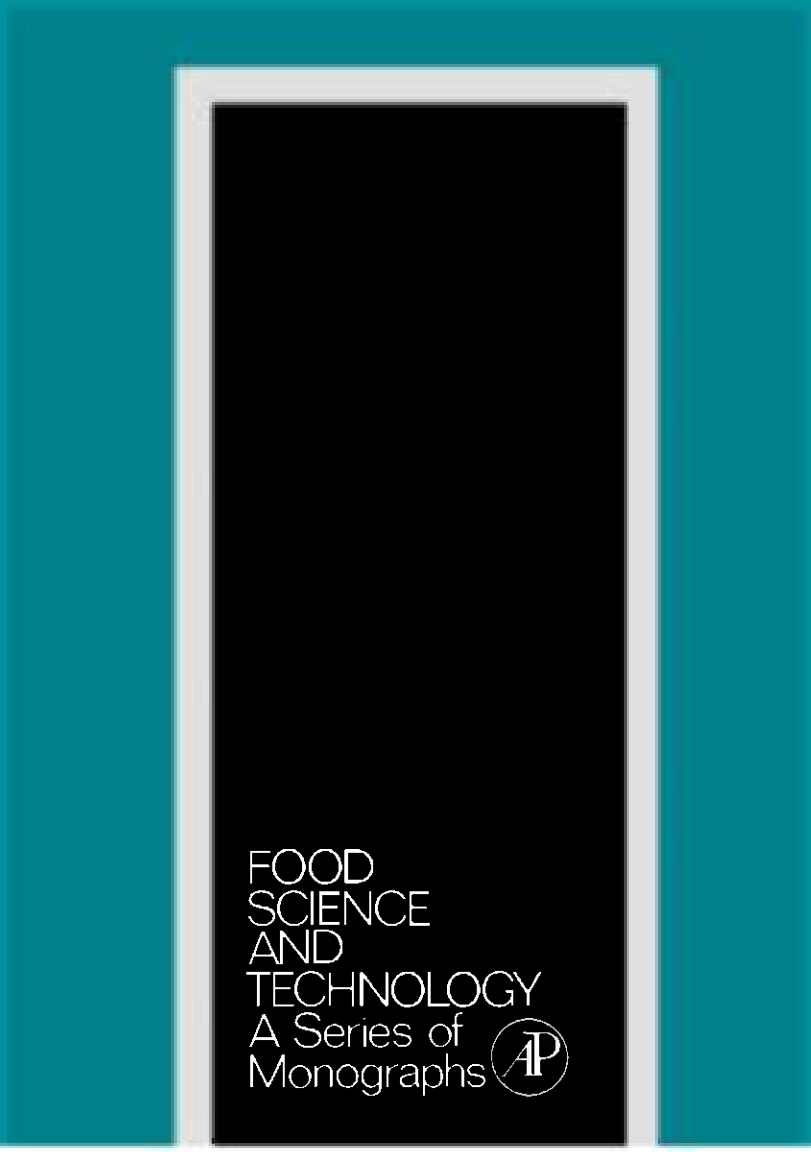
AFLATOXIN

Scientific Background, Control, and Implications

Edited by LEO A. GOLDBLATT



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Scientific Background, Control, and Implications

FOOD SCIENCE AND TECHNOLOGY

A SERIES OF MONOGRAPHS

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PREFACE

The presence of mold toxins is potentially the most serious quality problem which faces producers, manufacturers, and handlers of food and feed products. For many years, molds have been known to produce toxic metabolites but their effects were largely ignored, thus mycotoxicoses have been aptly called the neglected diseases. This situation has altered drastically with the developments relative to the "Turkey X" disease which appeared in England in 1960. The rapidity with which the cause was identified and the responsible compounds isolated and characterized (with the unparalleled collaboration of specialists in diverse scientific disciplines, utilizing the most advanced, powerful tools and techniques available), the demonstration of aflatoxin B_1 as an extremely potent carcinogen, the possible significance in problems of animal and human health, and the worldwide involvement focused intense attention upon mycotoxins.

