Insecticidal Mycotoxins Produced by *Aspergillus flavus* var. *columnaris*

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SUMMARY

Aspergillus flavus var. *columnaris* has the ability to produce a variety of toxic metabolic products, depending on the substratum upon which it is grown and on other conditions not understood. Some of these products are insecticidal. One such product, kojic acid, was found when the fungus was grown on a synthetic liquid medium. Other toxins were produced when the fungus was grown on a dog food-yeast-agar medium. These were water soluble and could be extracted from the culture medium when the fungus began to sporulate (3-4 days).

The toxins do not affect the hatching of house fly eggs, but do affect larval development. Lethal concentrations reduce the metabolic activity of maggots within a few hours, and death soon follows. Sub-lethal concentrations delay larval development and reduce the size of surviving flies. The milkweed bug is sensitive to the toxins in a comparable way, and development of the confused flour beetle can also be affected. No toxicity could be demonstrated to the earwig, two species of cockroach, the Indian meal moth, the greater wax moth, or to termites.

Although isolation and characterization of these toxins has not been completed, at least two heat-labile substances of higher molecular weight could be distinguished by gel filtration followed by ion exchange fractionation, and a heat-stable substance of lower molecular weight was indicated by gel filtration.

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Introduction

The remarkable ability of fungi, notably the genus *Aspergillus,* to produce toxic substances is attested by the discovery of the series of related chemical substances designated as aflatoxins (Goldblatt, 1969) and a series of unrelated metabolic products that have some toxic properties (Wilson, 1966; Feuell, 1969).

The aflatoxins (Büchi and Rae, 1969) are a group of acutely toxic and highly carcinogenic metabolites; they are oxygenated heterocyclic compounds. Aflatoxins B_1, B_2, G_1, and G_2 are distinguished by their fluorescence (blue or green) and chromatographic mobilities. Aflatoxins M_1 and M_2 (milk toxins) are derivatives of B_1. Aflatoxin B_{2a} and G_{2a} are hydroxy derivatives of aflatoxins B_2 and G_2. Aflatoxin P_1 is a phenolic derivative of B_1 (Dalezios et al., 1971).

Other metabolic products of *Aspergillus* growth (reviewed by Wilson, 1966; Feuell, 1969) include oxalic acid, kojic acid, a tremorgenic substance, aspergillic and related acids, β-nitro propionic acid, gliotoxin, helvolic acid, festuclavine, ergot alkaloids, terreic acid, nidulin, maltoryzine, xanthocillin, sterigmatocystin, and a series of ochratoxins. Still others will undoubtedly be added to this list.

With such an array of chemical products derived from *Aspergillus* metabolism, it is only reasonable to suspect that chemical toxins were involved when excessive mortality was observed in cultures of the larger milkweed bug, *Oncopeltus fasciatus* (Beard, 1959; 1968), and of the house fly, *Musca domestica* (Beard and Walton, 1965), when *Aspergillus* was present as an obvious contaminant on the food media but not as an invading pathogen of the insects.

Species of *Aspergillus* are among a relatively few hyphomycetous fungi which grow on insects. Probably more often than not they are saprophytic rather than parasitic, but a facultatively parasitic mode is possible (Steinhaus, 1949; Sussman, 1951; Madelin, 1963; Müller-Kögler, 1965). In the studies reported here no invasion of live insects was observed at any time.

When entomophagous fungi are parasitic, mycotoxins produced by them may play a significant role in pathogenicity. Burnside (1930) reported on the enteric invasion of honeybees by *Aspergillus,* as did

Toumanoff (1931) who postulated that toxins were the proximate cause of death. When fungal attack is through the integument, introduction of metabolic poisons into the body cavity represents a normal route of administration. This was the route simulated in the injection techniques used by Yendol et al. (1968) and Prasertphon and Tanada (1969) in their studies on mycotoxins of entomophthoraceous fungi. Also in this category are toxins (destruxins A and B) produced by *Aspergillus* and *Metarrhizum* as reported by Aoki, Kodaira, Roberts, Tamura, and others as reviewed by Tamura and Takahashi (1971).

Wounding of host insects may favor both fungal invasion (Hurpin and Vago, 1958) and exposure to toxins (Vey et al., 1967). Dresner (1950) observed that a toxin produced by germinating spores of *Beauveria bassiana* had a paralyzing and killing action on some insects by contact. This was not confirmed by Steinhaus and Bell (1953), but it suggested a different pathway of intoxication by a mycotoxin. Piericidins A and B, toxic metabolic products of *Streptomyces*, have pronounced insecticidal properties by topical application to some species of insects (Tamura and Takahashi, 1971).

Non-parasitic fungi may contaminate the food when this is the environment of insects, and so cause death. In this case depletion of essential nutrients is a possible explanation, but production of an insecticidal metabolite is more likely. In such an event, the toxic substance is principally an enteric poison introduced by ingestion of contaminated food. The mycotoxins reported here are in this category.

This study was prefaced by several scattered and, at first, unrelated observations and events. Initially, cultures of the larger milkweed bug would occasionally succumb in a manner suggesting a contagious disease, but no infections could be observed nor experimentally induced (Beard, 1959). Associated with the bug mortality was a moldy condition of the food media. This may well have been a complex of mold fungi, but *Aspergillus flavus* was identified as being present. Later Beard (1968) demonstrated that the bug malady could be explained as being due to mycotoxins in the cultures. This conclusion was based on the presence of toxic materials in such old food media as might be found in sick cultures and the similarity of behavior when demonstrated mycotoxins were added to the water supply of bugs in culture or infused into seeds fed to bugs. The original pathology has not been precisely reproduced, as a distinctive diarrhea has not been induced by experimental material. As will be discussed, mycotoxin production differs with different culture media and conditions of culture, and there is little question that the original intoxication resulted from a metabolic product differing from those products later encountered in experimental systems.

When house fly cultures declined at a time when *Aspergillus flavus* was conspicuously present (Beard and Walton, 1965) it was easily confirmed that the larval cultures were inhibited by the presence of a water-soluble toxic product of *Aspergillus* growth. The specific fungus involved was determined to be *Aspergillus flavus* var. *columnaris*, and

all subsequent experimental work was limited to this one variety and which hereafter will be designated as *Afc*.

With this background, search was initiated for the toxin* that could explain both the milkweed bug and fly maggot mortalities. Overlooking at the time the possibility of different toxins being produced on different media, a fluid growth medium was chosen for culturing *Afc* purely as a matter of convenience. The fact that an insecticidal product was formed justified this choice. A modified Diener's medium best served in producing the suspected toxic substance. The toxic material was associated with an easily extracted fraction having characteristic ultraviolet absorption peaks. Thus spectrophotometric methods, rather than bioassay, simplified the isolation and identification of the toxin which proved to be kojic acid (Beard and Walton, 1969). Kojic acid is a well known product of *Aspergillus* metabolism and had previously been suggested as having insecticidal properties (Beélik, 1956). These properties were manifested only when kojic acid was present in relatively large amounts, but the amounts produced on the modified Diener's medium were adequate to demonstrate insecticidal action. The rate of production of kojic acid seemed too slow to account for the house fly mortality that originally had implicated a mycotoxin, and when it was observed that kojic acid was not formed by *Afc* on the food medium used to rear house flies, it became obvious that kojic acid was an accidental surrogate of the substance originally sought. The fact, too, that kojic acid was the only insect toxicant produced by the modified Diener's medium confirmed that different toxic products can be derived from fungal culture under different conditions.

