Use of Chlorella in Mycotoxin and Phycotoxin Research

Miyoshi Ikawa, Daniel S. Ma, Gabrielle B. Meeker, and Robert P. Davis

Chlorella pyrenoidosa has been shown to be sensitive to a number of coumarin-related compounds, including aflatoxin. In a liquid system the growth of *Chlorella* was measured turbidimetrically and the concentration of toxin giving half-maximum growth determined. Aflatoxin B_1 was active at 4 μg. per ml. In an agar plate system paper disks containing test solutions were placed on agar plates uniformly seeded with *Chlorella* and zones of inhibition determined. Aflatoxin B_1 at 12.5 μg. per disk showed inhibition. By inoculating *Chlorella* agar plates with mold cultures, the production of toxic substances could be demonstrated by the appearance of zones of inhibition preceding the mycelial growth. Toxic substances present in mushrooms or in red and brown algae could be demonstrated by placing fresh specimens directly on the agar surface.

The toxicity of aflatoxin to a number of microorganisms has been studied. In general, most bacteria, fungi, and algae tested are relatively insensitive to the substance (Arai *et al.*, 1967; Burmeister and Hesseltine, 1966). Most sensitive of the bacteria tested were members of the genera *Bacillus, Clostridium, Streptomyces,* and *Nocardia.* To develop methods for the bioassay of toxic fungal and algal metabolites, studies were undertaken in the authors' laboratory on the effects of mycotoxins and related compounds on the growth of representatives of some of the major divisions of algae and classes of fungi (Ikawa *et al.*, 1967). The organisms studied included the blue-green alga *Lyngbya,* the green alga *Chlorella pyrenoidosa,* the euglenid *Euglena gracilis,* the phycomycetous mold *Rhizopus nigricans,* the ascomycetous mold *Aspergillus niger,* and the ascomycetous yeast *Saccharomyces cerevisiae.* Table I shows the inhibitory effects of several compounds on the growth of these organisms.

Lyngbya was much slower growing than the other organisms tried, and consequently the tests in which it was used took considerably longer. The *Rhizopus* and *Aspergillus* tests depended upon weighing the mycelia to determine growth and consequently were more tedious than the turbidimetric measurements used for the other organisms. Furthermore, since these three organisms seemed to show no particular advantages in terms of sensitivity to compounds, additional tests with these organisms were discontinued and further testing was done only with *Chlorella, Euglena,* and *Saccharomyces* (Ikawa *et al.*, 1967) (Table II). Although the list was limited in scope, it showed that coumarin-type compounds, as a group, seemed to exhibit a higher toxicity than the other types of compounds tested (aliphatic and aromatic acids, phenolic compounds, and γ-lactones). Of these three organisms, *Chlorella* appeared to be the most sensitive to the compounds in general and its possibilities for the detection and estimation of toxic mold metabolites have been further investigated.

EXPERIMENTAL

Materials. A sample of aflatoxin B_1 was supplied by Gerald N. Wogan, Massachusetts Institute of Technology, Cambridge, Mass., and a sample of ochratoxin A by S. Neshein, Food and Drug Administration, Washington, D.C. Toxin-producing strains of *Aspergillus flavus* (NRRL 2999), *Gibberella zeae* (NRRL 2830), and *Penicillium rubrum* (NRRL A-11,785) were supplied by C. W. Hesseltine, Northern Utilization Research and Development Division, Peoria, Ill. A strain of *Rhizopus nigricans* was isolated locally. A strain of *Fusarium tricinctum* was isolated from boiled rice exposed to the laboratory air. It was identified by W. F. O. Marasas, Department of Plant Pathology, University of Wisconsin, Madison, and has been shown to produce diacetoxyscirpenol (Jayaraman, 1967). An unidentified toxic fungus was also isolated from a mushroom. It assumes a white yeast-like growth or black mycelial growth, depending upon the growth medium. Mushroom species were gathered locally. Red and brown algae were collected from local coastal waters and tested on the same day.

