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Effect of dietary protein on plasma uric acid, body weight, and oxidative stress in broilers

Maribel Machin
West Virginia University

Effect of Dietary Protein on Plasma Uric Acid, Body Weight, and Oxidative Stress in Broilers.

Maribel Machín

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Hillar Klandorf, Ph.D., Chair
Ken Bleming, Ph.D.
Dale W. Porter, Ph.D.
Knox Van Dyke, Ph.D.

Division of Animal & Veterinary Sciences

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Abstract

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Uric acid is an important antioxidant due to its role as a free radical scavenger, and methods to elevate its plasma concentration may be important in animal health. In a preliminary study, the effect of dietary protein on plasma uric acid (PUA) was determined in 3-wk-old chicks. Twenty four broiler chicks were randomly assigned to 4 diets: low protein (LP) containing 10% casein, medium protein (MP) containing 20% casein, high protein (HP) containing 45% casein and a commercial diet (COD) for 2-wks. PUA concentration increased in chicks fed HP diet and declined in chicks fed LP. In the principal study, PUA and leukocyte oxidative activity (LOA) were also determined. Chicks were fed LP, MP, HP or COD diets for 4 wks. Feed restriction severely restricted body weighs gain in chicks fed synthetic diets. Changes in LOA were found linked to PUA concentrations in chicks fed MP, HP and COD diets.

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List of Abbreviations

ROS	Reactive oxygen species
LOA	Leukocyte oxidative activity
UA	Uric acid
NO	Nitric oxide
SMR	Simple metabolic rate
MLS	Life span potential
MR	Maillard reaction
PUA	Plasma uric acid
MS	Multiple sclerosis
EAE	Encephalomyelitis
LP	Low protein
HP	High protein
BCD	Basal cornstarch diet
BD	Before experimental diet
PBS	Phosphate buffer saline
PMA	Phorbol myristate acetate

Chapter 1: Introduction

The mechanism of free radical production and the way antioxidants function may differ between humans and birds. Even though birds have higher metabolic rates (Lindstedt et al., 1976), body temperature (3°C higher than mammals), and plasma glucose concentrations (2-6 times higher), they still have longer lifespans than mammals of comparable size (Holmes et al., 1995). The longevity of birds has long been a mystery, since the elevated metabolic rates and body temperatures of birds should promote oxygen free radical production and advanced Maillard products formation, factors which play an important role in the aging process (Kristal et al., 1992).

Antioxidants have been investigated and found capable of defending against reactive oxygen species (ROS), thus limiting damage to cells and tissues. Antioxidants may function as enzymes (superoxide dismutase, glutathione peroxidase and catalase) while others have non-enzymatic functions; vitamins, carotenoids, and low molecular weight antioxidants, such as uric acid. Uric acid (UA) is one of the most important antioxidants since it's a potent scavenger of free radicals and has a positive association with longevity among species (Ames et al., 1981; Cutler, 1984).

Antioxidants are determinants of longevity (Cutler, 1984a), therefore effective protection against free radical damage, including higher levels of circulatory antioxidants may be an explanation for the higher life span found in avian species compared to most mammals. This suggestion is supported by the observation that antioxidants such as UA, among others, are found in higher levels in birds than mammals (Iqbal et al., 1997) coupled with lower production of oxygen free radicals (Conlon et al., 1991).

A high proportion of UA exists as urate at physiological pH, which also has been found *in vitro* to contain antioxidant properties and contribute to the total antioxidant capacity of plasma (Benzie, 1996). In humans, UA is the major end product of purine metabolism, and is usually found in high levels because humans lack the enzyme uricase. In birds, uric acid is the major end product of nitrogen metabolism, and studies in which plasma uric acid (PUA) concentrations have been lowered, resulted in a dramatic increase in oxidative stress (Simoyi et al., 2002). There was also an increase in tissue aging, as evidenced by an increase in the glucoxidation product pentosidine and the shear force of the *Pectoralis major* muscle. This observation was consistent with the view that the increase in crosslink formation was caused by oxidants (Klandorf et al., 2001).

Concentration of urate in the plasma may be determined by the rate of endogenous urate synthesis and degradation, as well as the concentration of purine in the natural diet of the species (Wyngaarden et al., 1976; Roch-Ramel et al., 1976). Manipulation of diet may be used to control PUA levels in birds and mammals (Itoh et al., 1974; Okumura et al., 1997), which can be linked to measurements of oxidative stress. By increasing PUA, free radical induced aging can likely be reduced (Simoyi et al., 2002).

These results are important in human health, since uric acid plays a major role in the antioxidant defences of human beings (Davies et al., 1986). Studies with birds can help to clarify how these antioxidants defences differ between birds and mammals.

Based on the antioxidant role of uric acid in birds and mammals, it is hypothesized that an increase in dietary protein levels, will elevate PUA and subsequently decrease oxidative stress in broilers chicks.

Chapter 2: Literature Review

2.1 Oxidative Stress and Antioxidants

Biological oxidations include a series of reactions initiated by the removal of hydrogen from a substrate, followed by multiple transfers via reduction-oxidation reactions and finally attachment to an oxygen molecule.

Oxygen is a precursor of toxic reactive species in biological system, and is the final electron acceptor, since it has the highest redox potential, in the respiratory chain. A molecule of oxygen with only two atoms can behave as a free radical, a chemical species with one or more unpaired electron. Some reactive compounds, such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO\cdot$) can generate reactive oxygen species (ROS), which can produce oxidative damage to other macro-molecular components of the cell (Carmeli, 2000). These highly reactive molecules can damage enzymes and proteins, which contributes to tissue damage, and are implicated in tissue injury and a number of widely studied diseases. However certain ROS are important since they serve as signal messengers and trigger molecules (Halliwell et al., 1986), where they can activate adenylate cyclase (Tan el al., 1995) and iron regulatory proteins (Rouault, 1996).

One of the highly studied ROS is nitric oxide (NO), a normal product of arginine catabolism, which is important in the regulation of some physiological process. NO can serve as an important messenger. It has been proposed to signal important homeostatic roles, mediating inflammatory reactions and maintaining vascular tone (Freeman et al.,

1998). But it has a short half-life because can interact with oxygen thus generating oxo-nitrate radicals that can lead to free radical injury (Lancaster, 1996).

Many pathological conditions, including ischemia/reperfusion, inflammation and sepsis may induce tissues to produce NO and superoxide (Lancaster, 1996). The reaction of these two molecules can form peroxynitrite (Equation 1.1) during an inflammatory response and cause a variety of toxic effects, such as tyrosine nitration, compromising enzyme regulation and signal transduction.

$$NO^{\bullet} + O_2^{\bullet -} \leftrightarrow ONOO^{-} \qquad \text{(Equation 1.1)}$$

Peroxynitrite is a long life toxic oxidant that can be produced in vivo, and can be rapidly decomposed to peroxynitrous acid (ONOOH**)** at physiological pH, and subsequently decomposes to $^{\bullet}NO_2$ and $^{\bullet}OH$. Peroxynitrite is characterized by highly toxic effects. Moreover. Peroxynitrite can oxidize urate and structurally related purines. Peroxynitrite is also associated with many pathological diseases, including neurodegenerative disorders, such as Multiple Sclerosis, a progressive disorder of the central nervous system.