The following account reports the continued search for insecticidal mycotoxins produced by *Afc* when grown on the food medium used in raising house flies.

Materials and Methods

The fungus *Afc* was maintained on tubes of potato dextrose agar. Inoculum was obtained by washing the spores and hyphal fragments from the surface of a 5-8 day-old slanted tube culture into approximately 125 ml sterilized water. Approximately 5 ml of this suspension was placed onto each plate and the suspended fungal parts allowed to settle for 1 hour. The excess water was then poured off.

Initial experiments used a liquid medium containing 5 percent dog food (Gaines) and 5 percent yeast powder in water. The ingredients were ground in a Waring Blendor and placed in 250 ml Erlenmeyer flasks, 100 ml per flask, and sterilized. Each flask was inoculated with a 5 mm disc taken from a 5-8 day plate culture.

When the liquid culture technique did not result in satisfactory toxin

* Although reference will be made to the toxin as if it were a single entity, later discussion will disclose that a complex is involved.

production, a solid medium was utilized. The medium consisted of 10% dog food, 10% yeast powder and 1.5% agar. The dog food and yeast extract were ground in a Waring Blendor before addition of the agar. After sterilization, plates were poured (approx. 10 ml each) and inoculated as described above. The plates were incubated at room temperature unless noted otherwise.

Except as will be discussed, the house fly (*Musca domestica* Linn.) was the chief insect used. Stock cultures of flies were of a long-standing laboratory strain of mixed origins. They were kept in ventilated plastic containers and supplied with dried milk powder, sugar, and water. Eggs were collected on pelleted dog-meal (Gaines) moistened with a yeast suspension (7 g/l of water) and placed on similar medium (50 g dog-meal, 60 ml yeast suspension) for larval development. Eggs for testing were spread in a film of water on black filter paper and counted under a low power microscope.

The other insects used were of cultures maintained routinely in the laboratory.

In addition to using intact medium contaminated with *Afc,* water-soluble toxic materials were extracted from agar mats with *Afc* actively sporulating (showing definite yellow color). Such mats, usually of four-day cultures, were removed from petri dishes into a beaker. As a safety measure to reduce distribution of spores and to contain any aflatoxin that might be present, the plates were sprayed with chloroform, and some chloroform was added to the beaker into which the mats were placed. Water was added to the beaker and the agar mats were chopped, not homogenized, into small fragments. The final amount of water added was just enough to cover the mash. The beaker was then placed in the refrigerator for a few hours or overnight to permit the toxin to diffuse into the water. The mash was then placed in a cloth-lined potato masher type hand press, and the fluid was expressed. After centrifugation, the water fraction was filtered twice. This crude fraction was toxic to fly maggots and could be used for testing or for further purification.

The bioassay of the insecticidal mycotoxin used house fly maggots as the test organism. The technique applied previously (Beard and Walton, 1969) was satisfactory, but it called for more material than was sometimes available. As a modification of this technique, 1.6 gm of a mixture of finely ground dog-meal (4 parts by weight) and yeast powder (1 part) were placed in a 1-ounce plastic creamer. This was moistened with 3 ml of water or test solution. On the surface were placed 50 fly eggs, and the cup was capped with a paper lid perforated by two small holes. If humidity or metabolic water was excessive, crumpled absorbant tissue was added to provide a drier pupation site. If only the presence of toxin was to be detected, the success or failure of maggot growth was observed. If a quantitative measure was desired, the test fluid was serially diluted in decrements of ½ and each of six or seven concentrations tested. When crude extract was employed, this dosage series assured a range of from complete mortality to essentially normal fly

development. Evaluation was based on numbers of pupae resulting from the 50 eggs, or in some cases, the number of flies emerging.

Spectrophotometric records were made with a Bausch and Lomb Spectronic 505 instrument.

RESULTS AND DISCUSSION

Effects of Afc *contamination on maggot development*

Hatch of house fly eggs is not affected by the presence of toxic material, and for a few hours maggots on contaminated medium appear no different from controls. Then they become progressively more sluggish and their feeding more desultory. No obvious change in gross appearance can be detected during these early hours of exposure, but affected larvae exposed for 4 to 6 hours can be distinguished from healthy larvae in 80% of the cases by microscopic examination of the gut with transmitted light. This distinction is largely subjective as no well-defined criteria serve to differentiate the affected from the unaffected maggots. It is reasonable to suspect that the difference in appearance is associated with the amount of food ingested and the degree and rate of digestion.

In media covered by sporulating *Afc* some wandering maggots may get covered with conidia and adhere to the mycelium and conidiophores. After a period of activity in a characteristic flexing motion, the maggots shrivel and die. This type of death is believed to be physically induced (desiccation, etc.) and is questionably associated with any toxic action.

If maggots are removed from contaminated medium any time before six hours exposure and placed on uncontaminated food, they develop normally except that the longer exposure within this period can delay the completion of larval development one to three days. This suggests a type of sub-lethal toxic action rather than interrupted metabolism as this lengthened larval life cannot be simulated by chilling or starving young maggots for an equivalent period.

Eight hours of exposure to food contaminated with adequate concentrations of toxin seems to mark the approximate "point of no return." Such exposed larvae then begin to cease activity and gradually become shrunken and moribund. These signs and symptoms of intoxication can be confirmed by measurement of metabolism as indicated by respiration. Contaminated media and uncontaminated media were placed in respiration flasks and seeded with 100 fly eggs each, the eggs being of uniform age laid in late afternoon. The time of hatch is not known, but the next morning after the eggs hatched, the flasks were set up in a Gilson respirometer and the cumulative oxygen consumption was recorded; the results are illustrated in Figure 1. Other experiments had shown that under these conditions for this length of time fermentation and other biological processes did not materially affect the response attributable to maggot development. Activity of older maggots overwhelmed the

