Ammoniation of Aflatoxin B_1: Isolation and Characterization of a Product with Molecular Weight 206

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A second compound was isolated in pure form from a model system in which aflatoxin B_1 reacted with ammonium hydroxide under elevated temperature and pressure. The compound was identified as dihydro-4-hydroxy-6-methoxyfuro[2,3-*b*]benzofuran, mol wt 206, mp 145–146°C, λ_{max}(MeOH) 205, 227 (sh), 269, and 278 (sh) nm with 35000, 8130, 730, and 560, respectively. It is a nonfluorescent phenol which retains the difuran moiety but lacks both the lactone carbonyl and the cyclopentenone ring characteristic of aflatoxin B_1.

One of the most promising practical approaches to detoxifying mold-damaged oilseed meal is chemical inactivation of contaminated meals by treatment with ammonia gas under elevated temperature and pressure (Dollear, 1969; Masri et al., 1969). Gardner et al. (1971) reported that residual aflatoxins were reduced to less than 1 μg/kg by reacting moistened, contaminated cottonseed and peanut meals with ammonia gas at 40–59 psig for 30 min at 95–125°C. To better study the chemistry of the ammoniation process, Lee et al. (1974) utilized a model reaction system in which aflatoxin B_1 reacted with ammonium hydroxide at 100°C in a small Parr bomb. The major component of the chloroform-soluble fraction of the crude ammoniation product was identified as a nonfluorescent phenol of mol wt 286 (aflatoxin D_1) in which the lactone carbonyl moiety characteristic of aflatoxin B_1 was lacking. Kiermeier and Ruffer (1974) also reported that a compound of mol wt 286 with a structure identical with that proposed by Lee et al. for aflatoxin D_1 was produced by reacting aflatoxin B_1 with sodium hydroxide at 100°C. This article describes the isolation and characterization of a second major ammoniation product, a nonfluorescent phenol of mol wt 206 which is similar in structure to aflatoxin D_1, but lacks the cyclopentenone ring.

EXPERIMENTAL SECTION

Model reactions were conducted using 4-mg portions of aflatoxin B_1 with 2 ml of concentrated ammonium hydroxide in a small Parr bomb (22 ml) for 1 and 3 hr at 100°C. For each time interval, a total of 48 mg of B_1 was ammoniated and the crude reaction products lyophilized and fractionated into water and chloroform-soluble fractions (CSF), as described by Lee et al. (1974). Water and CSF represented 27 and 73%, respectively, of the crude product after 1 hr reaction, and 59 and 41% after 3 hr. Since previous data (Lee et al., 1974) showed that the water-soluble fraction contained only low molecular weight fragments (mass spectra) in the range of 57–157, the CSF were used for the isolation work.

Mass spectra of the CSF (Figure 1b) obtained by direct introduction probe into a CEC 21-110B mass spectrometer showed residual B_1 (mol wt 312), the previously identified aflatoxin D_1 (mol wt 286), masses of 229, 243, and 257, attributed to fragmentation of D_1 (Figure 1c), and a molecular ion mass 206 not present in spectra of either B_1 (Figure 1a) or D_1 (Figure 1c). Isolation and purification of this compound of mass 206 were attempted.

Isolation of Molecular Weight 206 Compound. The CSF was screened by high-pressure liquid chromatography (HPLC) with a Waters Associates Model ALC-202 instrument, fitted with a small particle (10 μ) porous silica gel column (Waters μ-Porasil, 4 mm i.d. × 30 cm long), using ACS chloroform (0.75% ethanol) as elution solvent at 1.0 ml/min flow rate, and uv detection at 254 nm. A typical chromatogram (Figure 2) shows four major components, one of which is aflatoxin D_1 (286), the mol wt 206 compound, and two other unidentified components. The CSF was partially purified by column chromatography on a mixed alumina–oxalic acid adsorbent (Steyn, 1970). The adsorbent, 60 g, was slurry packed in benzene into a column 22 mm i.d. × 500 mm long. Several grams of adsorbent were mixed with the CSF, the solvent was evaporated in a rotary evaporator, and the dry material was added at the top of the column. The column was eluted with benzene–chloroform (8:2, 1:1, 1:2.3), chloroform, and chloroform–methanol (1:1). Eluates were evaporated, dissolved in chloroform, spotted on plates coated with silica gel (Adsorbosil-1, Applied Science), and developed in chloroform–acetone (9:1 for ca. 40 min). Developed chromatograms were viewed under long-wave ultraviolet light to detect fluorescent compounds, and sprayed with a coupling reagent for visualization of phenols (Waldi, 1965), fast blue salt B (0.5% in water) and sodium hydroxide (0.1 *N*). With the coupling spray the phenol–aflatoxin D_1 turned pink whereas the new mol wt 206 compound turned a brilliant orange. The mol wt 286 compound was concentrated in the chloroform eluate and most of the mol wt 206 compound was eluted by the benzene–chloroform (1:1). The mol wt 206 compound was free of residual B_1, but was contaminated with several other faster moving components.

