Further Characterization of Tissue Distribution and Metabolism of \([^{14}C]\)Aflatoxin B\(_1\) in Chickens\(^1\)

JOHN R. CHIPLEY, MICHAEL S. MABEE, KENNETH L. APPELAGE, AND MARK S. DREYFUSS

\(\text{Department of Poultry Science, Ohio State University, Columbus, Ohio 43210}\)

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The distribution and metabolism of \([^{14}C]\)aflatoxin B\(_1\) in chicken tissues were further investigated. Previously dried and frozen ethyl acetate extracts of liver, heart, gizzard, breast, leg, blood, and fecal samples were obtained from either layer or broiler chickens fed subclinical levels of \([^{14}C]\)aflatoxin B\(_1\). Treatment of these extracts with either carboxypeptidase A, leucine aminopeptidase, pepsin, or trypsin revealed that an average of 50% of the \(^{14}C\) detected in the acetate extracts was a liberated peptide (or amino acid) conjugate of \([^{14}C]\)aflatoxin B\(_2\). When a prepared standard of B\(_2\) was made by incubation of B\(_1\) with cold dilute aqueous HCl, the \(R_f\) values and absorbance maxima were identical with those of the tissue extracts after enzymatic treatment.

Aflatoxins, secondary metabolites produced by certain strains of \textit{Aspergillus}, are recognized as food and feed contaminants having worldwide significance. The ubiquitous nature of these organisms suggests a potential hazard as far as human and animal intoxications are concerned.

Results of earlier research (1-4, 14) have indicated that there was no transfer of aflatoxin to edible tissues of chicken fed diets containing crude aflatoxins. However, we have recently reported the distribution and metabolism of \([^{14}C]\)aflatoxin B\(_1\) in broiler (8) and layer (9) chickens. In these studies, we found that both broilers and layers excreted approximately 90% of the \(^{14}C\) administered by crop intubation daily for 14 days. Significant amounts of \(^{14}C\) were detected in the blood, liver, heart, gizzard, breast, leg, and feces. Treatment of aqueous extracts for conjugated steroids by treatment with beta-glucuronidase revealed that approximately 32% of the \(^{14}C\) detected in the aqueous extract was a liberated glucuronide conjugate of \([^{14}C]\)aflatoxin M\(_1\), with the remaining \(^{14}C\) being uncharacterized. Aflatoxin M\(_1\) was also recently found to be the major metabolite in studies of the excretion and metabolism of orally administered aflatoxin B\(_1\) in rhesus monkeys (5).

With the above results in mind, the present study was designed to further characterize the metabolites of \([^{14}C]\)aflatoxin B\(_1\) in poultry meat.

\textbf{MATERIALS AND METHODS}

\textbf{Chemical assays of extracts.} Previously dried and frozen ethyl acetate extracts, isolated from broiler (8) and layer (9) chickens, were thawed and pooled extracts of either liver, heart, gizzard, breast, leg, blood, or fecal samples were each redissolved in 30 ml of sodium acetate buffer (0.1 M, pH 4.5). These solutions were then divided into 6-ml fractions for subsequent treatment with different enzyme preparations.

\textbf{Enzymatic treatment of extracts.} Carboxypeptidase A (EC 3.4.2.1), leucine aminopeptidase (EC 3.4.1.1), pepsin (EC 3.4.4.1), and trypsin (EC 3.4.4.4) were all obtained from Worthington Biochemical Corp., Freehold, N. J. Treatment of extracts using each of these enzymes was conducted according to procedures outlined by the manufacturer. Six milliliters of one of the above enzyme preparations plus 6 ml of indicated buffer and cofactors were added to each of the 6-ml fractions. For carboxypeptidase A, enzyme was added along with an equal amount of 0.025 M tris(hydroxymethyl)aminomethane buffer (pH 7.5) containing 0.5 M NaCl to the fractions. For leucine aminopeptidase, enzyme was added along with equal amounts each of tris(hydroxymethyl)aminomethane buffer (0.5 M, pH 8.5), MnCl\(_2\) (0.025 M), and MgCl\(_2\) (0.125 M) to fractions. For the pepsin assay, enzyme along was added to fractions. For trypsin assays, enzyme was added along with tris(hydroxymethyl)aminomethane buffer (0.046 M, pH 8.1) to fractions. All fractions were then incubated for 1 h according to procedures outlined by the manufacturer.

