BACTERIOLOGICAL REVIEWS, Dec. 1977, p. 822-855
Vol. 41, No. 4

Printed in U.S.A.

Biosynthesis of Aflatoxins

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INTRODUCTION

Fifteen years have passed since the discovery of aflatoxin, the most potent hepatocarcinogen known. These years can best be described as the era of the "mycotoxin gold rush," which led to the discovery of a large number of mycotoxins. These mycotoxins have been tabulated and reviewed extensively (49, 57, 114, 149, 150, 179, 242, 243, 280, 280a, 321). In addition to the above monographs, a large number of reviews dealing with the various aspects of aflatoxin and other mycotoxins have appeared (13, 39, 50, 51, 55, 95, 99, 115, 142, 143, 152, 174, 183, 205, 212, 218, 239, 255, 265, 317, 322-326). Many symposia on aflatoxins and other mycotoxins have been held, and the proceedings have appeared either in book form or in various journals.

Most of the reviews on aflatoxin mentioned above have not covered the biosynthesis of aflatoxins to any great extent. The reviews dealing with the biosynthesis of aflatoxins are by Detroy et al. (81), Roberts (257), and Berger and Jadot (26). Various theories for the biogenesis of aflatoxins have been discussed by the above authors. Recent advances in the study of aflatoxin biosynthesis have outdated these reviews in many respects. Moreover, ever since the discovery of aflatoxins, none of the published reviews have dealt with the relationship between aflatoxin formation and the primary metabolism of the *Aspergillus flavus* subgroup. We have investigated the relationship between primary and secondary fungal metabolism with particular reference to aflatoxin production by *A. flavus* and *Aspergillus parasiticus*. The results obtained provide some understanding of the profound changes taking place in the fungal physiology before, during, and after the onset of aflatoxin production; the mechanism of stimulation of aflatoxin production by zinc; and the comparative biochemical differences in the aflatoxigenic and non-aflatoxigenic strains.

The topics covered in the previous reviews (26, 76, 257) will not be covered here. The pres-

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ent review supplements the existing reviews by covering newly available information and by dealing mainly with the relationship between primary and secondary fungal metabolism.

CHEMICAL ASPECTS

Primary and Secondary Fungal Metabolites

Metabolites essential for growth and formed during cell multiplication, like proteins, nucleic acid, lipids, and carbohydrates, are termed primary metabolites. On the other hand, substances formed during the end of the exponential growth phase of the fungus and having no apparent significance in the fungal growth or physiology are termed secondary metabolites. Whereas primary metabolism is basically the same for all living systems, secondary metabolism is restricted to lower forms of life and is strain specific. The factors controlling secondary metabolism in fungi are complex and are not fully understood at present. The great chemical diversity of secondary metabolites has been covered extensively (49, 72, 296–298, 311–313, 317); these include such products as quinones, antibiotics, mycotoxins, alkaloids, and other pigments. The functions of secondary fungal products have been discussed (44, 74, 311, 312). The most commonly agreed postulate suggested that secondary metabolites are formed when large amounts of primary metabolic precursors, such as acetate, malonate, pyruvate, and amino acids, accumulate. The fungus gets rid of these precursors by diverting them into secondary metabolites (74, 227). The regulation of secondary fungal metabolism has been the subject of reviews by Berry (27), Bu'Lock (42–44), Turner (297, 298), Weinberg (311–313), and Demain (70–73).

Metabolites of the *A. flavus* Subgroup

The structures of the aflatoxins and other bisfuranoids produced by *A. flavus* and *A. parasiticus* are shown in Fig. 1 and 2. Figure 1 gives only the aflatoxins that have been obtained from fungal cultures and excludes biologically

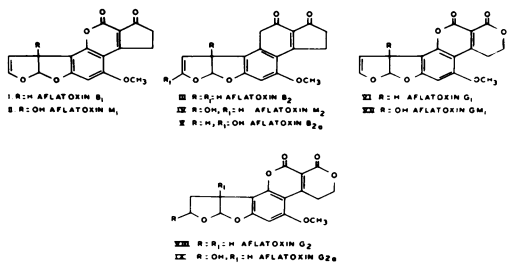

FIG. 1. *Structure of aflatoxins.*

reduced and hydroxylated aflatoxins, such as aflatoxicol and aflatoxins Q_1, H_1, and P_1. Apart from aflatoxins, a large number of metabolites produced by fungi of the *A. flavus* group have been characterized (57, 218). Wilson (316) has given a detailed account of the known toxins of *A. flavus* other than aflatoxins. These include oxalic, kojic, β-nitropropionic, and aspergillic acids. Yokotsuka et al. (329) have characterized 15 pyrazine compounds from *Aspergillus oryzae* strains.

Considerable work has been done on the isolation, characterization, and synthesis of polyhydroxyanthraquinones of the *A. flavus* subgroup and *Aspergillus versicolor* (11, 23–26, 119–120, 218, 257). Some quinones have been suggested as biogenetic precursors of aflatoxins and other bisfuranoids. Grove (119–120) has isolated asperantin, 4- and 5-hydroxyasperantin, asperflavin, anhydroasperflavin, and derivatives from *A. flavus*. Berger and Jadot (25) isolated two new metabolites, namely, deoxyaverufinon and dehydroaverufin, from the cultural filtrate of *A. versicolor*. The structures of some of the pigments, like averufin, averantin, averythrin, bipolarin, and norsolorinic acid, that have been postulated as biogenetic precursors of aflatoxins are shown in Fig. 3. In general, most of the anthraquinones of the aversin and versicolorins types are produced by *A. versicolor, A. nidulans,* and *Bipolaris* species (11). None of these species produce aflatoxins, thereby indicating a metabolic block in aflatoxin biosynthesis. Dutton and Heathcote (90) isolated two anthraquinones, averufanin and versicolorin C. Bennett et al. (22) reported the presence of norsolorinic acid in *A. parasiticus*. Benzoquinones, including two isoprenoid precursors of ubiquinone (Q_{10}), have been isolated from *A. flavus* (186, 304, 304a). Recently, averufin and versicolorin A have been isolated from *A. parasiticus* strains deficient in aflatoxin production (121, 192).

Kingston et al. (182) isolated griseofulvin, dechlorogriseofulvin, 6,8-di-*O*-methylnidurufin, and 3,8-dihydroxy-6-methoxy-1-methylxanthone from *A. versicolor*. Sterigmatocystin and some of its derivatives, namely, *O*-methylsterigmatocystin, aspertoxin, and versicolorins C and A, have been isolated from aflatoxin-producing strains of *A. parasiticus,* and their structures have been established (48, 63, 148, 192, 193, 258, 307). Schroeder and Kelton (268) observed that all the sterigmatocystin-producing cultures of *A. flavus* and *A. parasiticus* also simultaneously produced aflatoxins and *O*-methylsterigmatocystin. Less production of sterigmatocystin was observed in *A. parasiticus* cultures (12 μg) as compared with *A. flavus* (381 μg), thereby showing that the conversion of ste-

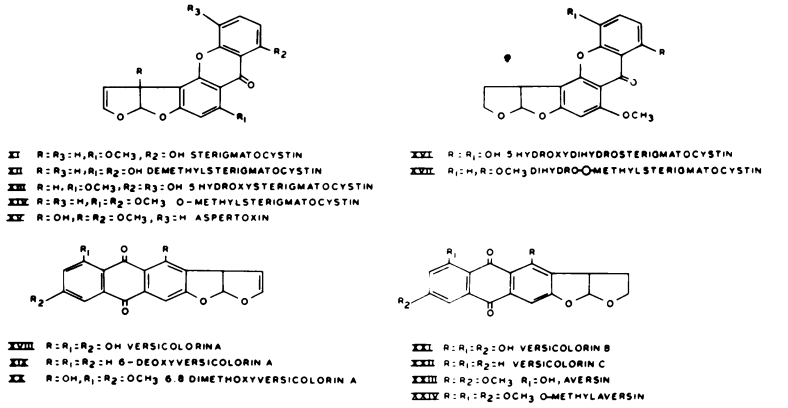

FIG. 2. *Structures of bisfuranoids.*

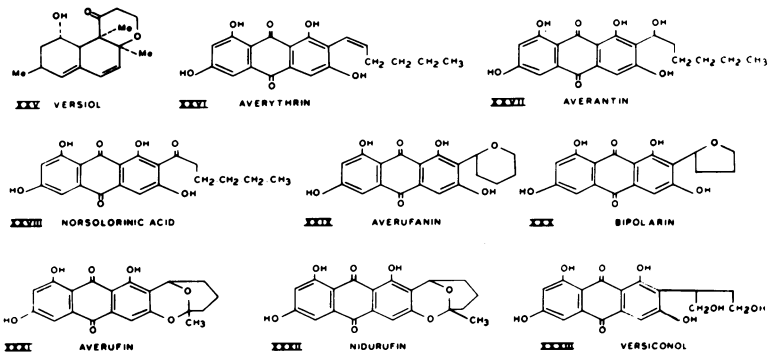

FIG. 3. *Structures of polyhydroxyanthraquinones and versiol.*

rigmatocystin into aflatoxin is more efficient in *A. parasiticus*. Elseworthy et al. (94) isolated demethylsterigmatocystin and 6-deoxyversicolorin A from *A. versicolor*. Recently, Fukuyama et al. (111) have isolated a new metabolite, versiol, that may be a biogenetic precursor of sterigmatocystin. The labeling pattern of sterigmatocystin and averufin derived from [^{13}C]acetate has been established by ^{13}C nuclear magnetic resonance and chemical degradation (23, 157, 231, 292). Roberts (257) has reviewed the chemistry, synthesis, and biosynthesis of sterigmatocystin.

Preparation of Labeled Aflatoxins

Isotope-labeled aflatoxins with high specific activities are useful for studies on biosynthesis. Adye and Mateles (2) were the first to obtain ^{14}C-labeled aflatoxins by incubation of sodium [^{14}C]acetate with mold mycelia in a nitrogen-free resting-cell medium. Later workers prepared labeled aflatoxins with high specific activities in resting-cell cultures (16, 29, 30, 79, 87, 163, 164, 200). Ayres et al. (14) showed that the resting-cell systems of *A. parasiticus*, after separation by thin-layer chromatography, yielded an aflatoxin that was contaminated with a highly radioactive impurity, the latter having chromatographic characteristics similar to those of aflatoxin B_1. Detroy and Hesseltine (79) and Gupta et al. (136) found that incorporation of labeled acetate into aflatoxins was favored by acid conditions and inhibited by higher concentrations of sodium acetate. Hsieh and Mateles (164) found that glucose was required for high incorporation efficiency, and 40 to 70 h of incubation gave high yields. High levels of exogenous acetate inhibited incorporation of radioactivity from labeled glucose into aflatoxins. By continuously adding labeled acetate, 2% of the label could be recovered in aflatoxins. Lijinsky (194) reported preparation of ^{3}H-labeled aflatoxins by catalytic tritium exchange. Substantial degradation of aflatoxins was observed with this technique. Ayres et al. (14) used rice medium to support fungal growth, and labeled precursors were added to sterilized rice to get radioactive aflatoxin. However, the incorporation efficien-

cies were only 0.1 and 0.05% for aflatoxins B_1 and G_1, respectively, using [1-^{14}C]acetate. Gupta et al. (136) obtained an incorporation efficiency of about 1% of sodium [1-^{14}C]acetate into aflatoxins. The mycelium was grown for 4 days on a sucrose-asparagine-salts medium (251) and suspended in the presence of [1-^{14}C]acetate in phosphate buffer for 2 h. Sucrose and asparagine were found to inhibit [1-^{14}C]acetate incorporation into aflatoxin. Ethanol was found to markedly inhibit ^{14}C incorporation into aflatoxins. Two of the tricarboxylic acid cycle intermediates, oxaloacetic and citric acids, gave good stimulation of incorporation, as did malic acid at low concentrations. Ethylenediaminetetraacetate enhanced the incorporation of both labeled acetate and malonate into aflatoxins. Ethylenediaminetetraacetate, L-cycloserine, and methionine sulfoxide stimulated incorporation of acetate into aflatoxins at all levels tried (from 0.1 to 10 mM). Malonic acid, malic acid, iodoacetic acid, sodium arsenite, 2,4-dinitrophenol, *o*-iodobenzoic acid, sodium fluoride, boric acid, *p*-aminosalicyclic acid, desoxypyridoxine hydrochloride, oxythiamine chloride, and β-phenylbutyric acid stimulated incorporation of labeled acetate into aflatoxins at low concentrations and inhibited this process at high concentrations. *p*-Nitrobenzoic acid, anthranilic acid, L-cysteic acid, dehydroacetic acid, sulfanilamide, sodium arsenate, salicylaldehyde, acetylpyridine, salicyclic acid, and sodium azide were inhibitory at all concentrations tried (137, 139, 141). Fumaric acid, malic acid, and alanine partly reversed the inhibition caused by arsenite and iodoacetic acid. Some inhibitors, like fluoride, arsenite, arsenate, salicylaldehyde, and iodoacetate, inhibited fungal growth and aflatoxin production as well as ^{14}C incorporation into aflatoxins. Moreover, a stronger inhibition was observed in the growth medium than was observed in the suspension medium (131). Hsieh and Yang (165), by continuously adding labeled acetate, obtained an incorporation efficiency of 3% in sterigmatocystin.

