Effect of Dietary dl-α-Tocopherol on Tissue α- and γ-Tocopherol and Pulmonary Hypertension Syndrome (Ascites) in Broilers

W. G. BOTTJE, G. F. ERF, T. K. BERSI, S. WANG, D. BARNES, and K. W. BEERS

Department of Poultry Science, Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, Arkansas 72701

ABSTRACT The objectives of this experiment were to determine the effects of high dietary levels of vitamin E on growth performance and pulmonary hypertension syndrome (PHS) mortality. Male broiler chicks (Cobb 500) were randomly assigned to one of four dietary treatments consisting of standard starter and grower diets supplemented with 0, 17, 46, and 87 mg dl-α-tocopherol acetate / kg. To encourage the development of PHS, air temperature in the house was 32 and 28 C for Weeks 1 and 2, dropped to 18 C during Week 3, and kept between 10 and 15 C during Weeks 4 through 7. Also, chicks were placed in floor pens on litter used for five previous flocks and ventilation reduced to increase dust and ammonia in the house. Ammonia levels increased from an initial 18 to 36 ppm on Day 42 with the increase in ammonia corresponding to an obvious increase in dust in the air. Lung and liver tissue obtained at 2, 5, and 7 wk of age were analyzed for tissue α- and γ-tocopherol by liquid chromatography. Dietary vitamin E had no effect on body weight, feed intake, or feed efficiency. Cumulative PHS mortality through 7 wk of age was 21% and was also unaffected by dietary treatment. Liver and lung α-tocopherol concentrations exhibited a dose-response increase to dietary tocopherol and there was a high correlation between lung and liver tissue α-tocopherol ($r = 0.72$, $P < 0.05$). Whereas γ-tocopherol concentrations in lung and liver were unaffected by dietary treatment, liver and lung exhibited age-dependent increases in both α- and γ-tocopherol. Despite dose-dependent increases in tissue α-tocopherol, supplementation of diets with up to 87 mg dl-α-tocopherol acetate had no effect on growth performance or PHS mortality in broilers under the conditions used in this study.

(*Key words*: tocopherol, lung, liver, pulmonary hypertension syndrome, ascites)

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INTRODUCTION

Vitamin E is a major lipid-soluble, chain-breaking antioxidant that scavenges lipid radicals released from damaged membranes (Tappel, 1962; Burton *et al.*, 1991). Vitamin E actually refers to a group of structural isomers of tocopherol, of which α-tocopherol is the best known, possessing roughly 90% of the antioxidant activity of associated with vitamin E (Burton *et al.*, 1982; Machlin, 1991). Vitamin E is essential in maintaining membrane integrity of the cardiovascular, nervous, and immune systems (Machlin, 1991; Combs, 1992). Whereas tocopherol deficiency is associated with a number of chronic health problems and causes severe membrane damage from oxidative stress (Machlin, 1991), tocopherol supplementation above recommended levels improved resistance to microbial infections (Nockels, 1979), apparently through enhancement of humoral immunity (Tanaka *et al.*, 1979; Lawrence *et al.*, 1985). Vitamin E was also reported to exert profound beneficial effects on cell-mediated immunity by enhancing levels of T-helper cells and raising the T-helper to cytotoxic T cell ratio in thymus and blood (Erf and Bottje, 1996).

Pulmonary hypertension syndrome (PHS), commonly called ascites, is a costly metabolic disease occurring in the poultry industry worldwide (Huchzermeyer and DeRuyck, 1986; Huchzermeyer *et al.*, 1988). Conditions contributing to the development of PHS, such as high altitude, rapid growth, cool temperatures, and poor ventilation, have been recognized for several years (Lopez-Coello *et al.*, 1986; Wideman, 1988). It is now apparent that a major contributing factor leading to the development of PHS and mortality from congestive heart failure is an inherent inability of broiler pulmonary vasculature to cope with relatively small increases in cardiac output (Wideman and Bottje, 1993; Wideman *et al.*, 1996).

