AN ABSTRACT OF THE THESIS OF

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(Name of student) (Degree)

in Food Science and Technology presented on January 7, 1972
(Major) (Date)

Title: BIOLOGICAL PREPARATION OF ^{14}C LABELED AFLATOXIN

Abstract approved: Russell O. Sinnhuber

Selected parameters that affect crude toxin production in both primary culture (growing cell culture) and resting culture were examined during efforts to produce sufficient crude toxin for purification. The parameters included carbohydrate and label source and concentration, cell concentration, incubation time and temperature and shaker speed. A higher yield of crude toxin was obtained with 0.005 M glucose plus 0.002 M acetate than with 0.08 M glucose alone. Acetate-1-^{14}C gave a higher specific activity of crude toxin than glucose-6-^{14}C. Maximum yields and incorporation were obtained with washed cells from 72 hour primary culture incubated with nitrogen free replacement medium for 12 hours at 30°C on a rotary shaker at 200 rpm.

A number of chromatography supports and solvent systems were examined in an attempt to purify crude toxin. Activated

silica gel H eluted from a column with a gradient of chloroform: methanol from a metering pump gave fractions of pure aflatoxin B_1.

Comparison of the ratios of the ultraviolet radiation absorbances of the toxin to pure reference aflatoxin B_1 was a criteria for chemical purity. Retention of specific activity after chromatography, hydrogenation to tetrahydrodeoxoaflatoxin B_1 and preparation of the hemiacetal and epimeric acetates indicated the label was incorporated in aflatoxin B_1.

A successful method for the preparation of ^{14}C labeled aflatoxin B_1 from resting cultures of Aspergillus parasiticus ATCC 15517 in a defined synthetic medium containing 0.02 M glucose and 0.005 M acetate is described. After purification ^{14}C labeled aflatoxin B_1 of specific activity 744 μci per mmole was obtained from 2 mci acetate-1-^{14}C of specific activity 1 mci per mmole.

Biological Preparation of ^{14}C Labeled Aflatoxin

by

Grant Louis Schoenhard

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

June 1972

APPROVED:

Professor of Food Science and Technology
in charge of major

Head of Department of Food Science and Technology

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Date thesis is presented January 7, 1972

Typed by Ilene Anderton for Grant Louis Schoenhard

ACKNOWLEDGMENT

The author wishes to express his appreciation to Professor Russell O. Sinnhuber for his guidance during the course of this investigation and preparation of the thesis. The author also wishes to acknowledge Dr. Donald J. Lee for his suggestions and assistance throughout this study.

The author thanks his wife, Alice, for her assistance in the preparation of the manuscript.

This research was supported by Public Health Service Training Grant FD 00010 and by Public Health Service Research Grant ES 00256. The author expresses his gratitude to this agency for making this study possible.

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BIOLOGICAL PREPARATION OF ^{14}C LABELED AFLATOXIN

INTRODUCTION

Aflatoxin B_1 is one of several structurally related mycotoxins of the Aspergillus flavus group of fungi. Their metabolites are toxic and highly carcinogenic. They have been found as contaminants in many foods consumed by man and domestic animals. The metabolic fate of the aflatoxins has been examined in an effort to elucidate their biological activity. The fate of the individual carbon atoms and site of action can best be determined through the use of labeled aflatoxin. Other investigators have attempted to prepare ^{14}C labeled aflatoxin B_1 from resting cell cultures of Aspergillus flavus in a defined liquid medium containing the labeled precursor. No conclusive evidence has been presented for the structural identity or purity of the labeled products. In fact, Ayres (11) found from cultures prepared by the same methods that the label was associated with a highly radioactive impurity with chromatographic properties similar to aflatoxin B_1. He was only successful in preparing ^{14}C labeled aflatoxin B_1 on rice cultures of Aspergillus flavus containing the labeled precursor. Unfortunately, the specific activity was low presumably because the label was diluted in the complex culture. A defined liquid medium could circumvent this problem. The objective of this

thesis was to prepare and isolate pure ^{14}C labeled aflatoxin B_1 from liquid cultures of Aspergillus parasiticus.

LITERATURE REVIEW

In 1960, 100,000 young turkeys (18), 14,000 ducklings, 5,000 partridge and pheasants (8) died in England from an unknown disease. Simultaneously an outbreak of trout hepatoma occurred in the United States (156). The aflatoxins, a group of structurally related compounds, were found to be the cause of both incidents (117, 57). The aflatoxins are toxic and carcinogenic metabolites of Aspergillus flavus and parasiticus (58, 61). They have been isolated from peanuts, cottonseed, rice, wheat, oats, corn (60), milk and the tissues of cows (2).

The metabolic fate of aflatoxin in animals has been examined with radioactively labeled aflatoxin (11, 15, 40, 80, 102, 103, 122, 123, 154, 155). After extensive purification Ayres (11) and Lijinsky (79) found that the label was not solely associated with the prepared ^{14}C and ^{3}H labeled aflatoxin B_1. They questioned the radiopurity of the aflatoxins used in the published metabolic fate studies.

Chemistry of Aflatoxin

Structure

Asao et al. (6), based on interpretation of ultraviolet, infrared, nuclear magnetic resonance and mass spectroscopic data, elucidated

the structures of aflatoxins B_1 and G_1. Aflatoxins B_2 and G_2 were determined to be the dehydro derivatives (144). The absolute configuration was determined by Brechbühler, Büchi and Milne (22). Racemic tetrahydrodeoxoaflatoxin B_1 was synthesized by Knight et al. (74, 75). The structure of B_1 was confirmed by the total synthesis of racemic aflatoxin B_1 (27). Table 1 shows the structures of the four major aflatoxins and related compounds.

Dutton and Heathcote (54) reported the hemiacetals, B_{2a} and G_{2a}, of aflatoxins B_1 and G_1 as mold metabolites. Pohland, Cushmac and Andrellos (96) suggested that B_{2a} was an artifact of mold culture acidity or the isolation procedures.

Aflatoxins M_1 and M_2 are metabolites of Aspergillus flavus and animal metabolites of aflatoxins B_1 and B_2 (70, 84). Racemic M_1 has been totally synthesized (25). During the structural determination of aflatoxins B_1 and M_1 the tetrahydrodeoxo derivatives were prepared (70, 145).

Heathcote and Dutton (67) and Stubblefield et al. (139) isolated parasiticol, also known as aflatoxin B_3, from several strains of Aspergillus flavus and parasiticus. Since the structure could result from the hydrolytic cleavage and subsequent decarboxylation of aflatoxin G_1, they speculated that parasiticol was either a precursor or degradation product of G_1 or B_1.

Heathcote and Dutton (67) confirmed the findings of others (88)

Table 1. Structure and predominant occurrence of aflatoxins and related compounds.

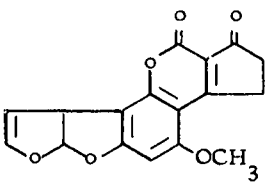
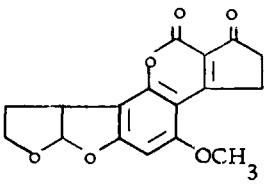
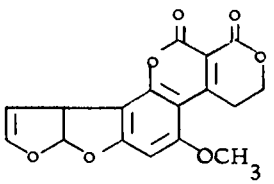
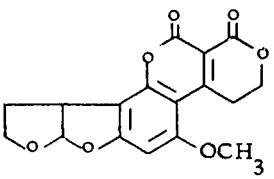
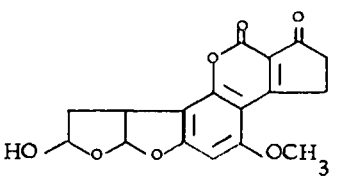
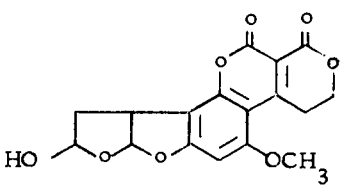
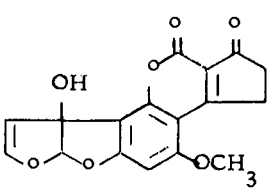
Compound	Structure	Predominant occurrence [a/]	References
Aflatoxin B_1		m	6
Aflatoxin B_2		m	6
Aflatoxin G_1		m	6
Aflatoxin G_2		m	6
Aflatoxin B_{2a}		m, d	54, 55
Aflatoxin G_{2a}		m, d	54, 55
Aflatoxin M_1		m, a	70

Table 1. (continued).

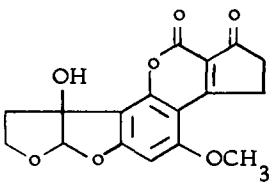
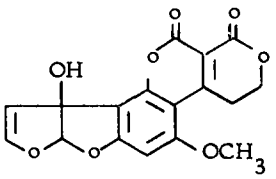
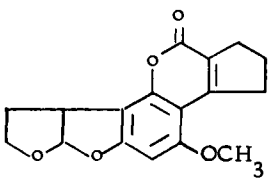
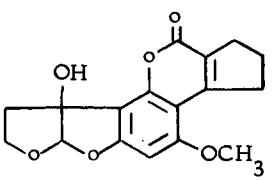
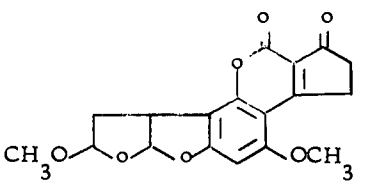
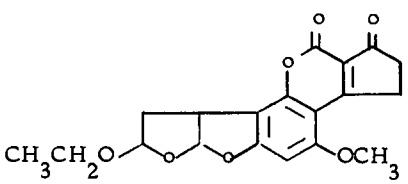
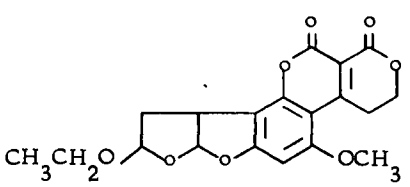
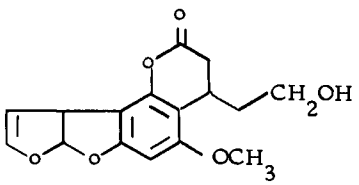
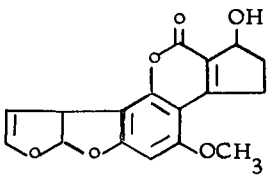
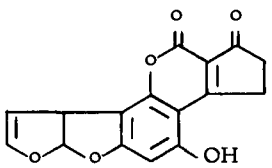
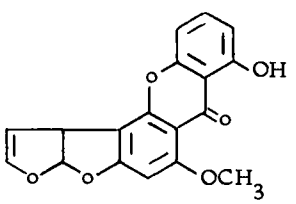
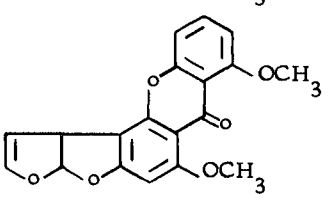
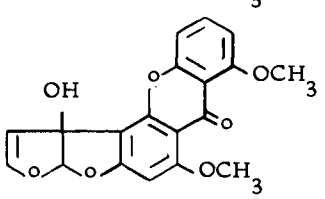
Compound	Structure	Predominant occurrence[a]	References
Aflatoxin M_2		m, a	70
Aflatoxin GM_1		m	67, 88
Tetrahydrodeoxo-aflatoxin B_1 ($THDB_1$)		d	70, 74, 75
Tetrahydrodeoxo-aflatoxin M_1($THDM_1$)		m, e	145
2-Methoxy, 3-hydro adduct of B_1		m, e	53
2-Ethoxy, 3-hydro adduct of B_1		m, e	53
2-Ethoxy, 3-hydro adduct of G_1		m, e	53

Table 1. (continued).

