

MORPHOLOGICAL AND BIOCHEMICAL ALTERATIONS
IN BACILLUS MEGATERIUM NRRL B-1368
AS PRODUCED BY AFLATOXIN B₁

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
LARRY R. BEUCHAT
1970



This is to certify that the
thesis entitled
Morphological and Biochemical Alterations
In Bacillus megaterium NRRL B-1368
As Produced by Aflatoxin B₁

presented by

Larry R. Beuchat

has been accepted towards fulfillment
of the requirements for
Ph.D. degree in Food Science

R. V. L. L. L.
Major professor

Date May 21, 1970

ABSTRACT

MORPHOLOGICAL AND BIOCHEMICAL ALTERATIONS IN BACILLUS MEGATERIUM NRRL B-1368 AS PRODUCED BY AFLATOXIN B₁

By

Larry R. Beuchat

Bacillus megaterium NRRL B-1368 cells and spores were produced on trypticase soy broth (TSB) and agar (TSA) containing 3.8 µg/ml aflatoxin B₁, analyzed for selected chemical constituents and morphological characteristics, and compared to cells and spores of B. megaterium produced on nontoxic trypticase soy medium. There was an initial 30% kill of cells after inoculation into toxic TSB and during the first 3 1/2 hr of incubation followed by a logarithmic growth phase in which the generation time was 75 min as compared to 20 min for the control culture. Morphologically abnormal cells were produced by B. megaterium in response to aflatoxin B₁. Filamentous forms were characterized by early granulation and unusually large and numerous deposits of poly-β-hydroxybutyric acid within the cells. Bulb-like structures occasionally formed at the terminal portions of these filaments after

14 hr of incubation, possibly indicating weakened cell walls in the filamentous forms.

Chemical analyses revealed an increase in protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) on both a per cell basis and a per cent dry weight basis when B. megaterium was grown in toxic TSB. There was a concurrent decrease in the total amounts of cellular protein, DNA, and RNA synthesized in toxic TSB. Amino acid and N-acetylglucosamine analyses of control and test cell walls showed little, if any, difference in cell wall composition.

Transfer of aberrant forms to nontoxic TSA yielded macrocolonies with daughter cells morphologically indistinguishable from untreated cells. Agar slide cultures of filamentous cells on nontoxic TSA also showed that normal size and shape daughter cells were formed, indicating that aflatoxin B₁ did not produce a permanent genetic alteration for the phenotypic expression of cell length in B. megaterium.

Pantoyl lactone was without effect as a reversing agent for the observed inhibition of cell septum formation and aflatoxin had no effect on apparent penicillinase formation.

B. megaterium completed the sporulation process with about 97% efficiency after 3 days on nontoxic TSA while 6 days were required to obtain 65% sporulation on toxic TSA. Germination of spores was not inhibited by approximately

4.0 µg/ml aflatoxin but outgrowth was. No significant differences were observed in the heat resistance, protein, DNA, RNA, or dipicolinic acid content of spores formed on toxic TSA and nontoxic TSA.

Electron photo-micrographs showed a decreased number of mesosomes in filamentous cells as compared to control cells. There were no observable morphological differences in spores formed on toxic or nontoxic TSA.

MORPHOLOGICAL AND BIOCHEMICAL ALTERATIONS
IN *BACILLUS MEGATERIUM* NRRL B-1368
AS PRODUCED BY AFLATOXIN B₁

By

Larry R. Beuchat

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science

1970

ACKNOWLEDGMENTS

My sincere thanks are extended to Dr. R. V. Lechowich for his guidance and assistance throughout the research program and during the preparation of this manuscript.

Special thanks are expressed to Thayne R. Dutson for his advice and assistance in the electron microscopy portion of this study.

Research was supported, in part, by the National Institutes of Health Training Grant No. FD-00001-09.

TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	ii
LIST OF TABLES	v
LIST OF FIGURES	vi
INTRODUCTION	1
LITERATURE REVIEW	4
Aflatoxin Chemistry and Formation	5
Occurrence of Fungi that Produce Aflatoxin	5
Structure and Chemistry of the Aflatoxins	7
Synthesis of Aflatoxin	10
Factors Influencing Production of Aflatoxin	12
Metabolism and Biochemical Effects of Aflatoxins	
in Living Cells	15
Effect of Aflatoxins on Plants	15
Aflatoxicosis in Vertebrates	16
Effects of Aflatoxins on Insects	22
Toxicity of Aflatoxins to Microorganisms	22
Methods for Detection of Aflatoxins	24
Problems in Methodology	24
Screening Tests	25
Presumptive Tests	27
Confirmatory Tests	28
Detoxification of Foods and Feeds Containing	
Aflatoxin	30
Physical Destruction	30
Chemical Inactivation	31
Implications of Aflatoxicosis in Man	31
METHODS AND MATERIALS	34
Production of Vegetative Cells and Spores of	
<u>Bacillus megaterium</u> NRRL B-1368	34
Growth Media	34
Quantitation of Aflatoxin B ₁	35
Production and Harvesting of Vegetative Cells	
and Spores	38
Observations on Vegetative Cell Division	40
Phase Contrast Microscopy	40

	Page
Macrocolony Formation	41
Direct Counts, Viable Counts, and Absorbancy Changes	41
Effects of Cell Septum Initiators on Cell Division	42
Effect of Penicillin on Cell Division	43
Staining Studies	43
Heat Resistance of Spores Formed in the Presence of Aflatoxin B ₁ and the Effects of Aflatoxin B ₁ on Spore Germination	44
Heat Resistance Studies	44
Germination Studies	44
Chemical Analyses of Vegetative Cells and Spores of <u>Bacillus megaterium</u> Formed in the Presence and Absence of Aflatoxin B ₁	45
Preparation of Vegetative Cell Walls for Amino Acid Analysis	45
Procedure for Amino Acid Analysis of Vegetative Cell Walls	46
Total Protein Analyses of Vegetative Cells and Spores	47
Total Ribonucleic Acid Analyses of Vegetative Cells and Spores	48
Total Deoxyribonucleic Acid Analyses of Vegeta- tive Cells and Spores	49
Dipicolinic Acid Analysis of Spores	51
Preparation of Vegetative Cells and Spores of <u>Bacillus megaterium</u> for Observation by Electron Microscopy	51
RESULTS AND DISCUSSION	54
Growth Media	54
Recovery of Aflatoxin Added to the Medium	55
Effects of Aflatoxin upon Growth of <u>Bacillus</u> <u>megaterium</u>	59
Growth Curves	59
Cellular Morphology as Affected by Aflatoxin	60
Pantoyl Lactone Studies	67
Penicillin Studies	70
Effects of Aflatoxin upon Sporulation of <u>Bacillus</u> <u>megaterium</u>	72
Heat Resistance Studies	73
Effects of Aflatoxin on Spore Germination and Outgrowth	76
Effect of Aflatoxin on Vegetative Cell Wall Composition	77
Effect of Aflatoxin on Vegetative Cell and Spore Composition	79
Electron Microscopy of Vegetative Cells and Spores	81
LITERATURE CITED	93

LIST OF TABLES

Table	Page
1. Summary of physical, chemical, and spectral data on aflatoxins	9
2. Absorbancy at 650 nm of spore suspensions in trypticase soy broth containing 3.8 µg/ml aflatoxin B ₁ (toxic) and trypticase soy broth (nontoxic)	77
3. Concentration in µ Moles per mg protein of lysine, glutamic acid, and alanine in cell walls of <u>Bacillus megaterium</u> formed in trypticase soy broth (control) and trypticase soy broth containing 3.8 µg/ml aflatoxin B ₁ .	78
4. Per cent protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) in vegetative cells and spores and per cent dipicolinic acid (DPA) in spores of <u>Bacillus megaterium</u> formed in trypticase soy medium (control) and trypticase soy medium containing 3.8 µg/ml aflatoxin B ₁ (treated)	80

LIST OF FIGURES

Figure	Page
1. Structure of aflatoxins	8
2. Growth curves for <u>Bacillus megaterium</u> cultured in trypticase soy broth (control) and trypticase soy broth containing 3.8 µg/ml aflatoxin B ₁ (treated)	57
3. Normal and aberrant forms of <u>Bacillus megaterium</u> produced during growth in trypticase soy broth and trypticase soy broth containing 3.8 µg/ml aflatoxin B ₁ , respectively	61
4. Micrographs showing bulb-like swellings at the terminal portions of filaments in a 14-hr culture of <u>Bacillus megaterium</u> grown in trypticase soy broth containing 3.8 µg/ml aflatoxin B ₁	63
5. Demonstration of capsular material in <u>Bacillus megaterium</u> cultured in (A) trypticase soy broth and (B) trypticase soy broth containing 3.8 µg/ml aflatoxin B ₁	65
6. Time-lapse phase contrast micrographs of dividing filamentous <u>Bacillus megaterium</u> cells which were formed in trypticase soy broth containing 3.8 µg/ml aflatoxin B ₁ and then transferred to nontoxic trypticase soy agar	68
7. Survivor curves at 92 C (197 F) for <u>Bacillus megaterium</u> spores formed in trypticase soy agar (control) and trypticase soy agar containing 3.8 µg/ml aflatoxin B ₁ (treated)	74
8. Ultrathin sections of <u>Bacillus megaterium</u> cells formed in trypticase soy broth	83
9. Ultrathin sections of <u>Bacillus megaterium</u> cells formed in trypticase soy broth containing 3.8 µg/ml aflatoxin B ₁	85

Figure	Page
10. Ultrathin sections of <u>Bacillus megaterium</u> spores formed in trypticase soy agar . . .	88
11. Ultrathin sections of <u>Bacillus megaterium</u> spores formed in trypticase soy agar con- taining 3.8 µg/ml aflatoxin B ₁	90

INTRODUCTION

The word mycotoxin is not present in the third edition of Webster's unabridged dictionary (1966). The word myco is defined as "a combining form from the Greek mykes, meaning fungus." Toxins are defined as "any of various poisonous substances that are specific products of the metabolic activities of living organisms . . . " A mycotoxin, then, is a poisonous material produced by a fungus. Mycotoxins are specific chemicals or mixtures of chemicals. It is aflatoxin B₁, a mycotoxin produced along with at least seven other aflatoxins by the Aspergillus flavus group of fungi, that was under study and will be reported on in this manuscript.

Aflatoxins are the most sensational and probably the most significant mycotoxins discovered to date. Aflatoxin-producing molds are ubiquitous and not particularly fastidious in their requirements for growth and toxin production. The toxin is lethal to all animal species thus far studied and has inhibitory effects on certain plants and microorganisms. Their extreme potency as carcinogens might be visualized in this way: an ounce of aflatoxin B₁, properly administered, could kill a million ducklings. Certainly the threat to human health

is real and the problem of aflatoxins in foods must be explored and solved from the standpoint of preventive contamination, decontamination, and detection.

Of great concern is the fact that aflatoxins are relatively stable compounds not significantly altered by cooking or ordinary decontamination measures used in food processing. Although aflatoxins are rarely found in agricultural commodities if proper harvesting, handling, and storage procedures are followed, it is desirable to have fast, accurate, and preferably inexpensive methods for detection of the toxin in suspect cases. Thin-layer chromatography in conjunction with confirmatory bioassays utilizing ducklings or embryonated chicken eggs are routinely used to quantitate aflatoxins. Problems associated with duckling and embryonated chicken egg bioassays include their expense and demand for technical personnel. An alternative confirmatory bioassay providing simple and rapid techniques is based on the growth inhibition of Bacillus megaterium. However, the microbial inhibition test is not as sensitive as other bioassay methods. Dozens of other mycotoxins may inhibit the growth of B. megaterium, including toxic metabolites of the A. flavus group. The objective of this study was to chemically and morphologically analyze the aberrant cells and the spores formed by B. megaterium in the presence of aflatoxin B₁ in an attempt to derive additional information which could

be used to supplement existing data available on growth inhibition. Such newly derived data might be used as additional confirmation for the presence of aflatoxin in agricultural commodities.

LITERATURE REVIEW

More than 100,000 young turkeys died in England within the course of a few months in 1960 from a disease termed "turkey X disease" (Blount, 1961). It was soon discovered that the disease was not restricted to turkeys as heavy mortality was also experienced with ducklings, pheasants, and partridges. Almost simultaneously an outbreak of trout hepatoma was reported in the United States (Wolf and Jackson, 1963). Although there was no known relationship between the outbreaks, a toxic secondary metabolite of the mold Aspergillus flavus (christened aflatoxin for A. flavus toxin) was shown to be commonly present. The impact of this "new" mycotoxin gave stimulus to much research. No less than 1500 scientific publications concerning aflatoxins have appeared in the literature within less than a decade. No attempt is made here to thoroughly review the literature as several recent reviews dealing with many aspects of this mycotoxin are available (Adrian and Lunven, 1969; Bilal, 1969; Boesenburg, 1969; Jones and Jones, 1969; Nesterin and Vissarionova, 1969; Scott, 1969b). In addition, an excellent book entitled "Aflatoxin,"

describing its scientific background, control, and implications, has recently been published (Goldblatt, 1969).

Aflatoxin Chemistry and Formation

Occurrence of Fungi that Produce Aflatoxin

Aflatoxicosis is probably not a new phenomenon, although studied in depth only recently. The A. flavus group of molds (A. flavus and A. parasiticus), which are now generally accepted as being solely responsible for the production of aflatoxins (Wilson et al., 1968), are ubiquitous and grow rapidly. It is their frequent role as contaminants in food of both animal and man that has attracted attention to their investigation. Interest has evolved to determine the suitability of nearly every type of food for the support of growth of A. flavus and subsequent production of aflatoxin. The Japanese, who consume a considerable amount of controlled and uncontrolled fermented foods in their diet, have carried out studies on the population of toxigenic fungi in foodstuffs (Kurata et al., 1965; 1968). Twenty-one strains of the A. flavus group were isolated from several kinds of flours including wheat, red bean, and soybean. Five of the twenty-one strains were found to produce aflatoxin. The toxin has not been detected in takadiastase (Nose and Ishibashi, 1967). More than 60% of 1,626 isolates of A. flavus from groundnut kernels and soils in Israel

produced aflatoxin in excess of 25 µg/g (Joffe, 1969). Surveys to establish the distribution and degree of occurrence of aflatoxin in groundnuts have also been made in South Africa (Sellschop et al., 1965) and the United States (Dickens, 1968; Schroeder and Ashworth, 1965). Aflatoxin-producing A. flavus have been isolated from red pepper (Christensen et al., 1967; Schindler and Eisenberg, 1968) and from fish food materials (Phelps, 1965). A. flavus has been isolated with regularity from molded soybeans and soy sauce but extracts of the molded products failed to reveal the presence of aflatoxins (Chong, 1966).

Most reports have pointed to members of the A. flavus group for being responsible for production of aflatoxin (Diener and Davis, 1966; Raper and Fennell, 1965; Senser, 1967), however a few researchers have indicated that certain members of the genus Penicillium (Hodges et al., 1964; Kulik and Holaday, 1966; Wilson et al., 1967) as well as Aspergillus ostianus also produce aflatoxin. One Streptomyces species was reported to produce the toxin (Mishra and Murthy, 1968). Wilson et al. (1968) more fully examined the toxin-synthesizing capacity of several of the fungi previously reported to produce aflatoxins and noted that pitfalls in analytical technique have led to confusion on fluorescent materials with R_f values

approximating those of the aflatoxins with the actual aflatoxins, thus producing falsely positive results.

Structure and Chemistry of the Aflatoxins

Figure 1 shows the structures of the eight reported aflatoxins. Table 1 lists various physical and chemical characteristics of the toxins. Of special interest are the melting and decomposition points which render the toxins stable under normal processing procedures used by the food industry. The coumarin-lactone type structure, common to all, is characteristic of many naturally occurring, physiologically active compounds but the bifuran structure, also common to all, is known to occur in only one other naturally occurring compound, sterigmatocystin (Goldblatt, 1968).

Methods for structural elucidation of the aflatoxins have involved the ultraviolet, infrared, nuclear magnetic resonance, and mass spectrograph (Asao et al., 1965). Isolation and characterization of aflatoxins B₁, B₂, G₁, and G₂, four of the eight known aflatoxins, were the first to be established through separation on silica gel chromatoplates using chloroform:methanol (98:2) as a developing solvent (Hartley et al., 1963). Ultraviolet light was used to determine the R_F values. Other researchers have worked independently to elucidate the chemical identity and biological activity of these

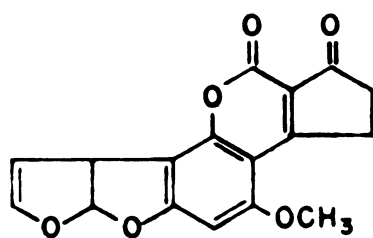
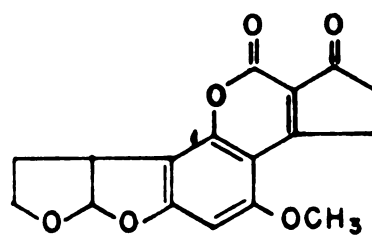
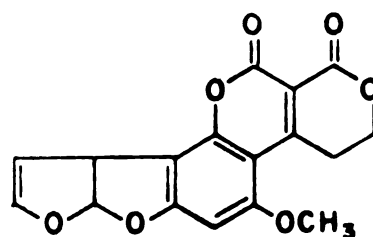
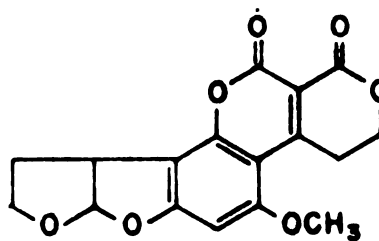
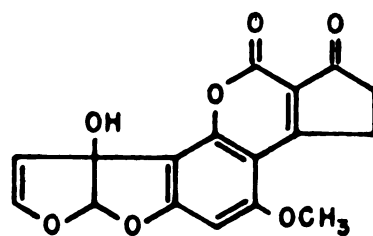
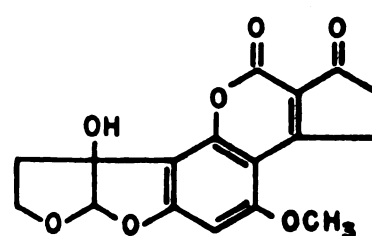
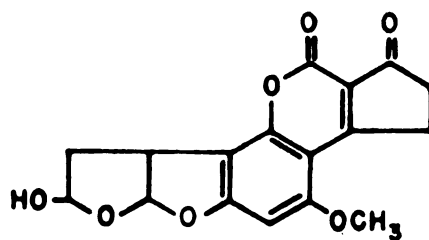
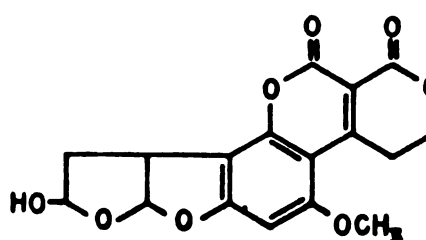
 B_1  B_2  G_1  G_2  M_1  M_2  B_{2a}  G_{2a}

Figure 1. Structures of aflatoxins.

TABLE 1.--Summary of physical, chemical, and spectral data on aflatoxins.

Aflatoxin	Ultraviolet Absorption		Fluorescence Emission (nm)	Molecular Formula	Molecular Weight	Melting Point	Reference
	265 nm	363 nm	color				
B ₁	13,400	21,800	blue	C ₁₇ H ₁₂ O ₆	312	268	Wogan, 1966
B ₂	9,200	14,700	blue	C ₁₇ H ₁₄ O ₆	314	286	Wogan, 1966
G ₁	10,000	16,100	yel-gr	C ₁₇ H ₁₂ O ₇	326	244	Wogan, 1966
G ₂	11,000	19,300	yel-gr	C ₁₇ H ₁₁ O ₇	330	237	Wogan, 1966
M ₁	11,600	19,000 ^a	blue	C ₁₇ H ₁₂ O ₇	328	-	Holzappel, 1966
M ₂	10,900 ^b	21,000 ^a	blue	C ₁₇ H ₁₄ O ₇	330	-	Holzappel, 1966
B _{2a}	17,600 ^c	20,400	blue	C ₁₇ H ₁₄ O ₇	330	-	Dutton and Heathcote, 1966
G _{2a}	18,600 ^d	18,000 ^e	green	C ₁₇ H ₁₄ O ₈	348	-	Dutton and Heathcote, 1966

* Decomposes

a 357 nm

b 264 nm

c 256 nm

d 223 nm

e 365 nm

aflatoxins (Chang et al., 1963; Van Dorp et al., 1963; Van Soest and Peerdeman, 1964; Wick et al., 1965; Brechbuehler et al., 1967).

Aflatoxins M_1 and M_2 were isolated from urine collected from sheep during two days after they had been dosed with aflatoxins B_1 , B_2 , G_1 , and G_2 , and resolved by paper chromatography (Holzapfel et al., 1966). Crystalline aflatoxin M_1 was later isolated from urine and milk (Masri et al., 1967). Aflatoxin M_1 is 4-hydroxyaflatoxin B_1 and aflatoxin M_2 is 4-hydroxyaflatoxin B_2 . The most recent aflatoxin to be isolated are aflatoxins B_{2a} and aflatoxin G_{2a} , 2-hydroxy derivatives of aflatoxins B_2 and G_2 , respectively (Dutton and Heathcote, 1966). Formulation was made by means of ultraviolet, infrared, mass, and nuclear magnetic resonance spectra, and toxicity was confirmed by use of duckling assays (Dutton and Heathcote, 1968). The chemical identity of aflatoxin B_{2a} (Poland et al., 1968) and G_{2a} (Poonoose, 1969) has been confirmed by their research reports.