The mol wt 206 rich fraction from the column separation was further purified by preparative HPLC on μ-Porasil. The 206 peak (Figure 2) was harvested and rechromatographed twice to obtain approximately 4 mg of pure crystalline material used in the subsequent characterization of this compound.

Characterization of Molecular Weight 206 Compound. The mass spectrum of the preparation purified with HPLC showed an intense molecular ion mass 206 and fragments with masses 121, 134, 149, 163, 177, and 191 (Figure 1d), entirely different from the fragmentation pattern of aflatoxin D_1 (Figure 1c). The nonfluorescent compound melted sharply, 145–146°C (Kofler hot stage), and no nitrogen was detected by the sensitive microtechnique of Brown and Hoffpauir (1951). The ultraviolet absorption spectrum, λ_{max}(MeOH) 205, 227 (sh), 269, and 278 (sh) nm (ε 35000, 8130, 730, and 580, respectively), was different from that of aflatoxin D_1, λ_{max}(MeOH) 227 and 324 nm (ε 15920 and 12440, respectively). The infrared absorption spectrum, KBr (Figure 3), showed a strong band at 3370 cm^{-1}, indicative of free hydroxyl, and a band at 1635 cm^{-1}, attributable to a vinyl ether grouping. The

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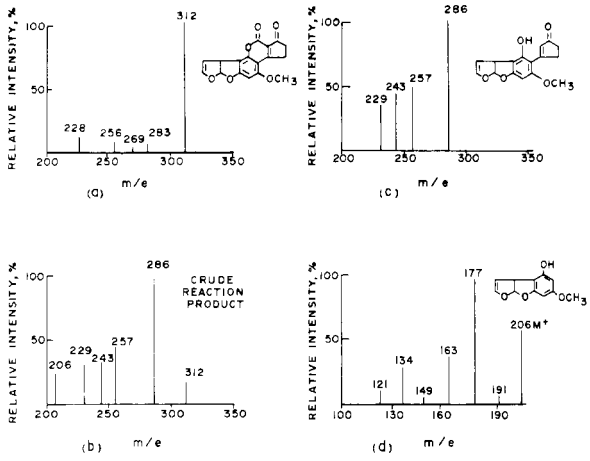

Figure 1. Relative intensities of peaks obtained from mass spectra: (a) aflatoxin B_1 (mol wt 312); (b) crude reaction product; (c) aflatoxin D_1 (mol wt 286); (d) mol wt 206 compound.

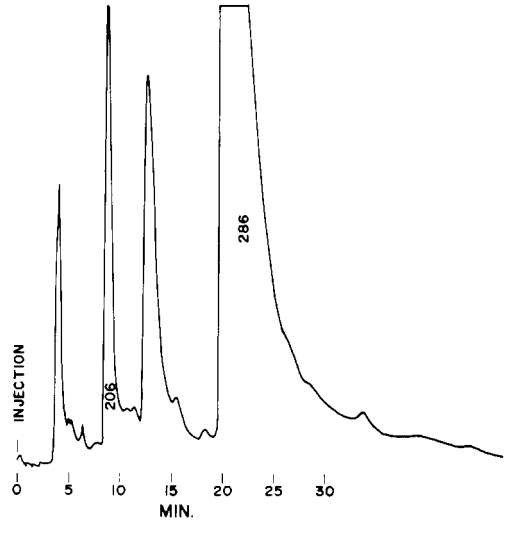

Figure 2. A typical HPLC chromatogram of the chloroform-soluble fraction.

