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Enzymes and Aflatoxin Biosynthesis

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INTRODUCTION

The enzymology of secondary metabolism has not received the same attention as that for primary (general) metabolism. There are traditional and other reasons for this bias, some of which will become apparent in this review.

With the advent of recombinant deoxyribonucleic acid technology (genetic engineering), it seems inevitable that this state of affairs should change. Many secondary metabolites have economic significance, and it would be very desirable to be able to manipulate their production by using this latest technology. Strictly speaking, it is not impossible to do this without a knowledge of the enzymes involved in their production, but in practice it is essential that we have thorough and detailed information on the enzymes (and their regulation) that we would desire to manipulate. The truth of this statement is well supported by the increased interest in the enzymes responsible for the formation of secondary metabolites of commercial importance (93, 116, 135). The field of antibiotics is a particularly good example of this groundswell and includes work on penicillin (103), tetracycline (104), tylosine (129), and bacitracin (158).

Another group of secondary metabolites of economic impact are the mycotoxins, although it is implicit in their nature that we should try to prevent, rather than promote, their formation. An important example of mycotoxins, the aflatoxins, have generated much interest in both their effects and their biosynthesis; investigations into the latter have led to elucidation of the pathway of their formation (16). In spite of agreement on the validity of this pathway, there is still much to be learned with respect to the enzymology of the process.

This review attempts to bring together what is known with regard to these enzymes and their regulation and associated processes and also to stimulate further interest in the subject.

FUNGAL SECONDARY METABOLIC ENZYMES

Characteristics

As far as has been ascertained, the properties of secondary metabolic enzymes are the same as those of their primary counterparts. They are formed by the usual protein biosynthetic machinery and in many cases are subject to feedback inhibition, induction, and catabolite repression (62). A more important likeness is the use of common coenzymes, and that directly links secondary metabolic processes with the overall metabolic state of the cell in terms of reduced coenzyme and energy charge. These conditions may dictate whether a secondary metabolic pathway is functional, either by activation through phosphate-nucleotide phosphate balance (62) or as a consequence of other thermodynamic equilibria, e.g., a high reduced nicotinamide adenine dinucleotide phosphate (NADPH)/$NADP^+$ ratio (high anabolic reduction charge [23]).

Secondary metabolic enzymes, however, do have some special characteristics of their own; they are only active or formed at the commencement and during the idiophase (12) when normal growth has ceased and differentiation has commenced (for review, see reference 172). In certain cases, they exhibit relative specificity (132); i.e., an enzyme catalyzes analogous reactions with a series of structurally related metabolites, often resulting in the generation of a metabolic grid (37), unlike primary metabolic enzymes which are usually absolutely specific. Secondary metabolic activity usually ceases because of synthase decay or feedback inhibition and enzyme repression (55) or both.

One group of enzymes, oxygenases (EC 1.13/1.14), are involved in oxidative modification of many secondary metabolites, and this includes the aflatoxins. Oxygenases catalyze the incorporation of molecular oxygen into their substrates. They are divided into two classes: monooxygenases that incorporate one atom from molecular oxygen,

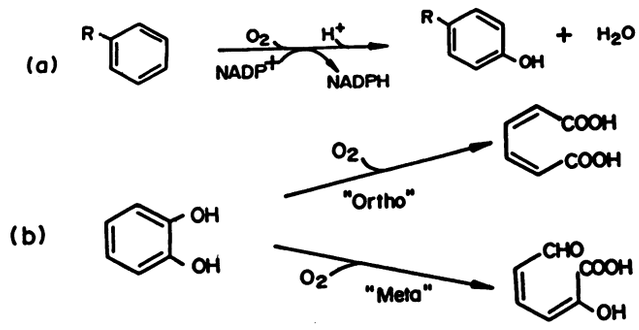

FIG. 1. Incorporation of molecular oxygen by oxygenases: (a) monooxygenases and (b) dioxygenases.

the other atom being reduced by NADPH (Fig. 1a), and-dioxygenases that incorporate both atoms of oxygen, sometimes with ring cleavage (Fig. 1b). The important detoxifying system, cytochrome P-450, is a monooxygenase containing the heme prosthetic group (201); others require metal ions. Dioxygenases often are involved in ring cleavage reactions and may act in concert with monooxygenases to degrade recalcitrant substances via ortho or meta fission (52) (Fig. 1b).

Another type of oxidative cleavage found in aflatoxin biosynthesis is the Baeyer-Villiger (BV) reaction. In this process an oxygen atom is inserted between two carbons, one of which has a carbonyl function, to yield an ester or lactone. An example found in a filamentous fungus is the conversion of progesterone via 4-androstene-3,17-dione to testololactone (Fig. 2) by *Penicillium lilicium* (153). As only one of the molecular oxygen atoms is found in the end product, enzymes catalyzing BV reactions can be regarded as monooxygenases.

