Ammonium Hydroxide Treatment of Aflatoxin B_1. Some Chemical Characteristics and Biological Effects

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When a mixture of aflatoxin B_1 and ammonium hydroxide is stirred at room temperature for 21 hr, aflatoxin B_1 is obtained after acidification and chloroform extraction. When a similar reaction mixture is stirred at room temperature for 8, 10, 11, 14, and 18 days, drying on a rotary evaporator at 60° yields a brown product, which upon acetone extraction gives a mixture of substituted *o*-coumaric acid and aflatoxin B_1 as evidenced by infrared and ultraviolet spectroscopy. The brown residue is nontoxic in a chick embryo bioassay.

Agricultural commodities containing aflatoxin B_1 treated with ammonia for either possible chemical inactivation or degradation at ambient temperature or under pressure with heat have been reviewed by Goldblatt (1969). Lee *et al.* (1974) obtained aflatoxin D_1 as the major product from the reaction of aflatoxin B_1 and ammonium hydroxide with heat under pressure. Sargeant *et al.* (1961) treated aflatoxins isolated from peanut meal with 5 *N* ammonium hydroxide and reported no toxicity in the chloroform extracts. Masri *et al.* (1969) treated agricultural commodities containing aflatoxins with ammonia at ambient temperatures; he showed commodities treated in this manner were nontoxic to 1-day-old ducklings and the assay procedure employed showed a substantial decrease in aflatoxin B_1. However, further work was not initiated to establish if the loss of toxicity resulted from either formation of an ammonium salt of the aflatoxins which was not extractable by the assay procedure used or whether other transformation products were formed.

In continuing studies we have some observations to report on the chemical action of ammonium hydroxide on aflatoxin B_1 at room temperature *in vitro*. We developed biological data, using chick embryo bioassay, about a brown reaction product that is produced.

REACTION OF AFLATOXIN B_1 AND AMMONIUM HYDROXIDE

21-Hr Reaction. For 21-hr reaction aflatoxin B_1 (2.6 mg) was stirred at room temperature with 25 ml of concentrated ammonium hydroxide (17 *N*). The reaction mixture was acidified with hydrochloric acid and extracted with chloroform. The chloroform extract was washed with water and concentrated to dryness. Thin-layer chromatography (tlc), infrared (ir), ultraviolet (uv), and emission spectroscopy showed the residue (2.4 mg) to be aflatoxin B_1.

8-, 10-, 11-, and 14-Day Reactions. In a glass-stoppered erlenmeyer flask a mixture of 10.3 mg of aflatoxin B_1 and 10 ml of ammonium hydroxide (17 *N*) was stirred at room temperature in the dark. A clear yellow solution which resulted in 0.5 hr had an emission maximum at 475 nm (excitation 400 nm). After reaction times of 8, 10, 11, and 14 days, a 1-ml sample was taken to dryness in a rotary evaporator at 60°. The residue was triturated with acetone, and the ir spectrum of the acetone-soluble material recorded.

18-Day Reaction. To obtain more of the reaction products of aflatoxin B_1 and ammonium hydroxide, the following experiment was run. Aflatoxin B_1 (10 mg) was allowed to react with 10 ml of 5 *N* ammonium hydroxide in the dark at room temperature with stirring. After 18 days, the reaction mixture was taken to dryness on a rotary evaporator at 60° and gave 8.5 mg of a solid. Acetone extraction gave 4 mg of a white solid (A) and a residue of 4.1 mg of a brown solid (B). The ir spectrum of solid A was recorded.

Solid A was then treated with diazomethane for 10 min and taken to dryness on a steam bath before the residue was analyzed by tlc and by ir, uv, and emission spectroscopy. Solid B was tested for toxicity.

TOXICITY TEST OF BROWN SOLID

Solutions of aflatoxin B_1 were prepared in ethanol and dimethyl sulfoxide, whereas the brown solid (B) because of insolubility in ethanol was dissolved only in dimethyl sulfoxide. Air cells of fertile white Leghorn eggs were injected with 0.05-ml test solutions of solid B at levels of 0.31, 0.031, and 0.05 μg/egg according to the technique of Verrett *et al.* (1964). Noninjected controls and eggs injected only with solvent were used with all experiments to provide background mortality data. Eggs were incubated and candled from the fourth incubation day on. After 20 days, embryos were killed by exposing the eggs to ether; all embryos were removed and examined grossly for teratogenic effects. Embryos selected randomly were stained by the method of Dawson (1926) and these specimens again examined for teratogenicity.

APPARATUS

Ir spectra were determined on a Beckman IR-8 spectrophotometer as films deposited onto KRS-5 plates (Wilks), uv spectra were determined on a Unicam SP 800 spectrophotometer, excitation–emission spectra were determined on an Aminco-Bowman spectrophotofluorometer, and mass spectra were determined on a CEC-Dupont Model 12-491 mass spectrometer.

For tlc, glass plates were coated with a 0.50-mm layer of silica gel slurry and dried. The developing solvent was chloroform–acetone (90:10, v/v) in an unequilibrated tank. Spots were visualized under long-uv light and quantitated with a Schoeffel spectrodensitometer (Model SD-3000).