Compound	Structure	Predominant occurrence[a]	References
Parasiticol or aflatoxin B_3		m	67
Aflatoxicol or aflatoxin R_O		f	48, 107
Aflatoxin P_1		a	40
Sterigmatocystin		m	109
O-methyl-sterigmatocystin		m	32
Aspertoxin		m	112, 113

[a] m = natural mold metabolite
d = chemical derivative
a = animal metabolite
e = extraction artifact
f = fungal transformation product

that Aspergillus flavus produced an hydroxylated G_1 analogous to M_1 and isolated two pigments, tetrahydroxyanthroquinone and versicolorin-C also a metabolite of Aspergillus versicolor (69). Versicolorin-C and aflatoxin B_2 were suggested to be biogenically related (68, 112). Both contain the same tetrahydrofurofuran ring structure.

Other metabolites of the Aspergillus species contain the dihydrofurofuran structure. Rather than a substituted coumarin, a substituted xanthone is fused to the 4,5 position of the furofuran ring in sterigmatocystin, O-methylsterigmatocystin and aspertoxin (32, 112, 113).

The 2-methoxy, 3-hydro and 2-ethoxy, 3-hydro adducts of B_1 and the 2-ethoxy, 3-hydro adduct of G_1 were obtained by Dutton and Heathcote (53) both as mold metabolites and as artifacts during isolation. The latter occurred when B_1 and G_1 were converted to their respective hemiacetals, B_{2a} and G_{2a}, and exposed to methanol or ethanol. Alcohol need only be present in trace amounts in the extracting chloroform to produce the compounds. To minimize photocatalyzed oxidation products, phosgene, chlorine and hydrochloric acid (133), ethanol is often deliberately added to chloroform.

Aflatoxicol, aflatoxin R_o, the reduction product of the aldehyde portion of aflatoxin B_1 to the alcohol has been reported (48, 107). Dazelios, Wogan and Weinreb (40) isolated aflatoxin P_1, the

0-demethylation product of B_1. The structure was confirmed by methylation with diazomethane to yield the parent aflatoxin B_1.

Physical Characteristics

Rodricks et al. (111, 114) determined ultraviolet absorptivity values and the ratios of the absorbances for the major spectral peaks of aflatoxins B_1, B_2, G_1 and G_2. The ratios of the absorbances provide a criteria for purity independent of sample weights. Table 2 summarizes the wavelengths of maximum absorbance of ultraviolet radiation and their extinction coefficients.

The fluorescence emission spectra of aflatoxins B_1, B_2, G_1 and G_2 were examined by Carnaghan, Hartley and O'Kelly (33) and Robertson, Pons and Goldblatt (106). Aflatoxins B_1 and G_1 exhibit distinct fluorescence and phosphorescence (90, 146). Characteristic fluorescence spectra were obtained with 10^{-2} to 10^{-4} μg/ml compared to 2 μg/ml for an ultraviolet spectra and 10^{-4} to 0.2 μg/spot (61) for thin layer fluorescence chromatography.

Büchi and Rae (24) summarized the nuclear magnetic resonance, infrared and mass spectral data for each of the aflatoxins. Haddon, Wiley and Waiss (64) reported a method for positive detection with mass spectrometry of 50 ng or less of aflatoxins B_1, G_1, G_2, M_1 and aspertoxin isolated by thin layer chromatography. NMR is not destructive but, as Rodricks (109) emphasizes, it requires

Table 2. Ultraviolet spectra data of the aflatoxins.

Compound	λ_{max}, nm (ϵ^a), solvent	References
B_1	223 (22,100) 265 (12,400) 360 (21,800) alcohol	114, 7, 144
B_2	222 (18,600) 265 (12,100) 362 (24,000) alcohol	114, 144
G_1	216 (27,400) 242 (9,600) 265 (9,600) 362 (17,700) alcohol	114, 7
G_2	214 (25,300) 244 (10,500) 265 (9,000) 362 (19,300) alcohol	114, 144
B_{2a}	228 (17,600) 256 (10,300) 363 (20,000) methanol	27, 54
G_{2a}	223 (18,600) 242 (10,100) 262 (8,700) 365 (18,000) methanol	54
M_1	226 (23,100) 265 (11,600) 357 (19,000) ethanol	70, 83
M_2	221 (20,000) 264 (10,900) 357 (21,000)	70
GM_1	235 (21,200) 262 (16,300) 358 (12,000)	67
$THDB_1$	255 (8,500) 264 (9,200) 332 (13,900) ethanol	24
$THDM_1$	254 (7,150) 263 (8,000) 328 (13,000) ethanol	70
2-methoxy, 3-hydro adduct of B_1	224 (------) 265 (------) 362 (------)	53
2-ethoxy, 3-hydro adduct of B_1	226 (14,600) 266 (12,140) 364 (12,580) methanol	53
2-ethoxy, 3-hydro adduct of G_1	223 (17,140) 244 (12,840) 266 (11,700) 366 (19,500)	53
Parasiticol or aflatoxin B_3	217 (17,300) 225 (sh) (12,600) 253 (6,800) 262 (7,400) 325 (9,700) methanol	139
aflatoxicol or aflatoxin R_o	254 (6,790) 261 (10,800) 325 (14,100) methanol	48

[a] ϵ given is from the first reference noted.

substantial amounts of toxin of high purity. Mathews (86) summarized the available optical rotation data and melting points. The latter are of limited value since all of the toxins decompose. X-ray crystallographic studies on B_1 and B_2 were reported by Cheung and Sim (34) and van Soest and Peerdeman (147).

Chemical Behavior

The aflatoxins contain both a lactone ring and a furofuran moiety. The lactone ring has been saponified to the alkali salt (93). Sreenivasamurthy et al. (128) found with acidification that ring reformation was 80% efficient.

The dihydrofurofuran moiety of B_1 was reduced to the tetrahydrofurofuran (7, 144) by catalytic hydrogenation. Similarly G_1 and M_1 have been reduced to G_2 and M_2 (7, 70). Hydrogenation of B_1 or M_1 until 3 moles of hydrogen were absorbed yielded the tetrahydrodeoxoaflatoxin B_1 or M_1 (70, 145). Addition of water to B_1 formed the hemiacetal, B_{2a} (4). At 37°C 0.01 N, 0.1 N and 0.5 N HCl in 2 hours converted 4%, 30% and 90% of B_1 to B_{2a} (96). At 37°C 1 N HCl gave a 90% conversion in 15 minutes. Pons et al. (101) observed the conversion of B_1 to B_{2a} and G_1 to G_{2a} at a pH less than 3 when heated above 40°C. Reaction of acetic acid thionyl chloride with aflatoxin B_1 produced the α and β acetates at the 2 position of the difurofuran moiety (132, 151).

Oxidations with ozone (56, 145), hydrogen peroxide (128, 141, 145), chromic trioxide in acetic acid (27) and NaOCl (141) have been examined as methods to detoxify aflatoxin B_1. Andrellos, Beckwith and Eppley (3) and Van Duuren, Chan and Irani (146) observed that ultraviolet radiation converted the aflatoxins to new compounds postulated to be the hydroxylated or hydro-peroxylated derivatives. Lillard and Lantin (81) found the products contained the intact coumarin moiety although Aibara and Yamagishi (1) indicated it may be dimerized.