Synthesis of Aflatoxin

Utilization of ^{14}C -labeled compounds has enabled researchers to hypothesize biochemical pathways leading to the formation of aflatoxins. The addition of methyl-labeled methionine to an A. flavus culture medium resulted in the appearance of ^{14}C in the methoxy function (Adye and Mateles, 1964). Addition of acetate-1- ^{14}C as the sole

source of carbon yielded aflatoxin labeled in the ring carbons. Aflatoxin B₁ produced by a phenylalanine-requiring mutant, A. flavus A77, grown in the presence of ¹⁴C-labeled (ring) DL-phenylalanine had a relative isotopic content of less than 0.01, indicating that phenylalanine was not a precursor of B₁ (Donkersloot et al., 1968). Similarly ¹⁴C-labeled (ring) shikimic acid was shown not a precursor, however indications were that acetate was a precursor. Through the use of acetate-1-¹⁴C and acetate-2-¹⁴C, a hypothetical scheme for biosynthesis of aflatoxin was recently proposed (Biollaz et al., 1968a; 1968b). Labeled acetate, mevalonate, and leucine were demonstrated to be incorporated into aflatoxins by the mitochondrial fractions of reconstituted homogenates from A. flavus, indicating that enzymes involved in biosynthesis of aflatoxins were in the mitochondria (Raj et al., 1969). This tends to confirm in part an earlier hypothesis (Moody, 1964) involving base-catalyzed ring fission and closure and implicating mevalonate as a precursor to aflatoxin B₁. Kojic acid was also proposed to have a role in the formation of aflatoxin (Heathcote et al., 1965).

Total synthesis of racemic aflatoxin B₁ (Beuchi et al., 1966; Beuchi et al., 1967) and racemic aflatoxin M₁ (Beuchi and Weinreb, 1969) was described. Synthesis of

racemic aflatoxin B₂ from racemic tetrahydro-4-hydroxy-6-methoxyfuro(2,3-b)benzofuran was also carried out (Roberts et al., 1968).

Factors Influencing Production of Aflatoxin

The influence of temperature on the production of aflatoxin has been studied in depth and mainly with reference to agricultural crops. The optimal temperature range for aflatoxin production on substrates having adequate moisture is between 20 and 35 C, depending on the strain and substrate used (Dickens and Pattee, 1966; Rabie and Smally, 1965; Schindler et al., 1967; Schroeder and Helne, 1967). Significant concentrations of aflatoxin were produced at 7.5 to 10 C, which include temperatures of household refrigeration (Van Walbeek et al., 1969). The ratio of production of different aflatoxins as well as the total quantity produced varies with incubation temperature. Fluctuation in temperature reduced the growth of A. parasiticus and the production and accumulation of aflatoxins when compared with cultures held continuously at a temperature equalling the average of the range of fluctuation (Schroeder and Hein, 1968).

Generally, growth substrates having less than 10% moisture or equilibrium relative humidities of less than 80% are not satisfactory for the support of growth and aflatoxin production (Diener and Davis, 1967; Jemmali et al., 1969; Love, 1968).

Atmospheric gases, especially carbon dioxide, exert a measurable effect on fungal growth and toxin production. Growth and sporulation of A. flavus appeared to be visually decreased when a growth chamber that contained 20% carbon dioxide was used (Landers et al., 1967; Sanders et al., 1968). It was suggested that alterations in atmospheric gas composition induced a change in the growth behavior of A. flavus with a resulting change in aflatoxin production (Pattee et al., 1966).

Only 80% as much aflatoxin was produced in the presence of normal light intensity as can be produced in the dark (Joffe and Lister, 1969). It is the author's opinion that the above concentration of aflatoxin is a measure of the aflatoxin not degraded by light during the incubation of the culture rather than the total production of aflatoxin.

Many studies were conducted on the effects of invasion of foods by aflatoxin-producing fungi and the subsequent public health hazards that could result when the foods are consumed by man or animals. Many foods have been reported to be suitable substrates for growth and aflatoxin production and the foods implicated include: fruit juices (Wildman et al., 1967), coconut (Arseculeratne et al., 1969), cassava (Nartey, 1966), pecans (Lillard et al., 1970), and cottonseed products (Mayne et al., 1966; McMeans et al., 1968). Other foods in addition to

those of vegetable origin such as dairy products including Cheddar cheese, casein, and butter (Lie and Marth, 1967, 1968; Rehm and Schmidt, 1969) and meats (Bullerman and Ayres, 1968; Bullerman et al., 1969a, b, c; Strzelecki, 1969), have served as growth media for aflatoxin production.

Since peanut meal was confirmed to be the toxic vehicle which resulted in the turkey kill in 1960, initial efforts in the examination of aflatoxin dealt with peanuts and peanut products. Surveys and investigations were conducted to determine the incidence of toxic fungi in peanuts (Bushnell, 1965; Tabor and Schroeder, 1967). Knowledge concerning the effects of kernel condition (Schroeder and Ashworth, 1965) and peanut varietal difference on aflatoxin accumulation (Rao and Tulpule, 1967) have resulted in a more controllable situation with regard to reducing the chances of aflatoxin being present in the finished product.

Fungi are the most common cause of post-harvest deterioration of seeds, cereal grains, and oilseeds. Invasion of these agricultural commodities by aflatoxin-producing fungi was studied by many groups (Boller and Schroeder, 1966; Shotwell et al., 1966; Stubblefield et al., 1967; Howell, 1968; Schroeder, 1968; Schroeder et al., 1969; Wheeler, 1969). Findings on the incidence of aflatoxin in peanuts include the corresponding presence

of broken or damaged nuts or shells, inadequate drying procedures, or water damage during rail and road transport.

A. flavus can produce aflatoxins on flue-cured tobacco leaves under favorable growth conditions (Pattee, 1969). However, aflatoxin was not detected in the leaves of four moldy tobaccos nor the smoke of regular nonfilter cigarettes (Tso and Sorokin, 1967).

Experimentation to determine the nutrient requirements for certain strains of the A. flavus group resulted in numerous media which can be used to produce the aflatoxins (Mateles and Adye, 1965; Ciegler et al., 1966; Basappa et al., 1967; Davis et al., 1967; Osiyemi et al., 1967; Davis and Diener, 1968). Barium reportedly inhibits aflatoxin synthesis (Lee et al., 1966) as does p-aminobenzoic acid, potassium sulfite, and potassium fluoride (Davis and Diener, 1967).

Metabolism and Biochemical Effects of Aflatoxins in Living Cells

Effect of Aflatoxins on Plants

Lipase and α -amylase formation were inhibited in germinating cottonseed by a mixture of aflatoxins B₁ and G₁ (Black and Altschul, 1965), leading investigators to believe that aflatoxin inhibits protein synthesis. Chlorophyll synthesis in the distal halves of cottonseed cotyledons (Jones et al., 1967), in water cress seeds

(Schoental and White, 1965), and in corn seedlings (Slowatizky and Mayer, 1969) was reduced in the presence of aflatoxin. The growth of peanut seedlings was greatly retarded when aflatoxin B₁ was applied to either the roots or to the cotyledons (El-Khadem et al., 1966). Root cells of Vicia faba (Sutton's prolific Longpod) seedlings showed an abnormal number of anaphases and considerable inhibition of mitosis following treatment with aflatoxin (Lilly, 1965).

Aflatoxicosis in Vertebrates

Trout hepatoma became a national concern in 1960 after isolated reports from different hatcheries confirmed that rainbow trout reared on dry rations for two or more years had developed massive liver tumors. Aflatoxin was found to be the common vector responsible for these tumors, the approximate LD₅₀ values being 0.81 mg/kg and 1.90 mg/kg for aflatoxins B₁ and G₁, respectively, in nine-month-old rainbow trout (Bauer et al., 1969). Dietary factors play an important part in the degree of tumor formation. Cyclopropenoid fatty acids were shown to act as powerful co-carcinogens when present in rainbow trout rations (Sinnhuber et al., 1968). Diets of aflatoxin-contaminated cottonseed meal and peanut meal and controls of a purified diet containing added aflatoxins produced microscopic tumors in trout within six months. Labeled carbon studies of aflatoxin B₁ in trout showed that

carcinogenicity depended upon the bifunctional activity of the molecule with a requirement for both the unsaturated furan and lactone structures (Ayres, 1969; Ayres et al., 1969). Coho salmon (Halver et al., 1966) and zebra fish larvae (Abedi and Scott, 1969) are also aflatoxin-sensitive.

Susceptibility estimates of most farm animals were derived from feeding peanut meal containing aflatoxin. Such feeding trials indicated a marked variation in susceptibility, but the young were more susceptible than mature animals. Lactating cows showed a significant reduction in milk yield when fed aflatoxin-containing diets. "Milk toxin," now recognized as aflatoxins M_1 and M_2 , is a conversion product of aflatoxin B_1 (de Longh et al., 1964; Allcroft et al., 1968). The milk toxins are produced in minor quantities by certain of the A. flavus group (Holzapfel et al., 1966).

Aflatoxin M_1 was detected in urine, feces, and milk of lactating ewes (Allcroft et al., 1966; Nabney et al., 1967).

Pig feeding experiments using meals containing moderate amounts of aflatoxin were designed to determine palatability and the development of acute aflatoxicosis with time (Armbricht et al., 1968; Pier et al., 1968). Postmortem examination of pigs which had consumed a diet containing 810 ng/g revealed severe liver degeneration

(Hintz et al., 1967). Daily administration of aflatoxin B₁ to piglets resulted in an increase of saturated and mono unsaturated fatty acids and a decrease in the percentage of polyunsaturated fatty acids in the liver (Veen, 1967). The serum alkaline phosphatase level and liver weight of growing pigs increased when their diets contained aflatoxin B₁ plus 250 µg/g Cu⁺⁺, while the liver vitamin A concentration decreased (Barber et al., 1968).

Fowl are the most susceptible farm animals to toxic effects of aflatoxins, although varying degrees of susceptibility have been reported. One-day-old chicks and White Leghorn hens were unaffected after consumption of a ration of 400 ng/g aflatoxin for eight weeks (Kratzer et al., 1969). Contrary to this report a ration containing 308 ng/g aflatoxin B₁ increased chicken mortality (Cottier et al., 1968). Toxin administered to the yolk sac or the air cell resulted in LD₅₀'s of chick embryos of 0.948 µg/egg and 0.025 µg/egg, respectively (Verrett et al., 1964). Toxic manifestations were found in the chick liver as with most vertebrates (Brown and Adams, 1965; Carnaghan et al., 1967; Terao and Miyaki, 1968; Adamesteanu and Adamesteanu, 1969). Ducklings are more sensitive than chicks to aflatoxin, single injection LD₅₀'s on a mg/kg basis were:

B₁ = 0.36, G₁ = 0.78, B₂ = 1.696, and G₂ = 2.45

(Carnaghan et al., 1963). Aflatoxins M_1 , M_2 , B_{2a} , and G_{2a} are somewhat less toxic. Liver alterations in ducklings exposed to aflatoxin include decreased glycogen and glycogenesis (Shank and Wogan, 1966), degeneration of mitochondria and endoplasmic reticula (Theron et al., 1965), and general bile duct hyperplasia with minor to acute lesions (Newberne et al., 1964; Chong and Ponnampalam, 1967; Czarnowski et al., 1969). Total serum protein is elevated (Datta and Gajan, 1965; Juhasz and Nemeth, 1968; Nemeth and Juhasz, 1968).

The acute toxicity and carcinogenicity of aflatoxins induced in laboratory animals led to research in animals with rats studied most extensively. The rat LD_{50} for aflatoxin B_1 was reported to be from 7 to 16 mg/kg (Butler, 1964), depending on injection route and sex. Females are less sensitive, although the toxin may cause fetal growth retardation during pregnancy (Butler and Wigglesworth, 1966). Hepatic carcinomas from the consumption of toxic meal are similar to those described for other vertebrates, but because of the intensive research with rats, information available concerning toxic effects on specific tissues and organelles is more abundant (Butler and Barnes, 1966; Newberne et al., 1967; Floyd et al., 1968; Monneron et al., 1968; Balasubramaniam et al., 1969; Epstein et al., 1969; Monneron, 1969; Williams and Rabin, 1969). Aflatoxin B_1 binds to native

and denatured deoxyribonucleic acid (DNA) and inhibits incorporation of cytidine into rat liver nuclear ribonucleic acid (RNA) (Sporn and Dingman, 1966; Sporn et al., 1966). The action of aflatoxin is thus similar to actinomycin (Lafarge et al., 1965; Lafarge et al., 1966; Wogan and Friedman, 1968). The toxin may be responsible for RNA polymerase inhibition, thus preventing transcription of DNA. Thermal hyperchromicity studies involving the interactions of aflatoxins with nucleosides resulted in different spectra, suggesting that purine bases bind aflatoxin to DNA (Clifford and Rees, 1967a, b; Moule and Frayssinet, 1968). Binding of aflatoxin to human plasma was demonstrated (Rao et al., 1968). Decreased protein synthesis in rat liver was reported by Wogan (1968). The extent of protein synthesis inhibition in rat liver was proportional to the spectral shift caused by aflatoxin interacting with DNA (Clifford et al., 1967). Aflatoxin B₁ inhibited the incorporation of ¹⁴C-labeled leucine by liver in vitro (Smith, 1963; Villa-Trevino and Leaver, 1968). Hepatic cholesterol synthesis decreased in the presence of aflatoxin (Kato et al., 1969). Toxic effects of aflatoxins upon specific enzymes in rat liver were studied. Synthesis and activity of RNA polymerase was reduced (Gelboin et al., 1966; Wilson et al., 1967) and an aflatoxin-4-hydroxylase was observed in the microsomal fractions of rat liver

(Behabert and Eteyn, 1969). Various acute effects arising in the liver were decreased or somewhat prohibited by applications of such chemicals as prednisolone (Madhavan, 1967), dimethyl sulfoxide (Green, 1968), and diethylstilbestrol (Newberne and Williams, 1969).

Mice appear to be relatively resistant to acute poisoning by aflatoxins as indicated by short-term feeding of heavily contaminated (0.6 $\mu\text{g/g}$ each of B_1 and G_1) peanut meal (Platinow, 1964). Inhibition of tumorigenesis on mice skin by 7,12-dimethylbenz(a)anthracene was inhibited by application of aflatoxin B_1 (Van Duuren et al., 1969).

Histological examination of rhesus monkeys fed diets containing aflatoxins caused fatty infiltration, biliary proliferation, and portal fibrosis (Tulpule et al., 1964; Madhavan et al., 1965). Acute hepatic lesions resulting from oral administration (0.45 to 3.78 mg/kg) of B_1 resembled human liver lesions during acute viral hepatitis (Svoboda et al., 1966; Svoboda and Higginson, 1966).

Human tissue cultures were used to determine the mechanism of action of aflatoxin. Human embryonic lung cells went through abnormal morphological patterns including vacuolization, giant cell formation (Legator et al., 1965), and an increase in thymidine kinase activity (Childs and Legator, 1966). Abnormal morphology and

decreased RNA and DNA synthesis were also observed in human embryonic liver cells (Zuckerman et al., 1967; Zuckerman et al., 1968).

Effects of Aflatoxins on Insects

Aflatoxin in A. flavus medium supernates was toxic to house fly (Musca domestica) larvae (Beard and Walton, 1965). In another laboratory the per cent egg hatchability produced by the yellow-fever mosquito (Aedes aegypti), the house fly, and the fruit fly (Drosophila melanogaster) was decreased by aflatoxin (Matsumara and Knight, 1967). Heliothis virescens larvae were also sensitive to aflatoxin (Gudauskas et al., 1967).

Toxicity of Aflatoxins to Microorganisms

A survey to test the sensitivity of 329 microorganisms to aflatoxin included 30 genera of bacteria, 34 genera of fungi, 4 genera of algae, and 1 protozoan (Burmeister and Hesseltine, 1966). A streptomycete, a clostridium, and 12 Bacillus species were inhibited when crude aflatoxin (30 µg/ml; 36% pure) was incorporated into the growth medium. Among the most sensitive bacteria was Bacillus megaterium, and has thus been proposed as an assay organism for aflatoxin in foods (Burmeister, 1967; Clements, 1968a, b; Jayaraman et al., 1968; Viitasalo and Gyllenberg, 1969). The technique is similar to antibiotic assays and a zone of growth inhibition around the

test material is compared to standards to estimate the concentration of aflatoxin in the test material. In another study aflatoxins were found to be inactive against common Gram positive and Gram negative bacteria at concentrations of 100 µg/ml (Arai et al., 1967). Aflatoxin B₁ inhibited the growth of several Aspergillus and Penicillium species on modified Czapek Dox broth (Lillehoj et al., 1967c). Aflatoxin uptake by Flavobacterium aurantiacum and the resulting toxic effects were studied (Lillehoj and Ciegler, 1967; Lillehoj et al., 1967a, b). The toxin inhibited DNA synthesis and, to a lesser extent, RNA and protein synthesis. Aflatoxin removed from the medium by F. aurantiacum cells could not be extracted but autoclaving of cells and cell wall preparations reversibly removed a fraction of the aflatoxin present in a test system. Aberrant forms of the bacterium were produced during growth in the presence of B₁ and G₁. Binding capacity studies indicated that B₁ has a greater affinity for DNA than B_{2a} (Lillehoj and Ciegler, 1968; Lillehoj and Ciegler, 1969). The incorporating activity of Escherichia coli DNA polymerase was significantly lowered by 5 µg/ml B₁ (Wragg, 1967; Wragg et al., 1967). The interaction of B₁ with polynucleotides of E. coli was studied spectrophotometrically and a requirement for the amino group of adenine or possibly guanine for binding of aflatoxin was apparent (King and Nicholson, 1969). The same authors demonstrated that

aflatoxin B₁ reduced the activity of DNA-dependent RNA polymerase in E. coli (King and Nicholson, 1967). Amino acid activation in E. coli preparations was inhibited by aflatoxin in vitro (Smith, 1965).

Treatment of virulent bacteriophage with aflatoxin B₁ rendered the phage incapable of lysing Streptococcus lactis cells (Jemmali, 1969), however the same toxin was reported to act as an inducing agent for lysogenic Staphylococcus aureus (Legator, 1966).

Dactylium dendroides, Absidia repens, and Mucor griseo-cyanus transformed about 50 to 60% of aflatoxin B₁ in shake cultures to a new fluorescent-blue compound (Detroy and Hesseltine, 1968, 1969). Tetrahymena pyriformis was also observed to make a similar conversion (Teunisson and Robertson, 1967).

Methods for Detection of Aflatoxins

Problems in Methodology

The main objective in aflatoxin methodology has been to develop a rapid and accurate procedure to analyze a large number of representative samples with adequate sensitivity to detect a few micrograms of toxin per gram of sample. Distribution of the toxin is not always homogeneous (Ayres et al., 1970) and methods satisfactory for one product may be completely inadequate for another. Consequently numerous screening, presumptive, and

confirmatory tests were developed for detection of aflatoxin in a wide variety of food materials. Methods for detection have been developed for milk (Purchase and Steyn, 1967; Roberts and Allcroft, 1968; Jacobson et al., 1969; Masri et al., 1969), wine (Schuller et al., 1967; Frank and Eyrich, 1968; Wang et al., 1968), fungal fermentation products (Denault and Underkofler, 1967), cocoa beans (Scott, 1969a), and green coffee (Levi and Borker, 1968; Scott, 1968). Procedures for aflatoxin analyses in oilseeds (Chen and Friedman, 1966; Gardner et al., 1968; Velasco, 1969), peanuts and peanut products (Jayaraman, 1965; Holaday, 1968; Juszkievicz and Stefaniakowa, 1969; Mayura and Sreenivasamurthy, 1969), stored corn (Johnson et al., 1969), cereal products (Lemieszek-Chodorwska et al., 1969), and malt feeds (Kuehnert and Buchhein, 1968) have also been reported.

Screening Tests

A rapid screening method requiring no special skills on the part of the examiner was described for aflatoxin detection in cottonseed (Whitten, 1969). Contaminated lots of cottonseed had stem ends that fluoresced a bluish color when exposed to longwave ultraviolet light. A greenish-yellow fluorescence was previously associated with the growth of A. flavus on seedcoat and cottonseed fiber constituents (Ashworth et al., 1967; Ashworth et al., 1968).

The color is believed to be formed under the influence of plant peroxidase on kojic acid produced by the fungus (Marsh et al., 1969).

Several quick chromatographic techniques were proposed for aflatoxin analysis of food materials. Precise quantitation, however, is unlikely to be achieved. The "milli-column" technique for detecting aflatoxins in peanuts involves filling a 4-mm i.d. glass tubing with silica gel, developing the column in a chloroform-methanol extract of the peanut sample, and then viewing the column under longwave ultraviolet light (Holaday and Passwater, 1969). Microslides were proposed for rapid thin layer chromatography (Eppley, 1969).

Analytical methods for screening zearalenone, aflatoxin, and ochratoxin (Eppley, 1968) and aflatoxin, ochratoxin, and sterigmatocystin in cereals and groundnuts (Vorster, 1969) involved the use and manipulation of various solvents for extraction and development on TLC plates.

Sampling mills which operate on the principle of size or color difference permitted effective separation of aflatoxin-contaminated peanuts prior to their incorporation into food materials (Pattinson et al., 1968; Dickens and Satterwhite, 1969; Whitaker and Wiser, 1969).

Presumptive Tests

Presumptive tests are more time consuming but provide better evidence than the previously described screening methods. Generally these physicochemical tests are not suitable for large samples. Preparation and extraction of the sample is usually performed in a Waring blender with solvents such as chloroform, methanol, benzene, acetonitrile (Yin, 1969), or aqueous acetone (Pons et al., 1966).

Pigments are removed from solvent extracts with copper carbonate (Wiseman et al., 1967). Cleanup procedures involve liquid-liquid partitioning followed by partitioning on a Celite column and concentration by evaporation.

