Aflatoxin-Impaired Ability to Accumulate Oxycarotenoid Pigments During Restoration in Young Chickens[1,2,3]

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ABSTRACT The mechanism by which aflatoxin causes paling in chickens was investigated by measuring its effect on the restoration of pigments in 3-wk-old birds made pale by feeding a white corn-soy diet. Pigment restoration was accomplished by feeding the same diet supplemented with lutein (70 μg/g of diet), which is the major oxycarotenoid pigment in chicken diets and tissues. The oxycarotenoids (free, monoester, and diester forms of lutein) in the toe web, liver, serum, and jejunal mucosa of control and aflatoxin-fed (2 μg/g of diet) birds were measured by HPLC at 0, 1, 2, 3, 6, and 9 days of repletion.

Aflatoxin caused a significant ($P<.05$) depression of all forms of lutein in the toe web. In the liver, aflatoxin decreased lutein significantly ($P<.05$) but increased lutein monoester and lutein diester. Lutein accumulation in serum and mucosa were inhibited significantly ($P<.05$) starting on Days 2 and 3, respectively. These data imply that the normal accumulation of lutein from the diet proceeded into and through the mucosa to the serum to depot sites in the liver and integument, where lutein was acylated to its monoester, which was acylated to its diester. Further, aflatoxin inhibited, apparently independently, the accumulation of lutein by the mucosa, serum, liver, and integument. Pharmacokinetic analysis of the data indicated that both acylation steps in the integument were sensitive to aflatoxin, but the passage of lutein from serum into the integument was not affected.

(*Key words:* aflatoxin, carotenoids, lutein, pale bird syndrome, pharmacokinetics)

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INTRODUCTION

Pale bird syndrome, which has been used as a synonym for generalized malabsorption in chickens, can be defined more accurately as the failure of chickens to realize the color potential of the carotenoids in their diet (Hamilton, 1984b). Factors known or suspected to cause paleness include mycotoxins (Osborne *et al.*, 1982), reoviruses (Rosenberger, 1984), coccidial infections (Ruff and Fuller, 1975), and nutritional imbalances (Marusich and Bauernfeind, 1981). One of the better studied causes is aflatoxin, which causes hypocarotenoidemia in the field and laboratory (Tung and Hamilton, 1973; Osborne *et al.*, 1982) and which has been associated with field outbreaks of pale bird syndrome (Tung and Hamilton, 1973; Hamilton, unpublished observations). All the mechanism(s) by which aflatoxin and other factors cause paleness in chickens are not known, but it can be hypothesized that the factors impair accumulation or enhance excretion of oxycarotenoids, which are the pigments responsible for the yellow color of chicken skin and egg yolks. Interference with the absorption of oxycarotenoids is considered the sole or at least major mechanism (Marusich and Bauernfeind, 1981), with the exception of *Eimeria tenella* infections, which cause copious hemorrhaging into the cecum during which serum carotenoids are lost (Ruff and Fuller, 1975).

In young chickens fed lutein, a dihydroxycarotenoid that is the major oxycarotenoid in poultry diets and tissues, aflatoxin caused several changes in carotenoid metabolism (Tyczkowski and Hamilton, 1987). Among the alterations were impaired esterification of lutein in the intestinal contents, decreased lutein concentrations in the intestinal mucosa, serum and

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integument, and abnormal ratios of lutein to its monoester and diester in the liver and integument. These observations, like most studies on factors causing paleness, were made at a single point in time under steady state conditions, i.e., when the tissue carotenoids were in equilibrium with dietary carotenoids (Marusich and Bauernfeind, 1981). Such experimental conditions are essential for many purposes; however, such conditions do not permit conclusions as to whether aflatoxin impairs the ability to accumulate or increases the excretion of oxycarotenoids.

An experimental approach to evaluating the mechanism by which aflatoxin and other factors cause paleness was derived from the work of Bartov and Bornestein (1969), in which was investigated the depletion and restoration of pigments occurring when highly pigmented and poorly pigmented chickens were fed pigment-free and pigment-augmented diets, respectively. Using this approach, it was found that aflatoxin had no significant effect on the depletion phase of oxycarotenoid metabolism. The mechanism by which aflatoxin causes paling in chickens was investigated in the present experiment by measuring its effect on the restoration of pigments in birds made pale by feeding a white corn-soya diet.