Table 1 summarizes some oxygenases found in filamentous fungi, although their occurrence in these organisms is not well defined in spite of several comprehensive reviews (95, 133, 144).

Isolation

One reason why studies on the enzymology of fungal secondary metabolism have had a slow start is the difficulties inherent in obtaining active cell-free fractions. The first problem is to determine when in the growth cycle to harvest and extract the cells. This is not difficult to ascertain, but it does require investigation to find when the trophophase switches to the idiophase and what triggers this event. The trigger can be quite complex, being controlled by a repressor, such as carbon and nitrogen source, or energy charge or one of several other regulatory systems (62).

The next step is to choose an appropriate method of cell disruption. The number of techniques available is substantial, and most of these have been used in the laboratory (Table 2) and industry (120) to make preparations from filamentous fungi. In the case of aflatoxin biosynthesis, Hsieh and co-workers used a rotating wire loop with glass beads to obtain a system that converted versiconal acetate to versicolorin A (198) and disruption with glass beads to prepare one that could convert sterigmatocystin to aflatoxin B_1 (170). Jeenah and Dutton have used preparations from powdered lyophilized mycelium that converted norsolorinic

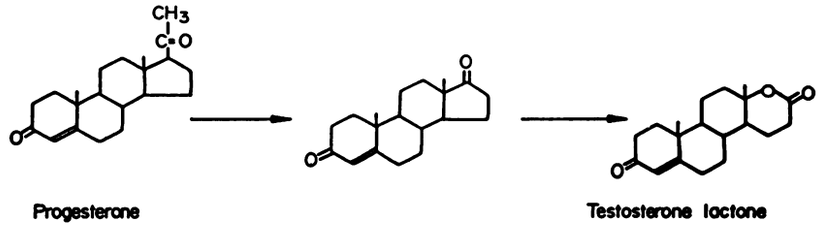

FIG. 2. Conversion of progesterone to testololactone by *P. lilicium* (153).

TABLE 1. Examples of oxygenases found in filamentous fungi

Reaction or enzyme	Substrate	Organism	Reference(s)
Cytochrome P-450	Aromatic ring	*Cunninghamella* spp.	44, 70, 71
Cytochrome P-450	Steroid	*Rhizopus nigricans*	31
Cytochrome P-450	Alkaloids	*Claviceps* sp.	5
Hydroxylation	Steroid	*Aspergillus ochraceus*	108
Hydroxylation	Steroid	*Aspergillus niger*	1, 102
Hydroxylation	Steroid	*Curvularia* sp.	145
Hydroxylation	Aromatic ring	*A. niger*	10, 29, 118, 178
Hydroxylation	Terpene	*A. niger*	76
Hydroxylation	Biphenyl	*A. parasiticus*	50
Hydroxylations	Various	Several	30, 173
Hydroxylation	Aromatic ring	*Penicillium patulinum*	139
Epoxidation	Cyclopenin	*Penicillium cyclopium*	197
BV	Steroid	*Cylindrocarpon* sp.	107
BV	Hydrocarbon	*Penicillium* sp.	4
BV	Hydrocarbon	*Fusarium lini*	181
Cleavage	Aromatic ring	*Penicillium patulum*	161
Cleavage	Various	Several	41
Cleavage	Quercetin	*A. flavus*	146
Lipoxygenase	Fatty acid	*Fusarium oxysporum*	159

TABLE 2. Examples of methods used to prepare cell-free extracts from filamentous fungi