Assay Organism. *Chlorella pyrenoidosa* (No. 252 from the Culture Collection of Algae, Department of Botany, Indiana University, Bloomington, Ind.) was used in these studies. The organism was maintained on agar slants consisting of the unbuffered medium containing 1% agar. It did not grow well on the surface of agar slants made up of the buffered high phosphate medium, even though the latter was suitable for the growth of the organism in liquid culture or within the buffered agar medium.

Unbuffered Liquid Culture. To 10 ml. of test solution containing graded concentrations of compound being tested were added 10 ml. of double-strength medium, which consisted of KNO_3 (1 gram), $MgSO_4 \cdot 7H_2O$ (0.25 gram), KH_2PO_4 (0.125 gram), K_2HPO_4 (0.125 gram), glucose (10 grams), and trace element

Department of Biochemistry, University of New Hampshire, Durham, N.H. 03824

Table I. Inhibition of Growth of Some Algae and Fungi by Several Compounds in Liquid Medium

(Mg. per ml. for half maximum growth)

Organism	Coumarin	Umbelliferone	Kojic Acid	Aflatoxin B_1
Lyngbya sp.	0.17	>0.5	0.92	
Chlorella pyrenoidosa	0.02	0.12	0.44	0.004
Euglena gracilis	0.12	0.33	>5.0	>0.01
Rhizopus nigricans	0.20	0.17	>5.0	>0.01
Aspergillus niger	0.26	0.31	>5.0	>0.01
Saccharomyces cerevisiae	0.19	0.50	>5.0	>0.01

Table II. Effect of Various Compounds on Growth of *Chlorella, Euglena,* and *Saccharomyces* in Liquid Medium

(μg. per ml. for half maximum growth)

Type	Compound	*Chlorella*[a]	*Euglena*	*Saccharomyces*
Acyclic compounds	Dimethyl sulfoxide	21,000	34,000	28,000
	Ethanol	14,000	16,000	32,000
	Oxalic acid[b]	>500[c]	160	400
	Fumaric acid	>500[c]	>500	>500
Aromatic acids and phenols	Salicylic acid	>500[c]	30	>500
	p-Hydroxybenzoic acid	>500[c]	300	>500
	Gentisic acid	>500[c]	>500	>500
	Resorcinol[b]	240	>500	>500
Saturated γ-lactones	γ-Butyrolactone[b]	>500	>500	>500
	γ-Valerolactone	>500	>500	>500
Unsaturated γ-lactones	α-Angelicalactone[b]	70	>500	230
	Gibberellic acid	>500[c]	>500	>500
Unsaturated aromatic δ-lactones	Coumarin	20	120	190
	7-Hydroxycoumarin	120	330	500
	4-Hydroxycoumarin	200	150	400
	Xanthotoxin	40	43	25
	Aflatoxin B_1	4	>10	>10
Other compounds	*p*-Benzoquinone[b]	250	50	50
	Santonin	>250	>250	>250
	Kojic acid	440	>500	>500

[a] Using unbuffered medium.
[b] Solutions sterilized by filtration.
[c] Solutions neutralized before testing.

solution (0.4 ml.) per 500 ml. The trace element solution consisted of 5 grams of $CaCl_2 \cdot 2H_2O$, 1 gram of $FeSO_4 \cdot 7H_2O$, 1 gram of $ZnSO_4 \cdot 7H_2O$, 100 mg. of $MnCl_2 \cdot 4H_2O$, 100 mg. of H_3BO_3, 10 mg. of NH_4VO_3, 10 mg. of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 10 mg. of $CoCl_2 \cdot 6H_2O$, 10 mg. of $CuSO_4 \cdot 5H_2O$, and 20 grams of Na citrate dihydrate per liter (Ikawa *et al.,* 1968). Since the buffering capacity of this medium is low, acidic substances were neutralized with sodium hydroxide before being tested. Failure to do so resulted in inhibition of growth due to low pH. After autoclaving, the system was inoculated with 0.1 ml. of a heavy suspension (absorbancy about 1.5 to 2) of *Chlorella.* After 2 to 3 days of growth at ca. 25° C. under constant illumination the absorbance of the culture medium was read at 650 mμ.

Buffered Liquid Culture. The procedure was the same as that described above, except that the KH_2PO_4 and K_2HPO_4 concentrations were increased to 2.5 grams each per 500 ml. of double-strength medium. Substitution of $NaH_2PO_4 \cdot H_2O$ for KH_2PO_4 did not affect the response of *Chlorella* to either coumarin or benzoquinone.