Antioxidants defend against the oxidizing properties of ROS by directly scavenging the initiating radical or terminating the radicals necessary for propagation, and turn them into harmless products. Antioxidants can function by removing or decreasing local oxygen concentrations, scavenging or preventing radical formation, repairing oxidative damage, or increasing the elimination of damaged molecules (Gutteridge, 1998).

2.2 Oxygen Radical and Aging

Oxygen radical accumulation is associated with the onset of many diseases because of their dangerous and highly toxics properties. In addition, oxygen radicals have been proposed to play a causative role in aging. Previous studies have proposed that the metabolic rate of some species might be related to their aging rate (Cutler, 1984b). This relation is based on the rate of toxic oxygen radical production in a tissue per gram basis, in other words proportional to the rate of oxygen utilized per weight of tissue, or its simple metabolic rate (SMR) (Cutler, 1984b). Since tissue is exposed to various sources of stress, it needs to have some protection, antioxidant. Antioxidants protect against free radicals toxic effects that can be harmful to the cells (Forman et al., 1981).

Some have proposed that aging is an accumulation of nonfunctional cells or metabolic processes. The possible consequences include the accumulation of altered proteins (Cutler, 1982). Alterations or modification of proteins can result from their reaction with ROS, leading protein to oxidation and inactivation. Thus a number of proteins accumulate in their inactive or less active forms as age increases. The relationship between oxidized protein and age has been widely studied. Many have found an increased age to be inversely proportional to catalytic activities of enzymes studied (Oliver et al., 1987, Starke-Reed et al., 1989, and Smith et al., 1991). Free radicals produce alterations of proteins and body processes and consequently lead the cell to not operate properly resulting in aging.

Another recent hypothesis is dysdifferentiation. Dysdifferentiation is where normal diploid cells continue passing thru numerous biochemical and morphological changes after the normal development processes has stopped (Cutler, 1984). These changes are

due to the interaction of active oxygen species with the genetic apparatus of cells, leading to improper gene regulation. Therefore, longevity should be proportional to the stability of cells against ROS or any mutagenic effects that drift the cell away from their proper state of differentiation (Cutler, 1982). Either way cell dysdifferentiation is produced, by free radicals or mutagenic agents, antioxidants are still proposed to be the major source of protection against anything that can affect proper cell functions.

2.3 Maillard Products

Damage to proteins and DNA can result during Maillard reactions (MR) and may be also associated with aging. Modification of proteins by glucose followed by MR can lead to the accumulation of glycosylated end products and the formation of glucose-derived crosslinked proteins leading to accelerated tissue aging.

MR are initiated in the presence of free amino groups which can react with a carbonyl group of reducing sugars and proceed in the formation of a shiff base derivative (Cerami et al., 1987). The product is highly sensitive to glycoxidation either producing an N-carbonyl lysine derivative (Wells-Knecht et al., 1993), or reacting with arginine residues to form pentosidine cross-linked adducts (Monnier, 1990). Amadori products can alter protein functions in several ways including changing enzyme conformation, inactivating enzymes and impairing ligand-binding activity, among others (Baynes et al., 1989b). Studies show that cellular and extracellular concentrations of initiators, propagators and end products of Maillard reactions (MR), are inversely correlated with species longevity (Monnier et al., 1991).

The glycosylation product, pentosidine, can be used as a sensitive indicator of oxidative stress and consequently as a biomarker of protein oxidation damage through MR (Baynes, 1991). Pentosidine is a skin crosslink formed within the matrix protein (Sell et al., 1992), and has been used in studies to measure the rate of aging in birds. Evidence for age-dependent chemical modifications of skin collagen by MR has been provided (Iqbal et al., 1999). In addition, pentosidine has also been used as a marker in studies involving the classification of diabetes as a disease of accelerated chemical aging of tissue proteins. High levels of pentosidine have been found in end stage renal disease patients (Sell et al., 1991). In insulin-dependent diabetes an increase in sugar precursor and a decrease in its renal clearance have been observed and both may play a role in the production of Maillard reactants (Monnier, 1990).

Maillard products can be harmful for proteins since they can be oxidized in the presence of Fe^{+2} and oxygen resulting in fragmentation. The products of fragmentation can produce carboxyalkylated proteins, which can affect protein function (Monnier et al., 1991).

Even though the factors mentioned before suggest that MR might have a strong relation with the aging process, there is some evidence to the contrary. For example as mentioned before, birds have higher metabolic rates and plasma glucose concentrations, and in combination with high body temperature should promote a higher rate of formation of Maillard products. However, birds can live for several decades compared to mammals of the same size. Birds longevity is one more time a mystery for all scientists, since based on the physiological characteristics mentioned before, birds should be more susceptible to the degenerative processes of aging. This shows again that birds apparently

have developed important defense mechanisms to diminish the damage produced by MR products and ROS.

2.4 Uric acid

Uric acid (UA) is the end product of purine metabolism and its synthesis is the result of adenine nucleotide degradation and accumulation of purine metabolites, such as hypoxanthine and xanthine (Hellsten, 1994**)** as show in figure 1. They are both converted to uric acid by xanthine oxidase, which is located in the endothelial cells of blood vessels.

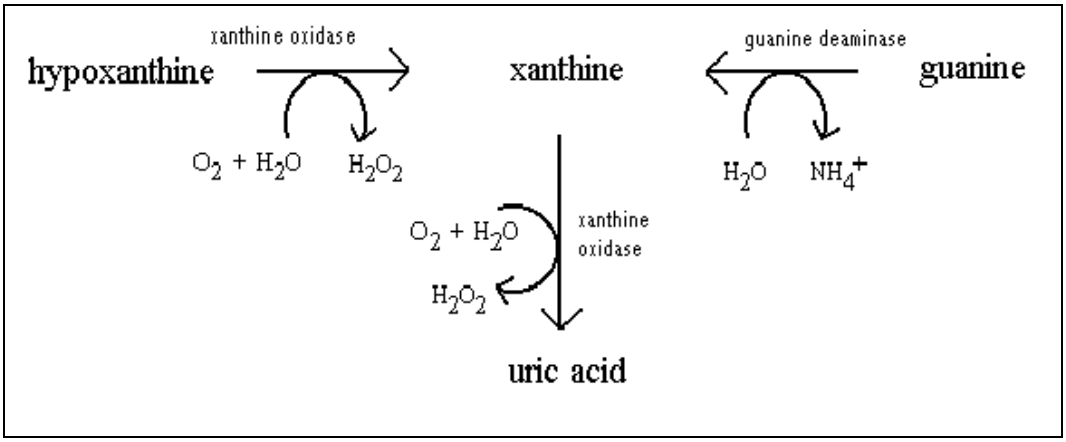

Figure 1. Hypoxanthine and xanthine degradation to uric acid by xanthine oxidase.

These free purines are reconverted to their nucleotides through salvage pathways where the enzymes adenine phosphoribosyltrasferase (APRT) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) are involved.

In addition to the antioxidant properties of UA discussed earlier, UA can also function as a neural stimulant (Cutler, 1982). High levels of UA can be found in humans since the enzyme uricase, which is responsible for further oxidation of UA, is not present.

This accounts for the difficulty of the human body in handling large purine loads and underlines in gout. Hyperuricemia and uricosuria are two characteristics of gout, where deposits of urate cause a painful inflammation in the joints. This condition can be controlled with allopurinol, an inhibitor of xanthine oxidase.

