Effect of Mycotoxins and Coumarins on the Growth of *Bacillus megaterium* from Spores

Albert R. Buckelew, Jr.,[1] Ashima Chakravarti, William R. Burge, Victor M. Thomas, Jr., and Miyoshi Ikawa*

The growth of *Bacillus megaterium* from spores is inhibited by a variety of toxic fungal metabolites and related compounds. Most of the mycotoxins possessing a carbonyl function conjugated with a double bond system, such as α,β-unsaturated lactones, pyrones, and quinones, were inhibitory. The notable exception was rubratoxin B. Of a number of 3-substituted 4-hydroxycoumarins tested, only those possessing a 3 substituent terminated by a large group were highly inhibitory. A number of compounds which act as uncouplers of oxidative phosphorylation were found to be inhibitory. It is suggested that, among others, toxic fungal metabolites which possess an α,β-unsaturated carbonyl system or which act as uncoupling agents of oxidative phosphorylation may be detected by the use of *Bacillus* spores.

Burmeister and Hesseltine (1966) showed that various species of *Bacillus*, and in particular *Bacillus megaterium*, were among the most aflatoxin-sensitive of a large number of microorganisms tested, and the use of *B. megaterium* as a possible rapid bioassay system for aflatoxin B_1 has been suggested (Clements, 1968; Jayaraman *et al.*, 1968). The sensitivity of *B. megaterium* to ochratoxin (Clements, 1968; Shotwell *et al.*, 1969) and to several other mold metabolites (Jayaraman *et al.*, 1968) has also been demonstrated. It has also been demonstrated that several *Bacillus* species are not sensitive to all mycotoxins (Jayaraman *et al.*, 1968; Burmeister and Hesseltine, 1970). In order to assess the applicability of *B. megaterium* as a rapid screening organism for mycotoxins in general and in order to gain an insight into the types of chemical structures or the types of modes of action that this organism is sensitive to, the effects of a variety of toxic mold metabolites and related compounds on the growth of *B. megaterium* from spores was investigated. Bacteria are, of course, sensitive to a wide range of antibiotics, which may act by interfering with such processes as respiration, oxidative phosphorylation, and the synthesis of cell walls, proteins, purines, and pyrimidines. To what extent mycotoxins interfere with these same processes may determine the extent of *B. megaterium* sensitivity to these compounds.

METHODS AND MATERIALS

Assay Procedure. The *Bacillus megaterium* spores and the procedure previously described were used (Jayaraman *et al.*, 1968). Sterile paper discs (Difco) 0.25 in. in diameter were used. Substances were dissolved in dimethyl sulfoxide (DMSO) and 20 μl of solution was absorbed per disc. Assays were run at 25°C for 15–21 hr instead of 12 hr, as previously described, because the zones of inhibition became much more discernible during the longer incubation period. Readings at 21 hr were consistent with readings at 15 hr. However, on comparing these results with those previously reported for 12 hr, ease of reading the zones seems to have been obtained at the sacrifice of some sensitivity, especially to dicumarol.

Chemicals. Dicumarol, gramicidins D and J, kojic acid, oligomycin, quinacrine hydrochloride, and L-thyroxine were obtained from Sigma Chemical Co., St. Louis, Mo.; aflatoxins B_1, B_2, G_1, G_2, carbonyl cyanide *m*-chlorophenylhydrazone, diacetoxyscirpenol, rubratoxin B, and valinomycin came from Calbiochem, Los Angeles, Calif.; emodin, *d*- and *l*-usnic acids, vulpinic acid, and xanthotoxin was obtained from K&K Laboratories, Plainview, N.Y.; and emodin,

Department of Biochemistry, University of New Hampshire, Durham, New Hampshire 03824.
[1] Present address: Department of Biology, Bethany College, Bethany, West Virginia 26032.

Table I. Inhibition of Growth of *B. megaterium* from Spores by Toxic Mold Metabolites and Related Substances

Compound	Net diam. inhibition zone (mm)[a] Concentration (mg/ml in DMSO) 1	0.1	0.01
(a) Unsaturated lactones with the lactone carbonyl conjugated to a double bond system			
Aflatoxin B_1	24	7	0
Aflatoxin B_2	14	8	0
Aflatoxin G_1	9	Tr.	0
Aflatoxin G_2	Tr.	0	
Aflatoxin B_1A[b]	10	0	
Dicumarol	23	11	0
Ochratoxin A	14	7	0
Rubratoxin B	0		
Vulpinic acid	24	6	0
Xanthotoxin (8-MeO-psoralen)	10	Tr.	
Zearalenone (F-2 crystals)	11	0	
(b) Compounds (other than lactones) with a carbonyl conjugated to a double bond system			
p-Benzoquinone	8	0	
Emodin	5	0	
Kojic acid	0		
Rotenone	Tr.	0	
Santonin	0		
d-Usnic acid	28	16	6[c]
l-Usnic acid	28	17	7[c]
(c) Other compounds			
Diacetoxyscirpenol	Tr.		
Oxalic acid	0		
Penicillin G, K salt	8	4	0
Polyporenic acid A	14	4	0
Polyporenic acid C	0		