The pathology of PHS includes observations of high numbers of inflammatory cells in tissues of birds with fulminant symptoms of PHS (Maxwell *et al.*, 1986). Because oxygen-derived free radicals play an important role in the genesis of tissue damage during inflammation (McCord, 1985; Halliwell and Gutteridge, 1990), radical-mediated oxidative stress may play an important

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role in the development of PHS. Demonstration of low tissue levels of vitamin E (α-tocopherol), vitamin C (ascorbic acid), and glutathione (GSH) and elevations in plasma lipid peroxides in birds with PHS give biochemical evidence of a role of oxidative stress in the pathophysiology of PHS (Enkvetchakul *et al*., 1993; Bottje and Wideman, 1995; Bottje *et al*., 1995). Attenuation of PHS mortality induced by low ventilation conditions was achieved by implanting birds with a vitamin E pellet that released a total of 15 mg of α-tocopherol from 0 to 21 d (Bottje *et al*., 1995). Birds provided the tocopherol implant exhibited lower plasma lipid peroxides than placebo-implanted and unimplanted birds maintained under similar low ventilation conditions. These results indicate a protective role of α-tocopherol in lowering PHS mortality through improved tissue antioxidant capacity. However, the use of a tocopherol implant by the poultry industry would not be practical due to the added costs of labor and implants. Therefore, the major objective of this study was to determine the effect of providing birds with high levels of vitamin E (α-tocopherol acetate) on PHS mortality and lung and liver α-tocopherol levels in broilers.

MATERIALS AND METHODS

Animals and Diet

Commercial broiler males (Cobb 500) were obtained from a local hatchery.[1] Chicks with low body weights were culled such that the average weight of the remaining birds was approximately 42 g. A total of 1,960 chicks were randomly assigned to one of four diets that were analyzed[2] to contain dl-α-tocopherol-acetate supplemented at 0, 17 (Control), 46, and 87 mg / kg feed. There were seven pens of each diet with an initial population of 70 birds per pen. The birds were fed starter diets (CP, 21.6%; ME, 3,120 kcal / kg) from 0 to 3 wk and grower diets (CP, 19.1%; ME 3,200 kcal / kg) from 3 to 7 wk. With the exception of α-tocopherol content, the diets were formulated to meet or exceed all other minimum NRC (1994) recommendations. Dietary components are shown in Table 1. The grower diets were pelleted and fed in crumble form. The birds were provided *ad libitum* access to feed and water throughout the study.

Protocol

To induce a high incidence of PHS mortality, a combination of cool temperatures and low ventilation was used as previously described (Wideman *et al*., 1995a,b). In addition, accumulation of dust and ammonia with low ventilation was facilitated by maintaining birds on old litter previously used for five flock cycles that was top-dressed with wood shavings. The chicks were brooded at 32 and 28 C for Weeks 1 and 2, respectively. During Week 3, air temperature was lowered from 28 to 18 C and kept between 10 and 15 C during Weeks 4 through 7 by adjusting thermostatically regulated ventilation fans. Initial ammonia concentrations on Day 1 at bird level were 18 ppm and measured as high as 36 ppm on Day 42. Ammonia was determined using a gas detector[3] and values represent the mean of six measurements made at bird level. The increase in ammonia corresponded to an obvious accumulation of dust within the house, but particulate matter in the air was not quantified.

Feed consumption per pen and body weights were recorded on Days 7, 14, 21, 35, and 49. All birds that died during the experiment were necropsied to identify PHS-related mortality. Symptoms of PHS included the presence of ascites fluid in the abdominal cavity, and cyanosis, and obvious pre-PHS symptoms such as right ventricular dilation, hydropericardium, and vascular congestion.

Tissue samples were obtained from birds (14 randomly selected per diet, 2 per pen) at 2, 5, and 7 wk. In addition, tissues were obtained from birds identified with PHS (six at Week 5 and eight at Week 7). After killing the bird by cervical dislocation, portions of the liver and both lungs were obtained and frozen in liquid nitrogen. The heart was removed and right ventricle (RV) and left ventricle plus septum (LV+S) weights were obtained. The ratio of RV to total ventricle (RV:TV) was calculated as an index of right ventricular hypertrophy, which is associated with the severity of pulmonary arterial pressure elevation in the animal (Burton *et al*., 1968).

TABLE 1. Starter and grower diet composition

Component	Composition expressed as a percentage of the diet	
	Starter	Grower
	(%)	
Corn	58.10	63.64
Soybean meal	30.23	27.85
Poultry by-product	5.00	2.50
Animal fat	3.58	3.00
Dicalcium phosphate	1.10	0.98
Calcium carbonate	1.03	1.38
Salt	0.40	0.32
Alimet 88%	0.26	0.03
Mineral premix[1]	0.10	0.10
Vitamin premix[2]	0.20	0.20

[1]Supplied per kilogram of diet: Mn ($MnSO_4 \cdot H_2O$), 100 mg; Zn ($ZnSO_4 \cdot 7H_2O$), 100 mg; Fe ($FeSO_4 \cdot 7H_2O$), 50 mg; Cu ($CuSO_4 \cdot 7H_2O$), 10 mg; I [$CaI(O_3)_2 \cdot H_2O$], 1 mg.

