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L-Gulonolactone Oxidase Activity, Tissue Ascorbic Acid and Total Antioxidant Capacity in Vitamin A-Deficient Chickens, *Gallus gallus*

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Experiments were conducted to determine the effect of vitamin A deficiency in chicks on tissue ascorbic acid, plasma oxidant status and antioxidant capacity, and renal L-gulonolactone oxidase activity (GULO) in broiler chickens. Chicks were reared in battery cages and fed a practical diet with vitamin A (control) or the same diet without supplemental vitamin A from day-old to 23 or 30 days of age. The treatments were arranged in a randomized complete block design with 6–8 replications. At termination body weight, feed intake, and tissue weights were recorded and tissues analysed for ascorbic acid, GULO activity, antioxidant activty, and oxidant status. Growth, feed intake, relative weights of bursa of Fabricius, liver, spleen and testis were significantly reduced in vitamin A-deficient chicks. Vitamin A deficiency depressed renal GULO activity by 20% ($P<0.08$) and 33% ($P<0.007$) in Experiment 1 and 2 respectively. Bursal, hepatic, splenic, testicular, and plasma ascorbic acid concentrations, plasma total antioxidant activity, and plasma oxidant status were not altered by the decrease in GULO activity. However, plasma advanced oxidation protein products were lower ($P<0.02$) in vitamin A-deficient chickens. The lack of effect on tissue ascorbic acid and antioxidant capacity suggest that metabolic changes associated with vitamin A deficiency may reduce ascorbic acid excretion. The reduction in plasma advanced oxidation protein products may be ascribed to lower metabolic activity because of hypothyroidism in vitamin A-deficient chicks. In conclusion, short-term vitamin A deficiency in broiler chicks reduced GULO activity without concomittant changes in tissue ascorbic acid.

Key words: chicken, L-gulonolactone oxidase activity, vitamin A, vitamin C

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Introduction

Poultry are renal synthesizers (Roy and Guha, 1958) of ascorbic acid (vitamin C) with kidneys contributing 100% of the vitamin synthesized and hence poultry diets are not normally fortified. However, the interrelationship of ascorbic acid (AsA) with other nutrients may not only affect its synthesis and metabolism but AsA supplementation may alleviate the deficiency of another vitamin (Dam *et al.,* 1948). Hypovitaminosis A occurs in poultry (Sato *et al.,* 1992; Cortes *et al.,* 2006) and the effects of vitamin A deficiency and infection are synergistic (Bang *et al.,* 1973). Ascorbic acid influences immune responses (Amaye-Amin *et al.,* 2000) of normal chickens and survival of vitamin A-deficient chickens (Kendler and Perek, 1968). Hence, the relationship between vitamins A and C is important in feeding poultry.

Vitamin A deficiency depleted tissue AsA, decreased AsA excretion (with greater excretion in males than in females), and supplemental AsA restored the values and extended longevity of vitamin A-deficient rats (Sure *et al.,* 1939; Sutton *et al.,* 1942; Mayer and Krehl, 1948; Boyer *et al.,* 1942; Mohanram *et al.,* 1976). Intracranial pressure (ICP) in vitamin A-deficient calves markedly decreased cerebrospinal fluid (CSF) AsA and administration of AsA raised CSF AsA and reduced ICP (Boyer *et al.,* 1942). In contrast, other researchers failed to detect a relationship between vitamins A and C and ascribed the earlier findings to inanition in vitamin A-deficient animals (Mapson and Walker, 1948). However, subsequent reports on synthesis of AsA, as measured by L-

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gulonolactone oxidase (GULO) activity, unequivocally showed that biosynthesis is decreased in vitamin A deficient rats (Malathi and Ganguly, 1964; Ghosh *et al.,* 1965).

