The Bioassay of Aflatoxins and Related Substances With Bacillus Megaterium Spores and Chick Embryos[1]

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Abstract

The "paper disc plate" method of antibiotic assay using *Bacillus megaterium* spores was tested for the assay of aflatoxins and related compounds. Significant inhibitions were obtained in the range of 1–4 μg/disc for aflatoxin B_1 and 4–8 μg/disc for aflatoxin G_1. Dicumarol was the most active of the compounds tested and inhibited at 0.1–1.0 μg/disc. Compounds were also tested using nine-day-old chick embryos. It was found that embryos incubated in egg cartons were much more sensitive to toxins than eggs incubated on cotton padding or in a commercial egg incubator. The LD_{100} of aflatoxin B_1 to carton-incubated embryos was 0.01 μg, while to embryos from the commercial incubator the LD_{100} was >5.0 μg. The results of the two bioassay procedures did not correlate completely with each other.

Introduction

THE NEED TO ASSAY for mycotoxins and related compounds becomes of prime importance when one considers that these compounds are capable of causing acute and chronic toxicity in animals. Chemical and physical methods depend upon a prior knowledge of the nature of a toxin and are generally useful only for specific types of compounds some of which may actually be nontoxic. Therefore, bioassay methods are indispensable since they evaluate true toxicity and are generally applicable to a wider range of compounds. Sargeant et al. (9) described a biological assay method for aflatoxins using day old Khaki Campbell ducklings. Armbrecht and Fitzhugh (2) worked out a similar bioassay method using Peking White ducklings. The latter authors reported an LD_{50} of aflatoxin of 1.40 mg/kg but observed liver damage after a single dose of 0.1 mg/kg. Platt et al. (8) described the use of five-day-old chick embryos for evaluating the toxicity of aflatoxin. They injected groundnut toxin through the air cell and recorded the death of the embryo as the indication of toxicity. Verrett et al. (11) standardized a chick embryo test using fresh White Leghorn eggs. They injected the toxin through the air cell or yolk sac, incubated the eggs for 20 days and compared the hatchability of the toxin-injected eggs with the control eggs. The LD_{50} of aflatoxin B_1 was reported to be 0.025 μg when injected through the air cell and 0.048 μg when injected through the yolk sac. Of other animal systems the use of calf kidney cells (6), cultured embryonic lung cells (7), and mollusc eggs (10) for the bioassay of aflatoxin have also been reported. It thus appeared that the chick embryo might be a simple and sensitive animal system for testing for toxic mold metabolites.

Surveying the effects of aflatoxin on microorganisms Burmeister and Hesseltine (3) demonstrated the sensitivity of various *Bacillus* species and Arai et al. (1) of *Streptomyces* and *Nocardia* species. Since microbiological methods are generally more rapid and easier to carry out than animal assay methods, it was decided to investigate the effect of aflatoxins and other compounds on *Bacillus megaterium* spores using the paper disc plate method of antibiotic assay (5) and compare the results of this microbial system with those of the chick embryo assay. Recently Clements (4), using this method, showed that 1 μg/disc of aflatoxin B_1 will inhibit the growth of *B. megaterium* spores.

Experimental Procedures

Chemicals and Reagents

The chemicals and reagents were obtained from the following sources: dicumarol, kojic acid (Sigma Chemical Co.); coumarin, 4-hydroxycoumarin, α-angelicalactone (Aldrich Chemical Co., Inc.); emodin, xanthotoxin (K & K Chemical Co., Inc.); penicillin G, streptomycin (Calbiochem); actinomycin D (Merck and Co., Inc.); chloramphenicol (Parke, Davis and Co.); dimethyl sulfoxide, propylene glycol (Fisher Scientific Co.); Tryptone, yeast extract (Difco Laboratories, Inc.); Trypticase Soy Broth (Baltimore Biological Laboratories); aflatoxin B_1, aflatoxin G_1, and crude aflatoxin (40.8% B_1, 16.3% B_2, 16.1% G_1, 1.1% G_2) (O. L. Shotwell, Northern Regional Research Laboratory, Peoria, Illinois); and warfarin (Wisconsin Alumni Research Foundation, Madison, Wisconsin).