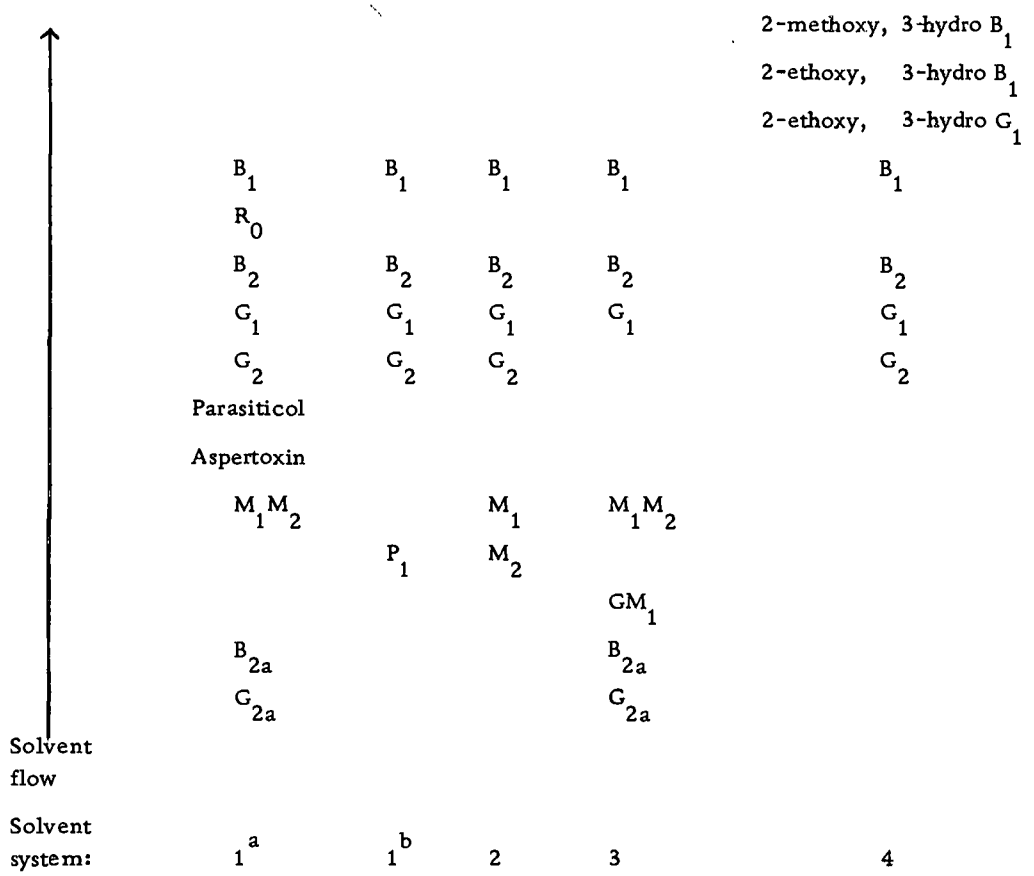
Isolation and Purification

The aflatoxins were first isolated from milk and groundnuts by Soxhlet extraction (104, 116, 117) and purified by liquid partition chromatography (92, 108). Pons and Goldblatt (99), Fishbein and Falk (58) and Stoloff (131) reviewed isolation procedures. Masri (83) examined extraction systems and found solvent systems containing benzene rather than chloroform gave cleaner initial extracts and simplified isolation of B_1 from liquid cultures of Aspergillus flavus.

Paper chromatography was first used to resolve the aflatoxins into the individual compounds (70, 116, 117). Thin layer chromatography has been employed with silica gel (46), alumina (23) or kieselguhr (10) as the support. Solvent systems with silica gel have been evaluated by Shih and Marth (124) and Stubblefield, Shannon and

Shotwell (136). Those reported to resolve aflatoxins B_1, B_2, G_1 and G_2 from each other and contaminants (12) include: chloroform: methanol 97:3 v/v (142), chloroform:methanol 99:1 v/v (124), chloroform:acetone 9:1 v/v (57), benzene:ethanol:water 46:35:19 v/v/v organic phase (134), toluene:ethyl acetate:10% formic acid 5:4:1 v/v/v (95). Figure 1 summarizes the supports and solvent systems which separate the aflatoxins. Stoloff et al. (135) developed a single extraction procedure for the four major aflatoxins and four other naturally occurring toxins: ochratoxin, zearalenone, sterigmatocystin and patulin. The aflatoxins were resolved from this extract on a silica gel TLC plate with benzene:methanol:acetic acid 18:1:1 v/v/v.

The characteristic intense fluorescence of the aflatoxins renders detection of submicrogram quantities on TLC plates. Ayres and Sinnhuber (13) originally demonstrated that fluorodensitometric measurements were feasible and more accurate than visual comparison to various levels of standards spotted on the same plate. Pons et al. (98, 100), Stubblefield et al. (140) and Beckwith and Stoloff (16) extended the application beyond aflatoxin B_1 from cottonseed extracts to the other aflatoxins from a variety of commodities. Quantitation was visually and densitometrically made against a standard whose concentration and stability were effected by the spotting solvent (94). Stoloff et al. (133) examined a series of



TLC adsorbents and solvent systems.

1^a. Adsorbosil-1 activated 105°C, 2 hours.
Acetone:chloroform:water 12:88:1.5 v/v/v (136)

1^b. Same as 1^a (40)

2. Adsorbosil-1 activated 105°C, 2 hours.
2-propanol:water:acetone:chloroform 1:1.5:12:88 v/v/v/v
or 2-propanol:acetone:chloroform 5:10:85 v/v/v (137)

3. Silica gel (Camag D5) activated 105°C, 2 hours.
Acetone:chloroform 1:4 v/v (118)

4. Cellulose powder MN 300 HR. Prepared as 15 g cellulose powder slurried with 70 ml of formamide:acetone 1:9 v/v.
Benzene saturated with formamide (53)

Figure 1. Compounds in order of increasing R_f in the indicated solvent system.

solvents and reported that standards were more stable in benzene.

Column chromatography was initially used to isolate the aflatoxins as a class from contaminated foods (38, 58, 131). Robertson, Pons and Goldblatt (106) resolved small quantities of aflatoxins B_1 and B_2. Stubblefield, Shotwell and Shannon (138) as part of an effort to obtain large quantities of pure B_1, B_2, G_1 and G_2 (126, 140) used three columns. B_1 was isolated from a silicic acid column eluted with chloroform:ethanol 99:1 v/v. The remaining aflatoxins were separated on two silica gel G columns in series using chloroform: acetone:ethanol 97.3:2.0:0.75 v/v/v.

Rodricks (110) found the aflatoxins were separated as a class from the other compounds in the extract with an acid alumina column eluted with benzene:chloroform 5:1 v/v followed by benzene:chloroform 1:1 v/v. Then, B_1, G_1 and G_2 were separated on a silica gel column with a benzene:chloroform gradient followed by a discontinuous gradient of chloroform:methanol. B_2 was separated from B_1 and G_1 with chloroform containing ethanol.

Wiley and Waiss (150) simplified the separation of aflatoxins B_1, B_2, G_1 and G_2 using silica gel H for TLC eluted with a continuous chloroform:methanol gradient from a non-pulsating metering pump. M_1 and M_2 were separated by Stubblefield, Shannon and Shotwell (137) on a silica gel eluted with hexane, hexane:chloroform 1:1 v/v, chloroform, and ethanol:chloroform 1.5:98.5 v/v in series.

Contaminants and degradation products with chromatographic behavior similar to the aflatoxins were reported to be a critical problem (59). Stefaniak (130) reported the interference of ethoxyquin during analytical TLC. Shotwell et al. (125) found a number of compounds from oats that could be mistaken for aflatoxins. A yellow pigment with absorbances at 223 nm and 362 nm was reported by Wiseman, Jacobson and Harmeyer (153). Addition of basic green copper carbonate absorbed the pigment and was selectively removed by filtration. The O-alkyl derivatives of aflatoxin B_1 comigrate on adsorption TLC but are separable with partition TLC (53). Bösenberg (20) observed the same behavior with ultraviolet radiation degradation products of B_1.

Biosynthesis of Toxic Metabolites by Fungi (Secondary Metabolism)

A large number of compounds have been isolated from microorganisms including the fungi that have 1. a restricted taxonomic distribution, 2. no obvious function in cell growth and 3. are synthesized by cells that have stopped dividing (149, 157). They were defined as secondary metabolites and their production termed secondary metabolism by Bu'Lock (29). He observed (28) that during cell growth as indicated by increases in nucleic acids, protein and cell number that secondary metabolite production did not occur. He

divided the dry weight curve (Figure 2) into two phases. The first phase corresponded to balanced cell growth and was named the trophophase. During the second phase, idiophase, idiosyncratic (i.e. species-peculiar) secondary metabolites were produced. Table 3 summarizes the differences observed between trophophase and idiophase.

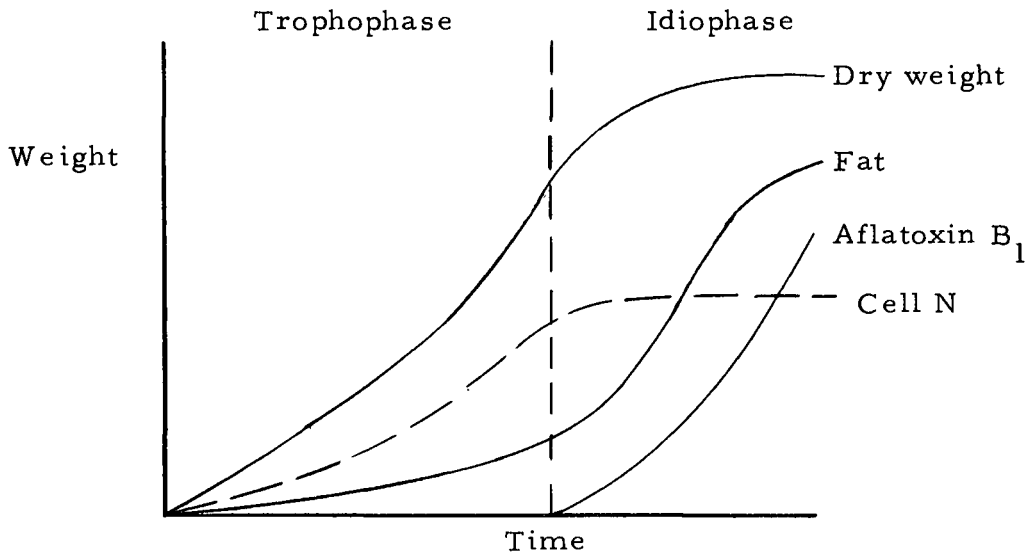


Figure 2. Dry weight curve (28, 50)

Detroy and Hesseltine (50) and Detroy and Ciegler (47) reported that aflatoxin B_1 synthesis occurs after dry weight mass, proteins and nucleic acids are no longer increasing, that is, after the trophophase and during the idiophase (Figure 2). Cycloheximide addition during the trophophase blocked all protein synthesis. At the

Table 3. Differences between trophophase and idiophase in P. urticae (30).

	Rate of dry wt. increase	Glucose uptake	N and P uptake	Mycelial SH %	Mycelial RNA %	Mycelial lipids %	Free amino-N %	Keto-acids %	Phenols
Trophophase	rising	rapid	rapid	high	high	low	high	----	absent
(transition)				maximal	maximal		falling	maximal	first
Idiophase	falling	cont'd	none	low	low	high	low	----	diverse

transitional phase it prevented B_1 synthesis but was ineffective during the idiophase. Addition of ethionine during the transition phase also prevented the synthesis of aflatoxin B_1 (6-methoxydifurocoumarone). Addition of ^{14}C-l-ethyl-ethionine competed with methionine and resulted in a new aflatoxin derivative, 6-ethoxydifurocoumarone. These authors suggested the possible formation of 6-demethoxyaflatoxin also known as P_1, in the absence of an alkyl donor.