Paper chromatography once was recommended for aflatoxin separation (Coomes and Sanders, 1963; Sreenivasamurthy et al., 1964) but was replaced by the more sensitive TLC method (Peterson and Ciegler, 1967; Nesheim, 1969; Stubblefield et al., 1969). Silica Gel G-HR (Brinkman Instruments, Inc., Westbury, New York), Adsorbosil-1 and Adsorbosil-5 (Applied Science Laboratories, State College, Pennsylvania) are the most commonly used brands of adsorbents. Polyamide-impregnated film TLC was reported to give increased separation of aflatoxin B₁ and G₁ (Lee and Ling, 1967). Various commonly used solvents include chloroform, acetone, methanol, toluene, isopropanol,

benzene, and formate in various ratios and combinations (Stoloff et al., 1968; Engstrom, 1969; Teng and Hanzas, 1969).

The accuracy of the physicochemical procedures described above ultimately depends upon the reliability of the method used for evaluating the amount of toxin present. Visual estimates were reported to be accurate within ± 20 to 30% (Nesheim, 1964; Pons et al., 1966) whereas instrumental methods for fluorodensitometric quantitation are claimed to have precision of ± 3 to 4% (Beckwith and Stoloff, 1969; Pons et al., 1968). A method utilizing colored Polaroid recordings of TLC plates for precise, permanent records was presented (Chang and Lynd, 1968).

Confirmatory Tests

Chemical and biological confirmatory tests require fairly pure samples obtained from time-consuming cleanup procedures.

Exposure of developed TLC plates to elemental iodine crystals was used to distinguish interfering materials from aflatoxins due to their continued fluorescence (Mislivec et al., 1968). Depending upon the concentration and purity of the sample, aflatoxin B₁ reacted with 2,4-dinitrophenylhydrazine to form a yellow to deep orange dinitrophenylhydrazone derivative (Crisan and Grefig, 1966; Crisan, 1968). Quantitation by both methods involved

visual comparison with standards. Modification (Stoloff, 1967) of a chemical confirmatory test (Andrellos and Reid, 1964) based upon derivative formation was adopted as "Official--First Action" by the Association of Official Analytical Chemists.

Biological assays proposed as confirmatory tests for aflatoxins are generally more expensive and more time-consuming than chemical confirmatory tests. In addition to the use of B. megaterium as a bioassay organism (see Toxicity of Aflatoxins to Microorganisms), numerous other biological systems were tested. Chlorella pyrenoidosa (Ikawa et al., 1968; Ikawa et al., 1969), brine shrimp (Brown et al., 1968; Brown, 1969), mollusks (Townesley and Lee, 1967), chicken embryo liver cells (Kiyoshi and Miyaki, 1968), chicken embryos (Verrett et al., 1964), and corn seedlings (Mayer et al., 1969) were examined for their possible use as confirmatory bioassays for aflatoxins.

Due to lack of specificity in most methods, proof of the presence of aflatoxin involves the use of physicochemical as well as biological procedures. Only three of the tests suggested to date were collaboratively studied and given "Official--First Action" status by the A.O.A.C. (Ayres et al., 1970). These include two methods for peanuts and peanut products (A.O.A.C., 1966, 1968, 1969), one for cottonseed (A.O.A.C., 1969), and one for confirmation of aflatoxin by derivative formation (A.O.A.C., 1967).

Detoxification of Foods and Feeds
Containing Aflatoxin

Physical Destruction

Inactivation or removal of aflatoxins from contaminated foods or feeds by methods presently investigated has generally left the food unfit to eat or, at best, with a decreased nutritional value. Early attempts to reduce toxicity of peanut meals by heating, cooking, or autoclaving were largely ineffective (Blount, 1961). Later reports, however, were more encouraging. Heating at atmospheric pressure at 60 to 80 C resulted in definite reductions in aflatoxin in cottonseed meals; heating cottonseed meals having 20% moisture at 100 C for 120 min resulted in an 80% reduction of aflatoxin B₁ (Mann et al., 1967). Sterilization by autoclaving wet groundnuts at 15 lb per in.² (121 C) produced a progressive reduction in toxicity with time (Coomes et al., 1966). However, heating dried fruits and various fruit juices in which A. flavus had grown and produced aflatoxin resulted in little toxin destruction (Frank, 1968). Buffered methanol containing aflatoxin received 30 Mrad of radiation which accelerated aflatoxin decomposition, but did not affect the biological toxicity of aflatoxin (Miyaka et al., 1966). Aflatoxins were reduced following roasting of contaminated peanuts (Sobolewski and Kmiecik, 1968; Lee et al., 1969). Natural or activated bleaching earth used

in peanut oil refining was responsible for detoxifying an oil containing 0.5 µg/g aflatoxin (Velan and Raymond, 1967).

Chemical Inactivation

Ammonia is one of the most effective chemicals for inactivation of aflatoxins in contaminated food materials. Agricultural products contaminated with aflatoxin were detoxified by mixing with 0.3 g of ammonia per kg of product and heated (Dollear et al., 1968; Masri et al., 1969). Contaminated cottonseed and peanut meal were treated with ozone and aflatoxins B₁ and G₁ were readily destroyed, especially at high moistures, high temperatures (100 C), and at longer treatments (Dwarakanath et al., 1968). Sodium hypochlorite (5%) is recommended for decontamination procedures in the laboratory (Fischbach and Campbell, 1965; Stoloff and Trager, 1965). A microbiological inactivation method using F. aurantiacum was proposed (Ciegler et al., 1966). Both growing and resting cells of this bacterium partially detoxified contaminated milk, peanuts, soy products, and corn without the development of new toxic products.

Implications of Aflatoxicosis in Man

Aflatoxins are probably the most potent hepatoma-inducing agents known. They are more toxic than benzopyrene and 1000 to 1500 times more effective than butter yellow, a

butter additive that is universally prohibited (Hanssen and Hagedorn, 1969). The Food and Agriculture Organization of the United Nations in cooperation with the World Health Organization established in 1966 the maximum concentration of aflatoxin in foodstuffs as 30 ng/g (Anonymous, 1966). In the United States, Switzerland, and the German Federal Republic a zero tolerance was set; a 5 µg/g maximum tolerance was set by Great Britain, Canada, and the Netherlands.

The ubiquitous nature of aflatoxin-producing molds, coupled with their nonspecificity on living cells of nearly every form of life, stimulated interest to determine the presence of the toxin in foods regularly consumed by man. In Uganda 15% of the samples of groundnuts sold for human consumption contained more than 1 µg/g aflatoxin B₁ (Lopez, 1967). Toxic strains of A. flavus were isolated from Nigerian foods (Bassir, 1964); aflatoxin was detected in commercial milk samples in South Africa (Purchase and Vorster, 1968); from peanut oil, peanut cakes, peanut butter, and dried sweet potatoes in Taiwan (Ling et al., 1968; Tung and Ling, 1968); and from unrefined peanut oil in Malaya (Chong and Beng, 1965). Tortillas made by a method commonly used in Mexico for centuries contained aflatoxin (Ulloa-Sosa and Schroeder, 1969). On the other hand, exhaustive studies of fermented Japanese foods failed to reveal the presence of aflatoxin (Manabe et al.,

1968; Murakami et al., 1967; Murakami et al., 1968; Yokotsuka et al., 1968).

Primary liver carcinoma is a relatively rare disease in Europe and North America but is much more common in certain parts of Asia and Africa (Foy et al., 1966; Purchase, 1967; Robinson, 1967). It is possible that there may be common links in the metabolic disorders leading to cirrhosis and to liver carcinoma in Africans, to hepatic fibrosis in aflatoxin treated rats, and to the hepatic lesions in acutely pyridoxine deprived baboons (Foy et al., 1966). High incidence of primary liver cirrhosis and carcinoma in the African may imply that their diets intermittently contain aflatoxin as a result of infection of porridges and brews by one or more molds. Other postulations were relative to the high incidence of carcinoma in areas where the consumption of fermented foods was high (Oettle, 1965). It is not suggested that aflatoxins are the only likely cause of hepatoma since many other factors may be implicated. The fact is that aflatoxins are highly suspect and should not be present in the human diet.

METHODS AND MATERIALS

Production of Vegetative Cells and Spores of *Bacillus megaterium* NRRL B-1368

Growth Media

Bacillus megaterium NRRL B-1368, obtained from Dr. C. W. Hesseltine, Chief of Fermentation Laboratory, United States Department of Agriculture, Northern Utilization Research and Development Division, Peoria, Illinois, was the organism used throughout this research. Any further reference to *B. megaterium* is reference to the above strain, unless otherwise indicated. The organism was cultured in Trypticase Soy Broth (TSB; Baltimore Biological Laboratories, Baltimore) which contains the following constituents on a per liter of distilled water basis: 17.0 g trypticase (pancreatic digest of casein); 3.0 g phytone (soy peptone); 5.0 g sodium chloride; 2.5 g dipotassium phosphate; 2.5 g dextrose; and adjusted to pH 7.1. Five-hundred milliliters of TSB were dispensed into 1000-ml Erlenmeyer flasks, autoclaved at 121 C for 15 min, and used for the production of vegetative cells analyzed during the research project. Fifty milliliters of the TSB medium containing 2% agar were added to 1000-ml pharmaceutical bottles and the agar was allowed to solidify along the flat side

surface in order to produce spore crops using a surface growth technique.

Besides using the "nontoxic" medium described above to culture B. megaterium, the same medium containing aflatoxin B₁ (crystalline, dried in situ from chloroform, grade B, lot 940032, Calbiochem, Los Angeles) was also used to culture the organism for vegetative cell or spore analyses. The objective in the aflatoxin addition was to culture the organism under conditions of strain severe enough to inhibit the rate of normal cell division but not to decrease viability. An approximate aflatoxin concentration of 4.0 µg/ml produced these effects.

Addition of aflatoxin was as follows. Crystalline aflatoxin B₁, in 10-mg quantities, was dissolved in 10 ml of chloroform. Two milliliters or 0.2 ml of the solution was dispensed aseptically into 500 ml of TSB or 50 ml of trypticase soy agar (TSA), respectively. The medium was then steamed for 15 min, driving off the chloroform and leaving aflatoxin in the medium. Care was taken to shield the toxin and toxic media from light. Theoretically the medium should have contained 4.0 µg aflatoxin per ml while the determined concentration was 3.8 µg/ml.

Quantitation of Aflatoxin B₁

The exact aflatoxin B₁ concentration in the chloroform solution added to the sterile TSB and TSA was determined as was the concentration in the medium before steaming

and before culturing. These two quantitations allowed the per cent recovery to be calculated. The concentration immediately after steaming but before culturing was determined to evaluate the stability of aflatoxin to heat. Concentration of aflatoxin in the spent broth upon termination of culturing was also established. To obtain this information, the following procedure was carried out. First the sample to be quantitated was treated ultrasonically with a Branson Sonifier Model S-75 sonic oscillator (Heat Systems-Ultrasonic Inc., Plainview, New York) operated at 5 amperes for 10 min. Fifty milliliters of the sample were poured into a 250-ml separatory funnel along with 50 ml of chloroform. The mixture was shaken vigorously for 1 min and allowed to separate into two phases before drawing the bottom (chloroform) layer off through a 2-cm layer of anhydrous sodium sulfate in a Butt tube. The partitioning was repeated with two additional 50-ml aliquots of chloroform after which the pooled chloroform extracts were evaporated to near dryness on a steam bath and the toxin was dissolved in sufficient chloroform to permit quantitative transfer to a vial. After evaporation to dryness, the vials were flushed with nitrogen and refrigerated until quantitated. The dry extract was dissolved in chloroform and spotted on TLC plates, along with an aflatoxin standard containing 1.0 μg B₁, 0.3 μg B₂, 1.0 μg G₁, and 0.3 μg G₂ per ml. The

aflatoxin standards were obtained from Dr. Leo A. Goldblatt, Chief of Oilseed Crops Laboratory, United States Department of Agriculture, Southern Utilization Research and Development Division, New Orleans, Louisiana. The TLC plates used were coated with Adsorbosil-5 (20 x 20 cm, 250- μ m layer) obtained from Applied Science Laboratories, State College, Pennsylvania. The plates were developed with chloroform:acetone:2-propanol (26:6:1, v/v/v) in unlined, unequilibrated tanks (Desaga Multiplate Glass Tank 25-10-25, Brinkman Instruments, Inc., Westbury, New York). After drying the plates for 15 min in the dark, measurement of the emitted fluorescence of aflatoxin B₁ upon exposure to longwave ultraviolet was made. The basic fluorometric densitometry system consisted of a Photovolt Model 530 densitometer equipped with a longwave ultraviolet lamp, No. 265 primary filter and 6 x 19 mm primary slit, a stage for TLC plates equipped with automatic drive, a No. 465 secondary filter and an ultraviolet sensitive phototube, and a No. 520-A multiplier photometer. A Varicord Model 43 recorder equipped with an automatic recording integrator (Integrgraph Model 49) was used to record the intensity of the fluorescent radiation.

Integrator area counts were used to calculate aflatoxin B₁ concentrations. The content of a sample was calculated according to the following equation:

$$\text{Aflatoxin B}_1 \text{ (}\mu\text{g/ml)} = \frac{(A_x) (V_s) (C_s) (S_d)}{(A_s) (V_x) (V)}$$

where:

(A_x) = average area count of duplicate B_1 sample spots

(V_s) = μ l of standard spotted

(C_s) = μ g of B_1 per ml of standard

(S_d) = volume of final extract in μ l

(A_s) = average area count of the B_1 standard spot

(V_x) = μ l of sample extract spotted

(V) = ml of original sample represented by the final sample extract (50 ml in this case)

Production and Harvesting of Vegetative Cells and Spores

A stock suspension of B. megaterium spores was heat shocked for 8 min at 80 C and inoculated into TSB. Transfers were made at 8, 6, and 4 hr into 1000-ml Erlenmeyer flasks containing 500 ml of medium and incubation was at 32 C in a New Brunswick Model G25 incubator gyratory shaker at 150 RPM. Vegetative cells were harvested by centrifugation at 9000 x g at a time late in the logarithmic growth phase. Cells were then washed seven times with 0.1 M phosphate buffer pH 7.1 and stored at -20 C until they were analyzed for chemical constituents. Cells used for electron microscopy studies were harvested in

early stationary phase and were not frozen, but stored overnight at 4 C.

Since it was impossible to get good sporulation in toxic liquid medium, TSA in the 1000-ml pharmaceutical bottles was employed for spore production. Two milliliters of a 2-hr-old culture of B. megaterium were inoculated on the surface of toxic or nontoxic agar slabs in pharmaceutical bottles and incubated at 30 C. Culture samples were observed at various times with an American Optical microscope with phase contrast optics (American Optical Co., Buffalo, New York) at 1000 x magnification. All phase dark and phase bright spore forms were counted as positive indices of sporulation. The per cent sporulation was computed by dividing the number of sporangia by the total number of cells counted and multiplying by 100. Spores were harvested from the nontoxic medium after 3 days and the toxic medium after 6 days with cold 0.1 M phosphate buffer (pH 7.1) and transferred to centrifuge bottles. Spores were washed twice with phosphate buffer, treated 1 hr at 37 C with 0.075% lysozyme, and again washed 5 times with phosphate buffer. Spore crops were stored at 4 C until chemical, germination, heat resistance, and electron microscopy studies were performed.

Observations on Vegetative
• Cell Division

Phase Contrast Microscopy

Cell division and morphological development of B. megaterium either grown in toxic or nontoxic TSB were followed. Cells were observed with either an American Optical microscope or a Leitz Ortholux microscope, both of which were fitted with photomicrographic equipment. Photographs were taken at several magnifications and times during the growth cycle.

The ability of elongated vegetative cells formed in toxic TSB to revert to normal cellular division and morphology when placed on nontoxic TSA was studied with phase contrast microscopy. Observations of cell division in one or more stationary B. megaterium cells were made on agar slides. A layer of liquified control TSA (4% agar) was placed near the center of a standard 3 x 1 in. glass slide and allowed to harden and dry for about 2 hr. The edges of the agar layer were trimmed to about 22 mm square and a loop of the vegetative cells was placed on the medium; a cover slip was placed on top of this and sealed with warm paraffin to prevent further drying. Agar temperature was maintained at 30 ± 1 C as measured potentiometrically with a 250-Watt infrared brooder lamp. Photographs were made throughout the progression of cell division.

Macrocolony Formation

Aberrant filamentous cells produced by B. megaterium in toxic TSB were surface plated on nontoxic TSA and incubated at 32 C. Colony formation was visually examined and morphology of daughter cells from different areas in the colony were examined by phase contrast microscopy to determine whether permanent genetic changes affecting phenotypic characteristics had occurred in the filament.

Direct Counts, Viable Counts, and Absorbancy Changes

The obvious differences in cell size of B. megaterium grown in toxic TSB versus cells grown in nontoxic TSB required the determination of viability and absorbancy changes during growth.

Direct microscopic counts were made on the nontoxic cultures at different times in the growth cycle using a Petroff-Hausser counting chamber. A 1 to 4 dilution of the suspension was added to 3 ml of 30% glycerol, the counting chamber filled with one loopful of the suspension, and the organisms in 80 small squares were counted. Total counts were calculated according to the following equation:

$$\text{Total Count} = \frac{(\text{Number Cells Counted})(\text{Dilution})(2 \times 10^6)}{(\text{Number Small Squares Counted})}$$

Viable counts were made at selected times during the growth cycle by making appropriate dilutions in sterile 0.2% peptone and plating in triplicate on TSA. Incubation was at 30 C and counts were made 48 to 72 hr after pouring.

Cellular growth as measured turbidimetrically was also correlated with viable counts of B. megaterium cultured on toxic and nontoxic TSA. Absorbancy of the culture at 660 nm was plotted versus time using a Bausch and Lomb Spectronic 20 spectrophotometer.

Effects of Cell Septum Initiators on Cell Division

Pantoyl lactone, a septum-inducing agent, was added to B. megaterium in toxic TSB to determine its effects on cell septum formation. The chemical was added at a concentration of 0.075 M initially or after 1 1/2 hr of growth to toxic and nontoxic TSB. Fifty milliliters of growth medium were added to 250-ml Erlenmeyer flasks and incubated at 30 C in a shaker-incubator at 150 RPM (New Brunswick Model G25). Absorbance at 660 nm and viable counts using TSA were made throughout the growth cycles.

The effects on cell division of 5 µg/ml Co^{++} , Fe^{+++} , Ba^{++} , Zn^{++} , Cu^{++} , and Cr^{++} in toxic TSB were investigated using the same culturing techniques as described for pantoyl lactone.

Effect of Penicillin on Cell Division

Both 15 and 30 units/ml of penicillin G were added to 50 ml of nontoxic and toxic TSB in 250-ml Erlenmeyer flasks. Agitated incubation was performed as previously described. Cultures were examined visually for turbidity and microscopically to determine the development of possible morphological changes due to the interaction of penicillin and aflatoxin or to penicillin alone.

Staining Studies

Six-hour cultures of B. megaterium grown in toxic and nontoxic TSB were stained for cell wall development. A smear was prepared and fixed by heat. Three drops of 0.34% aqueous cetyl pyridinium chloride were placed on the smear followed by the addition of 1 drop of saturated aqueous Congo red. The slide was washed with distilled water after 1 min and counterstained with methylene blue for 10 sec. The stain was then rinsed once more with distilled water, dried, and examined microscopically.

Sudan black B (0.3% in 70% ethanol) was flooded over a wet smear of B. megaterium cells to demonstrate the existence and relative amounts of poly- β -hydroxybutyric acid at different stages of growth. The smears were washed with distilled water, dried, and examined microscopically.

Heat Resistance of Spores Formed in
the Presence of Aflatoxin B₁ and the
Effects of Aflatoxin B₁ on Spore
Germination

Heat Resistance Studies

One tenth milliliter of stock B. megaterium spore suspension formed in toxic or nontoxic TSA was dispensed into 16 x 150 mm screwcap test tubes containing 9.9 ml of water tempered at 92 C (197 F). Aliquots were removed after 10, 20, 30, 50, and 70 min of heating, appropriately diluted, and plated in TSA. Incubation was at 30 C and counts were made after 48 hr. Decimal reduction times (D values) were calculated by plotting the viable count versus time of heating in min at 92 C on semi log paper.

Germination Studies

B. megaterium spores produced in TSA as described previously were heat shocked at 60 C for 10 min and diluted to yield approximately 10^7 viable spores per ml. One milliliter of the suspension was inoculated into 6 ml of TSB and to 6 ml of TSB containing 3.8 µg/ml aflatoxin B₁. The TSB media was tempered at 32 C. Absorbance at 650 nm was recorded each 15 min for the first 2 hr and at 3, 14, and 24 hr of incubation.

Chemical Analyses of Vegetative Cells
and Spores of *Bacillus megaterium*
Formed in the Presence and
Absence of Aflatoxin B₁

Chemical analyses described below were performed on vegetative cells produced in toxic and nontoxic TSB which were stored at -20 C and on spores formed in toxic and nontoxic TSA which were stored at 4 C. Except for a few minor changes in procedure, the method for a particular analysis of vegetative cells or spores was the same. Analyses were performed in triplicate on vegetative cells and spores formed both on toxic and nontoxic media.

Dry weights were determined in quadruplicate on cell and spore suspensions.

