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Evaluation of Bone Strength During Aflatoxicosis and Ochratoxicosis†

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Young chickens were fed graded levels of aflatoxin (0, 0.625, 1.25, 2.5, 5.0, and 10.0 μg/g of diet) or ochratoxin (0, 0.5, 1.0, 2.0, 4.0, and 8.0 μg/g of diet), and the breaking strength, displacement before failure, and diameter of their tibias were determined. Breaking strength was decreased at growth inhibitory levels of aflatoxin (2.5 μg/g) and ochratoxin (2 μg/g), whereas a reduction in diameter required higher levels (5.0 and 4.0 μg/g, respectively). Bones from birds with ochratoxicosis selected to have diameters equal to control bones had lower breaking strength. In an attempt to negate mathematically the effect of decreased diameter and bias in any selection process, stress at time of failure of the bones was calculated and found to be decreased by feeding aflatoxin but not ochratoxin. Total displacement of bones before breaking was increased significantly ($P < 0.05$) by both toxins at the highest levels administered, but this increase was primarily the result of an increase in displacement from the start of failure to complete failure. Increased displacement associated with both toxicoses was equal in bones selected to be of equal diameter or in bones from the same treatment but of different diameters. However, calculation of modulus of elasticity which is corrected for diameter revealed aflatoxin had no effect whereas ochratoxin tripled the effect. These data indicate that the material properties of bones can be altered during mycotoxicoses and suggest yet another way in which mycotoxins are detrimental to animal health.

Aflatoxin and ochratoxin are two mycotoxins of current concern to animal and public health. Aflatoxin is considered to be primarily a hepatotoxin (14), and ochratoxin is thought to be primarily a nephrotoxin (8) in young broiler chickens. Aflatoxin and ochratoxin both produce severe economic and physiological effects in broiler chickens such as altered nutrient absorption (D. J. Osborne, W. E. Huff, and P. B. Hamilton, Abstr. Poult. Sci. **55**:2075, 1976), impaired immunity (16; W. E. Huff, unpublished results), decreased growth rate (8, 14), impaired blood coagulation ability (2), and poor carcass pigmentation (6, 18).

Bone abnormalities are yet another area of economic importance to the animal industry for which evidence of mycotoxin involvement has accumulated. "Field rickets," a rachitic condition of chickens that does not respond to the addition of vitamin D to the diet (9), is considered to be caused by mycotoxin(s) (E. L. Stephenson, 1974, Abstr. Annu. North Carolina Nutrition Conference). Aflatoxin interacts with vitamin D deficiencies, and several natural outbreaks of rickets have occurred in chickens fed diets marginally deficient in vitamin D_3 and containing aflatoxin (4, 5). Further, we made the incidental observation during field outbreaks that the bones from chickens with aflatoxicosis or ochratoxicosis appeared more pliable than bones from control birds. These considerations prompted an investigation to document if possible the effect of dietary aflatoxin and ochratoxin on bone strength in chickens.

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MATERIALS AND METHODS

Husbandry. One-day-old male broiler chicks (Cobb × Cobb) from the North Carolina State University farm were used in these studies. The birds were housed in electrically heated batteries under continuous illumination with feed and water available ad libitum. The feed was a commercial broiler-starter ration from which all medications had been excluded. The experiments were terminated when the birds reached 3 weeks of age.

Ochratoxicosis. Ochratoxicosis was induced by in-

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corporating known amounts of ochratoxin into the diet fed to the birds. Ochratoxin was produced by growing *Aspergillus ochraceus* NRRL 3174 on wheat by the method of Trenk et al.(17), extracting by the method of Steyn and van der Merwe (15), purifying by thick-layer preparatory chromatography using silica gel with a solvent of benzene and acetic acid (9:1, vol/vol) as described by Eppley (3), and crystallizing from cold benzene. Crystalline ochratoxin was dissolved in 95% ethanol and mixed with a small portion of the diet which was dried and mixed with the rest of the diet to provide 0, 0.5, 1.0, 2.0, 4.0, and 8.0 μg/g of diet.