Biosynthesis of the Aflatoxins

Fungi that Produce Aflatoxin

The source of the aflatoxins was originally reported by Sargeant et al. (117) to be the fungus Aspergillus flavus Link ex Fries as identified by J. J. Elphick. Later Elphick determined the fungus was identical to Aspergillus parasiticus Speare var. globosus Murakami, nov. var. 15517 (9). The organisms producing the aflatoxins have been referred to as the Aspergillus flavus group because they have attributes of both flavus and parasiticus (51).

Borker et al. (19), Hesseltine et al. (68) and Diener and Davis (51) summarized the species of Aspergillus, Penicillium and Rhizopus reported to produce the aflatoxins. In most cases the presence of aflatoxin was only based on a visual comparison of its fluorescence and R_f to that of a standard on the same TLC plate. To

authenticate these reports Mislivec, Hunter and Tuite (87) examined for aflatoxin production 260 of the isolates which included 43 species of Penicillium and 7 of Aspergillus. The suspected toxins were subjected to 6 TLC solvent systems and iodine vapors for identification. After elimination of the false positives, aflatoxin was not found from any species other than flavus and parasiticus. These results were confirmed by Wilson et al. (152) who examined 29 species and found the A. flavus group was the only producer of the toxin.

Parameters that Affect Toxin Production

Increasing the temperature to 30° C increased the proportion of B_1 to other products (127) and reduced the time for maximum production (51). The maximum temperature for growth was different than for toxin production (119). Both were unaffected by diurnal cycles (120).

Glucose, sucrose, fructose, xylose, ribose and glycerol were the preferred carbon sources (42, 44, 45, 85). Gamma irradiation (63, 73), 1-4% ethanol (14), tryptophan, methionine, proline (89), zinc (5, 43, 78, 85, 91), iron and magnesium (43, 85) were all reported to increase aflatoxin production. Contradictory results have been reported for optimum pH and aeration (49, 85). Ciegler et al. (36) reported the necessity for mycelial pellet dispersion and minimum incubation in cultures. Degradation of the toxins was observed

only on cell lysis. Evidence did not support the hypothesis of an "aflatoxinase".

Biological Preparation of Labeled Aflatoxin

Ayde and Mateles (10) were the first to report the preparation of labeled aflatoxins. Mycelia were first grown on synthetic liquid medium containing nitrogen (Table 12). After 48 hours at 30° C on a rotary shaker the mycelia were collected and washed to remove nitrogen compounds and transferred to a resting medium (Appendix III, (2)) which also included a labeled precursor. After 24 hours at 30° C on a rotary shaker the medium was extracted with an equal volume of chloroform. The aflatoxins were purified using thin layer plates spread with kieselguhr G sprayed with 8 ml of formamide: water 1:2.5 v/v and developed with benzene saturated with formamide. The bands whose R_f corresponded to the aflatoxin standards were scraped off and eluted with methanol. The yields were determined by the absorption at 362 nm. L ^{14}C methyl methionine, DL ^{14}C alanine-3-phenylalanine, DL ^{14}C alanine-2-tyrosine and 1- ^{14}C acetate gave the highest relative isotopic content which they defined as the specific activity of the products divided by the specific activity of the precursor on a mole basis.

The same group (52) later reported the preparation of 57 μci of ^{14}C labeled aflatoxin B_1 from 1 mci of acetate-1- ^{14}C giving a

relative isotopic content of 1.48. Primary culture (Table 12) was incubated at 25° C for 72 hours at 200 rpm on a rotary shaker. The resting culture containing 0.005 M glucose and 0.002 M acetate was incubated at 25° C for 12 hours at 250 rpm.

After the preparation of labeled aflatoxin according to the procedure of Ayde and Mateles (10), Ayres, Lee and Sinnhuber (12) found by two dimensional TLC the label was not entirely associated with aflatoxin B_1 but also with a highly radioactive impurity. Detroy and Hesseltine (49) found that ^{14}C acetate incorporation was favored by acidic conditions. Above pH 5.0 aflatoxin synthesis decreased drastically and incorporation of radioactivity into lipids increased markedly.

Hsieh and Mateles (72) used the same primary culture as they had before (52) and increased the glucose and acetate concentration to 0.02 M and 0.005 M in the resting culture. Primary culture was carried out at 30° C at 200 rpm on a rotary shaker for 70 hours. A relative isotopic content greater than 8 was reported, which approaches the hypothetical limit of 9 based on the labeling pattern reported by Biollaz, Büchi and Milne (17).

Basappa, Sreenivasamurthy and Parpia (14) prepared ^{14}C labeled aflatoxin according to the procedure of Ayde and Mateles (10) without glucose. The resting culture was incubated at 25° C for 72 hours at pH 6. In contrast to Detroy and Hesseltine (49) who found

primarily lipid material above pH 5, Basappa et al. reported maximum yield of aflatoxin between pH 5 and 6.

Hsieh and Mateles (71) modified their previous resting culture medium to contain 0.167 M glucose. Labeled acetate was maintained at 0.01 M by continuous addition. Two percent of the label was incorporated into aflatoxin with a specific activity three times the labeled acetate.

Ayres, Lee and Sinnhuber (12) incubated Aspergillus flavus on rice with labeled precursors. Incontrovertible evidence for the chemical and radiopurity of the labeled aflatoxin B_1 was obtained by a variety of chromatographic techniques, recrystalization to constant specific activity and hydrogenation of aflatoxin B_1-^{14}C to tetrahydrodeoxoaflatoxin B_1-^{14}C.

Suggested Biogenic Pathways for the Aflatoxins

Kojic acid was reported produced by an aflatoxin producing strain of Aspergillus flavus (41). Kojic acid is also a metabolite in the synthesis of sterigmatocystin. Holker and Underwood (69) and Heathcote, Child and Dutton (66) suggested the two syntheses were related.

Degradation studies on labeled aflatoxin were reported by Biollaz, Büchi and Milne (17). Seven of the sixteen ring carbons came from 2-^{14}C acetate and the other nine were from 1-^{14}C acetate.

The methoxy carbon came from methionine in agreement with Detroy and Ciegler (47). From the results of their degradation studies Biollaz et al. (17) proposed a new hypothesis for the biogenesis of aflatoxin consonant with the hypothesis that O-methylsterigmatocystin and versicolorin-C are co-metabolites. They favored synthesis starting from a single polyacetate chain which is in agreement with the observation that many of the secondary metabolites are polyketides. Their hypothesis is in accordance with the postulation of Detroy and Hesseltine (50) for an aflatoxin synthetase analogous to that for fatty acid biogenesis. Detroy and Hesseltine speculate that the synthetase assembles acetyl CoA and malonyl CoA units into a polyketide chain stabilized by cyclization. Just as the formation of malonyl CoA from acetyl CoA is the limiting reaction for fatty acid synthesis, they suspect it is for B_1 also. Since citrate is known to stimulate this reaction a sudden buildup of TCA intermediates which occurs during the close of the trophophase, the transition, could induce the secondary enzyme. Lacking is a mechanism to limit fatty acid synthesis. The authors suggest cell free systems would aid the investigation.

EXPERIMENTAL

Preparation of ^{14}C labeled aflatoxin B_1 was first reported by Ayde and Mateles (10) and Donkersloot, Hsieh and Mateles (52). It was produced from resting cell cultures of Aspergillus flavus in a nitrogen free synthetic liquid medium. Only two purifications using preparative thin layer chromatography were used to isolate ^{14}C labeled aflatoxin B_1. No spectroscopic evidence was reported to substantiate the purity other than a single spectral observation at 362 nm.

Ayres, Lee, and Sinnhuber (12) sought ^{14}C labeled aflatoxin B_1 to utilize in metabolic fate studies with laboratory animals. They reported that the above resting cell preparation yielded, after additional purification, a highly radioactive impurity with chromatographic characteristics similar to aflatoxin B_1. For this reason they produced ^{14}C labeled aflatoxin B_1 on rice culture of Aspergillus flavus. Despite their success in obtaining pure ^{14}C labeled aflatoxin B_1, the specific activity was low. The labeled precursor was presumably diluted in the complex rice culture.

The objective of this thesis was to evaluate the preparation and purity of ^{14}C labeled aflatoxin B_1 from defined liquid medium rather than a solid substrate such as rice. In this manner the specific activity could be raised. To accomplish the objective various

parameters affecting the production of aflatoxin B_1 were examined. For each parameter the amount of crude or mixed toxins was determined. Purification of crude toxin was done to establish the presence of aflatoxin B_1. Purity of B_1 was determined by careful examination of each absorbance in the ultraviolet spectrum. Specific activity was compared before and after the preparation of derivatives.

Parameters Affecting Toxin Production

The concept that aflatoxin B_1 is a secondary metabolite of Aspergillus parasiticus was employed for the preparation of labeled aflatoxin B_1 from liquid medium. Its metabolism was considered to occur in two distinct phases. During the first stage, trophophase, cell growth occurred. It required a medium of salts, trace metals, an energy and nitrogen source. Idiosyncratic (species peculiar) metabolites including aflatoxin B_1 were produced in the second stage, idiophase. At this time cell growth and protein synthesis no longer occurred. For this reason nitrogen was not required in the medium. Evidence indicated (47, 50) that the proteins for aflatoxin B_1 synthesis were produced during the transition between the trophophase and idiophase.

To duplicate the conditions in the laboratory for the trophophase and idiophase, a primary and resting culture were employed. The mycelia were grown in the primary culture containing an energy and

nitrogen source. Once evidence that the proteins for aflatoxin B_1 synthesis were present the mycelia were washed free of exogenous nitrogen and placed in a resting culture. It contained a nitrogen free replacement medium with a labeled precursor for aflatoxin B_1. The success of this procedure depended on determining the culture age at which the maximum enzyme concentration for aflatoxin B_1 synthesis was available. An indication of the effect of various parameters on the presence of the required enzyme was determined by measuring the amount of crude toxin. Resting culture parameters were examined in the same manner. The crude toxin was subsequently purified to determine if it contained aflatoxin B_1.