In poultry, neither hepatic nor duodenal AsA was reduced in pullets depleted of vitamin A for eleven weeks (Rubin and Bird, 1943) and the researchers concluded that vitamin A deficiency does not interfere with AsA synthesis in mature chickens as it does in calves and rats. Vitamin A deficiency in cockerels reduced plasma AsA at 3 and 7 weeks of age but not at 4, 5 or 6 weeks of age (Nockels *et al.,* 1973) and excess vitamin A markedly reduced tissue AsA (Surai *et al.,* 2000). The limited information suggested that biosynthesis of AsA in poultry is not reduced in vitamin A-deficient chickens and showed that in extreme hypervitaminosis A, tissue AsA content is decreased. This study examined the relationship between vitamins A and C in chickens by determining whether GULO activity, tissue AsA, and antioxidant capacity are altered in vitamin A-deficient chickens.

Materials and Methods

Sixty and one hundred twenty eight commercial mixed-sex chicks (*Gallus gallus*) (Cobb×Cobb) from 45-week and 40-week old breeder flocks were used in the two experiments. Chicks were transported 30 miles, wing-banded, and distributed at random to pens in battery brooders with raised wire floors. The floor space in each pen was 0.75 m^2 and stocked with 5–8 chicks to 21–23 days of age. In experiment 1, one male was sampled from each pen at 23 days of age. In experiment 2, four males per pen were identified at 21 days of age and transferred to wire-floored grower pens with 1 m^2 floor space each. The brooder and grower units were maintained in an environment-controlled room with a mean room temperature of 26℃ during the study. Feed and water were provided *ad libitum.* The room lights were on for 23 hours each day. The chicks were observed daily in the morning and afternoon and humane endpoints were used to remove and euthanize chicks. Humane endpoints included non-weight bearing for more than 24 hours or inappetance for more than 48 hours.

The basal diet (Table 1) was a standard sorghum-soybean meal starter diet (Nockels and Kienholz, 1967) that met or exceeded all nutrient recommendations except for vitamin A. Commercial feed grade vitamin A was added to the vitamin A-deficient basal diet to produce the control diet with 8600 IU/kg vitamin A. Vitamin A was not detected in the basal diet. The study was conducted under a protocol approved by Clemson University Institutional Animal Care & Use Committee (IACUC) in an Association for Assessment & Accreditation of Laboratory Animal Care (AAALAC) approved facility.

At termination birds were handled individually for euthanasia and tissue collection in a laboratory away from the animal room. Tissues samples were collected at 23 days in experiment 1 and 30 days of age in experiment 2.

Table 1. **Composition of vitamin A-deficient sorghum-soybean meal diet fed to broiler chicks for 23 and 30 days of age**

Ingredients	(%)
Sorghum	54.5
Soybean meal	36.5
Poultry fat	5.5
Dicalcium phosphate	1.6
Limestone	1.0
Salt	0.35
Choline chloride (60%)	0.2
DL-methionine	0.2
Vitamin A-free premix[1]	0.1
Trace mineral mix[2]	0.05
Calculated nutrient composition Metabolizable energy, MJ/kg	13.2
Analyzed nutrient composition	
Crude protein	22.5
Calcium	0.98
Phosphorus	0.71

[1] Vitamin A-free premix supplied per kg diet: cholecalciferol, 825 IU; vitamin E, 10 IU; menadione sodium bisulfite, 1.5 mg; niacin, 50 mg; pantothenic acid, 13.8 mg; riboflavin, 5 mg; thiamine, 4.6; pyridoxine, 8.2 mg; folic acid, 1 mg; vitamin B_{12}, 15 μg; biotin, 200 μg.
[2] Trace mineral mix provided per kg diet: copper, 100 mg; iron, 100 mg; manganese, 100 mg; zinc, 100 mg; iodine, 0.5 mg; selenium, 0.1 mg.

