The Three Eras of Fungal Toxin Research

LEONARD STOLOFF, Bureau of Foods, U.S. Food and Drug Administration, Washington, DC 20204

ABSTRACT

The era of moldy food or feed toxicoses dates from 1711 when the role of the fungus *Claviceps purpurea* in the formation of the poisonous ergot grains on rye was established. Since that time numerous toxic incidents have been related to ingestion of moldy food or feed. The thrust of the research through this period was to isolate and identify the responsible mold(s). We are now well into the mycotoxin era, which dates from 1962 with the isolation and characterization of the aflatoxins. The absence of viable mold in the toxic peanut meal that sparked this era provides a suitable backdrop for the current research activity with isolated, well identified toxins of mold origin. We now have identified toxins with no obvious disease relationship. This brings us to the third era, the era of multiple challenges just getting under way. Since some of the first era toxicoses cannot be explained on the basis of the isolated toxins alone, we are beginning to realize that multiple factors may be involved.

There has been a complete turnaround since 1962 when Forgacs and Carll published their review (1) on mycotoxicoses ". . . the least investigated . . . among the various maladies afflicting animals and human beings . . . " Little did they know as they gathered their material that events were in the making that would completely alter the picture. Their comprehensive review ushered out the era of mold-associated toxicoses and set the stage for the era of mycotoxins. In reviewing mold-associated toxicoses, the first era of fungal toxin research, they started by eliminating from consideration not only poisonous mushrooms, which truly belong in a separate category, since they are consumed directly, but also ". . . grain where the toxic substance is formed during invasion of the living plant by the causal fungus . . ." By limiting the presentation to ". . . those maladies associated with ingestion of products . . . on which fungi proliferate as saprophytes and give rise to toxic products," they not only lost a historical perspective – the slow progress since the recognition in 1711 that ergoty grain was the result of mold damage – but contributed to the development of the erroneous view that toxicogenic molds were associated primarily with faulty harvesting and storage practices. Despite these shortcomings, Forgacs and Carll were unusually astute. Referring to antibiotics as an example, they noted the wide variety of biologically active compounds produced by molds, and reasonably assumed that the variety was not limited to antibiotics. They observed that not all strains of the same fungal species were necessarily toxicogenic and that toxin-producing strains could lose that capability on repeated subculture. They called attention to the selective properties of the usual employed laboratory culture media and the need to look at undisturbed field material for the dominant mold types, instead of counting colonies on a plate. And finally these authors called for accurate taxonomy and a multidisciplinary approach.

What, then, was the state of knowledge about mycotoxicoses when Forgacs and Carll prepared their review in 1960? Alimentary Toxic Aleukia, first recorded as a food intoxication in the Amur region of Siberia in 1913 and endemically thereafter in various other regions of Siberia and Russia, was attributed to toxins from the growth of *Fusarium sporotrichioides* on cereals held over the winter. A recurring disease of horses in eastern Europe was shown to be associated with the growth of *Stachybotrys atra* on their fodder. Facial eczema in ruminants, caused by photosensitization from excessive bilirubin in the blood, was related to liver damage from toxins occurring in dead pasture invaded by *Sporodesmium bakeri* in New Zealand or *Periconia minutissima* in the United States (Florida). Clear attribution of a disease to a particular fungus was not so easily made in the case of moldy corn toxicosis of swine or of moldy feed toxicosis in poultry. The disease symptoms in swine could be reproduced with cultures of *Aspergillus flavus, Penicillium rubrum,* or *P. purpurogenum* isolated from moldy corn. The disease symptoms in poultry could be reproduced with a much longer list of isolates, obtained from moldy feed and cultured on broiler mash: *Aspergillus clavatus, A. flavus, A. fumigatis, A. glaucus, Penicillium citrinum, P. purpurogenum, P. rubrum, Paecilomyces varioti,* an *Alternaria sp.,* and a number of unspeciated *Pencillia.*

