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Antimicrobial Activity of Aflatoxins

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ABSTRACT

Antimicrobial activity of a crude aflatoxin preparation and of aflatoxin B_1 was studied. They were found inactive against common gram-positive and gram-negative bacteria at a concentration of 100 μg/ml. Both samples of aflatoxin did, however, exhibit antimicrobial activity, though narrow and limited, against various strains of *Streptomyces* and *Nocardia*. The antibiotic action of aflatoxin B_1 was confirmed by bioautogram after thin-layer chromatography. Among seven strains of microorganisms, including aflatoxin-sensitive and -resistant strains, *N. asteroides* IFM 8 was found to reduce aflatoxin B_1, in addition to other minor fluorescent components in the crude preparation.

Although numerous data concerning biological activity of aflatoxins (12, 13), the toxic secondary metabolites of *Aspergillus flavus*, have been thus far accumulated, little information is available on their antimicrobial activity. Burmeister and Hesseltine (3) reported that crude aflatoxin preparation incorporated into the growth substrate inhibited 12 species of the genus *Bacillus*, one species of *Clostridium*, and one of *Streptomyces* among the 329 microorganisms tested at a concentration of 30 μg/ml. The crude preparation before chromatography on an alumina column

TABLE 1. *Antimicrobial activity of crude aflatoxin and aflatoxin B_1*[a]

Organism	Minimal inhibitory concn (μg/ml)	
	Crude aflatoxin	Aflatoxin B_1
Bacillus subtilis PCI 219	75[b]	100
B. cereus ATCC 10702	100[b]	100
Streptomyces vinaceus IFM 1017	25	10
S. olivoreticuli IFM 1018	10	—
S. lavendulae IFM 1025	25	10
S. roseochromogenes IFM 1029	50	25
S. virginiae IFM 1028	25	7.5
S. halstedii IFM 1032	50	25
S. netropsis IFM 1035	25	7.5
S. aureofaciens IFM 1042	100	50
S. antibioticus IFM 1049	25	10
S. griseoluteus IFM 1055	25	10
Nocardia leishmanii IFM 27	100	50
N. asteroides IFM 1	50	10
N. asteroides IFM 2	25	10
N. asteroides IFM 3	25	10
N. asteroides IFM 8	>100	100
N. coeliaca IFM 30	25	—
N. rangoonensis IFM 23	>100	100
N. brasiliensis IFM 65	50	—

[a] Medium: glucose-nutrient agar for *Bacillus* and Sabouraud agar (1% glucose) for *Streptomyces* and *Nocardia*. Incubation: 27 C for 72 hr for *Streptomyces*, 37 C for 24 hr for *Bacillus*, and 37 C for 72 hr for *Nocardia*.

[b] Growth was retarded down by concentrations as low as 50 μg/ml.

was also found by Miyaki and Aibara (*personal communication*) to exhibit antimicrobial activity against *Bacillus* species. The active principle in the preparation, however, was removed as the purification of the toxin proceeded.

Most of carcinogenic four- or five-membered lactones are known to possess some antimicrobial activity. Aflatoxins are the first unsaturated, six-membered cyclic lactones which have been found to be carcinogenic, and studies on the antimicrobial activity of aflatoxins are of some interest.

This paper deals with the antimicrobial activity of aflatoxin and the reduction of the toxins by a strain of *Nocardia asteroides*.

Materials and Methods

Crude aflatoxin material was prepared as described by Asao et al. (2). *A. flavus* MOOI (ATCC 15517), a gift from G. N. Wogan, Massachusetts Institute of Technology, was grown in submerged culture in Adye's medium (1), and the active principle was extracted with chloroform and precipitated with petroleum ether. It was further purified by chromatography on an alumina column (activity grade 1, M. Woelm Eschwege, Germany). By developing the column with chloroform, aflatoxin B_1 was selectively eluted. The preparation thus obtained was 55% pure by calculation with the extinction coefficient, and no fluorescent contaminant was revealed by thin-layer chromatography.