Aflatoxicosis. Aflatoxicosis was induced in a similar manner by adding known amounts of aflatoxin to the ration. Aflatoxin was produced by growing *Aspergillus parasiticus* NRRL 2999 on rice by the method of Shotwell et al. (12) as modified by West et al. (19). The moldy rice was steamed, dried, and ground to a fine powder which was analyzed spectrophotometrically for its aflatoxin content by the method of Nabney and Nesbitt (10) as modified by Wiseman et al. (20). The ratios of aflatoxin B_1:B_2:G_1:G_2 were 70:9:15:6 by the method of Pons et al. (11). The rice powder was then added to the diet, but never exceeding 1% of the diet, to provide 0, 0.625, 1.25, 2.5, 5.0, and 10 μg of total aflatoxins per g of diet.

Analyses. At the termination of the experiment the birds were weighed and killed by cervical dislocation, and both tibias were removed. The central diameter of the tibias was determined with calipers. Breaking strength of tibias was measured with an Instron universal testing machine, model 1130 (Instron Corp., Canton, Mass.). The bone was placed across two supports, and a moving force was applied on the bone at the midpoint between the supports. The force applied resulted from a constant rate of deformation. Both the force and the displacement required to break the bone were measured. Figure 1 is an idealized force diagram depicting the configuration curves produced by this procedure. The first phase of the curve represents the initial contact on the bone of a force which increases to a maximum at which time the bone begins to break. The second phase represents the continual decrease in resistance of the bone to the applied force until failure or complete breakage occurs. It should be noted that the bones continue bending until complete breakage.

Histopathology. Bones were split lengthwise, fixed in buffered Formalin, and shipped to an independent laboratory (Rex Hospital, Raleigh, N.C.) where thin sections were made, stained, and mounted. The slides were examined in a double-blind fashion by the microscopist.

Statistical considerations. The experimental design was completely randomized with four groups of 10 birds per treatment. The data were subjected on a group basis to an analysis of variance in which an *F*-ratio was calculated. If the *F*-ratio were significant, the least significant difference among the treatment means was calculated (1).

RESULTS

The effect of dietary aflatoxin and ochratoxin on the maximum force the bones would sustain before initiation of failure or bone breakage is presented in Fig. 2. Tibial strength as measured in this fashion was decreased significantly ($P < 0.05$) by aflatoxin at concentrations of 2.5, 5.0, and 10.0 μg/g and by ochratoxin at 2.0, 4.0, and 8.0 μg/g. These are the growth inhibitory levels in these experimental systems (8, 14). The extent of the decrease in bone strength was greater with ochratoxin, which caused a loss at its high-

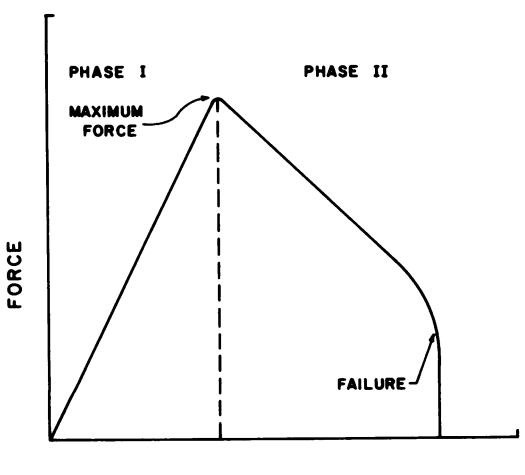

FIG. 1. *Idealized diagram of force and displacement associated with breakage of bones. The maximum force a bone will support represents the initiation of breakage, and complete breakage or failure of the bone occurs when the force returns to zero as the displacement increases.*

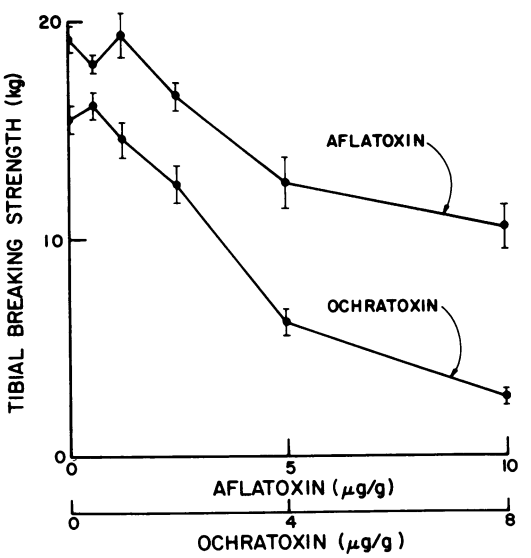

FIG. 2. *Breaking strength of tibias of birds fed graded concentrations of dietary aflatoxin or ochratoxin. Each point is the mean ± standard error of four groups of 10 birds fed the indicated level of toxin.*

est dose to about one-eighth of the control value, as compared with aflatoxin, which caused a loss of only about one-half.