One mycotoxicosis that was missed by Forgacs and Carll illuminates those factors that stimulate interest and activity. Vulvovaginitis of swine associated with moldy feed had been known since at least 1928 (2); the active compound, later named zearalenone, had been isolated by 1960 (3), and by 1962 had been fully reported in the open literature (4). However, little interest appears to have developed beyond those immediately involved. Compare this with Turkey X and related veterinary diseases, described in 1960, that ushered in the second era of fungal toxin research, the era of mycotoxins. Even though the mold association was not immediately evident, it was quickly established. The active compounds, aflatoxins, were isolated and characterized within the years 1963 to 1965, the same period in which the zearalenone reports appeared. The interest in aflatoxins would probably have been as specialized as in zearalenone except for two factors: animal studies showed the aflatoxins to be potent carcinogens, and the development of simple analytical methodology for low level detection allowed the demonstration of their widespread natural distribution in foods as well as feeds. In spite of considerable effort to find similar situations of widespread natural occurrence of a potent chronic toxin of mold origin, the aflatoxins are still unique, and for regulatory purposes, at least in the United States, aflatoxin is virtually synonymous with mycotoxin.

The search for analogies to the aflatoxin situation started as soon as the realization struck home that a residue of mold growth, capable of causing severe harm at a time remote from ingestion, could be present in food or feed with no associated overt evidence of the mold. Fortunately, the secondary products of mold metabolism had intrigued some organic chemists, particularly Raistrick, his colleagues, and their successors at the London School of Hygiene and Tropical Medicine, who, starting in 1931 (5) and continuing to this day, have published on the isolation and characterization of numerous mold metabolites. The search for antibiotics, and the lesser search for other products for exploitation by the fermentation industry, provided additional compounds for the list. Most of these compounds had been conveniently tabulated in the "Pfizer Handbook of Microbial Metabolites" (6) or in the "Handbook of Toxicology: Antibiotics" (7). A search of the literature turned up a few more compounds, particularly those isolated by the Japanese (8,9), who had been exploring

their own mycotoxin problem associated with moldy rice. Although predating aflatoxin, this work had made little impact on the Western world, probably because of the language in which it was originally published. From among these myriad compounds, the following criteria were used by the FDA mycotoxin research group to select those likely to cause harm as natural contaminants of food or feed: (a) at least one of the producing molds must be a common component of the microflora of food or feed, or have been implicated in a toxicosis; (b) the toxicological evidence must indicate a potential for harm from relatively low level ingestion, including but not restricted to carcinogenesis. The list of compounds of interest thus compiled in 1965 and revised in 1974 included:

Actinomycins	Islanditoxin	Penicillic Acid
Aflatoxins	Luteoskyrin	Rubratoxins
Unnamed butenolide	Rugulosin	Sporidesmins
Citrinin	Nidulin	Sterigmatocystins
Ergot Alkaloids	Ochratoxins	Trichothecenes
Gliotoxin	Patulin	Zearalenone

The first compounds selected for attention were: sterigmatocystins, because of its close structural relation to aflatoxin; patulin and penicillic acid, because of the number and ubiquity of the producing molds, the demonstrated carcinogenic potential of the compounds, and the relation of their structures to know carcinogens; and zearalenone, because its natural occurrence as a contaminant of corn had already been demonstrated, and toxicologists were basically suspicious of any compound with hormonal activity. The trichothecenes were later earmarked for attention when their relation to alimentary toxic aleukia in man had been established and their production by *Fusaria* common to American grains was demonstrated.

More recently, toxins produced by species of *Alternaria* have been earmarked for attention because of the frequent occurrence of members of this genus on foodstuffs and the characterization of some of their toxic products.

What is the current state of knowledge about these mycotoxins?

Two independent lifetime feeding studies with rats have shown sterigmatocystin to be heptocarcinogenic, but surveys of substrates suitable for sterigmatocystin-producing molds have uncovered no natural contamination of marketable food or feed. Although the analytical limit of detection for sterigmatocystin is two orders of magnitude greater than that for aflatoxin, the yield of sterigmatocystin from laboratory cultures is higher than that for aflatoxin by the same magnitude.

As expected from the number and prevalence of the producing molds, with very little survey effort patulin and penicillic acid were found to occur naturally in significant amounts (patulin in apple juice, penicillic acid in corn and dried beans), justifying a comprehensive toxicological study. Patulin has been carried through acute, subacute, short term (10) and metabolism (11) studies; long term studies are now almost complete. There is presently no evidence of any potential for harm from oral ingestion, except for those levels that can alter the gut microflora because of patulin's antibiotic properties. Short term (12) and metabolism (13) studies of penicillic acid have also been completed with no adverse evidence. The carcinogenicity of both compounds had been demonstrated by subcutaneous injection (14). Both penicillic acid and patulin react readily with sulfhydryl groups like those in the sulfur-containing amino acids, a possible explanation for the observed lack of oral toxicity and a deterrent to providing a total assay.