MATERIALS AND METHODS

Husbandry. Day-old male chickens (Dekalb XL) were obtained from Clay's Hatchery (Blackstone, VA) and maintained throughout the study in electrically heated brooders where feed and water were available *ad libitum* and the incandescent lighting was continuous. Body weights were taken as the birds were received and weekly thereafter.

Feed. The basal diet was manufactured by the North Carolina State University feed mill and contained white corn (62.04%), 48.5% protein soy meal (32.00%), cottonseed oil (2%), salt (.40%), defluorinated rock phosphate (2.50%), limestone (.60%), DL-methionine (.26%), trace mineral mix (.10%), and vitamin mix (.10%). The basal diet contained 1.08 μg total carotenoids/g by analysis (Horwitz, 1980). When supplementation of the basal diet with carotenoids was desired, Oro-Glo (Kemin Industries, Inc., Des Moines, IA), a stabilized and microencapsulated form of lutein prepared from marigold (*Tagetes erecta*), was added to the diet to supply 70 μg lutein/g diet. Analysis of a sample of the diet after saponification revealed that the oxycarotenoid fraction contained 97% lutein.

Aflatoxin. Aflatoxin was produced by the rice fermentation method of Shotwell *et al.* (1966) as modified by West *et al.* (1973), using *Aspergillus parasiticus* NRRL 2999. The moldy rice was steamed to kill the fungus, dried, and ground to a fine powder that was analyzed for aflatoxin content by the method of Hutchins and Hagler (1983). When aflatoxicosis was induced, weighed amounts of the rice powder that contained aflatoxin B_1, B_2, G_1 and G_2 (79:8:12:1) were added to the diet to provide 2 μg total aflatoxin/g diet. Accuracy of addition and stability of aflatoxin was established by analyzing feed taken from feeders at the end of the experiment.

Experimental Design. A completely randomized 2 × 6 factorial design was employed. Factors were presence and absence of aflatoxin and six time intervals, with four replicates of 10 birds/cell. One-half of the birds received aflatoxin (2 μg/g diet) throughout the experiment. The basal diet was fed to all birds the 1st 3 wk, unsupplemented with carotenoids. Then all birds were placed on diets supplemented with free lutein (70 μg/g diet). Birds that had received aflatoxin in the basal diet continued to receive aflatoxin (2 μg/g diet) in their lutein-supplemented diet. At 0, 1, 2, 3, 6, and 9 days after placing on the diets supplemented with lutein, four groups of ten control birds and four groups of ten birds fed aflatoxin were removed for analysis of carotenoids in their tissues.

Statistical Analyses. Group means over time were subjected to a two-way analysis of variance in which an F ratio was calculated. If the F ratio were significant ($P<.05$), the least significant difference among treatment means was calculated, as outlined by Bruning and Kintz (1977).

Sample Preparation. Birds were bled for serum, with 2.5 mL taken from each bird by cardiac puncture, killed by cervical dislocation, and the jejunum, liver, and one foot removed and combined on a group basis. After removing the jejunal contents and washing the jejunal walls thoroughly with water, the mucosa was removed by scraping. Livers were homogenized in deionized water (1:5, w/v). Toe webs were sampled with a circular, 3-mm diam punch (Heiman and Tighe, 1943; Marusich and Bauernfeind, 1981) and two circles of toe web/bird were combined on a group basis. All samples were stored at –20 C until analyzed.

Sample Extraction. Thawed samples of 2 mL of liver homogenates or serum, 1 g mucosa, and 20 circles of toe web were extracted with 30 mL hexane: acetone: toluene: absolute ethanol (10:7:7:6) in the dark at room temperature overnight.

Purification of Extract. The extract was added to 30 mL hexane, made to 100 mL with 10% aqueous Na_2SO_4, mixed, and let stand for 1 h; then a 10 mL aliquot was removed from the upper phase, which was evaporated under N_2. The residue from evaporation was purified by passing through a cartridge of silica gel (Sep-Pak, Millipore-Waters, Milford, MA) according to the method of Tyczkowski and Hamilton (1986a).