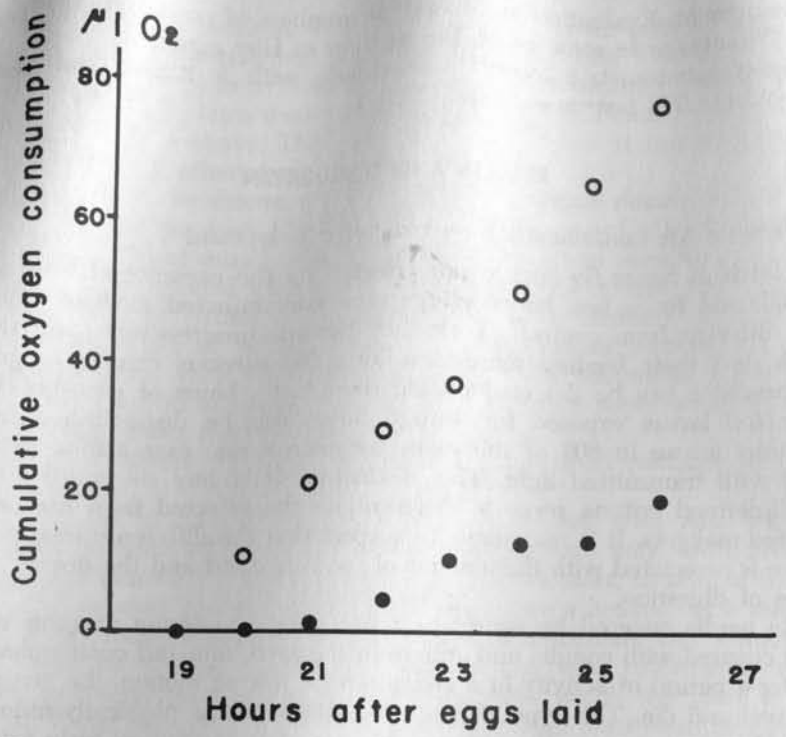

Figure 1. Cumulative oxygen consumption (microliters of oxygen) of maggots hatching from eggs placed in contaminated media (closed circles) and in uncontaminated media (open circles). Results of pooled samples of three flasks each seeded with 100 eggs.

system, and after the death of maggots, other systems could cause confusion. For these reasons observations were not continued longer than indicated.

Figure 1 illustrates a conspicuous difference in metabolic activity of maggots in contaminated and control media. In the experiment illustrated in Figure 2, 50 newly-hatched maggots rather than eggs were placed in each flask. Here there was no exposure to contaminants prior to placement. In this instance oxygen consumption was expressed at rate per hour, and for the first four hours the metabolism of maggots in contaminated media paralleled, and in fact exceeded, that of control maggots. Later the rate of oxygen consumption leveled off at a time when that of control maggots perceptibly increased. These data are consistent with the other direct observations on the maggots.

Even casual observation discloses that the presence of toxin in fly culture media reduces the number of maggots, retards the rate of development of the survivors, and diminishes the size of pupae resulting. This can be visualized more graphically in Figure 3 which is based on

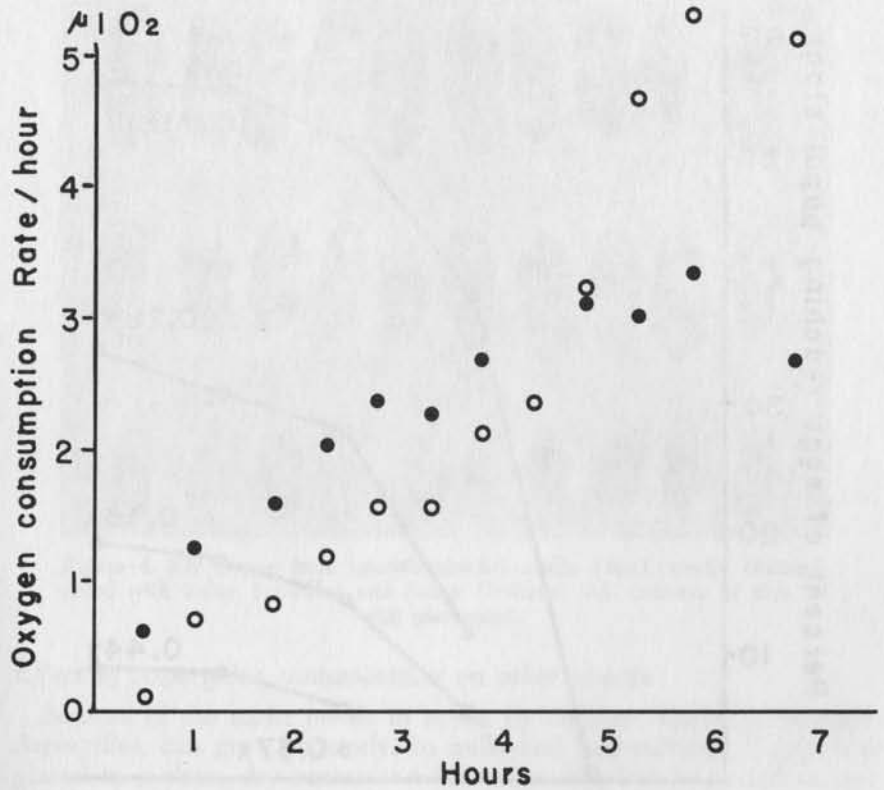

Figure 2. Rate of oxygen consumption (microliters of oxygen per hour) of recently hatched maggots placed in contaminated media (closed circles) and in uncontaminated media (open circles). Results of pooled samples of three flasks, each stocked with 50 maggots.

four concentrations of contaminant in six replicated cups for each concentration and in which 50 eggs per cup were introduced. Six similar cups without contaminated media served as controls. Actually more concentrations were employed, but those causing complete mortality were discarded, and the greatest dilution of crude extract that still caused complete mortality was designated as 1 or x; the other values are expressed as dilutions of this.

In addition to the effects of the toxin in reducing the numbers of maggots and delaying their development in contaminated cultures, is an effect on size of surviving flies. Figure 4 illustrates pupae obtained from uncontaminated media and from media inoculated 2 and 3 days prior to introduction of fly eggs (see data in Table 1). As an example of the magnitude of such differences, in another trial 50 pupae in a culture inoculated with *Afc* spores a day after the fly eggs were placed weighed 746 mg as compared with 495 mg for 50 pupae from cultures

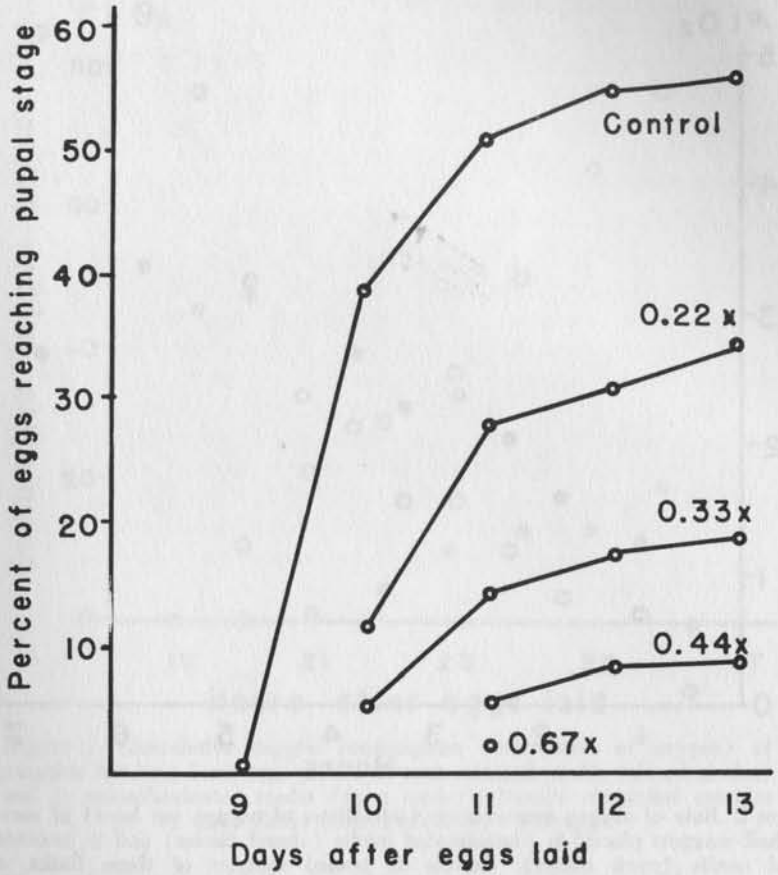

Figure 3. Effect of toxin concentration on maggot development from 300 eggs (each concentration). Concentrations based on proportion of x = minimum concentration permitting no survival.

inoculated with spores and eggs at the same time, and 469 mg for 50 pupae from cultures inoculated with spores one day before the addition of fly eggs.