Preparation of Vegetative Cell
Walls for Amino Acid Analysis

Cells were resuspended in 200 ml of cold distilled water. One-hundred milliliters were then sonicated for 7 min (filamentous cells formed in toxic TSB) or 12 min (control cells formed in nontoxic TSB) at a setting of 5 amperes. Sonication was about 98% complete as evidenced by phase contrast microscopy. The sonicated cell suspension was centrifuged at 10,000 x g for 10 min and the supernate was discarded. The pellet, containing cell walls, intact cells, and coarse debris, was resuspended in 50 ml cold water and centrifuged at 3,000 x g for 5 min. The pellet was discarded and the supernate, containing

cell walls and fine particles, was centrifuged at 10,000 x g for 20 min. The supernate was discarded and the pellet or crude cell wall fraction was washed four times in 1 M NaCl; after each washing the cells were centrifuged at 10,000 x g for 20 min and the supernatant fluid was discarded. The pellet was then suspended in cold water and centrifuged at 3,000 x g for 5 min. The supernate, containing cell walls, was centrifuged at 10,000 x g for 20 min and the pellet was dried in preparation for amino acid analysis. All centrifuging was done at 4 C.

Procedure for Amino Acid Analysis of Vegetative Cell Walls

Sufficient dried cell wall material, as determined by semimicro Kjeldahl analysis (Kabat and Mayer, 1948), was dissolved in 1 ml of 6 N hydrochloric acid in 10-ml ampoules to give a 0.4% protein solution. The tube was drawn to a small neck, cooled, and placed in a dry ice bath to freeze the contents. After freezing, the tube was evacuated, the sample was allowed to melt, and the tube was sealed at the constriction with an air-propane flame. The sealed tube was then placed in an oil bath in an oven at 110 C for 20 hr or 70 hr. Following hydrolysis and cooling, the hydrochloric acid was quickly removed on a vacuum evaporator and the sample was dissolved in 5 ml of 0.067 M citrate-hydrochloric acid buffer, pH 2.2.

An aliquot of 0.2 ml was removed from each sample for analysis on a Beckman Amino Acid Analyzer Model 120 C (Moore et al., 1958). The amino acid composition of the protein samples was expressed as μ Moles amino acid per mg of protein.

Total Protein Analyses of Vegetative Cells and Spores

Breakage of vegetative cells was by sonication for 7 or 12 min as described earlier. Spores were mechanically ruptured by means of a Bronwill Disintegrator (Type 2876, Bronwill Scientific Inc., Rochester, New York). The supernate and three washings from the glass beads used for disrupting the spores were pooled for the analysis. The Biuret reaction (Gornall et al., 1949) and the method of Lowry et al. (1951) were used to estimate the protein concentration in the supernatant fluids collected from the cell sonicates and in the spore disintegrates. Standard protein solution was made using crystalline bovine albumin (Nutritional Biochemicals Corporation, Cleveland, Ohio).

The total protein analysis described in this section and the RNA analysis described in the following section were performed using supernatant fluid collected by centrifugation following sonication of cells and on spore disintegrates. However, calculations were made to express

the resultant quantities of each component as a percentage of dry weight of the whole cell or spore.

Total Ribonucleic Acid Analyses
of Vegetative Cells and Spores

The RNA isolation procedure described by Clark (1964) was modified to determine the RNA content in the supernatant fluid collected from cell sonication and in the spore disintegrates. An equal volume of 88% phenol was added to the chilled supernate and the mixture was stirred at room temperature for 30 min. The emulsion was then cooled in an ice bath for 5 min before separating by centrifugation at $3,300 \times g$ for 15 min. The aqueous upper supernatant layer and most of any intermediate layer containing denatured protein was decanted from the brown phenol phase. Removal of denatured protein from the aqueous phase was accomplished by centrifugation at $8,000 \times g$ for 5 min. A volume of 20% (w/v) potassium acetate (pH 5.0), 1/10 the volume of the aqueous fraction, was added to the aqueous fraction followed by precipitation of RNA by the addition of two volumes of cold 95% ethanol. The suspension was chilled in an ice bath 5 min before collecting the precipitate by centrifugation at $1,000 \times g$ for 20 min. The precipitate was washed once each with ethanol:water (3:1, v/v), absolute ethanol, and anhydrous ether and then air dried. All centrifugation was performed at 0 to 5 C.

The dried precipitate was dissolved in 10 ml of 0.5 N potassium hydroxide and hydrolysis proceeded at room temperature for 24 hr. Sufficient 20% (v/v) hydrogen perchlorate was added to the chilled hydrolyzed solution to reduce the pH to about 2 followed by removal of precipitated potassium perchlorate, DNA, and/or protein by centrifugation at 1,000 x g for 10 min. The pH of the supernate was adjusted to 3.5 with 1.0 N potassium hydroxide.

A modification of the Ashwell (1957) procedure for colorimetric analysis of sugars was used to determine the RNA content of vegetative cells and spores. The acidified hydrolyzed sample of RNA was appropriately diluted in deionized water and suitable aliquots were mixed with 6 ml of orcinol acid reagent (0.1% FeCl_3 in concentrated HCl) and 0.4 ml of 6% (w/v) ethanolic orcinol. Total volume was brought to 10 ml with deionized water. The solutions were heated 20 min in a boiling waterbath, cooled, and absorbancies were measured at 660nm. A standard of 10^{-4} M nucleic acid (Ribonucleic acid, Nutritional Biochemicals Corporation, Cleveland, Ohio) was used.

Total Deoxyribonucleic Acid
Analysis of Vegetative
Cells and Spores

The DNA isolation procedure of Clark (1964) was used. Twenty milliliters of sonicated cell suspension or

disintegrated spore suspension was mixed with 80 ml of 15% (w/w) sodium lauryl sulfate in 0.14 M sodium chloride: 0.01 M sodium citrate. The mixture was homogenized and stirred 30 min. Two-hundred milliliters of absolute ethanol were added slowly and the precipitate was collected by centrifugation at 3,000 x g for 5 min. The precipitate was then washed three times with 5 ml of 1.4 M sodium chloride, collecting the pellet formed each time at 10,000 x g for 20 min and pooling the supernates. The pooled supernates were mixed with an equal volume of absolute ethanol; DNA was collected on a glass rod and air dried.

The diphenylamine reaction was used to quantitate the DNA obtained. Two milliliters of diphenylamine reagent (1.0 g diphenylamine dissolved in 100 ml of glacial acetic acid plus 2.75 ml of concentrated sulfuric acid) were added to 1.0 ml of sample dissolved and diluted in sufficient water to contain approximately 200 µg of DNA and the mixture was heated for 10 min in a boiling waterbath. The absorbancy was read at 600 nm upon cooling. Sperm deoxyribonucleic acid (Nutritional Biochemicals Corporation, Cleveland, Ohio) was used as a standard.

Dipicolinic Acid Analysis of Spores

Dipicolinic acid analysis was performed according to a modified Janssen et al. (1957) procedure. Spore suspensions containing 25 to 35 mg (dry weight) of spores were diluted to 2.5 ml with deionized water in a 10-ml volumetric flask. Hydrochloric acid (1 N) was added to the suspension to bring the pH to 2.0 and the hydrolysate was autoclaved for 1 hr at 121 C. The flask was then cooled and 3 ml of 4% trichloroacetic acid was added to precipitate interfering materials. Flasks were placed in an ice bath for 1 hr to complete precipitation followed by removal of the precipitate by centrifugation at 10,000 x g for 20 min. Aliquots of the supernate made to 4 ml with deionized water were mixed with 1 ml of color reagent (1% ascorbic acid and 1% $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ in 0.5 M sodium acetate) and the absorbancies were determined at 440 nm. Standard dipicolinic acid solution (100 $\mu\text{g}/\text{ml}$) was also monitored.

Preparation of Vegetative Cells and Spores of *Bacillus megaterium* for Observation by Electron Microscopy

Production of vegetative cells and spores was previously described. Cell types were fixed by adding sufficient cell or spore suspension to 13 x 100 mm screwcap test tubes to yield pellets about 0.5 mm thick upon centrifugation. Both cell and spore pellets were treated

identically. The pellets were suspended in a 50% aqueous solution of glutaraldehyde diluted to 6.25% with Sorensen's phosphate buffer, pH 7.2, and kept at room temperature for 2 hr. The samples were then washed three times in 1 hr with Sorensen's buffer and suspended in a 2% aqueous solution of osmium tetroxide diluted to 1% with Sorensen's buffer. The samples were dehydrated after 1 1/2 hr at room temperature for 10 min each in 25, 50, 75, and 95% ethanol and twice for 15 min each in 100% ethanol. The samples were treated for 30 min twice in propylene oxide and kept overnight in propylene oxide plus EPON complete mixture (1:1, v/v). The EPON complete mixture was prepared according to the following formula:

Solution A: 80 g EPON 812 (W.P.E. = 160) plus 94 g dodecenyl succinic anhydride

Solution B: 100 g EPON 812 plus 78 g nadic methyl anhydride

Solutions A and B were mixed in ratio A:B::6:4 and 4 drops of hardener, dimethyl amino methyl phenol, were added for every 10 ml of the mixture.

Samples were collected on the following day by centrifugation and dried with a stream of nitrogen. The samples were then suspended in the complete EPON mixture and kept overnight in a desiccator. Approximately 2 drops of the sample were placed on top of fresh EPON complete mixture in gelatin capsules, the desiccator was evacuated, and the samples were permitted to settle to the bottom of the

capsule overnight. Hardening was accomplished by heating the samples for 12 hr at 35 C, 12 hr at 45 C, and 12 hr at 65 C. The gelatin was removed from the hardened EPON upon cooling and ultrathin sections were made on an LKB ultratome (LKB, Type 4801 A, Rockville, Maryland) and examined with a Philips electron microscope (Model EM-100 B, The Netherlands).

RESULTS AND DISCUSSION

Growth Media

A sufficiently aflatoxin-sensitive bioassay micro-organism was sought to investigate the mechanism of toxicity of aflatoxin. Since most available data describe metabolic responses of higher organisms to the toxin, additional data are needed on simpler organisms.

Bacillus megaterium NRRL B-1368, previously found to be inhibited by 15 µg/ml aflatoxin (Burmeister and Hesseltine, 1966), was chosen for this purpose. Various morphological and biochemical responses of the vegetative cell to aflatoxin were evaluated and sporulation, germination, and outgrowth processes as affected by aflatoxin were studied. To date no reports are available involving the effects of aflatoxin on sporulation and germination.

The organism was initially cultured on several media to determine a suitable medium for both vegetative cell and sporulation studies. Media evaluated were nutrient broth (Difco), peptone broth (Difco) plus 10 µg/ml Mn, trypticase soy broth (Baltimore Biological Laboratories), and a special modified sporulating medium containing metal salts, glucose, and casamino acids (Webster and Lechowich, 1970). Trypticase soy broth

(TSB) was superior with respect to rate of cell growth for culturing vegetative cells and for sporulation (TSB plus 2.0% agar, TSA) and was therefore chosen as the growth medium.

Recovery of Aflatoxin Added to the Medium

The calculated aflatoxin concentration in the toxic TSB quantitatively obtained by fluorodensitometry against aflatoxin standards should have been 4.0 µg/ml prior to inoculation. The medium was steamed for 15 min to drive off the chloroform in which the aflatoxin was dissolved, and a 5% loss in aflatoxin B₁ concentration was observed. This loss could have been due to thermal inactivation or degradation to some other compound, although no additional fluorescent spots were detected by thin layer chromatography. This loss could also be due to the failure in quantitative removal of the toxin from the TSB by the partitioning procedures used for quantitation. The per cent recovery of aflatoxin from TSB before steaming was about 97%, indicating that the 5% loss was probably due to a combination of both phenomena.

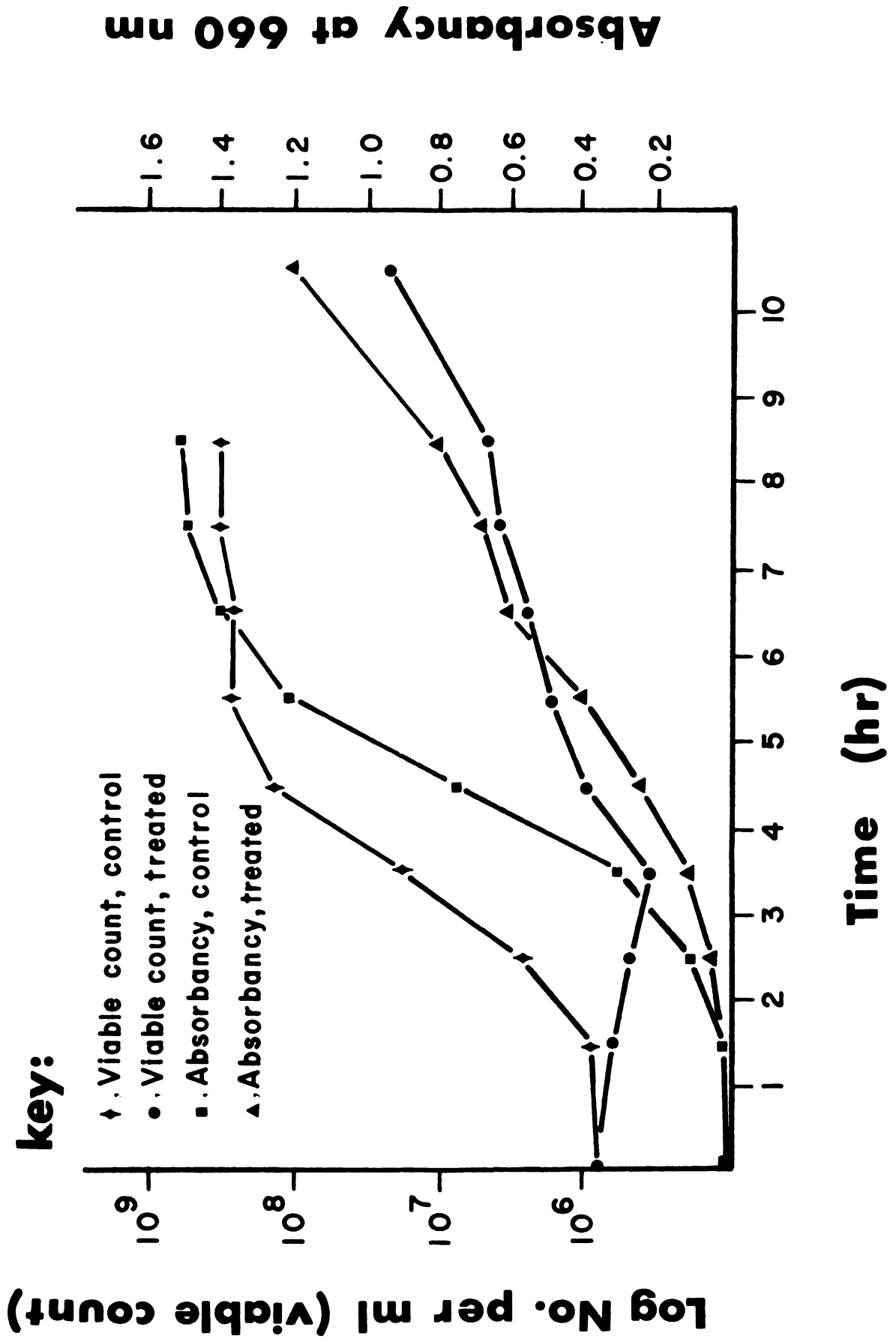
Only 1.7 µg aflatoxin B₁ per ml or about 45% of the original B₁ added to the TSB were detected after 10 1/2 hr of culturing B. megaterium at 30 C on a gyratory shaker. The decrease may have been caused by oxidation during the culturing period, utilization of the toxin by the organism,

and/or inefficient extraction of the toxin from the ruptured cellular components liberated by sonication. Decrease due to utilization of the toxin might be inferred from the observed initial 30% kill of B. megaterium during the first 3 1/2 hr after inoculation (Figure 2). The lethal effect was followed by an extended lag and possible adaptation period before logarithmic growth commenced. During such an adaptation period, mutation could have occurred to yield cells potentially capable of utilizing the toxin.

The apparent loss of aflatoxin B₁ is more likely principally due to chemical oxidation since a 2.4 µg/ml concentration (60% of that initially added) of B₁ was detected in noninoculated TSB after 10 1/2 hr at 30 C on a gyratory shaker or to inadequate extraction of the ruptured cellular components resulting from sonication. Lillehoj and Ciegler (1968) reported that repeated chloroform washing of macerated B. megaterium cells was required to quantitatively remove aflatoxin from cell walls, protoplasts, and nucleic acid.

Aflatoxin concentration in the sporulation medium was 3.2 µg/ml after 6 days of incubation.

Figure 2.--Growth curves for Bacillus megaterium cultured in trypticase soy broth (control) and trypticase soy broth containing 3.8 $\mu\text{g/ml}$ aflatoxin B₁ (treated).



Effects of Aflatoxin upon Growth
of Bacillus megaterium

Growth Curves

It is interesting to examine and study the growth curves of B. megaterium cultured in toxic and nontoxic TSB which are shown in Figure 2. There was a decrease in the viable number of cells during the first 3 1/2 hr of incubation in toxic TSB followed by logarithmic cell division at a rate considerably less than the growth rate of the control culture. Absorbance of treated cells at 660 nm measures increase in total cellular mass, and was observed to be more like the control culture, although the slope of the control culture was greater. The apparent differences when comparing the rates of growth and rates of cell division of control and treated cells are probably due to the difference in size of the viable units capable of forming colonies on nontoxic TSA. The aberrant single cell forms have increased length and mass after exposure to aflatoxin B₁ in comparison to cells grown in nontoxic TSB. This results in a concomitant decrease in the number of viable cell units and thus the observed slope differences. Generation times were calculated to be 75 min for the treated cells and 20 min for the control cells. The shape of the viable units also had an effect on cellular chemical composition which will be discussed later.

Cellular Morphology as
Affected by Aflatoxin

Figure 3 shows the filamentous cells formed in the presence of aflatoxin B₁ and normal B. megaterium cells. Filaments often reached 50 μ and intertwined to such an extent that made direct microscopic counts impossible. Massive accumulations of poly- β -hydroxybutyric acid and swelling, usually terminal, can be seen in mature aberrant cell forms (Figure 4). The bulb-like formations might indicate a weakening of cell wall structure with age or a greater sensitivity to osmotic change. Lending some support to this reasoning were the observed times required to rupture normal and treated cells. Control cells required 12 min and treated cells required only 7 min for satisfactory rupture by sonication. However, long filamentous cells would naturally be more susceptible to breakage and thus should require a shorter sonication treatment.

The affinity of cell wall or capsular material of the two cell types for the stains cetyl pyridinium chloride and Congo red was evaluated. Photographs of stained 6-hr cultures of control and treated cells are shown in Figure 5. The striated character of the distribution of poly-saccharide and the coincidence of major striations with septa of control cells is of interest. Although striations can be seen perpendicular and irregularly along the long

Figure 3.--Normal and aberrant forms of Bacillus megaterium produced during growth in trypticase soy broth and trypticase soy broth containing 3.8 µg/ml aflatoxin B₁, respectively. (A) control at 3 1/2 hr incubation, (B) 3.8 µg/ml B₁ at 3 1/2 hr, (C) control at 5 1/2 hr., (D) 3.8 µg/ml B₁ at 5 1/2 hr. Micrographs taken through a phase microscope X500.

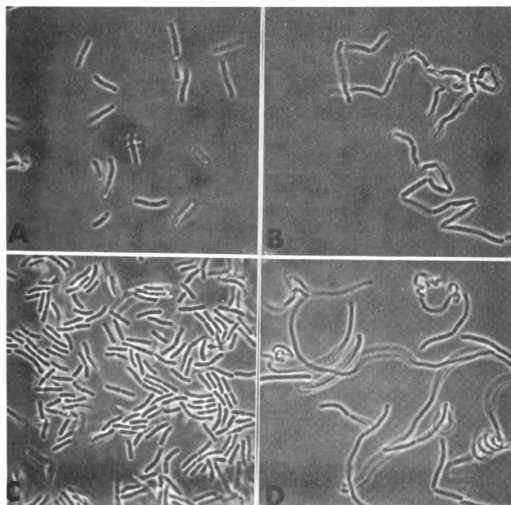


Figure 4.--Micrographs showing bulb-like swellings at the terminal portions of filaments in a 14-hr culture of Bacillus megaterium grown in trypticase soy broth containing 3.8 µg/ml aflatoxin B₁. Stained with 1% tannic acid and 0.4% basic fuchsin in 0.5% NaCl. Micrographs were taken at X500.

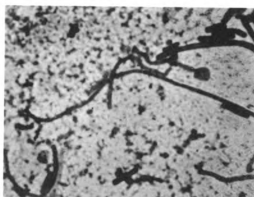
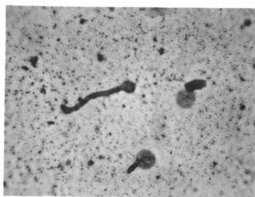
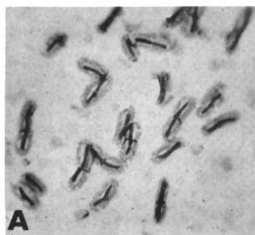


Figure 5.--Demonstration of capsular material in Bacillus megaterium cultured in (A) trypticase soy broth and (B) trypticase soy broth containing 3.8 µg/ml aflatoxin B₁. Note the striated character of the distribution of polysaccharide capsular material and the coincidence of striations with septa between dividing cells of the control culture. Few septa were observed in filaments of the treated culture. Micrographs were taken at X500.



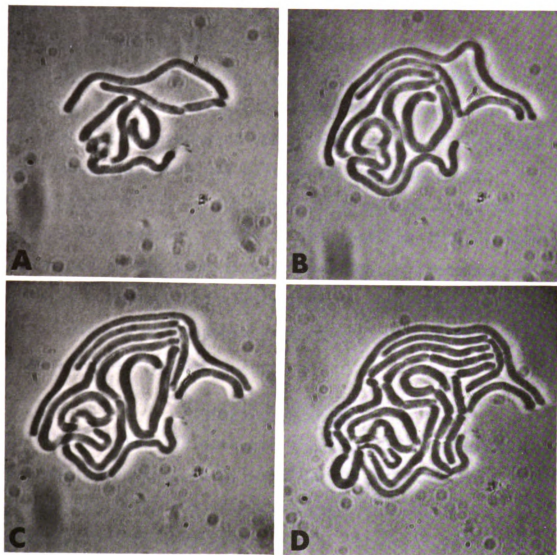
filaments, septa were rarely detected in these filaments by phase contrast microscopy or by electron microscopy. Thus the accumulation of polysaccharide near regions of septa formation must be independent of the inhibitory effect of aflatoxin on septum formation.