Because the two toxins inhibit growth, including skeletal growth, and because it is reasonable to expect smaller bones to have a lower breaking strength, the diameter of the bones as a function of dietary toxin was measured at the point of breakage (Table 1). Analyses of variance revealed that tibial diameters were significantly ($P < 0.05$) decreased at the two highest levels of aflatoxin (5.0 and 10.0 μg/g) and ochratoxin (4.0 and 8.0 μg/g). In addition, the control diameters of the ochratoxin experiment were smaller than those of the aflatoxin experiment which agree with the lesser breaking strength obtained with the ochratoxin controls (Fig. 2). These data suggested that decreased bone diameter could account for part but not all of the decreased bone strength because the three highest doses displayed decreased bone strength (Fig. 2) whereas only the two highest had decreased diameter.

To obtain a better idea of the relative importance of diameter in decreased bone strength during the mycotoxicosis, the data from the above experiments were examined with the idea of selecting bones of equal diameters from all treatments and comparing their breaking strength. Sufficient data from the ochratoxin experiment were available to permit analysis. When the toxin effect on diameter was negated in this fashion, there was still a significant ($P < 0.05$) decrease in breaking strength at the three growth inhibitory levels of ochratoxin. While this approach indicated an effect on intrinsic strength of bones separate from diameter, it required a selection of experimental material with a consequent possible bias. Therefore, a mathematical modeling approach was undertaken.

For this purpose the bones were assumed to be solid cylinders which fail according to the formula for theoretical stress at failure (13): $S_f = 98066.5\ (8\ F_f\ L)/(\pi\ D^3)$, where S_f is stress at failure (in Newtons per square meter), F_f is force at failure (in kilograms), L is effective length of bone (in centimeters), *and* D is diameter (in centimeters). Since F_f and D are the only variables in our experimental system, the formula reduces to $S_f = (k\ F_f)/(D^3)$, where k is a constant. Therefore, S_f encompasses and is corrected for diameter. Table 2 shows the stress at failure of bones from birds fed graded doses of aflatoxin and ochratoxin. Analyses of variance revealed that the three highest doses of aflatoxin caused a significant ($P < 0.05$) decrease in this parameter. Ochratoxin, however, did not produce a significant alteration in the stress of bones at failure.

Besides lowered breaking strength, another aspect of bone problems drawing comment is that of rubbery or flexible bones that bend excessively before breaking and which are considered diagnostic of rickets and other rachitic conditions. Our experimental system permitted measurement of the displacement or bending associated with the bones breaking from the applied force (Table 3). Both aflatoxin and ochratoxin at the two highest doses administered increased significantly ($P < 0.05$) the total displacement, with the increase approaching twofold. These increases in total displacement are the result mainly of an increase in phase II displacement (Table 4), which is the displace-

TABLE 1. *Effect of aflatoxin and ochratoxin on tibial diameters*

Mycotoxin	Dose (μg/g)	Diam (mm)
Aflatoxin	0.0	5.3 ± 0.2[a]
	0.625	5.2 ± 0.1[a]
	1.25	5.3 ± 0.1[a]
	2.5	5.3 ± 0.1[a]
	5.0	4.8 ± 0.1[b]
	10.0	4.5 ± 0.2[b]
Ochratoxin	0.0	4.7 ± 0.1[a]
	0.5	4.8 ± 0.1[a]
	1.0	4.5 ± 0.1[a]
	2.0	4.4 ± 0.1[a]
	4.0	3.5 ± 0.2[b]
	8.0	2.5 ± 0.1[c]

[a, b, c] Values for aflatoxin or ochratoxin with different superscripts differ significantly ($P < 0.05$). Values are mean ± standard error of four groups of 10 birds.