A male bird was sampled from each pen and blood sample collected by frontal cardiac puncture. After euthanasia tissues were collected, weighed, samples wrapped in aluminum foil and held in crushed ice during collection, and subsequently stored at −20℃ pending analyses. Blood samples were chilled in ice, plasma separated by centrifugation, and ascorbic acid determined within 4 hours of collection (Hooper *et al.,* 2000). Tissue samples were processed and assayed for ascorbic acid and plasma total antioxidant capacity (Maurice and Lightsey, 2007). L-gulonolactone oxidase (EC 1.1.3.8) activity was assayed using whole tissue homogenate that enhanced sensitivity as it avoids the large and variable losses incident to isolation of microsomes (Ayaz *et al.,* 1976). The procedure of Ayaz *et al.* (1976) was optimized for chicken tissue (Hooper *et al.,* 2000) and further refined by the addition of reduced glutatione to the incubation buffer (4.17 mM GSH). Ascorbic acid formation from L-gulonolactone was linear with time with negligible spontaneous product formation without the renal homogenate. Each sample was corrected for background AsA in the homogenate used. Ascorbic acid concentration in plasma, tissues, and GULO reaction mixture were measured by a rapid, highly specific, colorimetric micromethod that relies on the reduction of ferric iron by AsA followed by formation of a complex of ferrous iron product and α, α'-dipyridyl in an acidic solution (Zannoni *et al.,* 1976). Tissues samples were extracted with 5% trichloroacetic

acid and the extract assayed for AsA. The acidic extractant and use of metaphosphoric acid in the assay procedure avoided oxidation of AsA.

Total oxidation status was measured by the ability of oxidants in a sample to oxidize ferrous (44944 Lot 399964/1, Fluka Chemie AG) o-dianisidine (D 06770 Lot 63027−1, Pfaltz & Bauer Inc., CT) complex to the ferric state and form a colored complex with xylenol orange (X-0127 Lot 38H1772, Sigma Chemical Co., MO) in an acidic medium (Erel, 2005). The color intensity measured at 560 nm is related to the amount of oxidant molecules present in the sample and was calibrated with hydrogen peroxide. Advanced oxidation protein products in plasma were measured by spectrophotometry (Witko-Sarsat *et al.,* 1996). Plasma (400 μl) was diluted (1: 5 v/v) with phosphate buffered saline. To the diluted plasma 100 μl 1.16 M potassium iodide (P-410 Lot 700522, Fisher Scientific Company, NJ) solution in distilled water followed by 200 μl acetic acid were added and mixed. The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing the reagents. The assay was calibrated with chloramine-T (O-1779 Lot 794722 Fisher Scientific Co., NJ) and the reaction was linear in the range of 0.22−7.1 mM.

The study used mixed-sex pens stocked with five randomly assigned day-old chicks as experimental units arranged in a randomized complete block design with six replications in experiment 1 and eight randomly assigned day-old chicks per pen in a similar design with eight replications in experiment 2. A pen of birds constituted the experimental unit. At the conclusion of the study, one bird was randomly sampled from each pen for measurement, so the linear model of the observations from this study was $Y_{ij}=M+T_i+B_j+e_{ij}$ with ($i=1,2$; $j=1,6$ in Expt. 1 and $i=1,2$; $j=1,8$ in Expt. 2), where Y_{ij} = observed response for i^{th} dietary treatment in j^{th} block, M = general mean, T_i = i^{th} dietary treatment effect, B_j = j^{th} block effect, and e_{ij} = experimental error for i^{th} dietary treatment in j^{th} block. The data were subjected to analysis of variance and comparison of dietary treatments using $\alpha=0.05$ to determine statistical significance. Means with pooled standard errors are presented in tables along with actual p-values.

Results

Vitamin A-deficient chicks were stunted and many birds exhibited incoordination, a staggering gait, ruffled feather, and generalized weakness. The kidneys of vitamin A-deficient birds were slightly enlarged and some birds had white patches in the trachea. Despite these symptoms chicks moved around and consumed feed and water. Three birds were culled from the experimental group and one from the control group in experiment 1 and the numbers were five and two respectively in experiment 2.