[2]Supplied per kilogram of diet: vitamin A (retinyl acetate), 7,710 IU; cholecalciferol, 2,203 IU; vitamin B_{12}, 0.013 mg; riboflavin, 6.6 mg; niacin, 38.5 mg; pantothenic acid, 16 mg; menadione, 1.5 mg; folic acid, 0.881 mg; pyridoxine, 3.3 mg; thiamine, 1.5 mg; d-biotin, 0.07 mg.

[3]Values represent the means of four measurements.

[1]Randall Road Hatchery, Tyson Foods, Inc., Springdale, AR 72762.
[2]CEPS Central Analytical Laboratory, University of Arkansas, Fayetteville, AR 72701.
[3]Sensodyne Gas Tech Precision Gas Detector, Clearwater, FL 34620.

TABLE 2. Body weights, feed intake, feed efficiency, and cumulative mortality attributed to pulmonary hypertension syndrome (PHS) of birds fed a control (17 mg dl-α-tocopherol/kg) or diets supplemented with 0, 46, or 87 mg α-tocopherol acetate/kg of diet[1]

Variable	dl-α-Tocopherol acetate 0 mg/kg	17 mg/kg	46 mg/kg	87 mg/kg	Total
Body weight, g					
Week 1	40.8 ± 0.5	40.8 ± 0.6	41.2 ± 0.6	40.8 ± 1.1	
Week 1	130 ± 13	129 ± 11	128 ± 11	131 ± 9	
Week 2	330 ± 36	325 ± 21	325 ± 21	329 ± 16	
Week 3	654 ± 54	632 ± 41	638 ± 34	637 ± 27	
Week 5	1,537 ± 104	1,551 ± 88	1,487 ± 107	1,511 ± 78	
Week 7	2,638 ± 174	2,646 ± 134	2,679 ± 75	2,695 ± 64	
Feed intake, g					
Weeks 0 to 1	161 ± 11	164 ± 19	174 ± 34	148 ± 7	
Weeks 1 to 2	294 ± 38	295 ± 23	304 ± 39	303 ± 18	
Weeks 2 to 3	576 ± 14	565 ± 50	590 ± 112	538 ± 32	
Weeks 3 to 5	1,920 ± 139	1,908 ± 87	2,016 ± 322	1,940 ± 64	
Weeks 5 to 7	3,342 ± 498	3,557 ± 422	3,529 ± 296	3,733 ± 473	
Feed efficiency, g gain:g feed					
Weeks 0 to 1	0.55 ± 0.08	0.54 ± 0.10	0.51 ± 0.13	0.61 ± 0.06	
Weeks 1 to 2	0.69 ± 0.03	0.66 ± 0.04	0.65 ± 0.06	0.66 ± 0.02	
Weeks 2 to 3	0.56 ± 0.03	0.55 ± 0.04	0.54 ± 0.08	0.57 ± 0.05	
Weeks 3 to 5	0.45 ± 0.02	0.47 ± 0.04	0.42 ± 0.05	0.44 ± 0.03	
Weeks 5 to 7	0.33 ± 0.04	0.28 ± 0.04	0.31 ± 0.04	0.30 ± 0.06	
Cumulative PHS mortality					
Weeks 2 to 7	96/486 (20%)	115/476 (24%)	92/480 (19%)	104/486 (21%)	407/1928 (21%)

[1]Each value represents the mean ± SEM of seven observations. Data are presented uncorrected for mortality.

Chemical Analysis

Tocopherols (α- and γ-) analysis was accomplished using a modified method of Warren and Reed (1991) in hexane-extracted supernates of ethanol-precipitated tissue homogenates. Briefly, 500 mg of tissue was homogenized in 1 mL ice-cold deionized water containing ascorbic acid (1 g/L) and Tocol[4] (0.75 μg/mL) as an internal standard. Protein was precipitated in duplicate aliquots using ice-cold ethanol containing ascorbic acid (1 g/L). After extracting the sample homogenate twice with 2 mL hexane, the combined organic layer was evaporated under nitrogen. The tocopherols were redissolved in methanol/acetonitrile (1:3), centrifuged for 5 min at 12,000 × *g*, and 20 μL of the supernatant used for liquid chromatography. The results were compared with standards analyzed under identical conditions.