Zearalenone has been associated with invasion of corn by *Gibberella zeae* (= *Fusarium roseum*), an endemic field problem in some parts of the Corn Belt, which sporadically bursts forth with increased intensity when favored by weather conditions. The anabolic property related to the estrogenic activity has led to commercial exploitation of the compound in the form of a more active chemical modification for implanting in steers. This exploitation has provided some information on its short term toxicology and relative estrogenic potency and has furnished a ready source of the compound for further study. Experiments, with primates (15), which are continuing, currently show no cause for concern from the levels encountered in feed grade corn during one of the peak occurrence years. The major danger from zearalenone is to sows, which appear to be particularly sensitive to its estrogenic activity. There is a good possibility that many of the problems occur because of storage conditions that allow continued activity of the mold to increase zearalenone over field levels. Zearalenone contamination is not limited to corn. In a recent survey (16), zearalenone was found in wheat, related to an epidemic of scab, demonstrating that any grain susceptible to *Fusarium roseum* scab is a possible candidate for zearalenone contamination.

Although acute human fusariotoxicosis, presumably caused by trichothecenes, is documented (17) as having occurred in a number of areas of the world from grains used under adverse conditions, there is no evidence of any similar occurrence in the United States. However, without information on chronic toxicity the problem cannot be dismissed. To acquire this information, those trichothecenes that are likely to occur in crops grown in different parts of the world must be selected from among the 23 that have been isolated from various species of Fusaria. For this determination, "trichothecene profiles" of typical Fusaria from each area are needed. An approach to acquiring these profiles for *Fusarium* species found on grains in the United States is underway at the Food and Drug Administration's Bureau of Foods. Analytical method development will be focused on trichothecenes selected on the basis of these factors: (a) prevalence of the producing mold; (b) amounts produced in culture; and (c) acute toxicity. Surveys for these trichothecenes should then provide a rational basis for selection of those to be submitted for chronic toxicity study.

We have paid close attention to the Danish work in which ochratoxin and citrinin in barley invaded by *Penicillium viridicatum* were related to nephrosis in swine consuming that barley (18). Subsequent Danish controlled studies of ochratoxin toxicity in swine have received FDA support. The data derived from these experiments are insufficient for an assessment of risk, but do show that ochratoxin can reproduce the lesions seen in both market pigs and pigs exposed experimentally to naturally contaminated barley.

It is evident from the search for aflatoxin analogies that each mycotoxin situation has its own peculiarities. Even so, there are enough similarities that a review of the aflatoxin story could prove useful. What bits of wisdom can be distilled from a decade and a half of investigative effort that has produced more than 2,000 papers describing original research on the mycology, chemistry, toxicology, and control of aflatoxins?

From the reports of aflatoxin production by at least 13 different mold species, the practical etiology has boiled down to the initially discovered relationship with *Aspergillus flavus* and its close relative *A. parasiticus*. The first conjecture that the mold invasion could be the result of faulty storage practices is still valid but not the total story. There is developing evidence that, depending on the commodity and the technological sophistication of the marketing system, preharvest invasion by the aflatoxin-producing molds can be a significant cause of contamination.

At first, every commodity or manufactured product

susceptible to mold damage was considered a likely candidate for aflatoxin contamination; however, after considerable survey of those candidate commodities consumed in the United States (19), the most frequently contaminated were found to be peanuts, Brazil and pistachio nuts, corn, and cottonseed, with a strong regional source bias in the degree of contamination found. A much lower frequency and level of contamination has been found in almonds, walnuts, pecans, figs, and sorghum. Aflatoxin has also been identified, chemically and from the symptoms, in feeds associated with veterinary problems (20), incidents that could have been avoided had available technology been employed to prevent moisture accumulation and subsequent mold growth.

Other lessons in mycology: mold growth is not synonymous with mycotoxin; a laboratory demonstration of potential is not evidence of natural occurrence.

