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Lipid-soluble and water-soluble antioxidant activities of the avian intestinal mucosa at different sites along the intestinal tract

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Abstract

The antioxidant capacity of the avian intestinal mucosa is potentially important in protecting the gut wall from the harmful actions of reactive oxygen species originating from the diet, mucosal metabolism and the inflammatory response to enteric microbes. To assess this capacity, we determined the total lipid-soluble and water-soluble antioxidant activities of mucosal extracts, using tissue from different parts of the intestinal tract of the chicken. The lipid-soluble antioxidants, vitamin E and carotenoids, were also measured in the same samples. Total lipid-soluble antioxidant activity was highest in mucosa from the duodenum followed by the jejunum, with much lower activities in the ileum, ceca and colon. Total water-soluble antioxidant activity of the mucosa was at least an order of magnitude greater than the lipid-soluble activity under the assay conditions and did not differ significantly among the different parts of the intestinal tract. High concentrations of vitamin E were present in the mucosa of the duodenum and jejunum, with a trend to lower levels in the ileum and ceca, and significantly less in the colon. Similarly, the mucosa of the duodenum and jejunum contained the highest concentrations of carotenoids, with much lower levels in the ileum and colon. The different isoforms of vitamin E were absorbed from the digesta by the mucosa without any major selectivity. However, the liver was greatly enriched with α-tocopherol over the other isoforms, indicating a high degree of discrimination by this tissue. The results indicate major differences in the relative contributions of lipid- and water-soluble antioxidants in the mucosa along the different parts of the intestinal tract, most likely reflecting the sites of vitamin E and carotenoid absorption.

Keywords: Antioxidants; Carotenoids; Chicken; Intestine; Mucosa; Prooxidants; Vitamin E

1. Introduction

All cells of the vertebrate animal depend on a functional antioxidant capacity to provide protection against the harmful effects of free radicals and reactive oxygen species that are the inevitable consequences of aerobic life (Halliwell, 1999). To achieve optimum protection, the tissues deploy an integrated antioxidant system that consists of a diverse array of lipid-soluble (e.g. vitamin E, carotenoids), water-soluble (e.g. ascorbic acid, glutathione) and enzymic (e.g. glutathione peroxidase, superoxide dismutase) components. The key feature is that these various components act in synergy (Surai, 2002). For instance, water-soluble antioxidants such as ascorbic acid intercept free radicals in the aqueous phase (Meister, 1992), while vitamin E, located within the lipid bilayer of cellular membranes, breaks the chain reactions of fatty acid peroxidation (Brigelius-Flohe and Traber, 1999). The vitamin E that is sacrificed (i.e. converted to tocopheroxyls) during these reactions can be regenerated by redox reactions involving ascorbic acid, glutathione and carotenoids (Frieslaben and Packer, 1993). Crucially, the overall antioxidant capacity of this multi-component system must at least be sufficient to counterbalance the various free radical generating and prooxidant activities that threaten the viability of the cell.

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In relation to prooxidant–antioxidant balance, the intestinal mucosa occupies a unique situation (Halliwell et al., 2000). On the one hand, being the site of absorption of dietary antioxidants, the mucosa is the first of the body tissues to take up the feed-derived vitamin E, carotenoids and ascorbic acid. Thus, in the fed state, the mucosa is likely to play host to substantial amounts of these antioxidants, even though their residence within the tissue is transitory. However, by the same token, the gut wall will be repeatedly exposed to any prooxidants that are present in the diet. These could include natural substrates for peroxidation such as the highly susceptible polyunsaturated lipids (Kanner and Lapidot, 2001), as well as peroxides and their derivatives arising, for example, from reheated or partly deteriorated dietary oils (Kimura et al., 1984). Prooxidant metal ions (e.g. Fe, Cu) and mycotoxins in the diet will also place demands on the mucosal antioxidant defences (Srigiridhar and Nair, 2000; Gautier et al., 2001).