TABLE 2. *Effect of dietary aflatoxin and ochratoxin on bone stress at failure*

Mycotoxin	Dose (μg/g)	Stress at failure (N/m^2) × 10^6
Aflatoxin	0.0	63.3 ± 3.0[a]
	0.625	62.0 ± 1.6[a]
	1.25	65.4 ± 2.2[a]
	2.5	55.1 ± 1.9[b]
	5.0	55.4 ± 3.0[b]
	10.0	55.2 ± 2.2[b]
Ochratoxin	0.0	81.2 ± 5.2[a]
	0.5	71.9 ± 2.0[a]
	1.0	81.1 ± 3.9[a]
	2.0	73.0 ± 1.1[a]
	4.0	74.5 ± 1.9[a]
	8.0	75.5 ± 1.6[a]

[a, b] Values for aflatoxin or ochratoxin with different superscripts differ significantly ($P < 0.05$). Values are the mean ± standard error of four groups of chickens at 3 weeks of age.

TABLE 3. *Effect of aflatoxin and ochratoxin on total displacement*

Mycotoxin	Dose (μg/g)	Displacement (mm)
Aflatoxin	0.0	2.6 ± 0.3[a]
	0.625	2.7 ± 0.1[a]
	1.25	2.8 ± 0.1[a]
	2.5	2.9 ± 0.3[a]
	5.0	3.6 ± 0.2[b]
	10.0	4.1 ± 0.4[b]
Ochratoxin	0.0	2.5 ± 0.6[a]
	0.5	2.5 ± 0.3[a]
	1.0	2.4 ± 0.1[a]
	2.0	2.9 ± 0.1[a]
	4.0	4.8 ± 0.4[b]
	8.0	4.9 ± 0.6[b]

[a, b] Values for aflatoxin or ochratoxin with different superscripts differ significantly ($P < 0.05$). Values are mean ± standard error for four groups of 10 birds.

TABLE 4. *Effect of aflatoxin and ochratoxin on displacement from maximum force to failure*

Mycotoxin	Dose (μg/g)	Displacement (mm)
Aflatoxin	0.0	0.9 ± 0.2[a]
	0.625	1.0 ± 0.1[a]
	1.25	1.0 ± 0.2[a]
	2.5	1.3 ± 0.3[a]
	5.0	2.0 ± 0.3[b]
	10.0	2.7 ± 0.5[b]
Ochratoxin	0.0	0.7 ± 0.1[a]
	0.5	0.7 ± 0.1[a]
	1.0	0.7 ± 0.1[a]
	2.0	1.1 ± 0.1[a]
	4.0	3.3 ± 0.4[b]
	8.0	3.3 ± 0.6[b]

[a, b] Values for aflatoxin or ochratoxin with different superscripts differ significantly ($P < 0.05$). Values are means ± standard error for four groups of 10 birds.

ment or bending from the point of maximum force (start of failure) until complete failure. Aflatoxin increased this parameter threefold at the highest dose, whereas ochratoxin caused a fivefold increase. The toxic effects on displacement are easily visualized from idealized force diagrams (Fig. 3) for the breaking of bones from control and intoxicated birds. The illustration shows that tibias from birds fed toxin have shorter phase I displacement, lowered maximum force at failure, and a marked increase in phase II displacement.

In an effort to determine the role of bone diameter on its displacement, bones of the same diameter were grouped and their displacements from maximum force to complete failure were compared. The displacements of tibias of birds fed growth-inhibitory doses of either toxin were increased significantly ($P < 0.05$) over those of the tibias from control birds. A second type of grouping consisted of tibias within the same treatment but of different diameters. The displacement from maximum force to failure of small bones did not differ significantly from the displacement of large bones. While this approach indicated that the effect of the toxins on displacement of bones from maximum force to failure (Table 4) was independent of bone diameter, it required a selection of the experimental results with a consequent possible bias.

Another approach to the problem of bone flexibility and diameter was to calculate the modulus of elasticity of the bones, assumed to be solid cylinders, according to the formula (13): $E = 41{,}621\ (L^3 F_f)/(D^4 \delta)$; where E is modulus of elasticity (in Newtons per square meter), L is effective length (in centimeters), F is force at failure (in kilograms), D is diameter (in centimeters), and δ = phase I displacement (in centimeters). Therefore, E encompasses and is corrected for diameter although it describes events of phase I only. Table 5 shows the modulus of elasticity of bones from birds fed graded levels of aflatoxin and ochratoxin. Aflatoxin had no significant ($P < 0.05$) effect on the modulus of elasticity, whereas ochratoxin levels of 4 and 8 μg/g were effective. At 8 μg/g, the modulus was almost tripled, thus offering clear evidence that the bones from birds with severe ochratoxicosis are more brittle than normal.