Aflatoxin-induced mutation is unlikely, due to the reversion of filamentous cells formed in toxic TSB to normal morphology when transferred on nontoxic medium (Figure 6). Visual examination of colonies of daughter cells formed from filamentous cells streaked on TSA indicated no morphological differences from cells never exposed to aflatoxin. Transfer of filamentous cells to nontoxic TSB resulted in growth patterns identical to the control cells. Thus there does not seem to be permanent genetic damage in the filamentous cells formed in toxic TSB.

Pantoyl Lactone Studies

Mutant cells of Escherichia coli formed after exposure to ionizing radiation is thought to be genetically controlled (Adler and Hardigree, 1964). Such filaments do not give rise to macrocolonies. Pantoyl lactone, an agent that initiates cross plate formation, allows these filaments to divide normally and produce macrocolonies. Reversal of inhibition of division by D-serine, D-histidine, D-phenylalanine, and penicillin in an Erwinia sp. was observed to occur after the addition of pantoyl lactone

Figure 6.--Time-lapse phase contrast micrographs of dividing filamentous Bacillus megaterium cells which were formed in trypticase soy broth containing 3.8 µg/ml aflatoxin B₁ and then transferred to nontoxic trypticase soy agar. Note the formation of normal sized daughter cells. (A) 0 hr incubation at 30 C, (B) 1 hr, (C) 2 hr, and (D) 8 hr. Micrographs taken through a phase microscope X1950.



to the growth medium (Grula and Grula, 1962). Pantoyl lactone has two distinct roles in metabolism, one as a precursor to pantothenate and another undefined role in cell division. To determine the reversibility of aflatoxin inhibition of division in B. megaterium by pantoyl lactone, 0.075 M was added initially and after 1 1/2 hr incubation to control and aflatoxin-treated cultures. Data derived from turbidimetric measurements and viable cell counts of the growing cultures indicated that pantoyl lactone had neither an inhibitory nor stimulatory effect on the rates of growth or division in control and treated cultures. The apparent lack of effect may be due to the inability to distinguish formation of septa by turbidity and viable counts. Observations by phase contrast microscopy, however, did not reveal these morphological structures.

Penicillin Studies

Penicillin inhibits the formation of cell walls in Gram positive bacteria by interfering with the attachment of the tetrapeptide to the N-acetylglucosamine-N-acetylmuramic acid backbone. Cells formed in media containing penicillin may divide at a reduced rate, sometimes producing penicillinase which destroys the penicillin and permits reversion to normal cell growth and division. Data from experiments involving the addition of 15 or 30 units/ml of penicillin G to aflatoxin-containing and

nontoxic media indicated that the metabolic actions of penicillin and aflatoxin were apparently independent of each other. Media containing high or low concentrations of penicillin or penicillin plus aflatoxin resulted in similar growth lags, the lags being greater than those observed for aflatoxin B₁ alone. Once growth commenced, cells formed in TSB plus penicillin were morphologically normal with only occasional bulging. Filamentous cells formed in TSB plus penicillin and aflatoxin were morphologically indistinguishable from filaments formed in TSB with aflatoxin alone, with the exception of occasional bulging. Aflatoxin B₁ had no apparent effect on the inhibition or enhancement of apparent penicillinase formation in this particular strain of B. megaterium. Penicillinase was not assayed, however cultures containing penicillin alone and penicillin plus aflatoxin behaved similarly after similar extended lag periods. It is inferred that the formation of penicillinase was inducible during the lag periods. Lillehoj and Ciegler (1969a) also observed that simultaneous addition of 50 µg of B₁ per ml and penicillin to an inducible strain of B. cereus did not interfere with penicillinase synthesis. Exposure of the cells to toxin for 2 hr before incubation, however, reduced penicillinase production 45 to 60% compared to the control.

Effects of Aflatoxin upon Sporulation
of *Bacillus megaterium*

Sporogenesis in *B. megaterium* begins when a portion of an axial thread of chromatinic material becomes positioned at one end of the sporangium and a centripetally growing thin septum encloses the chromatin and a small amount of cytoplasm. The sporulation process is believed to be irreversible or committed at this stage. Since one of the morphological alterations in *B. megaterium* grown in toxic TSB was the apparent inhibition of septum formation, a study was made to determine the effect of aflatoxin on the initial septum formation phase of sporulation. Completion of septum formation is compulsory for the development of the mature spore.

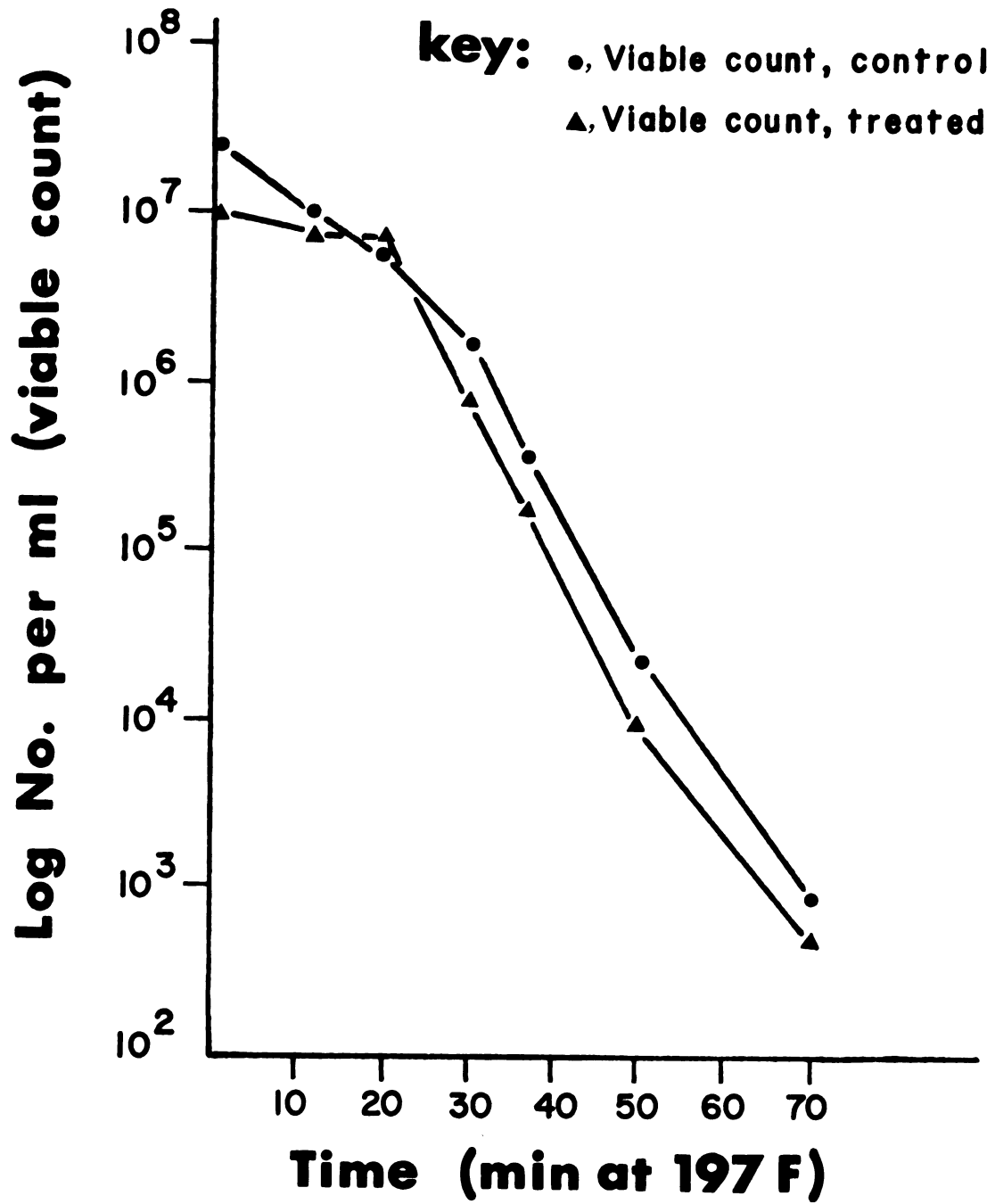
Sporulation was followed on toxic and nontoxic TSA. After 3 days, 97% sporulation occurred in the control culture as measured by direct microscopic count. Only 65% sporulation was obtained in the test culture after 6 days in the toxic medium. The 65% figure is only approximate due to the difficulties in counting caused by abnormal length of the filaments and the frequency of multiple spores being formed within these filaments. Old filaments were observed to lay down septa at close intervals to form a chain-like arrangement of cells, the length and width of which were nearly the same. The cells failed to separate, perhaps because of incomplete septum formation. Spores were often formed within these

Irregular partitionings of the filaments. Although the medium contained 3.2 µg/ml aflatoxin (16% decreased from initial) on a total volume basis when spores were harvested on the sixth day of incubation, it is probable that the content may have been decreased in the immediate area of colony formation due to oxidation and dilution brought about by increased cell mass. Aflatoxin concentration may have been decreased to a level which was not detrimental to either septum formation in dividing cells or septum formation as an initiator of sporogenesis.

Heat Resistance Studies

Once the spores were formed in a medium initially containing 3.8 µg/ml aflatoxin, were they physically, chemically, or morphologically different from spores formed on nontoxic medium? An examination of the wet heat resistance of spores formed on control and toxic TSA indicated no significant difference. Survivor curves for spores formed on toxic and nontoxic TSA are shown in Figure 7. The D_{197} (time in minutes to destroy 90% of the population at 197 F) for the control was 17.0 min; the D_{197} for the test culture spores was 16.9 min. The D values are extremely low, indicating little heat resistance. The chemistry and morphology of control and treated spores will be discussed later.

Figure 7.--Survivor curves at 92 C (197 F) for Bacillus megaterium spores formed in trypticase soy agar (control) and trypticase soy agar containing 3.8 µg/ml aflatoxin B₁ (treated).



Effects of Aflatoxin on Spore Germination and Outgrowth

Germination of spores is characterized by loss in heat resistance, permeability to dilute stains, loss in turbidity or dry matter, and darkening under phase contrast (Murrell, 1961). The loss in turbidity and phase darkening were followed to study the effect of aflatoxin on germination of spores which were formed on nontoxic TSA. Initiation of germination is usually brought about by chemical substances and can occur even if environmental conditions are unfavorable to germinal development. Germination initiation involves among other processes, the uptake of water with a coincident swelling and decreased refractility of the spore. Germinal development following initiation involves either rupture or adsorption of spore coats followed by the elongation of a germ cell and is generally defined as outgrowth. Results from germination studies using normal spores exposed to a suitable germination environment and the same environment plus aflatoxin indicated that the toxin had no effect on germination initiation but suppressed outgrowth for an extended period. Table 2 lists absorbancy readings at 650 nm of spore suspensions in toxic TSB and nontoxic TSB at various times during incubation at 30 C.

The degree of outgrowth inhibition, that is the percent of spores which initiated germination but failed to develop through the outgrowth stage, cannot be estimated

TABLE 2.--Absorbancy at 650 nm of spore suspensions in trypticase soy broth containing 3.8 µg/ml aflatoxin B₁ (toxic) and trypticase soy broth (nontoxic).

	Absorbancy (650 nm) with Time (hr)							
	0	$\frac{1}{4}$	$\frac{1}{2}$	$1\frac{1}{4}$	$1\frac{3}{4}$	3	14	24
Toxic TSB	.620	.569	.538	.523	.495	.469	.432	.553
Nontoxic TSB	.638	.569	.538	.523	.495	.469	.854	.957

due to experimental design. More than 90% of the spores turned phase dark in both control and treated spore suspensions. A lag of more than 14 hr was required for outgrowth of treated spores compared to about 5 hr for control spores. It would only require a small percentage of spores to progress through the outgrowth stage and begin dividing to increase the turbidity as an indication of growth. Results from the germination studies indicate, then, that the catabolic processes occurring during germination initiation are not inhibited by aflatoxin but the outgrowth phase, in which anabolic mechanisms are at their maximum rates, is inhibited.

Effect of Aflatoxin on Vegetative Cell Wall Composition

It is obvious upon examination of the aberrant cell forms resulting from growth of B. megaterium in toxic TSB that there is more cell wall material per living unit

and probably less cell wall material on a dry weight basis than in shorter control cells. This can be concluded by virtue of the shape of the cells, assuming there is no difference in cell wall thickness. But there have been no reports on the composition of cell walls of these filamentous forms. For this reason it was decided that the amino acid composition of cell walls of control and treated cultures would be analyzed. Although the cell wall preparations were not particularly clean, ratios of alanine, glutamic acid, and lysine, the three amino acids making up the tetrapeptide bridge in most Gram positive bacteria, were not significantly different when chromatograms of the extracts were compared. Table 3 lists the μ Moles amino acid (lysine, glutamic acid, and alanine) per mg protein in the vegetative cell walls of control and test cells. Although amounts of the constituents were greater in cell wall preparations of the control cells,

TABLE 3.--Concentration in μ Moles per mg protein of lysine, glutamic acid, and alanine in cell walls of *Bacillus megaterium* formed in trypticase soy broth (control) and trypticase soy broth containing 3.8 μ g/ml aflatoxin B₁ (treated).

	Control (μ Moles per mg protein)	Treated (μ Moles per mg protein)
Lysine	3.31	2.73
Glutamic acid	6.37	5.25
Alanine	6.37	5.28

approximate ratios of lysine:glutamic acid:alanine in test (1:2.1:1.9) and control (1:1.9:1.9) cell walls were similar. Apparent higher concentrations of the cell wall constituents in control cells was most likely due to improper preparation and cleanup; the presence of intracellular materials would tend to alter the relative concentrations of amino acids.

The constituents N-acetylglucosamine, N-acetylmuramic acid, and diaminopimelic acid were not run as standards, however, no significantly different peaks were observed. It was concluded that aflatoxin B₁ does not affect the chemical composition of cell walls formed in its presence.

Effect of Aflatoxin on Vegetative Cell and Spore Composition

Chemical analyses performed on whole cells and spores formed in toxic and nontoxic TSB revealed different results. Table 4 lists the total protein, DNA, and RNA of control and treated cells and spores along with the total dipicolinic acid (DPA) content of spores. Analyses of whole cells revealed that the percentages of protein, DNA, and RNA on a dry weight basis were greater when the cells were grown in toxic TSB. At the same time it should be noted that total synthesis of all three components was decreased on a total culture basis. The increase in percent protein, DNA, and RNA can be explained in part by considering cell shape. Assuming the concentration of the

TABLE 4.--Per cent protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) in vegetative cells and spores and per cent dipicolinic acid (DPA) in spores of Bacillus megaterium formed in trypticase soy medium (control) and trypticase soy medium containing 3.8 µg/ml aflatoxin B₁ (treated).

	Vegetative Cells		Spores	
	Control	Treated	Control	Treated
Protein	52.6%*	73.0%	57.1%	59.4%
DNA	4.1	5.3	2.85	2.73
RNA	11.2	14.6	10.5	10.3
DPA	-	-	6.8	6.3

* Per cent of each component is on a dry weight basis.

three components on a cytoplasmic volume basis to be equal in both filamentous and short cells, then the per cent of each component on a dry weight basis would be greater in the filamentous cells due to the decrease in per cent of cell wall material. Cell wall material, of course, would contribute no DNA nor RNA, and little protein to cell mass.

A similar imbalance in macromolecular biosynthesis was reported in E. aurantiacum grown in the presence of 50 µg/ml aflatoxin B₁ (Lillehoj and Ciegler, 1967). The imbalance, particularly in DNA production, might have been responsible for the decrease in viability of multiplying B. megaterium (Lillehoj and Ciegler, 1968). The decreased total synthesis of DNA, RNA, and protein as reported by Lillehoj and Ciegler is in agreement with the data presented here.

No report was made by Lillehoj and Ciegler, however, basing the percentage of these cellular components on a dry weight basis. Percentages of all three components were found to increase over controls when calculations were made using chemical analyses data derived in this laboratory.

Age of the cells undoubtedly affects chemical composition. Older cells become granular in appearance and probably had decreased protein, DNA, and RNA composition on a dry weight basis. The differences in chemical makeup which are reported in Table 4 may not be entirely attributable to cell shape.

Chemical analyses of spores formed in toxic and non-toxic media revealed no significant differences in protein, DNA, nor RNA content. Dipicolinic acid (DPA), unique to bacterial spores but not found in vegetative cells, was also quantitated in the control and test spores and reported in Table 4. Any difference in composition may be attributed to inefficient cleaning of the test spores as cleaning was particularly difficult due to the abundance of nonsporulating cells. More cell debris in the test spore preparation would tend to decrease the DPA percentage.

Electron Microscopy of Vegetative Cells and Spores

Reports involving the use of electron microscopy for the examination of possible changes in morphology in bacterial cells and spores formed in aflatoxin-containing media have not been made. Gross examination of cells

immediately reveals the inhibitory nature of aflatoxin on vegetative cell division. However, no detectable differences could be found with an ordinary light microscope when comparing spores formed in the presence or absence of the toxin. It was decided, therefore, to analyze these cells and spores using electron microscopy to determine whether unusual organelles were formed in aflatoxin-treated cells and spores or, on the other hand, to determine if the usual organelles were similar in kind and number to the control cells and spores. Organelles of specific interest were transverse septa and mesosomes in the vegetative cells and mesosomes in spores.

Mesosomes are intracytoplasmic membranes contiguous to the cytoplasmic membrane and contain enzymes involved in terminal electron transport and may function as control points in DNA replication. Other functions attributed to mesosomes include involvement in the formation of spore septa and transverse septum formation during cell division (Hendler, 1968).

Figures 8 and 9 show electron micrographs of vegetative cells grown in nontoxic and toxic TSB, respectively. Structural components are pointed out. Two normally dividing cells are shown in Figure 8, one in the early stages of centripetal deposition of a new septum and the second in a later stage. Filamentous cells are shown in Figure 9. Poly- β -hydroxybutyric acid deposits are more

Figure 8.--Ultrathin sections of Bacillus megaterium cells formed in trypticase soy broth. Cells are in different stages of cell division. The following abbreviations are used: CW, cell wall; CM, cell membrane; PHB, poly- β -hydroxybutyric acid; S, septum; M, mesosome; and NM, nuclear material. The marker represents 1.0 μ .

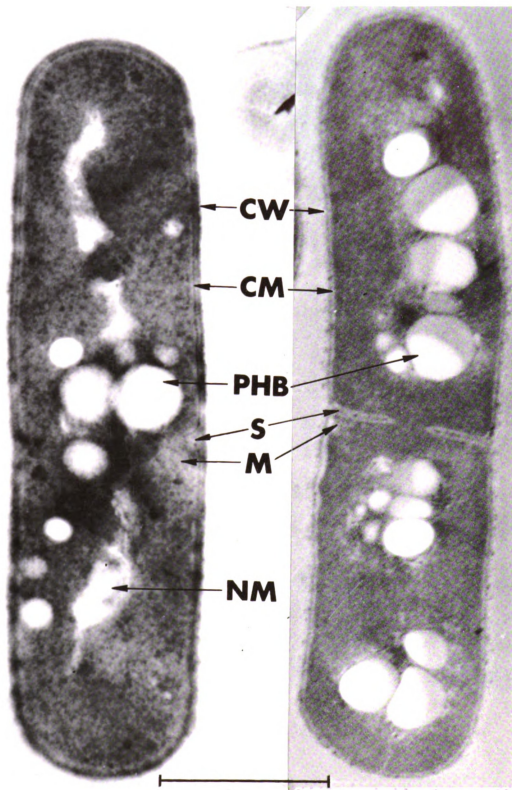
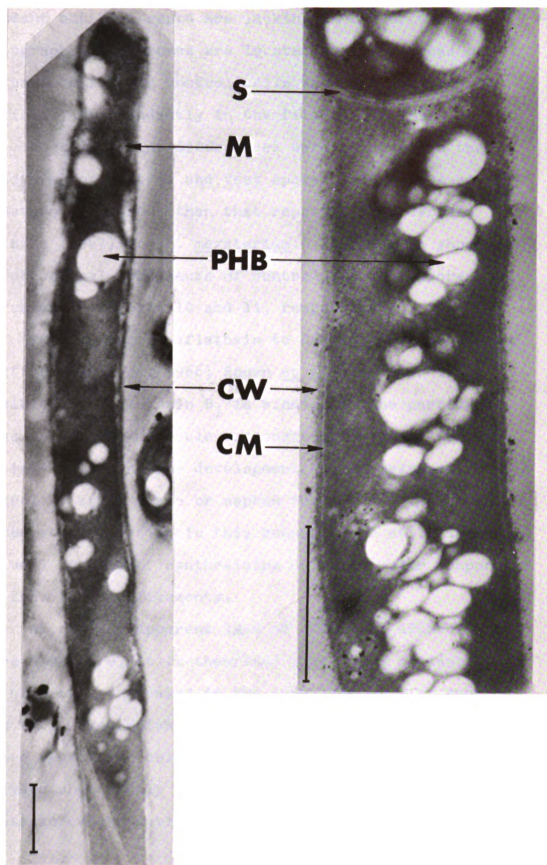


Figure 5.--Demonstration of capsular material in Bacillus megaterium cultured in (A) trypticase soy broth and (B) trypticase soy broth containing 3.8 µg/ml aflatoxin B₁. Note the striated character of the distribution of polysaccharide capsular material and the coincidence of striations with septa between dividing cells of the control culture. Few septa were observed in filaments of the treated culture. Micrographs were taken at X500.



abundant but cell septa are lacking in one of the two photographs. Mesosomes are located near the areas of septum formation in control cells but were not seen as distinctly or abundantly in the filamentous cells.

