se-containing proteins or protein subunits (Behne et al., 2000). The most abundant selenoprotiens in mammals are glutathione peroxidase (GSH-px) and thioredoxin reductase (TR) (Gladyshev et al., 1998). The expression of these selenoproteins is dependant on the concentration of available Se, hormones, and environmental conditions (Kohrle et al., 2000). The majority of the selenoproteins contain a single selenocysteine residue per polypeptide chain inserted by the UGA stop codon during protein synthesis (Tujebajeva et al., 2000; Patching and Gardiner, 1999). The presence of a selenocysteine at the active site of an enzyme can increase its activity 100-1000 fold (Burk, 2002).

Selenoproteins are important in many diverse systems such as redox signaling, regulation of apoptosis, immunomodulation, spermatogenesis, and embryonic development (Surai, 2002). Recent studies have attributed the impaired function of the cellular thioredoxin and glutathione systems as just one of the myriad effects attributed to Se-deficiency. However the majority of the selenoproteins still have undefined mechanisms of action. This lack of knowledge and the clear multifunctional role of selenoproteins have served as a strong impetus for increasing research focused on this area of protein chemistry.

Thioredoxin reductase (TR) was first isolated and characterized from calf liver and thymus (Holmgren, 1977) and then from rat liver cytosol (Luthman and Holmgren, 1982). TR is a selenoenzyme found as a dimer with a molecular weight of 116 kDa with

each subunit weighing approximately 58 kDa. TR from mammalian cells contains a selenocysteine residue in the conserved C-terminus. TR contains a FAD prosthetic group that is tightly bound to the enzyme and very sensitive to inhibition by heavy metals such as arsenic (Tamura and Stadman, 1996). This selenoenzyme is a flavoprotein that requires NADPH for reducing equivalents used to reduce free radicals (Ganther, 1999). TR also belongs to a family of oxidoreductases that share a sequence and mechanism of action similar to glutathione reductase (Gasdaska et al., 1995). It has been suggested that TR actually evolved in mammals from GR instead of its archaeic ancestor in bacteria. TR differs from GR by its selenocysteine insertion, but all other comparative aspects of the two enzymes are homologous (Mustacich and Powis, 2000).

There are three known forms of TR that are significant to the thioredoxin system. TR1 is predominantly a cytosolic selenoenzyme that was first purified from ^{75}Se-labeled human lung cancer cells (Tamura and Stadtman, 1996). TR1 is a dimer with two identical 56 kDa subunits. The second TR (TR2) is primarily located in the mitochondria and protects against oxidative stress (Behne and Kyriakopoulos, 2001). The sequence for TR2 is about 56% homologous with TR1. TR2 differs from TR1 in the N-terminal mitochondrial leader sequence (Miranda-Vizuete et al., 1999). A third TR (TR3) was purified from ^{75}Se- labeled mouse testis (Sun et al., 1999). TR3 has 70% homology with TR1. Based on these observations, it is possible that other TR forms might exist in the various tissues in an animal (Behne and Kyriakopoulos, 2001).

The mechanism by which thioredoxin system protects cellular components from oxidative damage is very important for cell survival. Mammalian TR contains a selenocysteine residue in the conserved C-terminal sequence Gly-Cys-SeCys-Gly, which

forms a selenylsulfide bond in the oxidized enzymes. NADPH reduces the selenylsulfide, which forms a selenolthiol. The selenolthiol serves as the active site in the reduction of thioredoxin (Sandalova et al., 2001). Thioredoxin, in turn, reduces oxidized proteins through a thiol-disulfide exchange reaction. In addition to reducing oxidized proteins, TR can catalyze the reduction of many other substrates such as lipid peroxides, thioredoxin peroxidase, glutathione, ascorbate, selenite, selenodiglutathione, and protein disulfide isomerase (Chae et al., 1994; Luthman and Holmgren, 1982). Certain compounds containing arsenic or gold can inhibit these functions (Smith et al., 1999). Inhibition of TR could prevent the regeneration of reduced thioredoxin from thioredoxin disulfide and allow oxidized proteins to accumulate inside the cell (Lin et al., 2001). The inhibition of these functions has been proposed as the means by which arsenic mediates its toxic and carcinogenic effects.