Carotenoid Analysis. The oxycarotenoids were separated by high pressure liquid chromatography according to Tyczkowski and Hamilton (1984). Oxycarotenoids were quantitated by the peak height method using lutein diester (A. L. Laboratories, Englewood Heights, NJ), lutein monoester (prepared from the diester by alkaline partial hydrolysis), and free lutein (prepared by alkaline complete hydrolysis of the diester) as standards. The absorbance of peaks eluted from the column was compared to the absorbance of standard solutions. The peaks were identified by their retention times, spectra, and behavior to saponification.

Pharmacokinetic Analysis. A one compartment, open model (Baggot, 1977) was used for analysis of the accumulation of integumentary lutein and its acylation to lutein monoester and lutein diester because of the changing rates of accumulation, because the integument can be considered a metabolic cul-de-sac without competing reactions, and because a monoexponential equation gave the best fit to the data based on analysis of residuals using the general linear model procedure (SAS Institute, Inc., 1985). The flow of oxycarotenoids into the integument was assumed (Tyczkowski and Hamilton, 1986a,b, 1987) to proceed according to the following three equations:

$$\text{serum lutein} \xrightarrow{K_1} \text{integumentary lutein} \xrightarrow{K_2} \text{integumentary lutein monoester} \xrightarrow{K_3} \text{integumentary lutein diester},$$

which have the rate constants designated as K_1, K_2, and K_3. The analysis further assumed that the compartment was empty at the start of depletion (a reasonable assumption based on the magnitude of the lutein inflow), that lutein entered

TABLE 1. *Effect absence (−) or presence (+) of aflatoxin on forms of lutein in toe web during pigment restoration of pale birds*[1,2]

Time	Lutein		Lutein monoester		Lutein diester		Total oxycarotenoids	
	−	+	−	+	−	+	−	+
(days)	(ng/cm²)							
0	10 ± 1^{f}	12 ± 1^{f}	15 ± 1^{g}	14 ± 0^{g}	54 ± 3^{f}	57 ± 1^{f}	79 ± 13^{f}	83 ± 10^{f}
1	209 ± 13de	220 ± 34de	25 ± 1^{f}	23 ± 1^{f}	57 ± 1^{f}	62 ± 1^{f}	291 ± 12^{e}	305 ± 23^{e}
2	182 ± 6^{d}	176 ± 11^{d}	23 ± 2^{f}	21 ± 2^{f}	77 ± 4^{ef}	67 ± 5^{f}	282 ± 5^{e}	264 ± 15^{e}
3	236 ± 9^{c}	267 ± 9^{c}	37 ± 3^{e}	32 ± 1^{e}	125 ± 2^{d}	102 ± 7de	398 ± 6^{d}	401 ± 11^{d}
6	394 ± 28^{b}	395 ± 23^{b}	57 ± 4^{c}	47 ± 2^{d}	337 ± 13^{b}	207 ± 5^{c}	788 ± 33^{b}	649 ± 25^{c}
9	489 ± 12^{a}	400 ± 45^{b}	90 ± 5^{a}	70 ± 4^{b}	560 ± 27^{a}	343 ± 20^{b}	1,139 ± 36^{a}	813 ± 40^{b}

[a−g] Values within forms of lutein (lutein, monoester, diester, or total) with no common superscripts differ significantly ($P \leq .05$).

[1] Values are mean ± SE of four groups of 10 birds.

[2] Plus sign indicates presence of 2 μg aflatoxin/g diet.

the compartment from the serum, and that depletion reactions, which are equivalent to restoration reactions in steady state conditions, are negligible during repletion.