Development of analytical methods for aflatoxin was facilitated almost from the start by the ready availability of reference standards: at first the original toxic peanut meal, then toxin concentrates from mold culture extracts. Within three years of their first separation, the toxins had been chemically characterized, and "pure" reference standards were being distributed. Spurred by the early discovery of the carcinogenic properties of the toxins and the importance of peanuts as a food, at least half a dozen laboratories in England, Canada and the United States were involved in devising analytical systems. Information was freely exchanged between laboratories, with no thought of publication priorities, so that within two years of the first demonstration of the oncogenic properties of the aflatoxins, a collaborative study of an analytical method had been completed. Key elements in the success of these efforts were: (a) the fortuitous property of intense fluorescence exhibited by the aflatoxins, (b) the rapid rise of thin layer chromatography as an analytical tool. By the time the 11th edition of Official Methods of Analysis of the AOAC (21) was ready for assembly in 1969, eight methods for aflatoxin analysis had been adopted. By this time, also, the spirit of cooperation had crystallized. On the recommendation of the American Oil Chemists' Society, a Joint Mycotoxin Committee to coordinate methods development efforts was established with the Association of Official Analytical Chemists, to be joined later by the American Association of Cereal Chemists. As of today, 22 methods for aflatoxins have been adopted by one or more of the organizations (22), and, through informal cooperation, by the International Union for Pure and Applied Chemistry (IUPAC) and by the European Economic Community. Spawned by the rapid pace of events, an informal "Mycotoxin College" has developed for rapid exchange of information across organizational lines.

Other lessons in methods development: the potential for cancer provides a tremendous impetus to the provision of resources, and cooperative effort can speed development, but the most rapid progress comes from the exploitation of fortuitous circumstances (serendipity).

By far the greatest volume of published work on the aflatoxins is concerned with their toxic and carcinogenic properties. Part of this effort can be attributed to the ready availability of the isolated toxins, part to the easy availability of funds during this period for any research on cancer, and part to the improvements in techniques for exploring biochemical reactions at the subcellular level. Most of this effort has been concerned with aflatoxin B_1, the most toxic and the most prevalent of the mold-produced aflatoxins. From all this, several things were learned about the hazard to man from aflatoxin ingestion (23).

The accumulated evidence shows that the original studies of carcinogenicity were performed with the animal species (the rat) – and the determination of aflatoxin potency was made with the rat strain (Charles River) – most sensitive to aflatoxin carcinogenesis. The evidence also shows that animal species differ greatly in susceptibility to aflatoxin carcinogenesis and that primates are not immune. The liver has been the primary target organ in all animals studied, although tumors could be induced in other organs with relatively massive doses. Limited studies on fetal risk from transplacental exposure indicate the possibility of either terata or liver tumors, but again relatively massive doses were required for an observable response. These observations make sense in light of the evidence that one or more reactive metabolites, and not aflatoxin B_1 itself, are the immediate carcinogens, and that animals differ in the way they metabolize aflatoxin. Comparative studies indicate that man metabolizes aflatoxin most like those animals of intermediate sensitivity to aflatoxin carcinogenesis. Epidemiological studies in areas of Asia and Africa, where relatively high rates of primary liver cancer are encountered, show a correlation between current liver cancer rates and current measures of dietary exposure to aflatoxin. Although suggestive of an association between the two factors, the argument has many flaws, including projection to past exposure from a very limited time frame and neglect of other known insults to the liver in the areas studied. Liver cancer rates in the United States actually show a negative correlation with expected exposure to aflatoxin (23).

Other lessons in toxicology: the truth is seldom as bad as the worst case situation based on limited information; it becomes very difficult to withdraw from a position based on the worst case.

Efforts to control aflatoxin contamination have been based on the solid triad of: (a) research into causes and cures, (b) education of farmers and manufacturers concerning the research findings, and (c) government regulation founded on the best available knowledge. The task was a cooperative one from the start, involving the U.S. Department of Agriculture, the Food and Drug Administration, and industry groups, often working through the professional societies represented in the Joint Committee referred to in the discussion on analytical methods. Research into causes started, obviously, only after surveys had demonstrated the existence of a contamination problem. Deviations from good commercial storage practices in the United States were the easiest to correct, but turned out to be the least significant contamination factor. The contribution of faulty on-farm storage practices is difficult to assess and probably varies with crop and region. Some cases of peanut contamination have been related to poor storage conditions (24), and the worst cases of corn contamination have been on-farm incidents with improperly stored grain (20).