There is a Se requirement for the formation of a functional TR. Radiolabeling of proteins by incubation of cDNA transfected cells with ^{75}Se in the form of sodium selenite demonstrated that Se is incorporated into the expressed TR protein (Fujiwara et al., 1999). Se supplementation can increase the activity of TR. Berggren et al. (1997) showed that Se increased the activity of TR in human cancer cell lines by increasing the amount of selenocysteine incorporated into proteins resulting in an increase in the specific activity of TR and TR protein levels. In humans, an increase in TR activity can be observed with human serum Se levels as low as 0.01 to 10 μM (Kitaoka et al., 1994). Mammalian TR increased activity with Se supplementation in nutritional and supranutrtional ranges. Berggren et al. (1999) showed that supranutritional levels of Se increased activity in the kidney, lung, and liver of the rat. They also demonstrated that an

increase in TR activity was not necessarily indicative of an increase in the actual TR protein concentrations. The demonstration of increased TR activity at supranutritional Se levels supports the hypothesis that TR is involved in Se-related suppression of cancer development and growth.

The examination of TR functions in chickens is a new research area and preliminary observations from this laboratory suggest that chicken TR may be different from mammalian TR (Chapter 2 and Chapter 3). It is important to look at TR in chickens because the growing poultry industry on a global basis has encountered increasing problems with oxidative stress. Thus, a means for controlling oxidative stress in poultry is economically important. The potential to correct an apparently easily amenable problem might be as simple as proper Se nutrition in poultry species. However, the technology developed from the study of TR in poultry extends beyond the poultry world because environmental pollutants that have the potential to initiate oxidative stress and possibly cause endangerment of avian species in the wild are constantly challenging wild bird populations.

Thioredoxin and thioredoxin reductase has been purified and sequenced in most mammals. However, avian work on this enzyme has not developed and is now far behind the work conducted in mammals. The purpose of this investigation was to attempt isolation, purification, and characterization TR from chicken liver. This work is vital to better understanding of the role played by the thiredoxin-thioredoxin reductase system in avian species.

Materials and Methods

Animals and Diets

This project was approved and conducted under the supervision of the North Carolina State University Animal Care and Use Committee that has adopted Animal Care and Use Guidelines governing all animal use in experimental procedures.

Ross male broilers were fed North Carolina Agricultural Research Service diets from day 1 to six weeks of age. The experimental diets consisted of Starter (3177 kcal/kg ME, 22.5% CP to 16 days of age), Grower (3168 kcal/kg, 19.5% CP from 16-35 days of age), and Finisher (3160 kcal/kg, 17.5% CP from 35-42 days of age). Feeds were either supplemented with 0.3 ppm of sodium selenite (SE), 0.3 ppm Sel-Plex® (SP) or Control (C). Control diet (C) had no supplemental selenium. Birds were given water and feed for *ad libitum* consumption.

Thioredoxin Reductase activity

Liver samples (0.5 to 1.0 gm) from birds were taken at various ages and homogenized with a polytron homogenizer[17] in cold 50mM TrisHCl 1mM EDTA pH 7.5 in a 1:3 ratio. The homogenates were centrifuged (Beckman L5-50) at 16,000 rpm for 30 min. The supernatant from each sample was removed and analyzed for TR activity using the DTNB assay previously described by Luthman and Holmgren (1982). The working buffer (100mM Na phosphate, 10mM Na_4EDTA, 0.2 mM NADPH, 0.2 mg bovine serum albumin (BSA)/mL, 1% ethanol, 5 mM DTNB) and 0.5 mM FAD (4.2 mg of FAD in 500mM Tris, pH 7.4) were added to a 2 mL cuvette and heated to 37°C before adding sample. Absorbency of duplicate samples was read every 30 seconds at 412 ηm for 3

min on a Beckman DU-70. Results were calculated based on the yield of 2 moles of 2-nitro-5-thiobenzoate per mol of NADPH.