The results of experiment 1 are presented in Table 2. Body weight, feed intake, and liver weight, were markedly reduced ($P<0.01$) in vitamin A-deficient chicks at 23 days of age. Compared with the control group there was a trend for reduction (27%) in relative weight of spleen in chicks fed the vitamin A-deficient diet ($P<0.09$). In experiment 1, GULO activity was 20% lower ($P<0.08$) in vitamin A-deficient chicks than in control chicks. The reduction in enzyme activity was not reflected in alterations in plasma or hepatic ascorbic acid and plasma total antioxidant capacity at 23 days.

The results of experiment 2, which was terminated at 30 days of age, are summarized in Table 3. The growth response was similar to that observed in experiment 1 and additional organ measurements showed that vitamin A deficiency significantly ($P<0.02$) reduced hepatic and testicular weights and induced regression ($P<0.02$) of lymphoid organs like spleen and bursa of Fabricius. *In vitro* synthesis of ascorbic acid by renal GULO was decreased ($P<0.01$) by 33% in vitamin A-deficient chicks. However, neither ascorbic acid concentration in tissues (bursa, spleen, and testis) nor plasma antioxidants and oxidant status were significantly altered by the reduction in GULO activity. Protein oxidation products were 17% lower in vitamin A-deficient birds.

Discussion

Chicks reared on a practical vitamin A-deficient diet exhibited clinical signs of deficiency. Our results demonstrated for the first time in chickens that vitamin A deficiency decreased the activity of GULO. L-gulonolactone oxidase is the the key enzyme in vitamin C synthesis since loss of GULO activity is the reason for inability to synthesize AsA in a wide range of animals (Smirnoff *et al.,* 2004). This reduction in GULO activity was not reflected in changes in tissue AsA under conditions of this study. Hence, the earlier conclusion, based on measurement of tissue AsA concentration (Rubin and Bird, 1943; Nockels *et al.,* 1973), that AsA synthesis is not impaired in vitamin A-deficient chickens must be rejected. The failure to observe changes in tissue AsA concentration in vitamin A-deficient chickens is similar to previous reports (Rubin and Bird, 1943; Nockels *et al.,* 1973) and may be due to a reduction in the turnover of AsA produced. Our finding questions conclusions about AsA biosynthesis on the basis of changes or lack of them in tissue AsA concentration. Interpretation of tissue AsA is further complicated when AsA is fed since dietary AsA inhibits GULO activity (Hooper *et al.,* 2002).

The decreased *in vitro* synthesis of AsA in vitamin A-deficient chickens is in agreement with the results reported for rats (Malathi and Ganguly, 1964; Ghosh *et al.,* 1965). Though chickens and rats differ in the site of biosynthesis of AsA (kidney versus liver) and exhibit dissimilar gender differences in GULO activity (Maurice and Lightsey, 2007) the response of GULO to age (Hooper *et al.,* 2000) and vitamin A deficiency are similar in the two species. Several reasons may account for the marked reduction in GULO activity previously reported in rats and observed in chickens in this study. Feed

Table 2. **Response of broiler chickens at 23 days of age to vitamin A deficiency**

Response variable	Control[1]	Vitamin A deficient[1]	±SEM	*P*-value
Body weight at 23 days, g	979[a]	716[b]	25.9	0.0008
Feed intake 0–23 days, g/day	62.3[a]	47.8[b]	1.18	0.0003
Livability, %	96.7	90.0		
Liver weight, g/100 g BW	2.53[a]	2.25[b]	0.053	0.0121
Spleen weight, mg/100 g BW	83.8	61.2	7.67	0.0914
Plasma ascorbic acid, mg ml^{-1}	12.3	11.4	0.95	0.5639
Plasma total antioxidant capacity[2]	496.7	551.4	21.22	0.1263
Hepatic ascorbic acid, $\mu g\ g^{-1}$	263.2	248.6	15.79	0.5364
L-gulonolactone oxidase activity[3]	404.2	323.0	25.38	0.0815

[a, b] Difference between means considered statistically significant if P-value < 0.05.
[1] The sorghum-soybean meal diet without vitamin A supplement constituted the vitamin A deficient diet. The control diet was the same diet with 8,600 IU/kg vitamin A added.
Each mean was based on six individual chicks.
[2] Expressed as nmol Trolox equivalents ml^{-1}.
[3] Expressed as nmol mg^{-1} protein $hour^{-1}$.

