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ISOLATION AND STRUCTURE OF AFLATOXINS M_1 AND M_2

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A toxic factor is excreted in the milk of cows fed aflatoxin containing groundnut meal[1]. de Iongh et al.[2] showed that the toxic factor is a blue-violet fluorescent compound which had an R_F value well below that of aflatoxin B_1. They also found that the lactating rat was able to convert aflatoxin B_1 into this 'milk toxin', while Butler and Clifford[3] have shown the presence of this component in the livers of rats given aflatoxin B_1. Allcroft et al.[4] presented chromatographic evidence that a compound excreted in the urine of sheep fed mixed aflatoxins was probably identical to the 'milk toxin' and suggested giving the compound the trivial name 'aflatoxin M'.

We have repeated the experiments of Allcroft et al. Two adult sheep (total weight, 67 kg) were each given intraperitoneally a dose (1 mg/kg) of mixed aflatoxins (B_1 73%, B_2 24%, G_1 2% and G_2 1%) dissolved in arachis oil (2 mg/ml). The urine collected during the 48 hr. after dosing was extracted continuously with chloroform for 36 hr. and the extract chromatographed on Merck's kieselgel. The material eluted with 2% methanol in chloroform contained aflatoxin M as shown by thin-layer chromatography using the systems described[4] by Allcroft et al. A pure concentrate (0.7 mg) of aflatoxin M was

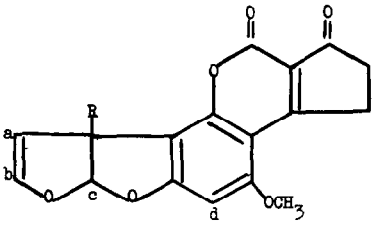
obtained by chromatography on Merck's kieselgel G chromatoplates using chloroform-methanol (97:3) as developing solvent. Chromatography of the concentrate on Whatman No. 1 filter paper impregnated with formamide-water (85:15) using ethyl acetate-benzene (9:1) as mobile phase gave two components; a blue-violet fluorescent substance (R_F 0.34) and a violet fluorescent substance (R_F 0.23) in the ratio of approximately 3:1. These components are designated aflatoxin M_1 (R_F 0.34) and aflatoxin M_2 (R_F 0.23).

de Iongh et al.[2] have presented chromatographic evidence that an extract of Aspergillus flavus culture on peanuts contained a component probably identical with aflatoxin M. We have extracted mouldy peanuts (7.5 kg) with hexane and then with acetone-water (86:14). The acetone extract contained aflatoxins B_1, B_2, G_1, G_2 as well as the unidentified blue-violet fluorescent component described by de Iongh et al. A concentrate of the latter substance was obtained by chromatography on formamide-impregnated cellulose powder (elution with chloroform) and then on Merck's kieselgel (elution with 2% methanol in chloroform). This concentrate (200 mg) was chromatographed on impregnated Whatman No.3 M M filter paper using the solvent system described above. A blue-violet and a violet fluorescent band at R_F 0.34 and 0.23 respectively were separately eluted (aqueous methanol), each yielding a pure crystalline compound. The compound with R_F 0.34 (11 mg) was shown to be identical with the aflatoxin M_1 extracted from urine. This was proved by paper and thin-layer chromatography, ultraviolet, infrared and mass spectra. Similarly, the compound with R_F 0.23 (4 mg) was identical with aflatoxin M_2.

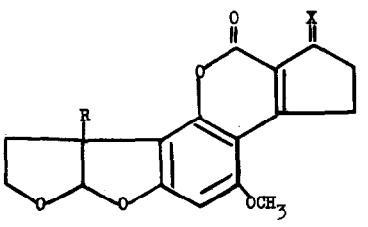
Aflatoxin M_1 (from methanol) had m.p. 299° (decomp.), $[\alpha]_D$-280° (c 0.1 in dimethylformamide)*, $\lambda_{max.}$ (ethanol) 226, 265 and 357 mμ (ε 23,100,

*Aflatoxin B_1 $[\alpha]_D$-480° (c 0.1 in dimethylformamide).