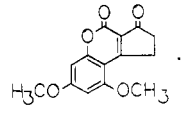
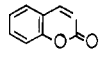
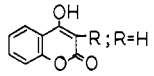
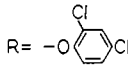
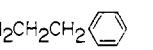
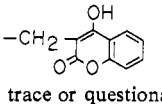
[a] Total diameter inhibition zone less disc diameter. Tr. = trace or questionable activity. (Net zone < 4 mm). [b] Synthetic aflatoxin B analog  . [c] No inhibition at 0.001 mg/ml.

Table II. Inhibition of Growth of *B. megaterium* from Spores by 4-Hydroxycoumarin Derivatives

Compound	Structure	Net diam. inhibition zone (mm)[a] Concentration (mg/ml in DMSO) 1	0.1	0.01
Coumarin		0		
4-Hydroxycoumarin (4-OHc)		0		
3-Methoxy- (4-OHc)	R=OCH_3	0		
3-Phenoxy- (4-OHc)	R= -O 	Tr.		
3-(2,4-Dichlorophenoxy)- (4-OHc)		18	0	
3-(*p*-Phenoxyphenethyl)- (4-OHc)	R= $-CH_2CH_2$ 	22	10	0
3-(γ-Phenylpropyl)- (4-OHc)	R= $-CH_2CH_2CH_2$	18	0	
3-(ω-Phenyloctyl)- (4-OHc)	R= $-(CH_2)_8$ 	13	10	4[b]
3-(Δ^8-Nonenyl)- (4-OHc)	R= $-(CH_2)_7CH=CH_2$	19	13	0
3-(α-Methyl-β-acetylethyl)- (4-OHc)	R= $-CHCH_2CCH_3$ (CH_3, O)	0		
3-(α-Phenyl-β-acetylethyl)- (4-OHc) (Warfarin)	R= $-CHCH_2CCH_3$ ( , O)	0		
3-(α-Phenyl-β-benzoylethyl)- (4-OHc)	R= $-CHCH_2C$ ( , O)	12	0	
Dicumarol	R= $-CH_2$ 	23	11	0

[a] Total diameter inhibition zone less disc diameter. Tr. = trace or questionable activity (net zone < 4 mm). [b] No inhibition at 0.001 mg/ml.

β-glycyrrhetinic acid, 4-hydroxycoumarin, and pentachlorophenol came from Aldrich Chemical Co., Milwaukee, Wis.

We are indebted to the following: T. G. Halsall, University of Oxford, England, for the polyporenic acid A; R. L. Harned, Commercial Solvents Corp., Terre Haute, Ind., for the nigericin; J. M. McGuire, Eli Lilly and Co., Indianapolis, Ind., for the monensin; C. J. Mirocha, University of Minnesota, St. Paul, Minn., for the F-2 crystals (zearalenone); J. V. Rodricks, Food and Drug Administration, Washington, D.C., for the aflatoxin B_1A; L. Stoloff, Food and Drug Administration, Washington, D.C., for the ochratoxin A; and Wisconsin Alumni Research Foundation, Madison, Wis., for the warfarin.

The coumarin derivatives listed in Table II have been previously described (Ikawa, 1948).

Isolation of Polyporenic Acid C. *Polyporus betulinus* fruiting bodies were collected from dead birch trees, *Betula papyrifera*, in the early fall and kept frozen at −20°C. Three kilograms of *P. betulinus* were thawed, cut into pieces, minced in a blender, and extracted twice with 3-l. portions of ethanol for a total of 4 days at room temperature. The residue was removed by filtration and 500 ml of water was added to the combined ethanol extracts. This mixture was kept at 4°C for 1 week. The insoluble material was collected by filtration, dissolved in 250 ml of ethanol, and sufficient 1 *N* NaOH was added to the solution to bring the pH to 11–12. The basic solution was extracted with ether to remove impurities. It was then made acidic (pH 1–2) with 1 *N* HCl, and the resulting mixture was again extracted with ether. The acid ether extract, which contained the polyporenic acids and other saponins, was taken to dryness on a rotary evaporator. Polyporenic acid C was isolated from the crude saponin mixture with Girard's reagent, using the method of Bowers *et al.* (1953). The sample of polyporenic acid A supplied by T. G. Halsall was further purified by recrystallization from ethyl acetate–hexane. Purification of polyporenic acids A and C was followed by thin-layer chromatography on silica gel G, using chloroform:methanol (85:15) as the solvent. The R_f values for polyporenic acids A and C in this system were 0.39 and 0.72, respectively. A spray for detecting steroids and triterpene acids consisting of 0.5% vanillin in H_2SO_4:ethanol (4:1) (Mathews, 1963) was used. Polyporenic acid A stained blue and polyporenic acid C stained pink with this reagent. Purified polyporenic acid A showed no uv spectrum, while polyporenic acid C showed absorption maxima at 234, 242, and 250 nm, agreeing with values reported in the literature (Bowers *et al.*, 1953).