Histopathological examination of sections of the epiphysis of bones from birds with aflatoxicosis and ochratoxicosis showed no abnormalities.

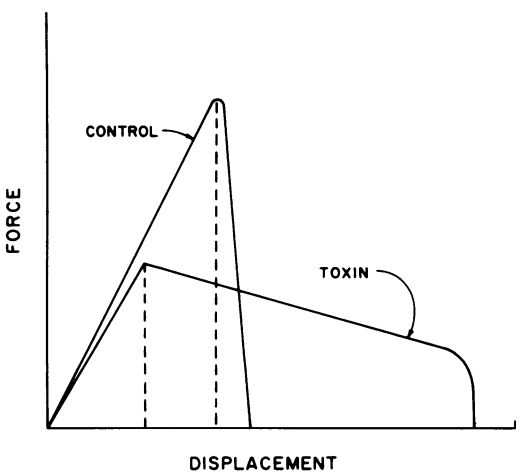

FIG. 3. *Idealized diagram of force and displacement associated with breakage of a bone from a typical control bird and from a typical bird fed toxin.*

TABLE 5. *Effect of aflatoxin and ochratoxin on the modulus of elasticity*

Mycotoxin	Dose (μg/g)	Modulus elasticity (N/m^2) × 10^8
Aflatoxin	0.0	4.70 ± 0.52[a]
	0.625	4.59 ± 0.16[a]
	1.25	4.52 ± 0.13[a]
	2.5	3.91 ± 0.08[a]
	5.0	4.70 ± 0.41[a]
	10.0	5.84 ± 0.76[a]
Ochratoxin	0.0	5.96 ± 0.65[a]
	0.5	5.75 ± 0.34[a]
	1.0	7.06 ± 0.52[a]
	2.0	6.58 ± 0.40[a]
	4.0	9.57 ± 1.55[b]
	8.0	16.16 ± 1.55[c]

[a, b, c] Values for aflatoxin or ochratoxin with different superscripts differ significantly ($P < 0.05$). Values represent the mean ± standard error of four groups of 10 birds.

DISCUSSION

It seems clear from these results that aflatoxin and ochratoxin have deleterious effects on bone properties. The breaking strength was decreased (Fig. 2), and the flexibility of the bones was increased (Tables 3 and 4). The question of whether these toxic effects act directly on the intrinsic properties of the bone or indirectly on a smaller bone produced by general growth retardation cannot be answered with quite as much certainty. The occurrence of decreased bone strength at dietary concentrations of the toxins too small to reduce the diameter of the bones (Fig. 2 and Table 1) seems clear evidence for a direct effect on intrinsic bone strength independent of diameter. This view was supported, in ochratoxicosis, by a decreased strength in selected bones of equal diameters. In addition, calculations of stress at failure which correct mathematically for differences in diameter revealed that this measure was decreased significantly at the dietary concentrations of aflatoxin which caused a decrease of breaking strength. However, ochratoxin had no effect on stress at failure. Nevertheless, the preponderance of the data support the view that the toxins have a direct effect on the intrinsic maximum force tolerated by the bones before breaking.

The role of aflatoxin and ochratoxin in the flexibility of bones is not entirely clear. Dietary ochratoxin but not aflatoxin caused an increase in the modulus of elasticity (Table 5). The elasticity referred to here is independent of diameter and is a measure of material stiffness before reaching maximum stress when phase II commences. Thus, the bones from birds with ochratoxicosis might be considered more brittle than normal. The increased rubberiness or flexibility noted in these experiments (Tables 3 and 4) is a phenomenon of phase II and does not appear until breakage or failure begins; in general, the rubberiness (increased displacement) is a manifestation of the breaking process. While the displacement, both total and from maximum force to failure, was dose related for the two toxins, the causal relationships are indefinite at this time. The possibility that the decreased diameters of the bones during ochratoxicosis and aflatoxicosis are determinants of the increased displacement cannot be dismissed completely even though bones of the same diameter from different treatments had different displacements and bones of different diameters from the same treatment had the same displacement. The possibility remains because the toxins altered the displacement only at doses that produced bones of reduced diameter. Attempts to correct mathematically for altered diameters have failed because of an insufficient theoretical framework; that is, the science of material properties has concentrated on phase I, and formulas for phase II have not been developed. The possibility that the increased displacement is a consequence of a direct effect on an intrinsic material property is more attractive even though differences in structure could not be detected in the histopathological examination.