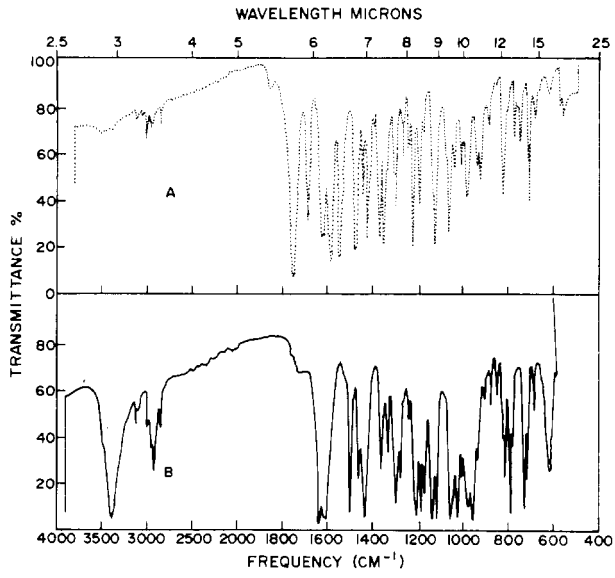

Figure 3. Infrared spectra (KBr): (A) aflatoxin B_1; (B) mol wt 206 compound.

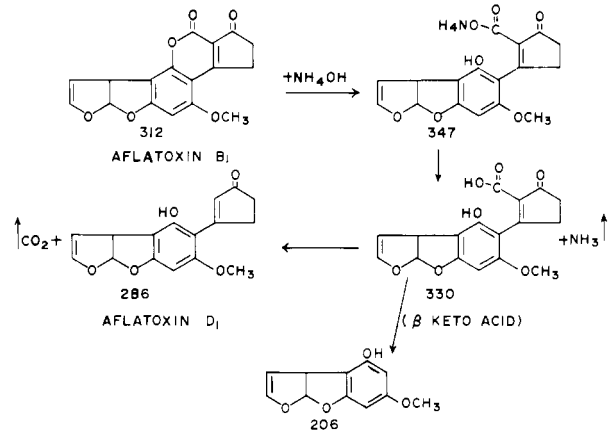

Figure 4. A proposed scheme for the ammoniation of aflatoxin B_1 to produce aflatoxin D_1 and mol wt 206 compound.

lactone carbonyl band at 1750 cm^{-1}, present in the aflatoxin B_1 spectrum (Figure 3), was absent in the spectrum of the mol wt 206 compound.

Phenolic hydroxide in the compound was further confirmed by preparation of an acetate (Stack et al., 1972) which had the correct mol wt 248 (mass spectra) for the presence of one hydroxyl group. The acetic acid addition derivative prepared from the acetate by reaction with acetic anhydride–hydrochloric acid (Pohland et al., 1970) had molecular weight 308 (mass spectra) indicating that the unsaturated difuran moiety of B_1 in the structure of mol wt 206 compound was retained. Nuclear magnetic resonance spectra were inconclusive, due to limited solubility of the compound in deuterated acetone or chloroform. However, signals were obtained at δ 6.9–6.3 (vinyl proton), 5.8 (ArH), and 3.7 ($-OCH_3$).

Knight et al. (1966) identified the compound tetrahydro-4-hydroxy-6-methoxyfuro[2,3-*b*]benzofuran, mol wt 208, from degradation of sterigmatocystin (a mold metabolite containing the furobenzofuran moiety). The physical data they reported [mp 153–154°C; λ_{max}(EtOH) 208, 227 (sh), 271, and 278 nm (ϵ 35600, 8400, 580, and 520, respectively); infrared absorption at 3350 and 1634 cm^{-1}; mass spectra molecular ion 208 with prominent peaks at masses 193, 179, 151, and 91] are similar to values reported here for the mol wt 206 compound. Their mol wt 208 compound is apparently the saturated analog of our mol wt 206 compound.

All of the experimental data indicate that the mol wt 206 compound produced by ammoniation of aflatoxin B_1 is dihydro-4-hydroxy-6-methoxyfuro[2,3-*b*]benzofuran; its structure is shown in Figure 1d. We propose that the compound arises from opening of the lactone ring of aflatoxin B_1 in the presence of ammonium hydroxide (Figure 4), followed by decarboxylation of the resultant β-keto acid to produce both aflatoxin D_1 and the mol wt 206 compound. Prolonged heating and pressure decrease the amount of aflatoxin D_1 produced while the formation of mol wt 206 compound, which lacks the cyclopentenone ring, increases.