In summary, the preparation of crude toxin using liquid medium was divided into: 1. the primary culture corresponding to the trophophase, 2. the harvest of mycelia to remove nitrogen to minimize further protein synthesis, 3. the resting or stationary culture corresponding to the idiophase and 4. the extraction and quantitation of mixed or crude toxin. The purification of aflatoxin B_1 from crude toxin will be described after the preparation procedures.

Primary Culture

The primary culture was prepared according to Appendix IV A Steps 1 - 3. The cultures were incubated on a rotary

shaker[1/] equipped with a water bath[2/]. Several incubation temperatures, shaker speeds and times (Tables 4 - 6) were individually examined to determine their effects on crude toxin production.

Harvest of Mycelia

Mycelial balls from the individual incubation flasks were collected, washed, chopped in a blender, and transferred to Erlenmeyer flasks (Appendix IV B). The method of disaggregating the mycelia was based on a compromise between two assumptions. 1. Label uptake from the resting medium would increase with increased surface area of cells exposed. 2. Too much blending could result in cell lysis.

Resting or Stationary Culture

Nitrogen free replacement medium was added to the culture flasks containing the freshly harvested mycelia. Presterilization of the medium was not necessary. Because of lack of nitrogen it would not support growth of microorganisms. Various incubation times, temperatures and cell concentrations (Tables 7 - 9) were

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1/ Model 75-732, Eberbach Corp., Ann Arbor, Michigan.

2/ Lauda Circulators, Model K2/R, Brinkman Instruments, Inc., Westbury, New York.

separately evaluated for the four replacement media given in Appendix III.

Extraction and Quantitation of Crude or Mixed Toxins

To determine the quantity of crude toxin present at any particular time during incubation, 1 ml of medium was removed. The toxin was extracted with 2 ml of chloroform in a separatory funnel. The absorbance of the chloroform extract was measured at 362 nm and the concentration of crude toxin approximated as described in Appendix VII using 20,000 as the extinction coefficient.

Preparation and Purification of Crude Aflatoxin B_1 from Resting Culture

The success of a resting culture depended on finding the parameters which produced sufficient crude toxin from which aflatoxin B_1 could be easily purified. After each preparation of crude toxin, attempts were made to isolate pure aflatoxin B_1. Those fractions having chromatographic characteristics of aflatoxin B_1 were carefully examined using ultraviolet spectroscopy from 370 nm to 220 nm. This was in contrast to previous workers using liquid media preparations who only examined the absorbance at 362 nm to quantitate the yields and did not authenticate the identity or purity spectroscopically.

Three preparations yielded sufficient crude toxin for several

purifications and ultraviolet spectroscopy. If, after several methods of purification, the purity did not increase but only resulted in a marked decrease in total toxin the crude toxin from another resting culture was evaluated. Following are the preparations which produced the greatest crude toxin. Purification procedures and methods of evaluation are included.

Resting Culture 1 - 23° C, 0.005 M Glucose

After 84 hours the mycelia were harvested and washed from the primary medium. One gram of wet mycelial cake was placed in each 300 ml indented Erlenmeyer flask. Twenty-five ml of replacement medium, Appendix III (3), was added. The medium contained 250 µci of acetate-1-^{14}C of specific activity 57.3 mci/mmole.[3/] The flasks were incubated at 23° C at 136 rpm on a rotary shaker for 12 hours. The yield of crude toxin was measured as described in Appendix VII.

Attempts were made to purify the extract using preparative TLC (Appendix V). The purity of the B_1 fractions was evaluated using ultraviolet spectroscopy (Appendix VII). The fate of the label was followed by preparation of the hemiacetal and epimeric acetates of the suspected ^{14}C labeled aflatoxin B_1 (Appendix VIII). The spots

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[3/] New England Nuclear, Boston, Massachusetts.

on the TLC plate whose R_f corresponded to authentic derivatives and the spaces in between were separately removed by scraping. The scrapings were counted to a 1% error in a dioxane cocktail (Appendix X).

Resting Culture 2 - 25° C, 0.005 M Glucose

Twenty-five ml of replacement medium, Appendix III (3), containing 500 μci of acetate-1-^{14}C of specific activity 25 mci/mmole[4/] were added to either 1 or 2 g of mycelia in 300 ml Erlenmeyer flasks. The mycelia were incubated on a rotary shaker at 136 rpm at 25° C for 12 hours beyond the primary culture harvest time of 72 hours.

Preparative TLC was used to isolate ^{14}C labeled aflatoxin B_1. Plates spread with MN-silica gel G-HR were developed with one of the following systems: Chloroform:acetone 9:1 v/v or 95:5 v/v; chloroform:methanol 93:7 v/v; and benzene:ethanol:water 46:35:19 v/v/v with 50 ml of the lower phase in the tank and 50 ml of the upper phase in the trough. Partition TLC was used with a 250 micron layer of kieselguhr G[5/] sprayed with 8 ml of formamide:water 1:2.5 v/v developed immediately with benzene saturated with formamide.

[4/] Calbiochem, Los Angeles, California.

[5/] Brinkman Instruments, Inc., Westbury, New York.

For each chromatography plate the streak corresponding to authentic B_1 was removed and eluted (Appendix V).

The purity was examined by using the ultraviolet absorbance ratios according to Appendix VII, exposure to iodine vapor and spraying with sulfuric acid or 2, 4-dinitrophenylhydrazine (39). The conversion of the suspected ^{14}C labeled aflatoxin B_1 to the hemiacetal and epimeric acetates was examined according to Appendix VIII.

An additional purification of the crude toxin was done by chromatography on a column packed with MN-silica gel H and eluted with a chloroform:acetone gradient using a proportioning pump (Appendix VI).

Resting Culture 3 - 30° C, 0.02 M Glucose

Unlabeled Trial for Preparation of ^{14}C Labeled Aflatoxin B_1

One hundred ml of replacement medium (Appendix III (4)) were added to 5 g of water washed and chopped mycelia in each 500 ml indented Erlenmeyer flask. The mycelia were incubated at 30° C on a rotary shaker at 200 rpm for 21 hours. The total incubation time from the start of primary culture was 108 hours. The details of the procedure are given in Appendix IV.

The aflatoxins were extracted with chloroform (Appendix IV D). The extract was concentrated and subjected to fractional column

chromatography as described in Appendix VI. The fractions from the column containing the aflatoxin B_1 were located with analytical TLC (Appendix V). The middle fractions of B_1 were combined and prepared for ultraviolet spectroscopy (Appendix VII).

Preparation of ^{14}C Labeled Aflatoxin B_1

^{14}C labeled aflatoxin B_1 was prepared and isolated in the same manner as the unlabeled trial (Appendices IV and VI). Each flask contained 500 μci of acetate-1-^{14}C of specific activity 25 mci/mmole[6/] and unlabeled acetate to give a final acetate concentration of 0.005 M and specific activity of 1 mci/mmole.

The chemical purity of the labeled aflatoxin B_1 was examined spectroscopically (Appendix VII). To determine if the label was primarily associated with aflatoxin B_1, a portion of the sample was spotted on the corner of a silica gel plate. The plate was developed two dimensionally, first with chloroform:acetone 9:1 v/v and then with chloroform:acetone 9:5 v/v. The plate was examined under ultraviolet radiation at 365 nm. The spot corresponding to aflatoxin B_1 was removed and counted in toluene gel.

To obtain further evidence that the counts were associated with the aflatoxin B_1, a portion of the labeled material was hydrogenated

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6/ Calbiochem, Los Angeles, California.

as described in Appendix IX. Tetrahydrodeoxoaflatoxin B_1, ($THDB_1$), the primary product after 90 minutes of hydrogenation was applied as a streak to a portion of a silica gel plate. To one end of the streak a spot of pure unlabeled B_1 was applied. To another portion of the plate a streak of $THDB_1$ from a similar hydrogenation of unlabeled aflatoxin B_1 was applied. Labeled aflatoxin B_1 obtained from the hydrogenator prior to hydrogenation was overlaid on the streak of unlabeled $THDB_1$. The plate was developed in chloroform: acetone 9:1 v/v. The streaks corresponding to both labeled and unlabeled $THDB_1$, B_1, B_2 and origin were each separately scraped and counted in toluene gel (Appendix X). In addition the remaining sections of the plate above each origin were scraped and counted.

For additional evidence that the counts were associated with the aflatoxin B_1, the hemiacetal and epimeric acetates were prepared (Appendix VIII), chromatographed, scraped and counted.

RESULTS AND DISCUSSION

Parameters Affecting Toxin Production

Towards the end of the trophophase in primary culture the proteins for the synthesis of aflatoxin B_1 appeared. The effects of several parameters on the enzyme system were determined by measuring the amount of crude toxin. Only after mycelia demonstrated crude toxin production were they harvested and used in the replacement medium. Various parameters of the resting culture (i.e. idiophase) were evaluated by crude toxin yield.

A liquid culture permitted the preparation of the nitrogen free conditions for an idiophase and the control of the levels of each nutrient. This is not possible with a solid substrate such as rice. Other advantages included rapid and semi-synchronous growth with a total culture time of 70 to 96 hours versus 10 days for a rice culture. The extraction of the toxins was less time consuming than with rice which requires three reflux steps of 4, 18 and 14 hours (11). The savings in time facilitated the use of crude toxin yields to monitor the cultures.

Primary Culture

Table 4 indicates that with moderate increases in temperature

crude toxin production increased before reversing itself. This was not an unusual finding. A change of optimum temperature of just a few degrees has been demonstrated to greatly influence the germination and growth of fungi (31, 76, 129, 143).

Table 4. Effect of temperature.

Temperature (°C)	Yield per flask (mg)
25	1.0[a]
28	1.5
32	0.5

[a] Average of four replicates.

A 27% increase in shaker speed as indicated in Table 5 increased the toxin production by 15.5%. Other speeds could not be examined because of equipment limitations. Table 6 indicates the results of increasing the incubation time.

Table 5. Effect of shaker speed.

Shaker speed (rpm)	Yield per flask (mg)
136 for 72 hours	1.5
144 for 25 hours 200 for 47 hours	1.7[a]

[a] Average of three replicates.

Table 6. Effect of incubation time.

Incubation time (hours)	Yield per flask[a] (mg)
72	1.2
84	1.6

[a] Average of three replicates.