Tocopherols (α- and γ-) were analyzed by reverse-phase HPLC using a Waters HPLC[5] with a C_{18} Nova-Pak[4] column (3.9 × 150 cm). The tocopherols were eluted using isocratic conditions with a mobile phase consisting of 25% methanol and 75% acetonitrile at a flow rate of 1 mL/min and monitored by fluorescence detection using emission and excitation wavelengths of 298 and 328 nm, respectively. Tocopherols were identified and quantified by comparison to the retention times and peak areas, respectively, of authentic standards. Extraction efficiency of the tocopherols was based on the theoretical response of the internal standard. All chemicals were obtained from Sigma Chemical Co.[6] with the exception of solvents (methanol, hexane, and acetonitrile), which were obtained from Fischer Scientific.[7]

Statistical Analysis

A two-way analysis of variance was performed to determine sources of variation due to treatment, week, and the treatment by week interaction. Comparisons of dietary treatment or week means were made by least squares means analysis and differences assessed by multiple *t* test. Mean values of birds with PHS are reported but were not used in statistical comparisons. All of the above statistical analyses were accomplished using General Linear Models procedures of SAS® software (SAS Institute, 1985). A probability level of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

A summary of growth performance and cumulative PHS mortality during the experiment is provided in Table 2. Under the conditions used in this experiment, supplemental dietary dl-α-tocopherol acetate ranging

[4]Hoffmann-La Roche Inc., Nutley, NJ 07110.
[5]Waters Corp., Milford, MA 01757.
[6]Sigma Chemical Co., St. Louis, MO 63178-9916.
[7]Fischer Scientific, Fairlawn, NJ 07410.

from 0 to 87 mg/kg of feed had no effect on body weight, feed intake, or feed efficiency. The lack of effect on growth performance is similar to reports in poults (Soto-Salanova and Sell, 1996), pigs (Cannon *et al.*, 1996), and rats (Parkkila *et al.*, 1996) using levels of dl-α-tocopherol supplementation at levels similar to those of the present study. The lack of effect on PHS mortality differs from a previous experiment in which providing chicks at 1 d of age with a vitamin E implant lowered PHS mortality (Bottje *et al.*, 1995).

A possible explanation for the lack of effect on PHS mortality in the present experiment might be the timing and amount of tocopherol provided to the birds. Previously (Bottje *et al.*, 1995), chicks were implanted at 1 d with a pellet containing 15 mg α-tocopherol. The implant, designed to release a constant amount of α-tocopherol over a 21-d period, would therefore provide 5 mg of α-tocopherol during the 1st wk in addition to what the bird received in the diet. Soto-Salanova and Sell (1996) demonstrated that a subcutaneous injection of 25 mg of d-α-tocopherol in poults at 1 d was as effective as at least 80 mg dietary dl-α-tocopherol fed from 1 through 21 d of age in enhancing tissue α-tocopherol concentrations. Based on this observation, parental administration of α-tocopherol was apparently threefold more efficacious than an equivalent amount provided in the diet. Using this assumption, chicks provided the α-tocopherol implant (Bottje *et al.*, 1995) would have received the equivalent of approximately 15 mg of dietary dl-α-tocopherol during the 1st wk, in addition to α-tocopherol consumed in the diet. This would contrast dramatically with the present study, in which birds on the 17 mg dl-α-tocopherol acetate/kg (Control) diet would consume roughly 20% of this amount in the diet during Week 1 (0.164 kg feed × 17 mg dl-α-tocopherol acetate/kg = 2.8 mg α-tocopherol). Birds fed the highest level of dl-α-tocopherol acetate would consume approximately 86% (0.148 kg feed × 87 mg dl-α-tocopherol acetate/kg = 12.9 mg α-tocopherol) of what birds had received by the α-tocopherol implant (Bottje *et al.*, 1995). However, it has also been estimated that α-tocopherol absorption from the gastrointestinal tract is only 60 to 80% efficient (Hollander, 1981), depending on the type of fat in the diet (Abawi *et al.*, 1985). Thus, using the assumptions outlined above, birds fed the 87 mg dl-α-tocopherol diet would actually receive only 50 to 70% of that provided by α-tocopherol implant in the previous study (Bottje *et al.*, 1995).