A further source of mucosal oxidative stress arises from the inflammatory and immune responses of the gut wall to the bacterial and protozoan populations that colonize the intestinal tract. Studies in mammals have shown that, in many cases, the interaction of microbes or their toxins with the mucosa triggers signal transduction cascades within the intestinal epithelial cells that result in the activation of the transcription factor NF-κB (Keates et al., 1997; Jobin and Sartor, 2000). Among the genes that are upregulated in mammalian intestinal cells by NF-κB are those coding for the proinflammatory cytokine interleukin (IL)-8 (Sharma et al., 1998; Nemeth et al., 2002) and for inducible nitric oxide synthase (iNOS) (Griscavage et al., 1996). Both of these gene products have major downstream consequences for the oxidative balance of the mammalian mucosa. IL-8 is a potent chemotactic cytokine that, on secretion from the intestinal epithelial cells, directs the infiltration of neutrophils into the gut wall and stimulates their activation (Ina et al., 1997). The "respiratory burst" performed by the activated mammalian neutrophils projects an intense stream of superoxide anions, hydroxyl radicals and hydrogen peroxide onto the invading bacteria (Zhang et al., 2002). Although effective in killing bacteria, these reactive oxygen species inevitably have collateral effects on the neighbouring mucosal cells.

Nitric oxide, synthesized by the action of iNOS, is an important signaling molecule that plays crucial roles in the regulation of cell function (Moncada et al., 1991). The low levels of nitric oxide produced by the intestinal cells under basal conditions perform protective roles and help to defend the tissue during the acute phase of inflammation (Lefer and Lefer, 1999). However, the sustained production of nitric oxide by iNOS during chronic inflammation exacerbates the tissue damage (Miller and Sandoval, 1999) due to its reaction with superoxide anions to form peroxynitrite, a highly cytotoxic oxidant (Grisham et al., 1999). Thus, the products emanating from IL-8 and iNOS expression interact synergistically to profoundly upregulate the prooxidant status of the mammalian mucosa.

Whether arising from the responses to gut microbes, the contents of the diet or as by-products of intestinal cell metabolism, the effects of prooxidant excess on the mammalian intestinal mucosa are severe, contributing significantly to the tissue damage that accompanies many diseases of the gut (McKenzie et al., 1996; Qiu et al., 1999). Reactive oxygen species trigger dramatic changes in intestinal epithelial cells, including collapse of the cytoskeleton, disruption of tight junctions and loss of mucosal barrier integrity (Rao et al., 1997; Lum and Roebuck, 2001; Banan et al., 2001). This oxidant-induced hyperpermeability of the gut wall results in a vicious cycle of sustained inflammation, oxidation and mucosal damage (Keshavarzian et al., 1992; Hollander, 1998). Enriching the diet with antioxidants is, therefore, proving to be an effective strategy for combating intestinal inflammatory diseases (Srigiridhar and Nair, 2000; Gonzalez et al., 2001).

The domestic chicken, selected for rapid and efficient growth, has a high food intake and consumes diets supplemented with vitamin E (Surai, 2002). On the other hand, poultry diets often include polyunsaturated oils and fishmeal that may contain peroxides (Engberg et al., 1996). Moreover, the gut of the chicken is home to a mixed population of microbes, including several that cause inflammatory lesions of the intestinal wall (Porter, 1998). Although the chicken's heterophils (the avian equivalent of neutrophils) are less reliant on the respiratory burst mechanism for their bactericidal action (Harmon, 1998), oxidative damage is nevertheless a major feature of intestinal inflammation in birds (Allen, 1997). Thus, in the chicken, the mucosa is potentially the focus for an array of intensive prooxidant and antioxidant activities, the outcome of which is pivotal for the viability of the intestine.

These considerations suggest that the antioxidant capacity of the mucosa of the chicken may be crucial for healthy intestinal function. To assess this capacity, we determined the total fat-soluble and water-soluble antioxidant activities of mucosal extracts obtained from various points along the intestinal tract. The fat-soluble activity was further characterized by determining the vitamin E isoforms and carotenoids of the mucosal tissue. The results demonstrate major variations in mucosal antioxidant profiles among the different parts of the digestive tract.

2. Materials and methods

Female chickens (*Gallus gallus* f. *domestica*) of the Ross 308 broiler strain were allocated at one day of age among six replicate floor pens, with 20 birds per pen. The birds were grown in a temperature and ventilation controlled environment with a lighting program appropriate to strain and age. The grower diet consisted of (wt.%) wheat (54.95), soybean meal (32.6), soybean oil (4.0), fishmeal (5.0), monodical

phosphate (1.0), limestone (0.9), NaCl (0.3), methionine (0.25) and vitamin–mineral premix (0.5). The total fat content of the diet was 7.73%. Linoleic acid, the main polyunsaturate, formed 2.57% of the diet. The concentrations (μg/g) of vitamin E and carotenoids in the diet were 84.8 and 0.43, respectively, measured as described below. The birds were provided with feed and water ad libitum. At 42 days of age, one bird from each replicate pen was sacrificed by cervical dislocation and the intestinal tract was removed and divided into duodenum, jejunum, ileum, ceca and colon. A 4 cm length of tissue was taken from the middle section of each of these intestinal regions and thoroughly cleaned free of digesta by gentle squeezing. The mucosa was carefully scraped from the luminal face of the gut samples and stored at −80 °C prior to antioxidant analyses. The remaining birds from each pen were for use in a related study. The study was approved by the SAC Animal Ethics Committee and conformed to UK regulations.