Method	Fungus	Enzyme or product	Reference(s)
Grind (buffer)	*Aspergillus niger*	Oxygenase	1, 118, 178
Grind (sand)	*A. flavus*	Kojic acid	11
Grind (sand)	*A. parasiticus*	Oxidase	154
Grind (sand)	*Penicillium cyclopium*	Alkaloids	157, 196
Grind (sand)	*Fusarium oxysporum*	Oxygenase	159
Grind (sand)	*Cylindrocarpon* sp.	Oxygenase	107
Freeze/grind	*Aspergillus ochraceus*	Oxygenase	81
Freeze/grind	Several	Ring fission	41
Freeze/grind	*Aspergillus tenuis*	Alternariol	78
Freeze/grind	*Penicillium patulum*	6-MSA	60
Freeze/grind	*Claviceps* sp.	Transferase	98
Freeze/blend	*A. niger*	Reductase	101
Freeze/grind	*Rhizopus leguminicola*	Slaframine	85
Freeze/lyophilize	*A. niger*	Dehydrogenase	113
Glass beads	*Penicillium lilacinum*	Oxygenase	43
Glass beads	*Fusarium oxysporum*	Desaturase	202
Glass beads	*Penicillium urticae*	Ascladiol	164
Glass beads	*Penicillium baarnense*	Dehydrogenase	21
Glass beads	*A. parasiticus*	VA	198
Polytron (beads)	*A. parasiticus*	AFB1	170
Omni mixer	*Rhizopus nigricans*	Hydroxylase	31
VirTis blender	*Pyrenochaeta terrestris*	Emodin	6
Potter-Elvehjem	*Penicillium brevicompactum*	Mycophenolic acid	49
Protoplast/lysis	*A. parasiticus*	AFB1	8
Protoplast/homogenization	*Cephalosporium acremonium*	Cephalosporin	114
Blendor/ammonia	*Penicillium stipitatum*	Stipitatic acid	180
French press	*Trichothecium* sp.	Trichodiene	69
French press	*P. patulum*	Patulin	161
X-press	*Aspergillus flaviceps*	Methyltransferase	77
Gaulin press	*P. patulum*	6-MSA synthetase	195
Gaulin press	*P. patulum*	Decarboxylase	125
Lyophilization	*A. parasiticus*	Various	34, 109
Lyophilization	*Claviceps* sp.	Ergot alkaloids	117
Lyophilize/grind	*P. patulum*	6-MSA synthetase	124
Lyophilize/sonicate	*Penicillium* spp.	Dehydrogenase	74
Lyophilize/acetone	*A. parasiticus*	Dehydrogenase	142
Lyophilize/beads	*P. patulum*	Patulin	84
Sonication	*Penicillium madriti*	Orsellinic acid	80
Sonication	*Aspergillus amstelodami*	Echinulin	3
Sonication	*Fusarium lini*	Oxygenase	181
Sonication	*Cephalosporium bainieri*	Oxygenase	71
Sonication	*Cephalosporium acremonium*	Epimerase	126
Acetone powder	*A. niger*	Phenol hydrolase	138
Acetone powder	*Pycnoporus* sp.	Synthase	140
Acetone powder	*Penicillium* sp.	Oxygenase	4

acid to averantin and sterigmatocystin to aflatoxin B_1 (109). Lyophilized powders are easy to prepare and are usually stable on deep-freezing; they have also been used to investigate primary metabolic enzymes in *Aspergillus parasiticus* (34).

The least damaging method of preparing cell-free extracts is lysis of protoplasts by osmotic or mechanical means. We found that this was the only technique that gave a system capable of biosynthesizing aflatoxin B_1 from acetate (8). A complete aflatoxin biosynthetic enzyme system made by grinding mycelium with sand has been reported (194); as far as I am aware, it has never been repeated, but the method was used to investigate the interconversion of aflatoxins (131).

Thus, for enzymes involved in aflatoxin biosynthesis, a gentle method of cell wall disruption is advisable. We found that the French press completely destroyed the ability of homogenates to convert sterigmatocystin to aflatoxin B_1; a similar effect was reported for the Hughes press in preparing other systems (84). Enzymes respond differently to a particular method of preparation; for instance, an aromatic dehydrogenase (161) from *Penicillium* sp. gave a higher level of activity when prepared by the French press than when prepared by either grinding or homogenizing the mycelium, which is in contrast to that mentioned above.

Purification

Earlier work on secondary metabolic enzymes used crude cell-free preparations or, at best, fractions produced by ammonium sulfate precipitation. The greater range of modern methods has resulted in purer preparations, although the isolation of pure enzymes is still rare.

The reason for this is not just apathy but often is inherent in the nature of the problem. Attention has already been drawn to the necessity of using gentler techniques, and the probable reason for this is the lower level of secondary metabolic enzyme in the cell. Although this may not be universal, the concentration of these enzymes is highly variable and in certain cases is limiting (cf. primary metab-

olism in which substrate/product concentration is regulated [38]). As low enzyme levels result in denaturing processes having a more marked negative effect, it is important at each stage of enzyme separation that optimization of concentration and stabilization is achieved. Suitable concentration methods are dialysis against a solid substrate, e.g., sucrose, and ultrafiltration. Precipitation with ammonium sulfate or protamine sulfate or streptamine sulfate or pH adjustment (21, 69, 74, 138) may remove much contaminating material. We found the latter method useful in the isolation of a methyltransferase system, although care has to be taken to avoid denaturation (R. K. Berry and M. F. Dutton, unpublished results).