One other response of maggots to sub-lethal concentrations of toxin is an avoidance reaction. Although this has not been evaluated, it may lead to the maggots completely leaving the culture medium and dying of desiccation or starvation, or it may be a temporary clustering at the margin of the food, the maggots later moving into the food and developing. Whether this phenomenon is associated with particular concentrations of a toxin complex or with one particular component among several in a complex has not been established.

Except for reduced size, the adult flies emerging behave normally and produce apparently normal offspring.

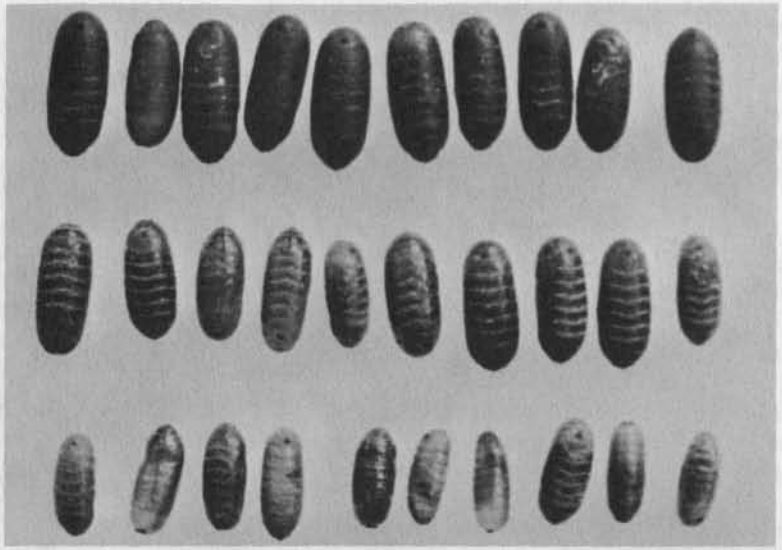

Figure 4. Fly pupae from uncontaminated media (top), media contaminated with 2-day (middle) and 3-day (bottom) *Afc* cultures at time of egg placement.

Effect of Aspergillus *contamination on other insects*

Because of the moist media in house fly cultures, fungi, particularly *Aspergillus,* can grow promptly. In milkweed bug cultures, if eggs are placed in a clean, dry container with water supplied so as not to wet the dried milkweed seed used for food, the seeds become moldy only after fluid waste products accumulate and humid conditions prevail. This means that the nymphal bugs run little risk of mycotoxic effects until they are well along in their development. This explains why the high mortality originally observed was associated with the fourth and fifth instars (Figure 5).

When crude extract is supplied as drinking water or infused into milkweed seeds which are then dried and fed to bugs upon hatching from the eggs, few bugs molt to the second stage. For example, when 200 eggs were placed in a cage, supplied crude toxin extract as a source of water, only 15 cast skins were found when all bugs were dead, this at a time when in a control cage 200 eggs had resulted in 63 third instar nymphs and 33 fourth instar, 195 cast skins being recovered. This again illustrates that the toxin delays development as well as being lethal.

The effect of the toxin on growth of the confused flour beetle, *Tribolium confusum,* was tested by incorporating 1% of test substance (dry weight) into a food medium consisting of 4 parts pulverized dog-meal and 1 part yeast powder. One gram of medium was placed in each of 5 creamers with 10 adult beetles randomly selected and unsexed. The test substance was a lyophilized active fraction of crude extract. Controls

Figure 5. Two fifth instar milkweed bugs (indicated by arrows) killed by mycotoxin and milkweed seed showing sporulating *Afc*.

were the same but without the test substance. After two months the medium was examined for cast skins as evidence of larval development. In two of the treated cups no development was seen. In the remaining three, a total of 139 cast skins were found. In the five control cups a total of 364 cast skins were found. The presence of the toxin was obviously deleterious, but it did not wholly prevent considerable development.

By offering contaminated food, no toxicity could be demonstrated against nymphs and adults of the earwig (*Forficula auricularia*), the American cockroach (*Periplaneta americana*), the German cockroach (*Blattela germanica*), the Indian meal moth (*Plodia interpunctella*) or the greater wax moth (*Galleria mellonella*). Termites (*Reticulotermes flavipes*) fed on cellucotton treated with crude toxic extract also showed no ill effects.

The conclusion is unavoidable that this mycotoxin is rather specific in its actions.

Crude extract and general nature of toxin material from Aspergillus *contaminated media*

Relation to aflatoxin

Some insecticidal properties have been attributed to the aflatoxins. They have been suspected as a cause of honeybee mortality (Foote, 1966) and to kill or affect reproduction in dipterous insects (Matsumura and Knight, 1967). Becker et al. (1969) reported on strains of *Aspergillus* that destroyed termites. Most, but not all, of the strains were effective producers of aflatoxin. The conclusion reached was that aflatoxin was the principal cause of termite deaths, but also involved were other toxins produced by strains that did not produce aflatoxin. It is important, therefore, to confirm or deny the identity of our toxin with the aflatoxins, especially since Gudauskas et al. (1967) assumed that our earlier report (Beard and Walton, 1965) referred to aflatoxins.

It was early concluded that the toxic substances found here are not aflatoxins, and tests for these, repeated from time to time, consistently gave negative results. Solubilities, fluorescence, and ultraviolet absorption spectra were the criteria used.

The aflatoxins are readily soluble in chloroform, and this solvent is routinely used in extracting aflatoxins. Chloroform extracts of our *Afc* cultures or chloroform partitioning from water extracts of such cultures failed to yield toxic substances when assayed against fly maggots. Moreover no chloroform extract of test cultures yielded anything to suggest aflatoxin when measured by spectrophotometry. Although some of the suspected toxic extracts and fractions fluoresced, the fluorescence was not that characteristic of the aflatoxins. By direct comparison with a mixture of aflatoxins, no extract or fractions of extract has shown similar ultraviolet absorption spectra, and no fraction has shown peak absorption corresponding to published spectra of the aflatoxins.

