Isolation and Structure of a Biologically Reduced Aflatoxin B_1

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A biologically reduced aflatoxin B_1 compound, produced by *Tetrahymena pyriformis* W from pure aflatoxin B_1, has been isolated and identified. The compound exhibited bright-blue fluorescence, and had an R_f of 0.52 on a silica gel tlc plate, as compared to 0.59 for aflatoxin B_1. It had a molecular weight of 314 by mass spectrometry; ultraviolet absorption λ_{max}^{MeOH} at 253, 261, and 328 mμ; and infrared absorptions at 3448, 1709, 1626, and 1606 cm^{-1}. The nmr spectrum was very similar to aflatoxin B_1 except for two additional signals. A multiplet at δ 2.40 assigned to a hydroxyl proton (confirmed by D_2O exchange), and a multiplet at δ 5.28, assigned to a methine proton attached to the same carbon as the hydroxyl. It was concluded that *T. pyriformis* W reduced the carbonyl in the cyclopentane ring of aflatoxin B_1 to a hydroxyl. The reduced aflatoxin appears to be identical to aflatoxin R_0.

The protozoan *Tetrahymena pyriformis* W is being used to assay the nutritional quality of peanut and cottonseed meals contaminated with aflatoxins in investigations being carried out to find means of inactivating the toxins. The effect of the aflatoxins on the organism and that of the organism on the aflatoxins was determined.

Teunisson and Robertson (1967) have reported that a culture of *Tetrahymena pyriformis* W, with or without nutrients, degraded aflatoxin B_1 (I of Figure 1) to a bright-blue fluorescent compound with a lower R_f (0.52) than B_1 (0.59) on thin-layer chromatographic plates. Recently, Detroy and Hesseltine (1968) reported the transformation of pure aflatoxin B_1 by *Dactylium dendroides* to a blue fluorescent hydroxylated compound. They assigned the name aflatoxin R_0 to the compound, and subsequently have determined its structure (Detroy, 1970). This report is concerned with the isolation of the reduction product (II of Figure 1) of aflatoxin B_1 produced by *T. pyriformis* W, and with the elucidation of its structure.

MATERIALS AND METHODS

Apparatus. Infrared spectra were measured in potassium bromide discs with a Perkin-Elmer spectrophotometer, Model 21. Ultraviolet spectra were determined in methanolic solution with a Beckman Model DK-2A spectrophotometer. Nuclear magnetic resonance (nmr) spectra were determined in deuterochloroform solution with a Varian A-60-A spectrometer, using tetramethylsilane as an internal reference. Mass spectrum analyses were made by the West Coast Technical Service, San Gabriel, Calif.

Thin-layer chromatography (tlc) was done on glass plates (20 $\times$ 20 cm) spread with 500 μ layers of Adsorbosil-1 silica gel. The developing solvent was chloroform:acetone:2-propanol (825:150:25). Spots were visualized under long-wave UV light.

Procedures. Aflatoxin B_1 was prepared by the procedure of Robertson *et al.* (1967). *T. pyriformis* W was propagated as previously described (Teunisson and Robertson, 1967) except that it was grown in 4-l. Fernbach flasks, 1 l. of broth each (two runs), or in 2.8-l. Fernbach flasks, 700 ml broth each (four runs). The depth of this amount of broth in each type of flask was about the same as that of 50 ml in the 300-ml De Long flask. Toxin was added in ethanol to four to six flasks in each run. The amount of inoculum was increased proportionately to the increased volume of broth. Approximately 2.0 mg of toxin in 20 ml ethanol was added to the 4/l. flasks and approximately 1.4 mg of toxin in 14 ml ethanol was added to the 2.8/l. flasks and incubated for approximately 30 hr. After incubation, the broth was extracted three times with chloroform. The combined chloroform extracts were concentrated under vacuum with a rotary evaporator. Then the mixture was chromatographed on a Merck silica gel (0.05–0.20 mm) column. The reduced aflatoxin B_1 was eluted with anhydrous ethyl ether, and the unchanged aflatoxin B_1 (50%), remaining on the column, was recovered by elution with 3% methanol in chloroform. The ethyl ether fractions containing the reduced aflatoxin were combined and concentrated with a rotary evaporator. The material was further purified by passing through a silica gel column (Merck, less than 0.08 mm) in which 20% methanol in benzene was the immobile phase and 1.75% methanol in benzene was the elution solvent (Robertson *et al.*, 1967). Based on tlc examination, the fractions containing the reduced aflatoxin were combined, concentrated with a rotary evaporator, and evaporated to dryness on a steam bath under a stream of nitrogen. The dried extract was dissolved in hot chloroform, and the compound precipitated by the addition of *n*-pentane. A white crystalline material was obtained by crystallization once from chloroform/hexane, m.p. 188°–194° (dec). A 30% yield of the conversion product was obtained for cultures incubated with aflatoxin B_1 for 30 hr.

