USDA Research Program on Mycotoxins[1]

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MY CONTRIBUTION TO THIS PROGRAM will be to acquaint you with the research activities in the US Department of Agriculture that have to do with the mycotoxins.

We will mention some of the practical steps the Department is taking to contain the mycotoxin problem while your research and ours is finding ways of eliminating it. We will confine our discussion to general implications of the research to date and avoid reporting specific research findings—a responsibility and privilege which is reserved for the researchers themselves.

Very little time need be spent before this group in reviewing the history of the mycotoxin problem. As you know, molds and mold damage in general have long been of interest to those concerned with agricultural products because of economic losses due to obvious decay and to more insidious adverse changes in the odor, taste, nutritive value, and appearance. Much research has been done over the years to find ways to minimize these losses. However, our action programs and our research were abruptly refocused a few years ago when the turkey poult incident in England and the succeeding scientific events identified a whole new family of problems—the problems of wholesomeness and safety of agricultural products contaminated with certain chemical by-products of certain molds.

Department Action in Response to the Mycotoxin Problem

The Department's interest began, quite understandably, with peanuts and aflatoxin—the particular mycotoxin produced by the mold *Aspergillus flavus*.

The Department made a survey of the incidence of aflatoxin in peanut stocks held by the Commodity Credit Corporation throughout the country. This study showed that there was a close relationship between damage in peanuts and the presence of aflatoxin. Shortly thereafter, the Department, working closely with the Department of Health, Education, and Welfare, embarked upon a four-part program in cooperation with peanut producers, shellers, and processors to assure that only high-quality peanuts were marketed for food, and that those not meeting these standards would be channeled into other uses.

The major points of this program were as follows: (a) changes in the price support program to better control the quality of marketed peanuts; (b) educational assistance to the peanut industry to acquaint them with the best known methods of safe growing, harvesting, drying, storing, shelling, and processing; (c) Federal-State inspection of all lots of shelled peanuts; and (d) a research program seeking ways to eliminate damage to peanuts due to molds.

Action Programs

An important first step was the Department's modification of its 1964 peanut price support program for the purpose of controlling the quality of peanuts marketed for edible use. The action taken recognized the fact that mold damage in peanut kernels is correlated with aflatoxin; that only a small proportion of the peanut kernels in the crop are damaged by mold; and that these kernels should not enter food trade channels. This program now includes provisions designed to keep contaminated peanuts out of food and feed channels and, at the same time, to minimize losses of peanut producers, shellers, or processors.

While all of the 1964 crop of peanuts has not been marketed by the shellers, the program is working well. The National Peanut Council has cooperated by adopting a Voluntary Code of Good Practices for purchasing, handling, storing, and processing peanuts. An excellent working relationship has been established between the Department—particularly the ASCS—the peanut industry, and the Food and Drug Administration.

Extensive chemical tests have been made on various grades and qualities of peanuts during the current year to check on aflatoxin. Based on these tests, manufacturers have rejected less than 1/10 of 1% of the better quality peanuts marketed for edible use. Based on tests of lower quality peanuts crushed by oil mills, the meal from peanuts representing not over 3% of the total crop has been restricted to nonfeed use because of aflatoxin.

Another aspect of the 1964 peanut marketing program has to do with the work of the Federal-State inspectors. The important feature involves positive lot identification—envisioned and planned jointly by the Department's inspection services personnel and the peanut industry. Positive lot identification, as the name implies, is intended to establish and maintain the identity of a lot of peanuts from the time it emerges from the milling operation until it is utilized in a manufacturing or processing plant. The system has served both to prevent the shipment of poor quality lots of peanuts to food outlets and to assure buyers that they are receiving the peanuts described on the certificate.

Concurrently, with improved inspection procedures, the Department's Consumer and Marketing Service undertook an aflatoxin testing program. Samples of shelled peanuts, peanut meal, and peanut butter are being analyzed for the presence of aflatoxin. This is accomplished by six laboratories located throughout the Eastern and Southern parts of the United States. This agency is responsible for the inspection and testing of peanut butter purchased for needy-family and school-lunch programs. They also test samples of CCC peanuts for aflatoxin. In addition, a large number of samples are run for commercial concerns interested in the control of aflatoxin in their products. To date, analyses have been made on 1,400 samples of peanut butter, 2,800 samples of shelled peanuts, and 200 samples of peanut meal.