Precursors of Aflatoxins

Adye and Mateles (2) demonstrated incorporation of ^{14}C-labeled methionine, phenylalanine, tyrosine, tryptophan, and acetate into aflatoxin B_1. Mateles and Wogan (212) showed that addition of unlabeled alanine or acetate drastically reduced the amount of label incorporated from tryptophan, thereby showing that tryptophan was degraded into alanine, pyruvate, and acetate. Many workers have shown that aflatoxins are acetate derived (2, 15, 30, 79, 87, 163, 164). Gupta et al. (136), Heathcote et al. (147), and Donkersloot et al. (87) found [2-^{14}C]acetate to be a better precursor than [1-^{14}C]acetate. Raj et al. (244) found that labeled acetate, leucine, and mevalonate were incorporated into aflatoxin molecules in the presence of a cell-free enzyme system prepared from a toxin-producing strain of *A. flavus*. Methionine specifically labeled the methoxy group of aflatoxins (2).

Later work indicated that labeled tyrosine, phenylalanine, and tryptophan were not readily incorporated into aflatoxins (87, 147). The earlier observations of Adye and Mateles (2) were explained as due to the formation of complexes between aflatoxins and added labeled amino acids that were very difficult to break down (224). Thus, the possibility of aflatoxins being derived by the shikimate pathway is ruled out. Moreover, labeled shikimate and mevalonate were not incorporated (87). Heathcote et al. (147) did not observe any incorporation of labeled malate, fumarate, succinate, malonate, and aspartate into aflatoxins. Acetoacetate showed rapid uptake into aflatoxins, and isoleucine and mevalonate were incorporated to some extent. The incorporation observed with isoleucine may again be due to its formation of a charge transfer complex with aflatoxin. The incorporation of labeled mevalonate fails to explain the resultant labeling pattern of aflatoxin B_1 in the bisdihydrofuran moiety of the molecule. The labeling pattern, as obtained by degradative studies, showed that, of 16 carbon atoms in the ring structure of aflatoxin B_1, 8 were derived from the carboxyl group (C-1) and 7 were derived from methyl group (C-2) carbon atoms of acetate (30). However, Biollaz et al. (30) were not able to recover the 3 skeletal carbon atoms, with the result that only 13 of the 16 carbon atoms of aflatoxin B_1 were defined. Recently, the labeling pattern of aflatoxin B_1 derived from [1-^{13}C]- and [2-^{13}C]acetate has been studied by ^{13}C nuclear magnetic resonance spectrum (167, 231, 284). The above workers were able to assign the label for all the carbon atoms of the aflatoxin B_1 molecule, including the skeletal carbon atoms, C-8, C-10, and C-12. Hsieh et al. (168) further showed that the labeling patterns of ^{13}C-labeled aflatoxin B_1, derived from [1-^{13}C]acetate, and ^{13}C-labeled averufin, which was also [1-^{13}C]acetate derived (102), were identical. Nine of the C atoms of aflatoxin were derived from averufin. The activities of the labeled carbon atoms were virtually equal throughout the molecule. The arrangement of the acetate units in aflatoxin and sterigmatocystin assigned by Pachler et al. (231) was different than that of Biollaz et al. (30) and Seto et al. (262). The aromatic rings of aflatoxin and sterig-

matocystin contain three acetate units and must be derived from an aromatic precursor. The resultant labeling patterns and origins of carbon atoms of aflatoxin B_1, averufin, and sterigmatocystin are depicted in Fig. 4 (102, 167, 168, 231, 284, 292).

It is clear from Fig. 4 that, although the usual alternation of the C-1 and C-2 acetate carbon atoms occurs throughout the molecule (the coumarin portion and the terminal dihydrofuran ring), the C-C bond joining the dihydrofuran moiety to the aromatic ring is formed between two carbon atoms that originate in acetate methyl groups (C-2). There are also two adjacent carbon atoms of C-1 acetate origin. Two different hypotheses have been advanced to explain this, namely, the single-polyketide (30) and the two-polyketide (147, 148) theories.

Holker and Kagal (156) suggested that sterigmatocystin may be biologically derived from versicolorin A or a related compound. Holker and Mulherin (157) and Tanabe et al. (292) showed that sterigmatocystin was acetate derived. It was proposed that sterigmatocystin was biogenetically derived from two separate polyketide units. Basappa et al. (16) failed to observe incorporation of labeled kojic acid into aflatoxins. Elseworthy et al. (94) showed that 5-hydroxydihydrosterigmatocystin was a biogenetic precursor of aflatoxins B_2 and G_2. It was further proposed that aflatoxin B_2 was a precursor of aflatoxin B_1. We have reported that aflatoxin B_1 is the biogenetic precursor of all the other aflatoxins (203). Heathcote et al. (148) converted labeled aflatoxin M_1 into other aflatoxins, such as B_1, B_2, G_1, G_2, M_2, GM_1, and M_{2a}. The conversion of aflatoxin B_1 into aflatoxins B_2, B_{2a}, G_1, G_2, and G_{2a} by *A. parasiticus* was also shown (148). None of the aflatoxins of the B or G series could be converted to aflatoxin M_1. It was proposed that aflatoxin M_1 is the biogenetic precursor of other aflatoxins.

Hsieh et al. (166) found that labeled sterigmatocystin was converted to aflatoxin B_1 by the resting mycelium of *A. parasiticus*. A cell-free preparation for the same conversion has been reported (278). Similarly, Lin and Hsieh (196) and Lin et al. (197) converted labeled averufin into aflatoxins B_1, B_2, G_1, and G_2. The conversion of versicolorin A and norsolorinic acid into aflatoxin has been achieved recently (169, 193). Bennett et al. (21, 22) have correlated the production of aflatoxins and pigments like versicolorins and norsolorinic acid. In a resting-cell suspension medium, higher concentrations of glucose stimulated aflatoxin and pigment formation. Additions of acetone and ethanol to the suspension medium resulted in opposite effects on the production of aflatoxin and versicolorin, showing the inverse relationship between the two pathways. Thus, norsolorinic acid, versicolorin A, sterigmatocystin, and averufin have been shown to be the biogenetic precursors of aflatoxins.

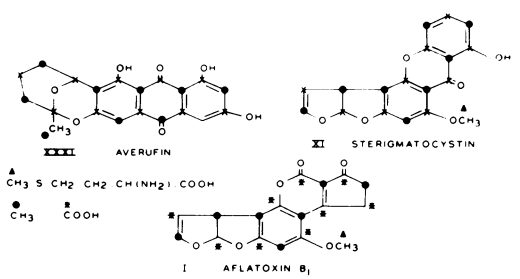

FIG. 4. *Labeling patterns of aflatoxin B_1 and its precursors.*

Mutant Strains in the *A. flavus* Subgroup

Accumulation of intermediates. Numerous workers have studied impaired aflatoxin biosynthesis by mutants of *A. flavus* and *A. parasiticus*. These mutants possess apparent blocks in the biosynthetic pathway for aflatoxins and may accumulate products that may be intermediate in the biogenetic scheme. It would be reasonable to assume that certain mutant strains lack an enzyme system for the conversion of accumulated compounds into aflatoxins (279). Donkersloot et al. (88) reported mutants of *A. parasiticus* impaired in aflatoxin biosynthesis that accumulated averufin, a possible intermediate. Lee et al. (191, 192) reported on mutants of *A. parasiticus* accumulating two possible intermediates, norsolorinic acid and versicolorin A.

Detroy et al. (82) showed that a nitrosoguanidine-derived mutant of *A. parasiticus* produced 100 to 200 times more norsolorinic acid than aflatoxin B_1, thereby indicating a possible metabolic block of aflatoxin biosynthesis. Schroeder et al. (270) observed that *A. parasiticus* in the presence of dichlorvos, a metabolic inhibitor of aflatoxin biosynthesis, accumulated a pigment, versiconal acetate, in the medium. Yao and Hsieh (328) were able to convert the above pigment into aflatoxins by incubating it with a dichlorvos-untreated mycelium. Furthermore, in the presence of dichlorvos, the conversion of labeled sterigmatocystin into aflatoxin B_1 was not affected, but averufin was not converted into aflatoxin B_1. In the presence of dichlorvos, the *A. parasiticus* mycelium converted averufin into versiconal acetate.

Any plausible biogenetic scheme for bisfuranoids must account for the coexistence of opti-

cally active sterigmatocystin, averufin, and versicolorin C in the same strains of *A. versicolor*, *A. nidulans*, and *Bipolaris* species (11, 26, 102, 148, 168). Moreover, sterigmatocystin, versicolorins C and A, and averufin have been isolated from aflatoxin-producing strains of *A. parasiticus* and *A. flavus* (102, 146, 192, 268). These anthraquinones are of polyketide origin and have been implicated in aflatoxin biosynthesis (26, 102, 231, 257).

Relative formation of aflatoxins B_1, B_2, G_1, and G_2. Different strains of the *A. flavus* subgroup show considerable variations in the type and amount of aflatoxin produced. The amounts and relative proportions of the four most common aflatoxins, B_1, B_2, G_1, and G_2, depend upon the strain, balance of nutrients in the medium, culture conditions, and isolation procedures used (6, 153, 154, 176, 230, 250, 252). Strains producing only the B group of aflatoxins, like B_1, B_2, M_1, and M_2, and no aflatoxin G are well documented (83, 84, 154, 204, 213, 214, 234, 267, 288). Some strains of *A. flavus* have been reported to produce only aflatoxin B_2 (234, 267). However, strains capable of producing only aflatoxin G_1 or G_2 have not been reported, so far, in the literature. This seems to suggest that aflatoxin G_1 is derived from aflatoxin B_1 by further oxidation (30). We have demonstrated the conversion of labeled aflatoxin B_1 to aflatoxin G_1 by *A. parasiticus* mycelial homogenate preparations. The results implied that aflatoxin B_1 was the precursor of all other aflatoxins (203). Elseworthy et al. (94) suggested that aflatoxins B_2 and G_2 were the precursors of aflatoxins B_1 and G_1. However, since only methyl side-chain ^{14}C-labeled compounds were used and since the methylating efficiencies of demethylated aflatoxins are not known, the results obtained by Elseworthy et al. (94) may only indicate preferential methylation of demethylated aflatoxins B_2 and G_2 in comparison with B_1 and G_1.

The proportions of minor aflatoxins, like M_1, B_{2a}, and G_{2a}, have been increased considerably under culture conditions in our laboratory. Lower pH and some trace metals, like manganese, magnesium, and vanadium, were found to favor formation of hydroxylated aflatoxins (201, 232). Similarly, conditions have been described under which aflatoxin G_1 is formed in larger amounts than is aflatoxin B_1. These include aeration, agitation, and higher temperature (131, 132, 134, 145, 250–252, 274, 275).

Relatively large amounts of aflatoxin G are formed in stationary cultures, and lower amounts are formed in shake cultures, thereby indicating rapid degradation of aflatoxin G_1 under aeration (56, 131, 134, 250, 275). Reddy (250) estimated that the newly synthesized aflatoxin B_1 is released from the fungal mycelium after 13 h, whereas Shih and Marth (273) observed that aflatoxin B_1 was released from the mycelium more slowly than were aflatoxins B_2, G_1, and G_2. Higher temperature favored faster release of aflatoxins from the mycelium into the medium. Glucose was found to inhibit release of aflatoxin during the early stages of the incubation period (273).

Several reports about aflatoxin production by mutants of the *A. flavus* subgroup have been published. An orange-yellow mutant of *A. parasiticus* was reported by Ellis (93) to produce nearly twice as much aflatoxin B_1 and G_1 as did the parent strain. Exposure to barium ions resulted in mutation of *A. flavus* with impaired or blocked aflatoxin synthesis (189). Murakami et al. (219) showed altered aflatoxin synthesis in mutants of *A. flavus* ATCC 15517 produced by ultraviolet irradiation of the parent strain. Detroy et al. (82) observed a drastic reduction in aflatoxin-producing ability in nitrosoguanidine-derived mutants of *A. parasiticus*. Leaich and Papa (187) reported a wide range of aflatoxin levels in ultraviolet-irradiated mutants of *A. flavus*. None of the isolates produced aflatoxin G, and some did not form aflatoxin B_2. The correlation between production of aflatoxin B_1 and production of aflatoxin B_2 was statistically significant. Appelgate and Chipley (5, 6) described conditions under which gamma-irradiated *A. flavus* strains produced as much aflatoxin B_2 as B_1. It has been reported that media, time of incubation selected, and gamma irradiation levels affected the daily and total amounts of aflatoxins produced, and the time of maximal aflatoxin production was shifted. The proportions of B_2 and G_1 could be increased by changing the dose levels (5, 6, 41).