Cytosolic Protein Determination

Total protein content was measured using a Bicinchoninic Acid kit[18] for protein determination using BSA as a standard. The standard curve was linear ($r^2 \geq 0.98$) up to 80μg BSA/mL of solution.

Preparation of TR for purification

Three different methods were used to prepare liver homogenate for purification. All steps were conducted at 0-4°C.

Method 1. Acid precipitation. This method was utilized to precipitate proteins that are not soluble at pH 5. Supernatant (S1) from chicken liver homogenate was kept on ice and glacial acetic acid was added until pH 5 was reached. S1 was then centrifuged at 10000 x g for 30 min. The supernatant of S1 (S2) was recovered and brought back to pH 7.5 with 1 M NH_4OH to flocculate proteins. The volume was then determined and approximately 75% of original proteins were removed.

Method 2. Ammonium sulfate. S1 from the original chicken liver homogenate was brought to 40% saturation with $(NH_4)_2SO_4$ for 35-45 min and precipitated proteins were removed by centrifuging samples for 10000 x g for 15 minutes. The supernatant (S2) was then collected and brought to 80% saturation with $(NH_4)_2SO_4$ for 35-45 min. Precipitated proteins were collected by centrifugation at 10000 x g for 15 minutes. The pellets were then dissolved in 50 mM Tris HCL, 1mM Na_4EDTA (pH 7.5) and vortexed. Resupended pellets were dialyzed overnight at 4°C with dialysis tubing with a cut-off

[17] Heat System Ultrasonics, Plainview, N.Y. 11803
[18] Sigma-Aldrich, St. Louis MO 63103

size of 12 kDa in 50 mM Tris HCL, 1mM Na_4EDTA (pH 7.5) to insure removal of excess $(NH_4)_2SO_4$. Another study was conducted in which S1 was saturated from 0 to 80 % with $(NH_4)_2SO_4$ and analyzed for activity to determine where chicken TR had been lost from solution.

Method 3. DTT reduction. During dialysis it is possible that proteins can become oxidized requiring a reduction event to reactivate them. Dialysate from ammonium sulfate preparation was collected and 2 mM of dithiothreitol was added and incubated for 30 min in a water bath at 37°C. The activity of both samples with or without DTT was compared.

Column Chromatography

Three different columns were used in the attempt to purify TR from chicken livers.

Column 1. DEAE Sepharose. Dialysate from either ammonium sulfate or acid precipitate preparations that had been or had not been reduced with DTT was loaded on to an anion exchange column with DEAE Sepharose[19]. The DEAE Sepharose had been soaked overnight in 100 mL of 50 mM Tris HCL, 1mM Na_4EDTA (pH 7.5). A linear gradient of 0 to 0.3 M of KCl was applied with a gradient mixer to the column along with the dialysate. The volume of the column is dependent on the volume of the dialysate (1:1). The gradient mixture should be 5x the bed volume. Approximate column size was about 20 ml. Fractions were collected, and TR activity and TR protein were analyzed as described above.

Column 2. Phenyl Sepharose. Dialysate from either ammonium sulfate or acid precipitate preparation that had been or had not been reduced with DTT was loaded on to

a hydrophobic column with Phenyl Sepharose[3]. The Phenyl Sepharose had been soaked overnight in 100 mL of 50 mM Tris HCL, 1mM Na_4EDTA (pH 7.5). A linear gradient of 0 to 30 % of $(NH_4)_2SO_4$ in 50 mM Tris HCL, 1mM Na_4EDTA (pH 7.5) was applied with a gradient mixer to the column along with the dialysate. The volume of the column is dependent on the volume of the dialysate (1:1). The gradient mixture should be 5x the bed volume. Approximate column size was about 50 ml. Fractions were collected, and TR activity and TR protein were analyzed as described above.