It would be difficult to exaggerate the importance of the discovery of the aflatoxins. The potency of these compounds as toxic agents and carcinogens (in rats, aflatoxin is by far the most active hepatocarcinogen known) make them powerful tools in investigations into the mechanism of toxicity and chemical carcinogenesis. In spite of the intensified interest on the part of medical researchers and of food and drug regulatory officials, it is still difficult to assess accurately the extent and severity of the mycotoxin problem. Reports of the synergistic effect of aflatoxins with components normally present in some foods and of the potentiation of another mycotoxin, rubratoxin, on aflatoxin activity present a new dimension in the potential for health hazards. It has been suggested that we may be on the threshold of vast developments, comparable to those with vitamins half a century ago, with respect to diseases caused by the presence of trace contaminants rather than by the absence of trace nutrients.

Because of the complexities involved and because they have been investigated so much more extensively than have other mycotoxins, this book is concerned primarily with the aflatoxins—their discovery and precautions to be observed, their elaboration, their chemistry and assay, their effects and metabolic fate, processing to ensure their removal or inactivation, and regulatory aspects. Nevertheless, the scope of several chapters has been

deliberately enlarged to encompass discussion of the more general problems of the types of mycotoxins that may be found in foods and feeds, of fungal spoilage of stored crops, and of implications of fungal toxins to human health.

It is hoped that this book will increase awareness of the problems of the aflatoxins, alert us to the need for accelerated programs directed toward other mycotoxins, and will be acceptable to scientists concerned with the production and processing of foods and feeds, with nutrition, and with animal and public health. This work is not intended as a substitute for the original literature cited in the references.

Because of the diverse scientific disciplines involved and their intricate interrelationship, experts who have themselves contributed significantly to the development of our knowledge in specific areas were enlisted to prepare the individual chapters. I am grateful to them, and wish to express to them my sincerest thanks and appreciation.

New Orleans, Louisiana
March, 1969

LEO A. GOLDBLATT

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CHAPTER I | INTRODUCTION

L. A. Goldblatt

I. INTRODUCTION

In 1960 more than 100,000 young turkeys on poultry farms in the south and east of England died in the course of a few months from an apparently new disease that was termed "turkey X disease" (Blount, 1961). It was soon found that the difficulty was not limited to turkeys. Ducklings and young pheasants were also affected and heavy mortality was experienced. On one farm alone some 5000 partridge and pheasant poults died and on another 14,000 ducklings died (Asplin and Carnaghan, 1961). Also, reports were received from Kenya and Uganda of severe losses of ducklings from a similar disease (Asplin and Carnaghan, 1961). Almost simultaneously, in the United States an outbreak of trout hepatoma was discovered in the spring of 1960 after a shipment of trout raised in a commercial hatchery was inspected at the California state border and many of the fish were found to have hepatomas (Wolf and Jackson, 1963). As is noted in Chapter X of this volume, trout hepatoma, which proved to be nationwide in distribution, became a national concern in 1960 and many laboratories mobilized to describe the etiology and determine the cause. There was then no known relationship between the various outbreaks, which were later shown to have in aflatoxin

a common denominator, but the reports of thousands of mortalities and hepatomas dramatized and focused attention on the practical problems. The importance of the stimulus thus given to research and related activities and of the resulting developments is difficult to exaggerate.

II. THE TURKEY X DISEASE PROBLEM

Details of the recognition of the problem of "turkey X disease" and of its solution are given in Chapter IX of this volume but it may be useful to sketch the development here. It affords a fascinating illustration of a multidiscipline approach to the solution of an important problem; of veterinarians, pathologists, microbiologists, nutritionists, and organic, physical, analytical and biochemists and still others, cooperating and employing classical as well as the most modern sophisticated techniques and instrumentation including column, paper and thin-layer chromatography, counter-current distribution and absorption, fluorescence, mass, nuclear magnetic resonance, and X-ray spectroscopy.

Turkey X disease is characterized by loss of appetite, lethargy, and a weakness of the wings. Affected birds die within a week and at the time of death assume a characteristic attitude of head, neck, and legs. The neck is arched, the head drawn back, and the legs extended fully backwards. Postmortem examination showed liver hemorrhages and liver necrotic lesions, and, frequently, swollen (engorged) kidneys. Histopathological examination revealed degeneration of the liver parenchyma cells and extensive proliferation of the bile duct epithelium cells. Ducklings and pheasants were similarly affected. Veterinary examinations for pathogenic microorganisms were generally negative and attempts to demonstrate virus infection were unsuccessful. It was soon generally agreed that no known microorganism had been isolated and that biological transmission was negative. Accordingly, there remained the possibility that the birds were being "poisoned." At first investigations were directed towards the possibility that the rations were contaminated with known toxic substances. Suspect feeds were examined for a large variety of organic and inorganic poisons and poisonous plant materials. However, these intensive efforts failed to disclose the presence of any known poisonous agent in significant amounts and all attempts to associate the disease with such poisons were fruitless (Blount, 1961).