Resting or Stationary Culture

The effect of two incubation times on the primary culture as indicated before was only significant as it affected the yields of aflatoxin at various resting culture times. Table 7 shows that, for the same resting culture incubation periods, mycelia from older primary cultures produced better yields of crude toxins. In both cases increasing the time of incubation of resting culture increased toxin production. This trend was found true in all cultures tried regardless of other variables. These findings agree with the hypothesis that, within a time period, increasing the primary culture increases the available proteins used in the synthesis of toxin. Further the rate of toxin production must be greater than breakdown.

For the only two cases where temperature was a comparable variable in resting culture the results confirmed the earlier results with the primary culture (Table 8).

Table 7. Effect of primary culture harvesting time and length of resting culture incubation time.

Primary culture age at harvesting (hours)	Resting culture incubation time (hours)	Yield per flask (mg)
74	12	0.5[a]
	18	0.6[a]
87	12	0.6[b]
	21	0.8[b]

[a] Average of four replicates.

[b] Average of seven replicates.

Table 8. Effect of temperature on resting culture.[a]

Temperature (°C)	Yield/ 25 ml medium (mg)	Gross counts/ 25 ml medium (dpm)	Specific Activity (μci/mmole)	I. E. (%)	R. I. C.
23	0.10	1.7×10^6	2.4×10^3	0.3	0.48
25	0.25	12.4×10^6	7.0×10^3	1.1	0.70

[a] Average of two replicates.

Table 9 indicates that doubling the concentration of mycelia gave a higher yield of crude toxin but did not give a greater relative isotopic content.

Table 9. Effect of cell concentration on resting culture.[a/]

Weight (g)	Yield/ 25 ml medium (mg)	Gross counts/ 25 ml medium (dpm)	Specific Activity (μci/mmole)	I. E. (%)	R. I. C.
1	0.25	12.4×10^6	7.0×10^3	1.1	0.70
2	1.25	48.2×10^6	5.4×10^3	4.4	0.54

[a/] Average of two replicates.

Tables 10 and 11 indicate the results of carbohydrate concentration, source and labeling. They are in accord with the findings of Hsieh and Mateles (72). They suggested that acetate-derived metabolites are generally synthesized extramitochondrially and that "acetate or a closely related metabolite controls the formation of the extramitochondrial CoASAc from glucose and that the extramitochondrial CoASAc pool is dominated by the CoASAc derived exogenous acetate" (72, p. 486). Romano and Kornberg (115) working with Aspergillus nidulans made similar observations. Further experimentation would be required for confirmation of these suggestions

and was not the purpose of this limited experiment.

Table 10. Effect of carbohydrate source and concentration.

Carbohydrate source	Concentration (g/l)	Concentration (M)	Yield/ 25 ml medium (mg)
Glucose	50.0	0.278	0.250
Glucose	15.0	0.083	0.025
Glucose + Acetate	0.90 0.16	0.005 0.002	0.250[a]

[a] Average of two replicates.

Table 11. Effect of carbohydrate source of label.

Carbohydrate sources and labeled precursors	Concentration (M)	Specific activity of precursor (μci/mmole)	Yield/ 25 ml (mg)	Gross counts/ 25 ml (dpm)	Specific activity (μci/mmole)	I. E. (%)	R.I.C.
Glucose $U^{14}C$	0.005	712	0.05	45.5×10^3	0.128×10^3	0.02	0.180
Acetate	0.002						
Glucose	0.005		0.25	12.4×10^6	6.96×10^3	1.12	0.696
Acetate-1-^{14}C [a]	0.002	10^4					

[a] Average of two replicates.

Extraction and Quantitation of Crude or Mixed Toxins

The amount of crude or mixed toxins measured in the chloroform extract was not indicative of the presence of aflatoxin B_1. It should be recognized that the yields of crude toxin obtained by the various parameters is only a preliminary indication of the actual aflatoxin B_1 present. Other aflatoxins and chloroform soluble materials may also absorb at 362 nm to give spurious results. Unfortunately previous workers frequently resorted to this single spectral value for quantitation of aflatoxin B_1. Consequently crude toxin must be subjected to further purification and establishment of identity before the yield has any real significance.

Preparation and Purification of Crude Aflatoxin B_1 from Resting Culture

Crude toxins obtained from three resting culture variations were further purified and examined for spectral purity and derivative formation.

Resting Culture 1 - 23° C, 0.005 M Glucose

After repeated preparative TLC of the crude toxin from this resting culture, an additional weak absorbance between 223 nm and 265 nm was observed in the ultraviolet spectrum. The ratios of the

absorbances of 220 nm/265 nm and 362 nm/265 nm did not agree with those established by Rodricks (114). Subjecting authentic B_1 to the same procedures did not result in loss of purity.

The fate of the label was determined after conversion to B_{2a} and the epimeric acetates, derivatives of B_1. For the B_{2a} preparation, 36% of the counts were with B_{2a}, 3.6% with unreacted B_1 and the remainder spread over the rest of the TLC plate. For the epimeric acetates, 56% of the counts were found in the lower R_f acetate, 14% in the higher R_f acetate, 3.5% with unreacted B_1 and the remainder spread over the plate.

The spectral and derivative data indicated that this resting culture produced impurities with characteristics similar to B_1. The proportion of B_1 to impurities was insufficient to achieve quantitative separation by the techniques employed without a disproportionate loss of B_1. Therefore, other resting cultures which gave higher yields of crude toxin were examined.

Resting Culture 2 - 25° C, 0.005 M Glucose

After submitting the crude toxin from this procedure to several preparative TLC procedures, alone or in series, the ratios of the absorbances still did not agree with the published data. When authentic B_1 was subjected to similar TLC procedures the spectral ratios were uneffected. Ayde and Mateles (10) reported partition

TLC on kieselguhr gave chromatographically pure aflatoxin B_1. This system was found to produce chromatographically pure aflatoxin B_1 from crude toxin from this culture. Exposure of the TLC plate in an iodine chamber or by spraying the plate with sulfuric acid or 2,4 dinitrophenol did not reveal anything besides B_1. However, the ultraviolet spectrum remained unacceptable. Ayde and Mateles did not report examining the spectra other than quantitating their yields at 362 nm. The results of column chromatography of the crude toxin were no better than the preparative TLC. As with Culture 1, the derivatives B_{2a} and epimeric acetates did not retain all of the label. These results of purification attempts suggested that this resting culture procedure did not yield crude toxin with sufficient aflatoxin B_1 to achieve purification without disproportionate loss of B_1.

Resting Culture 3 - 30° C, 0.02 M Glucose

Unlabeled Trial for Preparation of ^{14}C Labeled Aflatoxin B_1

Resting Culture 3 was found to produce sufficient crude toxin for purification. Aflatoxin B_1 was isolated by column chromatography (Appendix VI). The ultraviolet spectrum of the isolate was examined. The ratios of the absorbances agreed with those of Rodricks (114) which indicated the high purity of the aflatoxin B_1.

Preparation of ^{14}C Labeled Aflatoxin B_1

^{14}C labeled aflatoxin B_1 was prepared and isolated in the same manner as the unlabeled aflatoxin B_1. Examination of the ultraviolet spectrum indicated that the ratios of the absorbances were in close agreement with those of Rodricks and pure reference standard analyzed in the same manner. The specific activity remained constant before and after two dimensional TLC. From the four 100 ml cultures 1.37 mg of pure ^{14}C labeled aflatoxin B_1 was isolated. The specific activity was 1.64×10^9 dpm/mmole or 744 μci/mmole. The incorporation efficiency (I.E.) was 0.162% and the relative isotopic content (R.I.C.) was 0.744.

After hydrogenation followed by analytical TLC the number of counts found in the $THDB_1$ streak was equivalent to those in the original B_1 streak with less than 1% of the counts appearing on other parts of the plate. Preparation of the hemiacetal and epimeric acetates resulted in specific activity retention and 75% of the counts in the derivatives with the remainder associated with the side and degradation products which have been reported by the authors of the procedure (97). In total, the evidence supports the chemical and radioactive purity of the labeled aflatoxin B_1.

Since the successful preparation of the above labeled aflatoxin B_1, Hsieh and Mateles (71) described another procedure for the

preparation of labeled aflatoxins. They report aflatoxin synthesis was evident only during 40 to 70 hours of incubation and occurred in growing cells at pH 6. These findings are in contrast to their earlier reports (52, 72) and the conditions that successfully produced labeled aflatoxin for this thesis.

Hsieh and Mateles (71) submit as evidence for chemical and radiopurity two TLC purifications and absorbance at 362 nm for the material prepared in their above manner. They describe another procedure incorporating acetate-1-^{13}C and acetate-1-^{14}C. The resultant toxin was subjected to nuclear magnetic resonance (NMR) analysis to verify the labeling pattern. It is assumed but not reiterated by the authors that the preparation submitted to two TLC purifications and measured at 362 nm and the one made for NMR analysis were prepared under the same culture conditions. Resolution of this critical point is imperative particularly since the aflatoxin was produced in growing culture at a pH of 6.

Detroy and Hesseltine (50) and Detroy and Ciegler (47) through the use of protein synthesis inhibitors indicated that the proteins for aflatoxin synthesis only occurred late in the growing phase during the transition to idiophase. This is contradictory to Hsieh and Mateles' use of a growing culture and supports the hypothesis for synthesis in the idiophase. Furthermore, Detroy and Hesseltine (49) reported that lipid synthesis primarily occurred during the growing

culture. They found increasing the pH above 5.0 as Hsieh and Mateles did resulted in a seven-fold increase in lipid synthesis and a subsequent sharp decrease in aflatoxin synthesis in resting culture.

These contradictions coupled with the reports of false identification of unlabeled aflatoxin B_1 from various species of Aspergillus, Penicillium and Rhizopus (87, 152) and labeled aflatoxin B_1 (12) emphasize the importance of the purification methods and the criteria for chemical and radioactive purity.

SUMMARY AND CONCLUSIONS

Various parameters affecting the production of crude toxins from liquid cultures of Aspergillus parasiticus ATCC 15517 were examined. ^{14}C labeled aflatoxin B_1 was isolated from crude toxins produced under the following conditions:

1. Primary culture time, 72 hours.
2. Resting culture time, 12 hours.
3. 0.02 M glucose and 0.005 M acetate as sole carbon sources in resting culture.
4. Incubation temperature, 30° C.