Column 3. Sephadex G-200. Due to the bulk of protein the chicken liver, an attempt was made to remove large proteins with a Sephadex G-200[3] column. S1 from chicken liver homogenate was loaded on a size exclusion column equilibrated with 50 mM Tris HCL, 1mM Na_4EDTA (pH 7.5). The enzyme was tracked through fractions by monitoring TR activity and TR protein concentration. The active fractions were then pooled and applied to either the DEAE Sepharose column or the Phenyl Sepharose column.

Results and Discussion

TR has been characterized from bacteria, fungi, plants, and mammals. All have yielded a very active and stable enzyme that had the ability to reduce DTNB directly. The chicken TR proved to be quite different from any other organism. Absorbency at 412 ηm was very low compared to other TR activities (Table 1). The low absorbency at 412 nm was the first indication that chicken TR was different from mammalian TR. Acid precipitate, followed by ammonium sulfate fractions, and dialysate were run on the DEAE sepharose column. The TR activity was maintained through the ammonium sulfate saturation and fractionation, but very little TR activity was seen in the dialysate,

[19] Pharmacia, Piscataway, NJ

and the fractions off the DEAE column had no TR activity. The disappointing results from the first attempt to isolate chicken TR suggested that the large quantity of protein and hemoglobin in the homogenate and retained in successive steps of this procedure might have interfered with maintenance of TR activity through the column. The little TR activity found in the fractions off the anion exchange column was in red fractions with high concentrations of hemoglobin. The assay was conducted again, and the dialysate was reduced with DTT in case the enzyme had been oxidized. Incubation with the reducing agent had no effect on the activity. This indicated the possibly that chicken TR had been precipitated earlier with the ammonium sulfate saturation. An ammonium sulfate gradient was set up with 0, 10, 20, 30, 40, 50, 60, 70, and 80% saturation of 2 mL volumes of chicken homogenate. The results showed that chicken TR activity was the highest in the 30 % and 40% supernatant and 70% pellet (Table 2) and was clearly different from results of similar assays for mammalian TR.

The second attempt to purify chicken TR used the 40% supernatant and the 70% pellet resulting from ammonium sulfate saturation. The dialysate was dialyzed against 30% ammonium sulfate and loaded onto the Phenyl Sepharose hydrophobic column and employed the hydrophobic properties of TR to isolate the enzyme. The low TR activity was found again in the red fractions, and the enzyme seemed to be irreversibly degraded around 15% ammonium sulfate saturation of the column. Also, when active fractions were pooled and frozen, no TR activity could be recovered, which suggested that chicken TR enzyme was extremely susceptible to environmental conditions and potential irreversible oxidation. DTT reduction was tried again and no TR activities could be detected.

All fractions recovered from both columns had a large quantity of protein and very little activity. In an attempt to isolate TR from these proteins that may be inhibiting its activity, a Sephadex G-200 size exclusion column was used before any preparative steps after homogenization, and this did yield improved results. Sephadex G-200 was chosen because mammalian TR was found in a dimer that has a molecular weight of approximately 116 kDa. The Sephadex G-200 column excluded any proteins above 200 kDa. The active fractions were then saturated with 40% and 70% ammonium sulfate and dialyzed overnight against 30% ammonium sulfate in 50 mM Tris HCL, 1mM Na_4EDTA (pH 7.5). Low TR activity was maintained until the 70% pellet was dialyzed overnight. The fractions collected from the hydrophobic column had less protein, but no TR activity, even in the red fractions. At 4°C, chicken TR appeared to have been inactivated by oxidation and even DTT reduction did not improve TR activity.