Tissue levels of α- and γ-tocopherol for liver and lung in birds randomly selected from dietary treatments, and in birds specifically identified with overt symptoms of PHS, are shown in Figures 1 and 2, respectively. With a few exceptions, lung and liver exhibited both dietary- and age-related increases in tissue α-tocopherol levels in healthy broilers (Figure 1). At 7 wk, liver and lung α-tocopherol in the 87 mg/kg diet were approximately five times higher than in the Control diet. Liver and lung γ-tocopherol levels did not exhibit a response to

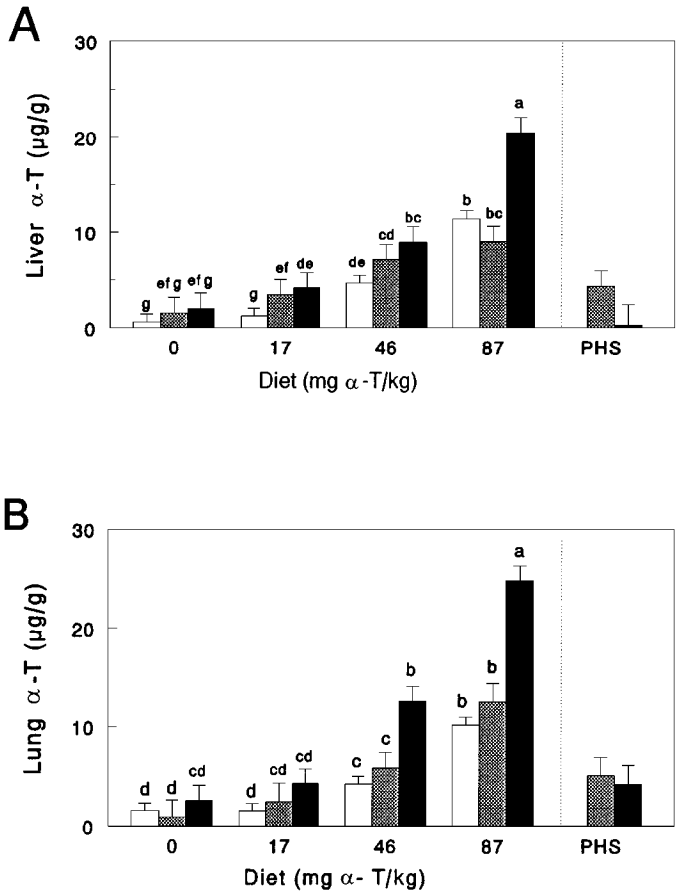

FIGURE 1. Liver (A) and lung (B) α-tocopherol levels at 2 wk (open bar), 5 wk (hatched bar), and 7 wk (solid bar) in birds fed diets supplemented with 0, 17, 46, and 87 mg α-tocopherol acetate/kg. Each value represents the mean ± SEM (n = 14). Also shown are tocopherol levels of birds with fulminant pulmonary hypertension syndrome (PHS) at 5 wk (n = 6) and 7 wk (n = 8). Dietary treatment means with different letters differ significantly ($P < 0.05$).

dietary treatment but generally exhibited an age-dependent increases regardless of dietary treatment (Figure 2). There was a high degree of correlation between lung and liver α-tocopherol levels in this study (Figure 3), as well as between lung and liver γ-tocopherol (data not shown).

Concentrations of tissue α- and γ-tocopherol for birds with PHS are presented in Figures 1 and 2 but were not compared statistically because only six birds were identified with overt symptoms of PHS at 5 wk (two each from diets containing 0, 46, and 87 mg dl-α-tocopherol acetate/kg) and eight at 7 wk (two from 0 mg, three from 17 mg, three from 46 mg, and one from 87 mg dl-α-tocopherol acetate/kg). The extremely low concentrations of α- and γ-tocopherol in the livers of PHS birds at 7 wk are similar to previous observations (Enkvetchakul *et al.*, 1993; Bottje *et al.*, 1995). In contrast, lung tocopherol levels were not depressed at 7 wk as observed in these earlier studies (Enkvetchakul *et al.*, 1993; Bottje *et al.*, 1995).