Quantitative HPLC on a μ-Porasil silica gel column using purified mol wt 206 and aflatoxin D_1 for calibration, ACS chloroform (206) and 3% methanol in ACS chloroform (D_1) elution solvents, at a flow of 1.0 ml/min, and detection at 254 nm gave linear calibration curves, peak heights converted to absorbance vs. micrograms injected, over a range of 0.2–0.8 μg (D_1) and 0.2–1.6 μg (mol wt 206) (Figure 5). Application of the HPLC method to the CSF of the crude product from the 1- and 3-hr ammoniations indicated that this product contained 3% (206) and 11% (D_1) after 1-hr reaction, and 10% (206) and 19% (D_1) after 3 hr. Small scale ammoniations, 4 mg of B_1, over 0.5–8

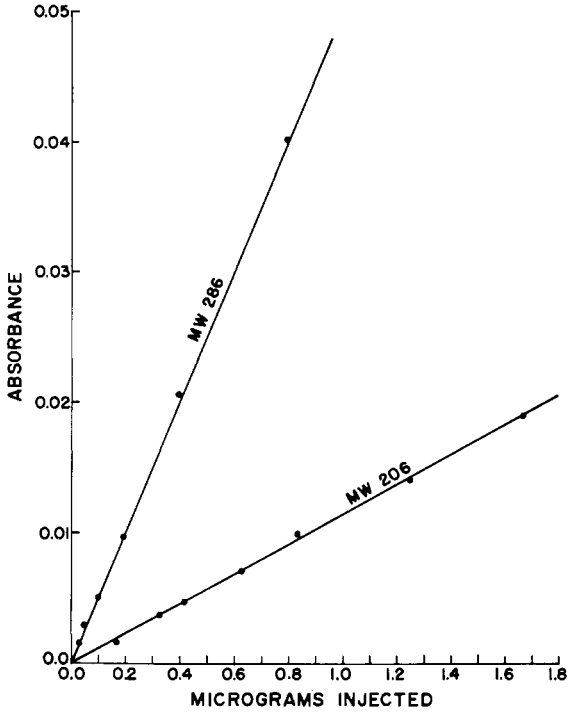

Figure 5. HPLC calibration curves for mol wt 286 and 206 compounds.

hr, indicated that the mol wt 206 compound gradually increased to 26% (8 hr), whereas aflatoxin D_1 reached a maximum of 23% (4 hr) and then gradually decreased to 3% (8 hr). The quantitative values reported here for aflatoxin D_1, mol wt 286, are not in good agreement with data obtained by Lee et al. (1974), who reported about 30% of D_1 in the crude product from 1-hr ammoniation of aflatoxin B_1. However, their data, obtained by absorptivity measurements on the chloroform-soluble fraction of the crude product, were undoubtedly subject to interferences.

ACKNOWLEDGMENT

We thank Joan W. Bennett for mold cultures used to prepare pure aflatoxin B_1, Elizabeth McCall for infrared spectra, Andrew G. Pierce and Gordon J. Boudreaux for NMR spectra, and Anthony O. Franz, Jr. for valuable technical help.

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Received for review July 7, 1975. Accepted October 20, 1975.

Rapid Screening Determination of Nitrate in Baby Food Using the Nitrate-Selective Electrode

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A nitrate-selective electrode was used to rapidly determine the nitrate nitrogen content of baby food. Interferences were eliminated by the use of two cation resins (Al and Ag). Of 53 samples analyzed, five (green beans, garden vegetables, spinach, squash, and beets) were over 20 ppm of nitrate nitrogen. The method is fast (15–20 min) but appears to read a few parts per million high for samples having a nitrate nitrogen content less than about 10 ppm.

The determination of nitrate in baby foods is important because infants are particularly susceptible to nitrate poisoning (Kamm et al., 1965). The current method (Kamm et al., 1965) is a colorimetric method requiring 2 to 3 days to do both nitrate and nitrite. The paper discusses the application of the nitrate-selective electrode method of Paul and Carlson (1968) to the analysis of baby food. The method reported here takes only about 10–20 min and was designed for use as a rapid-scanning method to identify samples over about 20 ppm of nitrate nitrogen. Such samples could then be analyzed by the more accurate method of Kamm et al. (1965).

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EXPERIMENTAL SECTION

Apparatus used included: nitrate ion electrode Model 92-07, Orion Research, Cambridge, Mass.; a pH meter with an expanded millivolt scale.

Samples. All baby food samples were Gerber's brand (an arbitrary choice) purchased at a commercial grocery. Samples were taken directly from the jar.

Reagents; Nitrate Standards. Knowns of 1, 4, 9, 18, and 45 ppm of nitrate nitrogen were made in 0.01 *N* KH_2PO_4 using sodium nitrate.

Resins. Both resins were Dowex 50W-X8 cation exchange resin. Conversion to the appropriate form was