The next step was to reevaluate the preparation steps and attempt to discover where TR activity might have been lost. Livers from 3 wk old broilers supplemented with 0.3 ppm of Sel-Plex®, an organic selenium supplement, were compared with livers from birds fed no supplemental selenium. The activity of TR was clearly higher in birds fed the organic selenium supplement, and the recovered TR activity from thawed samples was higher in the selenium supplemented birds (Table 1). Dietary supplementation of 1 ppm selenium (sodium selenite) to rats resulted in significantly increased TR activity compared to rats fed a basal diet with no supplemental selenium (Berggren et al., 1999). Se as organic selenium in the diet had a stabilizing effect on chicken TR, and it increased TR activity. Although this did improve chicken TR in crude preparations, it still did not

exhibit the large amount of activity or stability seen with mammalian TR (Berggren et al., 1999).

There was a large quantity of fat and protein in liver samples interfered with attempts to isolate chicken TR. TR activity was examined in 1 wk old broilers to attempt to eliminate the lipid-protein interference problem. The TR activity was much lower in the younger bird than in the 3 wk old broilers even when fed a Se supplemented diet (Table 1). This indicated an age-related influence on chicken TR. Age-related changes in the activity of antioxidant and redox enzymes have been noted in rats with glutathione and glutathione related enzymes (Kim et al., 2003). Five week old rats had significantly lower GST and GSH-px activity in the brain and liver than 9-month-old rats. In chickens, it has been reported that one primary antioxidant enzyme, GSH-px, increases activity as age of the bird increases (Kalytka and Donchenko, 1995), and it also appears that the second selenium-dependent antioxidant enzyme, TR, also follows an age dependent pattern of increasing activity. The age-dependency for activity of selenium-dependent antioxidant enzymes probably reflects the fact that more selenium, almost exclusively organic selenium, accumulates and becomes available for selenoprotein synthesis as age advances.

In another attempt to maintain TR activity in the preparation of chicken liver for purification, flavin adenine dinucleotide (FAD) cofactor was added to the DTNB assay. TR is a dimeric protein that requires FAD for activity, and in chicken TR, this cofactor may be tightly bound due to oxidation, and unable to contribute to the reduction of DTNB. A linear gradient of 5µl to 100 µl of 0.5M FAD was added to a 2 mL reaction of DTNB and chicken liver homogenate. The addition of 20 µl of FAD maintained the

same level of activity from homogenate to supernatant. Adding 20 μL did improve TR activity, which indicated that there is FAD dependence by chicken TR. A similar FAD-dependence had been noted with the cell-free production of active *E. coli* thioredoxin reductase when TR activity was sharply increased with the addition of 8 μM of FAD (Knapp and Swartz, 2004).

In the present study, attempted TR isolation from crude extracts from chicken liver used NADPH-dependent reduction by DTNB. In addition to being rapid and inexpensive, the assay permits quantification of TR activity independent of thioredoxin, which can complicate the assay (Holmgren, 1977). In crude extracts, glutathione and glutathione reductase can contribute to the NADPH-dependent reductase of DTNB. However, saturation with ammonium sulfate or acid precipitation can remove the bulk of glutathione and glutathione reductase so that the measurement is mainly TR. This is an essential preparative step before attempting to isolate chicken TR.

The activity of chicken TR may be sensitive to oxidation, proteolysis, or even inactivation by certain metals. However, reduction by DTT did not improve TR activity, indicating that assay conditions were not optimized for the chicken enzyme. Western blots done in a previous study showed that chicken TR does exist possibly in numerous isoforms. This may be the reason why chicken TR may be sensitive to the conditions of an assay developed for mammalian species. The addition of FAD to the DTNB assay appeared to be essential for quantification of chicken TR activity, which increased substantially. Structurally, both TR and GR are homodimers, at least in mammals, with one molecule of FAD. Both the NADPH- and FAD-containing domain of these enzymes can change conformation change during catalysis or oxidation (Lennon et al., 2000).

Since chicken TR appears to be labile, it is possible that the addition of FAD increased TR activity because of a conformational change was blocked by a stabilized FAD-containing domain.