11,600 and 19,000 respectively) and was shown by microanalysis and mass spectrum to have the empirical formula $C_{17}H_{12}O_7$, one oxygen atom more than aflatoxin B_1 (I, R = H)[5]. The close resemblance of the ultraviolet spectral data with that of aflatoxin B_1 suggested that the main chromophoric system was similar. Its infrared spectrum (in nujol) resembled that of aflatoxin B_1 and had carbonyl peaks at 1760 and 1690 cm^{-1}. However, absorption at 3425 cm^{-1} indicated that aflatoxin M_1 contained a hydroxyl group. This was confirmed by reaction with acetic anhydride-pyridine (under conditions which left aflatoxin B_1 unchanged) which gave an acetate, mol. weight 370 (mass spec.), $\nu_{max.}$ (chloroform) 1760, 1740 (sh) and 1692 cm^{-1}, $\lambda_{max.}$ (ethanol) 226, 265 and 357 mμ.



(I)



(II)

The above evidence led to the conclusion that aflatoxin M_1 is hydroxy-aflatoxin B_1 and it was formulated as (I, R = OH) on the basis of its nuclear magnetic resonance spectrum. The significant peaks in the spectrum (measured in deuteriodimethylsulphoxide) are as follows: methoxy group, τ 6.02; olefinic proton H_a -doublet at τ 4.36 (J = 3 c./sec.); olefinic proton

H_b - doublet at τ 3.17 (J = 3 c./sec.); H_c and H_d singlets at τ 3.54 and 3.22. The fragmentation of aflatoxin M_1 in the mass spectrometer is consistent with the formulation (I, R = OH) and, except for at peak m/e 310 arising from the loss of a molecule of water from the parent molecule, is largely similar to that of aflatoxin B_1, but shifted 16 mass units higher.

Aflatoxin M_2 (from methanol-chloroform) had m.p. 293° (decomp.), $\nu_{max.}$ (chloroform) 3350 (b), 1760 and 1690 cm^{-1}, $\lambda_{max.}$ (ethanol) 221, 264 and 357 mμ (ε 20,000, 10,900 and 21,000 respectively), and mol. weight 330 (mass spec.). It was identical with dihydro-aflatoxin M_1, obtained by hydrogenation (10 min.) of aflatoxin M_1 (Pd/C in acetic acid). The infrared spectrum of this compound lacked the vinyl ether bands at 3100, 1067 and 722 cm^{-1} which are present in the spectrum of aflatoxin M_1. Aflatoxin M_2 is, therefore, formulated as (II, R = OH, X = O).

Aflatoxin M_2 is recovered unchanged on treatment with chromium trioxide in acetone, confirming the tertiary nature of the hydroxyl group. Prolonged hydrogenation of aflatoxin M_1 (Pd/C in acetic acid) gave tetrahydro-desoxy-aflatoxin M_1 (II, R = OH, X = H_2), m.p. 249-251° (from benzene), $\nu_{max.}$ (chloroform) 3350 (b), and 1707 cm^{-1}, $\lambda_{max.}$ (ethanol), 254, 263 and 328 mμ (ε 7,150, 8,000 and 13,000 respectively) and mol. weight 316 (mass spec.). Its nuclear magnetic resonance spectrum (in deuteriochloroform) showed peaks due to the acetal and aromatic protons (singlets at τ 3.65 and 3.79). Furthermore, the portion of the spectrum representing the six cyclopentene protons was identical in detail with the corresponding region in the spectrum of tetrahydro-desoxy-aflatoxin B_1(II, R = H, X = H_2), m.p. 248-249° (lit.[5] m.p. 249-250°).

Aflatoxin M in extracts of infected peanuts can be detected by

chromatography on Merck's kieselgel G chromatoplates employing chloroform-methanol (97:3) as the developing solvent. It can be quantitatively assayed by comparing the intensity of the fluorescent spot at R_F 0.4 with that of standard aflatoxin M. The fluorescence of aflatoxins M_1 and M_2 is approximately three times the intensity of aflatoxin B_1.

The LD_{50} values of aflatoxin B_1, M_1 and M_2 on day-old Pekin ducklings were determined simultaneously using Weil's[6] technique. The value for B_1 was 12 μg, for M_1 was 16.6 μg and for M_2 was 62 μg per day-old duckling.

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