Chlorella-Agar Technique. Single-strength medium containing 2% agar and single-strength medium without agar were autoclaved separately and allowed to cool to 50° C. To the nonagar solution was added aseptically a thick-growing culture of *Chlorella* to an absorbancy (650 mμ) of ca. 0.6. It was then poured into an equal volume of the agar medium at 50° C. and the solutions were mixed and poured into sterile Petri dishes to cool. Plates were made up using both unbuffered and buffered media. The resulting plates are single-strength, contain 1% of agar, and are a very light yellow green in color. They were used on the same day that they were poured, so that the *Chlorella* growth was not excessive at the start of the experiment. The effects of any chance fungal or bacterial contaminants were also minimized by this procedure. Compounds and extracts were tested by making them up in dimethyl sulfoxide (DMSO). The solutions were sterilized by filtration through a Millipore filter unit. Fifty microliters of solution were applied to sterile, blank 1.3-cm. disks (Difco) and the treated paper disks placed on the agar surface. The plates were held at ca. 25° C. under constant illumination. After 2 to 3 days, inhibition zones appeared as colorless rings against a green background. DMSO did not exhibit any zones of inhibition. Therefore, many water-insoluble compounds could be tested at relatively high levels. Molds and yeasts were tested for the production of substances inhibiting *Chlorella* growth by inoculating the *Chlorella* agar plates with a piece of mycelium. The use of freshly prepared plates allowed fungal growth and toxin production to proceed before *Chlorella* growth became excessive. With organisms producing inhibitory substances, an inhibition zone in advance of the fungal colony appeared in 2 to 3 days. Fungi showing compact growth could be tested, but rapidly spreading organisms, such as *Rhizopus nigricans,* could not. The buffered medium tended to slow down fungal growth and any zones of inhibition could be more easily discerned under these conditions. Mushrooms and algae samples were tested by placing pieces of the organisms directly on the agar surface.

RESULTS AND DISCUSSION

Chlorella grows well at pH's between 3.7 and 7.5 (Nielsen and Willemoes, 1966). One of the factors studied was the buffering capacity of phosphate concentration and the effect of this concentration on the

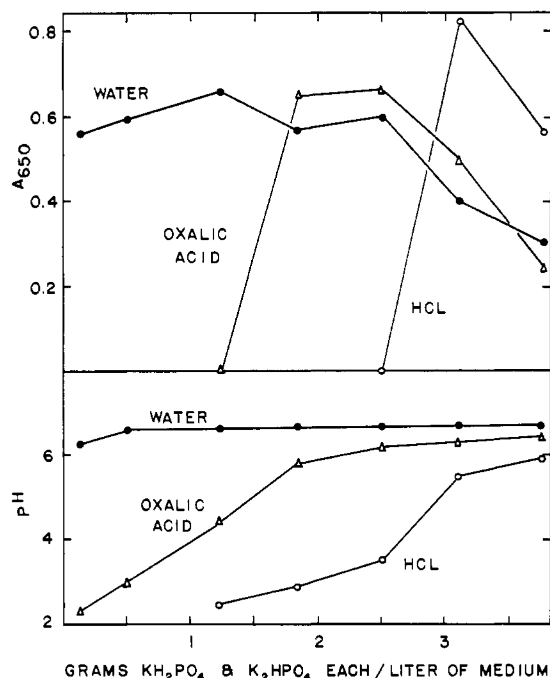

Figure 1. Effect of phosphate level and acids on growth of *Chlorella* and pH of medium
Double-strength medium was diluted with an equal volume of water, 1 mg./ml. oxalic acid solution, or 1 mg./ml. HCl solution. Abscissa gives final concentrations of KH_2PO_4 and K_2HPO_4 in single-strength medium