Feeding selenium supplemented diets has a significant impact on TR activity, both in the chicken and in mammals. Results from this investigation and in others (Chapters 2 and 3) show that dietary selenium supplementation influences TR activity. The selenocysteine insertion in TR is involved in a selenyl sulfide (Se-S) linkage that receives reducing equivalents from NADPH via the cysteine-cysteine dithiol center (Arscott et al., 1997; Nordberg et al., 1998). Therefore, the more Se that is available, the more Se-S linkages that can be formed and influence the reduced state of the enzyme. This is concept becomes quite evident when organic forms of Se such as Sel-Plex®, which is a selenomethionine rich source of Se, is supplemented in diets of poultry. The organic selenium in Sel-Plex® has been shown to be more available through the diet and better retained by chickens (Edens, 1996; 2001). Feeding birds increasing levels of Sel-Plex® caused a log increase in TR activity in previous studies (Chapters 1, 2 and 3). Feeding inorganic forms of Se has also been shown to increase TR activity in chickens, but not as high as Sel-Plex®. This project proved to be a very difficult challenge, but it has provided groundwork for future research with chicken TR. Future ideas include: loading dialysate directly on to a 2′, 5′ ADP column to stop inactivation and avoid the hemoglobin problem, using an antibody affinity column with rat antibodies to try and isolate chicken TR, and to use a possible chicken tumor cell line to examine activity and possibly isolate TR (TR is more active in mammalian tumor cells). The importance of TR in living systems is very evident. It could be very important in the chicken due to the

large amount of oxidative stressors they encounter. Understanding how these animals control oxidative stress could help increase production, livability, and profit, and better understanding of the interaction among selenium-dependent antioxidant enzymes must receive high priority in future research.

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Table 1. Thioredoxin Reductase absorbance at 412 nm for first minute from chicken tissues from male broilers. Different steps yielded different absorbance reading on spectrophotometer.

Step	ΔA_{412} nm per min	mg protein/mL
Crude TR activity (no Se or FAD)	0.1071	22.3
Crude TR of 3 week old broiler fed 0.3 ppm Sel-Plex®	0.1503	21.9
Crude TR of 1 week old broiler fed 0.3 ppm Sel-Plex®	0.0934	21.35
Supernatant with 20 µl of FAD+	0.1271	15.34
Dialysate without 0.2 M DTT	0.0194	11.8
Dialysate with 0.2M DTT	0.0160	11.8

TR activity was monitored using the DTNB assay that measures the NADPH-dependent reduction of the disulfide bond in 5,5'-dithiobis(2-nitrobenzoic acid) at 412nm. Results are given from the first minute when the activity is the highest. Reaction is run at 37° C for optimum activity.

Table 2. Thioredoxin Reductase absorbance at 412 nm for first minute from chicken tissues from male broilers. Different saturation with ammonium sulfate, indicates that TR falls out of solution at different concentration than mammalian conditions.

Step	ΔA_{412} nm per min in supernatant	ΔA_{412} nm per min in pellet
Supernatant (S1)	**0.1031**	**0**
30% $(NH_4)_2SO_4$ saturation	**0.1180**	**0.0197**
40% $(NH_4)_2SO_4$ saturation	**0.1191**	**0.0191**
50% $(NH_4)_2SO_4$ saturation	**0.0871**	**0.0357**
60% $(NH_4)_2SO_4$ saturation	**0.0694**	**0.0542**
70% $(NH_4)_2SO_4$ saturation	**0.0386**	**0.100**
80% $(NH_4)_2SO_4$ saturation	**0.0160**	**0.0597**

TR activity was monitored using the DTNB assay that measures the NADPH-dependent reduction of the disulfide bond in 5,5'-dithiobis(2-nitrobenzoic acid) at 412nm. Results are given from the first minute when the activity is the highest. Reaction is run at 37° C for optimum activity.