All test strains were obtained from the culture collection of our laboratories. They were maintained in appropriate culture media sealed with mineral oil. Prior to use, test bacteria were grown on nutrient agar for 24 hr at 37 C; *Streptomyces* species were grown on glucose-asparagine-agar for 1 week at 27 C, and *Nocardia* species were grown on potato extract-agar (Kelner-Morton; 7) for 1 week at 37 C. The usual agar streak method was employed for the determination of antimicrobial activity. Methanol solutions of aflatoxin were added to the agar plates to give a solvent dilution of 1:20, which was not inhibitory to any of the test organisms. Growth from *Streptomyces* or *Nocardia* cultures was homogenized, when necessary, with glass beads to make a suspension. Media used and incubation periods are indicated in Table 1. Thin-layer chromatography of aflatoxins was run with a solvent system of chloroform-methanol-formic acid (95:5:1) on a 250 μ thick layer of Kieselgel G (E. Merck AG, Darmstadt, Germany). For detection of antimicrobial activity (bioautogram), the layer was dried to remove formic acid, laid upside down on a potato extract-agar plate seeded with a suspension of *S. virginiae* spores, and covered with sterile filter paper. After 20 min at room temperature, the plate was removed with the filter paper. The treated agar plate was incubated at 27 C for 18 hr. Results had to be read before heavy growth with sporulation of the organism obscured the inhibition zone. To prepare the cell suspensions for reduction studies, the organisms were cultured on a rotary shaker at 27 C for 3 days for *Streptomyces* and 1 week for *Nocardia*. *Escherichia coli* was cultured without shaking at 37 C for 48 hr. The medium contained: glucose, 5 g; starch, 5 g; meat extract, 5 g; Polypeptone (BBL), 10 g; NaCl, 3 g; and distilled water, 1 liter (*p*H 7.2). The cells were collected by centrifugation. They were washed three times with saline and resuspended in 0.067 M phosphate buffer of *p*H 6.0 and 7.0. To 35 ml of suspensions containing 0.87 ml of packed cells was added 2.0 ml of 50% methanol solution of aflatoxin crude preparation or aflatoxin B_1 to give final concentrations of 9 and 12 μg/ml, respectively. The suspensions were then incubated on a shaker in a water bath at 37 C. For spectrophotometric determination, a 5-ml sample was withdrawn, extracted with 10 ml of chloroform, and applied to a Hitachi spectrophotofluorometer (model EPU-2). Fluorescence at 425 mμ was determined by using an excitation wavelength of 365 mμ.

Results

One strain each of *Staphylococcus aureus*, *S. citreus*, *S. albus*, *Streptococcus faecalis*, *E. coli*, *Salmonella paratyphi A and B*, *Shigella dysenteriae* Shiga, *Serratia marcescens*, *Sarcina lutea*,

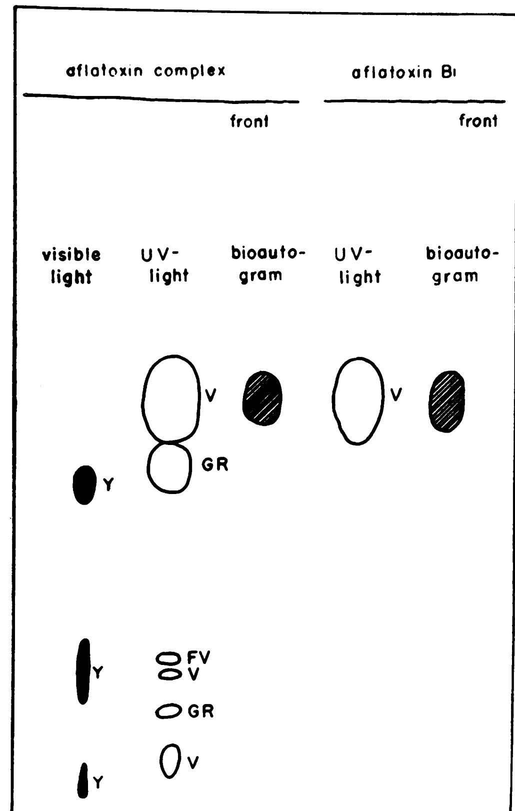

FIG. 1. *Comparison of chromatograms of crude aflatoxin as determined under visible and ultraviolet light, and by antimicrobial activity. Fluorescence: V, violet; FV, faint violet; GR, green; Y, yellow.*