There is now solid evidence that much of the contamination may have occurred before or during harvest of peanuts (24), field corn (25), cottonseed (26), almonds (27), walnuts (28), and pistachio nuts (29). The most severe observed occasions of aflatoxin contamination of peanuts and of corn have been related to conditions of heavy insect attack on plants stressed by drought. Aflatoxin contamination of cottonseed in the most severely affected area has been clearly related to pink bollworm damage. Aflatoxin contamination of almonds has been related to attack by the navel organe worm; in walnuts aflatoxin is most likely to be found in those varieties susceptible to "sunburn"; and aflatoxin has been found in some Iranian varieties of pistachio nuts freshly removed from the tree. Crops such as peanuts, corn, pecans and walnuts that require additional drying after harvest are also vulnerable to mold attack until adequately dried, because of mechanical damage from the harvesting process. At this stage nature plays an important role. Inclement weather and/or bumper crops can tax drying facilities.

Geographical considerations also appear to be important (19), probably as they affect weather conditions and agronomic practices. In the United States, aflatoxin contamination of peanuts is heaviest in the Georgia-Florida-Alabama area compared to the Virginia-Carolina or the Texas-Oklahoma areas; aflatoxin contamination of corn is heaviest in the Southeastern states and practically nonexistent in the Corn Belt; aflatoxin contamination of cottonseed is primarily a problem of the Southwest.

So far, research into causes has produced no cures for the preharvest contamination. Some of this unavoidable contamination can be reduced (30) by sorting out those kernels showing evidence of mold damage. Sorts by color, size, or weight have proven to be practical and effective for peanuts, almonds, walnuts, Brazil nuts, and pistachio nuts. Normal roasting of peanuts destroys about half of any aflatoxin present, and roasting has been advocated as a means for reducing aflatoxin contamination of corn to be used for feed. Oxidative degradation of aflatoxin is the probable mechanism. Peroxide and hypochlorite have been used directly on wet process peanut flour. Aflatoxin is even more susceptible to oxidative degradation when the lactone is converted to the acid salt, a fact that is utilized in the treatment of oilseed meals with gaseous ammonia under pressure at elevated temperature and in the treatment of corn with ammonia in solution. Normal refining effectively removes aflatoxin from oils made from contaminated oilseeds or grain germs, and normal milling procedures effectively divert to feed use that part of the corn kernel in which the major portion of the aflatoxin is found, except when the milled product is full-fat corn meal. Most of the aflatoxin from milled corn is found in the steep water, germ, gluten meal or bran, depending on the milling process. The traditional treatment of corn with lime water to make the masa for tortillas and other Latin American corn doughs removes a large proportion of any aflatoxins that may be present, and, although not tested, similar results would be expected from the alkali treatment of corn to make hominy.

The cooperative effort to determine causes and develop remedies for aflatoxins in peanuts has served as a model for other commodities subsequently found to be susceptible to contamination. The National Peanut Council, representing the consumer product manufacturers, and the Peanut Administrative Committee, representing the shellers, have worked closely with the U.S. Department of Agriculture and the Food and Drug Administration to develop and put into practice the controls to assure the minimum consumer exposure to aflatoxin. The system has worked well. Concern for consumer reaction motivates the manufacturers of the peanut products. Through organized action they maintain pressure on the sheller, who in turn pressures the farmer to prevent that part of the contamination he can control. The Food and Drug Administration has set guidelines above which contamination will not be tolerated and monitors the consumer products to see that the guidelines are met. These were first based on the limits of analytical capability. When progress in analytical capability outstripped the potential for manufacturing control, the Agency proposed to set the guideline on the basis of the best practical manufacturing capability. Final action on that proposal is pending.

