Isolation and characterization of toxins produced by *Fusarium roseum* var. *graminearum*

by

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TABLE OF CONTENTS

INTRODUCTION

The problem of mold metabolites in foodstuffs as a source of toxicosis has received an ever-increasing amount of attention within the last decade. Concurrently with this interest, or because of it, improved methods for the examination and detection of the toxic metabolites, or "mycotoxins", has developed.

Poisoning as a result of mycotoxins has occurred in man and animals for centuries. Only in the last 75 years have specific fungi, in their overgrowth of food, been incriminated as the causative agent. Well documented cases of moldy corn poisoning in pigs, estrogenism in pigs, and aflatoxicosis in turkeys may be cited as examples. Widespread outbreaks of human Fusariotoxicosis in Russia affected up to 80% of the local population in some cases.

The purpose of this study was to ascertain many of the essential characteristics of specified fungal toxins in hopes that it would contribute to their recognition in food. A Fusarium roseum isolate from a field outbreak of mycotoxicosis in swine was cultured on a natural substrate. This overgrown substrate was then extracted and purified. Subsequently one of the purified portions was closely examined to determine its physical characteristics, dose-response, and histopathology in laboratory animals.

LITERATURE REVIEW

Mycotoxicosis

The worldwide occurrence of fungi and their ability to thrive on a seemingly endless variety of substrates has been recognized for centuries. Since fungi have relatively simple nutritional requirements, they are natural invaders of feedstuffs that are improperly stored and, in many cases, may even invade living plant or animal tissue.

Occasional references have appeared in the literature for over 75 years as to the possibility of fungi being toxigenic. Only recently has the problem of mycotoxicosis been extensively studied and accorded the importance it deserves. Extensive reviews of the problem in animals and its potential effect in man have been published by Forgacs and Carll (32), Townsend (92), and Wogan (99).

It has been observed that hepatic carcinoma of man is more prevalent in the newly emerging nations of Africa than in more temperate climates (Alpert, 1). These temperate areas also tend to be more industrialized, and storage facilities are more nearly adequate. A correlation has been established between the overgrowth of food with fungi and the high incidence of carcinoma in many tropical countries.

In Japan, fermented foodstuffs, such as "miso", a fermented soybean paste, and "katsuobushi", fermented dry bonito, are part of the daily diet of most of the populace. A possible link between these foods and disease may be demonstrated by the high incidence of neoplasms (16.7%) as a cause of total deaths in the populace. Cardiovascular diseases accounted for 11% of the total deaths (Kinosita et al., 45).

Ergotism in man and animals has been known since the Middle Ages, especially in those countries of Europe and Asia where rye is extensively cultivated. The medieval accounts of "St. Vincent's Fire" and "Holy Fire" refer to both a gangrenous and convulsive form of the disease. Toxins which give rise to the manifestations are formed in a hard, dark mass on the head of the rye plant or other susceptible grass. This "sclerotium" is the result of invasion of the flowering plant by the parasitic fungus Claviceps purpurea. The fungus replaces the flower portion of the plant with a hard sclerotial mass composed of fungal hyphae. These dark sclerotia are ingested along with regular food and exert their action either on the peripheral circulation, on smooth muscle, or on the nervous system. Action of ergot on smooth muscle has led to its medicinal use in parturition or to control postpartum hemorrhage. The first official recognition of this use in difficult parturition was noted in Saratoga County, New York, in 1808 (Barger, 8). The sclerotium is a complex mixture of many other compounds and has many other medicinal uses, including the treatment of migraine headaches.

Moldy corn and fodder have often been incriminated as a cause of central nervous system distrubances in horses. Lesions of the disease, variously called enzootic cerebritis, leucoencephalomalacia, or "staggers", are usually confined to the meninges and cerebrum. One or sometimes both of the cerebral hemispheres presents a softened appearance with areas of liquefaction necrosis. Feeding experiments have revealed that a minimum of three weeks on moldy feed are required for the symptoms of drowsiness, staggering gait, and occasional delirium to develop (Biester and Schwarte, 11; Buckley and McCaullum, 15; Butler, 18; Schwarte, 81).

In 1891, Mayo (57) published a comprehensive account describing an outbreak of enzootic cerebritis near Manhattan, Kansas. Aspergillus glaucus was regularly found on affected corn, and cultured spore suspensions of this organism proved fatal when given intravenously to guinea pigs. Although he attributed death in the guinea pigs to fungal invasion, hyphae were demonstrable only in the liver. Alcohol and water extracts of the affected corn were nontoxic to horses. A. glaucus was also present in moldy silage that killed horses near Britt, Iowa, as reported by Beaumont (9). It was not proven to be the principle toxic organism as other species of fungi were also cultured from the feed. Samples of the affected silage were used to reproduce the disease at Iowa State College, with death of the horses occurring in from two to four days. However, the affected silage was apparently nontoxic to cattle.

The association of moldy sweet clover with a hemorrhagic disease of cattle was demonstrated by Schofield (80) in 1924. He succeeded in reproducing the disease in laboratory animals, including the rabbit. An Aspergillus species was isolated from moldy hay samples and was used to produce a lot of moldy hay under laboratory conditions. When this spoiled hay was fed to rabbits, many of the lesions produced by the original toxic feed were demonstrated. Roderick and Schalk (78) were not able to duplicate these results on a large scale using an adequate quantity of cultured hay to feed cattle.

In Russia, poisoning of horses and humans has been attributed to moldy straw that was overgrown by the dematiaceous fungus Stachybotrys atra (alterans). Since the organism flourishes over a wide range of temperature

and moisture conditions, it has ample opportunity to grow in improperly cured or stored hay.

Drobotko (29) observed that horses on infected feed developed irritation of the oral mucosa followed by desquamation and eventual ulceration. The oral lesions were accompanied by leukopenia and agranulocytosis. Extracts of the fungal growth caused a marked local irritation of the skin. Many laboratory personnel developed a rash, especially in the axillary region, following handling of the infected hay. Aerosol inhalation of the toxin has been reported with respiratory symptoms and a leukopenia developing in highly sensitive individuals. The disease has been termed Stachybotryotoxicosis by the Russian workers who have studied it most thoroughly.

The ability of fungal toxins to cause a secondary observable lesion by their effect on the liver is evident in the problem of facial eczema of sheep and cattle in New Zealand and Australia. Ingestion of toxic spores of the saprophytic fungus Pithomyces chartarum causes a primary damage to the liver, especially the bile ducts. This results in a retention of bile and reabsorption of phylloerythrin, a normal breakdown product of chlorophyll. Absorption of this porphyrin compound back into the circulation results in a photosensitization of exposed skin areas of cattle and sheep (Dodd, 27; Percival, 71; Worker, 101). Although the photosensitization is a visible result of the toxin ingestion, damage to the liver, especially in acute cases, is the primary cause of death. Recognition and understanding of the cause of the disease was delayed for years due to the sporadic occurrence and lack of a suitable biological assay. It is now established that the fungus primarily produces its toxin sporodesmium on dead perrenial rye

grass and only under restricted atmospheric conditions. Baby guinea pigs and rabbits are the animals of choice for assay of suspected toxic feeds (Dodd, 27; Percival, 71; Worker, 101).

A similar condition has been reported in cattle in Florida with photosensitization possibly being more manifest. The disease occurred primarily in animals grazing on bermuda grass that had been frosted or drought-killed several weeks previously. New plant growth started along with a subsequent luxuriant growth of fungi on the dead plant tissue. The predominant fungus observed on dead grass has been Periconia minutissima. In untreated herds of cattle, mortality ranged as high as 20%. Treatment with sodium thiosulfate effectively lowered the rate to near 3% (Kidder et al., 44).

Moldy corn was established as the source of toxin for an outbreak of toxicosis in pigs in 1951-1952 which occurred in Georgia and Florida. The outbreak followed a drought year when corn was badly stunted and soft varieties matured quite early. After the corn had fallen over, pigs were turned into the fields and used to glean the down grain which was overgrown with mold. Sippel et al. (85) estimated that over 2,000 pigs were affected, but probably only a small percentage of the overall total was reported.

A high mortality rate was observed with postmortem lesions showing primarily hemorrhage of most internal organs, inflammation of the digestive tract, and liver damage. Cultures of toxic corn samples resulted in the isolation of 13 strains of fungi. Two of these, Aspergillus flavus and Penicillium rubrum, proved toxic when they were cultured on corn and fed back to test pigs. Lesions similar to the original cases were demonstrated. Supernatant fluid from P. rubrum cultures was fatal to all of four treated horses within five days (Burnside et al., 17).

One of the most potent fungal toxins described to date, and certainly the most thoroughly studied, is aflatoxin. In 1961, Blount (12) called attention to an outbreak in turkeys that killed about 100,000 birds before it subsided. Studies revealed that all the turkeys had eaten feed which contained groundnut meal from South America. Examination of this and other suspicious lots of groundnut meal indicated a high level of toxin; on culture, Aspergillus flavus was the most common fungus isolated.

Extensive study during the ensuing ten years has provided a wealth of information about aflatoxin. It is a potent carcinogen in rats and, in higher concentrations, may be fatal in a matter of hours in many laboratory animals (Kraybill, 47; Sargeant et al., 79).

Intensive work in Japan within the last two decades had led to the characterization of several toxins produced by molds. Notable among these are islanditoxin and leutoskyrin produced by Penicillium islandicum (Uritani, 96). Both substances are highly toxic to laboratory animals, and islanditoxin has been found to possess carcinogenic ability.

Fusariotoxicosis

The genus Fusarium is placed in the class of imperfect fungi, although it has been found that many species have a perfect stage. In general, members of this genus produce branched conidiophores with terminal, spindle or sickle-shaped macroconidia. It is a difficult genus to subclassify due to variability within species and a tendency to mutate, thus presenting a different taxonomic picture from the wild isolate to the laboratory strain. Many of the parasitic species have a perfect stage, often in the genus Gibberella (Atanasoff, 4).

Many members of the genus are common soil fungi and are prominent as parasites on field crops. Involvement of this group of fungi in a "scab" condition on wheat was reported in Iowa in 1868 (Scott, 83). In a survey of central Iowa storage facilities in 1966, shelled corn samples were collected from 25 bins. Culture of these samples yielded 246 fungal isolates, seven of which were Fusarium spp. These seven isolates were cultured on rice, extracted with ether, and the extract administered to mice and ducklings. Three of the extracts killed ducklings, and one was fatal to mice (Richard, 76).

A survey of field crops in Canada has revealed that oats, barley, and wheat are routinely infected by Fusarium sp. (Gordon, 36). Over a four-year period, more than 3,700 seed samples were cultured. Oats had the highest number of isolates with nearly 80% of the samples originating in eastern Canada being positive compared to 39% of the samples originating from western Canada.

Prior to 1960, most of the research on fusariotoxicosis was conducted in Russia. Many widespread outbreaks have been experienced in their vast grain regions. The lack of satisfactory harvest and storage conditions at times no doubt contributed to the presence of toxins in foodstuffs. Fusarium roseum was almost invariably found as the predominant fungus in affected food in the Primorski region of Siberia in 1916 and in European Russia in 1923. In some areas, up to 88% of the harvested barley was affected (Dounin, 28; Naumov, 67). Toxic symptoms included headache, nausea, and vomiting. Bread made from affected barley was known locally as "drunken bread". In cases where flax seed was affected, linseed oil reportedly had an "inebriant" quality.

The most extensive outbreak reported in human medicine occurred in Russia during the period 1942-1945. Intensive study showed that two species of Fusarium, F. sporotrichioides and F. poae, were toxin producers. The poisoning was usually due to the consumption of millet that had overwintered in the fields during the wartime when labor was scarce (Gajdusek, 33; Joffe, 39, 40; Mayer, 55, 56). Symptoms and lesions varied with the length of exposure and amount of toxic food ingested. The mortality rate varied from 5-80% in different districts. For the clinical disease to occur, an exposure of two to three weeks was usually required; an occasional case would result from the use of a few hundred grams of the toxic flour. A necrotic angina, extreme leucopenia, and multiple hemorrhages were observed in most cases, often with a fatal termination. Treatment was usually symptomatic with rest, nutritional supplements, and removal from the offending food.

In addition to the Russian outbreaks, poisoning by Fusarium species has occurred in Japan and Korea. Uraguchi et al. (95) report that in 1954 in Tokyo, "25 reckless lads" suffered toxic symptoms, including nausea and dizziness, from eating infected rice. As recently as 1964, a severe outbreak of scab in barley in Korea was responsible for toxicity in man and domestic animals (Cho, 21).

A disease of livestock that showed many of the symptoms of ergotism but caused by Fusarium was described in Nebraska in 1904. Pigs were usually affected more severely than other farm animals, primary lesions being a sloughing of the hoofs and shedding of hair. On some farms, the mortality rate exceeded 95%. Cattle and horses were also affected, though not as severely or as frequently, showing typical symptoms of ergotism. Chickens

on affected feed would quite often lose their feathers, and their eggs would be infertile. It was reported that some farms had experienced the condition for up to nine years. In nearly all cases investigated, sick animals had been eating poor quality corn overgrown with a pink mold which was later identified as Fusarium moniliforme. Cultures of this fungus on a cracker and bran mixture were effective in reproducing the disease in swine (Peters, 72; Sheldon, 84).

The "fescue foot" syndrome describes a condition that occasionally occurs in cattle grazing on tall fescue pasture. Symptoms vary from a loss of weight to sloughing of extremities, the latter case sometimes being confused with ergotism. In examining samples of fescue hay from suspected pastures, strains of Fusarium tricinctum have been isolated that showed toxicity when administered to laboratory animals. When a purified compound from this strain was administered to test cattle, a sloughing of the tail was observed on one animal (Grove et al., 37; Yates et al., 102, 103).

The estrogenic effect of toxins from the genus Fusarium has been recognized for over 40 years. The condition is most frequently reported in hogs with symptoms similar to those produced by other estrogenic compounds, i.e., vulvovaginitis, with possible prolapsing of the vagina, rectal prolapse, and mammary swelling in the female. In the male, mammary swelling and preputial swelling have been noted. Abortion is sometimes observed in pregnant sows. F. graminearum has been frequently identified as the organism causing abortion with cultures of this organism reproducing the original symptoms and lesions. The toxin has also been identified as a metabolite of F. tricinctum and five varieties of F. roseum (Buxton, 19; Caldwell et al.,

20; Christensen et al., 23; Kurtz et al., 48; McErlean, 51; McNutt et al., 52; Mirocha et al., 62, 63; Stob et al., 88; Urry et al., 97).

The estrogenic metabolite has been purified by several investigators and studied extensively. It is variously called "zearalenone" or "F-2 toxin". When 24 kg gilts were given one mg oral doses for eight days, vulvar tumefaction was observed. Increasing the daily dose to five mg yielded the same results in five days. Histologic changes were confined to the mammary gland, ovary, cervix, and vagina.

Infertility problems have occurred in dairy cattle in England which had been fed a poor quality hay. In order to get the cattle to eat the hay, it was chopped and mixed with silage. F. moniliforme was isolated from cultures of the hay, and a level of 14 ppm of estrogenic toxin was reported (Mirocha et al., 61).

In 1928, the barley crop of several states in the Midwest, including Iowa, was severely affected by a scab condition with up to 40% of the grain reportedly overgrown with fungi. Numerous feeding experiments with this affected barley showed that it was toxic to farm animals, particularly hogs. When fed the barley as an exclusive diet, the pigs found it distasteful, tending to starve themselves. If they were forced by hunger to eat or if the barley was mixed with milk to induce them to eat, they developed nausea and diarrhea. The vomiting persisted in most cases as long as the hogs were on the diet, but the diarrhea usually subsided (Dickson et al., 26; Mains et al., 53; Mundkur, 65; Mundkur and Cochran, 66).

Gibberella saubinettii was isolated from affected grain and used to culture a quantity of normal barley. When this was fed to test pigs as 10% of their ration, an immediate weight loss was noted, but no vomiting

occurred. Water extracts of the cultured barley from field cases induced vomiting in test pigs.

Mature chickens apparently tolerated the feed quite well, but two-week-old birds lost weight and developed rough plumage possibly due to voluntary starvation. Little change was noted when cattle and sheep were fed the affected grain.

A large quantity of barley from the 1928 outbreak was shipped to Germany and caused similar trouble when fed to pigs in that country. G. saubinettii was again isolated from the barley and used to reproduce symptoms similar to the original outbreak (Anonymous, 2; Meissner and Schoop, 58). After the source of the toxicity was found, all barley entering Germany was subjected to animal testing before being used for food. In one report of 45 samples tested for toxicity, 25 were held suspect on the basis of swine feeding experiments. Interestingly enough, barley originating from Kansas, Texas, Oklahoma, and Colorado was later exempt from the feeding requirement, indicating the scab condition was apparently not as severe in the drier climates.

Fusarium-infested grain has also been tested by other investigators with varying results. Mature chickens seemed to be resistant to any toxic effects of the organism as test birds were essentially the same as those on normal barley (Titus and Godfrey, 91). However, in another series of tests, Gibberella saubinettii-infected barley proved inadequate, as test chickens died within three weeks (Vinson et al., 98).

In comparing the response of turkey poults to Fusarium toxins, Meronuck et al. (59) used corn invaded by F. roseum and F. oxysporum. The F. roseum isolate was known for its ability to produce estrogenic toxin.

Response of the poults included enlarged cloacae, swollen vents, and enlarged bursae of Fabricius. The F. oxysporum-invaded corn caused a marked weight loss when mixed as 10% of the ration and 100% mortality was observed when the proportion was increased to 40%.