and *Pseudomonas aeruginosa* was not inhibited with a concentration of up to 100 μg/ml of aflatoxins. As shown in Table 1, the crude preparation exhibited very limited antibiotic activity against *Bacillus subtilis* and *B. cereus*. Complete inhibition of *B. subtilis* was not obtained even at a concentration of 100 μg/ml of aflatoxin B_1, although growth was retarded at a concentration of 75 and 50 μg/ml. A similar effect on *B. cereus* was observed. Most test strains of *Streptomyces* and *Nocardia* were inhibited at a concentration of 25 to 50 μg/ml of the crude preparation. *Streptomyces olivoreticuli* was most sensitive, being inhibited at a concentration of 10 μg/ml. A comparable antimicrobial spectrum was obtained with purified aflatoxin B_1. *S. aureofaciens*, *N. asteroides* IFM 8, and *N. rangoonensis* were found to be resistant to both the crude preparation and B_1. The results of thin-layer chromatography as revealed by visible and ultraviolet light, and by bioautogram (antimicrobial activity), are presented in Fig. 1. Under visible light, the crude preparation had three yellow spots unrelated to those detected by other means. Under ultraviolet light, the preparation had several fluorescent spots in addition to two major spots, a violet fluorescent spot at the same height as B_1 and a green fluorescent one which is presumably G_1. By antimicrobial detection, both the crude preparation and B_1 had single inhibitory spots of an R_F value corresponding to that of B_1.

The fate of aflatoxins in the washed-cell suspensions of four resistant and three sensitive organisms was examined by thin-layer chromatography (Fig. 2). Complete disappearance of characteristic fluorescent spots was observed only with *N. asteroides* IFM 8; no change of pattern was noted with other organisms after 38 hr of incubation. The reduction of aflatoxin by *N. asteroides* IFM 8 was further followed quantitatively by fluorometric measurement and qualitatively by thin-layer chromatography and ultraviolet-absorption spectra. The results are shown in Fig. 3–7. Immediate quenching of fluorescence occurred both in *p*H 6.0 and in 7.0 buffers with

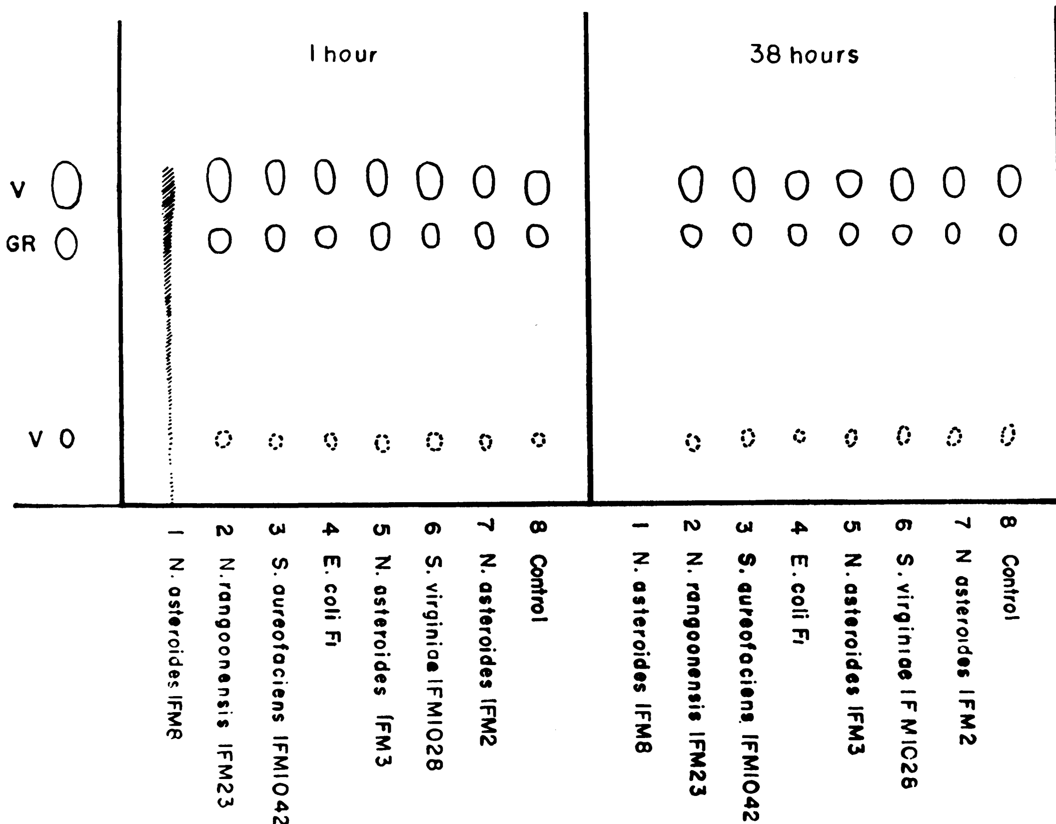

FIG. 2. *Reducing effect of Nocardia asteroides IFM 8 cells on aflatoxin shown by thin-layer chromatography. Fluorescence: V, violet; GR, green.*