One very marked early feature of the disease in turkeys was its geographical distribution. Almost 80% of the cases occurred within a radius of 80–100 miles of London. None was reported in Scotland or Wales, and very few in any Northern Counties. A careful survey of the early outbreaks

showed that they were all associated with feeds produced at one London mill. At that time no cases had been observed with feeds produced at any of six other mills of the same company. Suddenly six cases of turkey X disease appeared in another area with feed manufactured by a second of the company's mills. It was then discovered that a common ingredient of the feed from the two mills was a Brazilian peanut meal (Blount, 1961).

A more intensive investigation of the suspect peanut meal was then undertaken. It was quickly found that this peanut meal was highly toxic to poults and ducklings with symptoms typical of turkey X disease. Ducklings proved to be the more susceptible species and the hepatic lesions developed very rapidly (Asplin and Carnaghan, 1961). Proliferation of bile-duct epithelial cells was clearly visible within a few days of ingestion of toxic meal. This afforded the basis for a sensitive and relatively rapid biological method for detection of toxicity. In turn, this was used effectively to monitor the extraction and concentration of the toxin through classical procedures. These included extraction of the toxic meal with hot methanol, partitioning of an aqueous suspension of the dried extract into chloroform and finally concentration of the chloroform soluble residues by distribution in the system methanol : water : petroleum ether (10 : 1 : 10) whereupon the toxin passed into the aqueous-methanol layer. In this way a concentration of some 250-fold was achieved.

Chromatography of the 250-fold concentrate on neutral alumina afforded a crystalline almost colorless but still not quite pure toxin (Sargeant *et al.*, 1961a). Further purification of the toxin was achieved by paper chromatography. The crystalline product when chromatographed on Whatman No. 1 paper using *n*-butanol–5% acetic acid as developer gave a single spot at an R_f value of 0.7 which emitted a bright blue fluorescence when illuminated with ultraviolet light. As little as 0.1 μg of the crystalline toxin could be detected in this way. Further, the amount of fluorescent material, as estimated visually to be present in the spots produced in this way from numerous samples, correspond with the toxicity of the samples as determined biologically (Sargeant *et al.*, 1961b). Thus, for the first time the basis for routine chemical assay of the toxin was provided.

Speculations made during 1960 regarding the nature of the toxin included the suggestion that it might be of fungal origin. During an examination of a sample of toxic Brazilian peanut meal for the presence of fragments of poisonous plants it was noted that up to 20% of the pieces of peanut cotyledon tissue contained hyphae although none was present in a sample of nontoxic meal (Austwick and Ayerst, 1963). Although attempts at culture showed that these hyphae were dead, Sargeant *et al.* (1961b) subsequently succeeded in producing pure cultures of certain of the fungal species present in a highly toxic sample of peanuts from Uganda heavily contaminated with

fungi. A chloroform extract of a culture of one of the isolates grown on Czapek's solution agar was found to contain a fluorescent material with an R_f value of 0.7, when chromatographed on paper under the conditions developed previously, which was toxic to ducklings and produced the characteristic symptoms associated with turkey X disease. The toxin-producing fungus was identified as *Aspergillus flavus* Link ex Fries (Sargeant *et al.*, 1961b) and the toxin was given the name "aflatoxin" in view of its origin.

As is indicated elsewhere in this volume, see especially Chapters VII, IX, and X, it appears that feedstuffs containing toxins that have biological effects similar to those produced by aflatoxins have been in use for a number of years prior to the outbreaks of 1960. Also, Keppler and de Iongh (1964) have reported finding aflatoxin in 40-year-old samples of peanuts.