Other workers, too, have found *Afc* to be a poor producer of aflatoxin. Van Walbeek et al. (1968) found this fungus to produce only small amounts of aflatoxin B_2, but this it did on three substrates. We can only conclude that our strain of this *Aspergillus* when grown on the medium selected fails to produce aflatoxin. This is not to say, though, that the suspected toxins are not among those numerous metabolic products that have been identified by other workers (Feuell, 1969).

Extraction of crude toxin

Crude extract of contaminated culture media obtained as described above is amber colored, and the toxicity of the extract can be judged roughly by the depth of color. Apparently the color is produced in similar proportions to the toxic material whether or not color is associated directly with any toxin.

Efficiency of this water extraction is perhaps not high as the solid residue after extraction remains toxic to house fly maggots. Buffers with different pH and ionic strength are no better extractants than demineralized water. The fungal mats themselves have high buffering capacity so that reasonable differences in pH of eluant are equalized in the resultant extract. Phosphate buffers at pH 7.5 of 0.05 M and 0.15 M extract the toxic materials to the same degree, whether evaluated by bioassay or by ultraviolet absorption spectra of suspected fractions separated by gel filtration.

Water soluble residues after evaporation of chloroform extracts of

fungal cultures are non-toxic. Likewise if water extracts of the cultures are partitioned with chloroform, the toxic substances are limited to the water layer. Hexane behaves like chloroform and so is ineffective as an extractant for the toxins. Methanol apparently precipitates or denatures the toxins, and it, too, is ineffectual as an extractant. Dimethyl formamide also proved unsuitable.

Diffusion of toxin in culture media

Some early evaluations of agar (dog-meal and yeast) cultures of *Afc* for toxin content were highly variable. Some seemed highly toxic and some surprisingly seemed toxin free. Some fortuitous observations suggested that the toxin may be formed, but that it does not diffuse readily in the medium and so success or failure depended upon the concentration of the metabolic products in the portion of the medium sampled. This was confirmed by the following experiments.

Vertical diffusion was tested by preparing a deep medium of agar, dog-meal, and yeast in a beaker and inoculating the surface uniformly with *Afc* spores. After 5 days, when a continuous surface mat of sporulating fungus had developed, the cylinder of medium was carefully removed from the beaker and divided into six horizontal layers, each 6 mm deep, plus the fungal mat itself which separated from the medium. Each layer was then tested for toxicity by adding fly eggs directly to the medium. A vertical gradient of toxicity was evident as illustrated in Figure 6. In another similar test of a 3-day culture, only the top 5 mm contained material inhibitory to fly maggots. Fungal growth was found to parallel the distribution of toxin. After 3 days' incubation only the top 5 mm contained fungal growth when after 6 days fungal growth was found at the 25 mm depth.

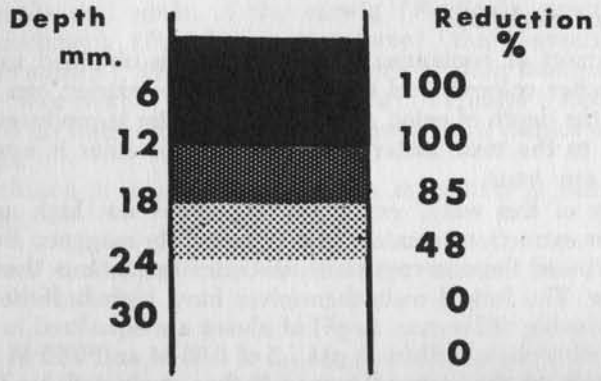

Figure 6. Diagram illustrating toxicity of vertical levels of media supporting 5-day culture of *Afc* on surface at time of seeding with fly eggs. Toxicity expressed as percent reduction of fly pupae compared to those obtained from uncontaminated media.

Failure of horizontal diffusion became evident when agar plates with discontinuous growth of *Afc* showed that maggots could survive in areas of medium not actually covered by fungal growth, but could not survive in areas covered by growth (Figure 7).

As a result of these observations, shallow agar plates and uniform surface inocula were used to maximize the toxin content in material for extraction. Thereafter the yield of toxin was consistent.

Time of toxin development

An estimate of the time of development of toxin was made in the following manner. The standard nutrient agar medium was poured into plastic creamers in uniform amounts. Ten replicates were used as controls and 50 were inoculated with spores of *Afc*. At this time the 10 control cups and 10 inoculated cups were each seeded with 50 fly eggs. On each succeeding day another 10 cups were likewise seeded with 50 eggs each so that in the series the fungus had from 0 to 4 days' advance growth before the eggs were introduced. The number of fly pupae resulting were as indicated in Table 1.

In contrast to the slow development of kojic acid, which reached peak amounts in 11 days (Beard and Walton, 1969), this toxin is produced quickly and so adequately accounts for the time sequence of mortalities observed in naturally contaminated fly cultures.

Stability of toxin upon standing

Stability of toxin when kept at room temperature, under refrigeration, or frozen was tested. A supply of crude extract was divided into 38 aliquots. Two of these were assayed at once, and two each for each storage condition were assayed after 1, 2, 5, 8, 16, and 32 days. The dilutions of crude extract in the assay were 0.5, 0.25, 0.12, 0.06, and 0.03, the data being expressed as the lowest of these concentrations that permitted no maggot development when 50 eggs were placed in each unit.

Although variation is obvious, it is not consistent with either time or condition of storage, and differences may be as great between replicates

Table 1

Days	*Total Pupae**	*% reduction from controls*
0	315	0
1	120	62
2	64	79
3	44	86
4	0	100
Control	315	