growth of *Chlorella* (Figure 1). Phosphate concentrations above 2.5 grams per liter of single-strength medium of both KH_2PO_4 and K_2HPO_4 start to inhibit *Chlorella* growth. This concentration of buffering salts was sufficient to neutralize the acidity of oxalic acid when present at a concentration of 500 μg. per ml. of single-strength medium but not sufficient to neutralize HCl present at this level. Since it is unlikely that an organic substance with more buffer-combining capacity than oxalic acid will be encountered, no difficulties due to a lowering of pH are likely to be encountered when substances are tested at a highest final concentration of 500 μg. per ml. Table III shows a comparison between the inhibitory effects of various compounds in buffered and unbuffered systems. As a rule, the buffered system seems to make the *Chlorella* more sensitive to the compounds. This may be due to the fact that the high phosphate concentration is already on the verge of having an adverse effect on *Chlorella* growth and any additional adverse effect by the compound then becomes more noticeable under these conditions. Both aflatoxin B_1 and xanthotoxin gave 50% growth inhibition at 10 μg. per ml. and were the most potent of the compounds tested. Figure 2 shows the effect of aflatoxin B_1 in the buffered and unbuffered systems. In this case no appreciable difference in toxicity was noted. Figure 3 shows the inhibitory effects of coumarin, 4-hydroxycoumarin, and 7-hydroxycoumarin and Figure 4 shows the effects of α-angelicalactone, kojic acid, and *p*-benzoquinone.

Warren and Winstead (1965) have suggested the use of agar plates seeded with *Chlorella* for the study of pathogenic fungi. They noted that if heavily seeded

Table III. Inhibitory Effects of Various Compounds toward *Chlorella* in Buffered and Unbuffered Liquid Media

(μg. per ml. for half maximum growth)

Compound	Unbuffered	Buffered
Resorcinol	240	170
α-Angelicalactone	70	35
Coumarin	20	70
4-Hydroxycoumarin	200	60
7-Hydroxycoumarin	120	30
Aflatoxin B_1	4	5
Xanthotoxin	40	8
p-Benzoquinone	250	30
Kojic acid	440	170

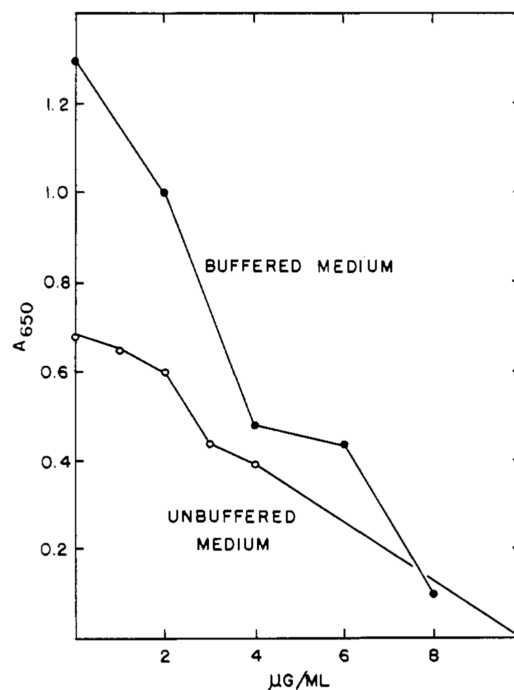

Figure 2. Effect of aflatoxin B_1 on growth of *Chlorella*

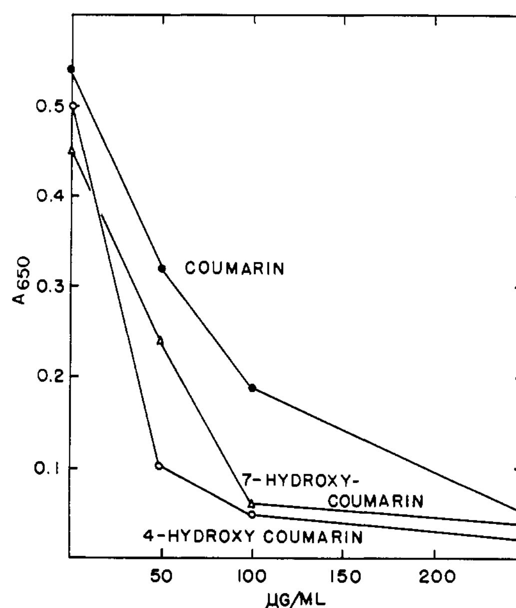

Figure 3. Effect of various coumarins on growth of *Chlorella* in buffered medium