In experiments reported by Roche and Bohstedt (77), barley from the 1928 outbreak apparently did not affect cattle or sheep. When rations of up to 70% of infected grain were fed to these animals, little change was noted in their condition. Finnish workers have found that feed cultured with F. roseum is tolerated in quantities up to 20% of the ration before being rejected (Rainio, 75).

Fusarium-infected grain has also been tested for its growth-promoting properties in poultry. In comparing the efficiency of two isolates over a protein-poor control mixture, improved growth rate was noted during a four-week period (Borcheks and Peltier, 14).

The toxins of fusariotoxicosis are in general long lived under normal storage conditions. Maximum time at which toxin could be detected in naturally infected food ranged up to four to six years in the case of the 1942-1945 Russian outbreaks (Gajdusek, 33; Joffe, 42). Insofar as the emetic principle from infected barley is concerned, toxicity has been variously reported at from 38 to 56 months (Christensen and Kernkamp, 25).

The genus Fusarium is normally considered as one of the "field fungi"; that is, it primarily grows on plants that have not yet been harvested (Christensen and Kaufmann, 22). Due to its minimum moisture requirements of 24-25% (wet weight basis), it normally would not grow on grain in storage with the possible exception of crib-stored corn that is exposed to wet weather. Koehler (46) was able to demonstrate growth of F. moniliforme at

18.4% moisture content but considered 23% as the minimal level to cause serious rot of corn.

In the laboratory, various natural and artificial substrates have been employed to experimentally produce Fusarium toxins. The tendency has been to use the same substrate as that from which the toxic isolate was obtained. Corn or corn and rice mixtures have produced high levels of toxin, while the Russian workers found that millet produced levels comparable to or exceeding those from the natural conditions. In a comparison of four grains, rice, oats, corn, and barley were cultured with F. nivale and found to produce descending amounts of toxin in the order listed (Caldwell et al., 20; Christensen et al., 24; Richard, 76; Stob et al., 88; Ueno et al., 94).

Several investigators have employed artificial media to advantage. In a comparison of emetic toxin produced on two media, Richard's solution and a beef extract-glucose-peptone mixture were tested. Emetic material was obtained from four of eight species. Other media employed include Sabouraud's agar, Sabouraud's maltose broth, and Czapek's broth with added peptone (Fahmy, 31; Keyl et al., 43; Prentice and Dickson, 73; Prentice et al., 74; Ueno et al., 94; Yates et al., 103).

As mentioned previously, the Fusarium group has a comparatively high moisture requirement in order to obtain minimal growth in its natural state. This requirement also holds true in artificial culture, and a 40-45% moisture content is routinely used in laboratory work with an upper extreme of approximately 70%. The usual procedure is to add the required amount of water to the preweighed grain or other substrate, allow to soak for at least one hour, and autoclave for at least 30 minutes (Caldwell et al., 20; Christensen et al., 23; Meronuck et al., 59).

Joffe (41) demonstrated that F. poae and F. sporotrichioides would grow at temperatures of -7° C and that toxin formation was most active at temperatures just below zero. In his work, he also found that sharp temperature fluctuations brought an intensive accumulation of toxin. Another observation was that nontoxic isolates of Fusarium did not possess cryophilic properties. Christensen et al. (23) used varying temperatures, i.e., three weeks at 25° C and two weeks at 12° C to obtain optimum production of an estrogenic metabolite from F. graminearum. In an extension of this work, a yield of 390 ppm of toxin was obtained by culturing at 12° C with no apparent yield when cultured only at 25° C.

Other investigators have reported optimum temperatures that vary according to the strain of fungus being tested. Strains of F. tricinctum produced a highly toxic scirpene compound at low temperatures (7-8° C) while a second toxic metabolic product of similar structure and toxicity was produced at 25° C from the same strain. In contrast, certain strains of F. nivale yielded maximal toxin output at 27° C with very little produced at low temperatures (Bamburg and Strong, 6; Berry and Magson, 10; Gilgan et al., 34; Ueno et al., 94).

Extraction procedures usually consist of exposing the overgrown substrate to water or organic solvents for varying periods of time. Since some of the toxins are water-soluble, a combination of procedures sometimes yields more than one toxin. For most effective use, an organic solvent should be able to remove a high level of toxin and yet be relatively easy to evaporate so that the residue might be readily available for dosing animals or for further testing and purification.

With these criteria in mind, Christensen et al. (23) compared ten organic solvents or combinations of solvents in extracting an estrogenic compound from a culture on a corn and rice medium. They employed a Soxhlet apparatus and found that methylene chloride was the solvent of choice for that particular toxin due to its ability to extract large amounts of toxin and its low boiling point.

A combination of procedures enabled other workers to eliminate water-soluble fractions leaving the remaining wet cake portion to be repeatedly extracted with anhydrous methanol. This methanol extracted fraction also had an estrogenic action (Stob et al., 88).

In surveying tall fescue pastures for toxic Fusaria, it was found that various solvents were effective in removing three toxins. When agar-plate cultures were used to produce toxin, either methylene chloride, ether, or an 80:20 ethanol:water mixture were used. From moldy grain or liquid cultures, ethyl acetate was employed for extraction (Keyl et al., 43).

In the Russian outbreaks, hundreds of fungal isolates were screened for toxicity after preliminary culture on millet or other substrates. It was found that either alcohol or ether satisfactorily extracted the crude substrate. These extracts were then concentrated and used for bioassay (Joffe, 40).

Hot water was employed to remove an emetic principle from scabby barley by Hoyman (38). The extract was further concentrated with methanol and ether to a syrupy consistency. Initial extraction with organic solvents was not attempted. In similar work in Russia on water-soluble toxins, Kvashnina (49) used saline to extract a toxic fraction that caused paresis, incoordination, and death of mice. In addition, a fatty

fraction from the affected feed and water-soluble fractions were further treated with ethyl ether. It was observed that strains that were originally toxic retained this activity in the ether fraction.

While most workers prefer to extract toxic material for not more than 24 hours, Mock and Robbers (64) used petroleum ether and continuous extraction for seven days.

The use of active charcoal in purifying liquid cultures has been reported by Japanese workers. The partially purified preparation is precipitated by heating a methanol suspension and using chloroform to further precipitate nontoxic fractions. In the case of infected grain, a preliminary removal of lipid material was obtained with hexane. The remaining material was evaporated, resuspended in methanol, and prepared for treatment with activated charcoal (Ueno et al., 94).

Early work on the description of the toxic principle from Fusarium and on the dose required to produce toxic symptoms was necessarily influenced by the purity of the substance being tested. Most of the extracts were composed of not only the toxin but many other compounds as well.

In their work on the estrogenic toxin, Mirocha et al. (60) were able to obtain a purified product by the combination of column chromatography, thin-layer chromatography, and crystallization. Silica gel was used in both chromatographic procedures. For thin-layer development, a 5% ethyl alcohol in chloroform solvent was used. For column separation, two solvents were used. Nontoxic metabolites were removed with petroleum ether after which the toxic fraction was eluted with methylene chloride. This fraction was concentrated in a countercurrent distribution apparatus, and

crystallization of the toxin was obtained with a chloroform:petroleum ether system.

Prentice and Dickson (73) separated emetic toxins from crude material by column chromatography. Cellulose powder was developed with benzene, benzene:n-butanol (2:1), and 95% ethanol. Active fractions from the cellulose column were further purified on a DEAE cellulose column.

Column chromatography enabled Bamburg and Strong (6) to purify toxins produced by F. tricinctum. Silica gel was employed in a preliminary purification, and one of the toxic fractions obtained was further purified on an alumina column.

Thin-layer chromatography has been used both as a purification procedure and as a means of detecting single compounds in partially purified preparation. Yates et al. (103) were able to identify two toxins from suspected fungal strains when silica gel plates were inoculated with toxic extracts and developed with ethyl acetate:toluene (3:1). The R_f values were on the order of 0.2 and 0.6, respectively, for toxins I and II. Detection limits of both toxins were about ten μgm.

Crystallization attempts have met with varying success. As noted previously, the estrogenic toxin of Mirocha et al. (60) apparently crystallizes easily with a chloroform:petroleum ether system. Grove et al. (37) used a toluene:Skellysolve B, toluene, benzene:Skellysolve B system to obtain hexagonal plates from an F. tricinctum extract. Yates et al. (102) crystallized a toxin from F. nivale with ethyl acetate:cyclohexane. In contrast, Bamburg and Strong (6) used various solvents over a period of several months but were unable to induce a "high temperature" toxin from F. tricinctum to crystallize.

Recent work on Fusarium toxins has allowed a comparison of selected characteristics among the various compounds. Mirocha et al. (60) in characterizing their estrogenic compound determined the molecular weight was 318. The melting point ranged from 163-165° C, and ultraviolet spectrophotometry showed maximal peaks of absorption at 314, 274, and 236 mμ.

F. tricinctum has demonstrated its ability to elaborate several toxins (Bamburg and Strong, 6; Gilgan et al., 34). Two of these compounds, diacetoxyscirpenol and a "T-2" toxin, have molecular weights of 306 and 466. Corresponding melting points are 171.5-173° C and 151-152° C. Further culture work with this fungus at higher temperature revealed a third toxic metabolite designated "HT-2". This compound had a molecular structure very similar to the T-2 toxin, lacking only one acetyl group of the low temperature toxin.

Yates et al. (102) reported in 1968 the isolation of a metabolite from F. nivale with a molecular weight of 141 and a melting point of 116-117.5° C. They also isolated a compound identical to the T-2 toxin mentioned previously. Further research on the identification of this strain of Fusarium showed that in reality it was an atypical strain of F. tricinctum (Grove et al., 34).

Elpidina (30) has partially determined the structural characteristics of an antibiotic from F. sporotrichiella, var. poae. Crystals had a melting point of 142-143° C and contained 59.7% carbon, 7.77% hydrogen, and 32.53% oxygen. The material proved capable of arresting tumor growth in experimental animals.

In two of the few Russian papers of fusariotoxicosis available, Olifson (69, 70) described a toxin from the fatty part of wheat and barley

wintered under snow. The compound had the characteristics of a six-membered lactone ring. Its ultraviolet absorption spectra had a maximum of 300-310 and minimum of 260-285 mμ.

Tatsuno et al. (89) prepared an isolate of F. nivale in Japan and described a toxic fraction with a molecular weight of 312, melting point of 222-223° C, and a single UV absorption spectrum at 220 mμ.

In the field of antibiotic research, test animals are essential to determine the potential for antibiotic substances to produce toxicity in the host animal. Arnstein et al. (3) determined that two antibiotic pigments isolated from F. javanicum could be tolerated by mice only to a maximum dose of ten mg when given intraperitoneally. Elpidina (30) found that a dose of 100-150 mg injected subcutaneously was necessary to kill mice with his antiblastic preparation from F. sporotrichiella.

Further experiments with mice have demonstrated paresis, incoordination, and death when extracts from Fusarium-infected feedstuffs were administered intraperitoneally or subcutaneously (Kvashnina, 49; Tatsuno et al., 89). The LD_{50} for nivalenol (from F. nivale) has been established at 41 μgm/10 gm in mice. Pathological findings include necrosis and degeneration of the proliferating cells in the gastrointestinal epithelium, bone marrow, lymph nodes, thymus, and testes.

Many of the fungal toxins studied have demonstrated their toxicity to animal skin, especially that of the rabbit and rat. Using this characteristic as a criterion of toxicity, Joffe (40) and co-workers examined hundreds of isolates of Fusarium and other fungal species in Russia.

Rabbits were used to test for dermal toxicity with responses varying from leukocytic infiltration to hemorrhage and necrosis of skin and, in

many cases, death of the rabbit. Oral administration to guinea pigs and rabbits of liquid filtrates, or powdered mycelium, from toxic strains resulted in death in from five to 24 days. Autopsies showed dilated blood vessels and hemorrhage in most organs and tissues.

Further outbreaks in Russia affecting domestic animals included one investigated by Loginov (50). He reported that newly weaned pigs were especially susceptible when they consumed bran from which F. sporotrichioides was isolated. An ether extract of the bran caused dermal necrosis and killed mice in six to seven hours. A similar condition caused by the same species of fungus gave clinical signs such as eruption of red spots on the skin one to six cm in diameter, edema of the eyelids and neck, and dyspnea (Marchenko and Resnyanshaya, 54).

In screening fungal isolates from tall fescue for toxicity, rabbits were used in a skin-bioassay test (Keyl et al., 43; Yates et al., 102). Extracts of cultures were evaporated and the residues resuspended in olive oil. Application of this suspension from toxic strains to the unabraded skin of rabbits resulted in reactions ranging from edema to necrosis. When ten-fold concentrates of F. nivale culture extract were injected intraperitoneally in mice, extensive visceral hemorrhage resulted.

Bamburg et al. (7) state that all the scirpene compounds cause a skin irritation. This becomes important in working with the compounds in the laboratory as workers have suffered from facial inflammation of the skin with subsequent desquamation and local irritation. The scirpene compounds described by these workers all proved irritating to rat skin; therefore, this animal was used for bioassay procedures. Gilgan et al. (34) found that 0.5 mg of crystalline toxin caused a marked response in rat skin tests.

In comparing the skin-necrotizing effects of various Fusarium toxins, Ueno et al. (93) utilized the rabbit, guinea pig, and mouse. They found the guinea pig was most sensitive, reacting to as little as 0.2 µgm of diacetoxyscirpenol. The skin showed a red ring within one-half day after application of this amount. When ten µgm and 100 µgm amounts were applied, the skin showed a marked hyperemia followed by crusting within a few days. Fusarenon-X and nivalenol, toxic metabolites from F. nivale, were not as effective, requiring from ten µgm to 100 µgm to show skin reaction.

In the work previously mentioned by Joffe (40), cats were fed various preparations from Fusarium infested substrates. They subsequently showed many of the effects of a general breakdown in the blood-vascular system that had been noted in man. Included was a decrease in percentage of hemoglobin, erythrocytes, leucocytes, and neutrophiles. The cat was considered to be the best model for reproduction of the disease as seen in man. Dogs and horses died after ingestion of one gm and 40 gm of the toxic cultures, respectively.

In an earlier report, Tistovich and Levinson (90) found that maize infested with Fusarium was toxic to dogs if fed as more than 10-12% of the ration. Pigeons, hens, and rabbits appeared to suffer no ill effects.

Scott (82) considered the duckling a valuable aid in screening procedures. He mixed one part of moldy feed with three parts of chicken meal as a ration and considered a meal toxic only if it killed all three test animals within 14 days. He reported that two of ten isolates of F. moniliforme and both strains of F. roseum tested fulfilled this criterion.

Weanling female rats and ovariectomized mice have been widely used as test animals for estrogenic toxins (Caldwell et al., 20; Christensen et al.,

23). The uterine weight of the rat increases significantly when the animals are either fed toxic feed or are injected with purified toxin. For injection purposes, the toxin may be suspended in propylene glycol and given intramuscularly. A similar response in uterine weight has been noted in ovariectomized mice when either fed cultured substrate or given subcutaneous injections of purified toxin.

Researchers working with the emetic principle formed by some strains of Fusarium are sometimes handicapped by the unresponsiveness of animals, other than pigs, to the toxin. Signs of toxicity usually consisted of nausea and incoordination, occasionally with diarrhea. Prentice and Dickson (73) have demonstrated that toxic material injected into the wing vein of pigeons will cause a rapid emesis. As little at 100 µgm of partially purified material will give this reaction. Hoyman (38) used the pigeon but administered the toxin orally and could not detect a greater sensitivity than with the pig.

Several laboratory animals were used to screen barley samples for emetic toxin in Korea in 1963 (Cho, 21). It was found that intraperitoneal injection of 0.3 ml of a concentrated water extract into suckling mice effectively demonstrated toxicity while chickens, rats, rabbits, and adult mice were resistant to the emetic principle. Pigs given at least ten ml of concentrated extract showed typical symptoms.

Burmeister and Hesseltine (16) have developed a rapid and easily performed screening test using the inhibition of pea seed germination as a measure of toxicity. They found that when pea seeds were soaked overnight in water containing as little as 0.5 µgm of "T-2" toxin per ml, germination was reduced to less than 50%. They also used a disc-assay technique to

check the toxin's ability to inhibit the growth of bacteria and fungi. Five of 11 fungal strains, but none of 54 strains of bacteria, were inhibited.

EXPERIMENTAL PROCEDURE

Animals

Chickens and weanling rats were used to test extracts for lethal toxicity. The chickens were day-old, broiler-type cockerels obtained through the courtesy of DeKalb Hatcheries, Roland, Iowa. Twenty-one-day-old white rats, male and female, were used for initial screening of toxic material.

White rats were also employed in testing certain extracts for estrogenic potential. Weanling female Dublin-Sprague Dawley Derived white rats were obtained from Flow Research Animals, Inc., Dublin, Virginia, for this purpose.

New Zealand White rabbits, weighing approximately 3.5 kg, and 350 gm white guinea pigs were employed in testing for dermal necrosis.

Chemicals and Glassware

All chemicals used were of reagent grade available through Chemistry Stores supply, Iowa State University. Glassware was thoroughly oven dried before use. All glassware used for storage or final weighing purposes was acid washed, rinsed thoroughly, and oven dried.