* From 500 eggs

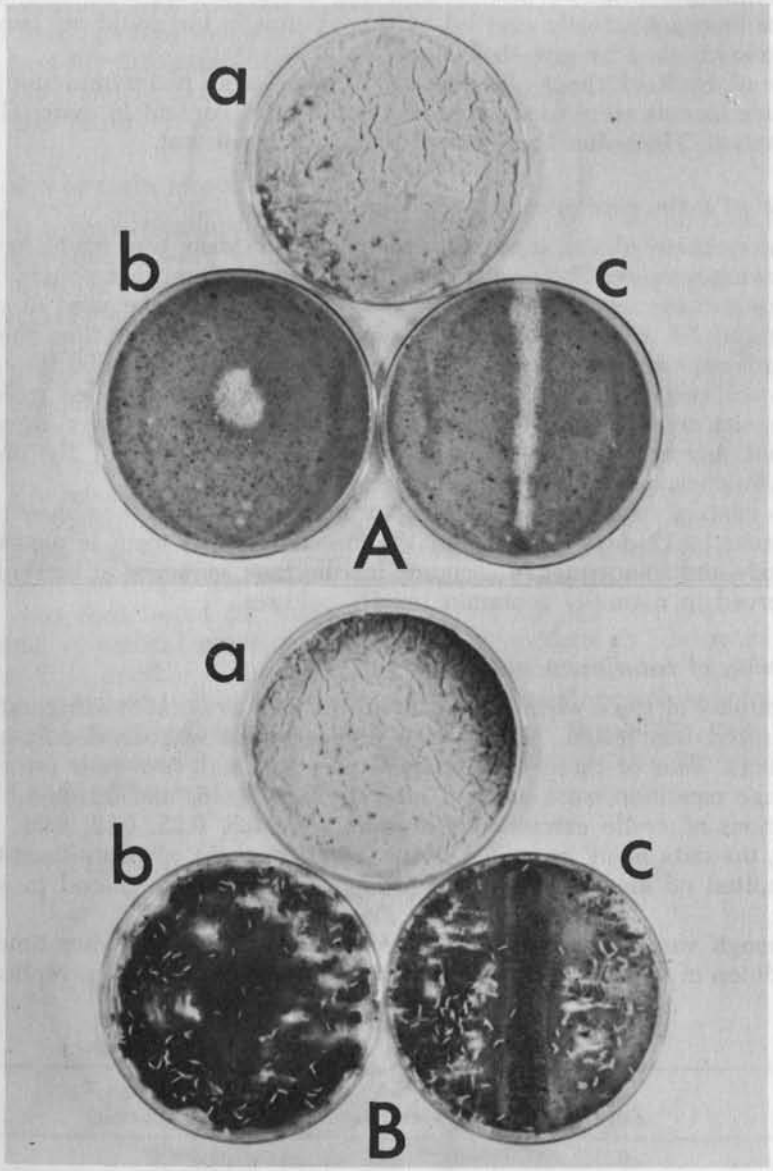

Figure 7. A. Cultures of *Afc:* a, uniform inoculum; b, center spot inoculum; c, streak inoculum. B. Same as A except seeded with fly eggs: a, no survival of maggots; b, c, *Afc* growth areas not fed upon, although maggot traffic has modified growth of fungus.

Table 2

Original	*Days later*					
	1	*2*	*5*	*8*	*16*	*32*
0.25						
0.25						
Room temperature	0.06	0.12	0.06	0.5	0.06	0.06
	0.06	0.12	0.12	0.25	0.12	0.12
Refrigerated	0.06	0.03	0.06	0.25	0.12	0.12
	0.12	0.03	0.5	0.25	0.12	0.12
Frozen	0.06	0.12	0.25	0.5	0.12	0.06
	0.06	0.25	0.25	0.12	0.25	0.03

as between the different test situations. It can only be concluded that the toxic substance is relatively stable even in the presence of bacterial growth as occurred prominently in that stored at room temperature and less so under refrigeration.

Sensitivity of toxin to heat

Media containing sufficient toxin to inhibit all maggot development, will, after heating to liquification, support the growth of maggots. If lesser amounts of toxin are present comparative values can be illustrated as follows. Two-day cultures of *Afc* on agar medium were heated over steam until the agar melted; after cooling, fly eggs were introduced. In four replicates, each with 50 fly eggs, the total number of pupae resulting was 16.2% less than similar but uncontaminated control media, as compared with 86.8% reduction in contaminated cultures unmodified by heat.

Obviously the toxin in crude extracts is heat labile, and the fungal culture has not so modified the nutrient balance that the media is unsuitable for growth of maggots. More will be said about heat stability in another connection.

Sensitivity of crude extract to proteolytic enzymes

Suspected of being a polypeptide or small protein, the toxic substance might be susceptible to enzymatic degradation. Extracts incubated with trypsin or chymotrypsin showed no loss of toxicity. When incubated with pepsin the material lost activity, but this was somewhat equivocal. As pepsin digests only in an acid medium, the requisite pH of 2 and subsequent neutralization may have influenced the assay as much as the pepsin treatment alone, although suitable controls indicated that modifying the pH in this way did not destroy toxicity.

Experiments to isolate and characterize the insecticidal mycotoxins

Over the period of time spent in trying to isolate and characterize the toxic material it has become obvious that the target has changed and

what once appeared to be a single toxin now appears to be a complex of several toxic components. What happened to account for shifting metabolic products is unclear. The change could have resulted from mutation of the fungus. Although the cultures were maintained so as to assure purity of strain, a mutant having greater survival value could have appeared and overrun the rest of the culture. Extrinsic factors such as possible change in formula of the commercial dog food used or some change in the environment might have led to different metabolic products. Photo-periodic change was one factor considered, and experiments on this will be reported.

In isolating the toxins the crude water extract from 4 or 5 day fungal cultures served as raw material. Precipitation by ammonium sulfate was thought to be ineffectual as toxic material was present in both filtrate and precipitate; however, the possibility of this as a means of distinguishing two substances acting similarly was not investigated. Adsorption onto hydroxylapatite gel was also not practicable, but gel filtration with sephadex or Bio-Gel was useful as an initial separation.

At first gel filtration yielded three easily recognized components. The first component to be eluted was the excluded proteins which were visibly evident as a cloudy solution. Following this were clear fractions that showed a characteristic ultraviolet absorption peak at 280 nm (illustrated in Figure 7) and that contained the toxic substance. The third evident eluate was a very large component amber in color. Thus even without spectrophotometric measurement or bioassay the toxic material could be purified considerably by saving that clear eluate between the visibly cloudy portion and the later appearing colored component neither of which showed toxicity. This intermediate material behaved on gel filtration columns as if its molecular weight approximated 6000-8000. It fluoresced with a pale yellowish color. Lack of purity became evident upon electrophoresis, when five or more bands could be distinguished. Before these different components could be separated, the first obvious shift in target material occurred.

The shift was not suddenly observed, so any events leading to a change could not be identified. The change resulted in the toxic material coming out with the protein components in the early fractions in the gel filtration. It is possible that the toxic component was essentially the same as before but possibly was now polymerized or aggregated to larger molecules. Its behavior continued much the same. It remained the only toxic portion of all the fractions; its electrophoretic pattern still showed more than five bands; its dominant feature was still a characteristic UV absorption peak at 280 nm; its effect on insects was not noticeably different. In other words, the only evident difference was that it behaved as a larger molecule than before, possibly in the range between 10,000 and 20,000.

The correlation between UV absorption at 280 nm and toxicity was established as follows. A series of fractionations of crude extract was made on Sephadex G-75 columns. The corresponding aliquots in each

Table 3

Tube number	*OD 280 nm*	*Relative toxicity*
13	.18	0
14	.23	0
15	.43	2 x
16	.44	2 x
17	.50	4 x
18	.57	4 x
19	.42	2 x
20	.38	2 x
21	.30	2 x
22	.25	x
23	*	x

x – minimum concentration causing complete mortality of maggots
* – obscured by overlap of changing peak

run were pooled, concentrated by lyophilization, and assayed for toxicity. The relative concentrations of toxin indicated in Table 3 are based on the maximum dilution that still permitted no survival of maggots.

Another association of this 280 nm peak and toxicity was made with three aliquots of the same sample of crude extract that had been frozen. The aliquots differed in their rates of melting. The first aliquot to melt was darker in color and when fractionated showed the highest absorption at 280 nm at the appropriate elution volume. The two succeeding aliquots diminished in color, absorption at 280 nm, and in toxicity as illustrated in Figure 7. This, incidentally, suggests a simple way of concentrating the crude extract.