Kinetic analysis required that the concentrations of metabolites at the various time intervals be corrected for mass flow through the reaction sequence. Because lutein monoester and lutein diester are products of lutein, the amount of lutein entering the integument from serum during restoration was not just the amount of integumentary lutein measured, but was the sum of measured lutein, lutein monoester, and lutein diester. Similarly, the measured lutein monoester was corrected for formation of lutein diester. The concentration of lutein diester required no adjustments because it is the end product of the restoration sequence. When these corrected concentration values were used, regressions of L_n [oxycarotenoid] on time gave linear responses. The slopes of these regression lines were defined as the rate constants for the accumulation of each form of integumentary lutein. Comparisons of the individual reaction rate constants between and within control and aflatoxin-treated groups were made by calculating the standard error between two linear regression coefficients and determining significance via a two tailed *t* test (Snedecor and Cochran, 1980).

RESULTS

Once restoration was started, total oxycarotenoids in the toe webs increased steadily in both control and aflatoxin-fed birds (Table 1). Aflatoxin had no significant ($P<.05$) effect on the accumulation of total oxycarotenoids until 6 and 9 days of restoration. The accumulation of lutein diester and lutein monoester forms of lutein also were retarded by aflatoxin on Days 6 and 9 of repletion. The accumulation of lutein was not retarded by aflatoxin until 9 days of repletion. During early restoration (0 to 1 day) lutein increased about 20-fold and lutein monoester increased about two-fold whereas lutein diester barely changed. From Day 3 to Day 9 in control birds, lutein and lutein monoester increased about two-fold whereas lutein diester increased about five-fold. At Day 9 in control birds, lutein, lutein monoester, and lutein diester were 22%, 29%, and 65% greater, respectively, than corresponding values in birds fed aflatoxin. The concentration of lutein diester, which exceeded that of lutein and of lutein monoester prior to restoration (Table 1, Day 0), was surpassed during the early surge of lutein into the toe web and did not exceed lutein again in control birds until 9 days of restoration.

Rate constants (Table 2) for the accumulation of the different forms of lutein were obtained by pharmacokinetic analysis of the toe web data (Table 1). There were no significant ($P<.05$) differences between K_1, K_2, and K_3 within treatments (i.e., in control birds or in birds receiving aflatoxin). However, when comparisons were made between control and aflatoxin-fed birds, K_2 (formation of lutein monoester) and K_3 (formation of lutein diester) were found to be depressed significantly ($P<.05$).

The pattern that emerged from analyses of livers (Table 3) was somewhat different. Total oxycarotenoids in the liver were not affected significantly by aflatoxin until Day 6, when they were higher in aflatoxin-fed birds than in control birds. On Day 9 the pattern was reversed, with the oxycarotenoids being about 10% lower in aflatoxin-fed birds than in control birds. Lutein increased steadily, but it was not depressed significantly ($P<.05$) by aflatoxin in comparison to amounts in controls until Day 9. Of the es-

TABLE 2. *Rate constants for reactions of integumentary pigment restoration during aflatoxicosis*

Rate[1]	Control	Aflatoxin[2]
	(day^{-1})	
K_1	$2.51 \times 10^{-1} \pm .03^a$	$2.08 \times 10^{-1} \pm .03^a$
K_2	$2.65 \times 10^{-1} \pm .01^a$	$2.05 \times 10^{-1} \pm .01^b$
K_3	$2.83 \times 10^{-1} \pm .01^a$	$2.15 \times 10^{-1} \pm .01^b$

[a,b]Values in a row with different superscripts differ significantly ($P<.05$).

[1] Designation for rate constants: K_1 = formation of lutein; K_2 = formation of lutein monoester; K_3 = formation of lutein diester.

[2] Diet contained 2.0 μg aflatoxin/g diet.

TABLE 3. *Presence (+) or absence (−) of aflatoxin and liver lutein forms during pigment restoration of pale birds*[1,2]