The tissue samples (0.5 g) were homogenized in 2 ml of a (1:1, v/v) mixture of NaCl solution (5%, w/v) and ethanol, followed by addition of 3 ml of hexane and further homogenization. After centrifugation, the hexane layer was collected and the extraction was repeated twice. The combined hexane extracts were dried under nitrogen and the residue was dissolved in 200 μl of methanol–dichloromethane (1:1, v/v). The remaining aqueous layer was also collected. The total antioxidant activities of the organic and aqueous extracts were determined separately using the spectrophotometric method described in detail by Re et al. (1999). The method is based on the inhibition by antioxidants of the absorbance at 734 nm of the radical cation 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate), using Trolox as a standard, and has been shown to be applicable to both aqueous and lipid-soluble antioxidant activities (Re et al., 1999). Units of antioxidant activity are given as μmol Trolox equivalents and expressed as units/g fresh tissue.

Aliquots of the organic extracts were also used for the measurement of vitamin E and carotenoids. Vitamin E (α- and γ-tocopherol and α-, γ- and δ-tocotrienols) was determined by HPLC using a Spherisorb type S30DS2, 3 μ C_{18} reverse phase column, 15 cm × 4.6 mm (Phase Separations, Clwyd, UK). Chromatography was performed using a mobile phase of methanol–water (97:3, v/v) at a flow rate of 1.05 ml/min. Fluorescence detection involved excitation and emission at 295 and 330 nm respectively. Standard solutions of tocopherols and tocotrienols in methanol were used for instrument calibration and tocol was used as an internal standard.

Carotenoids were analyzed by HPLC using a Spherisorb S30DS2 3 μ C_{18} reverse phase column, 25 cm × 4.6 mm (Phase Separations, Clwyd, UK). The mobile phase consisted of acetonitrile–methanol (17:3, v/v) and acetonitrile–dichloromethane–methanol (7:2:1, v/v) in a gradient elution. Detection was by absorbance at 445 nm. Peaks were identified by comparison with the retention times of a range of carotenoid standards (variously obtained from Sigma, Poole, UK; Fluka, Gillingham, UK; Apin, Abingdon, UK; and Hoffmann-LaRoche, Basel, Switzerland) as well as using coelution of individual peaks with known standards. Lutein and zeaxanthin were the only carotenoids detected and the results were expressed as μg total carotenoids/g tissue.

Variations of each antioxidant parameter among the different parts of the intestine were assessed using the general linear model procedure (SAS Institute, 1996, Statistical Software for Windows 6.12 Inc., Cary, NC). Significance was assumed at $P<0.05$. Percentage data were transformed to arcsine prior to statistical analysis.

3. Results

Total lipid-soluble antioxidant activity of the mucosa was greatest in the duodenum followed by the jejunum, with the lowest activities expressed in the ileum, ceca and colon (Fig. 1). The activity expressed by the duodenum was 3.3 times that of the colon. Total water-soluble antioxidant activity in the tissue exceeded the maximum fat-soluble activity by an order of magnitude and did not differ significantly among