Production of Toxin

Toxic material was obtained by the culture of a known toxic strain of Fusarium roseum, var. graminearum, (Mapleton 10 strain)[1] on an equal mixture of moistened, autoclaved corn and rice. This organism had been isolated from a field case of mycotoxicosis in pigs. All inoculations were

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[1]Kindly supplied by Dr. C. M. Christensen, University of Minnesota, St. Paul, Minn.

made from the same stock culture. Little loss in viability was noted over a three-year period of time.

Selected ears of mature, field-ripened corn were hand shelled, and the individual grains were carefully checked to remove any obviously moldy or deformed kernels. Polished rice was purchased through a local grocery in 25-lb lots.

500 gm portions of both rice and corn were measured into a 2,800 ml Fernbach flask, moistened with 400 ml of distilled water, closed with a cotton stopper, and allowed to soak for one hour. The mixture was then autoclaved at 121° C for 30 minutes and allowed to cool. The next day, the flask was shaken thoroughly and autoclaved for an additional 30 minutes. Inoculation of the cooled corn and rice mixture was made in a sterile hood using a soil culture of the fungus.

After a small portion of the inoculum was placed on the mixture, it was stoppered, shaken to distribute the spores, and placed in a 25° C incubator. The mixture was cultured for approximately two weeks with occasional shaking to prevent caking.

At the end of two weeks, the overgrown mixture was removed, air dried at room temperature in a fume hood, and chopped in a Waring blender. At this stage, the mixture was suitable for extraction or storage.

In a separate feeding experiment, three cultural procedures were used to determine if culture variations caused an increase or loss of toxicity. After initial inoculation, three lots of corn and rice were cultured on the following schedule: (a) two weeks at 25° C, (b) two weeks at 25° C, three weeks at 10° C, (c) two weeks at 25° C, one week at 10° C, two weeks at

25° C. After the incubation period had elapsed, the cultured substrate was dried, chopped, and mixed with commercial chicken feed at various levels.

Phase I of Extraction and Purification

Extraction and purification procedures were primarily modifications of the methods of Christensen et al. (23) and Richard (76). All procedures were conducted in a darkened room or shielded from direct light. If it was necessary to store material between steps, it was enclosed in a stoppered tube or flask, flooded with nitrogen, and tightly sealed. The container was then stored at 4° C until needed.

Extraction of the ground substrate was originally attempted using three organic solvents, viz., methylene chloride, chloroform, and methanol. Chloroform proved to be most adaptable due to the fact it extracted more toxic material than methylene chloride and was more easily removed by evaporation than methanol while nearly equaling it in extracting power.

Extraction of toxin from the chopped, dried substrate was accomplished in a Soxhlet apparatus. Large quantities of crude toxin could be obtained by using a size D Soxhlet apparatus and a 60x180 mm Whatman extraction thimble. 500 gm of substrate was weighed into a large beaker, 100 ml of water added, and mixed thoroughly. This moistened substrate was then packed into two extraction thimbles.

Overnight extraction with approximately 600 ml of chloroform per apparatus yielded a brown pigmented suspension. After combining the extracts, the chloroform was evaporated by use of a rotary evaporator[1] and a 50° C

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[1] Rinco Instrument Company, Inc., Greenville, Ill.

water bath. Evaporation continued until only a pigmented syrup-like residue remained.

100 ml of methanol was added to the extraction flask, the material transferred to a 200 ml volumetric flask, 30 ml of water added, and the volume brought to 200 ml with methanol.

Lipid materials were removed by extraction with hexane in a 500 ml separatory funnel. The 200 ml of toxic material was poured into the funnel and 100 ml of hexane added. After thorough agitation, the lower toxic layer was harvested, and the nontoxic hexane layer containing fats and oils was discarded.

To remove the toxic fraction from the methanol-water mixture, the alcohol concentration was further reduced by the addition of 80 ml of water. The material was then shaken four times in a separatory funnel with 50-ml quantities of methylene chloride. In order to expedite removal of the pigmented toxic portion, 20 ml of saturated sodium chloride was added.

The lower methylene chloride layer was removed and filtered through anhydrous granular sodium sulfate in order to remove any residual water. The methylene chloride was removed on a rotary evaporator, again leaving a light brown syrupy fraction. After restoring to a 10-ml volume with methylene chloride, the fraction was ready for further purification.

Phase II of Purification

The second step in purification, generally following the procedures of Stahl (86, 87), utilized thin-layer chromatography (TLC) using a silica gel

layer[1] on a glass plate. For preparative work, 20x20 cm plates were coated with a layer one mm thick. For analytical determinations, a 250 μ layer was used on either 5x20 cm or 10x20 cm plates.

A slurry of the silica gel was prepared with water using approximately 98 ml of water to 45 gm of silica gel. This served to cover three 20x20 cm preparative plates with very little waste. For analytical plates, approximately half the above amounts of silica gel and water covered enough five and ten cm plates to fill an applicator board.

Thin-layer plates were made with a standard Desaga applicator[2] and allowed to dry overnight. After drying, the plates were activated by heating at 120° C for one hour and were then ready to develop. If not used immediately, the plates were stored in a closed cabinet and activated just prior to use.

After the activated plates were cool, they were inoculated with the toxic preparation. For analytical purposes, micropipettes were used while preparative plates were inoculated with a pasteur pipette. A band of the sample was applied across the width of the plate about one cm from the lower edge.

Preliminary screening with analytical TLC plates using various solvents showed that the methylene chloride soluble preparation would separate into fluorescent bands when a mixture of ethyl acetate (80%) and cyclohexane (20%) was used as a solvent. It was also observed that when this separa-

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[1] Silica-Gel HR according to Stahl. Brinkmann Instruments, Inc., Westbury, N.Y.

[2] Brinkmann Instruments, Inc., Westbury, N.Y.

tion was carried out in an oxygen atmosphere, the fluorescent bands would apparently oxidize as they moved up the plate. This caused a diffusion of the various components and made separation impossible. Subsequent work for all purification procedures was carried on under an inert atmosphere of nitrogen.

After inoculation, the plates were immediately placed in a lined equilibrated developing chamber and tilted so that the solvent (solvent A, ethyl acetate:cyclohexane, 80:20, V:V) could not touch the plate. Through the use of a special lid, nitrogen was flooded into the chamber for ten minutes. The nitrogen was then shut off and the chamber closed and allowed to equilibrate for ten minutes. The chamber was then leveled to allow the solvent to contact the plate, and development proceeded for approximately 20 minutes or until the solvent front neared the top of the plate.

After the development was completed, the plate was removed from the chamber, allowed to dry for several minutes, and fluoresced under shortwave ultraviolet light.

Fluorescent bands in the silica gel were outlined with a spatula and scraped into 50 ml screw-cap tubes. The material in the silica gel was next eluted three times by adding 20 ml quantities of methanol, shaking for one minute, and centrifuging for ten minutes at 1,500 rpm to settle the silica gel.

The methanol extracts were collected and placed on a rotary evaporator until nearly all the methanol had been evaporated. Final evaporation was accomplished in a water bath at 50° C.

Silica gel was removed from the residue by suspending it in five ml of benzene containing 1% acetonitrile. After centrifuging, the supernatant

fluid was collected and the procedure repeated two more times. The collected supernatant was again dried, weighed, and prepared for toxicity checks and further purification.

Phase III of Purification

Toxic bands were further purified by again inoculating a preparative thin-layer plate and redeveloping in a second solvent (solvent B, chloroform:methanol, 85:15, V:V). Development with the second solvent, as well as elution and purification of fluorescent bands, was accomplished with the procedure described for phase II. After final evaporation of the benzene: acetonitrile supernatant fluid, the fractions were again weighed, checked for toxicity, and stored under nitrogen in a methylene chloride suspension.

To determine purity, analytical thin-layer plates were spotted with 50 µgm quantities of toxic material and developed following the aforementioned procedure using solvents A and B. After the plates dried, they were examined under shortwave ultraviolet light and fluorescent spots located. The next step consisted of spraying the plates with a dilute sulfuric acid preparation (Ziminski and Borowski, 104). The plates were again dried, heated in a 130° C oven for two hours, and examined for charred spots.

Preparation and Administration of Toxin

Oral toxicity checks were conducted using weighed amounts of toxin and a minimum of three chickens per dose. Day-old chickens were placed in cages within 18 hours after hatching and water supplied ad-lib. Food was withheld until treatment was completed.

Oral dosing of both crude and purified preparations was accomplished by weighing the dried material after all solvents had been evaporated. The

weighed material was resuspended in methylene chloride, chosen because of its low boiling point. By adding up to a known volume, usually 5-10 ml, a suspension was obtained with a calculated quantity of toxin per ml.

Measured amounts of the suspension were pipetted into a test tube and the methylene chloride evaporated. The dried material was then resuspended in 0.2 ml of ethanol.

The chickens could be dosed with up to 0.5 ml of an oily preparation without noticeable regurgitation. Depending upon the number of chickens to be dosed, an appropriate amount of olive oil was added to the tube of ethanol-suspended toxin, a cap applied, and mixed thoroughly[1] for approximately two minutes. The oily suspension was further mixed by drawing it into a 2½ ml plastic syringe and forcibly expelling back into the tube, repeating the process ten times.

Dosing was accomplished by the use of an 18-gauge curved animal dosing needle[2] on a 2½ ml syringe, placing the suspension in the posterior oral cavity. Approximately 20 minutes after dosing, food was supplied to treated and control chickens.

Toxicity of an extract was demonstrated by death of the test chickens, usually within 18 hours. If an animal survived for over two days, it usually recovered.

Birds were necropsied as soon as found after death and examined grossly for lesions. Selected tissues (liver, heart, and digestive tract) were removed and placed in 10% buffered formalin. The tissues were dehy-

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[1]Vortex Jr. Mixer. Scientific Industries, Inc., Queens Village, N.Y.

[2]Perfektum Scientific Instruments, New York, N.Y.

drated, embedded in paraffin, sectioned at a thickness of seven microns, and stained with hematoxylin and eosin.

An additional experiment was performed to determine the potential loss of toxic material that might occur during preliminary purification procedures and to determine the relative toxicity of each fraction. At each stage of purification, sufficient material to dose five chickens with each of three dosage levels was set aside and stored in the dark under nitrogen.

After the methylene chloride and water soluble portions were separated, all fractions were dried and prepared for oral dosing in chickens. Three levels were administered for all fractions except the water-soluble extract. Due to the small amount of this fraction available, only one level of dosage was administered. Death losses were recorded over a 48-hour period. Dead chickens were necropsied to determine if gross lesions varied from those of the purified toxin.

Test for Dermal Necrosis

The procedure of Ueno et al. (93) was modified to determine if toxin from band two of TLC possessed dermal necrotic ability. Measured amounts of the purified toxin, suspended in methylene chloride, were dried. The toxin was resuspended in 0.2 ml of ethanol and 0.1 ml of ethanol added for each animal to be tested. Dosage was calculated on a level of 5, 10, 20, 40, and 80 µgm daily for four days. The guinea pigs and rabbits to be tested were closely clipped and shaved along the dorsal area and the toxin applied daily. Control tests were prepared by evaporating an amount of methylene chloride equivalent to the largest amount used as a solvent for the toxin and then adding sufficient ethanol for dosing. Reactions were

graded as: (-) no reaction, (+) slight irritation, (++) reddening and swelling, (+++) scab formation, and (++++) necrosis and sloughing.

Test for Estrogenic Activity

To test an extract of the top band (band one) from the final phase of TLC for estrogenic activity, measured amounts of methylene chloride suspension were dried and resuspended in ethanol in the usual manner. Olive oil was added at the rate of 0.5 ml per rat and the suspension mixed thoroughly.

One group of five rats was given a daily dose of 0.4 mg of the purified extract from band one while a second group received 0.4 mg of crystalline zearalenone. For control purposes, a third group was given an evaporated methylene chloride, ethanol, olive oil suspension while a fourth group received no treatment.

The five rats in each group were treated daily for six days using oral dosing needles. On the eighth day, the rats were killed and necropsied. The uterus was carefully dissected out, weighed, and average uterine weights of each treated group compared to the average of the control rats.

Determination of Chemical Characteristics

Infrared spectra were obtained with a Beckman IR 4[1] spectrophotometer using a methylene chloride suspension of purified toxin. The suspension was spread as a film on a sodium chloride plate, air dried, and examined at a scanning speed of one inch per minute. Absorbance ranged from zero to 100% transmission using an expanded scale. The gain was 19.2 with a period

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[1]Beckman Instruments Inc., Fullerton, California.

of two. The slit schedule was 1.5 x standard, and the double beam mode was used.

Mass spectrophotometric analyses were run on an Atlas CH-4[1] spectrophotometer using the solid inlet. The analyses were made under the following conditions: source type, To 4; accelerating voltage, 3,000 V.; electrical energy, Rge; electric current, 19/9; collector type, SEV (1.9); slit width, 2/2; and inlet system, Vac Lock.

All the above tests were conducted by the toxicology section, Veterinary Diagnostic Laboratory and Chemistry Services, Iowa State University.

Feeding Experiments to Determine Toxicity

Feeding experiments with the toxic substrate were conducted by mixing the chopped mixture with commercial chick meal in 2.5, 5.0, and 10.0% amounts. The newly hatched chickens were placed on regular commercial feed for three to four days before being exposed to toxic food. Treated and control feed and drinking water were supplied ad-lib. Weights were taken at the start of the experiment and every four days thereafter until termination of the experiment.

During the course of the experiments, the chickens were closely observed, and dead chickens were necropsied as soon as possible after death. The digestive tract, lung, liver, spleen, heart, and kidney were preserved in buffered formalin and then prepared for histological examination. In addition, lung sections were stained with periodic acid-Schiff reagent to check for fungal elements.

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[1]Varian Associates, Park Ridge, Illinois.

Toxic feed was supplied at the prescribed percentages for 24 days at which time any surviving chickens on the 2.5% and 10% level were killed, necropsied, and tissues preserved in 10% formalin.

To determine if any permanent damage had occurred to tissues in the test chickens, those on the 5% level were placed back on normal feed. These birds were continued on this feed for approximately three more weeks, then killed and their tissues collected, formalinized, and prepared for histopathological examination in the manner previously described. Control chickens on a normal diet were also fed to the termination of the experiment in order to provide a comparison of weight gain for the treated chickens.

EXPERIMENTAL RESULTS

Toxic Potential of the Crude Extract

A crude chloroform extract (fraction one) of the overgrown substrate was partially purified and four fractions tested for toxicity. The protocol for this preliminary purification is presented in Figure 1. The toxicity of the various fractions is shown in Table 1.

Evaporation of chloroform from the overnight extract yielded a brown syrupy residue that was toxic to chickens. When doses of 100 mg were orally administered, four of five test animals died within 24 hours. Administration of 50 mg of this fraction one did not reveal toxicity.

Approximately 45% of fraction one was removed with hexane. Apparently none of the lethal toxin was present in this portion (fraction two) as doses of up to 400 mg did not affect test chickens.

Fraction three was soluble in water and proved to be toxic also. However, due to limited quantities, further characterization was not attempted.

Fraction four was soluble in methylene chloride and contained most of the lethal toxin. When oral doses as low as two mg were administered, one of five chickens died. Increasing the dose to four mg resulted in the death of all five test chickens.