Time	Lutein		Lutein monoester		Lutein diester		Total oxycarotenoids	
	−	+	−	+	−	+	−	+
(days)	(μg/g wet weight liver)							
0	.36 ± .02[f]	.30 ± .00[f]	.30 ± .03[f]	.31 ± .02[f]	...	.08 ± .02	.65 ± .04[g]	.68 ± .03[g]
1	2.99 ± .07[e]	3.03 ± .17[e]	.91 ± .10[e]	.96 ± .08[e]	.24 ± .04[e]	.21 ± .04[e]	4.14 ± .11[f]	4.19 ± .17[f]
2	4.98 ± .15[d]	4.46 ± .06[d]	1.13 ± .05[e]	1.69 ± .10[d]	.22 ± .04[e]	.80 ± .08[d]	6.33 ± .21[e]	6.96 ± .18[e]
3	7.66 ± .52[c]	6.94 ± .46[c]	1.54 ± .15[d]	2.69 ± .18[c]	.39 ± .08[e]	1.34 ± .11[c]	9.85 ± .80[d]	10.97 ± .30[d]
6	10.70 ± .91[b]	9.56 ± .34[b]	1.88 ± .13[d]	4.18 ± .21[b]	.24 ± .02[e]	2.06 ± .04[b]	12.81 ± .63[c]	15.82 ± .49[b]
9	16.40 ± 1.12[a]	10.88 ± .62[b]	2.32 ± .25[c]	3.60 ± .08[a]	.70 ± .05[d]	2.83 ± .30[a]	19.41 ± 1.30[a]	17.31 ± .49[b]

[a–g]Values within forms of lutein (lutein, monoester, diester, or total) with different superscripts differ significantly ($P \leq .05$).

[1] Values are mean ± SE of four groups of 10 birds. Lutein diester values of samples for 0 time without aflatoxin were lost during analysis, hence 0 time with aflatoxin values were not included in the analysis of variance.

[2] Plus sign indicates presence of 2 μg aflatoxin/g diet.

terified forms, lutein monoester accumulated to a greater extent in control birds than did lutein diester (Day 9 ratio of monoester:diester = 3.3:1.0). Lutein diester in control birds did not increase steadily with time but rather increased dramatically between Days 6 and 9. Contrary to the findings in toe webs, aflatoxin stimulated significantly ($P < .05$) the accumulation of both lutein monoester and lutein diester by Day 2, and when the experiment was terminated by Day 9, lutein diester was about 300% and lutein monoester was about 56% above corresponding values. The final ratio of monoester:diester in aflatoxin-fed birds was 1.27:1.0.

The lutein content of the serum (Table 4) increased 25-fold on the first day of restoration and then that day's value increased steadily another three-fold over the remainder of the experiment. Aflatoxin significantly ($P < .05$) inhibited lutein accumulation (about 25%) in serum, compared with values in controls, commencing on Day 2. Small amounts of lutein monoester and 3′-oxolutein were detectable in the serum, but they were not considered in the present analyses because lutein is the transport form.

The pattern of accumulation of lutein in the jejunal mucosa resembled that observed in the serum. The lutein content of the mucosa increased about 40-fold the 1st day of restoration (Table 5) whereas the increase in lutein from Day 1 to 9 was about two-fold. Aflatoxin inhibited by about 20% the accumulation of lutein in the mucosa at all time intervals, compared with that in controls, but the inhibition was significant ($P < .05$) only on Days 3, 6, and 9. It should be noted that after the first day, lutein

TABLE 4. *Effect of aflatoxin on serum lutein during pigment restoration of pale birds*[1]

Time	Aflatoxin (μg/g diet)	
	0	2
(days)	(μg/mL serum)	
0	.3 ± .0[h]	.3 ± .1[h]
1	7.6 ± .4[g]	6.5 ± .1[g]
2	11.7 ± .2[e]	9.0 ± .2[f]
3	15.7 ± .6[c]	11.9 ± .7[e]
6	18.0 ± .9[b]	14.3 ± .3[d]
9	23.0 ± .7[a]	17.1 ± .8[b]

[a–h]Values with different superscripts differ significantly ($P < .05$).

[1] Values are mean ± SE of four groups of 10 birds.

TABLE 5. *Effect of aflatoxin on free lutein of mucosa during restoration of pale birds*[1]

Time	Aflatoxin (μg/g diet) 0	2
(days)	(μg/g mucosa)	
0	.2 ± .0[g]	.2 ± .0[g]
1	9.7 ± .7[ef]	7.6 ± .3[f]
2	9.8 ± .4[ef]	7.6 ± .2[f]
3	13.6 ± 1.5[bc]	10.9 ± .7[de]
6	15.1 ± 1.5[b]	12.4 ± .5[cd]
9	18.6 ± .7[a]	15.3 ± .4[b]

[a–g]Values with no common superscripts differ significantly ($P<.05$).