Because PUA levels can be affected by diet, this approach has been utilized to manipulate endogenous concentrations of PUA. Rats fed with potassium oxonate (uricase inhibitor) have been used to study the effects of potassium oxonate on uric acid levels and uricase activity in the kidney (Mangoff et al., 1978). Where an increase in the excretion of UA was found associated with an increase in dietary potassium oxonate (Mangoff et al., 1978).

Many studies had been done using birds, since plasma uric acid (PUA) concentrations are twice that measured in humans. Studies with birds reveal that UA function as a potentially important protective agent against oxygen radicals having a positive correlation with Maximum Life Span (MLS). Elevated concentrations of antioxidant such as UA, has been suggested to be responsible for lower oxidative stress levels found in birds (Klandorf et al, 1999). In addition, studies have shown that a decrease in PUA concentrations are associated with an increase in leukocyte oxidative activity (LOA) in chicks fed allopurinol-supplemented diet (Simoyi et al., 2002).

2.5 Antioxidant Properties of Uric Acid

Antioxidant defense systems can reduce the detrimental effect of free radicals. Among the important antioxidant properties of UA, is its ability to shield transition metals ions and to inactivate potent biological oxidants (Rowley et al.,1985). In addition,

UA has been found to protect against free radical damage to DNA (Cohen et al., 1984), inhibit lipid peroxidation (Niki et al., 1986), and decrease oxidation of haemoglobin by nitrite (Smith et al., 1984).

UA scavenging functions are also observed in humans. Urate has been considered to be an efficient scavenger of the free radical peroxynitrite, because of its ability to react with peroxynitrous acid and the radicals generated from its decomposition. Propagating chain reactions and inhibiting the peroxynitrite-mediated tyrosine nitration (Santos et al, 1999). Peroxynitrite has also been identified as a major factor in the pathogenesis of experimental allergic encephalomyelitis (EAE) (Fox et al, 1985), and Multiple Sclerosis (MS) (Hooper et al., 1997). Peroxynitrite is toxic effects are mediated through the nitration of macromolecules in specific targets. For example, peroxynitrite can produce neural dysfunction by tyrosine nitration in the neuronal filaments of motor neurons. Peroxynitrite can also oxidize liposomes and LDL (Newcombe et al., 1994).

Hooper and collagues studied the scavenging properties of UA with peroxynitrite, its effect on NO production and peroxynitrite accumulation in vitro. They found that addition of a peroxynitrite donor to the cells caused cell death which was inhibited when UA was added. Low levels of PUA have been measured in MS patients, suggesting inadequate protection against peroxynitrite, resulting in progressive damage to the central nervous system (Hooper et al., 1998). Meanwhile, high serum UA levels were found to protect against the development of MS, and were effective in treatment of EAE before and after development of clinical symptoms. Continued administration of UA was necessary to maintain the EAE therapeutic effect (Hooper et al., 1998).

Urate can also inhibit oxidative reactions without being oxidized itself. For example, UA prevented ascorbic acid oxidation in human blood (Sevanian et al., 1985). This effect was due to UA binding transition metal ions, such as iron, and thus preventing ascorbic acid in reaction with iron (Davies et al., 1986).

Even though many sources suggest antioxidant effects of UA, some studies have revealed that urate may also have a prooxidant effect. UA may amplify the lipid oxidation of liposome and LDL induced by peroxynitrite (Thomas et al, 1998). Santos and co-workers reported uric acid reacts directly with peroxynitrite with a bimolecular rate constant of $4.8 \pm 0.1 \times 10^2$ M^{-1}/s, and also can react with peroxynitrous acid and its decomposition products. Oxidation of UA by peroxynitrite is accompanied by a rapid uptake of oxygen, which is indicative of an increase in free radical formation. Generation of free radicals is influenced by pH, where free radical formation and oxygen consumption increase in proportion to an increase in pH. However, there is no clear evidence of the prooxidant effects of urate. Additional research is needed before any firm conclusions regarding the role of UA in biological reactions can be established.

2.6 Effects of a Dietary Protein on Uric Acid Levels

UA is the principal product of nitrogen metabolism in birds and its tissue content can reflect both nutritional state and dietary protein intake. Starvation has been found to markedly decrease PUA concentrations (Bell et al., 1959), whereas even the intake of small quantities of food maintains its synthesis (Sturkie, 1961). A diet high in protein content will elevate PUA levels in birds (Okumura et al., 1997).

As previously mentioned, urate is the main product of purine metabolism or nitrogen metabolism in humans or birds, respectively, and anything affecting the concentrations of any of the intermediates involved in urate metabolism can affect its final concentration.

IMP and its conversion to adenosine monophosphate (AMP), are important in the control of purine catabolism. Purine metabolism is affected by dietary content, age of the animal, and the activity of the regulatory enzymes. Anything affecting the substrate or enzymes involved in the purine catabolism can affect UA concentrations (figure 2).

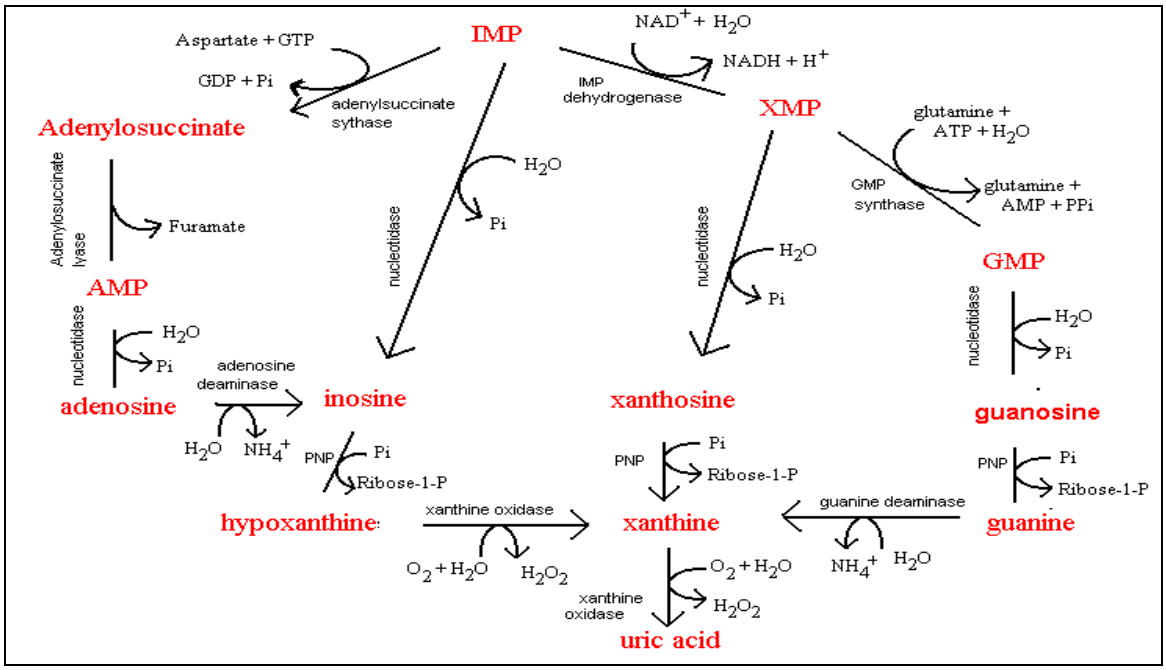

Figure. 2. Pathway of purine catabolism in animals. Inosine Monophosphate (IMP) is converted to adenosine monophosphate (AMP), guanosine monophosphate(GMP) and xanthosine monophosphate (XMP) by separate reaction pathways and subsequently degraded to uric acid. Inosine, xanthosine, and guanosine degradation involve the purine nucleoside phosphorylase (PNP). (Voet, D. and Voet, J., 1995)