The second stage of purification of the methylene chloride soluble material from phase I involved the use of preparative TLC plates developed in solvent A (ethyl acetate:cyclohexane, 80:20). Chromatographic separation produced three fluorescent bands possessing different Rf values which were toxic for test animals. A typical separation is illustrated in Figure 2. The most rapidly migrating compounds were subsequently referred to

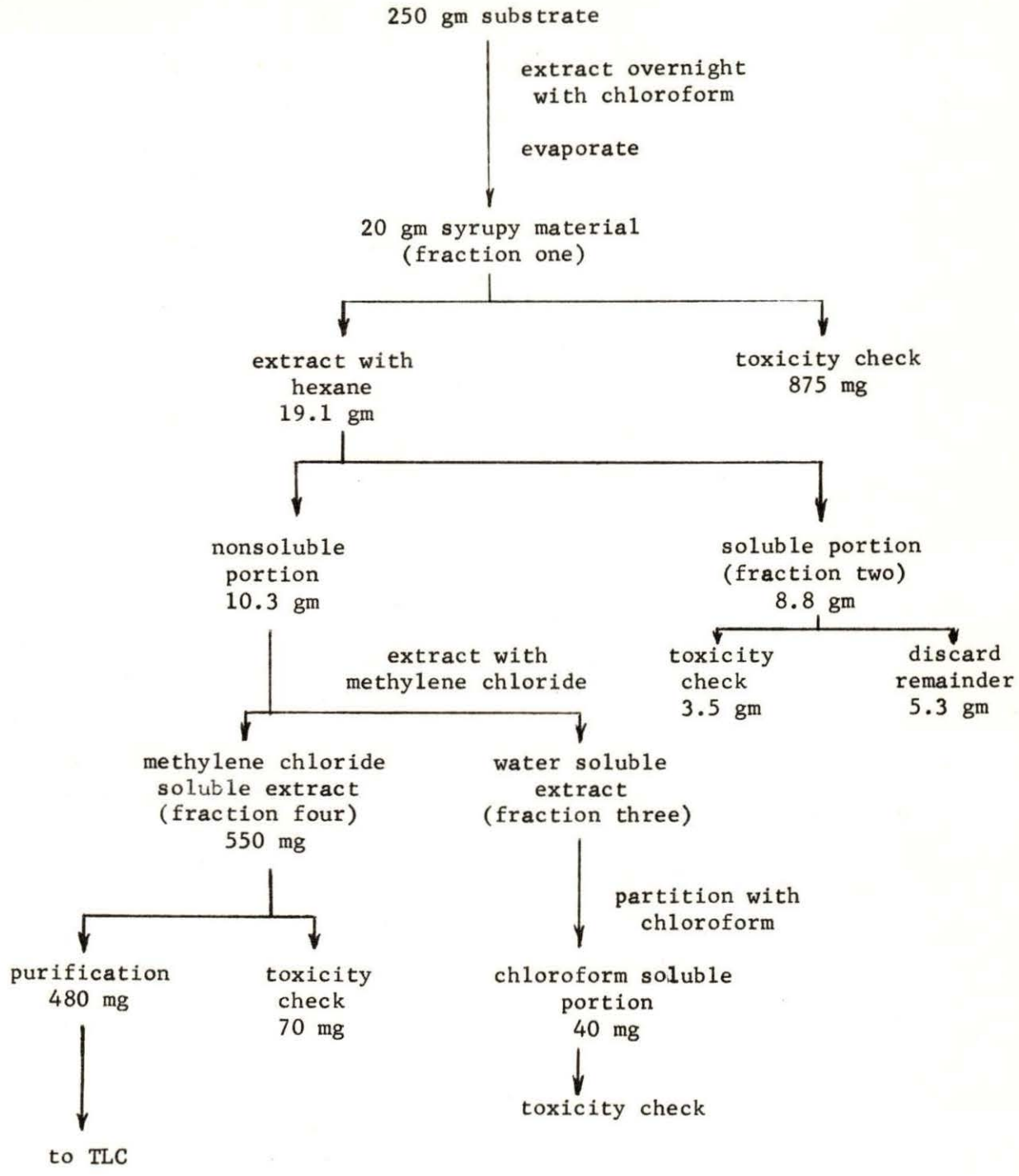


Figure 1. An outline to illustrate the yield of toxic and nontoxic portions during preliminary purification procedures

Table 1. The result of testing various fractions for toxicity during the initial purification process[a]

Fraction	Dose	Deaths
Crude chloroform extract (fraction one)	25 mg	0/5
	50 mg	0/5
	100 mg	4/5
Hexane-soluble extract (fraction two)	100 mg	0/5
	200 mg	0/5
	400 mg	0/5
Water-soluble extract (fraction three)	8 mg	5/5
Methylene chloride-soluble extract (fraction four)	2 mg	1/5
	4 mg	5/5
	8 mg	5/5

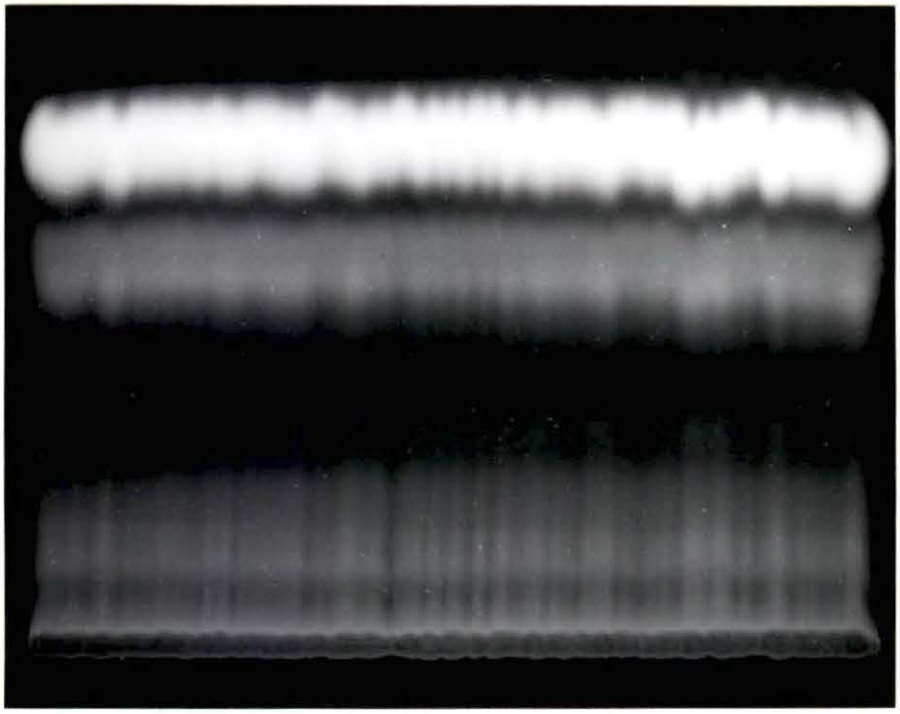
[a]Five day-old chickens were dosed orally with measured amounts of each extract.

as bands one, two, and three and subjected to further study. The broad area at the lower portion of the plate, referred to as band four, was not investigated further due to its poor separation on TLC.

The broad band at the top of the plate fluoresced a bright blue color under shortwave ultraviolet light but was not visible under normal light. This band was scraped from the plate, eluted with methanol, and compared with other toxins from Fusarium sp. on an analytical TLC plate. It was found to have an Rf value and fluorescence similar to zearalenone an estrogenic toxin.

The second band from the top was narrower than band one. It fluoresced a yellow-green color under ultraviolet light and had a lemon-yellow appearance under normal light. The toxic activity of this fraction was

Figure 2. Photograph of a typical separation of the methylene chloride-soluble extract by preparative TLC. The solvent was ethyl acetate:cyclohexane (80:20, V:V). Bands one, two, three, and four were removed and toxic material eluted from the silica gel carrier. The photograph was taken under shortwave ultraviolet light with an absorbing filter in front of the lens (Glazier et al., 35)


1
2
3
4

demonstrated by deaths of day-old chickens dosed with the extracted material.

Band three appeared as a thin, yellow-green fluorescing compound under ultraviolet light trailing band two by a distance sufficient to allow it to be removed and purified. Lethal toxicity was also demonstrated by this band in day-old chickens.

Phase III of purification was performed individually on each of the extracts from phase II and involved the use of solvent B (chloroform: methanol, 85:15, V:V). This further separated the toxic materials from phase II into individual bands. All three toxic compounds retained their fluorescing properties. Nontoxic compounds that might have possessed approximately the same Rf value in solvent A either moved more rapidly or slower with the second solvent.

By this procedure, it was possible to elute from the preparative TLC plates material that had a single fluorescent spot on analytical TLC plates. Material prepared by this procedure was used for subsequent experiments to determine biological and physical characteristics of each toxic compound.

Test for Estrogenic Activity

The purified extract from band one of TLC and crystalline zearalenone were orally administered to compare their estrogenic activity. The results of this experiment are listed in Table 2. The uterine weight of rats treated with band one (70 mg) was somewhat less than that of rats treated with zearalenone (90 mg). However, there was a significant increase in uterine weight over that of control rats (41 mg). Additional tests were not conducted with this compound as the primary interest was in band two toxin.

Table 2. This illustrates the estrogenic capabilities of a purified extract from band one of TLC as compared to zearalenone[a]

	Average body weight (gm)		Uterine weight[b] (mg)
	Start	Finish	
Control group A[c]	58	79	41
Control group B[d]	57	79	35
Zearalenone[e]	58	80	90
Band one[f]	58	80	70

[a]Five weanling female white rats were alloted to each group and dosed orally every day for six days. All animals were killed on the eighth day and uteri weighed.

[b]Represents the average weight of the uteri of the rats in each group.

[c]No treatment.

[d]Two ml of methylene chloride were evaporated followed by the addition of 0.2 ml of ethanol; this residue was then suspended in olive oil.

[e]0.4 mg of crystalline zearalenone daily.

[f]0.4 mg of purified extract from band one of TLC daily.

Response of Chickens to Band Two Toxin

Graded dosages of toxin from band two of TLC were administered orally to day-old chickens and the death losses recorded (Table 3). When 0.5 mg of the extract was administered, two of six chickens died within 24 hours. An increase in dosage to 0.7 mg for each chicken resulted in death of four of the six chickens. Results of this experiment indicated that the LD_{50} of the purified preparation was approximately 0.6 mg.

Table 3. Illustrating the response of day-old chickens to a single oral dose of purified toxin from band two of TLC

Dose	Deaths
0.5 mg	2/6
0.7 mg	4/6
0.9 mg	5/6
1.1 mg	6/6
Control[a]	0/6

[a]Two ml of methylene chloride was evaporated, 0.2 ml of ethanol added, and the residue suspended in olive oil.

Pathological Lesions in Chickens

Preparations of purified toxin representing bands two and three of TLC produced similar pathological lesions in treated chickens. Although lesions varied between animals, even within the same dosage range, the digestive tract was usually the site of most obvious damage. Figures 3 and 4 show typical gross pathology from administration of the two toxins.

The primary site of involvement in the digestive tract appeared to be the small intestine. A catarrhal enteritis was observed in the duodenal portion. Enteritis was usually observed in the posterior portion of the intestine but was not always constant in the region of the ileum and cecum. Anterior to the intestine, few changes were observed. However, in some of the more acutely poisoned cases, a stasis of the tract had apparently occurred. In these individuals, food was usually found in the crop or proventriculus but not in the ventriculus or intestine.

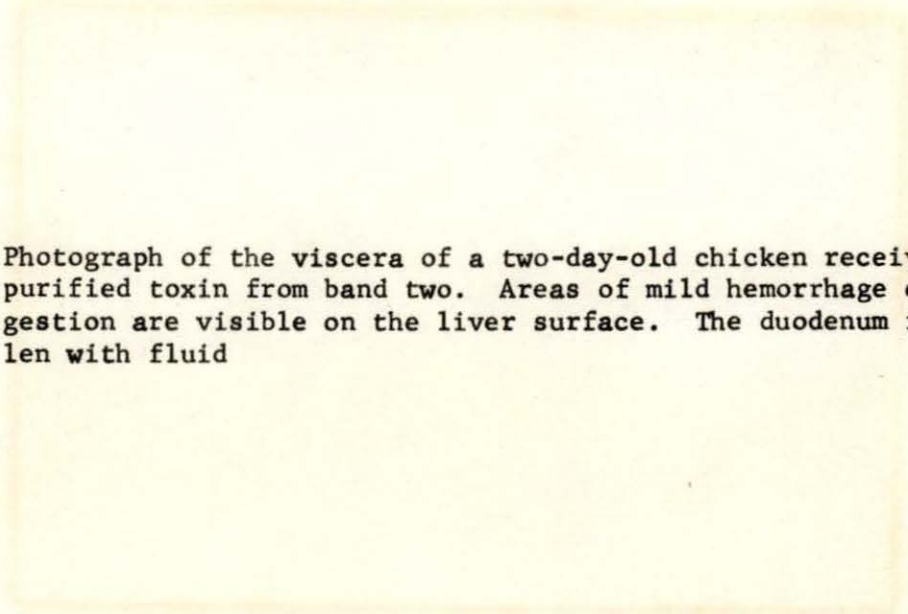


Figure 3. Photograph of the viscera of a two-day-old chicken receiving purified toxin from band two. Areas of mild hemorrhage or congestion are visible on the liver surface. The duodenum is swollen with fluid

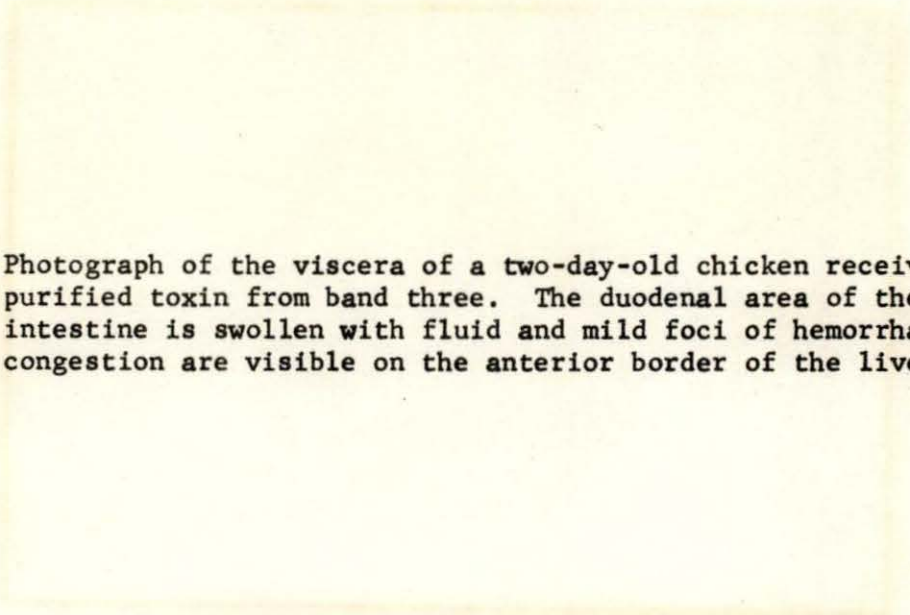
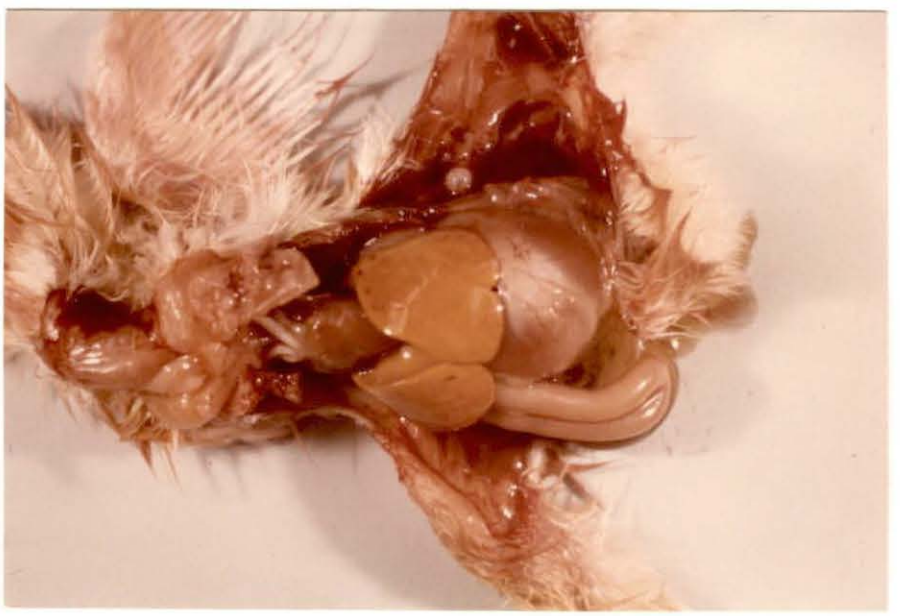


Figure 4. Photograph of the viscera of a two-day-old chicken receiving purified toxin from band three. The duodenal area of the small intestine is swollen with fluid and mild foci of hemorrhage or congestion are visible on the anterior border of the liver


2-B .8mg.
11-17-70



Liver lesions varied between chickens. Occasional cases were observed with massive sites of hemorrhage in one or more lobes, but more commonly, the involvement was much less severe. Mild foci of congestion or hemorrhage were often seen on the surface of the liver.

Chickens receiving lethal doses of toxin from bands two and three were necropsied and tissues saved for examination. Histopathologic sections from three portions of the digestive tract of chickens treated with band two are illustrated in Figures 5 through 10. All the described lesions were not constant in those chickens receiving the purified toxin. However, within a group, a certain consistency was noted in that at least some of the lesions were observed in any one affected bird.

As would be expected from the gross lesions, the intestinal tract and liver were primarily affected. In the duodenal portion of the small intestine, the tips of the villi were usually affected with hemorrhage and hyperemia visible in this area. This involvement of the villi was also evident in the jejunal region of the tract with similar lesions observed. Although the lesions were not as constant in the region of the cecum, edematous swelling and hemorrhage were still evident.

Examination of the liver sections revealed fatty infiltration and mild sinusoidal congestion. Occasional cases were observed in which the congestion was much more severe with gross hemorrhage evident.

Involvement of the digestive tract anterior to the intestine was inconsistent and usually mild with little observable cellular damage. This lack of microscopic lesions was consistent with the gross observations.