After purification, 1.37 mg of ^{14}C labeled aflatoxin B_1 of specific activity 744 μci/mmole was obtained from 2 mci acetate-1-^{14}C of specific activity 1 μci/mmole (after dilution with unlabeled acetate in liquid culture to give 0.005 M acetate).

^{14}C labeled aflatoxin B_1 was isolated from crude toxins by column chromatography on silica gel H eluted with a continuous gradient of chloroform to chloroform:methanol 98:2 v/v with a metering pump. The ultraviolet spectrum of the ^{14}C labeled aflatoxin B_1 was examined. The ratios of the absorbances at 220 nm/265 nm and 362 nm/265 nm compared to those of Rodricks (114) and reference standard were used as a criteria of chemical purity. Label integrity

was verified by chromatography, hydrogenation to the tetrahydro-deoxoaflatoxin B_1 and conversion to the hemiacetal and the epimeric acetates.

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APPENDICES

APPENDIX I

Safety Precautions and Procedures

1. Spores of Aspergillus parasiticus should only be handled in a hood with the worker wearing a mask, gloves and lab coat. Aspergillosis has been reported from several species of Aspergillus. Persons taking antibiotics or steroids should avoid exposure to spores.
2. Because of their reported toxicity and carcinogenicity workers should wear vinyl gloves and lab coats whenever handling glassware containing the toxins.
3. Crystalline aflatoxin develops a static charge and easily becomes airborne and should only be handled in a glove box.
4. Sodium hypochlorite (5%) is recommended for decontaminating aflatoxin exposed materials.
5. Spores to be destroyed should be treated with 5% sodium hypochlorite followed by autoclaving at 121° C at 15 psi for at least 20 minutes. Before autoclaving the chlorine should be removed by addition of sodium thiosulfate until starch iodide indicator paper no longer turns blue.

References: Goldblatt (62), Harrington (65) and Rodricks (110).

APPENDIX II

Preparation of Mold Spores for Inoculation

1. Prepare pour plates with Czapek's solution agar:

   35 g Czapek Dox broth

   20 g Bacto mycological agar

   1 l distilled water

2. Inoculate plates with spores of Aspergillus parasiticus ATCC 15517 to be recultured and incubate at 30° C until discrete colonies are obtained.

3. Using an isolated A. parasiticus colony as an inoculum, streak two plates heavily with a sterile loop. Incubate at 30° C until a good sporulation is obtained.

4. Rinse spores from the plates with 5 ml of sterile distilled water. Collect the spores in a sterile petri dish.

5. Add 100 g of garden soil (free of fertilizer and pesticides) to an Erlenmeyer flask and plug with cotton. Sterilize at 121° C, 15 psi for 30 minutes. After cooling inoculate soil with 1 ml portion of above spore suspension. Incubate at 30° C for 10 days.

6. Fill a 1 liter Roux culture bottle with 100 ml of Czapek's solution agar. Plug with cotton and sterilize at 121° C, 15 psi for

15 minutes. Allow to solidify on flat side of bottle by cooling.

7. Inoculate Aspergillus parasiticus spores on agar face of Roux bottle by streaking heavily with a sterile loop spores from the dirt culture. Add 5 ml of sterile water. Distribute evenly by tapping bottle.

8. Incubate at 27° C for 2-3 days flat side down. Turn the bottle over and incubate 5 more days.

9. Add 100 ml of sterile dilute soap solution (0.02% sodium dodecyl sulfate in distilled water) to each bottle and rinse spores from agar surface. Wash spores with two 150 ml portions of sterile distilled water by centrifugation.

10. Suspend spores in 150 ml of sterile distilled water and store suspension in sterile 50 ml bottles at 2° C. Spores may be kept in this manner for 3 months.

11. Inoculate pour plates of Czapek's agar from serial dilutions of the spore suspension in sterile water. After three days incubation at 30° C count comparable triplicate plates containing 3-30 spores. Calculate the number of viable spores per ml in the 50 ml bottles of spore suspension.

Reference: Mateles and Ayde (85).

APPENDIX III

Replacement Media

Compound	Medium 1 (g/l)	Medium 2 (g/l)	Medium 3 (g/l)	Medium 4 (g/l)
Glucose	50.0 (0.278 M)	15.0 (0.083 M)	0.9 (0.005 M)	3.6 (0.02 M)
Acetate			0.164 (0.002 M)	0.410 (0.005 M)
KH_2PO_4	10.0	5.0	5.0	5.0
$MgSO_4 \cdot 7H_2O$	2.0	0.5	0.5	0.5
KCl		0.5	0.5	0.5

Trace metals for each medium at the following concentrations (mg/l):

$Na_2B_4O_7 \cdot 10H_2O$, 0.7; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.5; $Fe_2(SO_4)_3 \cdot 6H_2O$, 10.0; $CuSO_4 \cdot 5H_2O$, 0.3; $MnSO_4 \cdot H_2O$, 0.11; $ZnSO_4 \cdot 7H_2O$, 17.6.

References: Ayde and Mateles (10), Donkersloot, Hsieh and Mateles (52) and Hsieh and Mateles (72).

APPENDIX IV

Biological Preparation of Aflatoxin

A. Primary Culture

1. Prepare stock solutions of glucose, salts and trace metals as indicated in Table 12.
2. For eight cultures of 100 ml each combine nine times the aliquots of stock per flask indicated in Table 12. of the salts and trace metals in a 1000 ml Erlenmeyer flask. Add water to make to 450 ml, stopper with a cotton plug and sterilize at 121° C for 15 minutes at 15 psi. Prepare and sterilize a separate 500 ml Erlenmeyer flask with nine times the aliquot of stock per flask of glucose indicated in Table 12.
3. Add one ninth of the volume of the above flasks to each of eight sterile 50 ml Erlenmeyer flasks. Make each flask to 100 ml with sterile water.
4. Measure the pH. If necessary adjust the pH to within the range of 4 to 5 with either sterile dilute HCl or NaOH.
5. Incubate the flasks at 30° C on a rotary shaker equipped with a water bath at 144 rpm for the first 24 hours and 200 rpm for the remainder of the time.
6. Cease incubation at 72 hours.

Table 12. Primary culture medium.

Compound	Stock solution	Aliquot of stock per flask	Final concentration per flask
	(g/250 ml)	(ml)	(g/l)
Glucose	50	25	50
	(g/100 ml)		
$(NH_4)_2SO_4$	4.0	10	4.0
KH_2PO_4	10.0	10	10.0
$MgSO_4 \cdot 7H_2O$	2.0	10	2.0
	(mg/100 ml)		(mg/l)
$Na_2B_4O_7 \cdot 10H_2O$	70	0.1	0.7
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	50	0.1	0.5
$Fe_2(SO_4)_3 \cdot 6H_2O$	100	0.1	10.0
$CuSO_4 \cdot 5H_2O$	30	0.1	0.3
$MnSO_4 \cdot H_2O$	11	0.1	0.11
$ZnSO_4 \cdot H_2O$	176	0.1	17.6

Reference: Ayde and Mateles (10).

B. Harvest of Mycelia

1. Collect separately the mycelia from each flask on a cheesecloth over a Buchner funnel attached to a suction flask equipped with a trap. Rinse well with distilled water.
2. Place the mycelia from one flask in a blender.[1/] Add 100 ml of distilled water and chop the mycelia 10 seconds on the "blend" setting.
3. Collect the mycelia as before on a fresh cheesecloth. Rinse well with distilled water.
4. Place the cheesecloth with the fresh wet mycelial cake on a piece of aluminum foil and weigh. Remove 5.0 g of mycelial cake and place in a clean indented 500 ml Erlenmeyer flask.

C. Resting Culture

1. Add solutions of glucose, acetate, salts, trace metals and water to the flasks containing the freshly prepared mycelia to give 100 ml of medium with the following concentrations: glucose 0.02 M, acetate 0.005 M, KH_2PO_4 5.0 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, KCl 0.5 g/l and trace metals as in the primary culture. When acetate-1-^{14}C is used the amount of unlabeled

[1/] Kenmore Blender Model 600, Sears, Roebuck and Co., Chicago, Illinois.

acetate should be adjusted so that the combined acetates are still 0.005 M.

2. Incubate the flasks at 30° C on a rotary shaker equipped with a water bath at 200 rpm.

3. Cease incubation between 12 and 20 hours and before the pH decreases below 3. The absorbance at 362 nm of 1 ml of medium extracted with 2 ml of chloroform should indicate a yield of greater than 0.500 mg per 100 ml using 20,000 as the molar extinction coefficient (Appendix VII).

D. Extraction

1. Filter the medium containing the crude toxins of each flask through a separate cheesecloth in a Buchner funnel attached to a suction flask with a trap attached. Rinse each flask and mycelial cake with 100 ml of chloroform.

2. Combine the filtrates in a 3 liter separatory funnel. Add chloroform equivalent in volume to 75 ml per flask. Mix the contents and allow the phases to separate.

3. Draw off the lower chloroform phase a little at a time and concentrate on a flash rotary evaporator[2/] at room temperature.

4. Isolate the aflatoxin B_1 using column chromatography (Appendix VI).

2/ Model PF-10DN, Buchler Instruments, Fort Lee, New Jersey.

APPENDIX V

Thin Layer Chromatography
Preparative and Analytical

1. Prepare adsorbent as indicated by the manufacturer.

   For MN-silica gel G-HR[3/]

   Shake 30 g silica gel with 60 g water for 1 minute. Spread plates 250 microns or 500 microns thick for analytical and preparative TLC respectively. Activate for 2 hours at 110° C. Cool in desiccator and preferably use immediately.

2. Quickly spot or streak under incandescent light either:

   a. For analytical purposes. 10 μl spots containing 50 μg per ml of aflatoxin B_1 give good separation and detection.

   b. For preparative purposes. More than two streak applications of 250 μl each is discouraged because the amount of degradation product found at the origin appears to increase with the length of time for sample application.

3. Develop in solvent of choice (see Literature Review and Experimental) in a chromatography tank protected from the light and sudden temperature changes. Chloroform:acetone 9:1 v/v

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3/ Brinkman Instruments, Inc., Westbury, New York.

unequilibrated gives excellent separation of the four major toxins from each other in this climate.