A few strains producing only one type of aflatoxin component could have a genetic block resulting in the absence of the enzyme system for interconversion of aflatoxins (203). Schroeder and Verrett (269) suggested that differential rates of metabolism of B_1 and B_2 in aflatoxigenic strains may account for the accumulation of either B_1 or B_2 in strains. The non-aflatoxigenic strains were assumed to metabolize aflatoxins at the same rate as they are formed.

We had suggested that production of different types of aflatoxins may be due to heterokaryosis, the association of genetically unlike haploid nuclei in a fungal mycelium (204). The production of different types of aflatoxins may be controlled by different haploid nuclei. One should be able to demonstrate the existence of such a mechanism by forcing heterokaryons among strains

that produce only one type of aflatoxin. Leaich and Papa (187) suggested that, in addition to heterokaryosis, the variable production of aflatoxins may involve complex polygenic effects.

Biogenetic Schemes for Aflatoxins

Various hypothetical schemes for aflatoxins have been proposed, but only one scheme explains the labeling pattern of aflatoxin B_1 as determined by Biollaz et al. (30) (Fig. 4). Biollaz et al. (29, 30) proposed a scheme starting with a polyhydroxynaphthacene. It was based on the fact that naphthacenes frequently occur in nature and are intermediates in the biogenesis of tetracyclines. Biollaz et al. (30) failed to observe any incorporation of labeled naphthacenequinones or benzanthraquinones into aflatoxins by resting cells of *A. flavus*. It was assumed that a C-18 polyketide was the precursor of polyhydroxynaphthacenes and aflatoxins.

The scheme proposed by Biollaz et al. (30) explained the labeling pattern of aflatoxin and sterigmatocystin, the uniform distribution of radioactivity in the carbon atoms of the ring structure, and the two adjacent C-1 and C-2 atoms at the fusion of the bisfuranoid ring with the aromatic ring in the two compounds. The carbon atoms in the ring structures of aflatoxin and sterigmatocystin are derived alternatively from C-1 and C-2 of labeled acetate, but, at the fusion of the aromatic and bisfuranoid rings, the two adjacent carbon atoms are derived from either C-1 or C-2 alone (257). The scheme also explained the coexistence of versicolorins, sterigmatocystins, and aflatoxins in the same fungal strains. The labeling pattern of sterigmatocystin has been determined (157, 231, 292) and is shown in Fig. 4. It was suggested by Holker and Mulherin (157) that sterigmatocystin is derived by the acetate-malonate pathway from two separate, preformed polyketide units.

According to Heathcote et al. (147, 148), most of the pigments isolated from the *A. flavus* subgroup are three-membered anthraquinones. No naphthacenequinone from *A. flavus* has been reported so far. Even the C_{20} quinones, like averantin, asperantin, averythrin, averufin, and norsolorinic acid, isolated from *A. flavus* and *A. parasiticus* have three-membered anthraquinone ring structures (11, 23–26, 119–120). Heathcote et al. (147) proposed that a C_4 unit, acetoacetate, is oxidatively coupled to a preformed polyhydroxyanthraquinone molecule to give versicolorin, which is then converted to aflatoxin via sterigmatocystin. It was assumed that the anthraquinone molecule was derived from a C_{14} polyketide unit.

Holker and Underwood (158) have proposed a possible route for the conversion of sterigmatocystin to aflatoxins. Thomas (294) proposed a hypothetical pathway for the biosynthesis of aflatoxins that involved averufin, versicolorin, and sterigmatocystin as intermediates. However, none of the above schemes explains the labeling pattern of aflatoxin B_1.

Lin and co-workers (196, 197) proposed a C_{20} polyketide as a precursor of aflatoxins. Roberts (257), Berger and Jadot (26), and Pachler et al. (231) suggested a common scheme for sterigmatocystin and aflatoxin starting with a C_{20} polyketide. The C_{18} polyhydroxyanthraquinone theory is no longer tenable, according to recent evidence (26, 231, 257). The scheme for the above conversion had been proposed by Thomas and had been outlined by Moss (218), Berger and Jadot (26), and Pachler et al. (231).

The biosynthetic schemes proposed by Biollaz et al. (30), Heathcote et al. (147, 148), and Roberts (257) do not imply the involvement of averufin. Therefore, a pathway different from either of the previously proposed pathways must actually be used in the biosynthesis of aflatoxin B_1. The currently favored biogenetic scheme for bisfuranoids envisages only one mode of folding of a single C_{20} polyketide precursor leading to averufin, versicolorin, sterigmatocystin, and aflatoxin (26, 167, 168, 182, 196, 197, 218, 231, 257). Kingston et al. (182) proposed a slightly modified scheme for the conversion of averufin to versicolorin A from that of Moss (218). The basic scheme, with minor modifications, is presented in Fig. 5. A C_{20} polyketide may condense to give the C_{20} homolog of bipolarin, which may condense to give averufin. Averufin can easily give rise to averantin, averythrin, and norsolorinic acid. Averantin can undergo condensation and loss of a C_2 unit to form dehydroversiconal and versicolorin A. Versicolorin to sterigmatocystin and, subsequently, to aflatoxin is the well-known sequence.

Under some conditions or in some particular strains, it is possible that the side chain of averufin may be removed completely, oxidatively, resulting in the formation of a tetrahydroxyanthraquinone that may couple with a C_4 unit, like acetoacetate, to form versicolorin A (147, 148). Moreover, if the nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), concentrations are high after the onset of aflatoxin production, in such reducing conditions, the terminal double bond may get reduced, and aflatoxin biosynthesis may proceed via versiconal, versicolorin C, and 5-hydroxydihydrosterigmatocystin. Such a sequence results in the formation of aflatoxins B_2 and G_2. The above scheme explains the formation of only aflatoxin

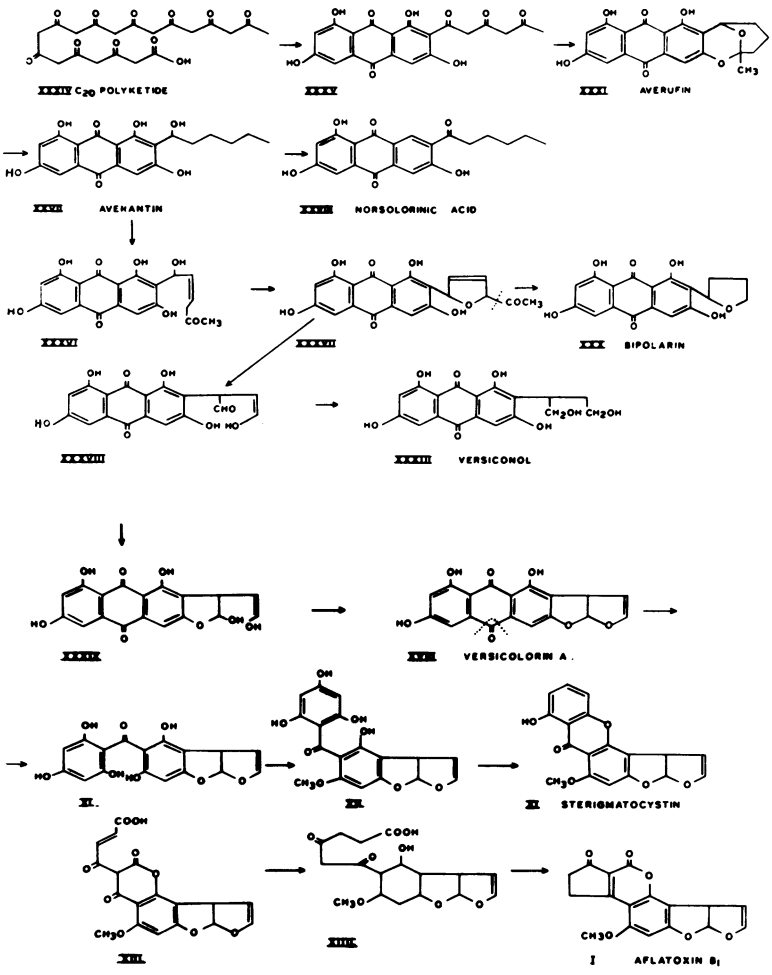

FIG. 5. *Biogenetic scheme for aflatoxin and other bisfuranoids.*

B_2 by some strains of the *A. flavus* subgroup and the accumulation of such intermediates as averufin, versiconal, and versicolorins. Most of the other aflatoxins can be formed from aflatoxin B_1 by oxidation and hydroxylation (203).

BIOCHEMICAL ASPECTS

Factors Affecting Aflatoxin Production

The factors affecting aflatoxin production have been extensively studied. In the present article, they will be discussed briefly, since many excellent reviews dealing with aflatoxin production in field crops, cereals, and nutrient cultures have appeared (55, 67–69, 81, 83, 84, 151, 153, 154, 176, 183, 212, 230, 266). In a chemically defined medium, low initial pH (4 to 5), glucose or sucrose as the carbon source, asparagine and ammonium sulfate as nitrogen sources, optimum levels of trace metals, and temperature ranging from 25 to 30°C have been recommended for optimum aflatoxin yields (84, 250, 252). Light was found to inhibit aflatoxin formation (177). High yields have been obtained on media containing crude natural extracts, like corn steep liquor, peptone, yeast extract, malt extract, peanut, or coconut (84, 315). Aflatoxin production has been reported to decrease with increasing carbon dioxide and decreasing oxygen concentrations (67). West et al. (314) observed increased aflatoxin formation by increasing the temperature from 15 to 21°C after 24 h and to 28°C after 48 h of incubation. The three most important factors controlling aflatoxin formation in field crops are relative humidity, moisture, and temperature (266).

Role of Trace Metals in Aflatoxin Biosynthesis

An important factor that regulates the biosynthesis of aflatoxin and that is not completely understood so far is the role of trace elements. Various reports are available regarding the role of trace elements in aflatoxin production in either synthetic medium or natural substrates. The inhibitory effect of barium and the stimulatory effect of cadmium on aflatoxin synthesis were first observed by Lee et al. (190). Lee and Townsley (189) were able to mutate an aflatoxigenic strain of *A. flavus* into a nontoxigenic one by successive exposure of cultures to barium ions. Tulpule (295) and Maggon et al. (206) reported the stimulatory effect of cadmium at low levels and its inhibitory effect at higher levels. Vanadium had an inhibitory effect on aflatoxin synthesis, although the fungal growth was not affected (206).

There are conflicting reports in the literature regarding the roles of cobalt, boron, molybdenum, calcium, iron, and copper. Cobalt, boron, and molybdenum were found by Mateles and Adye (211), Lee et al. (190), Davis et al. (69), and Reddy et al. (252) to have no significant effect on aflatoxin formation. However, Tulpule (295) observed that absence of cobalt from the medium significantly reduced aflatoxin yields. Maggon et al. (206) found that cobalt had no significant effect, whereas boron and molybdenum were stimulatory. Calcium and iron were reported to exert little influence on aflatoxin formation (190, 211). Similar results were obtained with copper (69). However, Tulpule (295) and Maggon et al. (206) reported reduced aflatoxin formation in the absence of copper, iron, and calcium from the culture media. Davis et al. (69) and Maggon et al. (206) observed that growth of the fungus as well as aflatoxin production were enhanced in the presence of magnesium. Marsh et al. (208) reported inhibition of aflatoxin formation in the low (in micrograms per milliliter) range of certain metals. At an added level of 25 μg of metal per ml, salts of iron, manganese, copper, cadmium, chromium, silver, and magnesium were observed to inhibit aflatoxin formation either partly or totally without influencing the mycelial weight.

Manganese appears to be a multifunctional metal in the metabolism of numerous fungal and bacterial systems. It is involved in cell wall synthesis as well as nucleic acid and fatty acid synthesis. Earlier workers observed that manganese exerted no effect or slightly reduced growth and aflatoxin formation (69, 190, 211). Detroy and Ciegler (77) did not observe any effect of manganese on aflatoxin production, although manganese deficiency caused incomplete or altered cell wall synthesis in *A. parasiticus*, resulting in a change of fungal morphology to a yeastlike form. Maggon et al. (206) observed different effects with varying concentrations of manganese in the medium. At low levels of manganese in the medium, inhibition of aflatoxin synthesis was observed, whereas higher levels resulted in enhanced aflatoxin formation; but at some intermediate concentration of manganese, there was no effect on aflatoxin biosynthesis.