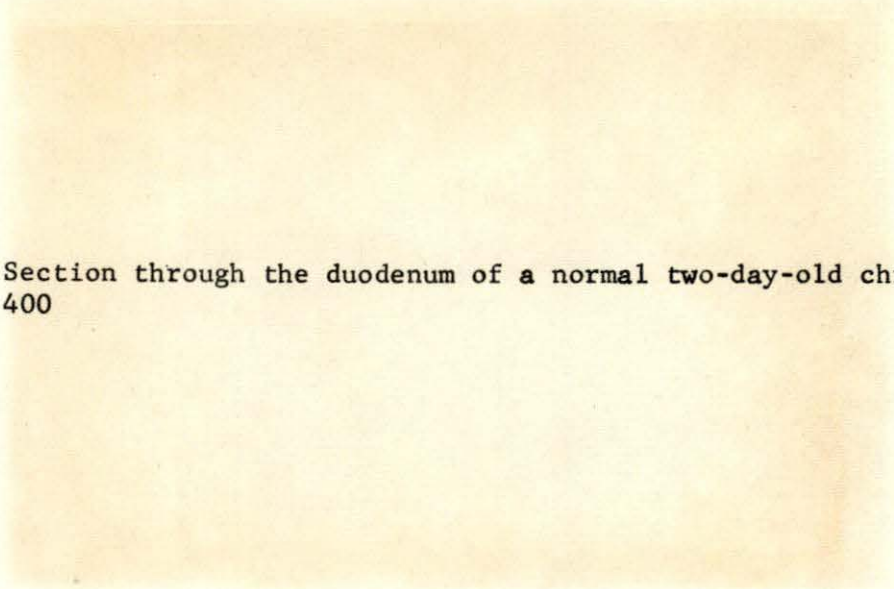


Figure 5. Section through the duodenum of a normal two-day-old chicken, X 400

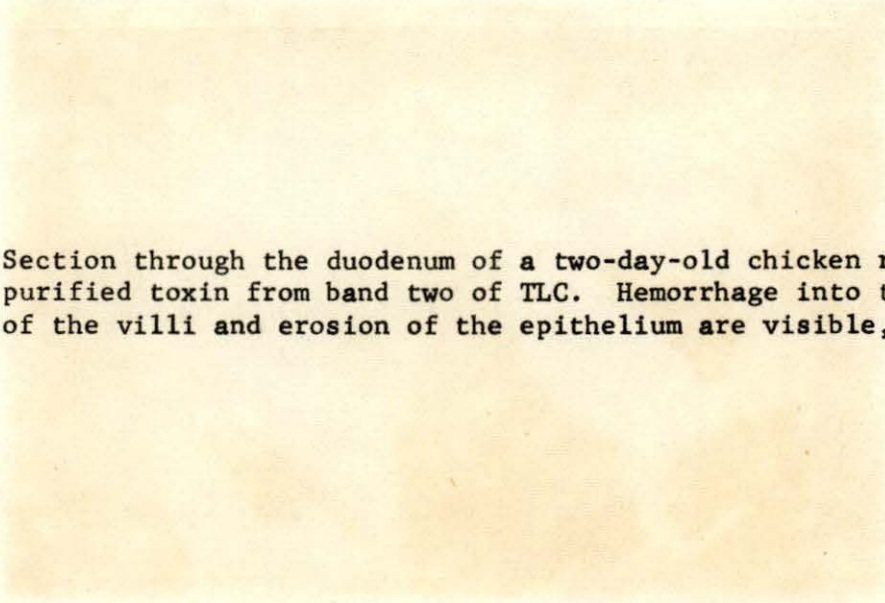


Figure 6. Section through the duodenum of a two-day-old chicken receiving purified toxin from band two of TLC. Hemorrhage into the tips of the villi and erosion of the epithelium are visible, X 400

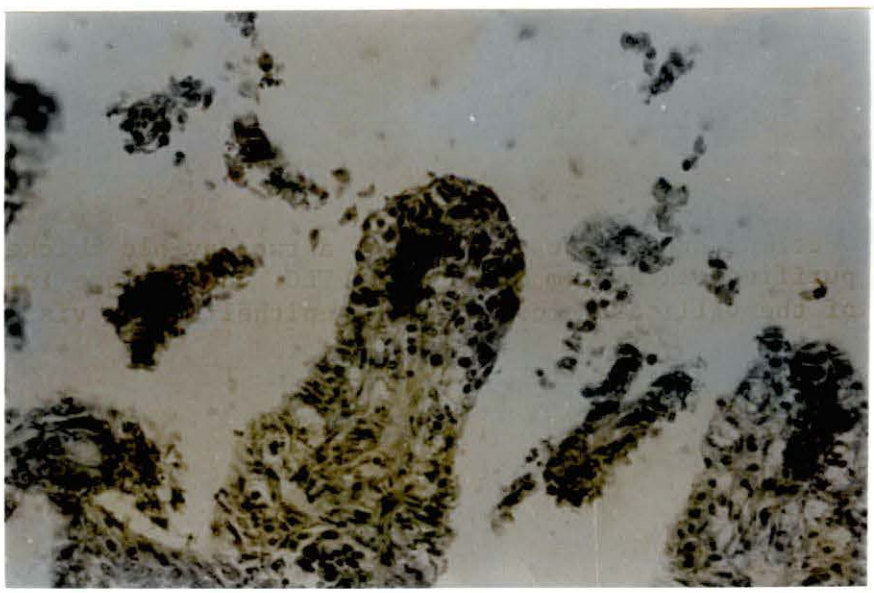
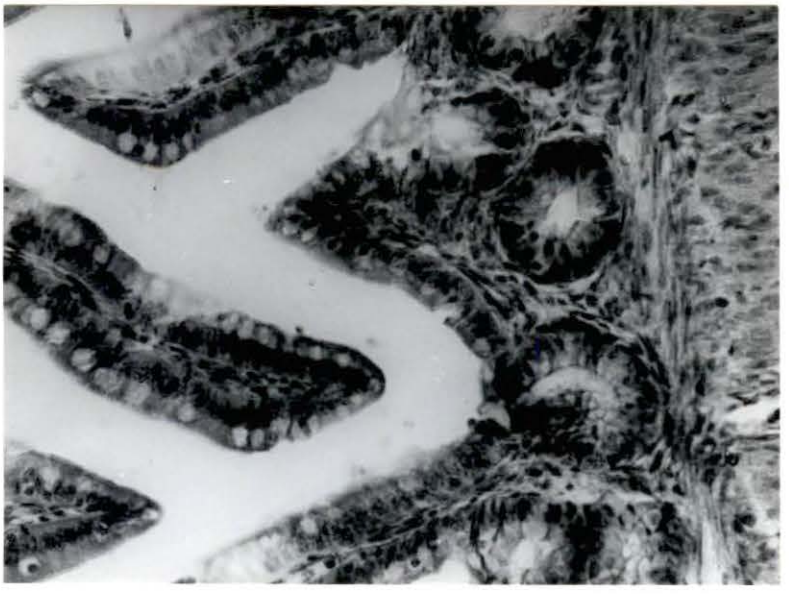
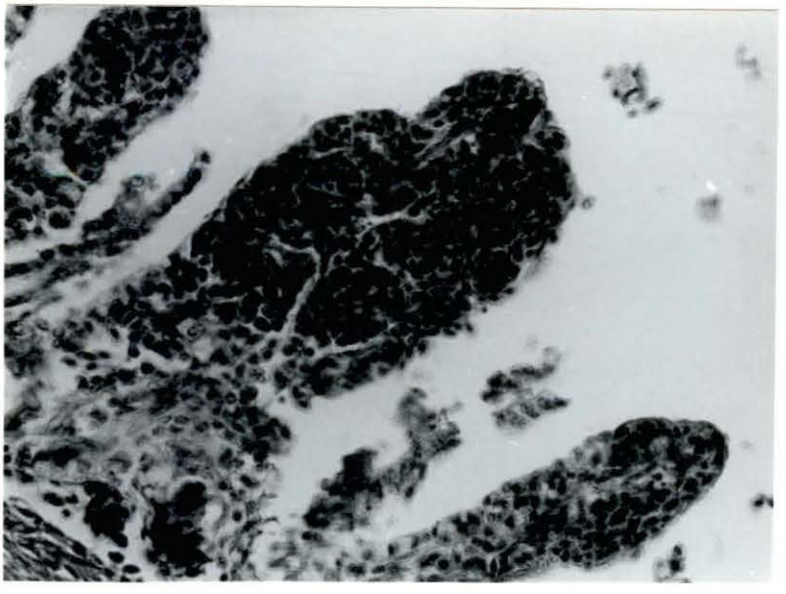


Figure 7. Section through the jejunal portion of the small intestine of a normal two-day-old chicken, X 400

Figure 8. Section through the jejunal portion of the small intestine of a two-day-old chicken which had received purified toxin from band two of TLC. Hyperemia and hemorrhage are visible in the villi accompanied by erosion of the epithelium, X 400





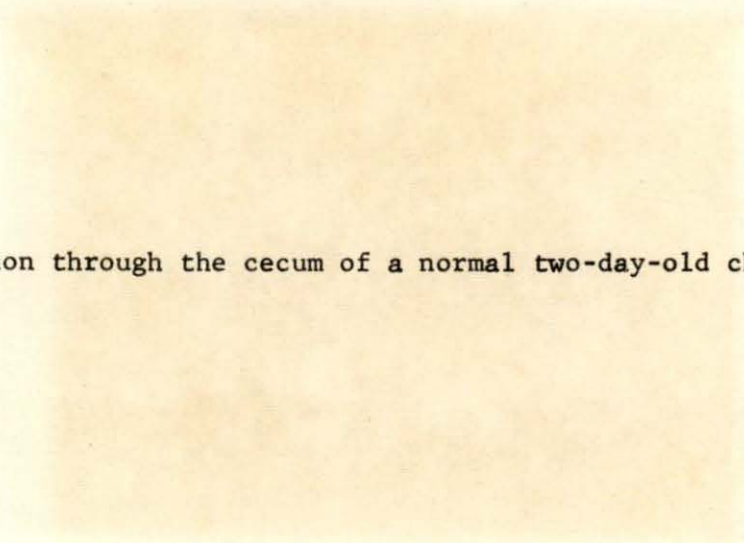


Figure 9. Section through the cecum of a normal two-day-old chicken, X 50

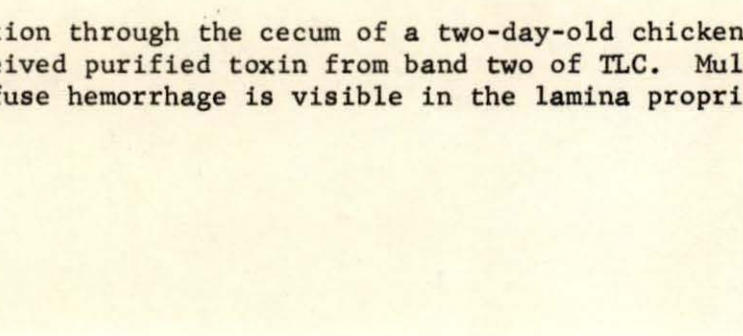
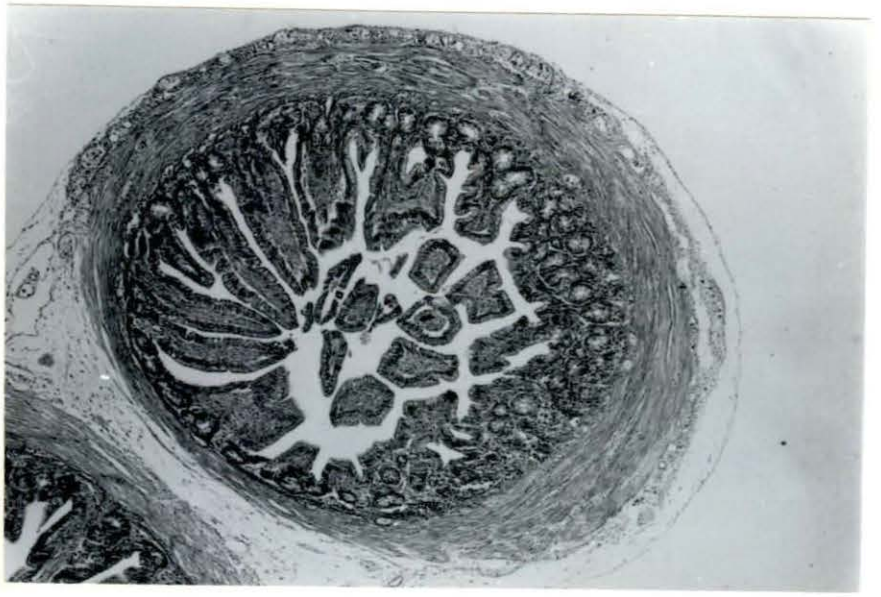
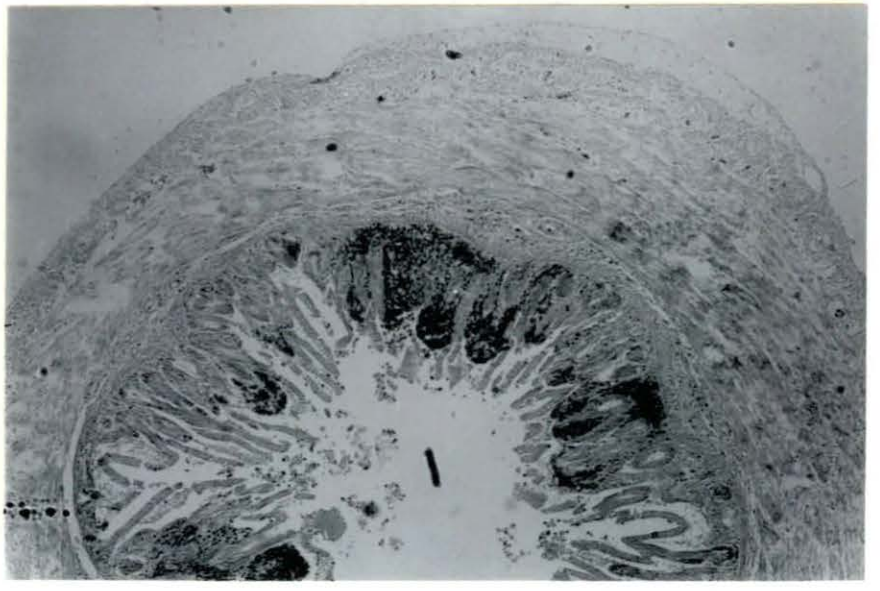


Figure 10. Section through the cecum of a two-day-old chicken which had received purified toxin from band two of TLC. Multifocal to diffuse hemorrhage is visible in the lamina propria, X 50





Test for Dermal Necrosis

Both the rabbit and guinea pig proved to be highly sensitive to purified toxin from band two. Applications of ethanol-suspended toxin on their skin caused a severe reaction as illustrated in Figure 11 and Table 4. Doses as low as five μgm daily for three days caused a visible reaction on the skin of both animals. As the dose was increased, the skin reacted with greater intensity. At the 20, 40, and 80 μgm levels, a distinct reddening and scabbiness was observed on the rabbit. At the 80 μgm level, the reaction of the guinea pigs was severe enough to cause a sloughing of skin over the affected area.

Chemical Considerations

Figures 12 and 13 and Tables 5, 6, and 7 illustrate the data obtained by mass spectrum analysis, infrared analysis, and analytical TLC of two compounds, zearalenone and band two toxin. Since initial infrared spectra of the two compounds showed many structural similarities, zearalenone was used as a working model for comparison.

It appears that many of the same functional groups exist in both compounds but probably have different positions in the structure. This is indicated by the following:

a. The benzene ring appears to have aliphatic groups attached instead of hydroxyl groups as seen by the infrared spectra.

b. The carbonyl group or groups are in an environment different from zearalenone as seen by infrared spectra.

c. The molecular weight of zearalenone is 318 while that of the band two is 348.

Figure 11. Photograph of the reaction of rabbit skin to purified toxin from band two of TLC. Toxin was applied on three consecutive days with site and dosage as follows: I - 5 µgm, II - 10 µgm, III - 20 µgm, IV - 40 µgm, V - 80 µgm, and VI - control

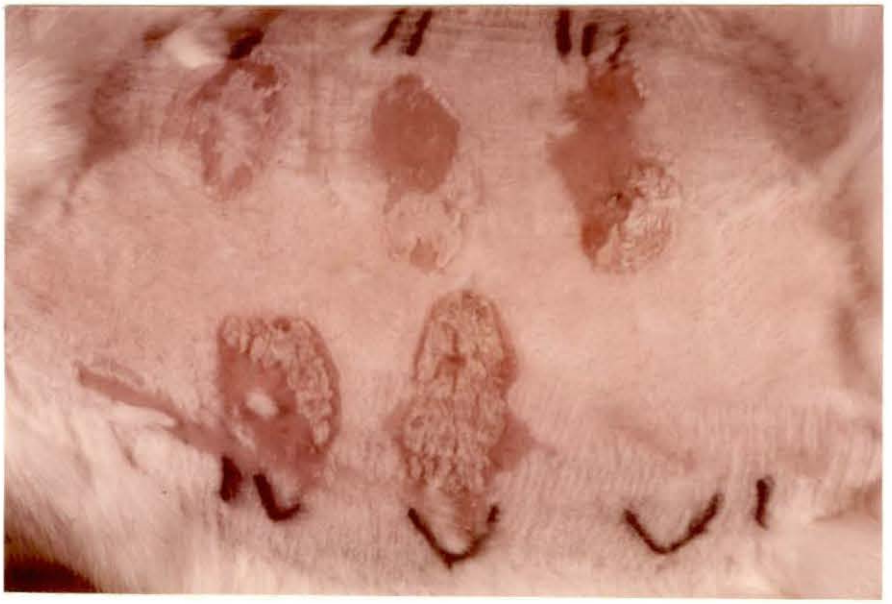


Table 4. The response of rabbit and guinea pig skin to purified toxin from band two of TLC[a]

Dose level	Rabbit	Guinea pig
5 µgm	+	+
10 µgm	++	++
20 µgm	+++	+++
40 µgm	+++	+++
80 µgm	+++	++++
Control[b]	-	-

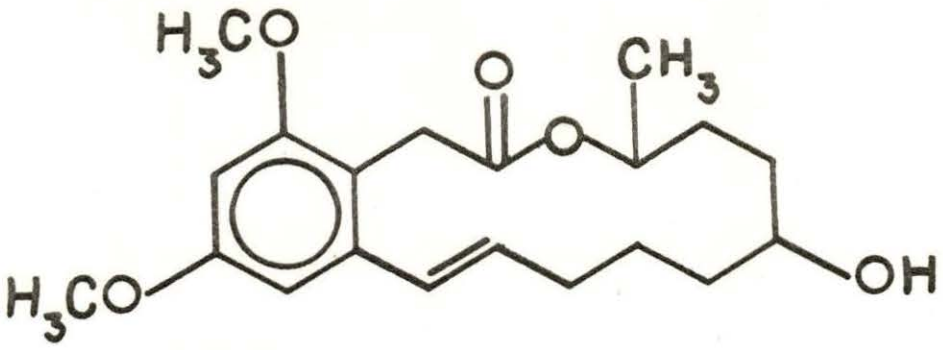
[a]Animals were clipped and shaved along the back. Measured doses of toxin were administered in an olive oil suspension daily for three days. Results were recorded seven days after the last day of treatment. Results were recorded as: - no reaction, + slight irritation, ++ reddening and swelling, +++ scab formation, ++++ necrosis and sloughing.