III. ISOLATION OF AFLATOXINS B_1, B_2, G_1, and G_2

With the availability of a simple chemical means for assay of the toxin and means for conveniently producing relatively large amounts of the toxin, a number of laboratories undertook such preparations, as described in the next chapter of this volume. Evidence was soon adduced by several groups of investigators that the toxin obtained after paper chromatography is still a complex mixture.

Nesbitt *et al.* (1962) succeeded in further resolving on alumina chromatoplates by means of chloroform : methanol (98.5 : 1.5) the fluorescent material described as having an R_f value of 0.7 on filter paper developed with *n*-butanol-acetic acid. Two spots were obtained, one having an R_f value of approximately 0.6 and having a blue-violet fluorescence and the other with a somewhat lower R_f value and having a green fluorescence. For convenience these were referred to as aflatoxin B and aflatoxin G, respectively. The two toxins were separated from each other by counter-current distribution in the system chloroform : carbon tetrachloride : water : methanol, 2 : 2.5 : 1 : 3 (200 transfers). Aflatoxin B was assigned the formula $C_{17}H_{12}O_6$ and aflatoxin G the formula $C_{17}H_{12}O_7$ on the basis of elementary analysis and mass spectrometric determinations.

Almost simultaneously other groups introduced the use of silical gel chromatography for the separation of aflatoxins. Smith and McKernan (1962) by chromatography on thin layers of silicic acid (Kieselgel G, Merck) of extracts of cultures of toxigenic strains of *A. flavus* obtained at least 12 fluorescent components. Five of these caused liver lesions when fed to ducklings. Two had a blue-green fluorescence in contrast with dark blue

fluorescence of the other three. They concluded that the hepatotoxic activity is associated with several discrete fluorescent substances and that with other means of separation the activity may prove to be even more complex in nature. Van der Zijden *et al.* (1962) reported the isolation in crystalline form of a toxin responsible for turkey X disease by chromatographing suitable extracts first on a column of silica gel (Mallinckrodt), followed by thin-layer chromatography on Kieselgel G (Merck), then column chromatography on Kieselgel G/Celite, and finally through a short column of alumina. The product obtained after crystallization from chloroform–methanol was designated as FB_1, but it was concluded that even this recrystallized material still contained some impurities. De Iongh *et al.* (1962) obtained a concentrate of the toxin after treatment of a chloroform extract with Girard T reagent. After decomposition of the derivative, two-dimensional thin-layer chromatography on silica gel G revealed a complex pattern of fluorescent spots. Two spots, designated as FB_1 and FB_2 contained material toxic to ducklings. One substance, FB_1, isolated in crystalline form had a blue-violet fluorescence. It was assigned the formula $C_{17}H_{12}O_6$ on the basis of elementary analysis and mass spectrometric determinations and certain structural features, including the presence of an OCH_3 group, on the basis of nuclear magnetic resonance spectra.

Isolation and characterization of four closely related toxins was first reported by Hartley *et al.* (1963). The four compounds separated on silica gel chromatoplates using chloroform:methanol (98 : 2) as developing solvent were designated aflatoxins B_1, B_2, G_1, and G_2 in order of decreasing R_f value. Aflatoxin B_1 appeared to be identical with the substance designated as FB_1 by van der Zijden and by de Iongh. The material called aflatoxin B

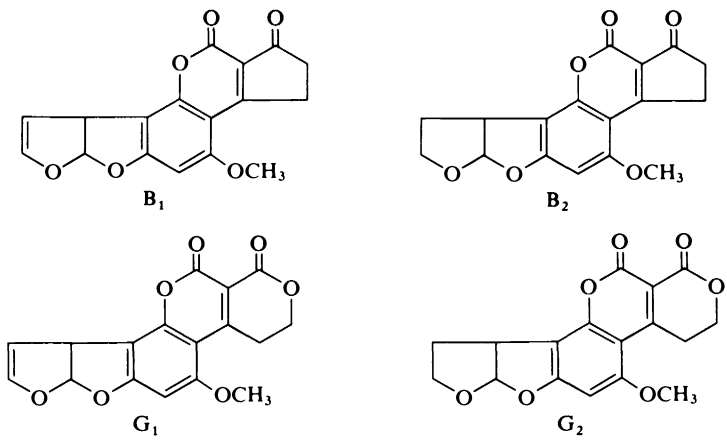

FIG. 1. Aflatoxins B_1, B_2, G_1, and G_2.