No significant differences were observed in the structures of control and test spores nor was the spore structure different than that reported by Freer and Levinson (1967) for B. megaterium. Photographs showing the structural components of control and test spores are shown in Figures 10 and 11, respectively.

The binding of aflatoxin to DNA has been documented (Clifford and Rees, 1966; Sporn et al., 1966). It is possible that aflatoxin B₁ is binding to the particular locus on the DNA molecule responsible, in part, for the initiation of mesosome development and/or mesosomal control of cross plate or septum formation. Inhibition of mesosomal function in this manner might still leave the cell capable of synthesizing other cellular components and thus to form filaments.

As for the apparent lack of effect of aflatoxin on spore structure, it is theorized that toxin concentration may have been decreased in the immediate area of sporulation to a level which may not have been inhibitory to either vegetative cell nor spore formation. However, analyses showed only a 16% reduction from the initial aflatoxin concentration in the total growth medium.

Figure 10.--Ultrathin sections of Bacillus megaterium spores formed in trypticase soy agar. The following abbreviations are used: P, protoplast; PCW, primordial cell wall; CX, cortex; ISC, inner spore coat; and OSC, outer spore coat. Markers represent 1.0 μ .

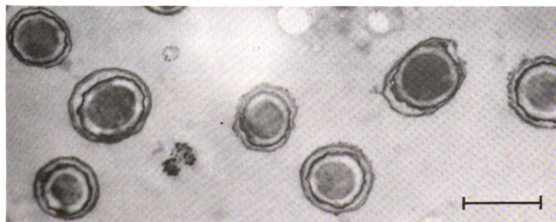
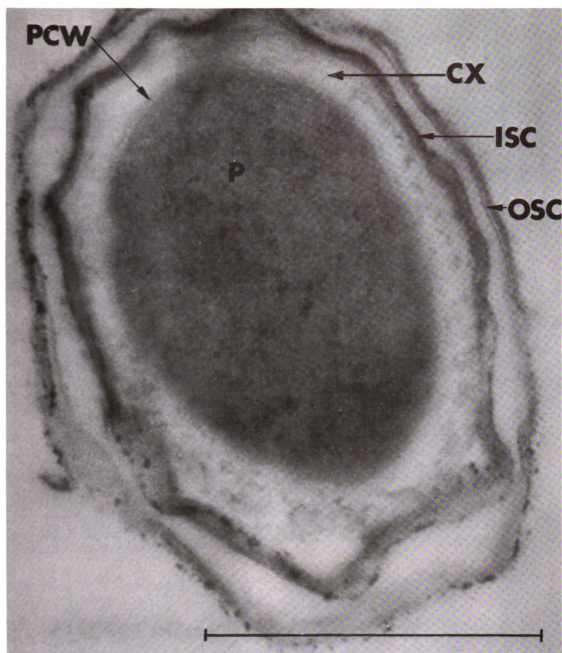
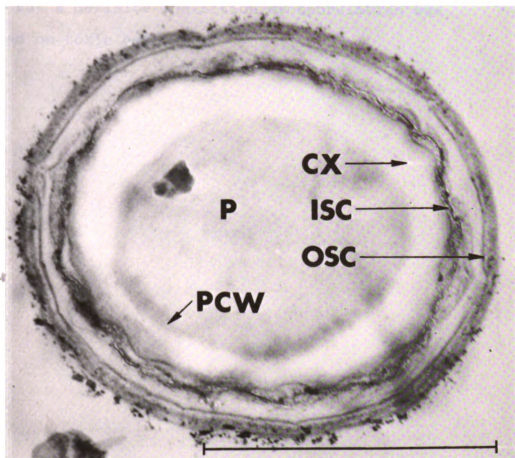


Figure 11.--Ultrathin sections of Bacillus megaterium spores formed in trypticase soy agar containing 3.8 $\mu\text{g/ml}$ aflatoxin B₁. The following abbreviations are used: P, protoplast; PCW, primordial cell wall; CX, cortex; ISC, inner spore coat; and OSC, outer spore coat. Markers represent 1.0 μ .



Chemical, heat resistance, and electron microscopy studies revealed no differences in control and test spores but a definite inhibition of sporulation was observed on toxin medium.

LITERATURE CITED

LITERATURE CITED

- Abedi, Z. H., and W. P. McKinley. 1968. Zebra fish eggs and larvae as aflatoxin bioassay test organisms. J. Assoc. Offic. Anal. Chemists. 51:902-905.
- Abedi, Z. H., and P. M. Scott. 1969. Detection of toxicity of aflatoxins, sterigmatocystin, and other fungal toxins by lethal action on zebra fish larvae. J. Assoc. Offic. Anal. Chemists. 52:963-969.
- Adamesteanu, I., and C. Adamesteanu. 1969. Aflatoxicosis in mammals and birds. Rev. Zootch. Med. Vet. 1:7-24.
- Adler, H. I., and A. A. Hardigree. 1964. Analysis of a gene controlling cell division and sensitivity to radiation in Escherichia coli. J. Bacteriol. 87:720-726.
- Adrian, J., and P. Lunven. 1969. Aflatoxins. I. Causal agents. II. Manifestations of toxicity. III. Methods of prevention and detoxication. Oleagineux. 24:31-35.
- Adye, J. C., and R. I. Mateles. 1964. Incorporation of labelled compounds into aflatoxins. Biochim. Biophys. Acta. 86:418-420.
- Allcroft, R., H. Rogers, G. Lewis, J. Nabney, and P. E. Best. 1966. Metabolism of aflatoxin in sheep: Excretion of the 'milk toxin.' Nature. 209:154-155.
- Allcroft, R., B. A. Roberts, and M. K. Loyd. 1968. Excretion of aflatoxin in a lactating cow. Food Cosmet. Toxicol. 6:612-625.
- Andrellos, P. J., and G. H. Reid. 1964. Confirmatory tests for aflatoxin B₁. J. Assoc. Offic. Anal. Chemists. 47:801-803.
- Anonymous. 1966. Alarm about aflatoxin. Nature. 212:1512.
- A.O.A.C. 1966. Nuts and nut products. J. Assoc. Offic. Anal. Chemists. 49:229-231.

- A.O.A.C. 1967. Nuts and nut products. J. Assoc. Offic. Anal. Chemists. 50:214-216.
- A.O.A.C. 1968. Nuts and nut products. J. Assoc. Offic. Anal. Chemists. 51:485-489.
- A.O.A.C. 1969. Nuts and nut products. J. Assoc. Offic. Anal. Chemists. 52:405-407.
- Arai, T., T. Ito, and Y. Koyama. 1967. Antimicrobial activity of aflatoxins. J. Bacteriol. 93:59-64.
- Arseculeratne, S. N., L. M. de Silva, S. Wyesundera, and C. H. S. R. Bandunatha. 1969. Coconut as a medium for the experimental production of aflatoxin. Appl. Microbiol. 18:88-94.
- Ambrecht, B. H., W. T. Shalkop, J. N. Geleta, and H. G. Wiseman. 1968. Swine feeding studies. Proc. 1967 Mycotoxin Res. Sem., pp. 103-108.
- Asao, T., G. Buchi, M. M. Abdel Kader, S. B. Chang, E. L. Wick, and G. N. Wogan. 1965. The structures of aflatoxins B and G₁. J. Amer. Chem. Soc. 87:882-886.
- Ashwell, G. 1957. Colorimetric analysis of sugars., pp. 73-105. In S. P. Colowick and N. O. Kaplan (eds.), Methods in Enzymology, Vol. 3. Academic Press, Inc., New York.
- Ashworth, L. J., J. McKinney, and J. L. McMeans. 1967. Seed coat and fiber constituents of cottonseeds that may influence aflatoxin analysis. J. Amer. Oil Chem. Soc. 44:394-396.
- Ashworth, L. J., J. L. McMeans, J. L. Pyle, C. M. Brown, J. W. Osgood, and R. E. Ponton. 1968. Aflatoxins in cotton seeds: Influence of weathering on toxin content of seeds and on a method for mechanically sorting seed lots. Phytopathology. 58:102-107.
- Ayres, J. C., H. S. Lillard, and D. A. Lillard. 1970. Mycotoxins: Methods for detection in foods. Food Technol. 24:161-166.
- Ayres, J. L. 1969. Studies of aflatoxin B₁ in rainbow trout (Salmo gairdnerii): Carcinogenicity and chemical structure. Tissue and subcellular distribution of carbon-14 from aflatoxin B₁-1⁴C. University Microfilms, Ann Arbor, Mich., Order No. 69-4656.

- Ayres, J. L., D. J. Lee, J. H. Wales, and R. O. Sinnhuber. 1969. Aflatoxin B₁ structure and hepatocarcinogenicity in rainbow trout. *Fed. Proc.* 22:304.
- Balasubramaniam, K., S. Wijesundera, S. N. Arseculeratne, and G. E. Tennekoon. 1969. Effect of aflatoxins on rat liver lysosomes. *Toxicon.* 7:159-161.
- Barber, R. S., R. Eraude, K. G. Mitchell, J. D. J. Harding, G. Lewis, and R. M. Loosmore. 1968. Effects of feeding toxic groundnut meal to growing pigs and its interaction with high-copper diets. *Brit. J. Nutr.* 22:535-554.
- Basappa, S. C., A. Jayaraman, V. Sreenivasamurthy, and H. A. B. Parpia. 1967. Effect of B-group vitamins and ethyl alcohol on aflatoxin production by Aspergillus oryzae. *Indian J. Exp. Biol.* 5:262-263.
- Bassir, O. 1964. The use of Nigerian foods containing cultures of toxic strains of Aspergillus flavus. *W. Afr. J. Biol. Appl. Chem.* 8:3-15.
- Bauer, D. H., D. J. Lee, and R. O. Sinnhuber. 1969. Acute toxicity of aflatoxins B₁ and G₁ in the rainbow trout (Salmo gairdnerii). *Toxicol. Appl. Pharmacol.* 15:415-419.
- Beard, R. L., and G. S. Walton. 1965. An Aspergillus toxin lethal to larvae of the house fly. *J. Invert. Pathol.* 7:522-523.
- Beckwith, A. C., and L. Stoloff. 1968. Fluorodensitometric measurement of aflatoxin thin-layer chromatograms. *J. Assoc. Offic. Anal. Chemists.* 51:602-608.
- Bilal, V. I. 1969. Aflatoxins. *Mikrobiol. Zh. (Kiev).* 31:123-128.
- Biollaz, M., G. Beuchi, and G. Milne. 1968a. Biosynthesis of aflatoxins. *J. Amer. Chem. Soc.* 90:5017-5019.
- Biollaz, M., G. Buechi, and G. Milne. 1968b. Biogenesis of bisfuranoids in the genus Aspergillus. *J. Amer. Chem. Soc.* 90:5019-5020.
- Black, H. S., and A. M. Altschul. 1965. Gibberellic acid-induced lipase and α -amylase formation and their inhibition by aflatoxin. *Biochem. Biophys. Res. Commun.* 19:661-664.
- Blount, W. P. 1961. Turkey "X" disease. *Turkeys.* 9:52, 55-58, 61, 77.

- Boesenberg, H. 1969. Aflatoxins. *Naturwissenschaften*. 56:350-352.
- Boller, R. A., and H. W. Schroeder. 1966. Aflatoxin producing potential of Aspergillus flavus-oryzae isolates from rice. *Cereal Sci. Today*. 11:342-344.
- Brechbuehler, S., G. Buechi, and G. Milne. 1967. The absolute configuration of aflatoxins. *J. Org. Chem.* 32:2641-2642.
- Brown, J. M. M., and L. Adams. 1965. Biochemical studies on aflatoxicosis. *Onderstepoort J. Vet. Res.* 32:119-145.
- Brown, R. F. 1969. Effect of some mycotoxins on the brine shrimp, Artemia salina. *J. Amer. Oil Chem. Soc.* 46:119.
- Brown, R. F., J. D. Wildman, and R. M. Epply. 1968. Temperature-dose relationships with aflatoxin on the brine shrimp, Artemia salina. *J. Assoc. Offic. Anal. Chemists*. 51:905-906.
- Buechi, G., D. M. Foulkes, M. Kurono, and G. F. Mitchell. 1966. The total synthesis of racemic aflatoxin B₁. *J. Amer. Chem. Soc.* 88:4534-4536.
- Buechi, G., D. M. Foulkes, M. Kurono, G. F. Mitchell, and R. S. Schneider. 1967. The total synthesis of racemic aflatoxin B₁. *J. Amer. Chem. Soc.* 89:6745-6753.
- Buechi, G., and S. M. Weinreb. 1969. Total synthesis of racemic aflatoxin-M₁. *J. Amer. Chem. Soc.* 91:5408-5409.
- Bullerman, L. B., and J. C. Ayres. 1968. Aflatoxin-producing potential of fungi isolated from cured and aged meats. *Appl. Microbiol.* 16:1945-1946.
- Bullerman, L. B., P. A. Hartman, and J. C. Ayres. 1969a. Aflatoxin production in meats. I. Stored meats. *Appl. Microbiol.* 18:714-717.
- Bullerman, L. B., P. A. Hartman, and J. C. Ayres. 1969b. Aflatoxin production in meats. II. Aged dry salamis and aged country cured hams. *Appl. Microbiol.* 18:718-722.
- Bullerman, L. B., P. A. Hartman, and J. C. Ayres. 1969c. Extraction and analysis of aflatoxins from cured and aged meats. *J. Assoc. Offic. Anal. Chemists*. 52:638-641.

- Burmeister, H. R. 1967. Microbiological screening process for aflatoxin. U. S. Patent No. 3,360,441.
- Burmeister, H. R., and C. W. Hesseltine. 1966. Survey of the sensitivity of microorganisms to aflatoxin. *Appl. Microbiol.* 14:403-404.
- Bushnell, D. G. 1965. The incidence of aflatoxin in the Rhodesian groundnut crop. *Rhodesia Agr. J.* 62:94-96.
- Butler, W. H. 1964. Acute toxicity of aflatoxin B₁ in rats. *Brit. J. Cancer.* 18:756-762.
- Butler, W. H., and J. M. Barnes. 1966. Carcinoma of the glandular stomach in rats given diets containing aflatoxin. *Nature.* 209:90.
- Butler, W. H., and J. S. Wigglesworth. 1966. Effects of aflatoxin B₁ on the pregnant rat. *Brit. J. Exptl. Pathol.* 47:242-247.
- Carnaghan, R. B. A., R. D. Hartley, and J. O'Kelly. 1963. Toxicity and fluorescence properties of the aflatoxins. *Nature.* 200:1101.
- Carnaghan, R. B. A., C. N. Herbst, D. S. P. Patterson, and D. Sweasy. 1967. Comparative biological and biochemical studies in hybrid chicks. II. Susceptibility to aflatoxin and effects on serum protein constituents. *Brit. Poultry Sci.* 8:279-284.
- Childs, V. A., and M. S. Legator. 1966. Induction of thymidine kinase by aflatoxin. *Life Sci.* 5:1053-1056.
- Chang, C. M., and J. Q. Lynd. 1968. Ultraviolet aflatoxin quantitation with Polaroid recordings. *Agron. J.* 60:582-584.
- Chang, S. B., M. M. Abdel Kader, E. L. Wick, and G. N. Wogan. 1963. Aflatoxin B₂: Chemical identity and biological activity. *Science.* 142:1191-1192.
- Chen, S. C., and L. Friedman. 1966. Aflatoxin determination in seed meal. *J. Assoc. Offic. Anal. Chemists.* 49:28-33.
- Chong, Y. H. 1966. Aflatoxins in groundnuts and groundnut products. *Far East Med. J.* 2:228-230.
- Chong, Y. H., and C. G. Beng. 1965. Aflatoxins in unrefined groundnut oil. *Med. J. Malaya.* 20:49-50.

- Chong, Y. H., C. G. Beng, J. T. Ponnampalam, and R. K. H. Lim. 1966. Moulded soy beans as a potential source of aflatoxin contamination. *Far East Med. J.* 2:298-300.
- Chong, Y. H., and J. T. Ponnampalam. 1967. The effect on ducklings of a diet containing moulded-soybeans. *Med. J. Malaya.* 22:104-109.
- Christensen, C. M., H. A. Fanse, G. H. Nelson, F. Bates, and C. J. Mirocha. 1967. Microflora of black and red pepper. *Appl. Microbiol.* 15:622-626.
- Ciegler, A., E. B. Lillehoj, R. E. Peterson, and H. H. Hall. 1966. Microbial detoxification of aflatoxin. *Appl. Microbiol.* 14:934-939.
- Ciegler, A., R. E. Peterson, A. A. Lagoda, and H. H. Hall. 1966. Aflatoxin production and degradation by Aspergillus flavus in 20-liter fermentors. *Appl. Microbiol.* 14:826-833.
- Clark, J. M. (ed.). 1964. Experimental Biochemistry, pp. 125-127, 132. W. H. Freeman and Company, San Francisco.
- Clements, N. L. 1968a. Note on a microbiological assay for aflatoxin B₁: A rapid confirmatory test by effects on growth of Bacillus megaterium. *J. Assoc. Offic. Anal. Chemists.* 51:611-612.
- Clements, N. L. 1968b. Rapid confirmatory test for aflatoxin B₁, using Bacillus megaterium. *J. Assoc. Offic. Anal. Chemists.* 51:1192-1194.
- Clifford, J. I., and K. R. Rees. 1967a. The action of aflatoxin B₁ on the rat liver. *Biochem J.* 102:65-75.
- Clifford, J. I., and K. R. Rees. 1967b. The interaction of aflatoxins with purines and purine nucleosides. *Biochem. J.* 103:467-471.
- Clifford, J. I., K. R. Rees, and M. E. M. Stevens. 1967. The effect of aflatoxin B₁, G₁, and G₂ on protein and nucleic acid synthesis in rat liver. *Biochem. J.* 103:258-261.
- Coomes, T. J., P. C. Crowther, A. J. Feuell, and B. J. Francis. 1966. Experimental detoxification of groundnut meals containing aflatoxin. *Nature.* 209:406-407.

- Coomes, T. J., and J. C. Sanders. 1963. The detection and estimation of aflatoxin in groundnuts and groundnut materials. Part I. Paper-chromatographic procedure. *The Analyst*. 88:209-213.
- Cottler, G. J., C. H. Moore, U. L. Diener, and N. D. Davis. 1968. Effect of continuous feeding of aflatoxin to chickens. *Highlights Agric. Res.* 15:7.
- Crisan, E. V. 1968. A 2,4-dinitrophenylhydrazine spray for the identification of aflatoxin B₁ on thin-layer chromatoplates. *Contrib. Boyce Thompson Inst.* 24:37-38.
- Crisan, E. V., and A. T. Grefig. 1967. The formation of aflatoxin derivatives. *Contrib. Boyce Thompson Inst.* 24:3-8.
- Czarnowski, A., W. Czarnowska, and Z. Pawlak. 1969. Toxicity of poultry feed containing aflatoxin. *Med. Vet.* 24:215-217.
- Datta, P. R., and R. J. Gajan. 1965. Plasma protein index of aflatoxin-fed ducklings. *Life Sci.* 4:1791-1795.
- Davis, N. D., and U. L. Diener. 1968. Growth and aflatoxin production by Aspergillus parasiticus from various carbon sources. *Appl. Microbiol.* 16:158-159.
- Davis, N. D., U. L. Diener, and V. P. Agnihotri. 1967. Production of aflatoxins B₁ and G₁ in chemically defined medium. *Mycopathologia et Mycologia Applicata*. 31:251-256.
- Denault, L. J., and L. A. Underkofler. 1967. A method for the detection and estimation of aflatoxin in fungal fermentation products. *Cereal Chem.* 44:1-7.
- Detroy, R. W., and C. W. Hesseltine. 1968. Isolation and biological activity of a microbial conversion product of aflatoxin B₁. *Nature*. 219:5157.
- Detroy, R. W., and C. W. Hesseltine. 1969. Transformation of aflatoxin B₁ by steroid-hydroxylating fungi. *Can. J. Microbiol.* 15:495-500.
- Dickens, J. W. 1968. Survey of aflatoxin in farmers' stock peanuts marketed in North Carolina during 1964-66. *Proc. 1967 Mycotoxin Res. Sem.*, pp. 5-7.

- Dickens, J. W., and H. E. Pattee. 1966. The effects of time, temperature, and moisture on aflatoxin production in peanuts inoculated with a toxic strain of Aspergillus flavus. Trop. Sci. 8:11-22.
- Dickens, J. W., and J. B. Satterwhite. 1969. Subsampling mill for peanut kernels. Food Technol. 23:90-92.
- Diener, U. L., and N. D. Davis. 1966. Aflatoxin production by isolates of Aspergillus flavus. Phytopathology. 56:1390-1393.
- Diener, U. L., and N. D. Davis. 1967. Limiting temperature and relative humidity for growth and production of aflatoxin and free fatty acids by Aspergillus flavus in sterile peanuts. J. Amer. Oil Chem. Soc. 44:259-263.
- Dollear, F. G., G. E. Mann, L. P. Codifer, H. K. Gardner, S. P. Koltun, and H. L. E. Vix. 1968. Elimination of aflatoxins from peanut meal. J. Amer. Oil Chem. Soc. 45:862-865.
- Donkersloot, J. A., D. P. H. Hsieh, and R. I. Mateles. 1968. Incorporation of precursors into aflatoxin-B₁. J. Amer. Chem. Soc. 90:5020-5021.
- Dutton, M. F., and J. G. Heathcote. 1966. Two new hydroxyaflatoxins. Biochem. J. 101:21-22P.
- Dutton, M. F., and J. G. Heathcote. 1968. The structure, biochemical properties and origin of the aflatoxins B_{2a} and G_{2a}. Chem. and Ind., pp. 418-421.
- Dwarakanath, C. T., E. T. Rayner, G. E. Mann, and F. G. Dollear. 1968. Reduction of aflatoxin levels in cottonseed and peanut meals by ozonization. J. Amer. Oil Chem. Soc. 45:93-95.
- El-Khadem, M., G. Menke, and F. Grossmann. 1966. Peanut seedling damage by aflatoxins. Naturwissenschaftler. 53:532.
- Engstrom, G. W. 1969. Solvent systems for thin-layer chromatography of aflatoxins. J. Chromatogr. 44:128-132.
- Eppley, R. M. 1968. Screening method for zearalenone, aflatoxin, and ochratoxin. J. Assoc. Offic. Anal. Chemists. 37:74-78.