Previous studies have shown how diet affects purine metabolism. A diet including hypoxanthine, AMP, guanosine monophosphate, IMP and adenine can have an effect on

uric acid levels in serum. Brulé and co-workers demonstrated this in a study where free purines (adenine, guanine, hypoxanthine and xanthine), their nucleosides (adenosine, guanosine and inosine) or nucleotides (AMP, GMP and IMP) were fed to rats. Since in rats, uric acid is further oxidized with the enzyme uricase, the final product of purine metabolism is allantoin. Hypoxanthine was found to have the greatest influence urinary allantoin excretion, follow by adenosine, xanthine, AMP and inosine. However adenine enriched diets increased levels of serum urea nitrogen, UA, creatine and allantoin, but decreased urinary excretion of allantoin. Adenine in free form was found to be more uricogenic than the nucleoside or nucleotide form. This suggests that the hyperuricemic potential of food is related to the availability of adenine (amount of free adenine) (Brulé et al., 1988).

A study done by Wegelin is an example of how age is also an important factor in purine metabolism. It was determined how IMP contributes to the availability of adenine nucleotides in chicken heart during aging (Wegelin et al., 1996). Purine salvage pathways predominated after birth, whereas AMP degradation was evident in pre-pubertal birds. In adult chickens, purine metabolism is limited to the recycling of purines as evidenced by the utilization of salvage pathways and exogenous sources of purine (Wegelin et al., 1996). A reduction in the activity of the enzyme involved in AMP degradation can reduce adenine nucleotide catabolism in chicken heart, and thus lower PUA concentrations (Wegelin et al., 1996). In addition, changes in the activity of enzymes involved in IMP degradation in the liver and kidney are affected by changes in the dietary protein content (Itoh et al., 1974).

The availability of the regulatory enzymes involved in the purine metabolism is a third factor important in the control of purine catabolism. This fact is shown in a study done by Okumura and co-workers, where high PUA concentrations were measured in birds fed diets high in casein (protein), which resulted in an increase in nitrogen metabolism (Okumura et al, 1968 and Siller et al., 1959). The increase in PUA was suggested to be due to an increase in the activity of the enzymes involved in the formation of UA from IMP.

The regulation of nitrogen elimination in uricotelic animals is dependent on changes in the activity of enzymes involved in the production of UA from Inosine Monophosphate (IMP). The activity of purine nucleoside phosphorylase, xanthine dehydrogenase and cytosol 5'-nucleotidase in chicken liver increases in response to an increase in dietary protein content (figure 3).

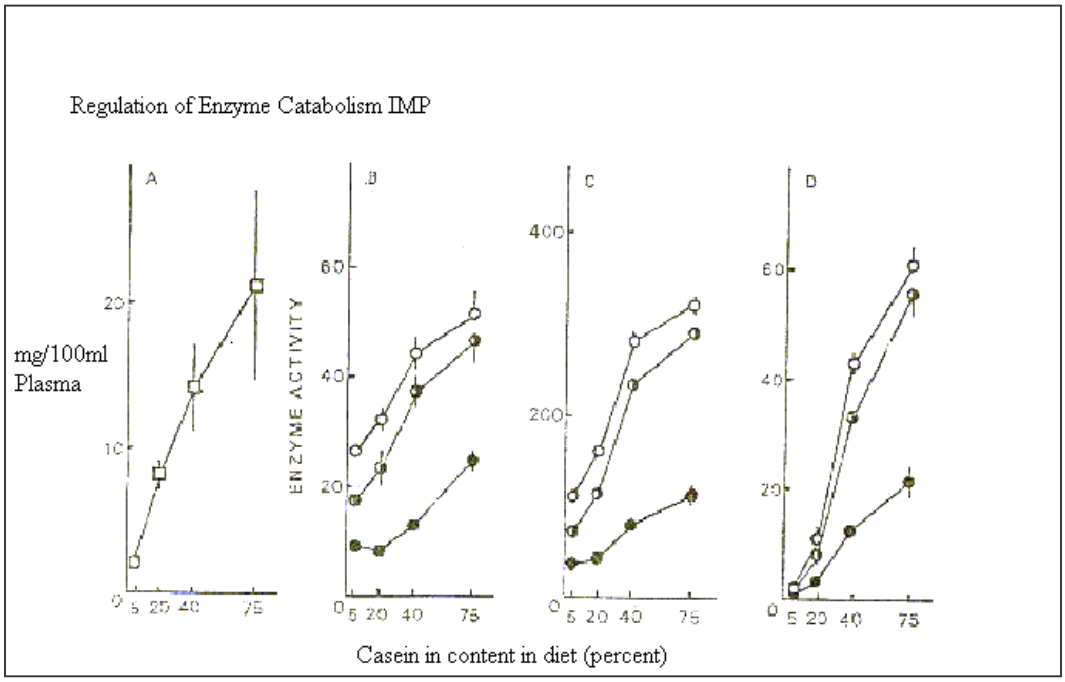

Figure 3. Effect of dietary casein on plasma uric acid concentration (A), cytosolic 5'- nucleotidase (B), purine nucleoside phophorylase (C), and xanthine dehydrogenase (D) activity in chicken liver. Units per mg protein × 10^3 (○); units per gram liver weight × 10 (●); units per 100g body weight (⊙). (Itoh et al., 1974).

However, following a period of adaptation to the high protein diet, the activity of these enzymes declined even though the diet remains high in protein content. Conversely, there was a reduction in the activity of the three enzymes when a low protein diet containing 5% casein was fed (Itoh et al., 1974). In contrast, in non-uricotelic animals, like rats, the enzyme activity in the liver did not change with high protein diets, but when low protein diet was fed, the activity of xanthine dehydrogenase decreased (Itoh et al., 1974; De Renzo, 1956).

Refeeding with different levels of dietary protein also affects PUA concentrations in chickens. For example, feeding White Leghorn cockerels a high protein diet (30 and 40% casein) resulted in high concentrations of PUA 2hrs after feeding. After 3hrs, only chickens fed a high protein diet maintained elevated PUA (Bell et al., 1959; Okumura et al., 1997). UA and ammonia levels in the liver and kidney also increased gradually with an increase in dietary protein level, whereas in birds fed diets containing less than 10% casein, ammonia levels did not increase.

Starvation maximally decreases PUA in birds (Siller, 1959), although even low portions of food can generate some UA (Sturkie, 1961). In addition, starvation states enhance xanthine dehydrogenase activity in the liver and increases tissue protein synthesis (Corte et al., 1967). This may explain the high PUA found in starved birds in Okumura study (Okumura et al, 1997).

The avian pancreas has also been suggested to synthesize and secrete UA. Although pancreatic cells were found to release UA, a study using pancreatic juice revealed no relationship between UA and pancreatic protein concentrations in birds. These results indicate that UA is independent of the protein concentration in pancreatic juice and that

the synthesis of UA is dependent on the activity of xanthine dehydrogenase (Herzberg et al., 1990).