[b]Control application consisted of methylene chloride vehicle which was evaporated before ethanol and olive oil were added.

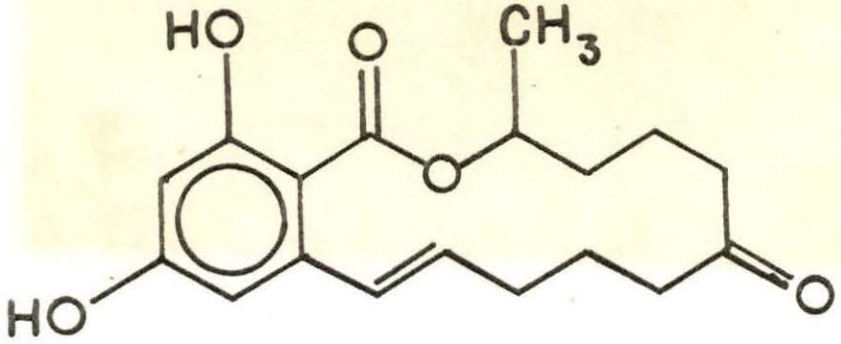
d. The Rf values of the two compounds differ when compared by analytical TLC.

e. Both compounds were isolated in the same fraction from the crude substrate.

Sufficient data was obtained by mass spectrum analysis, infrared analysis, and preliminary nuclear mass resonance that a structural formula for band two toxin could be postulated. As would be expected by the close resemblance to zearalenone, band two has a ring structure and may be represented by the following structural formula:



This may be compared to the structural formula of zearalenone (Urry et al., 97):



Feeding Experiments

Groups of chickens were fed substrate from three cultural procedures. This substrate was mixed in the feed at three dosage levels for each group. Figures 14-16 and Table 8 show the results of this experiment. Particularly noticeable was the slow rate of weight gain in all the chickens, with the exception of controls, during the first 12-16 days of the trial.

Figure 12. Mass spectrum analysis of zearalenone run at 70 electron volts

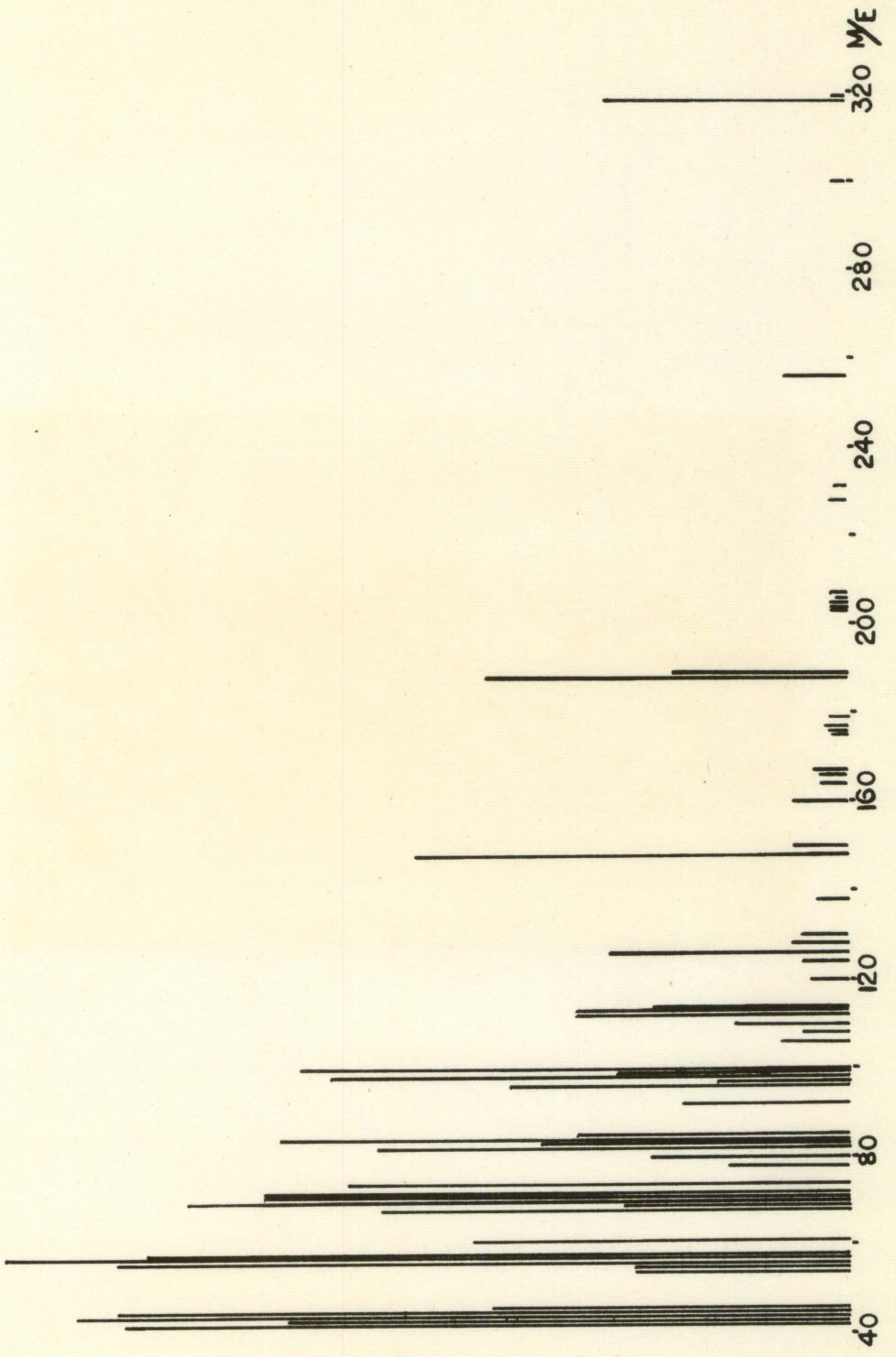

40
80
120
160
200
240
280
320
M/E

Figure 13. Mass spectrum analysis of band two toxin run at 16 electron volts

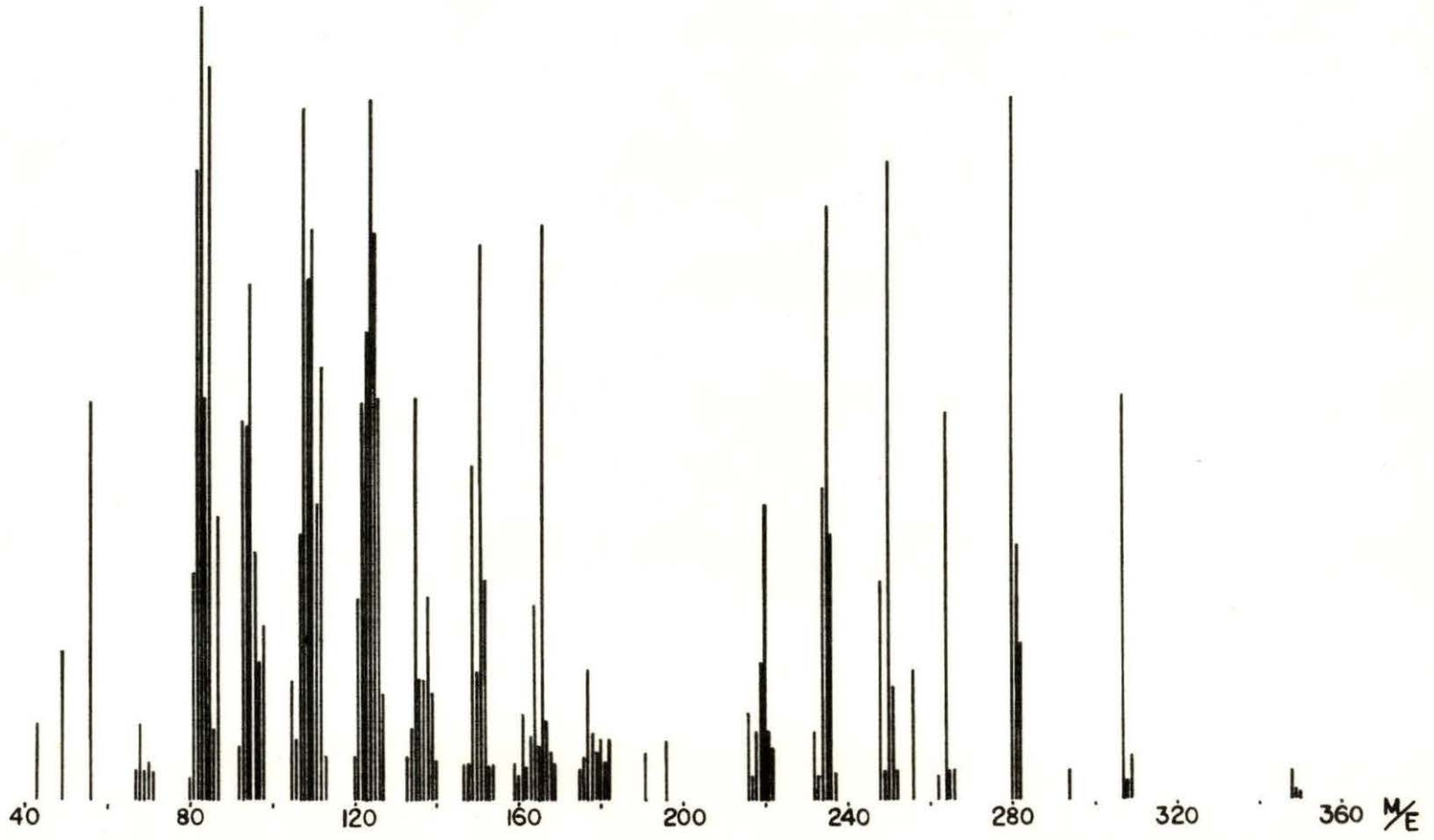

40
80
120
160
200
240
280
320
360
M/E

Table 5. Rf values of the three toxic fractions isolated as compared to zearalenone. The fractions were developed on analytical TLC plates of silica gel with solvents A and B

	Rf values	
	Solvent A	Solvent B
Zearalenone	5.8	6.7
Band one	5.8	6.7
Band two	4.0	6.0
Band three	2.5	5.0

Table 6. The infrared spectrum analysis of zearalenone

cm^{-1}	Relative intensity	Absorption
3356 ± 11	30	Hydroxyl stretch
2932 ± 9	36	Methyl and methylene stretch
1698 ± 3	39	Ketone stretch
1647 ± 3	70	-keto ester stretch
1616 ± 3	67	Skeletal stretch in benzene
1582 ± 3	52	Skeletal stretch in benzene
1449 ± 4	42	Methyl and methylene deformation
1383 ± 4	40	Methyl deformation
1355 ± 2	57	Carbon-oxygen stretch in phenol
1315 ± 2	67	In plane deformation for carbon-hydrogen in trans HC=CH
1263 ± 2	100	Carbon-oxygen stretch in benzoate
1202 ± 2	53	Phenol deformation
1172 ± 2	53	Benzene ring due to substituents
1123 ± 2	51	Carbon-oxygen stretch in benzoate
1056 ± 1	19	Lactone ring deformation
1019 ± 1	26	Benzene ring due to substituents
973 ± 1	31	Out-of-plane deformation for carbon-hydrogen in trans HC=CH
854 ± 1	22	Carbon-hydrogen out-of-plane deformation in benzene
736 ± 1	7	Skeletal vibration for $-(CH_2)_4-$

Table 7. The infrared spectrum analysis of band two from TLC

cm^{-1}	Relative intensity	Absorption
3472 ± 12	40	Hydroxyl stretch
2924 ± 9	76	Methyl stretch
2857 ± 8	50	Methylene stretch
1742 ± 3	89	Saturated ester stretch
1447 ± 2	44	Methyl and methylene deformation
1374 ± 2	57	Methyl deformation
1314 ± 2	34	In plane deformation for carbon-hydrogen in trans HC=CH
1244 ± 2	100	Carbon-oxygen stretch in the ester
1170 ± 2	50	Benzene ring due to substituents
1112 ± 2	44	Carbon-oxygen stretch in a secondary alcohol
1082 ± 1	53	Benzene ring due to substituents
1052 ± 2	38	Benzene ring due to substituents
1040 ± 2	40	Methyl rock on benzene ring
991 ± 1	30	Benzene ring due to substituents
963 ± 1	47	Out-of-plane deformation for carbon-hydrogen in trans CH=CH
930 ± 1	26	Lactone ring deformation
827 ± 1	13	Carbon-hydrogen out-of-plane deformation in benzene
735 ± 1	12	Skeletal vibration for - $(CH_2)_3$-

In chickens receiving the 10% level of toxic substrate, irritation of the oral mucosa developed within a period of one week. This was particularly evident on the palate and angle of the mouth. Grossly the affected areas showed an accumulation of soft, friable, dark colored material (Figure 17). Similar lesions were also observed in the vent area. This reaction was most apparent in chickens receiving substrate B.

Histopathological examination of the affected oral mucosa and adjacent area revealed a subacute fibrinopurulent glossitis and stomatitis. Erosion and ulceration of the epithelial surfaces was observed with an accumulation

Figure 14. Result of feeding experiment A showing the rate of weight gain. The substrate was cultured for two weeks at 25° C, then mixed with normal feed at 2.5, 5, and 10% levels and fed to chickens. On the 24th day, the surviving chickens on the 5% level were placed on normal feed until the termination of the experiment

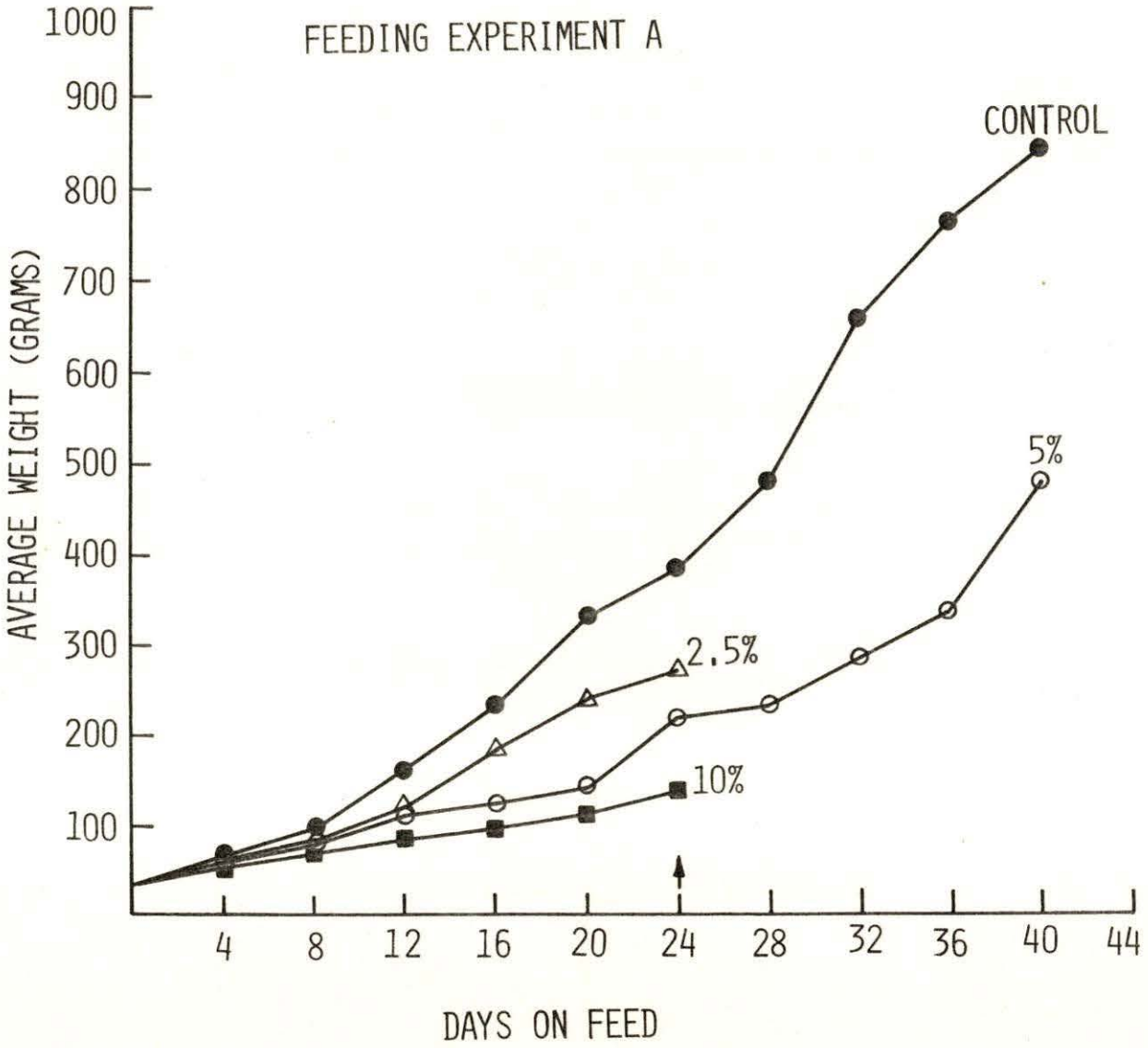

FEEDING EXPERIMENT A
AVERAGE WEIGHT (GRAMS)
1000
900
800
700
600
500
400
300
200
100
CONTROL
5%
2.5%
10%
4
8
12
16
20
24
28
32
36
40
44
DAYS ON FEED