RESULTS AND DISCUSSION

The cell population was less than previously obtained (Teunisson and Robertson, 1967). In the 4/l. Fernbach flasks, covered with polypropylene film, there were about 0.055 $\times$ 10^6 organisms/per ml. *After 1 day of incubation with* ethanol alone, or ethanol plus toxin, the estimated number per ml was about the same. In the 2.8-l. Fernbach flasks, also covered with polypropylene films, there were approximately 0.100 $\times$ 10^6 organisms/per ml (range 0.092 to 0.160 $\times$ 10^6 organisms per ml) in the 3-day old controls and in the cultures plus ethanol alone and alcohol plus toxin for 1 day.

On a silica gel thin-layer chromatographic plate developed with chloroform:acetone:2-propanol (825:150:25), the bio-

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logically reduced aflatoxin B_1 had an R_f of 0.52, as compared to an R_f of 0.59 for aflatoxin B_1 and 0.55 for B_2.

The molecular weight of the compound by mass spectrometry was 314. A peak at *m/e* 296 arising from the loss of a molecule of water from the parent molecule indicated the presence of a hydroxyl. In addition to these peaks, the compound had characteristic peaks at *m/e* 285, 281, 267, and 257 which were identical to the peaks of aflatoxin R_0 (Detroy, 1970). Reaction of the compound with acetic anhydride-pyridine which presumably gave an acetate which was less polar than aflatoxin B_1 or the reduced aflatoxin B_1 as shown by a higher R_f (0.85) on a tlc plate (developed with chloroform: acetone:2-propanol; 825:150:26) also indicated the presence of a hydroxyl.

The ultraviolet absorption spectrum of the compound exhibited λ_{max}^{MeOH} at 253, 261, and 328 mμ (ϵ 9,780; 10,790; 13,980) which was similar in shape to that of tetrahydrodesoxoaflatoxin B_1, a catalytic reduction product of aflatoxin B_1, which has λ_{max}^{MeOH} at 255, 264, and 332 mμ (Asao *et al.*, 1965).

The infrared spectrum (KBr disc) of the reduction product was similar to that of aflatoxin B_1, but it lacked the carbonyl absorption bands at 1760 and 1684 cm^{-1} possessed by B_1. It had an absorption band at 3448 cm^{-1}, which indicated that the compound contained a hydroxyl group, and strong absorption bands at 1709, 1626, and 1606 cm^{-1}, which also were very similar to the absorption of tetrahydrodesoxoaflatoxin B_1 (ν 1705, 1625, and 1610 cm^{-1}) (Asoa *et al.*, 1965). Detroy and Hesseltine (1969) observed that a shift in the infrared absorption bands from 1760 and 1684 cm^{-1} (B_1) to 1720 and 1610 cm^{-1} (R_0) and similar shifts in the infrared and ultraviolet spectra of tetrahydrodesoxoaflatoxin B_1 point to a reduction of the cyclopentane ring (C=O) to a (C—OH).

The nuclear magnetic resonance spectrum of the reduced aflatoxin B_1 (II) showed the following peaks: Olefinic proton H_a-triplet, δ 6.45; olefinic proton H_b-triplet, δ 5.48; H_c-multiplet, δ 4.77; H_d-doublet, δ 6.77; H_e-singlet, δ 6.37; (O-CH_3) protons (f)-singlet, δ 3.85; and H_g-multiplets, δ 3.27. These chemical shifts and splitting patterns of the protons were identical with those of aflatoxin B_1. In addition, the spectrum of aflatoxin B_1 had a multiplet at δ 2.65, whereas the reduced aflatoxin had changed in this part of the structure and had a signal at δ 2.23 (multiplet, H_h). One additional signal at δ 2.40 (multiplet, H_j) can be assigned to a hydroxyl proton and this was confirmed by exchange with D_2O; another at δ 5.28 (multiplet, H_i) may be assigned to a methine proton attached to the same carbon atom as the hydroxyl group. The chemical shifts and splitting patterns for these protons show the specific structure and orientation indicated in II of Figure 1. The above evidence indicates strongly that *T. pyriformis* W has reduced the cyclopentane ring (C=O) of aflatoxin B_1 to a (C-OH). The similarity of the ultraviolet, infrared, and mass spectra of the compound with the spectra of aflatoxin R_0 indicates that the two compounds are identical.

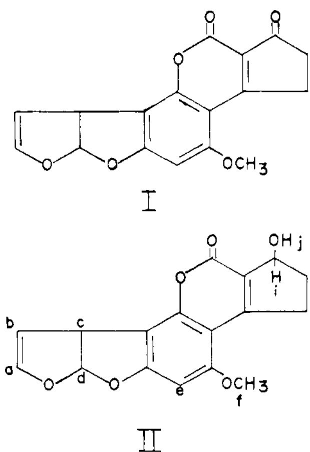

Figure 1. Structure of aflatoxin B_1 (I) and its reduction product (II) formed by ***Tetrahymena pyriformis*** **W**

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