An equally important aspect of the cooperative program of Government and industry to prevent contaminated peanuts from being used for food or feed has involved educational assistance. Farmers are being encouraged to use the best known practices for improving the quality of their peanuts, particularly better handling practices at the time of harvest. Educational programs are also emphasizing maintenance of quality through all handling and processing procedures.

Mycotoxin Research Programs

The fourth phase of the Department's program involves research. The major commodities being investigated are peanuts, cottonseed, cereal grains, and soybeans. The Department's research has four broad objectives:

1. To find practical methods for preventing, or at least minimizing, mold growth in agricultural commodities by determining at what stage in the growth, harvesting, and subsequent handling or storage of a commodity that mold growth and contamination by toxic substances occur.

2. To develop rapid methods for the detection of aflatoxin and solve any special problems in the analysis of each commodity, so as to make possible prompt and continuing surveillance of commodities in marketing channels.

3. To discover practicable processes for removing or destroying toxins in agricultural products that are contaminated.

4. To elucidate the metabolic fate of aflatoxin in domestic animals.

This research on mycotoxins is now under way in seven

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divisions of the Agricultural Research Service and eight State agricultural experiment stations. It is most convenient for me to discuss specific work being done in our divisions under three categories that reflect the organization of ARS—farm research, marketing research, and utilization research.

Farm Research

Mycotoxin investigations in this area are carried out by the Crops Research Division and the Agricultural Engineering Research Division, in cooperation with certain State agricultural experiment stations.

Studies are being made to determine whether *Aspergillus flavus* is present in peanuts, cottonseed, and soybeans before harvest and at what time after harvest these commodities may become contaminated in the field under various climatic conditions.

Studies on peanuts are being made to determine the effects of different digging, curing, and harvesting methods on quality and freedom from contaminating molds. In other studies, selected fumigants will be applied immediately after digging and during subsequent farm operations to find whether mold growth can be prevented under field conditions that are favorable to the growth of molds. Long range research is being undertaken in an attempt to breed varieties of peanuts with pods and seeds that are relatively more resistant to molds that produce toxins.

Research is also being undertaken to develop new and modified harvesting equipment to minimize mechanical damage which makes the peanuts more susceptible to damage from molds. Other work is being done on developing new and improved equipment and techniques for separating trash, soil, and immature peanuts to reduce the load in the subsequent drying operation and lessen contamination with molds from foreign matter.

Preliminary results are indicating that a small percentage of peanut seed and a much higher percentage of peanut pods are infected prior to digging with strains of *A. flavus*, most of which have the capacity to produce aflatoxin.

In the case of soybeans, arrangements have been made for a survey at the farm level, in the three major areas of soybean production, of the succession of fungi associated with developing and maturing soybean pods and seed. We hope to identify the principal fungi and to determine their toxin-producing potential.

Work has been started to identify potential toxin-producing organisms in and on cottonseed at harvest. Collections of seed-cotton have been made from the 1964 crop at several locations across the US cotton belt. Examinations to date have revealed the *Fusarium, Alternaria,* and *Aspergillus* species are among those that are common causes of internal infection. It will be recalled that previous workers have associated these fungi with the formation of mycotoxins under some conditions. No quantitative data are yet available on the frequency of occurrence of *Aspergillus flavus* in cottonseed at harvest, but the frequency seems to be low in collections examined to date.

Several of these fungi have been grown on living cotton bolls in a simulated boll-rot type of pure-culture incubation. Chemical and bioassay tests are contemplated to de-

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termine the toxin-producing potential of the associated fungi.

Marketing Research

Research in this area is carried out by the Market Quality and the Transportation and Facilities Research Divisions. Studies include the effects of curing, storage, and other off-farm handling conditions on the production of aflatoxin; development of methods for the rapid detection of molds and fungal metabolites; and improvement in techniques for storing, cleaning, shelling, and grading of peanuts. Some of this work also applies to cottonseed and rice, particularly the effects of ambient conditions of storage on molding and aflatoxin production in cottonseed.

Preliminary results are indicating that aflatoxin was not observed in freshly dug peanuts in 1964, although *A. flavus* was found to infect most of the samples from pods having visible openings. The development of aflatoxins in the windrow was quite variable. Under hot, humid conditions (over 90F and 70% relative humidity), significant amounts of aflatoxin were found in a small percentage of samples. But under cool conditions (70F), no aflatoxin development occurred even after a week or more in the windrow, regardless of rain and high humidity. It is significant to note that when aflatoxin did occur soon after harvest, it was associated with broken pods.