Figure 15. Result of feeding experiment B showing the rate of weight gain. The substrate was cultured for two weeks at 25° C, then for three weeks at 10° C after which it was mixed with normal feed at 5 and 10% levels and fed to chickens. On the 24th day, the surviving birds on the 5% level were placed on normal feed until the termination of the experiment

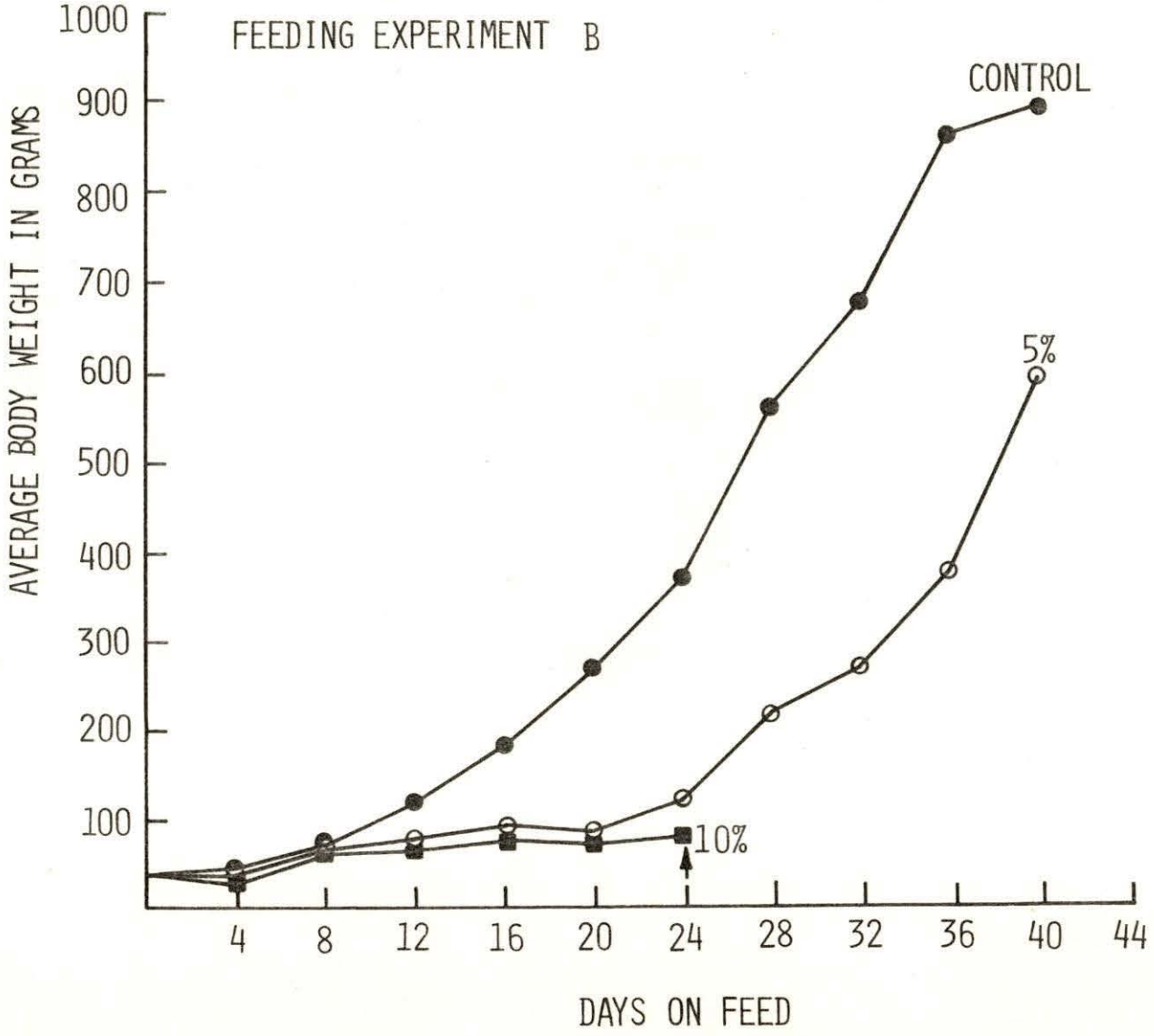

FEEDING EXPERIMENT B
CONTROL
5%
10%
AVERAGE BODY WEIGHT IN GRAMS
1000
900
800
700
600
500
400
300
200
100
4
8
12
16
20
24
28
32
36
40
44
DAYS ON FEED

Figure 16. Result of feeding experiment C showing the rate of weight gain. The substrate was cultured for two weeks at 25° C, then at 10° C for one week, and again for two weeks at 25° C. It was then mixed with normal feed at 2.5, 5, and 10% levels and fed to chickens. On the 24th day, surviving chickens on the 5% level were placed on normal feed until the termination of the experiment

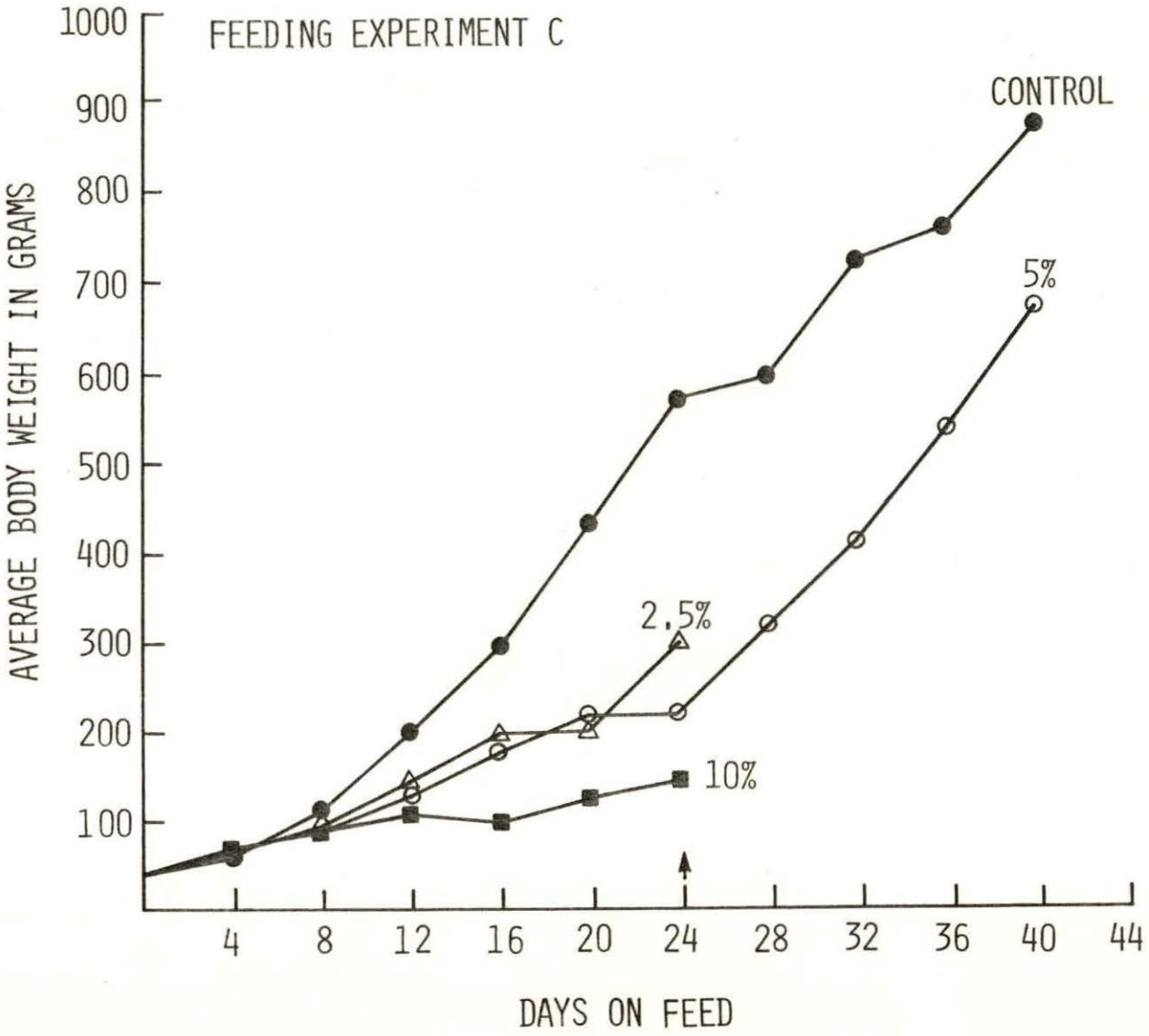

FEEDING EXPERIMENT C
CONTROL
5%
2.5%
10%
AVERAGE BODY WEIGHT IN GRAMS
1000
900
800
700
600
500
400
300
200
100
4
8
12
16
20
24
28
32
36
40
44
DAYS ON FEED

Table 8. Weights in grams of chickens fed various levels of cultured substrate from three cultural procedures[a]

	Days on feed									
	4	8	12	16	20	24	28	32	36	40
Experiment A										
2 wks. @ 25° C										
Control	53	99	164	237	330	382	486	654	779	839
2.5%	58	90	122	185	245	272				
5.0%	56	85	109	124	143	219	236	282	328	404
10.0%	56	67	89	100	110	143				
Experiment B										
2 wks. @ 25°										
3 wks. @ 10° C										
Control	45	68	114	182	267	370	562	678	870	898
5.0%	42	62	75	91	85	112	214	269	385	601
10.0%	37	62	64	79	71	81				
Experiment C										
2 wks. @ 25°										
1 wk. @ 10°										
2 wks. @ 25° C										
Control	61	108	199	290	439	570	593	728	760	881
2.5%	59	93	146	197	199	300				
5.0%	61	95	139	175	221	218	323	410	544	675
10.0%	63	88	101	99	125	147				

[a]Day-old chickens were fed normal feed for three days. They were then randomly separated and fed various levels of cultured substrate mixed with the feed. After 24 days, those birds on the 5% level were fed normal feed. Control birds received normal feed throughout the experiment. The average weight in grams of surviving chickens is represented in the columns below the days on feed.

of fibrin, inflammatory cells, and food debris on the surface of the affected area (Figures 19 and 20).

Treated chickens, particularly those receiving the 10% level, were less active after several days on the test, tending to become listless and

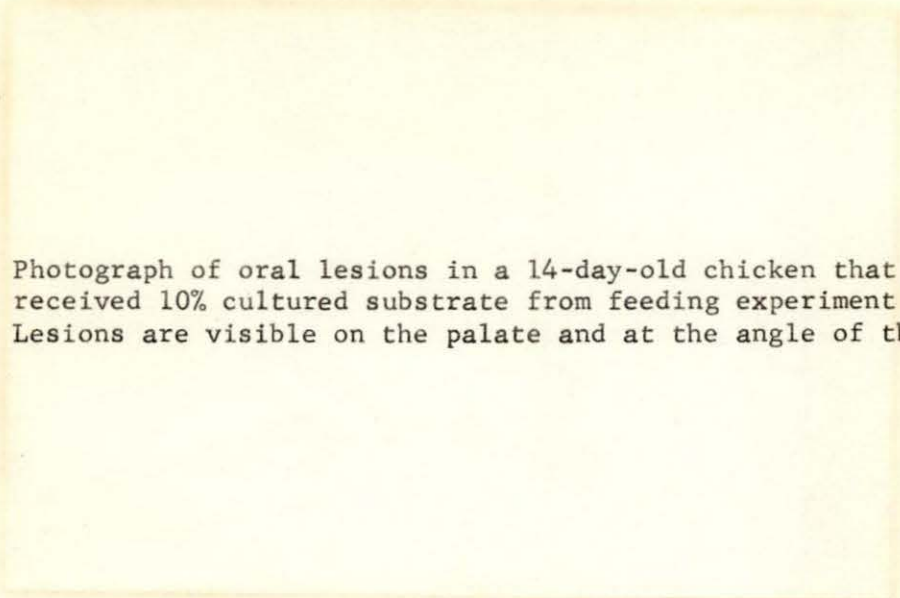


Figure 17. Photograph of oral lesions in a 14-day-old chicken that had received 10% cultured substrate from feeding experiment B. Lesions are visible on the palate and at the angle of the mouth

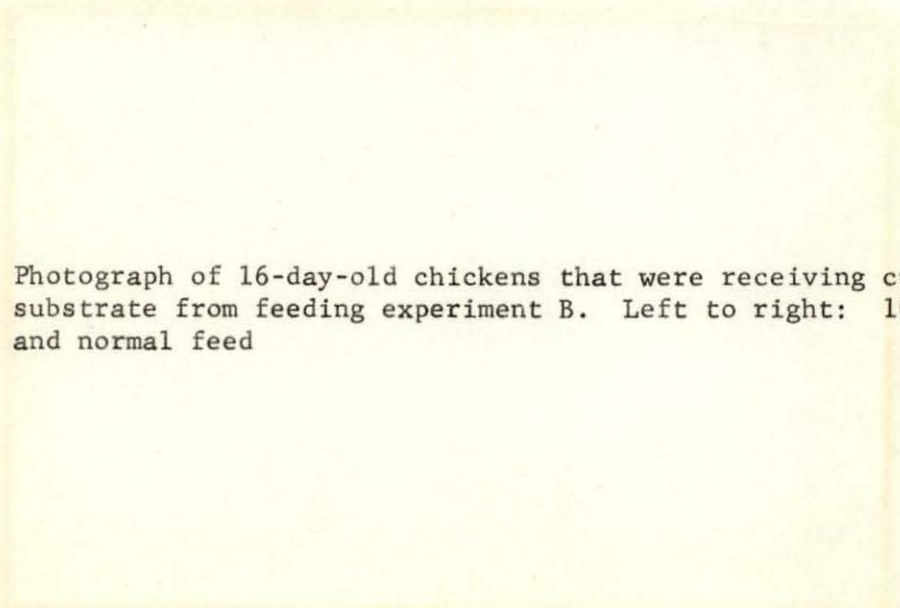
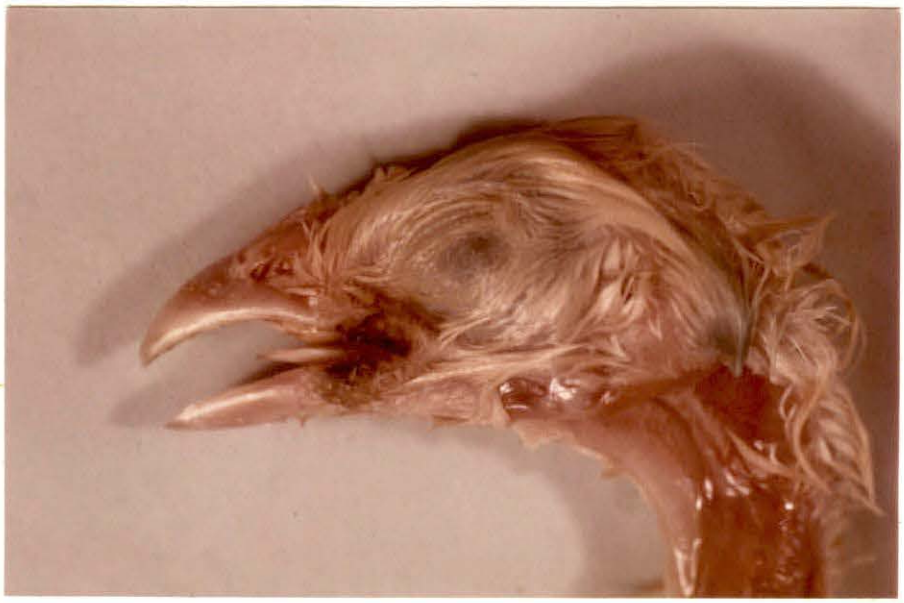
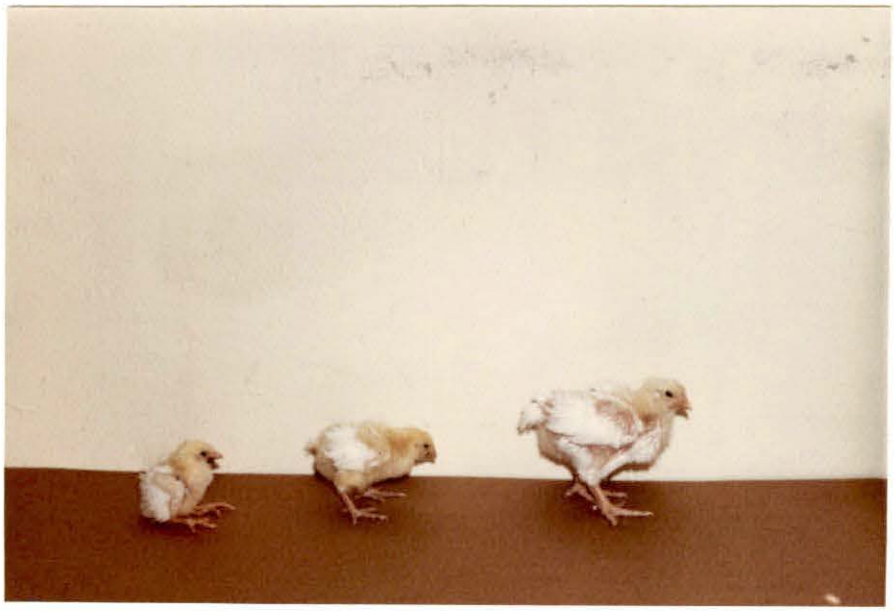