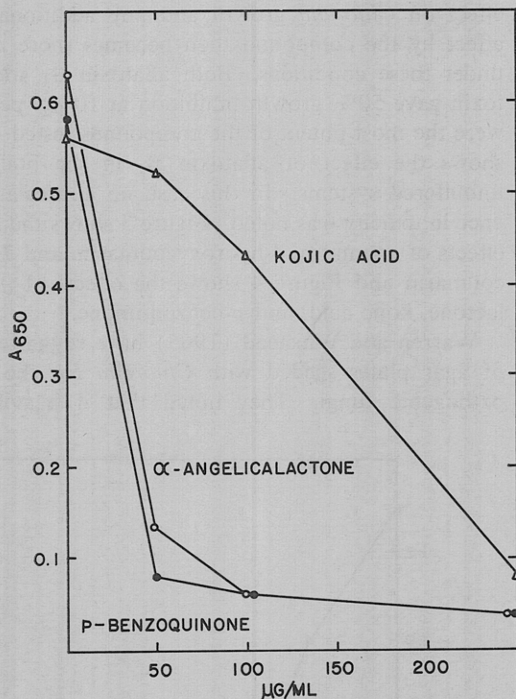

Figure 4. Effect of kojic acid, *p*-benzoquinone, and α-angelicalactone on growth of *Chlorella* in buffered medium

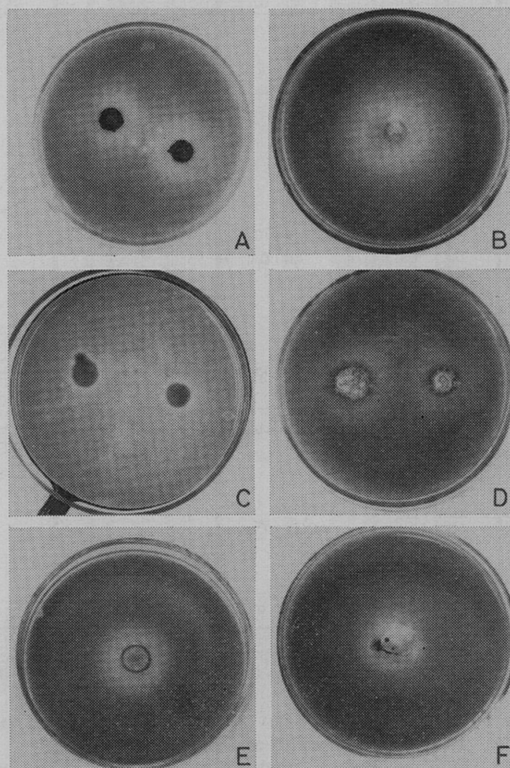

Figure 5. Effect of various fungi on growth of *Chlorella* in buffered agar plates

A. Aspergillus flavus
B. Fusarium tricinctum
C. Penicillum rubrum
D. Gibberella zeae
E. Unidentified fungus (see text)
F. Polyporus betulinus

plates were used, some fungal colonies caused a clearing or yellowing of the green agar plate. If lightly seeded plates were used, some colonies caused an inhibition of *Chlorella* growth, while some actually were growth-promoting and caused a darker green color to appear around and under the colony. The applicability of this method for the detection of toxin-producing fungi and algae and for the estimation of mycotoxins was further investigated in our laboratory. Figure 5 shows the results of inoculating buffered *Chlorella* agar plates with toxin-producing strains of *Aspergillus flavus, Penicillium rubrum, Fusarium tricinctum,* and *Gibberella zeae.* In each case a definite zone of inhibition is discernible around the colony. Figure 5 also shows an unidentified fungus which gave very clear zones of inhibition. An advantage of using buffered rather than unbuffered plates was that in the highly buffered plates the growth of the fungus was generally more compact and there was less spread of mycelia to obscure any zones of inhibition. *Rhizopus nigricans* mycelia could not be contained under any circumstances.