The resistance or susceptibility of natural commodities to aflatoxin production has been explained as the presence or absence of adequate amounts of trace metals. According to Bassir and Adekunle (17), the low yields of aflatoxin obtained on some natural substrates like orange, pineapple, lemon, and mango were due to the absence of molybdenum from these samples. Furthermore, the high yields of aflatoxin obtained on some other materials, like papaya, sugarcane, banana, and carrot, were attributed to the presence of magnesium, iron, and zinc in these substrates, apart from the carbohydrates. These natural substrates are adequate because, apart from fulfilling the carbohydrate prerequisite for toxin production, they also have a preponderance of the trace elements, like magnesium, zinc, iron, and molybdenum, that are relevant to toxin production (227, 228). Maggon and Venkitasubramanian (202) attributed the observed enhanced aflatoxin yields with some particular peanut fractions to the presence of inorganic constituents like copper, manganese, zinc, and iron. Schroeder (266) proposed that the enhanced levels of aflatoxin detected in media enriched with corn steep liquor were due to the presence of zinc in the corn extract. Lillehoj et al. (195) demonstrated an association between the presence of *A. flavus* infection in corn and higher levels of trace metals. Furthermore, it was observed that enrichment of corn with manganese, copper, and cadmium or chromium increased aflatoxin yields. Some of the chelating agents that selectively bind with zinc or other stimulatory metals have been reported to inhibit aflatoxin production. These include phytic acid, dimethyl sulfoxide, 2-mercaptoethanol, and tolnaftate (19, 121–124, 137–140; S. N. Khan, unpublished data).

The exact step in the biosynthetic scheme at which barium, copper, or iron acts is not known at present. Pigment formation is reduced considerably in the absence of iron and copper. It is likely, therefore, that copper and iron are essential for the cyclization of the polyketide to aflatoxins and other pigments. The step at which

barium inhibits aflatoxin biosynthesis is not known. Gupta et al. (139) observed enhanced [^{14}C]acetate incorporation into aflatoxins in the presence of Zn^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, and Ca^{2+} ions by resting resuspended mycelia of *A. parasiticus*. The results indicate that the inhibitory action of Ba^{2+} on aflatoxin production may be due to the inhibition of fungal growth and metabolic precursor pools for aflatoxin formation. Weinberg (311) has reviewed the role of trace metals and proposed sites of action of metals in the biosynthesis of secondary metabolites.

Recently, Hutner (171) has suggested that the excretion by many fungi of chelating agents, such as citric, kojic, aspergillic, and gluconic acids, exaggerated the needs for trace elements. Thus, the very high trace metal requirement for aflatoxin production by fungi of the *A. flavus* subgroup may be partly explained by the above argument.

Role of Zinc in Aflatoxin Production

Zinc seems to play a key role in the biosynthesis of many secondary fungal metabolites, including aflatoxins. At least 20 enzymes, including deoxyribonucleic acid and ribonucleic acid polymerase and reverse transcriptase, have been found to be zinc dependent (12, 64, 237). The metal has been observed to stabilize nucleic acids, ribosomes, lysosomes, microtubules, and cell membranes (54, 291). Cocucci and Rossi (61) showed that zinc deficiency in the medium results in altered morphology, growth, and metabolism in the fungus *Rhodotorula gracilis*. The uptake and retention of zinc has been studied in microorganisms (28, 98). The stimulatory effect of zinc on aflatoxin production is well documented, but the zinc levels reported by different workers have ranged from 0.4 to 2 mg per liter of medium (69, 190, 211, 295). Reddy and co-workers (250–252) observed that, for optimum aflatoxin production by *A. parasiticus* in a chemically defined medium, zinc levels of 10 mg/liter were adequate. For a local-soil isolate of *A. flavus*, the zinc requirement was as high as 50 mg/liter (206). The low zinc requirements of the fungus reported by earlier workers may be due to preexisting levels of metals in crude media used by them, containing yeast extract, malt extract, peptone, etc. Recent work in our laboratory has indicated that the absence of zinc completely blocks fungal growth and aflatoxin production. Moreover, zinc has been found to stimulate the general metabolism of *A. parasiticus*, including fungal growth, protein and nucleic acids synthesis, nicotinamide adenine coenzymes, adenosine 5′-triphosphate (ATP) levels, α-keto acids, etc. The levels of lipids and inorganic phosphate (P_i) were lower in the presence of zinc (125, 126, 303). High levels of P_i have been shown to be inhibitory for secondary biosynthesis (311, 313) due to either deprivation of essential trace metals or feedback inhibition of phosphorylated intermediates into biosynthetic pathways. Since aflatoxin and lipid biosyntheses are closely related (76, 79–81, 131–134, 274, 275) and since acetyl coenzyme A (CoA) is the common precursor for both, inhibition of lipid synthesis in a zinc-supplemented medium may result in enhanced aflatoxin biosynthesis. During the exponential phase of growth, zinc supplementation resulted in higher oxygen uptake and higher ATP and nicotinamide adenine dinucleotide (NAD) levels, thereby indicating higher metabolic activity of the fungus (106–108, 125, 126, 303). During stationary phase, zinc supplementation resulted in high adenosine 5′-monophosphate (AMP) levels, which have been shown to be inhibitory for lipid synthesis and, therefore, favorable for aflatoxin biosynthesis. We have observed that the glycolytic enzymes of *A. parasiticus* are zinc dependent, and these enzymes showed high activities on day 2 of growth and decreasing activity thereafter (121, 130, 303). The tricarboxylic acid cycle enzymes were observed not to be zinc dependent. The decreasing activities of glycolytic enzymes with culture age may indicate depletion of the carbon source in the medium. The higher activities of the glycolytic enzymes indicate a higher breakdown of glucose for energy and metabolic activities and accumulation of pyruvate, which we assume to be essential for the onset of aflatoxin biosynthesis.

Wold and Suzuki (319) have reported the regulatory role of zinc in citric acid production by *Aspergillus niger*. The fungus has two distinct phases, a growth phase, when cells proliferate without accumulation of citrate, followed by an accumulating phase, when citrate is produced without cell proliferation. In a low-sucrose (0.4 to 0.8%)-salts medium, these phases were controlled by zinc concentrations in the medium. High zinc levels (1 to 2 μM) maintained the growth phase, whereas, at lower zinc levels (less than 1 μM), fungal growth became limited and it passed into the accumulating phase. Addition of zinc to accumulating cultures resulted in their reversion to the growth phase. Other trace elements, like iron, manganese, and calcium, had no such effects. Zinc is involved in carbohydrate metabolism in fungi, and phosphofructokinase regulates glycolysis in *A. niger* (281, 282). At a high level of zinc, the fungal physiology is directed towards growth. The chain of events triggered during zinc deficiency culminates in

growth termination, reduction in cell adhesiveness, and citrate accumulation. It was considered likely that zinc was affecting the induction of enzymes of secondary metabolism. It is probable that zinc is playing a similar role in the metabolism of *A. parasiticus*.

Promoters and Inhibitors of Aflatoxin Biosynthesis

Besides environmental factors and trace metals, several metabolic promoters and inhibitors of aflatoxin biosynthesis have been reported. Several workers have reported enhanced aflatoxin production in the presence of amino acids in the medium or in a natural substrate. Basappa et al. (15) reported enhanced aflatoxin production in the presence of thiamine and ethyl alcohol. Gupta et al. (135) observed high aflatoxin yields by replacing the sucrose in a chemically defined medium with lauric and sebacic acids. Most of the carboxylic acids containing 2 to 10 carbon atoms, like acetic and propionic acids, were inhibitory to fungal growth and toxin production. Most of the higher fatty acids formed by the breakdown of the lipids present in natural substrates had a significant influence on aflatoxin production by *A. parasiticus*. Naik et al. (222) observed stimulation in aflatoxin biosynthesis in the presence of methionine, proline, and tryptophan, whereas Reddy et al. (251, 252) reported the stimulatory effects of asparagine, aspartic acid, and alanine. The stimulatory effect of various amino acids may be either secondary, due to their effect on the growth and general primary metabolism of *A. parasiticus* and *A. flavus*, or a direct effect on aflatoxin biosynthesis. Recently, Leaich and Papa (187) observed that mutants of *A. flavus* having different requirements for amino acids, such as methionine, lysine, and arginine, showed little differences in aflatoxin production levels. Work in our laboratory has revealed that asparagine and aspartic acid stimulate [1-^{14}C]acetate incorporation into aflatoxins (136), thereby showing that the stimulatory effect observed may be due to catabolism of these amino acids to pyruvate and acetyl CoA. Shih and Marth (275) reported that selective inhibition of the terminal respiration of *A. parasiticus* by sodium azide enhanced both aflatoxin and lipid biosyntheses, due to accumulation of acetyl CoA.

Bennett et al. (22) studied the effect of additions of acetone, acetic acid, ethanol, and sodium acetate on the production of aflatoxins and versicolorin pigments in resting cells of *A. parasiticus*. In the presence of glucose, acetone (0.25 M) mildly inhibited aflatoxin yields and stimulated versicolorin formation. Lower acetone concentrations (0.1 to 0.001 M) stimulated aflatoxin production. Acetic acid was found to be inhibitory for aflatoxin biosynthesis (22, 135). Although ethanol has been reported to enhance aflatoxin production (15, 22), it was not a useful substrate for toxin formation (16). It has been reported that acetate can serve as the sole carbon source and as a precursor of aflatoxin and that it stimulated aflatoxin biosynthesis (22, 163). Bennett et al. (22) have suggested that acetone enhances aflatoxin biosynthesis by an improved uptake and utilization of glucose.

A systematic study of various metabolic inhibitors and promoters of aflatoxin biosynthesis on growth and suspension media has been carried out in our laboratory (136–141). The results obtained are presented in Table 1. The metabolic inhibitors studied showed large variations in their effects on the suspension and growth media. Some compounds, like avidin, diphenylamine, and sulfanilamide, showed very little effect, probably because of permeability factors. In suspension medium, methionine sulfoxide, cycloserine, and iodobenzoic acid stimulated incorporation of acetate into aflatoxin at all levels used. L-Cysteic acid inhibited incorporation of acetate into aflatoxins at all levels tested. Some compounds, like malonate, iodoacetate, arsenate, and 2,4-dinitrophenol, stimulated incorporation at low levels and showed inhibition at higher ones. The mechanism of action of these inhibitors on fungal metabolism seems to be varied. Malonate and arsenate probably interfere with the tricarboxylic acid cycle, iodoacetate and fluoride probably interfere with glycolysis, and dinitrophenol and arsenate probably interfere with oxidative phosphorylation (309, 310). Malonate and iodoacetate probably interfered with the polyketide pathway. Gupta et al. (139) observed inhibition of [^{14}C]acetate incorporation into aflatoxin and inhibition of its biosynthesis by ethanol.

Ethylenediaminetetraacetate was observed to inhibit aflatoxin formation, but it stimulated incorporation of [^{14}C]acetate into aflatoxins. This may be due to the permeability effect. Moreover, incorporation into aflatoxin G was stimulated about 60%, as compared with 40% in aflatoxin B, by ethylenediaminetetraacetate (10 mM). Aminosalicyclic acid and *p*-phenylbutyric acid stimulated ^{14}C incorporation into aflatoxin B specifically, without affecting aflatoxin G, whereas sodium fluoride inhibited [^{14}C]acetate incorporation into aflatoxin B. It is clear from Table 1 that many inhibitors totally blocked fungal growth and aflatoxin formation when added to the growth medium. When such inhibitors were added to the suspension medium, only

TABLE 1. *Metabolic inhibitors and promoters of aflatoxin biosynthesis (131, 136–141)*[a]

Inhibitor	Concn (mM)	Inhibition (%) of:			Remarks	Known metabolic inhibitory action (309, 310)
		Fungal growth	Aflatoxin B and G formation	^{14}C incorporation into aflatoxins in suspended *A. parasiticus* (%)		
Ethanol	500	NT	17	71		
	1,000	NT	50	78.5		
Iodoacetic acid	10.0	NT	80	91.5	Stimulatory at 0.1 mM	Glycolysis, fatty acid synthesis
Sodium arsenite	0.20	NT		−75.5		α-Keto acid oxidases
	1	100	100	22		
	10	100	100	59.5		-SH group enzymes
o-Iodobenzoic acid	0.20	NT		−60		Succinic dehydrogenase
2,4-Dinitrophenol	0.01	6.7		−29	Stimulation: AFG, 48%; AFB, 16%	Stimulator of succinic dehydrogenase, oxidative phosphorylation
				8.5		
Sodium fluoride	1	20	37	23	Stimulation: AFB, 39%; AFG, 7%	Glycolysis, tricarboxylic acid cycle
	10	100	100			
Ethylenediaminetetraacetate	1	34	−52	−43	Stimulation: AFG, 60%; AFB, 40%	Chelating agent
	10	100	100	−50		
p-Aminobenzoic acid	1	NS	No effect	No effect		
	10	43	74			
p-Aminosalicylic acid	1	10	−10	−14	Stimulation: AFB, 26%, AFG, 8%	CoA, folic acid
	10	NS	88	58.3		
p-Nitrobenzoic acid	1	NS	−20	44.5		CoA, folic acid
	10	100	100	54.5		
L-Cysteic acid	1			62.5		Aspartate decarboxylation, alanine transamination
	10			78		
Dehydroacetic acid	1	NA	80	65		Fungal growth
	10	100	100	73		
β-Phenylbutyric acid	10	100	100	−7		Mevalonate biosynthesis
Anthranilic acid	10	NS	85	20.5		
Salicyclaldehyde	1	100	100	34.5		Aromatic biosynthesis
Salicylic acid	1	NS	38	45.5		CoA, ATP
Sodium pyrophosphate	10	NS	50	8		
Sodium azide	1	—	—	87.5		Electron transport system

[a] Abbreviations: AFG, aflatoxin G_1; AFB, aflatoxin B_1; NT, not tested; NS, not significant.

partial inhibition of [^{14}C]acetate incorporation into aflatoxins was observed (Table 1) (136–141). Davis and Diener (66) were the first to report inhibition of aflatoxin formation by *p*-aminobenzoic acid, potassium sulfite, and fluoride. Groll and Luck (118) showed that preservatives, like sorbic acid and sorbyl palmitate, considerably retarded aflatoxin formation by *A. flavus* in bread. Alderman and Marth (4) reported considerable inhibition by citrus oils of aflatoxin formation in *A. parasiticus* cultures.