4. Locate the fluorescence of the toxins with a 365 nm ultraviolet source.[4/][5/]

5. To remove toxins outline the fluorescent regions with a sharp object and, with two spatulas which have sharpened flat ends, one in each hand, scrape into each other in the manner one would sweep dust into a dustpan.

6. For labeled regions place the scrapings directly into toluene gel (Appendix X).

7. To examine spectroscopically add the scrapings to a column previously packed with one half inch of coarse anhydrous Na_2SO_4 on the bottom and an inch of fine anhydrous Na_2SO_4 on top in methanol. Elute with methanol and concentrate for spectroscopy according to Appendix VII.

8. Alternatively, to elute and remove silica gel or other support, place in a conical test tube, fill with methanol, mix on a Vortex mixer[6/] and centrifuge in a clinical centrifuge.[7/]

[4/] Chromato-vue cabinet, U. V. Model C-5, Ultraviolet Products, Inc., San Gabriel, California.

[5/] Blak-Ray UVL-22, San Gabriel, California.

[6/] Delux Mixer, Cat. No. 58220, Scientific Products, Evanston, Illinois.

[7/] Model CL, International Equipment Co., Needham Heights, Mass.

APPENDIX VI

Column Chromatography

1. Activate MN-silica gel H[8] at 150° C for 2 hours. Cool in a desiccator and use immediately. Quickly weigh 10 g into a 250 ml beaker. Add 200 ml chloroform and mix as a slurry.
2. Pour the slurry into a plumb 500 X 12 nm column.[9] A 1000 X 12 nm glass extender[10] is attached to the top of the column with an "O" ring[11] and clamp[12] so that the entire slurry can be added at once. The bottom of the column is fitted by a clamp with a fritted disc[13] in an adapter which has a Teflon stopcock.[14]

8/ Brinkman Instruments, Inc., Westbury, New York.

9/ Jacketed Chromaflex extender, K-422430, Kontes Glass Co., New Jersey.

10/ Jacketed Chromaflex extender, K-422430, Kontes Glass Co., New Jersey.

11/ "O" rings, type M, K 758280, Kontes Glass Co., New Jersey.

12/ Ground joint clamp, KG75050, Kontes Glass Co., New Jersey.

13/ Fritted disc, K952050, Kontes Glass Co., New Jersey.

14/ Chromaflex adapter, K-422380, Kontes Glass Co., New Jersey.

3. Tap the column gently and continuously to ensure even packing. Once the packing is well below the joint between the extender and the column to be used, wrap the joint with towels to absorb the chloroform and remove the extender.

4. When the column has packed as thoroughly as possible by gravity flow, fill the headspace of the column with chloroform. Clamp on "O" ring adapter with an attached 0.044 inch (id) Teflon tube to the top of the column. Connect the free end of the tube to a metering pump.[15] Pump solvent through the column at 60 ml per hour until the height of packing material remains constant.

5. Detach the "O" ring adapter and add acid washed and fired sand to a height of about 10 mm above the silica gel. Remove the chloroform to the surface of the sand.

(Carry out the remaining operations in subdued incandescent light).

6. Concentrate the sample to 1 ml in chloroform in a pear shaped flask on a flash rotary evaporator at room temperature. Carefully apply the sample to the sand layer with a pipette. Force the sample into the sand and onto the top of the silica gel layer with air pressure applied by hand with a suction bulb to the top of the column. Rinse the pear shaped flask with 1 ml of

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[15] Cheminert metering pump, Model CMP-1, Chromatronix, Inc., Berkeley, California.

chloroform and apply to the column in the same manner.

7. Carefully fill the column with chloroform taking care not to disturb the sample layer. Reattach the metering pump to the column.

8. Add 600 ml of chloroform to a 1 liter Erlenmeyer flask containing a large Teflon coated stirring bar. Add 600 ml of chloroform:methanol 98:2 v/v to a second 1 liter Erlenmeyer flask. Attach the two Erlenmeyers with a glass siphon. Attach the chloroform containing flask to the metering pump and stir it with a magnetic stirrer.

9. Turn on the metering pump at a flow rate of 60 ml per hour. Collect fractions at the rate of three per hour for the first eight hours and six thereafter. Depending on the activity of the packing material and the tightness of the pack pure aflatoxin B_1 may begin to elute at nine hours.

10. Estimate the tubes containing aflatoxin B_1 by quick examination with a handheld ultraviolet light. Determine the exact composition of the tubes by analytical TLC (Appendix V).

11. Combine the center fractions of aflatoxin B_1 and determine purity and concentration by ultraviolet spectroscopy (Appendix VII).

12. If labeled, this fraction may be counted to a 1% error with a toluene cocktail (Appendix X).

APPENDIX VII

Determination of Concentration and Purity by Ultraviolet Spectroscopy

The following data are necessary for determination:

Aflatoxin B_1 M.W. 312 in methanol (ethanol also acceptable)

λ, nm	ε	ratios of the absorbances
223	22, 100	220/265 = 1.77
265	12, 400	362/265 = 1.76
360	21, 800	

Crude toxin in chloroform

λ, nm	ε
362	20, 000

1. After removing all other solvents add the selected solvent to give an approximate concentration of 8-10 μg/ml of aflatoxin B_1.
2. Measure the absorbance at the above wavelengths with a suitable recording spectrometer, Beckman DK-1 or DB-G.[16/]
3. The concentration in μg/ml is calculated from

$$\mu g/ml = \frac{(A)\ (MW)\ (1000)\ (CF)}{\epsilon}$$

where A is the absorbance; MW is the molecular weight of the toxin; CF is the correction factor for the spectrometer;[17/]

16/ Beckman Instruments, Inc., Fullerton, California.

17/ Calculated by the method of Rodricks and Stoloff (111).

ϵ is the molar absorptivity for the wavelength at the absorbance and the designated solvent system.

Reference: Rodricks et al. (114).

APPENDIX VIII

Preparation of Aflatoxin B_1 Derivatives: The Hemiacetal and Epimeric Acetates

1. Place a chloroform extract containing 1 μg (0.1 μg for control) of aflatoxin B_1 in a one dram vial. Remove chloroform by evaporation under nitrogen on a steam bath.
2. Dissolve residue in 1.0 ml benzene:acetonitrile 98:2 v/v.
3. Transfer 0.25 μg of the aflatoxin B_1 to each of two half dram vials.
4. For the hemiacetal add 100 μl of water and a drop of concentrated hydrochloric acid to one of the vials.
5. For the epimeric acetates add 250 μl of acetic anhydride and one drop of hydrochloric acid to the other vial.
6. Seal the vials with foil lined caps. Mix well on a Vortex mixer. Heat 10 minutes on a steam bath with occasional agitation.
7. Cool, then open vials. Evaporate contents to dryness under nitrogen on a steam bath.
8. Dissolve products in 20 μl of benzene:acetonitrile 98:2 v/v.
9. Spot 10 μl of both the sample and control hemiacetals and acetates next to each other on a MN-silica gel G-HR plate prepared for analytical TLC (Appendix V). In addition, spot 20 ng of the presumptive aflatoxin B_1 and next to it 20 ng of

resolution reference standard.

10. Develop with chloroform:acetone 9:1 v/v.

11. Examine the plate under 365 nm ultraviolet radiation. The derivatives have dominant fluorescence. The hemiacetal has an R_f approximately 10% of unreacted aflatoxin B_1. The epimeric acetates appear as two spots of about equal size near or slightly behind the B_1 to G_2 area. The lower R_f spot is the brightest.

Reference: Pohland, Yin and Dantzman (97).

APPENDIX IX

Hydrogenation of Aflatoxin B_1

1. Dissolve 1 mg of aflatoxin B_1 in 30 ml of ethanol in a 125 ml T 24/40 round bottom flask fitted with a syringe septum. (If labeled aflatoxin is to be used adjust the proportion of unlabeled and labeled toxin to facilitate with ease counting to a 1% error when monitoring the course of the reaction.) In addition, add 50 mg of 5% Pd/C catalyst[18] and a Teflon stirring bar. Attach the flask to a Brown hydrogenation apparatus.[19]
2. To the hydrogen generator flask add 25 ml of glacial acetic acid and a large Teflon stirring bar. Attach the flask to the apparatus. Start the stirrer and inject 5 ml of 1 M $NaBH_4$ (39.5 g $NaBH_4$ per liter stabilized with 5 g of NaBH) to purge the system.
3. After purging the system once more, start the stirrer in the hydrogenation flask and open the stopcock for the $NaBH_4$ reservoir over the generator flask.
4. At half hour intervals withdraw 1 ml aliquots from the hydrogenation flask. Filter through #1 filter paper to remove the

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[18] Engelhard Industries, Inc., Newark, New Jersey.

[19] Delmar Scientific, Maywood, Illinois.

catalyst. Rinse the filter paper twice with 2.5 ml of chloroform.

5. Evaporate the filtrate to dryness and make to 500 μl with chloroform or benzene:acetonitrile 98:2 v/v. Carry out analytical TLC according to Appendix V.

Reference: Ayres (11).

APPENDIX X

Liquid Scintillation Counting Fluor Solutions

Name	Fluor solution components[a]	Solution volume (ml)	Sample		^{14}C counting[b]	
			Volume (ml)	Type	Efficiency[c] (%)	Background (cpm)
Toluene	1 l toluene, 4 g PPO, 40 mg POPOP	15	0-1	Organic	82	26
Toluene gel	1 l toluene, 4 g PPO, 40 mg POPOP, 40 g Cab-o-sil	15	0-1	TLC scrapings	82	26
Dioxane	980 ml dioxane, 4 g PPO, 200 mg POPOP, 60 g napthalene, 100 ml methanol, 20 ml ethylene glycol	15	0-3	Aqueous	65	23

[a] PPO: 2,5-diphenyloxazole (Scintillation grade); POPOP: 1,4 bis[2-(5-phenyloxzoyl)] (Scintillation grade); napthalene, dioxane, toluene, methanol and ethylene glycol (reagent grade); Cab-o-sil: Godfrey L. Cabot, Inc.

[b] Nuclear Chicago Liquid Scintillation Spectrometer.

[c] Efficiency determined by optimal settings of unquenched sample. Channels ratio and internal standard used to determine efficiency of samples.

References: Bray (21) and Wang and Willis (148).