Isolation of Bacillus megaterium spores

One liter of sterile Trypticase Soy Broth containing 70 μg/liter of $MnCl_2$ was inoculated with a 3-ml 24-hr culture of *B. megaterium* (University of New Hampshire Department of Microbiology strain) in Trypticase Soy Broth, and incubated at 38 C on a gyratory shaker (New Brunswick Scientific Co.) at 100 rpm for 48–72 hr. The optimum time for harvesting the spores was determined by microscopic examination of the culture broth at 12-hr intervals. When the sporulation appeared maximum, the broth was centrifuged at 12,000 × *g* for 20 min. The resulting pellet was resuspended in 100 ml of sterile water and recentrifuged under the same conditions. The spores were washed a total of four times to free them of nutrient broth. The spore pellet was then resuspended in 100 ml of sterile water and centrifuged at 500 × *g* for 20 min. The spores now remained in suspension and cell debris formed a pellet. The spore suspension was recentrifuged at 500 × *g* to remove further amounts of cell debris and the resulting spore suspension was then incubated for two days at 38 C on a gyratory shaker, after which, it was centrifuged again at 500 × *g* to further eliminate vegetative growth. The supernate was used for the assays. The spore count of the suspension was determined by the pour plate method and the final concentration was adjusted to 10^8 spores/ml.

[1] Presented at the AOCS-AACC joint meeting in Washington, D.C., 1968.

The spore suspension was stored at 5 C and was stable for over a year under these conditions.

Spore assay medium

The assay medium consisted of Tryptone (0.5 g), yeast extract (0.25 g), glucose (0.1 g), agar (1.0 g), and distilled water (to 100 ml). The pH was adjusted to 6.2.

Assay procedure

The assay medium (99 ml) was sterilized by autoclaving at 15 lb pressure for 15 min. The medium was brought to 80 C in a water bath and 1 ml of a spore suspension containing 10^8 spores added. After a period of 20 min at 80 C to activate the spores, 10-ml aliquots of the medium containing the spores were pipetted aseptically into sterile Petri plates and allowed to solidify. Stock solutions of the compounds to be tested were made in dimethyl sulfoxide (1 mg/ml) and diluted initially by factors of 10 to determine their approximate toxicities. The solutions were sterilized by membrane filters (Millipore Swinnex-13, Type HA). Sterile paper discs (Difco Concentration Discs, Sterile Blanks ¼ in.) were just touched to the surface of the test solution so that the discs absorbed the solution only by capillary action. The excess toxin solution adhering to the disc was removed by touching it to the side of the container. The discs absorbed 20 μl of the test solution consistently. Five discs of five different concentrations were placed in each plate and each plate was run in quintuplicate. After incubation at 25 C for 12 hr the diameters of the inhibition zones were measured. Results of replicate determinations generally varied between ±1 mm of the mean. Control discs containing 20 μl of dimethyl sulfoxide gave no inhibition.

Chick embryo test

Fresh White Leghorn eggs (University of New Hampshire poultry farm) weighing 60–65 g were incubated at 37 C under the following conditions prior to their use for assay: in a Thelco constant temperature incubator (Precision Scientific Co.) in the laboratory in egg cartons, the eggs being turned manually twice daily during the period of incubation; in the above incubator in enamel trays containing cotton padding, the eggs being turned manually twice daily; and in a Jamesway Model 252 commercial egg incubator where the eggs were turned automatically once an hour and humidity and temperature were automatically controlled.

The eggs were incubated for eight days and candled to detect defective eggs and to locate the air cell, which was marked with pencil on the outside of the shell. The air cell portion was surface sterilized with 90% alcohol and an opening of 1 mm diameter was drilled in the shell in this area without rupturing the membrane. In a sterile disposable syringe (Jelco 1 cc tuberculin) 50 μl of the toxin were injected slowly through the opening into the air cell and just above the inner membrane, 10 eggs being used for each level of toxin. The puncture was sealed with plastic cement and the eggs were allowed to sit in a vertical position for about 3 hr then incubated in a slanted position. One day after injection the eggs were candled to observe whether the embryos were still alive or not. In cases of doubt the eggs were opened and the activity of the embryos determined. The toxic level of toxin was determined as the level giving 100% mortality. Stock solutions of the toxins were made in propylene glycol (1 mg/ml) and diluted initially by factors of 10 to determine their approximate toxicities. Solutions were sterilized by membrane filters before injection. Propylene glycol (50 μl) when injected into groups of control eggs showed no toxicity.