Using the buffered plates, a number of fresh mushroom species were tested by placing sliced, fresh tissue on the agar surface. Out of 26 species tested only one, *Polyporus betulinus,* showed definite inhibition and two, *Amanita pantherina* and *Lactarius chrysorheus,* were doubtful. The remainder were nontoxic and included *Agaricus arvensis, Amanita brunnescens, A. citrina, A. muscaria, A. rubescens, A. vaginata, A. verna, Amanitopsus vaginata, Boletinus pictus, Boletus edulis, Chantharellus cibarius, Clitopilus orcellus, Coprinus micaceus, Hypholoma incertum, Hypomyces lactifluorum, Laccaria laccata, Lactarius sp., Russula delica, R. emetica, R. sanguinea, Schleroderma aurantium, Strobilomyces strobilaceus,* and *Suillus granulatus.* Figure 5 shows the results with *P. betulinus.* Included in the species which were noninhibitory to *Chlorella* are the poisonous species *Amanita verna* and *A. muscaria.* It appears that mushrooms poisonous to man are not necessarily inhibitory to *Chlorella.*

Using unbuffered *Chlorella* agar plates, a number of red and brown algae samples collected from the local marine environment were tested for inhibitory substances by laying freshly collected specimens on the surface of the agar. Of the various species tested only two red algae, *Chondrus crispus* and *Euthora cristata,* showed zones of inhibition (Figure 6). The red algae which were found to be nontoxic included *Ceramium*

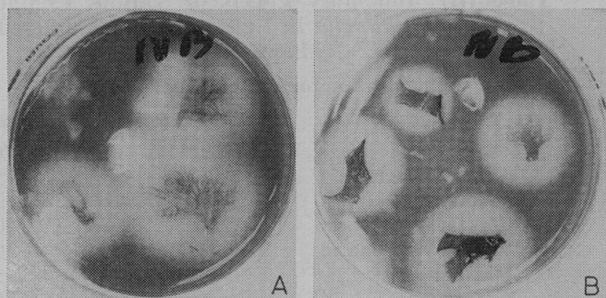

Figure 6. Effect of red algae *Euthora cristata* (*A*) and *Chondrus crispus* (*B*) on growth of *Chlorella* in unbuffered agar plates

Table IV. Effect of Various Acids on Growth of *Chlorella* on Buffered Agar Plates

Acid	Concentration[a] 1*M*	0.8*M*	0.6*M*	0.4*M*	0.2*M*	0.1*M*
	Zone Diameter Less Disk Diameter, Cm.					
Hydrochloric	1.2	0.9	0.8	0.1		
Oxalic	2.0	1.5	0.8	0.4	0.0	
Citric	2.1	1.6	1.5	0.8	0.0	
Succinic	0.2	0.0				
Fumaric	0.8	0.6	0.3	0.0		
Malic	0.7	0.6	0.4	0.0		
Salicylic	1.5	0.9	0.8	0.6	0.3	0.0
p-Hydroxybenzoic	1.0	0.6	0.4	0.1		

[a] Solutions in dimethyl sulfoxide.

rubrum, Cystoclonium purpureum, Phycodrys rubens, Phyllophora brodiaei, P. membranifolia, Polysiphonia lanosa, Porphyra umbilicalis, Ptilota serrata, and *Rhodymenia palmata.* The brown algae tested and found to be nontoxic included *Agarum cribrosum, Ascophyllum nodosum, Fucus distichus, F. vesiculosus, F. spiralis,* and *Laminaria digitata.*

Since *Chlorella* is sensitive to low pH's, the inhibitory effect of acids in the *Chlorella* agar plate method was investigated (Table IV). Even with the highly buffered plates most of the acids at 0.6*M* showed zones of inhibition, whereas at 0.2*M* most showed no inhibition. At 0.4*M* concentrations oxalic, citric, and salicylic acids showed some inhibition. It appears that at concentrations greater than 0.2*M* some nonspecific inhibition due to pH may be encountered. Assuming an average molecular weight of 100, it may be roughly estimated that a total acid concentration of 2% may result in the appearance of zones of inhibition.

Several of the fungi found to inhibit *Chlorella* growth were grown on rice and the cultures extracted with ethanol. Residues from the ethanol extracts were dissolved in dimethyl sulfoxide (DMSO) and tested on *Chlorella* agar plates (Table V). All of the extracts inhibited growth when applied in a 20% solution. However, only the *Rhizopus nigricans* extract inhibited growth when the concentration was reduced below 10%.