In previous studies (Enkvetchakul *et al.*, 1993; Bottje *et al.*, 1995), broilers fed diets supplemented with dl-α-

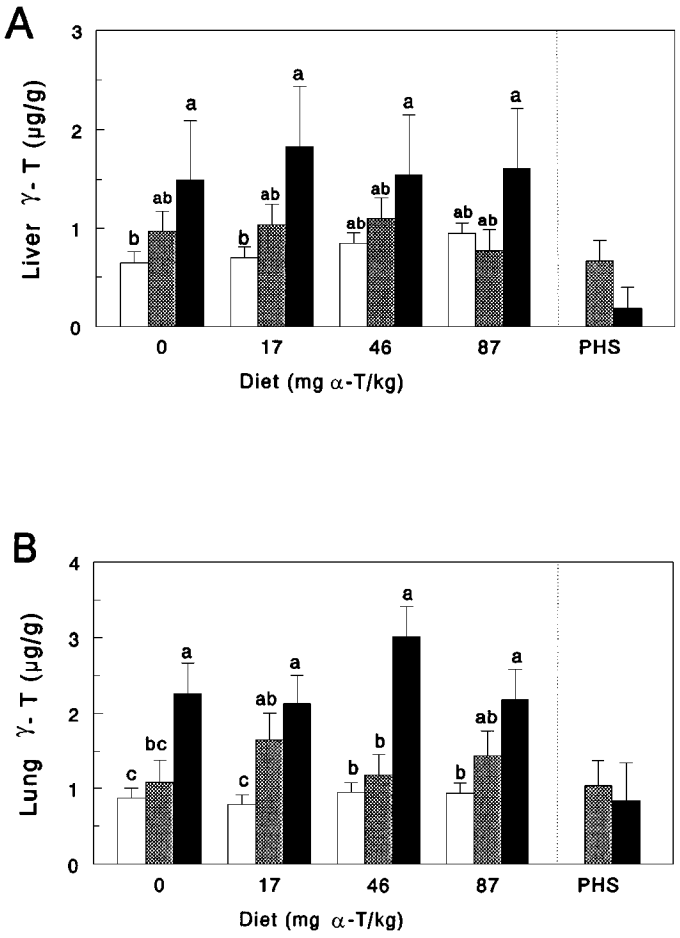

FIGURE 2. Lung (A) and lung (B) γ-tocopherol levels at 2 wk (open bar), 5 wk (hatched bar), and 7 wk (solid bar) in birds fed diets supplemented with 0, 17, 46, and 87 mg α-tocopherol acetate/kg. Each value represents the mean ± SEM (n = 14). Also shown are γ-tocopherol levels of birds with fulminant pulmonary hypertension syndrome (PHS) at 5 wk (n = 6) and 7 wk (n = 8). Dietary treatment means with different letters differ significantly ($P < 0.05$).

tocopherol acetate at approximately 11 mg/kg did not exhibit age-dependent increases in tissue α-tocopherol, but had much higher α-tocopherol levels (8 to 12 mg/g) than the Controls (1.5 to 4.5 mg/g) (Figure 2). However, lung α-tocopherol levels in this study and the earlier studies were similar; i.e., approximately 2 to 4 mg/g. The reason for the difference in hepatic α-tocopherol metabolism between these studies is not evident. As the type of fat or amount of unsaturated fat provided in the diet may affect fat-soluble vitamin absorption (Hollander, 1981; Abawi *et al.*, 1985), it is possible that there were differences in type or quality of dietary fat source between these experiments. Another notable difference between these experiments that could impact hepatic tocopherol metabolism might be attributed to environmental conditions in which the birds were housed. For example, in the present study, birds were maintained on old litter and exposed to cool temperatures in addition to low ventilation conditions, whereas birds in the previous studies (Enkvetchakul *et al.*, 1993; Bottje *et al.*, 1995) were housed in environmental chambers on new litter and exposed only to low ventilation to induce PHS. Thus, differences in environmental conditions might account in part for differences in liver α-tocopherol levels. However, this argument does not account for the lack of difference in lung α-tocopherol concentrations, which would be expected to have been lower in the present study, in which more severe environmental conditions were used to induce PHS, especially as similar conditions induced an oxidative stress in lung lining fluid of broilers (Bottje *et al.*, unpublished data).