Dietary protein level not only affects UA concentrations but also growth rate. A diet low in protein retards growth, because the chick does not consume the necessary quantity of nitrogen required for growth and maintenance. Chicks fed with a high protein diet have a growth advantage relative to chicks fed a low protein diet. When chicks previously fed a low protein diet, are offered a high protein diet, growth is resumed (MacRoberts et al., 1965).

It is thus apparent that disease and nutrition have pronounced effects on PUA levels, and that an increase in PUA may afford protection against the damage produced by free radicals.

2.7 Uric Acid End Products

Once UA has been formed in the body, there are several possible routes for its elimination, renal excretion being the most important one. Uricolysis occurs primarily in the gastrointestinal tract where bacteria degrade the UA via a uricotelic process. In other mammals, UA can be further oxidized to allantoin in a reaction catalyzed by the enzyme uricase. It has been demostrared that allantoin is also produced during the non-enzymatic oxidation of urate by several oxidants in humans (Santos et al, 1999**).**

Allantoin and other oxidation products of UA can be used as an indicator of free radical reactions in vivo, since high concentrations have been measured in patients suffering from conditions in which oxidant generation is increased, such as rheumatoid arthritis (Halliwell et al, 1985). It was proposed that this increase in allantoin results

from the oxidation of UA by the mixture of the released haemoglobin into the synovial fluid from an inflamed rheumatoid joint (Halliwell et al, 1988) as well as H_2O_2 produced by activated phagocytic cells (Howell et al., 1960).

In humans, plasma allantoin increases in conditions associated with increased oxidative stress and declines with antioxidant supplementation (Grootveld et al., 1987; Naidoo et al., 1998). In agreement with these findings, Benzie (1999) found high plasma allantoin concentrations in non-insulin-dependent-diabeties (NIDDM) with early peripheral vascular disease (Benzie et al., 1999). Subjects with NIDDM were found to have lowered antioxidants and increased oxidative stress (Benzie et al., 1999), which contributed to the development of diabetic complications (Armstrong et al., 1996).

The reaction of UA with ROS can produce a mixture of products that may be considered as oxidative stress markers in studies of free radical production in vivo (Grootveld et al., 1987). Even though humans and birds are phylogenetically different, studies with birds can help us understand how their antioxidant defense differs from mammals and to develop nutritional strategies to enhance antioxidant capacity in humans.

2.8 Research objective

The objective of this study is to test the hypothesis that uric acid is an important antioxidant in birds, which can be link to oxidative stress. Dietary protein has an effect on uric acid concentrations in broilers chicks. Thus, an increase in PUA concentration and low leukocyte oxidative activity are expected in chicks fed high protein diets.

Chapter 3: Materials and Methods

3.1 Birds and Management

3.1.1 Preliminary Study

Birds and Management. A preliminary study was performed to determine the magnitude of the effect of high protein diet on plasma uric acid (PUA) levels. Twenty four 3-wk-old mixed sex broilers (Ross x Cobb) were randomly allotted into 4 groups (n = 6): a low protein (LP) fed 10% casein, a medium protein (MP) group fed 20% casein, a high protein (HP) fed 45% casein and a commercial diet (COD) group fed with a commercial diet, for 2 wk. Cornstarch was filler for purified diets containing less than 45% casein. Each dietary treatment had two replicates consisting of two floor pens containing 3 birds. Diets were prepared according the NRC (1994) guidelines. Chicks were provided free access to feed, water and kept under standard conditions for the duration of the study. The composition of the purified diet is listed in Table 1.

Broilers were weighed and blood samples were collected from the brachial vein before chicks were transferred to their respective diets (Wk 0) and then weekly for the duration of the study. Heparinized blood samples were placed in an ice bucket until centrifugation at 1,500g for 20 min at 0-4°C. Plasma was stored at -20°C until analyses.

Table 1. Treatments diet composition.

Treatment ***Components***	**LP** **%**	**MP** **%**	**HP** **%**
Casein	10.0	20.0	45.0
Soybean oil	3.0	3.0	3.0
Cellulose	3.0	3.0	3.0
Akley premix	0.25	0.25	0.25
Coban	0.10	0.10	0.10
Cornstarch	80.25	70.25	45.70
Dicalcium Phosphate	1.75	1.75	0.74
Calcium carbonate	1.4	1.40	1.96
Salt	0.25	0.25	0.25

Percent of each component for each treatment diet; low protein (LP) diet (10% casein), medium protein (MP) diet (20% casein), and high protein (HP) diet (45% casein).

3.1.2 Principal Study

Birds and management. In this study the effect of dietary protein on PUA and leukocyte oxidative activity (LOA) was determined. Twenty-four 3 wk old broilers chicks (Ross x Cobb) were randomly divided into four groups (n = 6) and fed LP (10% casein), MP (20% casein), HP (45% casein), and COD for 4 wk. Broiler chicks were weighed and blood samples were collected at Wk 0 and then weekly intervals for the duration of the study. No measurements were made 1 wk after transfer, which was an adaptation period for the chicks to the new diet.

3.2 Determination of Plasma Uric Acid and Glucose Concentration

PUA concentrations were determined using a commercially available Uric Acid Reagent kit (Sigma Diagnostic; St. Louis, MO). Plasma glucose concentration was

determined using aYSI – 2700 Select Biochemistry analyzer (Yellow Springs Instrument Co; Yellow Springs, OH).

3.3 Chemiluminescence Measurement of Leukocyte Oxidative Activity

Luminol Chemiluminescence has been effectively used to detect the production of reactive oxygen species in cells or tissues (Van Dyke, 1987). In this study luminol-based Chemiluminescence was used to estimate the LOA as described by Iqbal and co-workers (Iqbal et al., 1999). One milliliter of blood from 3 – 7 wks old broilers (n = 6) was carefully layered on 5 ml in mono-polyresolving medium in a 13 x 100 mm # 10 Falcon tube, and leukocytes were isolates by centrifugation at 300 x g for 5 min. To a 3µl luminometer tube, was then added 100µl of leukocytes, 100µl luminol (10^{-5} M) solution, 200µl phosphate buffer saline (pH 7.4), and 100µl phorbol myristate acetate (PMA). Luminol reacts with hypochlorite with the production of photons. LOA is determinate using Berthold luminometer (Multi-Biolumat LB 9505C model) with a temperature set to 37 °C. Results are report as integrated light units (ilu).

3.4 Statistics

Data were analyzed using analysis of variance to perform analysis of variance. Differences between means were considered significant at ($P < 0.05$). The Student-Newman-Keuls Multiple Range test was used to estimate the significance of difference between means.

Chapter 4: Results

4.1 Preliminary Study

4.1.1 Body Weight

Body weights of birds fed LP diet were significantly lower than that of birds fed MP, HP, or control diet at wk 2 (Table 2). Chicks fed COD diet had significantly higher body weight than those fed LP, MP, and HP diet ($P < 0.05$) at wk 1 and wk 2.

Table 2. Body Weights of Broiler Chicks fed different diets; Preliminary study.