There appears to be a critical range of peanut kernel moisture, above and below which toxin production by *A. flavus* decreases sharply. Reduction of peanut moisture below the critical level necessary for germination of most fungal spores appears to be the best single means of controlling mold development on peanuts. Yet to be determined, however, are the optimum time-temperature-moisture relationships for drying peanuts to obtain the prompt reduction of moisture to minimize mold infection while maintaining desirable qualities such as taste and appearance. In this regard, preliminary experiments employing alternate heating and cooling show that aflatoxin development is arrested and, at the same time, desirable qualities in the dried peanuts are maintained even when the moisture reduction per hour is doubled.

An experimental continuous-type dryer has been designed that will provide much greater flexibility in drying-air temperatures, airflow rates, exposure time, and drying methods than presently available drying equipment. This new dryer will be used this coming harvest season in continuing studies to determine optimum drying treatments for arresting aflatoxin development, with particular emphasis on drying high-moisture, freshly dug peanuts. Variables in conventional drying as they affect aflatoxin contamination are also under study.

Some additional interesting information is emerging from Marketing Research studies.

For example, a survey is being made of the aflatoxin-producing capabilities of *A. flavus-oryzae* isolates from peanuts. Results to date reveal that most are potential aflatoxin producers. The total aflatoxins produced and the relative proportion of the four principal toxins produced on natural substrates is a function of strain and substrate; likewise, the relative amounts of aflatoxins excreted into the substrate in relation to that retained in the mycelium is a function of the same factors.

In farmers' stock peanuts stored for a year under ambient conditions, kernels from damaged pods were generally found to be contaminated but kernels from sound pods were free of toxin. In view of the generally higher aflatoxin content of stored samples compared to freshly harvested lots, the likelihood of an aflatoxin build-up in peanuts during storage is being investigated.

One potent *A. flavus* strain has been found to produce almost equal quantities of aflatoxins B_1, B_2, G_1, and G_2. An effort is being made to develop a rapid uniform method for the evaluation and classification of the toxin-producing ability of fungal isolates. A rapid screening test for detection of aflatoxin contamination is also being sought.

A large number of fungi, many typical of the microflora of stored agricultural grain, oilseeds, and seeds, are being examined for aflatoxin-producing capability. Strains of four aspergilli and three penicillia have been found to be aflatoxin producers.

Finally, in the Marketing Research Divisions, a thorough survey is under way on the incidence of aflatoxin contamination in cottonseed received at oil mills. Weekly sampling of cottonseed and cottonseed meal is to be made at fifty mills for a minimum of ten weeks.

Utilization Research

Three of the Utilization Divisions—Southern, Northern, and Western—are conducting extensive research programs directed toward solving the mycotoxin problem.

At the Southern Utilization Research Laboratory in New Orleans, considerable attention has been devoted to development and refinement of analytical methods for the aflatoxins in both peanut and cottonseed products. There are able spokesmen of the Southern laboratory here, so our remarks in this regard will be brief. Their technique, which utilizes a mixture of acetone, hexane, and water, rapidly and quantitatively extracts the aflatoxins from peanuts, while removing relatively little extraneous material. The readily purified extracts, dissolved in chloroform, are assayed for aflatoxin content by thin-layer chromatography on plates coated with silica gel. In raw peanuts, as little as 2 ppb (2 μg/kg) of aflatoxins can be detected by use of this procedure.

They have found, however, that this procedure results in intense pigmentation of gossypol derivatives when applied to whole cottonseed or kernels. This imposes severe limitations on the use of the techniques. The Southern laboratory has developed a method which makes use of 70% acetone for rapid extraction of aflatoxins essentially free of lipid contamination. Interfering gossypol pigments in crude extracts are removed by precipitation as insoluble lead derivatives. At the present time, as little as 1 ppb (1 μg/kg) of aflatoxins can be detected in cottonseed.

The aqueous acetone procedure has subsequently been found to be applicable to a variety of agricultural products, in that lipids and other interfering substances are not extracted.

The second area of research that is being given intensive attention at the Southern laboratory concerns the development of practical methods for removing or destroying the aflatoxins in contaminated cottonseed and peanut products. Attention has been given the application of the acetone-hexane-water solvent system for removing aflatoxins, and it is quite effective. It is potentially useful for making aflatoxin-free oil and meal out of contaminated cottonseed and peanuts, and for removing aflatoxin from meals produced by other processes.