Microscopic Examination of Germinating Spores. Samples (1-ml) of spore suspensions in 0.5% tryptone, 0.25% yeast extract, 0.1% glucose broth, pH 6.2, containing 10^8 spores were examined in the presence and absence of inhibitors under phase microscopy for changes in refractivity and appearance and for staining by methylene blue. Inhibitors were added at 100 μg/ml.

RESULTS AND DISCUSSION

The effect of a number of mycotoxins and related substances on the growth of *B. megaterium* is shown in Table I. Although other classifications are possible, the compounds have been grouped into (a) unsaturated lactones where the carbonyl group is in conjugation with a double bond system, (b) compounds (other than lactones) with a carbonyl group in

conjugation with a double bond system, and (c) other structures. A large number of mold metabolites contain the (a) and (b) structures (Shibata *et al.*, 1964) and a considerable number of mycotoxins have also been shown to possess these structures (Lillehoj *et al.*, 1970; Feuell, 1969). Judging from the results in Table I, most of the mycotoxins tested which possess the α,β-unsaturated carbonyl structure (a and b groups) inhibit the growth of *B. megaterium* from spores. Most of the compounds in these groups which were non or slightly-inhibitory are generally less toxic to animals. The notable exception was rubratoxin B. No explanation is apparent for the inactivity of this highly potent mycotoxin. Of the aflatoxins tested, B_1 and B_2 were the most active and G_2 displayed the weakest activity. Of fungal products possessing other structures (c), the antibiotic penicillin G and the fungal steroid polyporenic acid A inhibited *B. megaterium*. Diacetoxyscirpenol had trace activity at most under the conditions used. This latter result would tend to confirm the findings of Burmeister and Hesseltine (1970), who found several *Bacillus* species insensitive to the related T-2 toxin of *Fusarium*.

Since a number of the compounds in group (a) were coumarin derivatives and since an assortment of 3-substituted 4-hydroxycoumarins was readily available, the effect of these substances on the growth of *B. megaterium* was tested. The results are given in Table II. It is apparent that some of the compounds are very highly active and that those derivatives where the 3 substituent is terminated by a rather large group are the most active. The antibacterial activity of dicumarol has previously been reported (Goth, 1945) and other studies on 3-alkyl- and 3-acyl-4-hydroxycoumarins have indicated maximum antibacterial activity with a chain length of 10 carbon atoms (Stahmann *et al.*, 1948; Ukita *et al.*, 1951).

The modes of action of most of the compounds in Table I are still not known. The most thoroughly studied is penicillin, which acts as an inhibitor of bacterial cell wall synthesis. It is noted, however, that dicumarol (Martius and Nitz-Litzow, 1953), usnic acid (Johnson *et al.*, 1950), and polyporenic acid A (Whitehouse *et al.*, 1967), all three of which strongly inhibited the *B. megaterium*, have been described as uncoupling agents of oxidative phosphorylation. Polyporenic acid C, which had no inhibitory properties, is not an uncoupling agent (Whitehouse *et al.*, 1967) even though it is related structurally to polyporenic acid A. Consequently, a number of substances reported to be uncoupling agents of oxidative phosphorylation and several ionophorous antibiotics were tested (Table III). Here again a variation in response was obtained ranging from inactive, marginally active, to highly active. Most of the compounds did, however, show some activity. The inactivity of oligomycin under the conditions used might be explained by the fact that this compound also inhibits coupled respiration and acts at a different site in the phosphorylation process than uncouplers such as dinitrophenol or dicumarol (Lehninger, 1970). Therefore it appears that the *B. megaterium* assay will detect many mycotoxic substances whose mode of action is as uncoupling agents of oxidative phosphorylation. It also appears likely that some of the compounds in Tables I and II whose modes of action have not yet been determined function in this manner.

Examination of germinating spores in the presence of dinitrophenol, polyporenic acid A, sodium deoxycholate, carbonyl cyanide *m*-chlorophenyl hydrazone, and gramicidin D by phase-contrast microscopy and staining with methylene blue revealed that the initial stages of germination proceeded normally when compared with controls. The spores showed an initial loss of refractivity followed by uptake of stain, but in the presence of the uncouplers there was no elongation or outgrowth from the spores.