the crude preparation. The relative intensity of fluorescence dropped to 20 and 14% of the original, respectively. It was also revealed by thin-layer chromatography that the spots corresponding to aflatoxin G_1 and minor fluorescent compounds disappeared first, whereas that of aflatoxin B_1 persisted for 24 hr at *p*H 6.0 and 6 hr at *p*H 7.0. This was also the case with aflatoxin B_1. On the other hand, the reduction of fluorescence was slow and not so significant with B_1 as with the crude preparation. Changes in the spectral patterns of the aflatoxin crude preparation and B_1 in cell suspensions at both *p*H values are shown in Fig. 6 and 7. Absorbance at 362 mμ was reduced with time, whereas the absorbance at 265 mμ increased at first and then gradually decreased. Characteristic absorption maxima at 265 and 362 mμ were lost after 12 hr. Consequently, the relative intensity of these two absorption maxima was reversed after the contact of *Nocardia* cells.

Discussion

The difficulty of determining the antimicrobial activity of a partially purified material like aflatoxins is evident when one considers that some antibiotics inhibit the growth of bacteria at the concentration of 6.0×10^{-8} μg/ml (xanthomycin, *Staphylococcus aureus*). Moreover, aspergilli of the flavus group are known to produce several antimicrobial agents, such as kojic acid (10), aspergillic acid (11) and other pyrazine compounds (5, 8), and flavacidin (9), making it necessary to use caution in attributing the antibiotic action to aflatoxin in the crude preparation. In our experiments, the antimicrobial activity of the toxin against various strains of actinomycetes increased as the preparation was

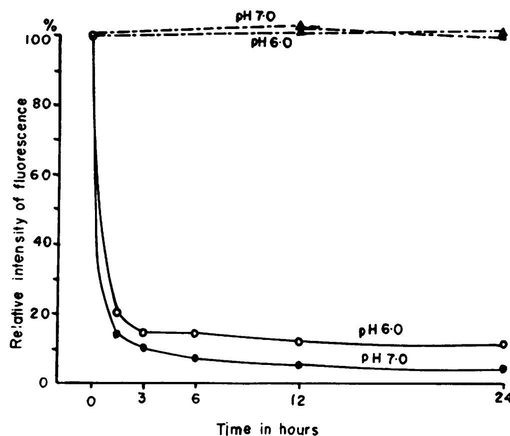

FIG. 3. *Rate of reduction of aflatoxin crude preparation by Nocardia asteroides IFM 8 as determined fluorometrically.*

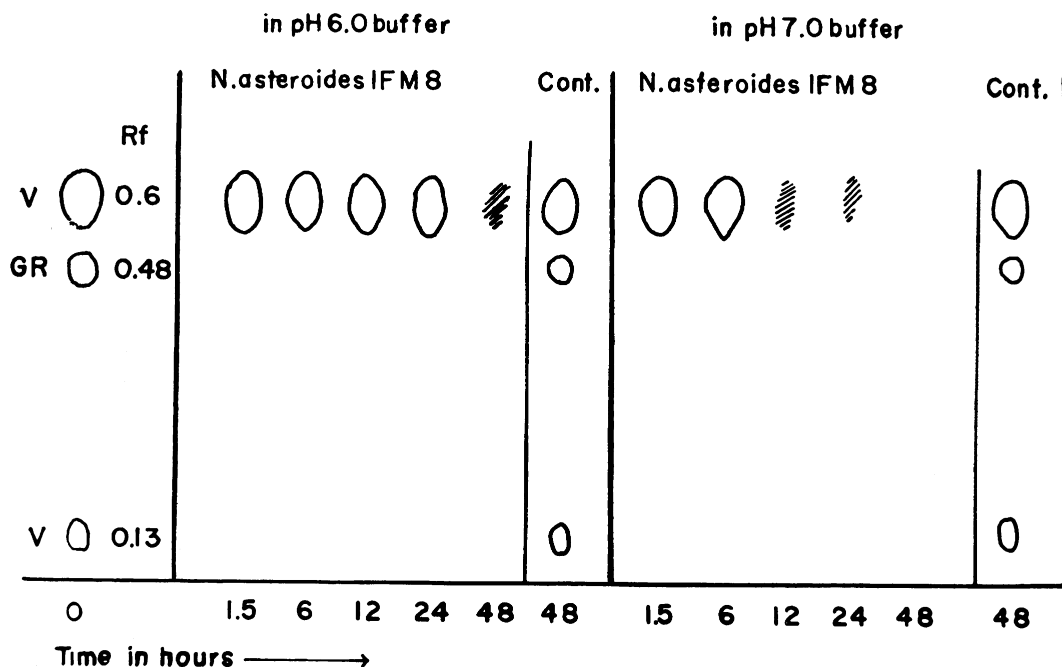

FIG. 4. *Reduction of aflatoxin crude preparation by Nocardia asteroides IFM 8 as revealed by thin-layer chromatography. Fluorescence: V, violet; GR, green.*