RESULTS AND DISCUSSION

Solutions of coumarin in ammonium hydroxide fluoresce green, a color that indicates formation of an ammonium salt. When the ammonium hydroxide solution is evaporated to dryness, coumarin is regenerated. Since the coumarin nucleus is part of the aflatoxin B_1 structure, comparable changes would be expected in B_1 on treatment with ammonium hydroxide. Coomes *et al.* (1966) have shown aqueous hydrolysis opened the lactone ring of aflatoxin B_1 to form a substituted *o*-coumaric acid which absorbs at 324 nm. Addition of base produces a bathochromic shift to 360 nm. The same spectral shifts were observed after 0.5-hr treatment of aflatoxin B_1 with am-

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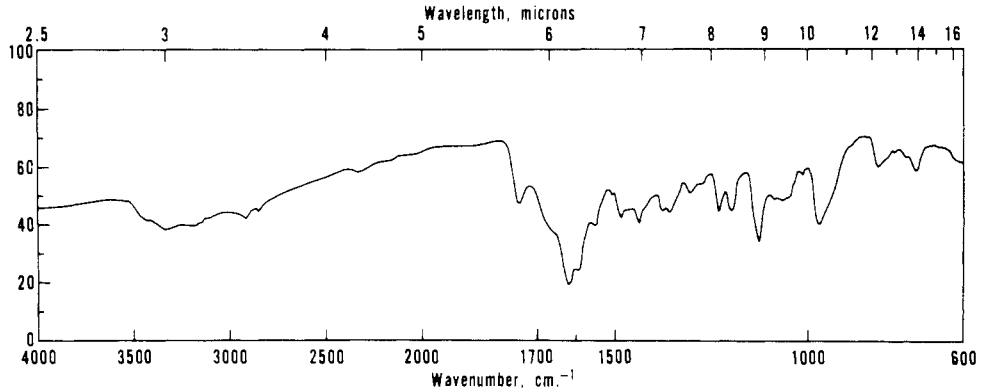
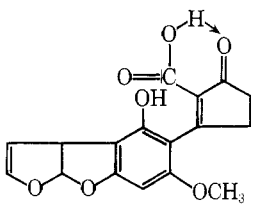


Figure 1. Infrared spectrum of acetone extract of residue from 8-, 10-, 11-, or 14-day reaction of ammonium hydroxide and aflatoxin B_1.

monium hydroxide (17 *N*). The resulting solution absorbed at 358 nm and on evaporation to dryness an absorption peak is observed at 324 nm. Acidification of an aflatoxin solution that had been reacted for 21 hr with ammonium hydroxide resulted in almost complete recovery of unchanged toxin as determined by tlc, ir, uv, and emission fluorescence. The sequence of spectral changes and recovery of aflatoxin B_1 are consistent with the reversible opening of the lactone ring by ammonium hydroxide.

Acetone extracts of the residue from evaporation of the aflatoxin B_1 and ammonium hydroxide reaction mixture gave identical ir spectra after 8, 10, 11, and 14 days. A typical spectrum is shown in Figure 1.

The medium band at 1750 cm^{-1}, the strong band at 1620 cm^{-1}, and absorption at 3320 cm^{-1} suggest that the ketone carbonyl in the cyclopentane ring of aflatoxin B_1 has undergone either a transformation (*e.g.*, formation of an imine) or an intramolecular association of the free acid with the carbonyl ketone as shown in structure **1**.



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To establish if structure **1** is correct, aflatoxin B_1 was allowed to react with 5 *N* ammonium hydroxide for 18 days. The reaction mixture when taken to dryness gave a solid. Acetone extraction gave a white solid (A), whose ir spectrum was the same as Figure 1, and mass spectral analysis showed peaks at 330, 312, 297, 285, and 267. These peaks are in agreement with structure **1**. Treatment of A with diazomethane then gave a product whose ir spectrum showed a new band at 1700 cm^{-1}, in addition to bands at 1750 and 1680 cm^{-1}. The methyl ester is unstable and the 1700-cm^{-1} band typical of an ester carbonyl rapidly disappeared and instead a broad band appeared at 1680 cm^{-1}. After the 1700-cm^{-1} band disappeared, the ir spectrum was equivalent to that of aflatoxin B_1. In addition the uv and emission spectra and tlc were indistinguishable from those of B_1. This series of observations indicates lactone ring opening in the presence of ammonia. Evaporation removes ammonia to give a mixture of the lactone and free acid. The free acid can then hydrogen bond to the ketone carbonyl of the cyclopentone ring of aflatoxin B_1.

The acetone-insoluble brown residue B, which is soluble in water and methanol, absorbs at 265 nm in uv and a broad plateau extending from 300 to 362 nm. When excited at 360 nm, product B has a fluorescence emission at 445 nm. Lack of volatility of residue B precluded mass spectral analysis. The toxicity of crude B was determined by the chick embryo bioassay. The LD_{50} reported by Verrett *et al.* (1964) for aflatoxin B_1 injected through the air sac is 0.025 μg/egg. Product B in dimethyl sulfoxide produced no mortality or teratogenic effects after 20 days at levels of 0.31, 0.031, and 0.005 μg/egg.

We have shown that when solutions of aflatoxin B_1 and ammonium hydroxide are stirred at room temperature for 18 days and taken to dryness on a rotary evaporator at 60°, approximately 50% of the total is isolated as product(s) nontoxic to the chick embryo. The other 50% is a mixture composed of some aflatoxin B_1 and of the substituted *o*-coumaric acid.

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Received for review July 8, 1974. Accepted November 18, 1974. The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.