Another promising solvent system, which has been applied only on a laboratory scale, includes extraction of cottonseed or peanuts with aqueous acetone (ca. 25% water) to remove aflatoxins prior to extraction of oil by hexane or mechanical pressing. In the case of cottonseed, gossypol pigments and aflatoxins are efficiently removed by aqueous acetone, but oil is substantially insoluble in the system. Other solvent systems are being explored.

Chemical treatments to inactivate aflatoxins in situ are also being investigated on a laboratory scale. To be practical, such treatments must be economic and readily introduced into currently used processes, and they must not destroy the nutritive value of the meals. Some appear promising but they require further investigation.

The Northern Utilization Research Laboratory has de-

veloped an experimental procedure to produce aflatoxin by growing *A. flavus* on moist whole polished rice. Yields of total aflatoxins are in the range of 1.5 g of aflatoxin per kilogram of fermented substrate. Aflatoxin B_1 is consistently produced on rice at almost a gram per kilogram. Aflatoxin is very readily recovered with a high degree of purity from this substrate. Optimum temperature for aflatoxin production is from 28C to 31C, but temperatures of 37C or higher decrease yields markedly. Aflatoxin is formed within 48 hr after inoculation and appears in the substrate, mycelium, and conidia. A search is also underway in the Northern laboratory for enzymes which destroy or inactivate the aflatoxins.

The Western Utilization Research Laboratory is investigating the physiological action and metabolic fate of aflatoxins in selected animals. Preliminary, acute experiments with the C^{14}-labeled aflatoxin in rats (2 mg per 400 g rat by stomach tube) indicate that most of the detectable aflatoxin is eliminated unchanged in the urine and feces during a 48-hr period, at the end of which the animals are moribund. The cause of death in these experiments was massive visceral hemorrhage. Small amounts of aflatoxin were found in kidney, liver, and fat, but very little in carcass muscle. Balance studies are planned to permit quantitative estimates of the distribution.

A swine-feeding trial is being conducted by the Western laboratory in collaboration with the Department of Animal Husbandry of the University of California, Davis, using aflatoxin-contaminated peanut meals furnished by the Southern laboratory.

Aflatoxin residues in tissues will be measured. Hematological and serum enzyme changes during the course of the feeding are also being measured to determine whether changes in liver function occur prior to overt histopathological damage.

Fifty-eight weanling Duroc Jersey pigs (gilts and barrows), divided into six experimental groups, were fed for 90 days on rations containing 15% peanut meal. The aflatoxin content of the meals was 0, 10, 50, 340, 750, and 1500 ppb.

I am told that no significant differences have appeared between the animals on the 0 control diet and the highest aflatoxin level (1500 ppb in the meal) with respect to mean daily weight gain, mean feed intake, feed efficiency, serum transaminase (SGOT), serum alkaline phosphatase, serum nonprotein nitrogen, serum copper, serum vitamin A, erythrocyte count, leukocyte count, hemoglobin (acid hematin), hematocrit, and differential leukocyte count (neutrophils, lymphocytes, monocytes, eosinophils, and basophils). In addition, a comparison of the serum protein spectrum (electrophoretic) showed no difference between the control and high-level groups.

The animals in this part of the trial were slaughtered from April 1 to 13, and pertinent organ weights were obtained as well as tissue samples for total histopathology. Blood and tissues are being lyophilized and analyzed chemically for aflatoxin residues. The lyophilized tissues will be fed to ducklings as 20 percent of their diet for 30 to 60 days to reveal the possible effect of nephrotoxins from the endotoxin portion of the *A. flavus* mycelium.

The second part of the trial will commence about June 1, 1965, at which time fortified peanut meals containing graded dose levels of 1500 to 5000 ppb of aflatoxin B_1 will be fed.

Although not directly related to physiological action or metabolic fate studies on aflatoxin, a two-year chronic toxicity trial is under way at the Western laboratory. This involves the feeding of treated and untreated peanut meals to rats. The meals are fed as 35% of the total ration. The aflatoxin content of the meal is 0 ppb, 75 ppb, and 1000 ppb. As of March 28, 1965, the trial had been in progress 333 days with 2 recent deaths in the treated group and 1 in the control group due to pneumonia. Growth curves and food intake curves are normal for all meals at this point. Autopsy of the 2 rats which died revealed cysts on the liver. Histopathological examination is now in progress.

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