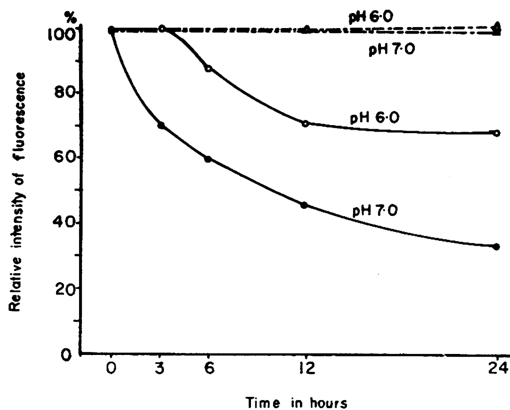

FIG. 5. *Rate of reduction of aflatoxin B_1 by Nocardia asteroides IFM 8 as determined fluorometrically.*

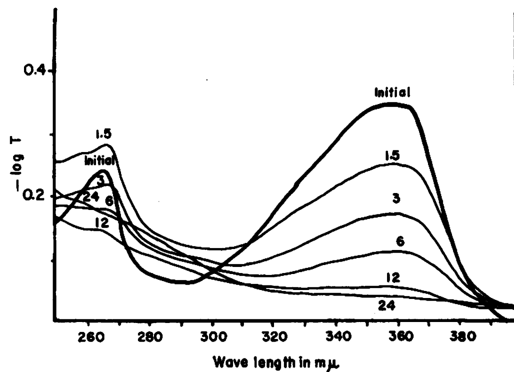

FIG. 6. *Changes in shapes of spectral patterns of aflatoxin crude preparation by Nocardia asteroides IFM 8 (pH 7.0).*

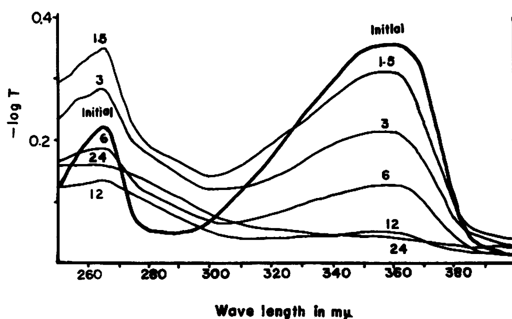

FIG. 7. *Changes in shapes of spectral patterns of aflatoxin B_1 by Nocardia asteroides IFM 8 (pH 7.0).*

further purified, patterns of antimicrobial spectra of crude preparation and purified B_1 were almost identical, and the location of the active principle on a thin-layer chromatogram corresponded exactly to that of aflatoxin B_1. This evidence supports the assumption that aflatoxin B_1 is the major antimicrobial principle in the preparation. However, other aflatoxins may have antimicrobial activity, but their amounts in the crude preparation are not large enough to be manifested by the bioautogram. The antimicrobial spectrum of aflatoxin is narrow and limited, inhibiting only various strains belonging to the family *Actinomycetaceae*. There are also some strains of *Streptomyces* and *Nocardia* which are resistant to the agent. Specific resistance of these strains, however, is mostly irrelevant to the production of degrading enzyme.

The reduction of aflatoxin by *N. asteroides* IFM 8 was more rapid and complete at neutral *p*H than at *p*H 6.0, and the disappearance of fluorescence occurred sooner with the crude preparation than with B_1. It is not yet clear whether this is due to the fact that fluorescent components other than B_1 in the crude preparation are much more sensitive to the microbial action or to the presence of some additional quenching agent in the crude preparation.

Since Geiger and Conn (6) reported the mechanism of antibiotic action of clavacin and penicillic acid, the antimicrobial activity of unsaturated lactones has been correlated to their reaction with sulfhydryl compounds. Dickens and Jones (4) studied the carcinogenic activity of a series of reactive lactones and formulated basic types of chemical structure in relation to carcinogenicity. Although selection of compounds for carcinogenic test was made on the basis of their antibiotic activity, the relationship between carcinogenicity and antibiotic action of these unsaturated lactones is not yet clear. As the compounds studied in their experiments were four- and five-membered ring lactones, the current data on aflatoxin at least provide some information on these two types of biological activity.

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