Treatment	Wk 0 (Kg)	Wk 1 (Kg)	Wk 2 (Kg)
Low protein	0.76 ± .03	0.90 ± .03[a]	0.95 ± .03[a]
Medium protein	0.86 ± .03	1.07 ± .05[a]	1.16 ± .04[b]
High protein	0.75 ± .05	1.03 ± .08[a]	1.21 ± .12[b]
Commercial diet	0.82 ± .06	1.34 ± .09[b]	1.95 ± .09[c]

Data are means (n = 6) ± Standard error of the mean (S.E.). Before experimental diet (Wk 0), Wk 1 and Wk 2 are the number of wk after transfer to experimental diet. Means with different letter differ significantly among groups ($p< 0.05$).

4.1.2 Plasma uric acid and glucose concentrations

PUA concentration was directly proportional to dietary protein levels. The highest PUA concentration was found in chicks fed with HP diet compared to those fed

LP diet ($P < 0.005$), MP diet ($P < 0.025$) or C ($P<0.025$) at each sampling time (Figure 4). No significant difference was found between MP and COD at wk 2.

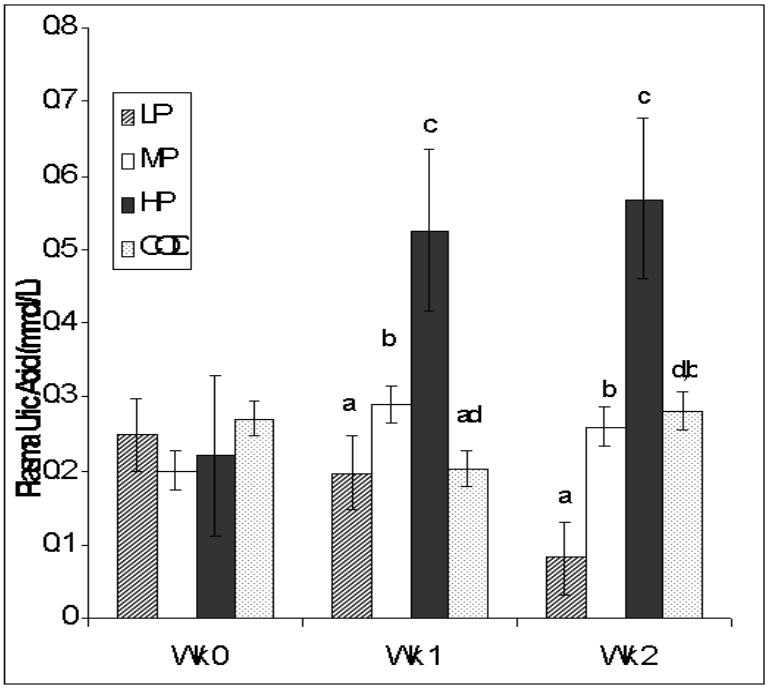

Figure. 4. Plasma uric acid concentration of broiler chicks fed low protein (LP), medium protein (MP), commercial diet (COD), and high protein (HP) diet; Preliminary Study. Values are (n =6) means ± S.E. Before experimental diet (Wk 0), 1 and 2 wk after dietary treatment. Means within column with a different subscript differ significantly ($P < 0.05$).

Plasma glucose concentration did not differ between chicks fed with different treatment diets at wk1. However, there was a decrease in plasma glucose levels in chicks fed LP diet at wk 2 ($P < 0.05$) as show in Table 3.

Table 3. Plasma glucose levels, in broiler chicks fed low protein (LP), medium protein (MP), commercial diet (COD), and high protein (HP) diet; Preliminary study.

Treatment	**Wk 0** (mmol/L)	**Wk 1** (mmol/L)	**Wk 2** (mmol/L)
Low protein	14.07 ± .36	12.24 ± .61	9.30 ± .39[a]
Medium protein	13.51 ± .49	11.63 ± .46	11.23 ± .38[b]
High protein	11.94 ± .43	10.48 ± .46	11.03 ± .43[b]
Commercial diet	12.38 ± .66	12.76 ± .7	10.35 ± .36[a,b]

Before transfer to experimental diet (Wk0), wk 1 and wk 2 after transfer. Data are (n =6) means ± S.E. Means with different letter differ significantly among groups ($p < 0.05$).

4.2 Principal study

4.2.1 Body weight

Body weights were lowered at wk 2, 3, and 4 after treatment diet in chicks fed LP. Chicks fed HP gained weight faster than those fed MP. Differences were significant at wk 3 and wk 4 ($P < 0.025$) as show in Figure 5. Similar to the preliminary study, all groups of birds fed with purified diet had a slower growth rate when compared to chicks fed COD diet.

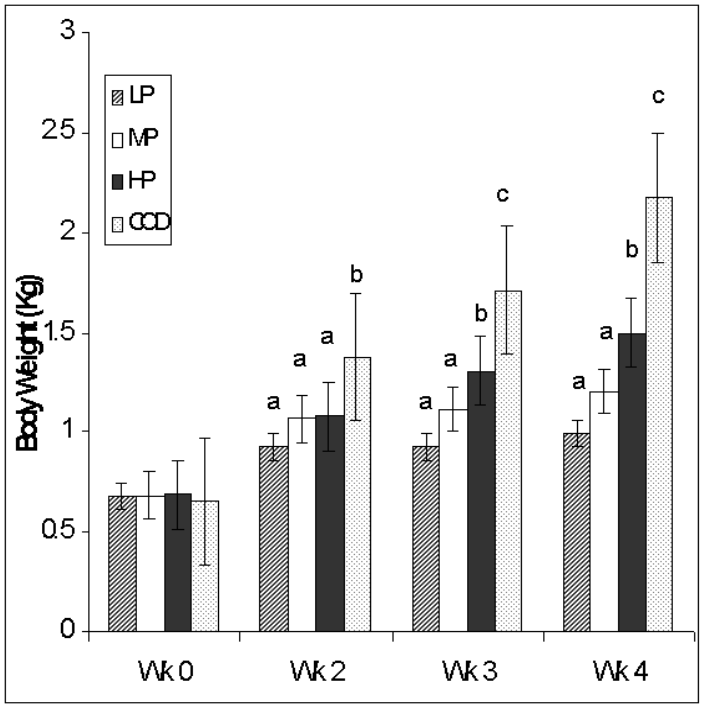

Figure 5. Body weight of chicks fed low protein (LP), medium protein (MP), commercial diet (COD), and high protein (HP) diets; Principal study. Before transfer to experimental diet (Wk 0), 2, 3 and 4 wk after transfer. Data are (n =6) means ± S.E. Means with different letters differ significantly at each experimental period ($P < 0.05$).

4.2.2 Plasma uric acid concentrations

The PUA concentration in chicks fed HP was significantly higher than that in chicks fed LP, MP or C diet in wk 3. By the fourth wk, PUA concentration in chicks fed HP diet had double compared to that of chicks fed MP or COD ($P< 0.005$). PUA decreased in chicks fed LP until wk 3 ($P < 0.005$) and then increased in wk 4 (Figure 6).