Different grades of Sephadex or Bio-Gel, or longer columns, did not resolve the toxic component any further. A separation into five recognizable components was made with an ion exchange column of DEAE cellulose and eluted step-wise with phosphate buffer of increasing molarity (Figure 8). If the toxic fraction obtained from gel filtration and concentrated by lyophilization was passed through this anionic exchange column, the toxic component is adsorbed, and a large non-toxic component with a UV absorption peak at 265 is removed with the 0.015 M eluate. Elution with 0.06 M brings out a component that is toxic, has a UV absorption peak at 280 nm and does not fluoresce. Another toxic substance can be eluted with buffer of still higher molarity. Eluates of 0.1 M, 0.15 M, and 0.25 M removed probably three separate components, but these are less well separated and would require recycling with perhaps somewhat different molar concentrations of eluant for better resolution. Which of these components alone or in combination are toxic has not been determined. Figure 9, which shows the spectrophotometric curves in the 280 nm region for each of the five components illustrates how their pooled effect could result in a seemingly characteristic curve for the initial fractionation.

So far, two toxins at least would seem to be present, but at some

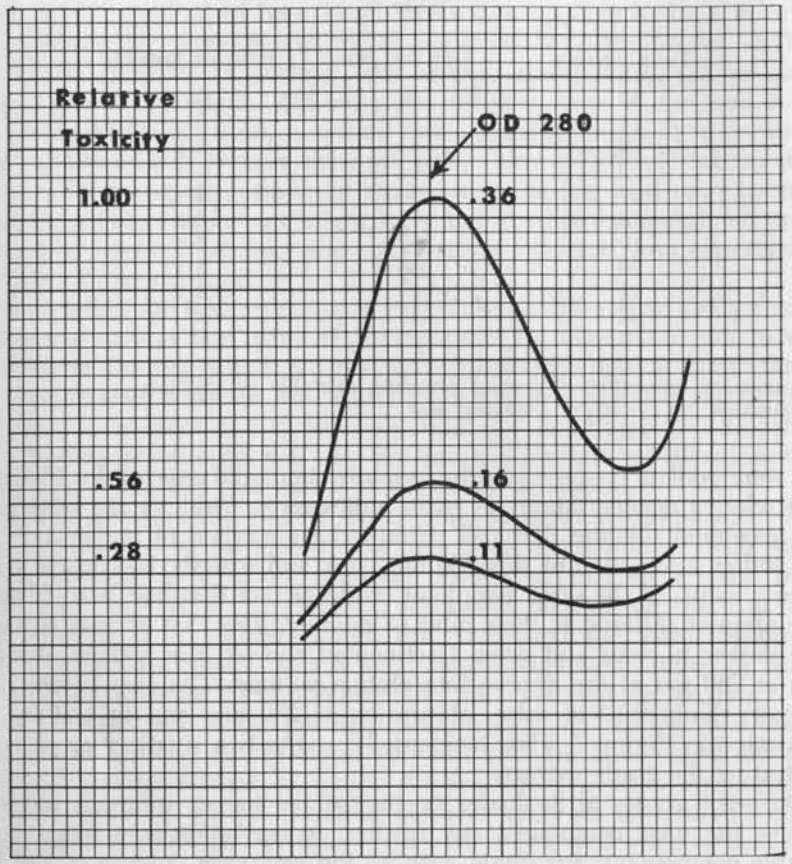

Figure 8. UV spectrophotometric curves in region of OD 280 nm of three concentrations of active fraction of crude toxin. OD at 280 nm and relative toxicity are indicated, the latter based on the highest concentration as 1.

point a still different toxic substance became suspected. When it appeared that we were getting a single entity, quantitative estimates of yield were attempted. The yield of the fraction obtained by gel filtration followed by 0.06 M elution of the product adsorbed on DEAE cellulose was only about 6% of that expected from the crude extract if this fraction were the sole toxic component. Some loss can be expected from the techniques employed, and a second toxin is presumed to be still adsorbed on the cellulose, but even so 6% seemed too low. When a further check was made on the heat lability of the toxin it was found that some toxicity remained after the crude extract was boiled. Moreover it was found that the concentrated residue of fractions from gel filtration after removal of the 280 nm fraction was also toxic. These pointed to another toxic component associated directly with the strongly colored segment which had a UV absorption peak at 330 nm with a very high extinction

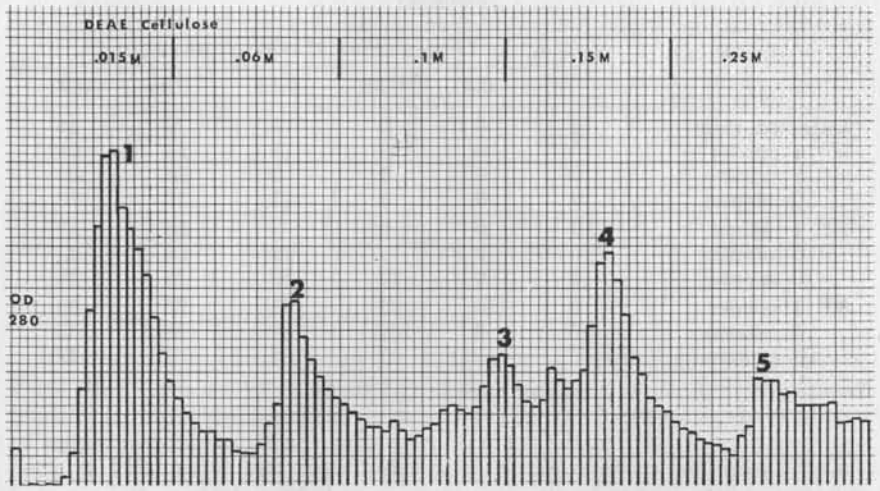

Figure 9. Optical density (280 nm) measurements of elution samples from DEAE cellulose column at different molarities of phosphate buffer eluant. Original sample was of the active fraction derived from gel filtration of crude extract.

coefficient. This region extracted by molecular sieving previously had been shown to have no toxicity.

Heat sensitivity of modified crude extract

Because of the above observations, the heat sensitivity of this apparently different crude toxin was examined. Six ml of crude toxin in a test tube with a thermometer used as a stirring rod were heated in a water bath. The temperature of the solution within the tube was raised to the desired point and then held for 1, 5, or 10 minutes at 50°, 60°, or 80° C. On the basis of previous experience it would have been expected that 50° would have no effect, 80° would destroy toxicity, and 60° would have an intermediate effect. The test extracts were serially diluted and assayed as usual. The results, expressed as number of pupae resulting from 50 eggs, are given in Table 4.

These data indicate that this crude extract is not so heat sensitive as that previously tested, and the likelihood is greater that a separate heat stable toxic substance is present than that the original toxic substance is only partially destroyed by heat.

Effect of photoperiod on toxin production

One significant change in the toxic products of *Afc* grown on the standard medium coincided with a seasonal change. This suggested photo-period as a possible regulator of fungal metabolism, especially as light does seem to affect sporulation of *Afc*. The effect of different light conditions on toxin production was tested as follows.