Table 3. **Response of broiler chickens at 30 days of age to vitamin A deficiency**

Response variable	Control[1]	Vitamin A deficient[1]	±SEM	*P*-value
Body weight at 30 days, g	1354[a]	1008[b]	23.3	0.0008
Liver weight, g/100 g BW	2.60[a]	2.22[b]	0.088	0.0121
Livability, %	96.9	92.2		
Spleen weight, mg/100 g BW	112.9	77.1	9.51	0.0206
Testis weight, mg/100 g BW	26.9[a]	19.2[b]	2.09	0.0208
Bursa weight, mg/100 g BW	215.3[a]	128.7[b]	13.1	0.0004
Plasma ascorbic acid, mg ml^{-1}	11.8	12.5	0.75	0.5639
Plasma total oxidant status[2]	54.2	57.4	4.66	0.6341
Plasma total antioxidant capacity[3]	519.4	544.9	17.92	0.3314
Plasma oxidation protein products[4]	37.5[a]	31.2[b]	1.78	0.0255
Bursal ascorbic acid, $\mu g\ g^{-1}$	307.3	308.8	10.33	0.9148
Splenic ascorbic acid, $\mu g\ g^{-1}$	254.3	265.7	8.41	0.3525
Testicular ascorbic acid, $\mu g\ g^{-1}$	361.0	410.5	24.44	0.1743
L-gulonolactone oxidase activity[5]	426.1[a]	286.3[b]	31.58	0.0074

[a, b] Difference between means considered statistically significant if P-value < 0.05.
[1] The same diets as in experiment 1. Each mean was based on eight individual chicks.
[2] Expressed as mmol H_2O_2 equivalents ml^{-1}.
[3] Expressed as nmol Trolox equivalents ml^{-1}.
[4] Expressed as mM chloramine-T equivalents ml^{-1}.
[5] Expressed as nmol mg^{-1} protein $hour^{-1}$.

provides the substrate for AsA synthesis and a reduction in feed intake could reduce the flux of glucose via the glucuronate pathway and impact AsA synthesis. This explanation must be rejected since GULO activity was not impaired in pair-fed controls (Malathi and Ganguly, 1964; Ghosh *et al.,* 1965). Hence, decreased GULO activity in chickens could not be due to reduced feed intake. Additional support for this is the fact that kinetics of glucose metabolism is not impaired in vitamin A-deficient chickens (Phillips and Nockels, 1977). Further, feed deprivation for 72 hours caused a 16% reduction in chicken GULO activity while the reduction was 35% in rats after 24-hour starvation (Hooper *et al.,* 2000).

Another possible explanation for impaired GULO activity may be inadequate cofactors. However, addition of various cofactors to the reaction mixture failed to enhance GULO activity (Malathi and Ganguly, 1964) and in the present study the diets were fortified with a vitamin-trace mineral premix. Other reasons for decreased GULO activity in vitamin A-deficient chickens include renal damage and hypothyroidism. The former is evident only after prolonged feeding (nine weeks) of a vitamin A-deficient diet to broiler chickens (Chandra *et al.,* 1984) and hence the latter may be advanced as a possible explanation for the reduction in GULO activity. Hypothyroidism is an early sign of vitamin A deficiency in chickens (Nockels *et al.,* 1984). Reduced AsA synthesis in vitamin A-deficient chickens may be an adaptive response as sur-

vival time is decreased in such chickens fed AsA (Kendler and Perek, 1968). The reduction in GULO activity may be due to hypothyroidism-induced impairment in the glucuronate pathway. Uridine 5′-diphosphate-glucuronyltransferases and β-glucuronidase are decreased in hypothyroid rats (van der Heide *et al.,* 2004). There are a number of questions that need to be addressed to explain the effect of vitamin A deficiency on endogenous AsA synthesis, the response to dietary AsA in hypothyroid chickens (Takahashi *et al.,* 1991), and ascorbate metabolism in altered thyroid states in chickens.