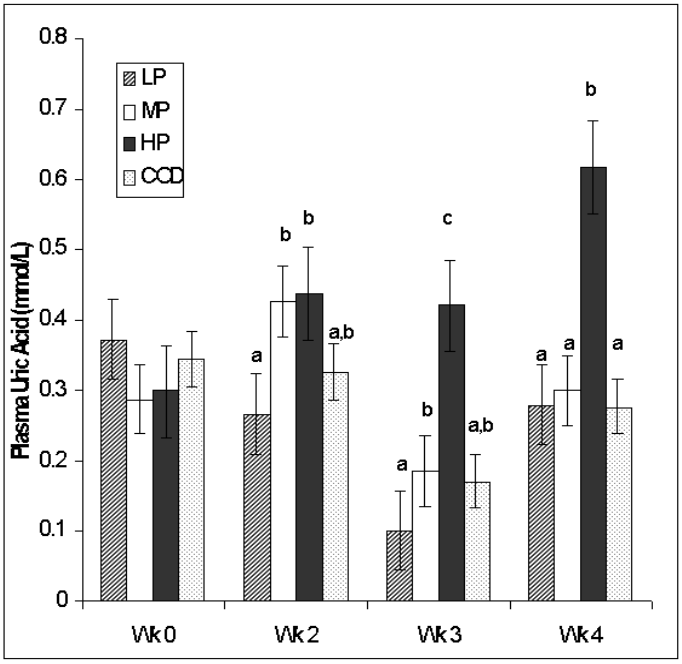

Figure 6. Plasma uric acid levels, in low protein (LP), medium protein (MP), commercial diet (COD), and high protein (HP) fed chicks; Principal study. Before transfer to experimental diet (Wk 0), 2, 3 and 4 wk after treatment. Data are (n =6) means ± S.E. Means with different letters differ significantly at each experimental period (P < 0.05).

4.2.3 Measurements of Leukocyte Oxidative Activity

There was a marginal decline in LOA in all treatments, throughout the sampling period (Figure 7). However, in chicks fed HP diet, a relation between PUA and LOA was observed. Where low LOA was found where PUA was in high concentrations ($P< 0.05$) as demonstrated in Figure 8.

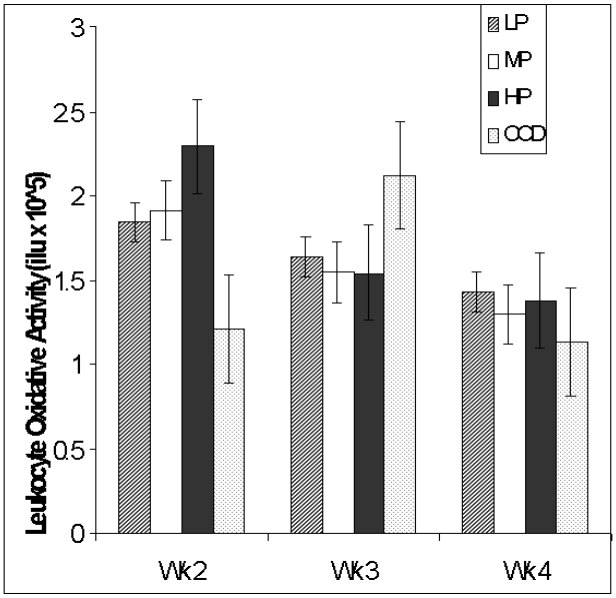

Figure 7. Leukocyte oxidative activity in low protein (LP), medium protein (MP), commercial diet (COD), and high protein (HP) fed chicks; Principal study. Wk 2, 3, and 4 after transfer. Data are (n =6) means ± S.E. No significant difference was found between the means among each wk.

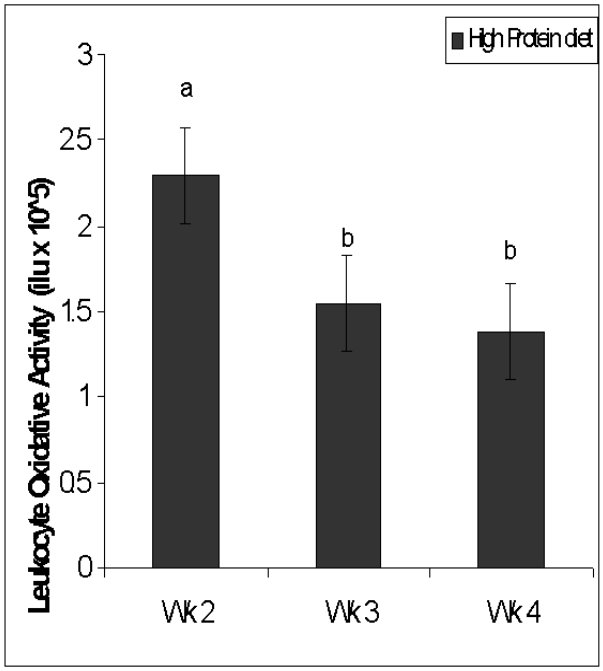

Figure 8a. Leukocyte oxidative activity in chicks fed high protein diet at wk 2, 3 and 4 after treatment. Data are (n =6) means ± S.E. Means with different letters differ significantly (P < 0.05).

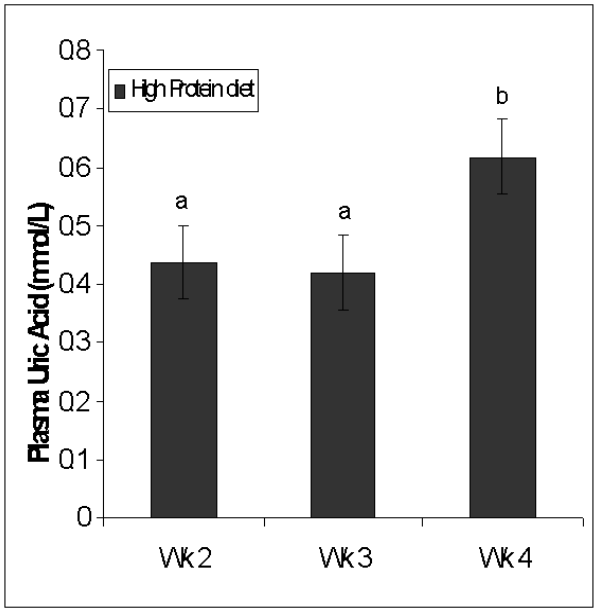

Figure 8b. Plasma uric acid concentrations in chicks fed high protein at wk 2, 3, and 4 after treatment. Data are (n =6) means ± S.E. Means with different letters differ significantly (P < 0.05).

Changes in LOA linked to PUA concentrations were also found in chicks fed COD, as show in figure 9.

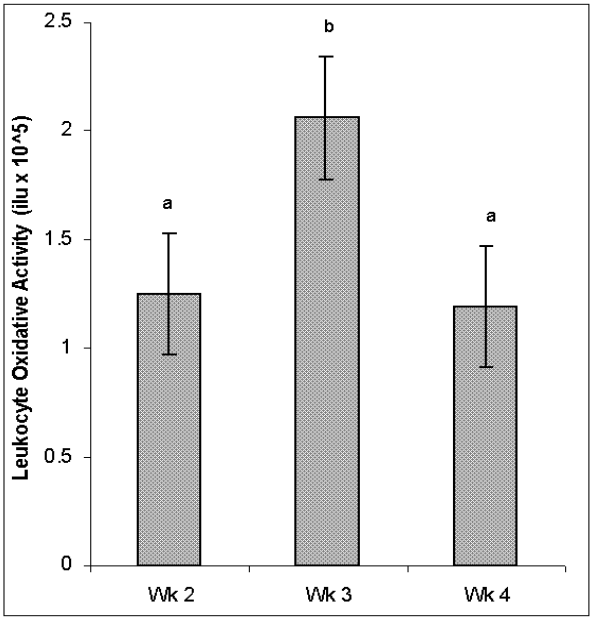

Figure 9a. Leukocyte oxidative activity in chicks fed normal commercial diet at wk 2, 3, and 4 after treatment. Data are (n =6) means ± S.E. Means with different letters differ significantly (P < 0.05).