Table III. Inhibition of Growth of *B. megaterium* from Spores by Uncouplers of Oxidative Phosphorylation and Ionophorous Antibiotics

Compound	Ref[a]	Net diam. inhibition zone (mm)[b] Concentration (mg/ml in DMSO) 1	0.1	0.01
Carbonyl cyanide *m*-chlorophenylhydrazone	*c*	22	12	Tr.
Deoxycholic acid, Na salt	*d*	8	0	
2,4-Dinitrophenol	*c*	Tr.		
18β-Glycyrrhetic acid	*e*	18	6	0
Gramicidin D	*f*	Tr.	Tr.	
Gramicidin J	*f*	4	0	
Monensin, Na salt		24	18	9[g]
Nigericin, Na salt	*f*	23	16	6[g]
Oligomycins (15% A, 85% B)	*f*	0		
Oleic acid	*c*	0		
Pentachlorophenol	*c*	35	18	0
Quinacrine HCl (Atabrin)	*c*	0		
Salicylic acid	*c*	0		
L-Thyroxine	*c*	4	0	
Valinomycin	*f*	Tr.		

[a] Reference (secondary or primary source) to mode of action as an uncoupler of oxidative phosphorylation of one type or another. [b] Total diameter inhibition zone less disc diameter. Tr. = trace or questionable activity (net zone < 4 mm). [c] Weinbach and Garbus (1969). [d] Lee and Whitehouse (1965). [e] Whitehouse *et al.* (1967). [f] Gottlieb and Shaw (1967). [g] No inhibition at 0.001 mg/ml.

LITERATURE CITED

Bowers, A., Halsall, T. G., Jones, E. R. H., Lemin, A. J., *J. Chem. Soc.* 2548 (1953).

Burmeister, H. R., Hesseltine, C. W., *Appl. Microbiol.* **14,** 403 (1966).

Burmeister, H. R., Hesseltine, C. W., *Appl. Microbiol.* **20,** 437 (1970).

Clements, N. L., *J. Ass. Offic. Anal. Chem.* **51,** 611, 1192 (1968).

Feuell, A. J., "Aflatoxin," L. A. Goldblatt, Ed., Academic Press, New York, N.Y., 1969, p 187.

Goth, A., *Science* **101,** 383 (1945).

Gottlieb, D., Shaw, P. D., Eds., "Antibiotics I. Mechanism of Action," Springer-Verlag New York, New York, 1967, p 764.

Ikawa, M., Ph.D. dissertation, University of Wisconsin, Madison, Wis. (1948).

Jayaraman, A., Herbst, E. J., Ikawa, M., *J. Amer. Oil Chem. Soc.* **45,** 700 (1968).

Johnson, R. B., Feldott, G., Lardy, H. A., *Arch. Biochem. Biophys.* **28,** 317 (1950).

Lee, M. J., Whitehouse, M. W., *Biochim. Biophys. Acta* **100,** 317 (1965).

Lehninger, A. L., "Biochemistry," Worth Publishers, New York, N. Y., 1970, p 386.

Lillehoj, E. B., Ciegler, A., Detroy, R. W., "Essays in Toxicology," Vol. 2, F. R. Blood, Ed., Academic Press, New York, N.Y., 1970, p 1.

Martius, C., Nitz-Litzow, D., *Biochim. Biophys. Acta* **12,** 134 (1953).

Mathews, J. S., *Biochim. Biophys. Acta* **69,** 163 (1963).

Shibata, S., Natori, S., Udagawa, S., "List of Fungal Products," University of Tokyo Press, Tokyo, 1964.

Shotwell, O. L., Hesseltine, C. W., Goulden, M. L., *J. Ass. Offic. Anal. Chem.* **52,** 81 (1969).

Stahmann, M. A., Owens, J. H., Ikawa, M., Mahoney, C. L., Scheel, L. D., Bubl, E., Volpert, E., Abstracts 113th Meeting American Chemical Society, Chicago Ill., 17c (1948).

Ukita, T., Mizuno, D., Tamura, T., Yamakawa, T., Nojima, S., *J. Pharm. Soc. Jap.* **71,** 234 (1951).

Weinbach, E. C., Garbus, J., *Nature (London)* **221,** 1016 (1969).

Whitehouse, M. W., Dean, P. D. G., Halsall, T. G., *J. Pharm. Pharmacol.* **19,** 533 (1967).

Received for review June 1, 1971. Accepted September 23, 1971. This work was supported in part by a grant (CSRS-616-15-4) from the U.S. Department of Agriculture and presented in part at the Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., May 1969. Published with the approval of the Director of the New Hampshire Agricultural Experiment Station as Scientific Contribution No. 487.