As surveys by the U.S. Department of Agriculture and the Food and Drug Administration established susceptibility and developed level and incidence data, trade associations for the respective commodities were advised of the findings and their relation to the FDA guideline. The response has varied in relation to the proximity of the group to the ultimate consumer product. Importers of Brazil and pistachio nuts have agreed to a voluntary program of inspection at port of entry with essentially 100 percent coverage. Domestic shellers of almonds, pecans, and walnuts have supported research into causes and methods for control. Corn millers have waged an intensive educational campaign, which unfortunately does not appear to have reached the unorganized "crossroads" millers. Although cottonseed millers, through their trade organization, have supported research on causes and removal of aflatoxin contamination and have disseminated information on control practices, application of the control has varied with the local regulatory situation for animal feed. At the start of the production chain, education of the individual independent farmer is one of the more refractory problems.

To summarize the lessons in aflatoxin control: producers, as a group, can be depended on to act responsibly. Anticipation of consumer reaction is a positive driving force for industry, that, like all forces, varies with the square of the distance between the objects. Postharvest, or preventable, causes of contamination can be avoided through education in good manufacturing practices; preharvest contamination is a sizable factor that is presently unavoidable. Conventional processing for food use generally tends to reduce contamination levels.

Even as exploration of the potential hazard from individual mycotoxins has progressed, there have been expressions of curiosity about the possibility of synergistic action from one or more mycotoxins occurring together (considering groups of related mycotoxins, such as the aflatoxins, as one mycotoxin). This curiosity first took the form of acute toxicity tests with combinations of two mycotoxins, with no rational basis for assuming their co-occurrence in a natural contamination situation. Multimycotoxin screening tests that were developed had no more rational basis. Thus, there was a transition into the third era of fungal toxin research, the era of multiple challenges.

There are at least four possible ways in which multimycotoxin contamination might occur: (a) by commingling of products contaminated with different mycotoxins, (b) by growth of mixed populations of toxicogenic molds, (c) by sequential growth of different toxicogenic molds on the same substrate, and (d) by multimycotoxin production by a single mold species.

The probability of various types of mixed mycotoxin contamination from commingling can be calculated from incidence level data which at present are available only for aflatoxin and zearalenone. This calculation has been made for corn from the Corn Belt, during a year when 3.6% of the lots tested had more than 1,000 ng/g zearalenone (31), mixed with an equal amount of corn from the Southeast when 20% of the samples had more than 40 ng/g total aflatoxins (19). Assuming no restrictions to random selection, there is a 0.7% chance of the resulting mixture having >20 ng/g total aflatoxins and >500 ng/g zearalenone. Since market forces also interact, the chance is likely to be even less that these probably innocuous levels will be exceeded.

The production of aflatoxin by *Aspergillus flavus* or *A. parasiticus* in mixed culture situations has received some attention. One study allowed *A. flavus* to compete with the normal mixed mycoflora of grain (32); a marked depression in aflatoxin production over the pure culture situation was noted. In another study (33), *A. flavus* was cultured with five other toxicogenic molds. Although, under the conditions used, *A. flavus* was the dominant mold, the presence of the other molds caused a marked depression of aflatoxin production. The inhibitory effect was traced to a filterable, heat stable product of the growth of two of the other toxicogenic molds, *Penicillium expansum* and *P. citrinum*. In these studies of mixed toxicogenic cultures, aflatoxin was the only mycotoxin that could be detected. The effects seen with mixed cultures of *A. parasiticus* with various yeasts and bacteria provided similar observations (34).

The developing evidence so far does not seem to support

the possibility of multimycotoxins, if one is looking at multiple molds as the source. But there is nothing in nature that restricts each mold to one toxin or group of toxins. We already know that *Fusarium roseum* can produce both zearalenone and trichothecenes (35). Some strains of *Penicillium viridicatum* can produce ochratoxin and citrinin (36); other strains of this same mold produce xanthomegnin and viomellein (37). A number of isolates of *Aspergillus ochraceus* were found capable of producing both penicillic acid and ochratoxin A (38-40), and isolates of *Penicillium expansum* produced both patulin and citrinin (41). *Penicillium islandicum* is a virtual toxin factory (42), producing at least five separate metabolites identified as capable of causing harm, including cancer, to laboratory animals. Although the optimum conditions for production of each toxin do not usually coincide, both ochratoxin and citrinin have been found in naturally contaminated barley invaded by *P. viridicatum* (18).

Perhaps from these observations the multiple order permutations of the third era investigations can be contained within reasonable bounds.

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