- Eppley, R. M. 1969. Use of microslides for the rapid thin layer chromatography of aflatoxins. J. Assoc. Offic. Anal. Chemists. 52:311.
- Epstein, S. M., B. Bartus, and E. Farber. 1969. Renal epithelial neoplasms induced in male Wistar rats by oral aflatoxin B₁. 29:1045-1050.
- Fischbach, H., and A. D. Campbell. 1965. Note on detoxification of the aflatoxins. J. Assoc. Offic. Anal. Chemists. 48:28.
- Floyd, L. R., T. Unuma, and H. Busch. 1968. Effects of aflatoxin B₁ and other carcinogens on nucleolar RNA of various tissues in the rat. Exp. Cell Res. 51:423-438.
- Foy, H., T. Gillman, A. Kondi, and J. K. Preston. 1966. Hepatic injuries in riboflavine and pyridoxine deficient baboons. Possible relations to aflatoxin induced hepatic cirrhosis and carcinoma in Africa. Nature. 212:150-153.
- Frank, H. K. 1968. Toxic molds on fruit products. Lebesm.-Wiss. Technol. 1:14-18.
- Frank, H. K., and W. Eyrich. 1968. Detection of aflatoxins and occurrence of aflatoxin-simulating substances in food. Z. Lebensm.-Unters. Forsch. 138:1-11.
- Freer, J. H., and H. S. Levinson. 1967. Fine structure of Bacillus megaterium during microcycle sporogenesis. J. Bacteriol. 94:441-457.
- Gabliks, J., W. Schaeffer, L. Friedman, and G. Wogan. 1965. Effect of aflatoxin B₁ on cell cultures. J. Bacteriol. 90:720-723.
- Gardner, H. K., S. P. Koltun, and H. L. E. Vix. 1968. Solvent extraction of aflatoxins from oilseed meals. J. Agric. Food Chem. 16:990-993.
- Gelboin, H. V., J. S. Wortham, R. G. Wilson, M. Friedman, and G. N. Wogan. 1966. Rapid and marked inhibition of rat liver RNA polymerase by aflatoxin B₁. Science. 154:1205-1206.
- Goldblatt, L. A. (ed.). 1969. Aflatoxin. 472 pp. Academic Press, New York.
- Gornall, G., G. Allen, C. J. Bardowill, and M. M. David. 1949. Determination of serum proteins by means of the Biuret reaction. J. Biol. Chem. 177:751-766.

- Green, S. 1968. Antineoplastic activity of aflatoxin B₁. *Nature*. 220:931-932.
- Grula, M. M., and E. A. Grula. 1964. Cell division in a species of Erwinia. IV. Metabolic blocks in pantothenate biosynthesis and their relationship to inhibition of cell division. *J. Bacteriol.* 83:989-997.
- Gudauskas, R. T., N. D. Davis, and U. L. Diener. 1967. Sensitivity of Heliothis virescens larvae to aflatoxin in ad libitum feeding. *J. Invert. Pathol.* 9:132-133.
- Halver, J. E., L. M. Ashley, and G. N. Wogan. 1966. Acute aflatoxicosis in rainbow trout and Coho salmon. *Fed. Proc.* 25:662.
- Hansen, E., and G. Hagedorn. 1969. Studies of aflatoxin B₁ and its behavior during food processing. *Z. Lebensmittel-Untersuch. u.-Forsch.* 141:129-145.
- Hartley, R. D., B. F. Nesbitt, and J. O'Kelly. 1963. Toxic metabolites of Aspergillus flavus. *Nature*. 198:1056-1058.
- Heathcote, J. G., J. J. Child, and M. F. Dutton. 1965. The possible role of kojic acid in the production of aflatoxin by Aspergillus flavus. *Biochem. J.* 95:23p.
- Hendler, R. W. (ed.). 1968. Protein Biosynthesis and Membrane Biochemistry. 344 pp. John Wiley and Sons, Inc., New York.
- Hintz, H. F., A. N. Booth, A. F. Cucullu, H. K. Gardner, and H. Heitman. 1967. Aflatoxin toxicity in swine. *Proc. Soc. Exp. Biol. Med.* 124:266-268.
- Hodges, F. A., J. R. Zust, H. R. Smith, A. A. Nelson, B. H. Armbricht, and A. D. Campbell. 1964. Mycotoxins: Aflatoxin isolated from Penicillium puberulum. *Science*. 145:1439.
- Holaday, C. E. 1968. Rapid method for detecting aflatoxins in peanuts. *J. Amer. Oil Chem. Soc.* 45:680-682.
- Holaday, C. E., and R. A. Passwater. 1969. Millicolumn chromatography and fluorometry. *Fluorescence News*. 4:1-2.
- Holzappel, C. W., P. S. Steyn, and I. F. H. Purchase. 1966. Isolation and structure of aflatoxins M₁ and M₂. *Tetrahedron Lett.* 25:2799-2803.

- Howell, R. W. 1968. The effects of crop production factors on occurrence of Aspergillus flavus in soybeans. Proc. 1967 Mycotoxin Res. Sem., pp. 10-11.
- Ikawa, M., D. S. Ma, G. B. Meeker, and R. P. Davis. 1969. Use of Chlorella in mycotoxin and phycotoxin research. J. Agric. Food Chem. 17:425-429.
- Ikawa, M., E. J. Herbst, G. L. Briand, and S. M. Daniel. 1968. Studies on bioassay methods for mycotoxins and related compounds. Proc. 1967 Mycotoxin Res. Sem., pp. 41-46.
- de Jongh, H., R. O. Vles, and J. G. van Pelt. 1964. Milk of mammals fed an aflatoxin-containing diet. Nature. 202:466-467.
- Jacobson, W. C., W. C. Harmeyer, and H. G. Wiseman. 1969. Determination of aflatoxin B₁ and M₁ in milk. J. Dairy Sci. 52:881.
- Janssen, F. W., A. J. Lund, and L. E. Anderson. 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science. 127:26-27.
- Jayaraman, A., E. J. Herbst, and M. Ikawa. 1968. The bioassay of aflatoxins and related substances with Bacillus megaterium spores and chick embryos. J. Amer. Oil Chem. Soc. 45:700-702.
- Jayaraman, A., V. Sreenivasamurthy, and H. A. B. Parpia. 1965. Extraction of aflatoxin from peanut products by dialysis. J. Assoc. Offic. Anal. Chemists. 48:1256-1260.
- Jemmali, M. 1969. Inhibition of a virulent bacteriophage by aflatoxin B₁. Ann. Inst. Pasteur. 116:288-291.
- Jemmali, M., J. Poisson, A. Guilbot, and F. Lagrandeur. 1969. Aflatoxin production in cereal products. Effect of various conditions. Ann. Nutr. Aliment. 23:151-166.
- Joffe, A. Z. 1969. Aflatoxin produced by 1,626 isolates of Aspergillus flavus from groundnut kernels and soils in Israel. Nature. 221:492.
- Joffe, A. Z., and N. Lister. 1969. Effects of light, temperature, and pH value on aflatoxin production in vitro. Appl. Microbiol. 18:517-518.

- Johnson, R. M., W. T. Greenaway, and C. Golumbic. 1969. Sampling stored corn for aflatoxin assay. *Cereal Sci. Today*. 14:25-29.
- Jones, H. C., H. S. Black, and A. M. Altschul. 1967. Comparison of the effects of gibberellic acid and aflatoxins in germinating seeds. *Nature*. 214: 171-172.
- Joner, N. R., and B. D. Jones. 1969. Aflatoxins. *Voeding*. 30:330-336.
- Juhasz, S., and I. Nemeth. 1968. Electrophoretic study of the serum proteins in aflatoxin-poisoned young ducks. *Kiserl. Orvostud.* 20:642-650.
- Juszkiewicz, T., and B. Stefaniakowa. 1969. Identification and quantitative determination of aflatoxin in peanut, *Arachis hypogoea*, and in mash and feed mixtures. *Med. Wet.* 25:110-114.
- Kabat, E. A., and M. M. Mayer. 1948. *Experimental Immunochemistry*, pp. 282-291. Charles C. Thomas, Springfield, Illinois.
- Kato, R., K. Onoda, and Y. Omori. 1969. Effect of aflatoxin B₁ on the incorporation of acetate-¹⁴C into cholesterol¹ by rat liver. *Experientia*. 25:1026.
- King, A. M. Q., and B. H. Nicholson. 1967. Effect of aflatoxin B₁ on a deoxyribonucleic acid-dependent ribonucleic acid polymerase in vitro. *Biochem. J.* 104:69-70p.
- King, A. M. Q., and B. H. Nicholson. 1969. Interaction of aflatoxin B₁ with polynucleotides and its effect on ribonucleic acid polymerase. *Biochem. J.* 114:679-687.
- Kiyoshi, T., and K. Miyaki. 1968. New bioassay method for aflatoxins, using cultured chick embryo liver cells. *Shokuhin Eiseigaku Zasshi*. 9:285-288.
- Kratzer, F. H., D. Bandy, M. Wiley, and A. N. Booth. 1969. Aflatoxin effects in poultry. *Proc. Soc. Exp. Biol. Med.* 131:1281-1284.
- Kuehnert, M., and H. J. Buchheim. 1968. Proposal for the use of thin-layer chromatography to determine mycotoxins (mainly aflatoxins) in germinated malt feeds. *Monatsh. Veterinaarmed.* 23:887-890.

- Kulik, M. M., and C. E. Holaday. 1966. Aflatoxin: A metabolic product of several fungi. *Mycopathol. Mycol. Appl.* 30:137-140.
- Kurata, H., H. Tanabe, K. Kanota, A. Udagawa, and M. Ichinoe. 1968. Studies on the population of toxigenic fungi in foodstuffs. IV. Aflatoxin producing fungi isolated from foodstuffs in Japan. *J. Food Hyg. Soc. Japan.* 9:29-34.
- Kurata, H., S. Udagawa, M. Ichinoe, Y. Kawasaki, M. Takada, M. Tazawa, A. Koizumi, and H. Tanabe. 1968. Studies on the population of toxigenic fungi in foodstuffs. III. Mycoflora in milled rice harvested in 1965. *J. Food Hyg. Soc. Japan.* 9:23-28.
- Lafarge, C., C. Frayssinet, and A. M. de Recondo. 1965. Inhibition of liver RNA synthesis by aflatoxin in rats. *Bull. Soc. Chim. Biol.* 47:1724-1725.
- Lafarge, C., C. Frayssinet, and R. Simard. 1966. Preferential inhibition of nucleolar RNA synthesis by aflatoxin in rat liver cells. *C. R. Acad. Sci., Paris, Ser. D.* 263:1011-1014.
- Landers, K. E., N. D. Davis, and U. L. Diener. 1967. Influence of atmospheric gases on aflatoxin production by Aspergillus flavus in peanuts. *Phytopathology.* 57:1086-1090.
- Lee, H. T., and K. H. Ling. 1967. Application of polyamide-impregnated film thin layer chromatography for the detection of aflatoxins. *T'ai-Wan I Hsueh Hui Tsa Chih.* 66:92-100.
- Lee, L. S., A. F. Cucullu, A. O. Franz, and W. A. Pons. 1969. Destruction of aflatoxins in peanuts during dry and oil roasting. *J. Agric. Food Chem.* 17:451-453.
- Legator, M. S. 1966. Biological effects of aflatoxin in cell culture. *Bacteriol. Rev.* 30:471-477.
- Legator, M. S., S. M. Zuffante, and A. R. Harp. 1965. Aflatoxin: Effect on cultured heteroploid human embryonic lung cells. *Nature.* 208:345-347.
- Lemieszek-Chodorowska, K., A. Snyckerski, W. Hryszczyńska, Z. Pawlak, H. Biskupek, S. Machowski, T. Rosiak, and J. Grzesiak. 1969. Preliminary assays of some cereal products for the presence of aflatoxin B₁. *Rocz. Panstw. Zakl. Hig.* 20:173-176.

- Levi, C. P., and E. Borker. 1968. Survey of green coffee for potential aflatoxin contamination. J. Assoc. Offic. Anal. Chemists. 51:600-602.
- Lie, J. L., and E. H. Marth. 1967. Formation of aflatoxin in Cheddar cheese by Aspergillus flavus and Aspergillus parasiticus. J. Dairy Sci. 50:1708-1710.
- Lie, J. L., and E. H. Marth. 1968. Aflatoxin formation by Aspergillus flavus and Aspergillus parasiticus in a casein substrate at different pH values. J. Dairy Sci. 51:1743-1747.
- Lillard, H. S., R. T. Hanlin, and D. A. Lillard. 1970. Aflatoxigenic isolates of Aspergillus flavus from pecans. Appl. Microbiol. 19:128-130.
- Lillehoj, E. B., and A. Ciegler. 1967. Inhibition of deoxyribonucleic acid synthesis in Flavobacterium aurantiacum by aflatoxin B₁. J. Bacteriol. 94: 787-788.
- Lillehoj, E. B., and A. Ciegler. 1968. Aflatoxin B₁ binding and toxic effects on Bacillus megaterium. J. Gen. Microbiol. 54:185-194.
- Lillehoj, E. B., and A. Ciegler. 1969a. Aflatoxin B₁ inhibition of macromolecular synthesis in Bacillus cereus. Bacteriol. Proc., p. 12.
- Lillehoj, E. B., and A. Ciegler. 1969b. Biological activity of aflatoxin B_{2a}. Appl. Microbiol. 17: 516-517.
- Lillehoj, E. B., A. Ciegler, and H. H. Hall. 1967a. Aflatoxin B₁ uptake by Flavobacterium aurantiacum and resulting toxic effects. J. Bacteriol. 93: 464-471.
- Lillehoj, E. B., A. Ciegler, and H. H. Hall. 1967b. Aflatoxin G₁ uptake by cells of Flavobacterium aurantiacum. Can. J. Microbiol. 13:629-633.
- Lillehoj, E. B., A. Ciegler, and H. H. Hall. 1967c. Fungistatic action of aflatoxin B₁. Experientia. 23:187-188.
- Lilly, L. L. 1965. Induction of chromosome aberrations by aflatoxin. Nature. 207:433-434.

- Ling, K. H., C. M. Tung, I. F. Sheh, J. J. Wang, and T. G. Tung. 1968. Aflatoxin B₁ in unrefined peanut oil and peanut products in Taiwan. T'ai-Was I Hsueh Hui Tia Chih. 67:309-314.
- Lopez, A., and M. A. Crawford. 1967. Aflatoxin content of groundnuts sold for human consumption in Uganda. Lancet., p. 1351.
- Love, T. D. 1968. Relation of temperature, time, and moisture to the production of aflatoxin in fish meal. Fishery Ind. Res. 4:139-142.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
- Madhavan, T. V. 1967. Effect of prednisolone on liver damage in rats induced by aflatoxin. J. Pathol. Bacteriol. 93:443-447.
- Madhavan, T. V., P. G. Tulpule, and C. Gopalan. 1965. Aflatoxin-induced hepatic fibrosis in rhesus monkeys. Arch. Pathol. 79:466-469.
- Manabe, M., S. Matsuura, and M. Nakano. 1968. Fluorescent compounds in fermented foods. I. Chloroform-soluble fluorescent compounds produced by aspergilli. Nippon Shokuhin Kogyo Gakkaï Shi. 15:341-346.
- Mann, G. E., L. P. Codifer, and F. G. Dollear. 1967. Effect of heat on aflatoxins in oilseed meals. J. Agric. Food Chem. 15:1090-1092.
- Marsh, P. B., M. E. Simpson, R. J. Ferretti, G. V. Merola, J. Donoso, G. O. Craig, M. W. Trucksess, and P. S. Work. 1969. Mechanism of formation of fluorescence in cotton fiber associated with aflatoxins in the seeds at harvest. J. Agric. Food Chem. 17:468-472.
- Masri, M. S., R. E. Lundin, J. R. Page, and V. C. Garcia. 1967. Crystalline aflatoxin M₁ from urine and milk. Nature. 215:753-755.
- Masri, M. S., J. R. Page, and V. C. Garcia. 1969. Modification of method for aflatoxins in milk. J. Assoc. Offic. Anal. Chemists. 52:641-643.
- Masri, M. S., H. L. E. Vix, and L. A. Goldblatt. 1969. Detoxifying foods or feeds contaminated with aflatoxins. U. S. 3,429,709 (Cl. 99-2; A 23kb), 25 Feb. 1969, Appl. 08 Oct. 1965; 4 pp.

- Mateles, R. I., and J. C. Adye. 1965. Production of aflatoxins in submerged culture. *Appl. Microbiol.* 13:208-211.
- Matsumura, F., and S. G. Knight. 1967. Toxicity and chemosterilizing activity of aflatoxin against insects. *J. Econ. Entomol.* 60:871-872.
- Mayer, A. M., A. Poljakoff-Mayber, P. Robinson, and I. Slowatizky. 1969. Simple bioassay for detection of aflatoxin in milk. *Toxicon.* 7:13-14.
- Mayne, R. Y., W. A. Pons, A. O. Franz, Jr., and L. A. Goldblatt. 1966. Elaboration of aflatoxin on cottonseed products by Aspergillus flavus. *J. Amer. Oil Chem. Soc.* 43:251-253.
- Mayura, K., and V. Sreenivasamurthy. 1969. Quantitative method for the estimation of aflatoxins in peanuts and peanut products. *J. Assoc. Offic. Anal. Chemists.* 52:77-81.
- McMeans, J. L., L. J. Ashworth, and W. A. Pons. 1968. Aflatoxins in hull and meats of cottonseed. *J. Amer. Oil Chem. Soc.* 45:575-576.
- Mishra, S. K., and H. S. R. Murthy. 1968. An extra fungal source of aflatoxins. *Current Sci.* 37:406.
- Mislivec, P. B., J. H. Hunter, and J. Tuite. 1968. Assay for aflatoxin production by the genera Aspergillus and Penicillium. *Appl. Microbiol.* 16:1053-1055.
- Miyaka, K., K. Aivara, and T. Muira. 1966. Resistance of aflatoxin to chemical and biological changes by gamma-irradiation. *Microbiol. Probl. Food Preserv. Irradiat.; Rep. Panel, Vienna.*, pp. 57-64.
- Monneron, A. 1969. Experimental induction of helical polysomes in adult rat liver. *Lab. Invest.* 20:178-183.
- Monneron, A., C. Lafarge, and C. Frayssinet. 1968. Induction by aflatoxin and by lasiocarpine of the accumulation of perichromatin granules in rat liver. *C. R. Acad. Sci., Paris, Ser. D.* 267:2053-2056.
- Moody, D. P. 1964. Biogenetic hypothesis of aflatoxin. *Nature.* 202:188.

- Moore, S., D. H. Speckman, and W. H. Stein. 1958. Chromatography of amino acids on sulfonated polystyrene resins. *Anal. Chem.* 30:1185.
- Moule, Y., and C. Frayssinet. 1968. Effect of aflatoxin on transcription in liver cell. *Nature.* 218:93-95.
- Murakami, H., H. Sagawa, and S. Takase. 1968. Nonproductivity of aflatoxin by Japanese industrial strains of Aspergillus. III. Common characteristics of the aflatoxin-producing strains. *J. Gen. Appl. Microbiol.* 14:251-262.
- Murakami, H., S. Takase, and T. Ishii. 1967. Nonproductivity of aflatoxin by Japanese industrial strains of Aspergillus. I. Production of fluorescent substances in agar slant and shake cultures. *J. Gen. Appl. Microbiol.* 13:323-334.
- Murakami, H., S. Takase, and K. Kuwabara. 1968. Nonproductivity of aflatoxin by Japanese industrial strains of Aspergillus. II. Production of fluorescent substances in rice koji, and their identification by absorption spectrum. *J. Gen. Appl. Microbiol.* 14:97-110.
- Murrell, W. G. 1961. Spore formation and germination as a microbial reaction to the environment. *Symp. Soc. Gen. Microbiol.* No. XI., pp. 100-150.
- Nabney, J., M. B. Burbage, R. Allcroft, and G. Lewis. 1967. Metabolism of aflatoxin in sheep: Extraction pattern in the lactating ewe. *Food Cosmet. Toxicol.* 5:11-17.
- Nartey, F. 1966. Aflatoxins of Aspergillus flavus grown on cassava. *Physiol. Plantarum.* 19:818-822.
- Nemeth, I., and S. Juhasz. 1968. Effect of aflatoxin on serum protein fractions of dayold ducklings. *Acta. Vet. Acad. Sci. Hung.* 18:95-105.
- Nesheim, S. 1964. Mycotoxins: Studies of the rapid procedure for aflatoxins in peanuts, peanut meal, and peanut butter. *J. Assoc. Offic. Anal. Chemists.* 47:1010-1017.
- Nesheim, S. 1969. Conditions and techniques for thin layer chromatography of aflatoxins. *J. Amer. Oil Chem. Soc.* 46:335-338.
- Nesterin, M. F., and V. Ya. Vissarionova. 1969. Aflatoxins. *Prikl. Biokhim. Mikrobiol.* 5:121-126.