TABLE I

Inhibitory Effect of Various Compounds on the Germination of *B. megaterium* Spores

Compound	Conc. μg/disc	Diameter inhibition zone (mm)
Aflatoxin B_1	1–2	15–20
Aflatoxin G_1	8	NI[a]
Aflatoxin Mix	4–8	14.5–19
Dicumarol	0.1–0.2	16–22
Coumarin	16	NI
Kojic Acid	16	NI
Xanthotoxin	4–16	14.5–14.75
Warfarin	10	NI
Penicillin G	0.2–2.0	12–20
Actinomycin D	0.2–2.0	17–22
Streptomycin	2.0	12
Chloramphenicol	0.2–2.0	12–22
Emodin	5–20	15–16.5
Benzoquinone	5–20	14.5–24.5
4-Hydroxycoumarin	5–20	NI
Oxalic Acid	10	NI

[a] NI = No inhibition.

Results and Discussion

Table I shows the effect of aflatoxins and related substances on the germination of *B. megaterium* spores. The inhibitory effects are compared with those of some of the standard antibiotics. Dicumarol inhibited at 0.1 μg/disc and was the most active of the compounds tested. Aflatoxin B_1 inhibited the germination of the spores at 1 μg/disc, agreeing with the value reported by Clements (4). Aflatoxin G_1 and aflatoxin mixture were less toxic than aflatoxin B_1 toward the spores. However, in the case of chick embryos, as shown in Table II, the aflatoxin mixture was about as potent as aflatoxin B_1 even though the mixture contained only 41% of aflatoxin B_1. In the chick embryo test dicumarol was not nearly as toxic as aflatoxin B_1. The effect of different methods of incubation on the sensitivity of the embryos is also shown in Table II. Eggs incubated in egg cartons in the laboratory were more sensitive than otherwise incubated embryos. In many cases the difference was over 100 fold. It may be that there are certain inhibitory principles in the egg carton that bring about a stress in the embryo resulting in the increased sensitivity. The hatchability of carton-incubated eggs was only 4% even though the embryos were alive up to 20 days, indicating a weakness in the embryos incubated under these conditions. A paper factor which inhibits the hatching of insect eggs has been described (12). If there is an in-

TABLE II

Toxicity of Various Compounds to 9-Day-Old White Leghorn Chick Embryos

Compound	Lethal dose (μg/egg)		
	A[a]	B[b]	C[c]
Aflatoxin B_1	0.01	>5.0	>5.0
Aflatoxin G_1	1.0	>2.5	>2.0
Aflatoxin Mix	0.01	5.0	>5.0
Dicumarol	1.0	>100	>100
Coumarin	1.0	>100	>100
Kojic Acid	0.1	>500	>500
Xanthotoxin	0.25	>100	>100
α-Angelicalactone	1000	.....	.....
Warfarin	.....	>100	.....

[a] A = Incubated in egg cartons in the laboratory.
[b] B = Incubated in trays in the laboratory.
[c] C = Incubated in automatic egg turning incubator.

hibitory factor present in egg cartons, its nature is not yet clear.

There appears to be no complete correlation between the results with the *Bacillus* spores and those with the chick embryos. This is not surprising since two entirely different systems are involved. Some toxins, such as aflatoxins, appear to be toxic to a wide range of biological systems including animal, plant and microbial, but others are much more restricted in action. Assays involving microorganisms are certainly desirable because they are generally rapid and convenient to carry out. However, since compounds toxic to higher animals are not always toxic to microorganisms, the use of microorganisms for the detection of substances toxic to higher animals has yet met with only limited success.

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