Many chelating agents that selectively bind to stimulatory trace metals for aflatoxin biosynthesis, like zinc, have been observed to inhibit aflatoxin formation. Bean and Rambo (19) reported inhibition of aflatoxin biosynthesis in *A. parasiticus* by dimethyl sulfoxide. We have observed inhibition of aflatoxin biosynthesis and [^{14}C]acetate incorporation into aflatoxin by phytic acid, 2-mercaptoethanol, and tolnaftate (122–124; S. N. Khan, unpublished data).

Ethionine and dichlorvos have been reported to inhibit aflatoxin biosynthesis (76, 162, 248, 270). Detroy and Freer (78) studied the regulatory role of methionine in aflatoxin biosynthesis. The addition of extracellular ethionine to *A. parasiticus* cultures grown on a chemically defined medium supplemented with methionine inhibited aflatoxin formation by about 50%. Replacement of the methionine with cysteine in the medium reduced aflatoxin synthesis by about 80 to 100%, under similar conditions. Other cysteine or methionine analogs were inhibitory to the extent of 40 to 90% for aflatoxin production and for incorporation of [^{14}C]formate into aflatoxins. The results obtained indicated a block in methylation, with formate as a C_1 donor. Leary (188) used dialkyl enol phosphates for controlling production of aflatoxin and other mycotoxins. Recently, Turner et al. (299) reported that 5,7-methoxyisoflavone inhibited fungal growth and aflatoxin production.

RELATIONSHIP BETWEEN PRIMARY AND SECONDARY FUNGAL METABOLISM

General Aspects

At present, it is generally accepted that the rapid growth during the exponential phase results in the accumulation of precursors that are subsequently diverted toward biosynthesis of secondary products, like mycotoxins, pigments, and antibiotics (74). The molecular events that initiate and abruptly terminate secondary metabolism are poorly understood. The biochemical functioning of the fungal cell has been explored, and work has focused mainly on gene-enzyme relationships, simple and complex regulatory mechanisms, and the spatial organization of enzymes and metabolites within the cell. Many reviews on the regulation and metabolic organization of the fungal cell have appeared recently (40, 44, 70–73, 89, 103, 104, 198, 238, 283, 320).

During the onset of secondary metabolism, the biosynthesis of primary metabolites is either blocked or inhibited (44, 70–73). Enzymes of secondary metabolism appear to be repressed during the exponential phase (42–46, 73), but the type of repression involved is not clear. Some secondary metabolites, like penicillin and ergot alkaloids, are formed when the accumulation of primary shunt products (polyols, lipids, polyphosphates, and nonstructural carbohydrates) stops (103, 290). Turner (297) and Bu'Lock (44) have described other secondary products that are formed during the primary shunt product phase. Many reviews have underlined the possible reasons and events for the onset of secondary metabolite synthesis (42–44, 70–73, 103, 209, 254, 256, 280, 280a, 282, 297, 298, 327).

Rehacek and Kozova (253) observed that, in submerged cultures of *Claviceps purpurea,* cytodifferentiation preceded biochemical differentiation. The alkaloid-producing phase was characterized by reduced cell proliferation, maximum acetyl-CoA carboxylase activity, maximum amounts of total fatty acids and higher cell pool tryptophan levels, and minimum tryptophan synthetase activity. Sekiguchi and Gaucher (272) correlated fungal sporulation and secondary metabolism in *Penicillium urticae.* It was observed that sporulation, like secondary biosynthesis, occurred due to nutrient (nitrogen) limitation, and it required the presence of enzymes for a specific and limited period of time. It was observed that patulin and its precursors were not a prerequisite to conidiogenesis in *P. urticae.* Environmental and developmental factors, such as calcium levels and conidiogenesis, indirectly affect the production of patulin pathway metabolites.

Growth Phases

During the exponential, or logarithmic, growth phase, many precursors of primary metabolism accumulate (like acetyl CoA, tricarboxylic acid cycle intermediates, phosphatides, etc.) (255, 297). Small amounts of secondary metabolites, like mycotoxins, may start appearing due to the presence of some nondividing cells. In *A. parasiticus,* this phase lasts up to 50 h of growth after inoculation (80). A slightly higher temperature is required for optimal growth of many fungi during this phase (70). *A. parasiticus* shows maximum growth at 35°C (274, 275), and that is the temperature for optimum exponential-phase growth. The enzymes of secondary metabolism appear to be repressed during this phase (233).

The transition phase is the period of changeover from the actively growing to the stationary phase, where cellular growth stops. During this phase, the nitrogen and phosphorous in the medium are limited; hence, the enzyme of secondary metabolism become derepressed. The depletion of nitrogen or phosphorous results in a declined rate of fungal growth and specific changes in the enzymatic machinery of the cell (40, 215). Accumulation of primary intermediates occurs due to cessation of cell division. The nature and quantity of intermediates depends on the culture conditions used (42, 70, 71, 198, 256). Primary metabolism (vegetative activity) is blocked. In *A. parasiticus,* this phase occurs from 50 to 70 h, and enzymes of aflatoxin synthesis appear to be derepressed during this period (80). Preliminary results obtained in our laboratory indicate that phosphoenolpyruvate may play an important role in the repression of aflatoxin synthase during the exponential growth phase. It has been observed to inhibit sodium [1-^{14}C]acetate incorporation into aflatoxin in a suspended system.

Demain (70–73) has characterized the stationary phase, or idiophase, as a period of decline in the rate of growth, carbohydrate utilization, and oxygen uptake and a period of secondary metabolite production. Generally, the mycelial dry weight during this phase remains more or less constant. Bu'Lock (43, 44) described some cases where the mycelial dry weight continues to increase during the stationary phase. The rate of growth, however, is lower than that during the exponential phase, and this increase has been attributed to the accumulation of polyols, polyphosphates, lipids, and nonstructural carbohydrates and secondary products (32, 33, 40,

43, 44, 103, 289).

The supply of nitrogen and phosphorous in the medium is exhausted and carbon is limiting during the stationary phase, and, hence, there is a decline in cellular growth. The main metabolic activity of the fungus is the synthesis of secondary metabolites. The pathways of protein and nucleic acid biosynthesis are blocked, and the bulk of secondary biosynthesis occurs during this stage (73, 76, 80, 81). The mechanism of onset of secondary metabolism is not clear.

During this phase, reduced temperature is required for optimum production of secondary metabolites and is generally lower than that required during the exponential phase. Shih and Marth (274) observed that *A. parasiticus* gave maximum aflatoxin yields at 25°C, a temperature lower than the optimum growth temperature by about 10°C. According to Detroy and Hesseltine (80), *A. parasiticus* enters the stationary growth phase after 70 h, and this phase continues till the exhaustion of the carbon source. Supplementation of the carbon source, however, fails to provide continuous aflatoxin formation. It has been postulated that the oxygen requirements of the organism during different growth phases would be different, higher during the exponential phase and lower during the stationary phase (70).

According to Weinberg (312), secondary metabolism does not occur in continuous cultures if balanced growth is maintained constantly by the supply of nitrogen and phosphate sources. The relationship between the exponential and stationary growth phases has been discussed (71, 308). It has been shown by Pirt and Righelato (241) that the decline in the growth phase is not responsible for triggering the onset of penicillin biosynthesis. Many workers have shown that aflatoxin production is not related to fungal growth (76, 80, 204, 247).

Borrow et al. (32, 33) divided the stationary phase into the storage phase, during which cell weight continues to increase due to the accumulation of fat and carbohydrates and during which production of secondary metabolites commences, and the maintenance phase, during which dry weight is constant but uptake of glucose and production of secondary metabolites continues.

Since most of the aflatoxin production in our laboratory is carried out in culture media having limited nitrogen or phosphate, it may be assumed that exhaustion of either of these may trigger the onset of aflatoxin formation. We have observed accumulation of pyruvate and other α-keto acids before the onset of aflatoxin production and pyruvate stimulation of sodium [1-^{14}C]acetate incorporation into aflatoxins. It has been postulated that accumulation of pyruvate and other α-keto acids inhibits fungal growth, shifting it to the stationary phase, and may be essential for the onset of aflatoxin biosynthesis (129, 303).

The decline phase is marked by a decrease in the number of viable cells. This decline is due to autolysis of the cells. The carbon source in the medium gets exhausted. The reduction of secondary products shows a decline after peak yields, and many workers have shown declines in aflatoxin levels (256).

Energy Requirements and Aflatoxin Formation

A wide range of organic compounds can be used as energy sources by fungi. Landers et al. (185) observed reductions in aflatoxin yields by *A. flavus* when the oxygen concentration was reduced in the environment.

Respiration. It has been established that the degree of aeration of *A. parasiticus* is important in controlling biosynthesis of aflatoxins. Aspergilli produced less aflatoxin and lipid when aeration was increased during incubation (62, 134, 145, 274, 275). Incubation with agitation enhanced mold growth, glucose uptake, and production of aflatoxin and lipid during the first 3 days. More growth occurred in stirred cultures than in stationary cultures, but stationary cultures gave maximum yields of aflatoxins (134, 274, 275). Shih and Marth (274) demonstrated the need for a limited, but not excessive, oxygen supply for the synthesis of aflatoxins and lipids. Oxygen is also necessary for catabolic repression of secondary enzymes by glucose.

In many instances, a high oxygen concentration is necessary to support microbial growth and production of fungal metabolites (40, 256). It should be noted that the critical dissolved-oxygen tension and the minimum necessary for optimum yields of secondary products are distinct parameters. The availability of oxygen affects the production of secondary metabolites by fungi (40).

Glycolysis. The presence of greater activity of the Embden-Meyerhof (EM) pathway in nonaerated cultures suggests that the environment becomes less aerobic in the late exponential phase of growth, when oxygen is gradually used up by the mold and oxygen uptake declines (121, 129, 256, 275). During the transition phase, acetate oxidation via the tricarboxylic acid cycle and NADPH oxidation by oxygen via the NAD electron transport system would be minimal or blocked, resulting in an accumulation of acetate and NADPH, which, in turn, would result in

enhanced formation of aflatoxins. Increased NADPH concentration favored aflatoxin formation.

To sum up, it can be postulated that there is marked correlation between glucose catabolism, which proceeds predominantly via the EM pathway, and the formation of secondary metabolites (27, 40, 42, 73, 256). Carter and Bull (52) observed that melanogenesis by *Aspergillus nidulans* cultures was largely associated with the EM pathway, whereas cultures using the hexose monophosphate (HMP) pathway were only slightly pigmented. Martin and McDaniel (209) observed that acetate stimulated the production of the macrolide antibiotic candicidin by *Streptomyces griseus* in the presence of glucose but not in the absence of the sugar. It was postulated that the reduced cofactor, NADPH, required for reduction of the polyketide chain was provided by glycolysis, and the intermediates of the HMP pathway were used for the aromatic moiety of candicidin.

Hostalek et al. (161) studied the activities of ATP glucokinase and polyphosphate glucokinase in *Streptomyces aureofaciens* during the chlortetracycline-producing phase. The enzyme ATP glucokinase was active only during the exponential phase and increased in parallel with the rate of chlortetracycline synthesis. The antibiotic synthesis takes place at the expense of glycolysis, using up the high-energy phosphate of polyphosphates (161). In chlortetracycline biosynthesis, interrupted aeration during the exponential phase resulted in a shift of metabolism from the HMP pathway to the EM pathway, accompanied by an inhibition of antibiotic formation (160). Excess of phosphate inhibits enzymes of the HMP pathway, and a shift to the EM pathway takes place. The virtue of the HMP pathway is the preponderance of NADPH needed for condensation reactions during polyketide synthesis (70, 160).

It seems probable that, for aflatoxin biosynthesis, proper proportions of both EM and HMP pathway metabolites are required. We have shown that enzymes of glycolysis in *A. parasiticus* are zinc dependent (130). Zinc stimulates glycolysis by the EM pathway in *A. parasiticus*, leading to pyruvate accumulation (see Table 2). In the absence of zinc, fungal glycolysis is mostly through the HMP pathway, in which sufficient amounts of pyruvate do not accumulate (121). Mason and Righelato (210) showed that excess glucose over the growth requirements of *Penicillium chrysogenum* was metabolized to organic acids, mainly malate and gluconate.