\textbf{Preparation of B\(_2\) standard.} It has been previously reported (6) that treatment of aflatoxin B\(_1\) with cold, dilute, aqueous mineral acid produces aflatoxin B\(_2\). This procedure was repeated in the present study with B\(_1\) (approximately 100 \(\mu\)g [dry weight]) being dissolved by mechanical agitation in 100 ml of 0.05 M HCl and incubated in darkness at 5 C for 16 h. Afterward, the solution was extracted with 3 \(\times\) 50 ml of chloroform and the pooled chloroform extract dried under vacuum. The dried material was then dissolved in 0.5 ml of absolute methanol and 10 \(\mu\)litters spotted on Silica Gel G-HR thin-layer chromatog-
phy (TLC) plates and developed according to procedures outlined by Ma bee and Chipley (8). Blue fluorescing spots corresponding to B2a were scribed and scraped from the plates with a vacuum zone collection apparatus (Brinkman Instruments, Westbury, N.Y.), eluted from silica gel with chloroform, and taken to dryness. The dried material was then redissolved in 1.0 ml of absolute methanol. \( R_f \) values and absorbance maxima were determined by the methods of Dutton and Heathcote (6) and compared with values reported by these authors for B2a.

**Extraction and characterization of fractions.** After incubation, each fraction was extracted with 3 \( \times \) 15 ml of chloroform. The chloroform extracts were then dried under nitrogen and stored at 5°C. The dried material was redissolved in 1.0 ml of absolute methanol and 10 \( \mu \)litters spotted on TLC plates and developed along with the B2a standard. Blue fluorescing spots corresponding to the standard were scribed and scraped from the plates, eluted with chloroform, dried, and \( R_f \) values and absorbance maxima in absolute methanol were determined. Afterward, 0.5 ml of the methanol solutions were reduced to dryness in liquid scintillation counting vials and the radioactivity was assayed according to the procedure outlined by Ma bee and Chipley (8).

**RESULTS**

**Synthesis of B2a.** Under the conditions reported in the present study, approximately 50% of aflatoxin B1 was converted to B2a when treated with cold dilute HCl as measured by spectrophotometric and TLC methods. Aflatoxin B1 had an \( R_f \) value of 0.45, whereas that of B2a was 0.18. The light absorption maxima at 228, 256, and 363 nm were identical to those reported for B2a by Dutton and Heathcote (6).

**Characterization of \([^{14}C]B2a\) recovered from excreta, organs, and tissues.** Enzymatic treatment of ethyl acetate fractions isolated from broiler and layer chickens with the enzymes listed above, followed by chloroform extraction, revealed that an average of 50% of the total radioactivity observed in the ethyl acetate fractions before enzyme treatment was now located in the chloroform extract. Values of about 40% liberation for the more specific peptide-hydrolyzing enzymes, carboxypeptidase A and leucine aminopeptidase, and 55% liberation for the less specific enzymes, pepsin and trypsin, were obtained for all of the excreta, organs, tissues, and blood fractions tested in the present study. TLC of chloroform extracts yielded faint bluish spots with \( R_f \) values of 0.18 to 0.20. When these spots were removed from TLC plates and concentrated, their absorbance maxima were identical to those described for B2a by Dutton and Heathcote (6). Subsequent liquid scintillation spectrometry of the eluted fluorescing spots revealed that approximately 90% of the radioactivity observed in the chloroform extract was confined to the isolated fluorescing material. A summary of the total distribution of radioactivity reported earlier for broiler (8) and layer (9) chickens, as well as of the results of the present study, is presented in Fig. 1.

**DISCUSSION**

The results obtained in the present study indicate that both broiler and layer chickens can metabolize the majority of aflatoxin B1 when administered at relatively low levels. Aflatoxin conjugates are the predominating form of metabolite produced. Absorbance maxima, \( R_f \) values, and radioactive-fluorescing spots on TLC are suggestive evidence that \([^{14}C]aflatoxin\ B1\) is metabolized to a major extent (see Fig. 1) to a peptide (or amino acid) conjugate of B2a and to a lesser extent to a glucuronide conjugate of M1. These metabolites are soluble in aqueous (sodium acetate) extracts. They were found in pooled samples of each of the biological tissues, organs, and excreta that were assayed in approximately the same ratios as shown in Fig. 1.