Figure 18. Photograph of 16-day-old chickens that were receiving cultured substrate from feeding experiment B. Left to right: 10%, 5%, and normal feed





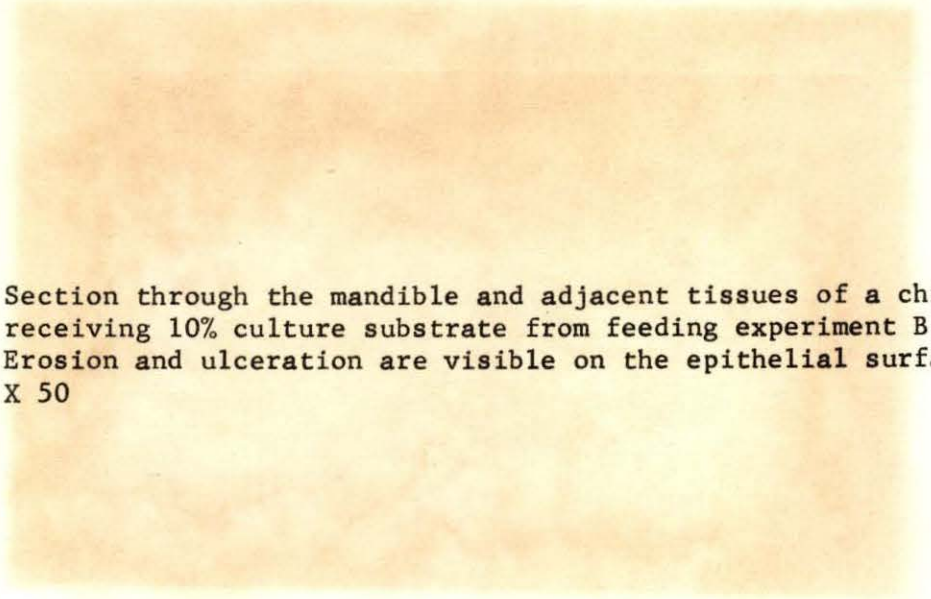


Figure 19. Section through the mandible and adjacent tissues of a chicken receiving 10% culture substrate from feeding experiment B. Erosion and ulceration are visible on the epithelial surface, X 50

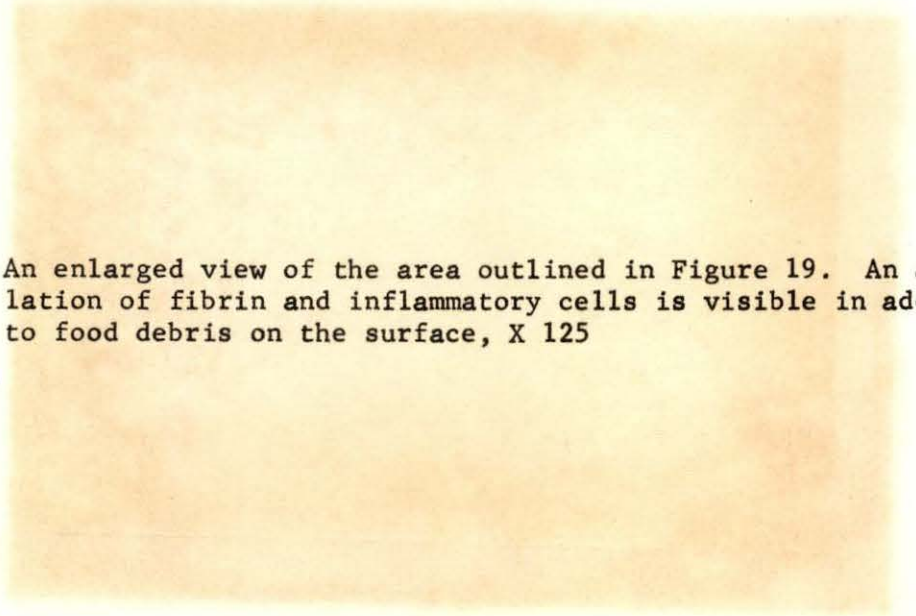
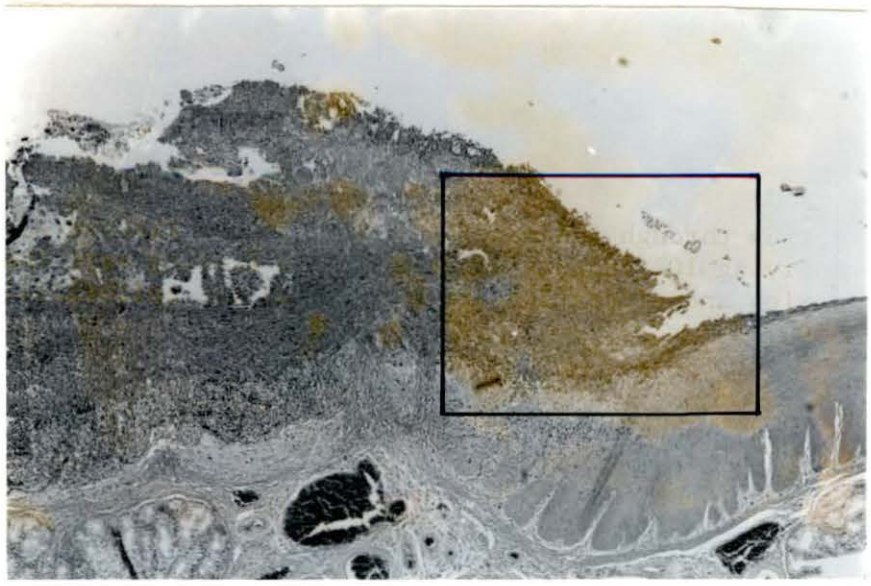


Figure 20. An enlarged view of the area outlined in Figure 19. An accumulation of fibrin and inflammatory cells is visible in addition to food debris on the surface, X 125



depressed as the experiment progressed. At the 24-day period, the survivors showed a marked reduction in thriftiness and size (Figure 18).

Tissues of chickens that showed severe retardation at the 10% level were examined histopathologically. No marked lesions of the gastro-intestinal tract or of the liver were observed. Similarly, periodic acid-Schiff stains of lung tissue failed to reveal invasion by hyphal elements.

Chickens consuming the 5% ration of all three substrates exhibited a much milder reaction in the oral cavity than the 10% level. Little or no reaction was observed at the 2.5% level.

When the chickens on the 5% level were placed back on the control feed at the end of the 24-day feeding period, they showed a rate of gain comparable to control birds. Any involvement of the oral mucosa that might have been present at this time apparently healed spontaneously when the treated feed was removed.

When substrates A and C were fed at the 2.5% level, a substantial difference in weight was noted between treated chickens and those on the control diet. Thus for substrate A, a difference of 110 gm was noted while for substrate C, the difference was 270 gm.

In addition to a difference of weight gain between different dosage levels, the tests also revealed a difference between the three cultural procedures. For instance, at 24 days those chickens receiving the 10% level of substrates A and C were approximately the same weight, i.e., 143 vs. 147 gm. Surviving chickens receiving substrate B averaged only 81 gm. This difference was also evident in the 5% groups where chickens on substrates A and C averaged 219 and 218 gm, respectively, as compared to 112 gm for substrate B.

Representative chickens on the 5% level were examined histopathologically at the end of the experiment. Little or no alteration was noted from control chickens.

DISCUSSION

The particular strain of Fusarium roseum studied proved to be highly toxic to at least two laboratory animals. It was observed that both chickens and young rats were very susceptible to purified toxins produced by culture of this fungus on a corn and rice mixture. The toxin was also readily assimilated from crude substrate as many animals would die overnight following ingestion of the ground corn and rice mixture. For this reason, the chickens used in the feeding experiments were held on normal feed for three to four days before exposure to the toxic feed mixture. This helped to prevent consumption of an abnormally large amount of treated food when it was first placed before them.

The feed from experiment B, which has been cultured for a longer time at 10^{o} C, proved to have a greater ability for retarding growth in feeding experiments. This effect could have been a result of several factors. Among them are: (1) a greater degree of irritation to the oral mucosa with a subsequent decrease in feed intake, (2) greater involvement of body tissues not detected by the methods used, and (3) decreased palatability.

In comparing the weight of chickens fed in experiment A with experiment C, there was little evidence that the toxin level was enhanced by the fluctuating temperatures. Holding the temperature at a low level (experiment B) apparently was a greater stimulant to toxin production.

The marked irritation of the oral mucosa of the test chickens undoubtedly contributed to a decreased feed intake. Whether this enforced starvation was the cause of death of those chickens on the higher level is not certain since it was not definitely determined which tissue or body system

was primarily affected. Other potent mycotoxins, such as aflatoxin, have a definite effect on RNA and DNA systems of body metabolism (Wogan and Pong, 100).

The heavy exposure to fungal hyphae and spores experienced by the chickens on the 10% ration should have provided an ideal environment for invasion of body tissues, particularly in the more debilitated chickens. An apparent absence of invading hyphae, as shown by the periodic acid-Schiff stain, demonstrated the low virulence of this particular fungus for body tissue.

Extraction and attempted purification of the substrate from experiment B resulted in TLC plates with an unexpectedly low level of well-defined band two toxin. This would indicate that the toxin had assumed perhaps an even more potent form possibly as a result of metabolic change at the low temperatures.

Preliminary purification procedures, primarily concerned with removing hexane-soluble oils from the crude extract, proved to be quite efficient. Subsequent purification on TLC plates would have been rendered much more difficult if these oils had not been removed.

The use of thin-layer chromatography proved to be a reasonably efficient, though time-consuming, means of purification of the toxins. In order to purify a quantity of toxin sufficient for dosing larger animals, a modification of technique, possibly incorporating column chromatography, would be desirable. In early experiments to develop purification technique, column chromatography was investigated. This procedure was abandoned since the material eluted from the column seemed to be detoxified.

The purified extract from band one apparently is closely related structurally to zearalenone. Nelson et al. (68) list seven derivatives of "F-2" toxin (zearalenone) which have been identified. Most of these derivatives have absorption and fluorescent characteristics similar to F-2 toxin. The similarity of band one toxin in TLC fluorescence, RF value, and molecular weight would indicate its close association. The variance in uterine stimulation when compared to zearalenone (70 vs 90 mg) may have been dose-related. Other researchers have reported a higher uterine weight gain when zearalenone is injected intramuscularly (Christensen et al., 23).

The inability of the estrogen-treated rats to gain weight, as compared to the untreated, may also be a dose-related variance. Mirocha et al. (60) reported a relative decrease in body weight gain as dosages of zearalenone were increased sufficiently to stimulate greater uterine growth.

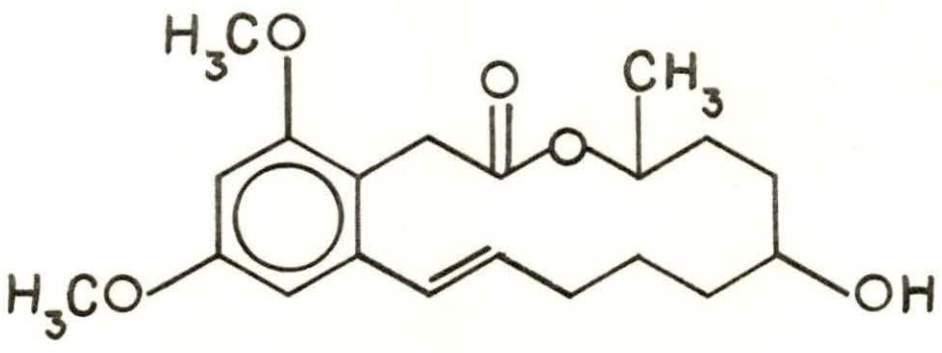
The most prominent of the lethal toxins was that purified from band two. This fraction was assumed to be primarily responsible for deaths in animals feeding on the cultured substrate or dosed with crude extracts. Its highly irritant action on the intestinal epithelium undoubtedly contributed to the deaths of animals tested. However, interference with other body structures cannot be excluded.

The LD_{50} for chickens of approximately 0.6 mg places the band two toxin in a class with the more potent mycotoxins. Wogan and Pong (100) state the LD_{50} in 50 gm ducklings for aflatoxin B_1 is 18.2 μgm while for 21-day-old male rats, it is 5.5 mg/kg. Further work with ducklings, rats, and other laboratory animals is indicated to compare band two toxicity with other known toxins. When a sufficient supply is available, it should be tested on larger animals such as pigs.

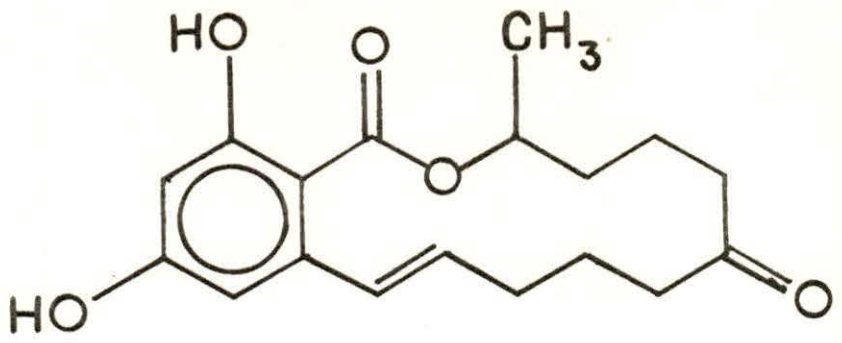
The reaction of guinea pig and rabbit skin to very low doses of purified band two toxin demonstrates the need for care in extraction and purification procedures. Laboratory personnel should wear protective gloves, especially when handling the highly concentrated material.

During the course of testing band two for dermal toxicity, several compounds were tested as vehicles for applying the toxin to skin. Ethanol was chosen over olive oil, acetone, and methanol because it was relatively nonirritating and did not disperse as rapidly as the other substances tested. An attempt was made to inject the ethanol suspension subcutaneously, hoping it would tend to concentrate the toxin in a smaller area. However, this proved unsuccessful as the ethanol apparently dispersed in the subcutaneous tissue. This irritation of the skin might be used as a biological screening test for determining toxicity of suspected feeds.

Infrared spectrum analysis of purified band two toxin had established many similarities between the compound and zearalenone. Additional examination by mass spectrum analysis and preliminary nuclear mass resonance yielded more information on its molecular characteristics. The proposed structural formula for band two toxin:



compared to the structure of zearalenone:



reveals a marked similarity. A substitution of aliphatic groups on the benzene ring and a slight shifting of the remaining structure is noted. This presumably accounts for the marked difference in biological action, Rf value, and fluorescent appearance. The aliphatic groups would also account for most of the difference in molecular weight (348 vs. 318).

Since a relatively small amount of band three toxin was available, it was not investigated as thoroughly as band two. Preliminary screening with the quantity available revealed a lower toxicity and an inconsistency in its dose response. The toxic dose for chickens of this material varied from 1.5 to 3.0 mg, depending upon the particular lot of substrate extracted and purified. A lower Rf value and structural variation from band two indicated that it was a separate compound, possibly the next step in the metabolic pathway of the toxin.

The small amount of water-soluble toxic material obtained in the preliminary procedures is interesting and should be investigated further. Gross pathological lesions in chickens were similar to those of bands two and three, but its variance in solubility from the latter compounds would indicate it is a separate toxin. Purification of a sufficient amount to

determine structural characteristics would be desirable. Comparison of the structure of the three lethal compounds might indicate only minor variations.

The broad band at the bottom of the preparative TLC plate should be examined further to determine if the toxicity it demonstrated is due to another compound. This band was eluted and the partially purified material used to dose chickens orally. Death was noted in chickens receiving as little as eight mg of the preparation. On necropsy, lesions similar to bands two and three were observed. The toxicity may be due to portions of band two or three that did not travel with the solvent or to another separate compound. A modification of technique to purify this portion should be attempted in order to more fully elucidate its toxic potential.

SUMMARY

A field isolate of the fungus Fusarium roseum var. graminearum was cultured on a suitable substrate, and its toxic potential was investigated in chickens and rats.

The growth of newly hatched chickens was retarded when they consumed rations containing as little as 2.5% of the cultured substrate. This retardation would appear to be the result of a combination of factors, viz: irritation of the oral cavity, transient erosion of the intestinal epithelium, and possible involvement of other body systems.

A technique is described to facilitate the extraction and purification of the crude material so that at least one estrogenic and three lethal toxins may be demonstrated biologically.

The estrogenic compound possesses physical and toxic properties similar to zearalenone, a known toxin produced by several species of the genus Fusarium.

Under the conditions described, a lethal toxin was produced in sufficient quantity to allow a thorough study of its physical and biological characteristics.

This lethal toxin appears to be one which has not previously been described from Fusarium roseum.

Two apparently different additional lethal compounds were demonstrated, but the limited quantities did not permit extensive characterization.

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