We found no significant effect of vitamin A deficiency on tissue AsA and this finding is in contrast with reports of tissue depletion of AsA in vitamin A-deficient calves (Boyer *et al.,* 1942) and rats (Sure *et al.,* 1939; Sutton *et al.,* 1942; Mayer and Krehl, 1948; Boyer *et al.,* 1942; Mohanram *et al.,* 1976). This species difference may be explained by the degree of vitamin A depletion, duration of experiments, effect of deficiency on expression of AsA transporters (Kuo *et al.,* 2004), and possible differences in rates of recycling and excretion of ascorbic acid. Urinary excretion decreased in rats fed vitamin A-deficient diets (Sutton *et al.,* 1942; Mapson and Walker, 1948; Malathi and Ganguly, 1964) with maximum reduction in severe deficiency (Malathi and Ganguly, 1964) and the response was influenced by sex with greater reduction in females (Sutton *et al.,* 1942). There is no comparable information in chickens or any avian species but it is conceivable that chickens respond in a similar manner when AsA biosynthesis is reduced. This possibility coupled with reduced metabolic rate in vitamin A-deficient chickens, arising from a hypothyroid state (Nockels *et al.,* 1984), and duration of experiments may account for the failure to observe a reduction in tissue AsA.

The lack of effect of vitamin A deficiency on plasma antioxidant capacity and oxidant status may reflect the summation of adaptive responses to modulate oxidative homeostasis at the time of measurement. The finding that plasma oxidation protein products were decreased in vitamin A-deficient chickens was consistent with hypothyroidism induced in avitaminosis A (Nockels *et al.,* 1984), the associated reduction in growth rate (stunted birds) and higher metabolism in the fast-growing control birds (Freeman, 1983). The apparent discrepancy between plasma antioxidant capacity, oxidant status, and protein oxidation products needs to be clarified. The importance of the three measures is unclear and we do not know if the effect is specific for a certain tissue at the time of measurement or the sensitivity of the methods and time course changes.

Body weight and relative organ weights were markedly reduced in vitamin A-deficient birds. In the present study the depression in body weight measured at 23 and 30 days of age in broiler chickens was similar to observations made in White Leghorn cockerels at 6–11 weeks of age (Nockels and Kienholz, 1967) but at variance with near normal growth observed in Light Sussex chickens (Lowe *et al.,* 1957). Our findings with respect to liver weight are similar to earlier reports (Lowe *et al.,* 1957) and the regression in bursa of Fabricius confirmed observations in White Leghorn cockerels (Nockels and Kienholz, 1967). Vitamin A deficiency reduced the weight of testes in our study by about 30% whereas it increased testes weight in Leghorn cockerels (Nockels and Kienholz, 1967). It should be noted that measurements were made at much later ages in the Leghorn cockerels but this may not be a factor since hypothyroidism reduced testicular size (Arneja *et al.,* 1989) and hypothyroidism is an early sign of vitamin A deficiency in chickens (Nockels *et al.,* 1984). Our overall findings of decreased growth, reduced hepatic and testicular weight, and regression of lymphoid organs in vitamin A-deficient chickens are similar to that produced by an exogenous stressor (Maurice *et al.,* 2007) but differed in that in vitamin A deficiency there was not a concomitant decrease in tissue AsA despite the reduction in GULO activity.

In summary, our findings demonstrated that vitamin A deficiency reduced in vitro ascorbic acid synthesis, as measured by L-gulonolactone oxidase activity, reduced growth and relative weight of liver, testes, spleen, and bursa of Fabricius. These changes induced by vitamin A deficiency did not influence tissue ascorbic acid, plasma total antioxidant capacity, and plasma oxidant status. The results of this study warrant further examination of the interaction of dietary components and ascorbic acid synthesis and metabolism.

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