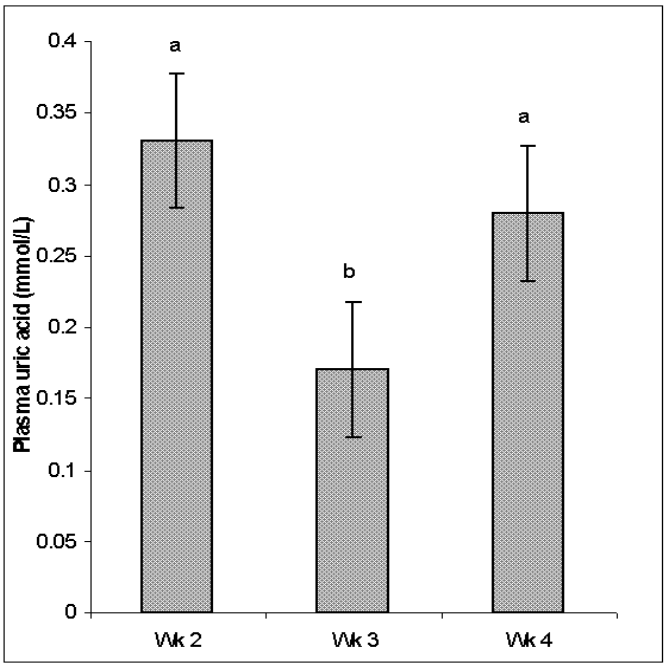

Figure 9a. Plasma uric acid concentrations in chicks fed normal commercial diet at wk 2, 3, and 4 after treatment. Data are (n =6) means ± S.E. Means with different letters differ significantly ($P < 0.05$).

Chapter 5: Summary and Conclusions

5.1 Summary

Severe growth restriction is one of the conditions that may be observed in chicks fed with LP diet. This may be a result of low nitrogen availability resulting from amino acid deficiency in the diet, thus not meeting the requirements for growth and maintenance (McRoberts, 1965). In our study, chicks fed with treatment diets showed this behavior. Whereas restriction in body weight gain was observed in chicks fed purified diets, with almost no growth in chicks fed LP diet.

It has been observed in previous studies that diet restriction increased the concentration of antioxidant enzymes and decreased oxidative stress (Youngman et al., 1992). This agrees with the results observed in our study, where growth restriction mask the antioxidant effect of elevated uric acid concentrations in birds fed the high protein diet. Also low PUA concentrations and leukocyte oxidative activity were observed in chicks fed LP diet, which were under severe diet restriction. This reduction in oxidative stress allowed to an increase in PUA observed at the end of the study.

PUA concentrations were found to increase in chicks fed HP diet. The increase was expected based on previous studies where the activity of the enzymes (purine nucleoside phosphorylase, xanthine dehydrogenase and cytosol 5'-nucleotidase) involved in uric acid formation from Inosine Monophosphate (IMP) increased or decreased when were fed with a high (75% casein) or low (5% casein) protein diet, respectively (Itoh et al., 1974).

Studies investigating the role of uric acid as an antioxidant demonstrated its biological roles in protection against the toxic effects of oxygen radicals (Simoyi et al., 2002). In our study, a relation between PUA and LOA was not observed in chicks fed LP diet. However its was observed in chicks fed with MP, HP or COD.

5.2 Conclusion

PUA concentrations were found to be directly proportional to dietary protein content. Feed restriction (voluntary or involuntary) severely restricted body weights gain.

Although no significant changes were found in oxidative stress associated with PUA in chicks fed LP diet, PUA concentrations were linked to changes in LOA in the remaining groups.

UA still believed to be an important antioxidant in broilers. The role of uric acid as an antioxidant and its effect on oxidative stress in broilers chicks deserves further research.

Chapter 6: Future studies

Based on the results of this study, it would be interesting to determine the effect of HP and LP diets on oxidative stress for a complete growth cycle. Weather LOA in chicks fed HP continues to decrease after this time will be of interest. Additionally, to study different antioxidants possibly related to uric acid function would also interesting. Uric acid may be working with other substances that could be responding more in situations were uric acid concentrations are low.

It will be interest to measure the uric acid end product allantoin and establish its role as an indicator of oxidative stress. Further it will be of interest to determine how uric acid and allantoin vary over the lifespan of a bird and in different disease states. Studies investigating the antioxidant properties of uric acid as control to combat neurological diseases remains an important objective in order to discover new methodologies to deal with these debilitating diseases.

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Biography

The author of this thesis was born in San Juan, Puerto Rico on July 24, 1977. Graduated from Notre Dame High School in Caguas, Puerto Rico on 1995, and she began her college education at the University of Puerto Rico, Mayagüez Campus. Graduated in 2000 with a Bachelor in Science, Animal Industry. After graduation she continue her studies at West Virginia University under the supervision of Dr. Hillar Klandorf.

As an under graduate she had the opportunity to work with Dr. VanCleve at the University of Puerto Rico, Mayagüez Campus, as a research assistant detecting the onset of estrous and recording standing events associated with estrus using the HeatWatch System. In the summer of 1999, she participated with the HACU internship program, working for the United State Department of Agriculture at Salinas, CA. And on fall of 2001, worked as teaching assistant at the West Virginia University, Biology Science Department.

Maribel Machin

m_machin@hotmail.com
9560 Muirkirk Rd 101
Laurel, MD 20708
(240)-568-4053

EDUCATION :

West Virginia University
MS Animal & Veterinary Science
G.P.A. 3.41/4.00
Completion date: 06/2002

University of Maryland, College Park
Graduate visitor student: 08/01-12/01

University of Puerto Rico, Mayagüez Campus
BS Animal Industry
G.P.A. 3.23/4.00 Major: 3.32/4.00
Graduation date: 12/1999

EXPERIENCE:

03/24/02-Present. **United State Department of Agriculture, Agricultural Research Service.** Biology Science Laboratory Technician.

08/2000- Present. **West Virginia University, Morgantown WV**
Graduate Research: Effect of Dietary Protein on Plasma Uric Acid, Body Weight, and Oxidative Stress in Broilers. Experience with laboratory equipment: HPLC (Waters 2690), Spectrophotometer (DU 640), vacuum centrifugation, Biochemistry analyzer (YSI-2700 Select), and Multi-Biolumat (LB 9505 C), among others. Knowledge in the collection and statistics analysis of data.

01/2001-05/2001. **Biology Science Department (WVU), Morgantown WV**
Teaching assistant, General Biology 4 Laboratory. *Explorations in Biology*.

05/1999 –08/1999. **United State Department of Agriculture**, **Salinas CA.**
HACU Internship Program. Worked for the Farm Service Agency. Farm inspections assistance and farmers record evaluations for disaster lost recovery loan programs, and clerical work.

SKILLS:

Fully Bilingual (English, Spanish), good oral and writing communication skills, team worker and capable of handing several priorities, experience managing and working with animals, knowledge of safety laboratory procedure and safety regulations. Computer knowledge: Window 2000, Word Processing, Word Perfect, Microsoft Office (Word, Excel, Power Point), and Internet.