Table 4

	Concentration of crude toxin					
	1	*0.5*	*0.25*	*0.12*	*0.06*	*0.03*
Unheated toxin	0	0	0	19	19	25
50° 1 minute	0	0	0	13	24	28
5 minutes	0	0	0	0	24	29
10 minutes	0	0	0	13	29	28
60° 1 minute	0	0	0	3	28	23
5 minutes	0	0	0	18	26	23
10 minutes	0	0	19	20	21	24
80° 1 minute	0	28	25	23	23	28
5 minutes	0	0	31	40	38	34
10 minutes	0	0	37	33	39	27
	water control: 20; 33; 24					

Eight standard culture plates inoculated with *Afc* were held in each of four conditions of light, namely continuous light, a 16-hour light period alternating with 8 hours of darkness, 8 hours of light alternating with 16 hours of darkness, and continuous dark. The same temperature of 20° C was maintained in each.

After 4 days of incubation each group of eight cultures was macerated in 100 ml of demineralized water and placed in the refrigerator for 7 hours. The material was then centrifuged and filtered, the filtrate being assayed as crude extract in the usual manner. The results are given in Table 5.

Table 5

	Concentration of crude toxin					
	1	*0.5*	*0.25*	*0.12*	*0.06*	*0.03*
Continuous light	0	0	0	6	1	14
16 hour light	0	0	0	6	20	20
8 hour light	0	0	0	8	14	23
Continuous dark	0	0	0	4	27	21

Clearly the crude extracts showed no difference in toxicity attributable to photo-period. The material was further tested by fractionating each lot of crude extract on Bio-Gel P-30. Thirty ml of crude extract were fractionated by five passages of 6 ml. Corresponding aliquots of the five series were pooled, evaporated at room temperature, each sample then being redissolved in 4 ml of water, three of which were added to 1.6 gm of dog-meal:yeast powder and tested for toxicity. The tube numbers (after the void volume) showing sufficient toxicity to prevent all maggots from developing in the assay units are as follows:

Although these data show no significant differences due to photoperiodic effects, the presence of two distinct toxic fractions is obvious.

Table 6

Continuous light	*16 hour light*	*8 hour light*	*Continuous dark*
3-10 incl.	1-10	1-10	1-8
37-50	41-44	41-52	39-50

If the first can be resolved into at least two toxins by DEAE cellulose, then at least three insecticidal entities are present in the crude extracts.

Significance of insecticidal mycotoxins

Insecticidal mycotoxins can be viewed in at least three perspectives. First, they can be viewed as the mode of attack of a pseudo-parasite leading to a saprophytic life. In these studies *Afc* has shown no evidence of being a primary pathogen, but by this chemical means resulting from its own growth on a separate medium, it can inactivate the insect which can then become a substrate for further growth of the fungus. Although fortuitous and dependent upon the environment of the insect as initial substrate, this is a mode of pathogenic action that explained in milkweed bug cultures an otherwise puzzling pathologic condition.

Secondly, mycotoxins can be viewed as products of fermentation having potential commercial value. Antibiotics of fungal origin were once laboratory curiosities, but fermentation engineering turned them into manufactured products having far reaching significance. The *Afc* toxins here encountered are unlikely to become commercial insecticides. Without a knowledge of their specific activity, their potency cannot be appraised. The toxicity of kojic acid was shown to be low, and low toxicity is suspected for other mycotoxins. Only a highly toxic compound against insects could be a candidate insecticide. More important is that any products of *Aspergillus* would be suspected of undesirable side-effects—guilty by association with the aflatoxins, even if innocent by itself.

Thirdly, such products of living organisms share features of chemistry and parasitology and so bridge the gap between chemical control and biological control. Although chemical entities are responsible for the death of the insects under discussion, the chemical substances are not applied in fixed amounts, but increase in concentration according to the metabolic activity of the fungus, and as the fungus extends its growth so spreads the chemical substances. In the cultural conditions of the laboratory where effects of the toxins were first observed, and presumably under natural conditions, the system is a complex of interacting components. If the fungus gets an early start so that toxin production reaches a level detrimental to the insects in their more vulnerable periods, the fungus takes over and no, or very few, insects develop. If the fungus is delayed in getting established, fly maggots can, by their feeding activity, retard fungal growth and actually overcome and destroy

the existing fungus. In between the fungus-only and fly-only extremes, mutual co-existence is possible with zones for each organism independent of each other or the toxic effects may be sub-lethal so that fly maggots develop but more slowly and diminished in size. These interactions attest to the biologic features of the system, the chemistry being only a part of the mechanism by which the system works.

Apart from the insecticidal features of these mycotoxins, there is wide interest in the metabolic products of *Aspergillus* as they are contaminants of foods and feeds. This interest has developed from the initial observations and discoveries of aflatoxins and their lethal effects on poultry to a widespread research effort to understand the *Aspergilli* as poison producers when grown on foods destined for consumption by humans and domestic animals.

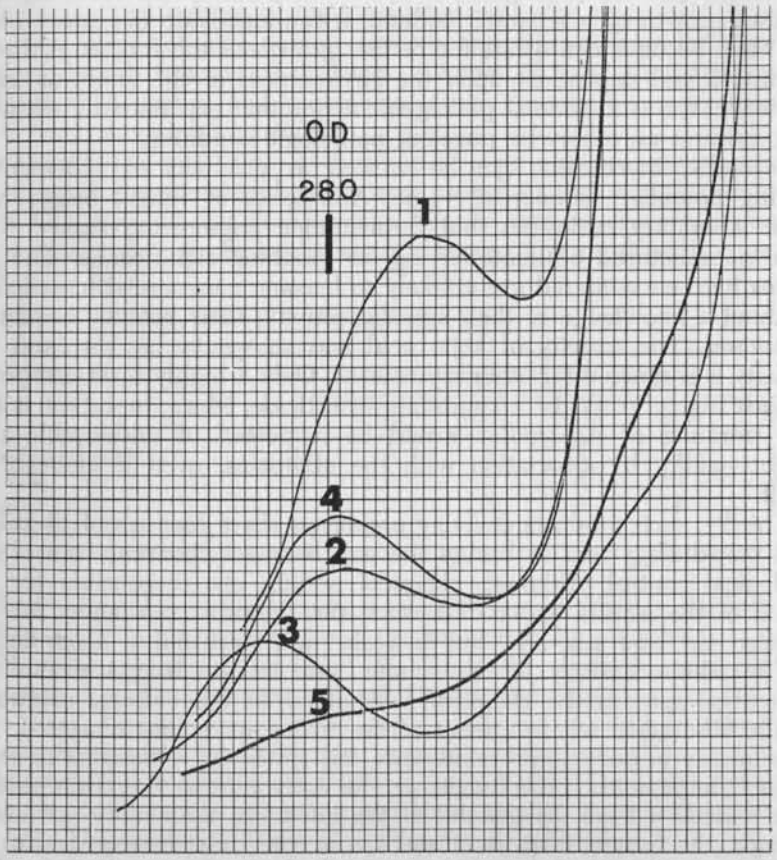

Figure 10. UV spectrophotometric curves in region of 280 nm of samples represented by the five peaks shown in Figure 9.

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