Demain (71) has discussed the regulation of secondary fungal metabolism by glucose catabolites. The unfavorable effect of glucose on antibiotics, alkaloids, and aflatoxins is well known (71, 163).

The role of cyclic AMP (cAMP) in the mediation of catabolic repression in fungal systems is not clear at present. Glucose repression of cAMP levels along with several enzymes of yeast, *Tetrahymena pyriformis*, and *Escherichia coli* has been reported during the log phase of growth (59, 263, 320). The above reports point to a specific role of cAMP during fungal growth and differentiation (44). Abou-Sabe et al. (1) proposed that the level of cAMP is regulated by the intracellular amounts and/or transport of glucose and not by extracellular concentrations. Zonneveld (332) reported that manganese deficiency had a definite effect on cAMP levels and that external glucose played only an indirect role. It was further proposed that cAMP levels are regulated by growth characteristics. Bu'Lock (44, 45) proposed that increased cAMP levels during the stationary phase may lift "catabolic repression" of enzymes of secondary biosynthesis. The involvement of cAMP in regulation of growth and citric acid accumulation in *A. niger* was studied by Wold and Suzuki (320). cAMP affected the rates of growth and acidogenesis when added to *A. niger* cultures growing at low, but not at high, zinc levels. It stimulated growth and cell adhesiveness in the growth phase, inhibited growth and cell adhesiveness during phase transition and the accumulating phase, and enhanced citrate production in the stationary, acidogenic phase. Thus, the growth and citrate-accumulating phases of *A. niger* could be differentiated with respect to cAMP metabolism. The major role of cAMP was to stimulate the physiological activity of the cells, regardless of the phase; it enhances thallogenesis by stimulating glycolysis in the growth phase and acidogenesis in the accumulating phase. Zinc and cAMP are regulatory partners in *A. niger;* zinc determines the physiological state and is required for cell proliferation, and cAMP regulates the physiological activity. cAMP participates in the regulation of metabolism and the growth and development of fungi (44, 45, 281, 319, 320).

Hsieh and Mateles (163) suggested that the acetyl CoA's derived from glucose and acetate are formed in separate pools in *A. flavus* and *A. parasiticus*. The formation of acetyl CoA from glucose takes place in the mitochondria by pyruvate dehydrogenase oxidation of pyruvate, whereas the formation of acetyl CoA from acetate, through the action of acetate thiokinase, takes place extra-mitochondrially. Therefore, the mixing of acetyl CoA derived from glucose

with that derived from acetate requires its transport across the mitochondrial membrane, which is rather impermeable to acetyl CoA. Hsieh and Mateles (163) showed that aflatoxins are acetate derived and are formed extra-mitochondrially. According to Paigen and Williams (233), the group of enzymes that bring carbohydrates into the EM, HMP, and Entner-Doudoroff (ED) pathways are very sensitive to catabolic repression. Occasionally, an excess of oxygen inhibits metabolite formation (70). It has been postulated that aeration encouraged respiration and growth and thereby reduced the amount of carbon available for lipid and aflatoxin biosynthesis (132–134).

Recently, we have studied the oxygen uptake of *A. parasiticus* during the exponential and stationary phases in comparison with a non-aflatoxigenic strain of *A. flavus* and during zinc deficiency (127–129). In *A. flavus* and with zinc deficiency, the oxygen consumption in the exponential growth phase was significantly lower than that of *A. parasiticus* with zinc-supplemented medium. The oxygen uptake declined during the stationary phase and the period of aflatoxin production. The decline in oxygen consumption was more significant in *A. parasiticus* in a zinc-supplemented medium (Table 2). Higher oxygen uptake indicates higher metabolic activity, which is necessary for accumulation of primary precursors at the end of the exponential growth phase. The non-aflatoxigenic *A. flavus* strain had lower oxygen uptake throughout the growth period than did *A. parasiticus*.

The most common glycolytic pathway in fungi is the EM pathway, which usually accounts for at least 50% of the glucose assimilated. The EM pathway becomes increasingly important under anaerobic conditions, whereas the HMP pathway becomes increasingly important under aerobic conditions and less active in the absence of free oxygen (60, 256). Generally, fungi assimilate glucose 60% by the EM pathway and 40% by the HMP pathway. The HMP pathway is the biosynthetic source of pentoses, which are required for nucleic acid synthesis. Unlike the reduced NAD, which is generated from the EM pathway, the NADPH, formed from the HMP pathway, apparently does not generate ATP through the cytochrome system. Therefore, the energy release through aerobic glycolysis occurs only from the EM cycle. The HMP pathway is more active during the exponential growth phase than during the stationary phase (60). Almost all fungi are aerobic, and our observations of higher oxygen uptake during the exponential phase support the postulate of Cochrane (60).

Shih and Marth (274, 275) observed that *A. parasiticus* formed more aflatoxin and lipids during lower aerobiosis, when greater involvement of the EM pathway occurred, thereby suggesting that the degree of aerobiosis governs aflatoxin formation. Moreover, aflatoxin formation was greater when oxidative phosphorylation of the mold was restricted by sodium azide, an inhibitor of terminal electron transport and respiration. Sodium azide slightly stimulates anaerobic glycolysis, resulting in accumulation of acetate, a precursor for aflatoxins and lipids. Part of the NADPH derived from the HMP pathway may be oxidized via the NAD cytochrome system. Therefore, the decline in the electron transport system results in an accumulation of NADPH, which may be reduced and utilized for synthesis of secondary metabolites (275).

Tricarboxylic acid cycle. The tricarboxylic acid cycle plays a central role in catabolic as well as anabolic processes in fungi. Recent results obtained in our laboratory indicate that at the end of the exponential growth phase, the toxic levels of α-keto acids accumulate before the onset of aflatoxin biosynthesis (126, 127). Using an aflatoxigenic strain in a zinc-replete medium, the levels of α-keto acids, particularly pyruvate, were significantly higher during the exponential growth phase. On transition to the stationary phase, the levels of α-keto acids were lower in the aflatoxigenic strain in zinc-replete medium as compared with those in a non-aflatoxigenic strain in zinc-deficient medium (Table 2). Pyruvate accumulation, along with other α-keto acids, to toxic levels is suggested as causing the shift in growth phase from exponential to stationary with the onset of aflatoxin biosynthesis. Moreover, the results obtained indicate the utilization of tricarboxylic acid cycle intermediates for the biosynthesis of aflatoxins and other secondary metabolites. This is supported by increased levels of these acids under conditions that inhibit aflatoxin formation. Borichewski (31) reported that α-keto acids, particularly pyruvate, were growth-limiting factors for *Thiobacillus thiooxidans*. Detroy and Hesseltine (80) suggested accumulation of tricarboxylic acid cycle intermediates as an essential prerequisite for the onset of aflatoxin biosynthesis.

It is probable that the accumulation of pyruvate during the exponential growth phase of the toxigenic strain coincides with the onset of aflatoxin production. Even zinc deficiency in the medium had no significant effect on pyruvate levels (Table 2) (126, 127). High levels of pyruvate activate the tricarboxylic acid cycle to form other tricarboxylic acid cycle intermediates, like acetaldehyde, oxaloacetate, acetyl CoA, and af-

TABLE 2. *Biochemical composition of A. parasiticus NRRL 3240 and A. flavus NRRL 3537 during late exponential (day 2) and early stationary (day 4) growth phases in relation to aflatoxin biosynthesis (121, 125–130)*

Strain	Medium	Days of growth	Mycelial component estimated																				
			Aflatoxins B and G (mg/100 ml of medium)	Dry wt (g/100 ml of medium)	Oxygen uptake (μatom/h)	Ribonucleic acid (g/100 g of mycelial dry wt)	Deoxyribonucleic acid (g/100 g of mycelial dry wt)	Protein (g/100 g of mycelial dry wt)	Lipids (mg/g of mycelial dry wt)	NAD (μg/g of mycelial dry wt)	NADP (μg/g of mycelial dry wt)	Riboflavine 5'-phosphate (μg/g of mycelial dry wt)	Flavine adenine dinucleotide (μg/g of mycelial dry wt)	ATP (μmol/g of mycelial dry wt)	ADP (μmol/g of mycelial dry wt)	AMP (μmol/g of mycelial dry wt)	P_i (μmol/g of mycelial dry wt)	Energy charge	Acetaldehyde (μmol/g of mycelial dry wt)	Pyruvic acid (μmol/g of mycelial dry wt)	Oxaloacetate (μmol/g of mycelial dry wt)	α-Ketoglutaric acid (μmol/g of mycelial dry wt)	Malic acid (μmol/g of mycelial dry wt)	Citric acid (μmol/g of mycelial dry wt)
A. parasiticus	Zn deficient	2	1.07	0.68	13.75	2.92	0.41	40.32	41.76	566.30	157.20	24.61	18.79	2.24	2.51	6.51	43.51	0.31	3.63	3.76	2.69	0.68	5.89	0.17
		4	5.70	2.66	14.21	1.86	0.37	26.13	72.98	301.15	98.97	50.08	29.48	21.93	5.59	20.73	35.42	0.51	64.59	5.38	4.34	1.22	3.55	0.42
	Zn replete	2	2.84	1.04	17.10	3.28	0.70	57.24	49.51	886.95	841.75	21.48	17.94	8.58	3.73	3.82	35.51	0.65	57.67	4.17	1.92	1.01	6.02	0.05
	(SLS[a])	4	15.61	3.24	12.39	2.22	0.41	29.00	55.98	447.75	221.35	32.95	24.10	25.64	13.35	59.63	27.73	0.33	29.55	3.05	3.41	0.82	3.36	0.12
A. flavus	Zn replete	2	0	1.74	8.90	2.66	0.91	35.95	52.51	1,005.80	585.05	27.81	41.00	1.28	2.08	0.56	29.80	0.59	22.87	1.66	1.18	0.66	3.26	0.20
	(SLS)	4	0	2.94	8.20	1.50	0.37	42.62	55.98	291.80	67.85	44.95	12.55	26.28	1.31	4.87	39.12	0.83	30.04	3.12	5.12	0.92	6.29	0.18
Assay procedure (reference)			(221)		(300)	(306)	(7)	(117)	(105)	(58)	(58)	(47)	(47)	(286)	(96)	(96)	(100)	(8, 9)	(110)	(110)	(110)	(110)	(170)	(97)

[a] SLS, Sucrose-low-salts medium (123, 135).

latoxin. During the stationary phase in *A. parasiticus*, as most of the pyruvate is used up for the synthesis of aflatoxins, less is available for the synthesis of α-keto acids, and, hence, low levels of these acids are observed (Table 2). Our results are supported by the observations of Bryzgalova and Orlova (38), who reported that, under conditions favoring synthesis of oxytetracycline by a strain of *Streptomyces rimosus*, sufficient organic acids were not formed. On the other hand, conditions unfavorable for oxytetracycline biosynthesis lead to production of more keto acids.

Glucose inhibition of enzyme synthesis can be achieved both by reducing the concentration of active internal inducer molecules and by inhibiting the biosynthesis of enzyme protein (233).

Foster (109) considered that secondary metabolites are "shunt metabolites," formed from normal intermediates when primary pathways (e.g., the tricarboxylic acid cycle) are shut down and new pathways arise. Many workers have shown accumulations of tricarboxylic acid cycle intermediates at the end of the exponential growth phase. In *A. niger*, the activities of aconitase and isocitrate dehydrogenases are reduced at the start of the stationary phase, while that of the condensing enzyme continues unabated, resulting in the excretion of high levels of citric acid (70). Bu'Lock (42) observed a marked decrease in tricarboxylic acid cycle activity at the termination of the exponential phase, probably due to catabolic repression by glucose. It stands to reason that, as growth rate declines, the resultant accumulation of amino acids and nucleotides would further shut off protein and nucleic acid synthesis by end product regulation. A decline in the activity of the tricarboxylic acid cycle should lead to accumulation of acetyl CoA, glycolytic intermediates, pyruvate, and citrate (70, 73). The well-known activation of acetyl-CoA carboxylase by citrate and α-glucose phosphate would result in the production of malonyl CoA (249). This would further shut off the tricarboxylic acid cycle, since malonate is an inhibitor of succinic acid dehydrogenase. Malonyl CoA is a precursor of polyketide secondary metabolites, which include tetracycline, pigments, 6-methylsalicyclic acid, mycotoxins, and aromatic products formed during the stationary phase (70, 73, 81, 136, 211, 298).

We have studied the effect of tricarboxylic acid cycle intermediates on aflatoxin biosynthesis and on the incorporation of [^{14}C]acetate into aflatoxins, and we have studied the levels of tricarboxylic acid cycle intermediates and enzymes during the exponential and stationary growth phases. Reddy (250) observed inhibition of aflatoxin formation in *A. parasiticus* when tricarboxylic acid cycle intermediates were used as the carbon source in the medium. With citric and succinic acid, a considerable inhibition of aflatoxin biosynthesis was observed, but growth was reduced by only about 33%. Later work by Gupta and co-workers (131, 136) showed stimulation of [1-^{14}C]acetate incorporation into aflatoxin by tricarboxylic acid cycle intermediates in *A. parasiticus*. Oxaloacetate gave maximum stimulation, followed by citrate, malate, fumarate, pyruvate, lactic acid, and other acids.