Table V. Effect of Fungal Extracts on Growth of *Chlorella* on Buffered Agar Plates[a]

Extract of	[H⁺][b] 20%	Concentration, % in DMSO 20	10	5	2	1	0.5	0.2
		Zone Diameter Less Disk Diameter, Cm.						
Fusarium tricinctum	0.28*M*	1.2	0.6	0.3	0.0			
Rhizopus nigricans	0.15*M*	2.4	1.7	1.5	1.2	0.6	0.2	0.0
Unidentified fungus	0.24*M*	0.9	0.3	0.1	0.0			
Polyporus betulinus	0.31*M*	0.5	0.3	0.2	0.0			

[a] Fungi cultured at ca. 25° C. on boiled rice and after 1 week of growth cultures were autoclaved and extracted in their entirety with ethanol. Ethanol extracts concentrated in vacuo to dryness and residues weighed and taken up in dimethyl sulfoxide. 50-μl. aliquots of filter-sterilized solution placed on 1.3-cm. sterile paper disks.
[b] Titratable acidity present in a 20% solution.

Table VI. Effect of Various Compounds on Growth of *Chlorella* on Buffered Agar Plates[a]

	Concentration, Mg. per Ml. in DMSO 1.0	0.5	0.25	0.1
	Zone Diameter Less Disk Diameter, Cm.			
Coumarin	0			
4-Hydroxycoumarin	0.2	0.1	0	
Dicumarol	1.3	0.8	0	
Warfarin	0			
Aflatoxin B_1		2.7	0.9	0
Xanthotoxin	0			
Ochratoxin A	0			
Emodin	0			
p-Benzoquinone	0			
Santonin	0			

[a] 50 μl. of sterile soltuion in DMSO placed on 1.3-cm. diameter sterile paper disks.

It showed inhibition even at 0.5%. The amount of titratable acidity in the 20% solutions (Table V) indicates that part of the inhibitory activity of the 20% concentrations may be due to acidity, since it has been shown that 0.4*M* concentrations of acids may inhibit growth (Table IV). It is doubtful, however, that the inhibitory effects observed with *Rhizopus* extracts at concentrations below 10% are due to pH.

Table VI shows the activity of pure compounds when tested by the *Chlorella*-agar method. Aflatoxin B_1 was by far the most active of the compounds tested. Of the other compounds only dicumarol showed inhibition at 1 mg. per ml. All the others were inactive at this level.

ACKNOWLEDGMENT

The authors thank A. C. Mathieson, Department of Botany, University of New Hampshire, for aid in identification of the algae.

LITERATURE CITED

Arai, T., Ito, T., Koyama, Y., *J. Bacteriol.* **93**, 59–64 (1967).
Burmeister, H. R., Hesseltine, C. W., *Appl. Microbiol.* **14**, 403–4 (1966).
Ikawa, M., Borowski, P. T., Chakravarti, A., *Appl. Microbiol.* **16**, 620–3 (1968).
Ikawa, M., Herbst, E. J., Briand, G. L., Ma, D. S., Summary of Proceedings of 1967 Mycotoxin Research Seminar, U. S. Department of Agriculture, Washington, D. C., p. 25, June 8-9, 1967.
Jayaraman, A., University of New Hampshire, unpublished results, 1967.
Nielsen, E. S., Willemoes, M., *Physiol. Plantarum* **19**, 279–93 (1966).
Warren, J. R., Winstead, N. N., *Phytopathology* **55**, 244–5 (1965).

Received for review October 18, 1968. Accepted January 6, 1969. Symposium on Natural Food Toxicants, Division of Agricultural and Food Chemistry, 156th Meeting, ACS, Atlantic City, N. J., September 8-13, 1968. Work supported in part by Grant 616-15-4 from the Cooperative State Research Service, U. S. Department of Agriculture, and Grant UI-00708 from the National Center for Urban and Industrial Health, U. S. Public Health Service, and published with the approval of the Director of the New Hampshire Agricultural Experiment Station as Scientific Contribution No. 459.