Patterson and Roberts (11) reported that livers of chicks, guinea pigs, and mice metabolized aflatoxin B1 into small amounts (5 to 10%) of M1, whereas the major metabolite (90%) was the aflatoxin hemiacetal B2a. Patterson (Abstr. Int. Union of Pure and Appl. Chem. Symp., p.

| Pooled Lyophilized Sample | Chloroform (10%) |
|---------------------------|------------------|
| Sodium Acetate (81%)      | Ethyl acetate    |
| Ethyl acetate dry         | Dissolve in sodium acetate and treat with enzymes. Extract with chloroform. |
| β-glucuronidase (31%)     | Carboxypeptidase A |
| Leucine aminopeptidase    | Pepsin           |
| Trypsin                   | Aflatoxin M1     |
| Aflatoxin B2a             |

**Fig. 1. Distribution of radioactivity in acetate and chloroform extracts. Percentages in parentheses indicate amounts of radioactivity recovered at that respective step. β-glucuronidase and aflatoxin M1 are data from Ma bee and Chipley (8, 9).**
7, 1972) has also shown that B$_{28}$, which is relatively harmless when taken orally, was an acute hepatotoxin whenever formed from B$_1$ by liver microsomal enzymes.

Pohland et al. (13) investigated the acid-catalyzed addition of water to the vinyl ether double bond of aflatoxin B$_1$. The hemiacetal (B$_{28}$) produced was biologically inactive to chicken embryos and tissue cultures at concentrations substantially higher than the minimal lethal dose of B$_1$. These authors also found that B$_{28}$ was highly unstable, probably as a result of its existence in a phenolate form.

Patterson and Roberts (12) reported that a nicotinamide adenine dinucleotide phosphate reduced form-linked cytoplasmic enzyme system of duck liver reduced aflatoxin B$_1$ to the cyclopentanol, aflatoxicol. Isolated liver microsomes were found to contain an enzyme that hydrates the vinyl ether double bond of aflatoxin to form its hemiacetal (B$_{28}$). They further stated that yields of hemiacetal were difficult to assess because of strong protein (or amino acid) binding, which probably involves the formation of Schiff bases with free amino groups. Aflatoxin itself and aflatoxicol readily bound to protein (or amino acids) less readily than was the hemiacetal. This would account for the conjugated metabolites found in the presently reported study.

Implications. The conjugated metabolites reported in this study are examples of the "detoxication" process described by Harper (7). In this process, toxic substances (aflatoxin B$_1$) are converted into nontoxic forms (aflatoxin conjugates of B$_{28}$ and M$_1$) that are most efficiently removed by excretory routes by tissues. He also stated that conjugation might be accomplished by the combination of the metabolite to a variety of compounds including amino acids, glucuronic acid, "active" sulfate, and acetate. This appears to be the case in the present study.

Conjugated aflatoxins can be liberated by animal systems in the presence of the appropriate enzyme. Since aflatoxins M$_1$ (8, 9) and B$_{28}$ were successfully liberated from conjugates with liver, stomach, and pancreatic enzymes in vitro, a similar reaction could take place in tissues of animals administered aflatoxin conjugates. The liberated or unconjugated aflatoxin would probably then undergo reconjugation as a part of the detoxication process in an animal's system, resulting in possible deposition in the animal's tissues.

In a recent report, Patterson (10) has reviewed the role of metabolism as a factor in determining the toxic action of aflatoxins in different animal species. He stated that once toxin has entered liver cells, the agency causing tissue injury in a particular animal species is dictated by the rate and pattern of aflatoxin metabolism. When it is metabolized slowly, untransformed toxin is probably the active molecular species with chronic liver damage the probable result. When it is metabolized rapidly, metabolites rather than original toxin appear to be involved. He also reported that acute liver damage may be caused by the intracellular formation of aflatoxin hemiacetal (B$_{28}$) in many species.

Once again, the results of the present experiment indicate that classical nonpolar extraction procedures cannot be relied upon for the isolation and identification of aflatoxins in animal tissues.

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