The levels of tricarboxylic acid cycle enzymes showed considerable variation during zinc deficiency and in nontoxigenic strains (126, 127). The aflatoxigenic strain in a zinc-supplemented medium had maximum levels of succinic dehydrogenase, malate dehydrogenase, and malic dehydrogenase (decarboxylating) at the end of the exponential growth phase, whereas pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase reached maximum levels on day 4 of growth, and then the levels decreased rapidly. The toxigenic strain in a zinc-replete medium had higher levels of pyruvate and succinic acid dehydrogenases during the exponential growth phase and lower levels of the other four enzymes investigated as compared with a non-aflatoxigenic strain or zinc-deficient cultures. To sum up, during stationary phase, the nontoxigenic strain showed higher tricarboxylic acid cycle activity, and it showed less of the activity during the exponential phase. In the aflatoxigenic strain, the tricarboxylic acid cycle activity declined during the period of aflatoxin formation (Table 3). Jechova et al. (175) observed that, when *S. aureofaciens* starts forming chlortetracycline, the activity of malate dehydrogenase (decarboxylating) decreases, and this enzyme served as a source of NADPH. The other source of NADPH is the HMP pathway, and it is probable that more NADPH is formed via this route. We have observed stimulation of [1-^{14}C]acetate incorporation into aflatoxins by 100 μM levels of NADPH in a suspension system (unpublished data). In our studies, we observed high levels of malate dehydrogenase (decarboxylating) in the nontoxigenic strain during the stationary phase, which would result in formation of more lipids (Table 3). Shih and Marth (275) reported stimulatory effect of NADPH on aflatoxin production.

Pool of adenine nucleotides. The reviews by Demain (71) and Holzer (159) deal with the regulation of secondary metabolism in fungi by the adenylic acid system. According to Atkinson (9, 10), the energy charge regulation of biosynthetic pathways involves activation and inhibi-

TABLE 3. *Enzymes of the glycolytic and tricarboxylic acid cycles in relation to aflatoxin biosynthesis*

Strain	Medium	Days of growth	Enzyme activity (U/mg of protein)										
			Hexokinase (EC 2.7.1.1)	Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)	Pyruvate kinase (EC 2.7.1.40)	Aldolase (EC 4.1.2.13)	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	Pyruvate dehydrogenase (EC 1.2.4.1)	Isocitrate dehydrogenase (EC 1.1.1.42)	α-Ketoglutarate dehydrogenase (EC 1.2.4.2)	Succinic dehydrogenase (EC 1.3.99.1)	Malate dehydrogenase (EC 1.1.1.37)	Malic dehydrogenase (decarboxylating) (EC 1.1.1.40)
A. parasiticus	Zn deficient	2	14.80	0.15	0.59	5.56	13.72	1.80	2.71	3.14	3.20	63.30	9.60
		4	5.40	0.13	0.23	1.35	14.56	7.14	4.74	5.00	3.70	100.00	7.10
	Zn replete (SLS[a])	2	25.50	0.43	1.92	12.54	28.54	1.96	2.00	1.55	3.97	242.50	8.60
		4	7.60	0.20	0.79	2.31	20.11	3.85	3.56	2.32	3.80	67.44	5.60
A. flavus	Zn replete (SLS)	2	24.90	0.36	1.02	9.12	9.55	1.84	10.21	3.96	2.51	285.80	9.10
		4	3.35	0.16	0.66	2.30	11.22	3.71	4.86	4.73	3.02	94.10	14.10
Assay used (reference)			(178)	(75)	(235)	(277)	(302)	(287)	(226)	(287)	(180)	(330)	(226, 101)

[a] SLS, Sucrose-low-salts medium (123, 135).

tion of enzymes of primary metabolism by relative levels of adenylates (ATP, adenosine 5'-diphosphate [ADP], AMP, and P_i) in the cell. A high energy charge inhibits some enzymes and activates others. A high concentration of P_i might increase ATP formation and lead to a high energy charge in the cell.

High amounts of P_i have been reported to inhibit production of secondary metabolites, such as antibiotics and pigments (70, 71, 73, 313). Janglova et al. (173) examined the ATP content of two strains of *S. aureofaciens,* one strain giving high yields of chlortetracycline and the other being a low producer. In both strains, the ATP concentrations rapidly increased during growth (exponential phase) and then rapidly decreased and remained almost constant during most of the stationary phase. The low chlortetracycline-producing strain had two to four times as much ATP as the high antibiotic-producing strain (173).

We have investigated the comparative levels of adenylates in aflatoxigenic and non-aflatoxigenic strains in zinc-replete and zinc-deficient media. The results are presented in Table 2 (125, 128). The ATP levels of *A. parasiticus* in a zinc-supplemented medium were high during the exponential growth phase and declined during the stationary phase. The reverse was true for levels of ADP and AMP. The P_i content was low in a zinc-replete medium throughout the growth period, and the energy charge was high during the exponential phase but low during the stationary phase. Generally, the high levels of AMP in the cells lowered the energy charge values (91, 92). The non-aflatoxigenic strain had low levels of ADP and AMP throughout the growth period, as compared with the aflatoxigenic strain. The P_i and energy charge values of the nontoxigenic strain were high during the phase of stationary growth. The ATP level of the nontoxigenic strain was lower on day 2 of growth but increased gradually during the stationary phase.

Chapman et al. (53) reported that an energy charge of 0.8 or higher is required for growth of *E. coli,* and when the energy charge value was less than 0.5, growth ceased. Therefore, the lower energy charge value in the zinc-deficient cultures of *A. parasiticus* results in slow synthesis and accumulation of primary metabolic precursors, leading to reduced aflatoxin yields.

Forrest (106, 107) reported that a critical concentration of ATP is required for the exponential phase of growth, and, at low ATP levels, a shift to the stationary phase takes place. According to Janglova et al. (173), high levels of ATP during the stationary phase are inhibitory for

chlortetracycline biosynthesis. The high levels of ATP in zinc-deficient cultures during the stationary phase (Table 2) may, similarly, be inhibitory to aflatoxin formation. According to Atkinson (8–10) the level of AMP is a sensitive control signal of the state of ATP supply. When the AMP content is low, most of the acetyl CoA is diverted to synthesis of lipids. High AMP content, on the other hand, is inhibitory for lipid synthesis and, therefore, favorable for aflatoxin formation.

Low production of aflatoxins, a high lipids formation rate, and low AMP levels in zinc-deficient cultures, as compared with high aflatoxin, low lipid content, and high AMP levels in zinc-supplemented cultures, have been observed in our laboratory (Table 2), thereby supporting the postulate of Atkinson (8–10). The low levels of AMP and high levels of lipids in the nontoxigenic strain can be explained similarly. From our observations of the low levels of ATP in the toxigenic strain during the stationary phase (Table 2), it can be postulated that high levels of ATP are inhibitory for aflatoxin biosynthesis. This is in agreement with the observations of many workers (106, 173, 217, 236). The accumulation of excess of P_i and high energy charge value in the nontoxigenic strain can be assumed to be unfavorable for aflatoxin biosynthesis (53, 92, 112, 311–313).

Curdova et al. (65) investigated the relationship between energy metabolism and chlortetracycline biosynthesis of *S. aureofaciens*. The energy charge values in a culture of a low-antibiotic strain were almost identical to those of a highly productive strain, but the total of adenylates was about 10 times higher. In the stationary phase, both strains showed a drop in energy charge values followed by a rise to the original level. An increase in the concentration of P_i in fermentation medium caused a suppression of antibiotic formation in the low-chlortetracycline-producing strain and a further rise in the total adenylate levels. The energy charge values acquired a complex character, owing to the participation of high-molecular-weight polyphosphates as energy donors and the adenylate system and to the probable lack of such a regulatory mechanism as the adenylate kinase reaction (20, 65, 161).

The drop in the energy charge values in the stationary phase was attributed to increased levels of ADP and AMP and not to a lowering of high energy bonds. During the exponential phase, ATP and ADP were utilized for protein synthesis. The increase in AMP levels was not at the expense of ATP and ADP.

The rise in AMP levels was attributed to the higher proportions of dying, inactive cells and a higher synthesis of AMP. The levels of adenylates, enzymes of the Krebs cycle, and sugar phosphorylation each showed a two-phase character by exhibiting two maxima.

There was a considerable fluctuation in the concentrations of the nucleotides; the ratio $(AMP \times ATP)/ADP^2$ was considerably lower than in other organisms, where it is usually about 1. This fluctuation led Curdova et al. (65) to conclude that *S. aureofaciens* does not possess any regulatory mechanism, such as the adenylate kinase reaction, that would stabilize the mutual production of individual adenylates. In such a case, the energy charge level will be unstable and insufficient for characterization of energy relations in the cell. The energy metabolism of such species depends on polyphosphates and polyphosphate hexokinase reactions (20, 65). Since the results obtained by us are very similar (125, 128), it is probable that a regulatory mechanism by the adenylates may not be functioning in *A. parasiticus*.

Nicotinamide Nucleotide Coenzyme Pool

Sanwal (261) had discussed the regulatory roles of nicotinamide nucleotide coenzymes in microbial metabolism and summarized the evidence for the inhibition of the tricarboxylic acid cycle by NADPH. It is known that glucose represses several tricarboxylic acid cycle enzymes and that the enteric microorganisms have high levels of NADPH when aerobic growth occurs in glucose. The level of NADPH can be considered as an indicator of the state of glycolysis. The inhibition of citrate synthase would block further production of NADPH, but this inhibition is reversed by acetyl CoA and oxaloacetate (311, 312). The results obtained in our laboratory (125) show a marked reduction of nicotinamide nucleotide coenzymes in a zinc-depleted medium (Table 2). This showed that zinc, by stimulating synthesis of these coenzymes, ultimately stimulates fungal metabolism. The toxigenic strain had high levels of NADP and low levels of NAD during the entire growth period (Table 2). Our results (125) are in agreement with the findings of many workers who have observed reduced coenzyme levels during zinc deficiency (144, 172, 260, 318). A high level of NAD is required for oxidative reactions of the organism. Miller et al. (216) observed that oxytetracycline required only 3 mol of NADPH, as compared with 14 mol required for the synthesis of palmitic acid. Jechova et al. (175) reported that low activity of the NADPH-generating system was a prerequisite to tetracycline biosynthesis. The formation of triacetic acid lactone in *E. coli* from

acetyl CoA has been shown to be dependent on the absence of NADPH (35, 36).

Shih and Marth (275) have observed increased aflatoxin formation in *A. parasiticus* in the presence of increasing levels of NADPH. Moreover, sodium azide, an inhibitor of terminal electron transfer resulting in accumulation of NADPH, increased synthesis of both aflatoxins and lipids. Singh and Hsieh (278) showed that the conversion of sterigmatocystin to aflatoxin in a cell-free extract of *A. parasiticus* was NADPH dependent.

Protein and Nucleic Acid Metabolism

Many reports have appeared about the termination of nucleic acid and protein syntheses during the stationary growth phase or the period of onset of secondary metabolism (32, 33, 42–46, 70–73, 76, 80, 311–313). Detroy and Hesseltine (80) and Detroy and Ciegler (76) showed continued aflatoxin biosynthesis when protein and nucleic acid biosyntheses declined in *A. parasiticus* during the stationary growth phase.

We have observed (121, 129) that during the period of aflatoxin biosynthesis (stationary phase) the levels of proteins and nucleic acids remain constant or decline. A similar pattern is observed during zinc deficiency, when aflatoxin formation is lowered considerably. In a non-aflatoxigenic strain, continuous synthesis of protein was observed, even during the stationary-phase period (Table 2). This shows that synthesis of primary metabolites is not shut off completely in non-aflatoxigenic strains.

Schmidt et al. (264) studied the activities of various enzymes of aflatoxigenic and non-aflatoxigenic strains of *S. flavus* and *A. parasiticus*. Buffered mycelial extracts were prepared from 20 isolates of *A. flavus* and 4 of *A. parasiticus*, and enzyme profiles were obtained by polyacrylamide gel electrophoresis. *Aspergillus* species were most readily distinguished by bands of α-esterase, β-esterase, and peroxidase. No distinct differences in enzyme patterns were detected between aflatoxigenic and non-aflatoxigenic strains. It was postulated that the enzymes of aflatoxin biosynthesis are, probably, either present in too small concentrations to be detected or poorly resolved by electrophoresis (264).