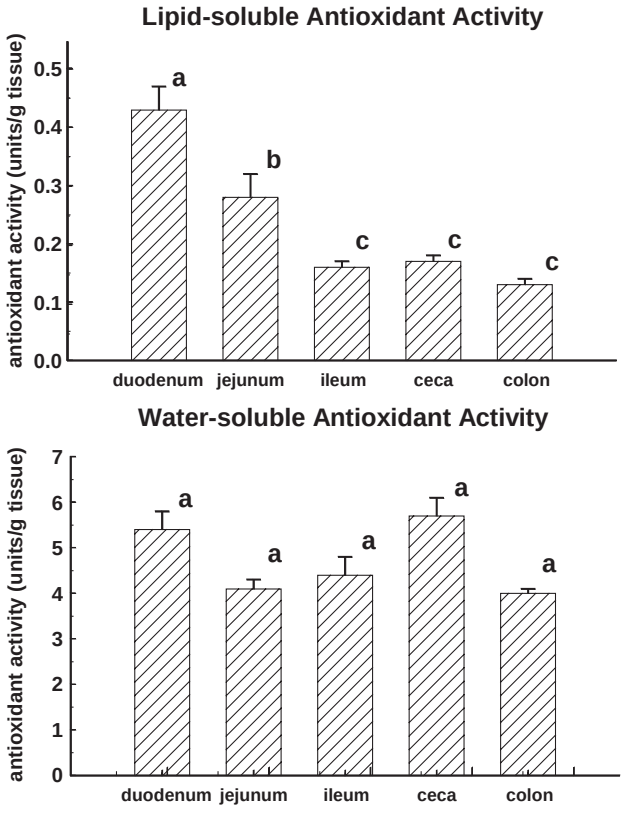

Fig. 1. Total lipid-soluble and water-soluble antioxidant activities (units/g tissue) of mucosa from different parts of the intestinal tract. Means ± S.E.M. ($n=6$) are shown. For comparisons among the various parts of the intestine, values that do not share a common superscript (a, b, c) are significantly ($P<0.05$) different.

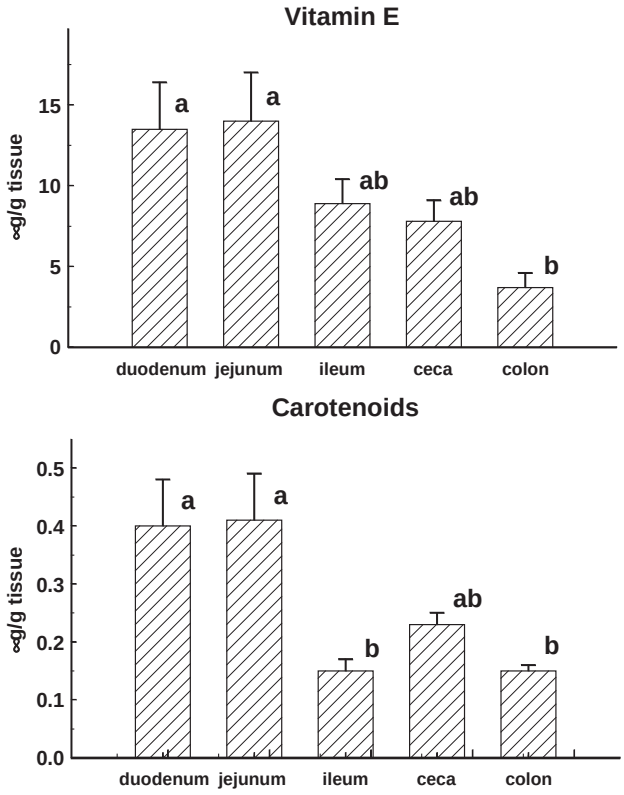

Fig. 2. Concentrations of vitamin E and carotenoids (μg/g tissue) in mucosa from different parts of the intestinal tract. Means±S.E.M. ($n=6$) are shown. For comparisons among the various parts of the intestine, values that do not share a common superscript (a, b) are significantly ($P<0.05$) different.

the different parts of the intestine (Fig. 1). The highest mucosal concentrations of total vitamin E were observed in the duodenum and jejunum, with a non-significant trend towards lower values in the ileum and ceca and a significantly lower concentration in the colon (Fig. 2). The vitamin E concentration in the duodenum was 3.6 times higher than that of the colon. The mucosae of the duodenum and jejunum contained the highest concentrations of carotenoids, with significantly lower levels in the ileum and colon (Fig. 2). The concentration of carotenoids in the duodenum was 2.7 times greater than that of the colon.

The vitamin E supplied in the diet consisted mainly of α-tocopherol and γ-tocopherol in approximately equal proportions, plus contributions from α-, γ- and δ-tocotrienols (Table 1). The proportions of these isoforms in the digesta from the duodenum were the same as in the original diet. The duodenal mucosa contained α- and γ-tocopherols in the same proportions as in the diet and digesta. However, the mucosa contained different proportions of the tocotrienols, with less of the α-form and commensurately higher proportions of the γ- and δ-tocotrienols. Thus, although α- and γ-tocopherols appear to be absorbed without discrimination by the mucosa, there appears to be some selectivity in the uptake of tocotrienols. The most dramatic change in the proportions of vitamin E isoforms occurred in the liver, where there was a great predominance of α-tocopherol over γ-tocopherol, with no detectable tocotrienols.