[1] Values are mean ± SE of four groups of 10 birds.

accumulated at a faster rate in the serum than in the mucosa.

DISCUSSION

The restoration model coupled with use of HPLC led to several findings about the metabolism of lutein. The accumulation of lutein during the first day of restoration varied from tissue to tissue, with the order being mucosa > serum > liver > integument. If a precursor-product relationship is assumed, and the relationship follows from consideration of the anatomy, it would appear that free lutein is absorbed by and through the mucosa into the bloodstream, where it is transported to the liver and then deposited in the integument, where it accumulates. In both liver and integument, lutein existed as the free alcohol, monoester, and diester. Initially, lutein accumulated mainly as the free alcohol in both organs. As the time of restoration was extended in the toe web, the monoester and then the diester accumulated. By the end of the experiment, lutein diester predominated in the toe web. Much the same pattern occurred in the liver. These data were consistent with lutein being deposited as the free alcohol in the liver and integument and then being acylated to form the monoester, which served as substrate for a second acylation reaction resulting in formation of the diester.

The results of pharmacokinetic analysis were consistent with the aforementioned mechanism for metabolism of integumentary lutein proposed earlier (Tyczkowski and Hamilton, 1987). The pharmacokinetic model employed required that the rate constants governing the sequential reactions satisfy the expression $K_1 \geq K_2 \geq K_3$. Failure to meet this requirement, i.e., if $K_3 > K_2$ or $K_3 > K_1$ or $K_2 > K_1$, would cause one of the intermediate metabolites (free lutein or lutein monoester) to deplete over time, and diverge from the observed increase of all forms of lutein over time (Table 1). Failure to observe significant differences between the rate constants in control birds or in aflatoxin-fed birds led to two conclusions. First, integumentary restoration proceeded according to the proposed model. Second, the similarity in reaction rates was not due to a rate-limiting reaction within the integument. Instead, the rate-limiting reaction in pigmentation of normal chickens occurred prior to integumentary deposition. During aflatoxicosis the situation was reversed, and the rate-limiting reaction(s) were in the integument.

The results imply that aflatoxin causes paleness in birds, at least partially, by interfering with the accumulation phase of pigmentation. Aflatoxin in the present experiment inhibited, apparently independently, the accumulation of lutein by the mucosa, the serum, the liver, and the integument. In the integument, the acylation of monoester to diester was more sensitive to inhibition by aflatoxin than was the acylation of free lutein to monoester; this process was in turn more sensitive to aflatoxin than was the accumulation of free lutein (as judged by time of onset of a significant effect), by the magnitude of the effect, and by the depression of the rate constant. In the liver, aflatoxin inhibited the accumulation of free lutein while stimulating the accumulation of monoester and diester. The differential effects of aflatoxin on the accumulation of the three forms of lutein in the liver and toe web imply that the formation of lutein diester from lutein required two distinct steps, each of which was influenced by aflatoxin.

The model and pharmacokinetic analyses used here are not affected by the well-known growth inhibitory properties of aflatoxin (Hamilton, 1984a). Specifically, the appetite of chickens is not affected by aflatoxin (Burditt *et al.*, 1983), hence the poor feed conversion ratios caused by aflatoxin (Smith *et al.*, 1971; Combs, 1975; Osborne and Hamilton, 1981) mean that birds overconsume on a body weight basis. Therefore, the decreased rate of restoration in birds fed aflatoxin is not a consequence of lower intake of oxycarotenoids. Further, the chemical and pharmacokinetic analyses are independent of body weight.

The present results have several broad implications. The failure of integumentary total lutein to reach a plateau and the failure to reach normal ratios of free lutein:diester after 9 days of restoration imply that a longer period of feeding was required to reach equilibrium with dietary carotenoids; such an equilibrium is a goal of practical poultry husbandry (Bartov and Bornstein, 1969; Marion *et al.*, 1985). The opposite effects of aflatoxin on esterification of lutein in the liver and toe web imply the possibility of distinguishing paleness caused by different agents by determining the carotenoid pattern in tissues.

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