Lipid Biosynthesis

The correlation of aflatoxin and lipid biosyntheses has been established beyond doubt, but whether it is direct or indirect seems to be controversial. Detroy and Hesseltine (79) studied the formation of lipids and aflatoxins under different environmental conditions. Acid pH favored ^{14}C incorporation into aflatoxins, whereas neutral or slightly alkaline pH favored synthesis of lipids. Under high acetate concentrations, aflatoxin formation was completely blocked, whereas lipid synthesis increased several-fold. It was concluded that an inverse relationship existed between aflatoxin and lipid biosyntheses. A similar pattern for lipid and alternariol formation has been reported by Tanenbaum (293).

A similar inverse correlation between wax ester and aflatoxin formation has been shown by us (207). A comparative study of [^{14}C]acetate incorporation into phospholipids by a toxigenic and a nontoxigenic strain yielded similar results. Recent work by Gupta (121) in our laboratory has supported the above view. It has been observed that increased synthesis of lipids occurs in conditions unfavorable for aflatoxin formation. Increased lipid formation was observed in cultures grown in zinc-deficient medium and in a non-aflatoxigenic strain (Table 2). Coccuci and Rossi (61) observed a similar increase of lipid synthesis in *R. gracilis* during zinc deficiency.

Rambo and Bean (246) compared eight isolates of *A. flavus* and *A. parasiticus* and found no correlation of total lipids, fatty acids, or sterols with aflatoxin production. Gupta et al. (133) have shown that aflatoxigenic and non-aflatoxigenic strains had similar patterns of phospholipid biosynthesis, and the differences observed by Raj et al. (245) were due to differences in the labeling of the fatty acid moieties of phospholipids. Gupta et al. (132–134) further showed that the toxigenic strain had a higher total lipid content than the nontoxigenic strain. The differences between the observations of Gupta et al. (132) and Gupta (121) may be due to the different strains used.

Shih and Marth (274, 275) have observed a similar pattern for the formation and depletion of lipids and aflatoxins in *A. parasiticus*. A similar or dissimilar pattern for lipids and aflatoxins could be obtained at different temperatures (274). It was suggested that the formations of both lipids and aflatoxins shared some similar biosynthetic steps. Certain conditions favored lipid synthesis and inhibited aflatoxin production, whereas, under several other conditions, similarities between formations of the two products were noted. Cultural conditions regulate the amounts of final products formed (275).

Nitrification by Aflatoxigenic Strains

Reddy and co-workers (250–252) observed stimulation by asparagine of aflatoxin formation in *A. parasiticus*. Little aflatoxin was obtained in the absence of asparagine and in presence of inorganic nitrogen sources, like ammonium sulfate, acetate, chloride, etc. Asparagine could be

effectively replaced by aspartic acid and alanine. Glycine and glutamic acid gave about 65 and 43% aflatoxin yields, respectively, as compared with asparagine. A number of other amino acids supported very little aflatoxin formation.

Shih et al. (276) reported the existence of the capacity for nitrification and aflatoxin production by strains of *A. flavus* and *A. parasiticus*. A lower pH of the yeast extract source was found to be favorable for aflatoxin synthesis, whereas a higher pH favored nitrification.

CONCLUSIONS AND SPECULATIONS

The study of the biosynthesis of aflatoxins has been dominated by the use of labeled precursors, identification of accumulated products in mutant strains unable to form aflatoxin, and resting-cell suspension experiments. Useful information can be obtained from such methods only if it is known that added compounds can cross the permeability barrier. The permeability question could, of course, be answered if labeled substrates were used, but synthesis of a large number of possible intermediates in the labeled form would be a tremendous task. The simplest approach would be the use of cell-free preparations capable of carrying out some or all of the reactions of aflatoxin biosynthesis.

There is little literature dealing with enzymatic biosynthesis of acetate-derived secondary fungal metabolites at the cell-free level. According to Tanenbaum (293), this is due to the difficulties involved in finding reproducible methods for the release of active extracts from the fungal mycelium, the difficulties involved in obtaining total biosynthesis from the acetate-malonate pathway, and the lack of knowledge concerning the polyketides that serve as immediate precursors. It is difficult to establish whether the labeled compound is a normal obligatory intermediate in the biosynthesis of secondary metabolites (42, 296). This is mainly due to nonspecific enzymes; the compound may be in equilibrium with a true intermediate, and the relative nonspecificity of enzymes of secondary biosynthesis may give rise to several different pathways (44, 296).

The encouraging results obtained with the development of cell-free systems for many secondary fungal products, like actinomycins, tetracyclines, penicillins, cephalosporins, fusidic acid, 6-methylsalicyclic acid, and alternariol, point to the possibility of a similar development in the aflatoxin biosynthesis (34, 37, 85, 86, 103, 240, 271, 293, 305, 308, 331). Singh and Hsieh (278) obtained partial success in developing a cell-free system for the conversion of sterigmatocystin to aflatoxin.

The overall scheme for the biosynthesis of aflatoxins is purely speculative and not well established, unlike those of fatty and amino acids biosynthesis. It is probable that the search for intermediates is fruitless, since several steps of the reaction sequence may be carried out on protein-bound intermediates, e.g., acyl carrier protein in fatty acid biosynthesis and polyketide carrier protein in the case of aflatoxin biosynthesis. The number of steps involved in aflatoxin biosynthesis from acetate is probably more than 50 (81). The polyketide theory of aflatoxin biosynthesis suffers from its demands for numerous binding sites and structural geometry to orient the residues and make them reactive. Direct evidence for a specific carrier protein in polyketide biosynthesis is not available. However, it is well known that polyketide synthesis shows an all-or-nothing characteristic, being a system that is not accessible to intermediates not bound to the carrier protein. The only intermediates susceptible to exogenous dilutions are acetyl and malonyl CoA.

The sequence of events leading to aflatoxin formation probably is as follows. Exhaustion of nitrogen or phosphorous in the medium at the end of the exponential growth phase is accompanied by inhibition of nucleic acid and protein synthesis and accumulation of the primary metabolic precursors. α-Keto acids, particularly pyruvic acid, accumulate to toxic levels, thereby inhibiting cellular division and growth. During the transition phase, from exponential to stationary, the syntheses of nucleic acids, proteins, and lipids continue, but at reduced rates. The enzymes of aflatoxin biosynthesis are derepressed during the transition phase. The activity of the tricarboxylic acid cycle continues unabated and reaches a maximum during the transition phase.

The fungus responds to toxic levels of pyruvate and other intermediates by shifting to the stationary growth phase. This is the period of onset of aflatoxin production. The synthesis of nucleic acids and proteins is terminated, and tricarboxylic acid cycle activity declines. Lipid synthesis continues unabated during the stationary phase, thereby showing the close relationship with aflatoxin biosynthesis. Studies with incorporation of labeled acetyl CoA, malonyl CoA, acetoacetyl CoA, hydroxymethylglutaryl CoA, and α-ketobutyryl CoA into aflatoxins, fatty acids, and sterols may reveal the common steps in the biosynthetic pathways of aflatoxin and lipids. It is likely that increased utilization of α-keto acids in aflatoxin biosynthesis also results in increased gluconeogenesis and transaminase activity during the stationary phase.

Information is also required about the induction of various fungal enzymes by zinc and can be obtained by growing the fungus on a zinc-deficient medium and then suddenly adding adequate zinc levels during the various growth phases for induction and assay of enzyme activities. Similar studies with asparagine-deficient medium are also required. One other area is the potential chemical induction of aflatoxin formation by non-aflatoxigenic strains and the mechanisms of stimulatory action of pyruvate and ethyl acetoacetate and of inhibitory action of barium, mercaptoethanol, and tolnaftate. Recently, Floss and co-workers (103, 104, 184) challenged the popular view that secondary metabolites are "metabolic accidents" due to a regulatory defect in primary metabolism. Floss et al. (104) failed to observe any correlation between ergoline (alkaloid) and tryptophan biosyntheses and showed that tryptophan biosynthesis was well regulated in *Claviceps* species. Drew and Demain (89) arrived at a similar conclusion about the regulation of cephalosporin biosynthesis by methionine.

The pathways of secondary metabolism in fungi are related to the primary metabolic pathways and use the same precursors. The polyketide route, the terpenoid route, and the pathways utilizing essential amino acids are the three important pathways of secondary metabolites in fungi (296, 297). The polyketide route is generally associated with higher fungi (81, 297). The well-known antibiotics penicillin and cephalosporins are derived from amino acids, and the study their biosynthesis in cell-free systems has been reported (18, 37, 225). Gibberellins and fusidic acid are the well-known examples of a large number of terpenoid-derived compounds isolated from fungi (297).

Most of the fungal metabolites are formed by the polyketide route, and the majority of polyketides are produced by fungi. There are two distinct phases in polyketide biosynthesis. The initial phase is the assembly of the polyketide chain and its modification, usually by aromatization or reduction, to give a stable product; this phase involves protein-bound intermediates. In many cases, the first stable product may be the major metabolite, but, generally, it undergoes further modifications during the second phase to give the major metabolite (44, 297, 298). The initial steps in aflatoxin biosynthesis from the condensation of one acetyl CoA and nine malonyl CoA molecules to a C_{20} polyketide or polyhydroxyanthraquinone, such as averufin or averufanin, probably takes place in the polyketide carrier protein. It is known that the fatty acid synthetase of fungi is generally found in a particle distinct from the ribosomes and that it sediments at high centrifugation speed and is nonmembranous (163, 249, 278, 280, 280a, 298). It is probable that the enzymes of aflatoxin biosynthesis and other polyketides are similarly arranged in discrete particles in the postmicrosomal fraction. It is probable that the reduction of the polyketide chain in aflatoxin biosynthesis is stepwise, as in fatty acid biosynthesis. The first reduction occurs at the triketide level, and dehydration follows before the next condensation step. Dimroth et al. (85, 86) and Scott et al. (271) have obtained evidence for such a sequence in the case of purified 6-methylsalicyclic acid synthetase. The intermediates, up to the C_{20} polyhydroxyanthraquinones on the polyketide carrier protein, may be stabilized by metal chelation (81, 311). The stimulatory metals for aflatoxin formation, like zinc, iron, magnesium, and copper, may have such a role. The later steps, from averufin to versicolorin, sterigmatocystin, and aflatoxin, may take place at the same site, or these enzymes may be membrane bound in either the cellular or the mitochondrial membrane. The methylation step lies beyond versicolorin but before sterigmatocystin. Harris et al. (144) showed that the methylation step was late in the biosynthetic scheme for griseofulvin, after the formation of aromatic rings and in the presence of a relatively stabilized methyl pool. Detroy and Freer (78) postulated that in aflatoxin biosynthesis the polyketide chain is methylated before aromatization and that C methylation and methionine formation are essential for aflatoxin biosynthesis. The hydroxylation and lactonization of the aflatoxin B_1 molecule may take place in the mitochondria. The NADPH dependence of sterigmatocystin-to-aflatoxin conversion indicates that the enzyme catalyzing the reaction is, most probably, an oxygenase, like the one in patulin biosynthesis (103, 220, 278). Increasing evidence shows that the biosynthesis of fungal polyketides is favored at a slightly alkaline pH (7.5 to 8.0). The mixed-function oxidases involved in the biosynthesis of patulin, ergoline, 6-methylsalicyclic acid, and aflatoxin have pH optima of 7.5, 7.8, and 7.5 to 7.8, respectively (85, 86, 103, 220, 271, 278). The conversions of versicolorin A, norsolorinic acid, and other intermediates into aflatoxins have been achieved (169). This shows the basic soundness of the hypothetical biogenetic scheme of aflatoxins outlined in Fig. 5. The characterization, localization, activation, and regulation of "aflatoxin synthetase" must be studied in detail to have definite ideas about the terminal steps of aflatoxin biosynthesis. Strains forming only one type of aflatoxin (like B_1 or B_2) should prove

valuable for biosynthetic studies because of less complications. Most of the biosynthesis work on aflatoxins has been done in batch cultures, in which the developing fungal population continuously modifies its environment and, by depleting the nutrient substrate, itself becomes modified by the changes in growth rate and metabolism of the cells produced. Batch cultures develop with time, and changes are related to the ages of the cultures. The continuous culture, on the other hand, is "ageless," and the reproduction period or the cell cycle can be measured. However, secondary biosynthesis is absent in continuous cultures (312). The use of synchronization cultures for studying the biosynthesis of aflatoxins is recommended. In synchronization cultures, the cells are in the same metabolic state, and aspects of cultivation related to multiplication could be followed. The developmental and cellular activities involved in the multiplication of cells can be studied.

The results obtained by using different methods of cultivation could reflect the varying trends in cell metabolism in relation to growth. Strict control of the cellular environment during growth is necessary for all cell studies. The cell cannot be assumed to behave in a constant manner, but is modulated under varying conditions.

ACKNOWLEDGMENTS

We are grateful to several colleagues who had the patience to read this article and participate in critical discussions that helped to improve it. In particular, we wish to mention B. D. Sanwal, K. Santhanam, K. G. Mukerji, U. K. Sinha, K. K. Kohli, and A. Dixit.

We also thank The Department of Science and Technology for financial support and the Council of Scientific and Industrial Research, New Delhi, for the placement of one of us (K.K.M.) in the Scientist's Pool.

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