4. Discussion

The lipid-soluble antioxidants, vitamin E and carotenoids, that were detected within the intestinal mucosa of the chicken, could originate from two possible sources. Uptake of dietary antioxidants from the intestinal lumen across the apical plasma membrane of the mucosal enterocytes is probably the most important source, at least in the fed state (Kayden and Traber, 1993; Furr and Clark, 1997). In addition, these antioxidants, as components of plasma lipoproteins, could conceivably be recycled to the mucosa by the circulation, with uptake in this instance occurring across the baso-lateral surface of the enterocytes (Traber and Kayden, 1984). Similarly, the water-soluble antioxidant, ascorbic acid, could enter the mucosa at the apical surface as a dietary component from the intestinal lumen or, alternatively, could arrive via the circulation at the baso-lateral face of the enterocytes following its synthesis in the avian kidney (Chatterjee et al., 1975). Glutathione, another important water-soluble antioxidant, could enter the mucosa from either the lumen or the circulation, in addition to its biosynthesis within the intestinal cells (Meister, 1992).

The absorption of vitamin E (Kayden and Traber, 1993) and carotenoids (Furr and Clark, 1997) by the intestinal mucosa, and the subsequent transfer of these antioxidants to the circulation, are closely entwined with the fate of dietary fat. In the duodenum, hydrolysis of dietary triacylglycerol by pancreatic lipase releases free fatty acids and monoacylglycerols that spontaneously aggregate with phospholipids and bile salts to form mixed micelles. Vitamin E and carotenoids, released from the food matrix during digestion,

Table 1
Proportions of vitamin E isoforms in diet, duodenal digesta, duodenal mucosa and liver of the chicken, wt.% of total vitamin E

	α-Tocopherol	γ-Tocopherol	α-\tocotrienol	γ-Tocotrienol	δ-Tocotrienol
Diet	42.3±5.7[a]	41.4±5.1[a]	9.1±0.9[a]	6.0±0.5[a]	1.2±0.1[a]
Digesta	41.2±4.2[a]	39.7±3.5[a]	10.0±1.1[a]	7.7±0.9[a]	1.4±0.2[a]
Mucosa	44.4±9.2[a]	32.6±6.2[a]	3.0±0.8[b]	14.8±2.0b	5.2±1.5b
Liver	73.1±8.5[b]	26.9±3.3[a]	0.0[c]	0.0[c]	0.0[c]

Values are means±S.E.M. ($n=6$).
Values within a column without a common superscript are significantly different ($P<0.05$).
Concentrations (μg/g fresh wt., $n=6$) of total vitamin E in the diet, digesta, mucosa and liver were 84.8±4.8, 20.9±2.1, 13.5±1.1 and 36.8±3.8, respectively.

also partition into the mixed micelles. Movement of free fatty acids, monoacylglycerols, vitamin E and carotenoids from the micelles into the mucosal cells of the small intestine is then achieved by passive or facilitated diffusion. On entry into the enterocytes, the fatty acids are re-esterified with monoacylglycerols to regenerate triacylglycerols. These are then packaged together with phospholipids, sterols, apoproteins, vitamin E and carotenoids to form large spherical lipid-rich lipoprotein particles (known as chylomicrons in mammals and portomicrons in birds). The lipoproteins, containing the fat-soluble antioxidants, are subsequently exocytosed from the baso-lateral surface of the enterocyte for release into the circulation (Phan and Tso, 2001).

In birds, the duodenum and jejunum are the main sites of lipid absorption (Krogdahl, 1985) and, therefore, of vitamin E (Sklan et al., 1982) and carotenoid (Tyczkowski and Hamilton, 1986) uptake. In the present study, the concentrations of both vitamin E and carotenoids in the duodenal and jejunal mucosae were found to exceed those in the mucosa of the lower parts of the intestinal tract. The high levels of these two lipid-soluble antioxidants at these mucosal sites most likely represent diet-derived vitamin E and carotenoids in a state of transit across the enterocyte layer. Their concentrations within the mucosa will reflect the relative rates of their uptake from mixed micelles at the apical surface and their release, as components of portomicrons, at the baso-lateral face.