The present experiments have yielded data indicating that the material properties of bones can be altered during mycotoxicoses. They have also indicated some methods that are applicable to such studies and indicated some areas where additional methods are needed. Obviously, there is a need for comparison of natural rachitic conditions with those known to be caused by mycotoxins so the role of mycotoxins in the leg problems confronting the animal industry can be assessed.

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LITERATURE CITED

1. **Bruning, J. L., and B. L. Kintz.** 1968. Computational handbook of statistics. Scott, Foresman and Co., Glenview, Ill.
2. **Doerr, J. A., W. E. Huff, H. T. Tung, R. D. Wyatt, and P. B. Hamilton.** 1974. A survey of T-2 toxin, ochratoxin and aflatoxin for their effects on the coagulation of blood in young broiler chickens. Poult. Sci. **53**:1728–1734.
3. **Eppley, R. M.** 1968. Screening method for zearalenone, aflatoxin, and ochratoxin. J. Assoc. Off. Agric. Chem. **51**:74–78.
4. **Hamilton, P. B.** 1977. Interrelationship of mycotoxins with nutrition. Fed. Proc. **36**:1899–1902.
5. **Hamilton, P. B., H. T. Tung, R. D. Wyatt, and W. E. Donaldson.** 1974. Interaction of dietary aflatoxin with

some vitamin deficiencies. Poult. Sci. **53**:871–877.

6. **Huff, W. E., and P. B. Hamilton.** 1975. Decreased plasma carotenoids during ochratoxicosis. Poult. Sci. **54**:1303–1310.
7. **Huff, W. E., R. D. Wyatt, and P. B. Hamilton.** 1975. Nephrotoxicity of dietary ochratoxin A in broiler chickens. Appl. Microbiol. **30**:48–51.
8. **Huff, W. E., R. D. Wyatt, T. L. Tucker, and P. B. Hamilton.** 1974. Ochratoxicosis in the broiler chicken. Poult. Sci. **53**:1585–1591.
9. **Hurwitz, S., and A. Meshorer.** 1973. Field rickets in turkey poults: plasma and bone chemistry, bone histology, intestinal calcium-binding protein. Poult. Sci. **52**: 1370–1374.
10. **Nabney, J., and B. F. Nesbitt.** 1965. A spectrophotometric method of bruising in the chicken. Poult. Sci. **50**: 795–800.
11. **Pons, W. A., A. F. Cuccullu, L. S. Lee, J. A. Robertson, A. O. Franz, and L. A. Goldblatt.** 1966. Determination of aflatoxins in agricultural products: use of aqueous acetone for extraction. J. Assoc. Off. Agric. Chem. **49**:554–562.
12. **Shotwell, O. L., C. W. Hesseltine, R. D. Stubblefield, and W. G. Sorenson.** 1966. Production of aflatoxin on rice. Appl. Microbiol. **14**:425–428.
13. **Singer, F. L.** 1951. Strength of materials. Harper Brothers, New York.
14. **Smith, J. W., and P. B. Hamilton.** 1970. Aflatoxicosis in the broiler chicken. Poult. Sci. **49**:207–215.
15. **Steyn, P. S., and K. J. van der Merwe.** 1966. Detection and estimation of ochratoxin A. Nature (London) **211**: 418.
16. **Thaxton, J. P., H. T. Tung, and P. B. Hamilton.** 1974. Immunosuppression in chickens by aflatoxin. Poult. Sci. **53**:721–725.
17. **Trenk, H. L., M. E. Butz, and F. S. Chu.** 1971. Production of ochratoxin in different cereal products of *Aspergillus ochraceus*. Appl. Microbiol. **21**:1032–1035.
18. **Tung, H. T., J. W. Smith, and P. B. Hamilton.** 1971. Aflatoxicosis and bruising in the chicken. Poult. Sci. **50**: 795–800.
19. **West, S., R. D. Wyatt, and P. B. Hamilton.** 1973. Improved yield of aflatoxin by incremental increases of temperature. Appl. Microbiol. **25**:1018–1019.
20. **Wiseman, H. G., W. C. Jacobson, and W. C. Harmeyer.** 1967. Note on removal of pigments from chloroform extracts of aflatoxin cultures with copper carbonate. J. Assoc. Off. Agric. Chem. **50**:982–983.