There is also a question regarding whether synthetic dl-α-tocopherol acetate is as effective as the natural α-tocopherol form (Huang *et al.*, 1982; Chung *et al.*, 1992). These researchers suggested that the alcohol form may have greater biological activity than the acetate ester commonly used in supplementation of poultry diets. The use of the alcohol form in the previous study (Bottje *et al.*, 1995), which lowered PHS mortality, as opposed to the acetate ester form used in the present study, which did not lower PHS, could lead one to speculate that α-tocopherol is protective against PHS and the acetate ester is not. However, this is undoubtedly too simplistic an explanation. As antioxidant biochemistry is very complex with numerous interactions between endogenous antioxidants, it is likely that antioxidant protection against metabolic diseases will not be accomplished by any one antioxidant, but rather will depend upon a protective network of several antioxidants working in concert. If this is the case, then it could be hypothesized that the requisite interaction of several antioxidants needed for protection against PHS was present in the earlier study (Bottje *et al.*, 1995), but not the current study. Further experimentation will be required to identify what the ideal antioxidant protective network may be required in broilers to attenuate PHS mortality.

The effect of diet on the right ventricular weight ratio as an index of pulmonary hypertension is shown in Figure 4. It should be emphasized that the randomly selected birds appeared to be clinically healthy, i.e., they exhibited no overt symptoms of PHS, whereas birds

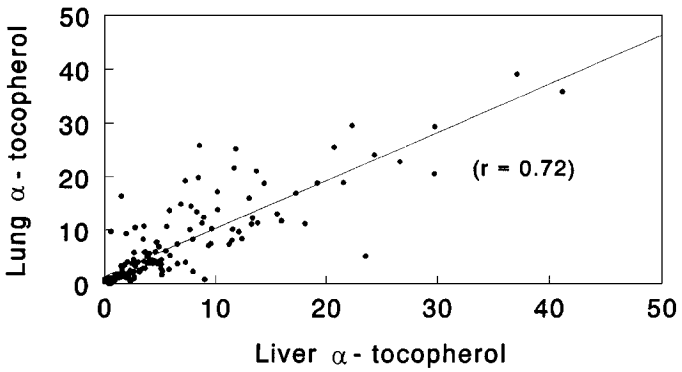

FIGURE 3. Relationships between liver and lung α-tocopherol concentrations.

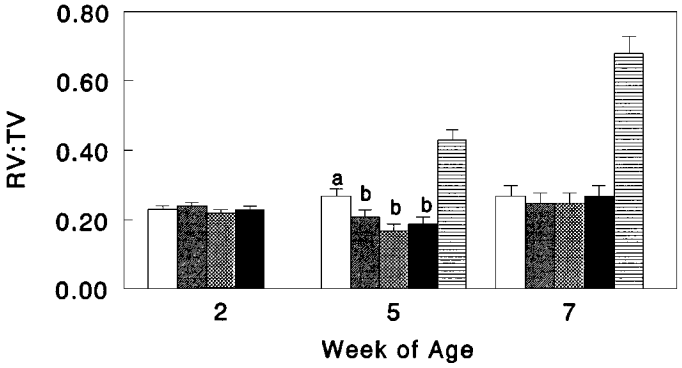

FIGURE 4. The right ventricular weight ratio (RV:TV) in birds fed diets containing 0 (open bar), 17 (hatched bar), 46 (cross hatched bar), or 87 (solid bar) mg α-tocopherol/kg and in birds with PHS (straight line bar) at 2, 5, and 7 wk of age. Each value represents the mean ± SEM (n = 14). Also shown are values for birds with fulminant pulmonary hypertension syndrome (PHS) at 5 wk (n = 6) and 7 wk (n = 8). Means with different letters differ significantly ($P < 0.05$).

with PHS were specifically selected because they had fulminant PHS symptoms. In Figure 4, it can be seen that at 5 wk, birds fed diet supplemented with α-tocopherol acetate had lower right ventricular ratios than birds fed 0 mg/kg α-tocopherol acetate or birds with PHS. Thus, at least at 5 wk of age, there is indication that diets containing supplemented vitamin E was associated with a lower right ventricular weight ratio, suggesting an attenuation of events leading to the development of PHS. At 5 and 7 wk, birds with PHS had elevated right ventricular weight ratios compared to those of clinically healthy birds.

In summary, the results of the present study indicate that supplementation of diets with α-tocopherol acetate ranging from 0 to 87 mg/kg exerted a dose-response effect on liver and lung α-tocopherol concentrations. Dietary supplementation of α-tocopherol, however, had no effect on growth performance in broilers and was ineffective in lowering PHS mortality induced by cool temperature in combination with high levels of dust and ammonia resulting from low ventilation conditions utilized in this experiment.

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