- Newberne, P. M., C. E. Hunt, and G. N. Wogan. 1967. Neoplasms in the rat associated with administration of urethan and aflatoxin. *Exp. Mol. Pathol.* 6: 285-299.
- Newberne, P. M., and G. Williams. 1969. Inhibition of aflatoxin carcinogenesis by diethylstilbestrol in male rats. *Arch. Environ. Health.* 19:489-498.
- Newberne, P. M., G. N. Wogan, W. W. Caroton, and M. M. Abdel Kader. 1964. Histopathologic lesions in ducklings caused by Aspergillus flavus cultures, culture extracts, and crystalline aflatoxins. *Toxicol. Appl. Pharmacol.* 6:542-556.
- Nose, K., and K. Ishibashi. 1967. Absence of aflatoxins in Takadiastase. *Sankyo Kenkyusho Nempo.* 19:74-80.
- Oettle, A. G. 1965. The aetiology of the liver in Africa: A critical appraisal of previous ideas with an outline of the mycotoxin hypothesis. *S. Afr. Med. J.* 39:817-824.
- Osiyemi, F. O., E. A. Bababunmi, and O. Bassir. 1967. Aspergillus flavus and its metabolites. I. Production of aflatoxin in cultures of Aspergillus flavus. *W. Afr. Biol. Appl. Chem.* 9:31-34.
- Pattee, H. E. 1969. Production of aflatoxins by Aspergillus flavus cultured on flue-cured tobacco. *Appl. Microbiol.* 18:952-953.
- Pattee, H. E., S. L. Sessoms, and J. W. Dickens. 1966. Influence of biologically modified atmospheres on aflatoxin production by Aspergillus flavus growing on peanut kernels. *Oleagineux.* 21:747-748.
- Pattinson, I., P. Crowther, and H. E. Noubey. 1968. The separation of aflatoxin infected groundnut kernels. *Trop. Sci.* 10:212-221.
- Peterson, R. E., and A. Ciegler. 1967. Separation of aflatoxins by two-dimensional thin layer chromatography. *J. Chromatog.* 31:250-251.
- Phelps, R. A. 1965. Occurrence of mold toxins in fish food materials. Trout Hepatoma Conf., March 18-19.
- Pier, A. C., S. J. Cysewski, G. W. Engstrom, J. L. Richard, R. W. Dougherty, and J. R. Thurston. 1968. The clinical pathology of acute aflatoxicosis of swine. (Summary). *Proc. 1967 Mycotoxin Res. Sem.*, pp. 102-103.

- Platonow, N. 1964. Effect of prolonged feeding of toxic groundnut meal in mice. *Vet. Rec.* 76:589.
- Pohland, A. E., M. E. Cushmac, and P. J. Andrellos. 1968. Aflatoxin B₁ hemiacetal. *J. Assoc. Offic. Anal. Chemists.* 51:907-910.
- Pons, W. A. Jr., A. F. Cucullu, A. O. Franz, and L. A. Goldblatt. 1968. Improved objective fluorodensitometric determination of aflatoxins in cottonseed products. *J. Amer. Oil Chem. Soc.* 45:694-699.
- Pons, W. A. Jr., A. F. Cucullu, L. S. Lee, J. A. Robertson, A. O. Franz, and L. A. Goldblatt. 1966. Determination of aflatoxins in agricultural products: Use of aqueous acetone for extraction. *J. Amer. Oil Chem. Soc.* 49:554-562.
- Pons, W. A. Jr., J. A. Robertson, and L. A. Goldblatt. 1966. Objective fluorometric measurement of aflatoxins on TLC plates. *J. Amer. Oil Chem. Soc.* 43:665-669.
- Poonoose, M. C. 1969. Aflatoxin G₁ hemiacetal. *Current Sci.* 38:364-365.
- Purchase, I. F. H. 1967. Fungal metabolites as potential carcinogens, with particular reference to their role in the aetiology of hepatoma. *S. Afr. Med. J.* 41:406-413.
- Purchase, I. F. H., and M. Steyn. 1967. Estimation of aflatoxin M in milk. *J. Assoc. Offic. Anal. Chemists.* 50:363-364.
- Purchase, I. F. H., and L. J. Vorster. 1968. Aflatoxin in commercial milk samples. *S. Afr. Med. J.* 42:219.
- Rabie, C. J., and E. B. Smalley. 1965. Influence of temperature on the production of aflatoxin by Aspergillus flavus. *Symp. Mycotoxins in Feedstuffs, Agric. Aspects, Pretoria.*, pp. 18-29.
- Raj, H. G., L. Viswanathan, and H. S. R. Murthy. 1969. Biosynthesis of aflatoxins by cell-free preparations from Aspergillus flavus. *Experientia.* 25:1141-1142.
- Rao, K. S., and P. G. Tulpule. 1967. Varietal differences of groundnut in the production of aflatoxin. *Nature.* 214:738-739.

- Rao, V. N. V., K. Valmikinathan, and N. Verghese. 1968. Binding of aflatoxin by plasma albumin in vitro. Biochim. Biophys. Acta. 165:288-289.
- Raper, K. B., and D. I. Fennell. 1965. The Genus Aspergillus, pp. 357-404. The Williams and Wilkins Co., Baltimore.
- Rehm, H. J., and I. Schmidt. 1969. Production of aflatoxins in butter and margarine. Zeitschrift Lebensmitteluntersuchung Forsch. 140:164-165.
- Roberts, B. A., and R. Allcroft. 1968. Semiquantitative estimation of aflatoxin M₁ in liquid milk by thin-layer chromatography. Food Cosmet. Toxicol. 6:339-340.
- Roberts, J. C., A. H. Sheppard, J. A. Knight, and P. Roffey. 1968. Mycological chemistry. XXII. Total synthesis of (±)-aflatoxin-B₂. J. Chem. Soc. 1:22-24.
- Robinson, P. 1967. Infantile cirrhosis of the liver in India, with special reference to probable aflatoxin etiology. Clin. Ped. 6:57.
- Sanders, T. H., N. D. Davis, and U. L. Diener. 1968. Effect of carbon dioxide, temperature, and relative humidity of production of aflatoxin in peanuts. J. Amer. Oil Chem. Soc. 45:683-685.
- Schabert, J. C., and M. Steyn. 1969. Substrate- and phenobarbital-inducible aflatoxin 4-hydroxylation and aflatoxin metabolism by rat liver microsomes. Biochem. Pharmacol. 18:2241-2252.
- Schindler, A. F., and W. V. Eisenberg. 1968. Growth and production of aflatoxins by Aspergillus flavus on red pepper (Capsicum frutescens L.). J. Assoc. Offic. Anal. Chemists. 51:911-912.
- Schindler, A. F., J. G. Palmer, and W. V. Eisenberg. 1967. Aflatoxin production by Aspergillus flavus as related to various temperatures. Appl. Microbiol. 15:1006-1009.
- Schoental, R., and A. F. White. 1965. Aflatoxins and 'albinism' in plants. Nature. 205:57-58.
- Schroeder, H. W. 1968. Aflatoxin development in undried rough rice. Proc. 1967 Mycotoxin Res. Sem., pp. 92-95.

- Schroeder, H. W., and L. J. Ashworth. 1965. Aflatoxins in Spanish peanuts in relation to pod and kernel condition. *Phytopathology*. 55:464-465.
- Schroeder, H. W., and H. Hein, Jr. 1967. Aflatoxins: Production of the toxins in vitro in relation to temperature. *Appl. Microbiol.* 15:441-445.
- Schroeder, H. W., and H. Hein, Jr. 1968. Effect of diurnal temperature cycles on the production of aflatoxin. *Appl. Microbiol.* 16:988-990.
- Schroeder, H. W., R. A. Stermer, R. A. Boller, and K. R. Beerwinkle. 1969. Market quality research. *Rice J.* 72:50-53.
- Schuller, P. L., T. Ockhuizen, J. Werringloer, and P. Marquardt. 1967. Aflatoxin B₁ and histamine in wine. *Arzneim-Forsch.* 17:888-890.
- Scott, P. M. 1968. Note on analysis of aflatoxins in green coffee. *J. Assoc. Offic. Anal. Chemists.* 51:609.
- Scott, P. M. 1969a. Analysis of cocoa beans for aflatoxins. *J. Assoc. Offic. Anal. Chemists.* 52:72-74.
- Scott, P. M. 1969b. The analysis of foods for aflatoxins and other fungal toxins--a review. *J. Can. Inst. Food. Technol.* 2:173-177.
- Scott, P. M., W. Van Walbeek, and J. Forgacs. 1967. Formation of aflatoxins by Aspergillus ostianus Wehmer. *Appl. Microbiol.* 15:945.
- Sellschop, J. P. F., N. P. J. Kriek, and J. C. G. du Preez. 1965. Distribution and degree of occurrence of aflatoxin in groundnut products. *Symp. Mycotoxins Foodstuffs, Agric. Aspects, Pretoria.*, pp. 9-17.
- Senser, F. 1967. Occurrence and the determination of toxin-producing fungi of the Aspergillus flavus group. *Deut. Lebensm.-Rundsch.* 63:140-144.
- Shank, R. C., and G. N. Wogan. 1966. Acute effects of aflatoxin B₁ on liver composition and metabolism in the rat and duckling. *Toxicol. Appl. Pharmacol.* 9:468-476.

- Shotwell, O. L., C. W. Hesseltine, R. D. Stubblefield, and W. G. Sorenson. 1966. Production of aflatoxin on rice. *Appl. Microbiol.* 14:425-428.
- Sinnhuber, R. O., D. J. Lee, and J. H. Wales. 1968. Dietary factors and hepatoma in rainbow trout (Salmo gairdnerii). II. Carcinogenesis by cyclopropenoid fatty acids and the effect of gossypol and altered lipids on aflatoxin-induced liver cancer. *J. Nat. Cancer Inst.* 41:1293-1301.
- Sinnhuber, R. O., J. H. Wales, J. L. Ayres, R. H. Engebrecht, and D. L. Amend. 1968. Dietary factors and hepatoma in rainbow trout (Salmo gairdnerii). I. Aflatoxins in vegetable protein feeds. *J. Nat. Cancer Inst.* 41:711-718.
- Slowatizky, I., and A. M. Mayer. 1969. Effect of aflatoxin on greening of etiolated leaves. *Isr. J. Bot.* 18:31-33.
- Smith, R. H. 1963. The influence of toxins of Aspergillus flavus on the incorporation of ^{14}C -leucine into proteins. *Biochem. J.* 88:50-51p.
- Smith, R. H. 1965. The inhibition of amino acid activation in liver and Escherichia coli preparations by aflatoxin in vitro. *Biochem. J.* 95:43-44p.
- Sobolewski, T., and S. Kmiecik. 1968. Determination of aflatoxins in peanuts and peanut cake imported into Poland. *Przem. Spozyw.* 22:155-156.
- Sporn, M. B., and C. W. Dingman. 1966. Chromatin. II. Effects of carcinogens and hormones on rat liver. *Cancer Res.* 26:2488-2495.
- Sporn, M. B., C. W. Dingman, H. L. Phelps, and G. N. Wogan. 1966. Aflatoxin B_1 : Binding to DNA in vitro and alteration of RNA metabolism in vivo. *Science.* 151:1539-1541.
- Sreenivasamurthy, V., A. Jayaraman, and H. A. B. Parpia. 1964. A rapid paper chromatographic method for the detection of aflatoxin in groundnuts. *Indian J. Technol.* 2:415-416.
- Stoloff, L. 1967. Collaborative study of a method for the identification of aflatoxin B_1 by derivative formulation. *J. Assoc. Offic. Anal. Chemists.* 50:354-360.

- Stoloff, L., A. C. Beckwith, and M. E. Cushmac. 1968. The spotting solvent for aflatoxins. J. Assoc. Offic. Anal. Chemists. 51:65-67.
- Stoloff, L., and W. Trager. 1965. Recommended decontamination procedures for aflatoxin. J. Assoc. Offic. Anal. Chemists. 48:681-682.
- Strzelecki, E., H. S. Lillard, and J. C. Ayres. 1969. Country cured ham as a possible source of aflatoxin. Appl. Microbiol. 18:938-939.
- Stubblefield, R. D., G. M. Shannon, and O. L. Shotwell. 1969. Aflatoxins: Improved resolution by thin layer chromatography. J. Assoc. Offic. Anal. Chemists. 52:669-672.
- Stubblefield, R. D., O. L. Shotwell, C. W. Hesseltine, M. L. Smith, and H. H. Hall. 1967. Production of aflatoxin on wheat and oats: Measurement with a recording densitometer. Appl. Microbiol. 15:186-190.
- Svoboda, D. J., H. Grady, and J. Higginson. 1966. Aflatoxin B₁ injury in rat and monkey liver. Amer. J. Pathol. 49:1023-1051.
- Svoboda, D. J., and J. Higginson. 1966. Ultrastructural lesions in rat and monkey liver due to aflatoxin. Fed. Proc. 25:361.
- Tabor, R. A., and H. W. Schroeder. 1967. Aflatoxin-producing potential of isolates of the Aspergillus flavus-oryzae group from peanuts (Arachis hypogaea). Appl. Microbiol. 15:140-144.
- Teng, J. I., and P. C. Hanzas. 1969. Developer for thin-layer chromatography of aflatoxins on silica gel plates. J. Assoc. Offic. Anal. Chemists. 52:83-84.
- Terao, K., and K. Miyaki. 1968. Different susceptibilities of chick embryo liver cells in vitro to aflatoxin, actinomycin D, and mitomycin C. Z. Krebsforsch. 71:199-207.
- Teunisson, D. J. 1961. Microbiological assay of intact proteins using Tetrahymena pyriformis W. I. Survey of protein concentrates. Anal. Biochem. 2:405-420.

- Teunisson, D. J., and J. A. Robertson. 1967. Degradation of pure aflatoxins by Tetrahymena pyriformis. Appl. Microbiol. 15:1099-1103.
- Theron, J. J., N. Liebenberg, and H. J. B. Joubert. 1965. Histochemistry and electron microscopy of acute liver lesions induced by aflatoxin B₁ in ducklings. Nature. 206:908-909.
- Townsley, P. M., and E. G. H. Lee. 1967. Response of fertilized eggs of the mollusk Bankia setacea to aflatoxin. J. Assoc. Offic. Anal. Chemists. 50:361-363.
- Tso, T. C., and T. D. Sorokin. 1967. Examination of aflatoxin B₁ in leaf tobacco and in cigaret smoke condensate. Beitr. Tabakforsch. 4:18-20.
- Tulpule, P. G., T. V. Madhavan, and C. Gopalan. 1964. Effects of feeding aflatoxin in young monkeys. Lancet. 1:962.
- Tung, T. C., and K. H. Ling. 1968. Aflatoxin in foods in Taiwan. J. Vitaminol. (Kyoto). 14:48-52.
- Ulloa-Sosa, M., and H. W. Schroeder. 1969. Aflatoxin decomposition in the process of making tortillas from corn. Cereal Chem. 46:397-400.
- Van Dorp, D. A., A. S. M. Van Der Zijden, R. K. Beethuis, S. Sparreboom, W. O. Ord, K. DeJong, and R. Keuning. 1963. Dihydro-aflatoxin B, a metabolite of Aspergillus flavus. Remarks on the structure of aflatoxin B. Rec. Trav. Chim. 82:587-592.
- Van Duuren, B. L., A. Sivak, C. Katz, and S. Melchionne. 1969. Inhibition in two-stage carcinogenesis on mouse skin. Cancer Res. 29:947-952.
- Van Soest, T. C., and A. F. Peerdeman. 1964. X-ray study of dihydroaflatoxin B₁. Koninkl. Ned. Akad. Wetenschap., Proc., Ser. B. 67:469-472.
- Van Walbeek, W., T. Clademenos, and F. S. Thatcher. 1969. Influence of refrigeration on aflatoxin production by strains of Aspergillus flavus. Can. J. Microbiol. 15:629-632.
- Veen, W. A. G. 1967. Changes in the fatty acid pattern of the liver of young piglets under the influence of aflatoxin B₁. Acta. Physiol. Pharmacol. Neer. 14:448-459.

- Velan, M., and M. Reynaud. 1967. Fate of aflatoxin during the course of refining of peanut oil by distillation. *Rev. French. Corps Gras.* 14:305-310.
- Velasco, J. 1969. Determination of Aflatoxin B₁ in cottonseed meals. *J. Amer. Oil Chem. Soc.*¹ 46: 105-107.
- Verrett, M. J., J. P. Marliac, and J. McLaughlin. 1964. Use of the chicken embryo in the assay of aflatoxin toxicity. *J. Assoc. Offic. Anal. Chemists.* 47: 1003-1006.
- Viitasalo, L., and H. G. Gyllenberg. 1969. Toxicity of aflatoxins to Bacillus megaterium. *Lebensm. Wiss. u. Technol.* 2:113-114.
- Villa-Trevino, S., and D. D. Leaver. 1968. Effects of the hepatotoxic agents retrorsine and aflatoxin B₁ on hepatic protein synthesis in the rat. *Biochem. J.* 109:87-91.
- Vorster, L. J. 1969. A method for the analysis of cereals and groundnuts for three mycotoxins. *Analyst.* 94:136-142.
- Wang, H. H., T. H. Snih, C. M. Chiou, T. I. Fan, H. L. Chuang, C. L. Tan, and C. C. Shieh. 1968. Determination of aflatoxin in wine products of Taiwan. *Chung Kuo Nung Yeh Hua Hsueh Hui Chih.* 6:69-75.
- Webster, F. H., and R. V. Lechowich. 1970. Partial purification and characterization of dihydrodipicolinic acid synthetase from sporulating Bacillus megaterium. *J. Bacteriol.* 101:118-126.
- Wheeler, B. 1969. Examination of cereals and feeds for aflatoxins. *Irish J. Agric. Res.* 8:172-174.
- Whitaker, T. B., and E. H. Wiser. 1969. Theoretical investigations into the accuracy of sampling shelled peanuts for aflatoxin. *J. Amer. Oil Chem. Soc.* 46:377-379.
- Whitten, M. E. 1969. Screening cottonseed for aflatoxins. *J. Amer. Oil Chem. Soc.* 46:39-40.
- Wick, E. L., G. N. Wogan, T. Asao, G. Buechi, M. M. Abdel-Kader, and S. B. Chang. 1965. The structures of aflatoxins B₁ and G₁. *J. Amer. Chem. Soc.* 87:882-886.

- Wildman, J. D., L. Stoloff, and R. Jacobs. 1967.
Aflatoxin production by a potent Aspergillus flavus
Link isolate. Biotech. Bioeng. 9:429-437. .
- Williams, D. J., and B. R. Rabin. 1969. Effects of
aflatoxin B₁ and steroid hormones on polysome
binding to microsomal membranes as measured by the
activity of an enzyme catalyzing disulfide inter-
change. Fed. Eur. Biochem. Soc. Lett. 4:103-107.
- Wilson, B. J., T. C. Campbell, A. W. Hayes, and R. T.
Hanlin. 1968. Investigation of reported aflatoxin
production by fungi outside the Aspergillus flavus
group. Appl. Microbiol. 16:819-821.
- Wilson, B. J., T. M. Harris, and A. W. Hayes. 1967.
Mycotoxin from Penicillium puberulum. J. Bacteriol.
93:1737-1738.
- Wilson, R. G., J. S. Wortham, and H. V. Gelboin. 1967.
Effect of methylcholanthrene, phenobarbital, and
aflatoxin on RNA polymerase of rat liver. Advan.
Enzyme Regul. 5:385-395.
- Wiseman, H. G., W. C. Jacobson, and W. C. Harmeyer. 1967.
Note on removal of pigments from chloroform extracts
of aflatoxin cultures with copper carbonate. J.
Assoc. Offic. Anal. Chemists. 50:982-983.
- Wogan, G. N. 1966. Chemical nature and biological effects
of the aflatoxins. Bacteriol. Rev. 30:460-469.
- Wogan, G. N. 1968. Biochemical responses to aflatoxins.
Cancer Res. 28:2282-2287.
- Wogan, G. N., and M. A. Friedman. 1968. Inhibition by
aflatoxin B₁ of hydrocortisone induction of rat
liver tryptophan pyrrolase and tyrosine transaminase.
Arch. Biochem. Biophys. 128:509-516.
- Wolf, H., and E. W. Jackson. 1963. Hepatomas in rainbow
trout: Descriptive and experimental epidemiology.
Science. 142:676-678.
- Wragg, J. 1967. Alteration of DNA polymerase activity in
aflatoxin B-treated Escherichia coli. Fed. Proc.
26:740.
- Wragg, J. B., V. C. Ross, and M. S. Legator. 1967.
Effect of aflatoxin B₁ on deoxyribonucleic acid
polymerase of Escherichia coli. Proc. Soc. Exptl.
Biol. Med. 125:1052-1055.

- Yin, L. 1969. Acetonitrile as an extracting solvent for aflatoxins. J. Assoc. Offic. Anal. Chemists. 52: 880.
- Yokotsuka, T., T. Kikuchi, M. Sasaki, and K. Oshita. 1968. Aflatoxin G-like compounds with green fluorescence produced by Japanese industrial molds. Nippon Nogei Kagaku Kaishi. 42:581-585.
- Zuckerman, A. J., K. R. Rees, D. Inman, and V. Petts. 1967. Site of action of aflatoxin on human liver cells in culture. Nature. 214:814-815.
- Zuckerman, A. J., K. R. Rees, D. R. Inman, and I. A. Robb. 1968. The effects of aflatoxins on human embryo liver cells in culture. Brit. J. Exptl. Pathol. 49:33-39.