The total in vitro fat-soluble antioxidant activity of the mucosal extracts was far higher in tissue from the duodenum and jejunum compared with samples from the ileum, ceca and colon, consistent with the distribution of vitamin E and carotenoids. Since the concentrations of vitamin E in the diet, digesta and mucosa exceed those of carotenoids by some 40–200-fold and, given that in vitro antioxidant activities of vitamin E tend to be at least as high as those of carotenoids (Surai, 2002), the fat-soluble antioxidant activities of the mucosa in this study will be due almost entirely to the action of vitamin E.

Studies in humans and other mammals have shown that the different structural isoforms of vitamin E are absorbed by the mucosa and incorporated into chylomicrons without discrimination (Kayden and Traber, 1993). Thus, the proportions of the various isoforms in the chylomicrons closely reflect their dietary profile. However, following uptake of chylomicron remnants (and their associated vitamin E) by the liver, a highly selective discrimination occurs in favor of α-tocopherol (Traber et al., 1994). Consequently, α-tocopherol becomes the major form of vitamin E in the plasma, even though γ-tocopherol usually predominates in the diet (Kayden and Traber, 1993). This selectivity is achieved by the action of α-tocopherol transfer protein (α-TTP) in the cytosol of the hepatocytes. α-TTP binds and retains α-tocopherol with high specificity and incorporates this isoform into very-low density lipoproteins for release into the plasma (Kayden and Traber, 1993). The other isoforms, including γ-tocopherol and the tocotrienols, are selectively excreted via the bile (Kayden and Traber, 1993) or degraded to metabolites for excretion in the urine (Brigelius-Flohe and Traber, 1999).

The present results for the chicken indicate little change in the proportions of the vitamin E isoforms among the diet, digesta and mucosa apart from some variation in the mucosal tocotrienols, in general agreement with the evidence for non-discriminatory uptake (Kayden and Traber, 1993). However, the liver vitamin E profile was dominated by α-tocopherol, consistent with the selective excretion or degradation of the other isoforms. We have previously reported the highly selective excretion of tocotrienols into the bile of the chicken embryo (Surai and Speake, 1998).

Under the in vitro assay conditions used in the present study, the total water-soluble antioxidant activities of the mucosa of the chicken were greater than the values for total fat-soluble antioxidant activities by at least an order of magnitude. We did not identify the agents responsible for the water-soluble activity in this work. However, we have found that the intestine of the zebra finch (*Taeniopygia guttata*) contains the water-soluble antioxidants ascorbic acid, glutathione and uric acid at concentrations that are 20–50 times greater than the concentration of vitamin E (Karadas, F., Surai, P.F., Blount, J.D., unpublished data).

Do the results of the present work provide any indication of the ability of the intestinal mucosa of the chicken to withstand the prooxidant hazards that inevitably assail the gut wall? Optimal protection is best served by the complementary action of a range of antioxidants (Frieslaben and Packer, 1993). The results imply that substantial levels of water-soluble antioxidants are active within the mucosal cells from all parts of the intestinal tract. Vitamin E and carotenoids are also present along the length of the gut lining and are particularly concentrated at their sites of absorption in the duodenum and jejunum. Thus, the mucosa can call upon the protection of an integrated antioxidant system throughout its length, although the relative contributions of water-soluble and fat-soluble components vary according to site. Since vitamin E and carotenoids are co-absorbed with dietary fat, these lipid-soluble antioxidants are well placed to protect dietary polyunsaturates during transfer across the mucosa and to neutralize any lipid peroxide radicals present in the digesta. With regard to the effects of intestinal bacteria, the most clinically dramatic enteric disease of the chicken is necrotic enteritis, caused by the bacterium *Clostridium perfringens* (Porter, 1998). The α-toxin produced by this bacterium induces duodenal inflammation and tissue necrosis by activating a signaling cascade involving protein kinase C within the enterocytes (Bunting et al., 1997). Conceivably, the preferential localization of lipid-soluble antioxidants in the duodenal mucosa may confer some protection against the prooxidant consequences of infection at this site. Moreover, in addition to its antioxidant role, vitamin E has been shown to ameliorate

the inflammatory response in mammalian tissues by inhibiting the activation of both NF-κB and protein kinase C (Deveraj and Jialal, 1998; Ricciarelli et al., 1998). The potential beneficial effects of supplementary vitamin E in preventing inflammatory lesions of the chicken intestine are possibilities for future investigation.

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