Many other stabilizing agents have been used in enzyme isolation, including the following: glycerol (162), Polyclar AT (removes phenols) (GAF Corp.) (161), thiol reducing agents (74), and di-isopropylfluorophosphate (inhibits autolysis) (79).

AFLATOXINS

The aflatoxins are fungal metabolites produced exclusively by strains of *Aspergillus flavus* (Link ex Fries) and *A. parasiticus* (Speare). They may be classed as secondary metabolites, which according to Weinberg (200) are "natural products that have a restricted taxonomic distribution, possess no obvious function in cell growth and are synthesized by cells that have stopped growing." Although this gives a definition of secondary metabolites, it would be of value to those untutored in the subject to consult a suitable review on secondary metabolism, e.g., that by Drew and Demain (62). Unfortunately, many textbooks on this subject are written from a chemical slant and often gloss over the more controversial biological aspects. Several points of view have been expressed on the subject of function, and the reader is directed to reviews by Bu'Lock (39) Zahner (205), Bennett and Christiansen (14, 16), and Campbell (42) for the more contrasting ones.

Three structural variations of the aflatoxin molecule give rise to a family of eight aflatoxins found in cultures of *A. parasiticus* (*A. flavus* is considered by some authorities to produce the B series only [100]). (i) The B series have a cyclopentenone ring structure, replaced by a lactone in the G series. (ii) The 1 series has a double bond in the terminal furan ring of a bisfuran moiety, absent in the 2 series (Fig. 3). (iii) The M series has a hydroxyl group on the tertiary carbon at the fusion of the two furan rings (Fig. 3). Putting together these features in all possible combinations, the resultant metabolites are: aflatoxin B_1 (AFB1); aflatoxin B_2 (AFB2); aflatoxin G_1 (AFG1); aflatoxin G_2 (AFG2); aflatoxin M_1 (AFM1); aflatoxin M_2 (AFM2); aflatoxin GM_1; and aflatoxin GM_2. Related metabolites are aflatoxin B_{2a} (AFB2a) and aflatoxin G_{2a} (67), aflatoxicol (57), and parasiticol (177) (aflatoxin B_3 [96]) (Fig. 3).

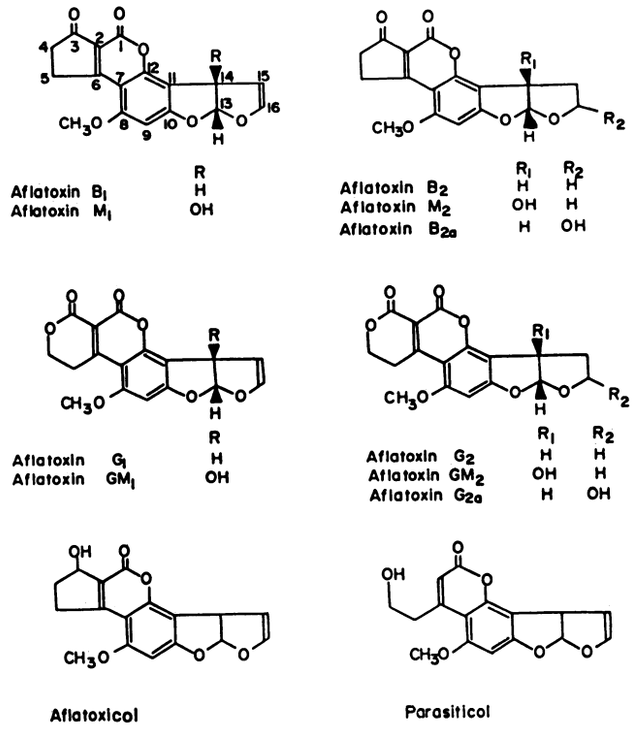

FIG. 3. Structures of the aflatoxins and closely related metabolites.

Biosynthesis and Primary Metabolism

All secondary metabolism stems from primary metabolism; therefore, the metabolic state of the latter will ultimately affect the former. Aflatoxin production is affected by catabolic activity (86, 87), reduced coenzymes level (23), energy charge (155), and metal ions (134). The role of these factors is difficult to define because of the complexity of the metabolic state of the organism. An added problem is that the metabolic activity of the fungus may be compartmentalized (12), and it is becoming clear that more powerful techniques such as recombinant deoxyribonucleic acid technology (15), immunohistology (91), and nuclear magnetic resonance spectroscopy of cells (160) will have to be applied.

Studies on the effect of nutrition on aflatoxin production started soon after their discovery (58). In general, zinc (134), magnesium (53), asparagine (156), proline (152), and high sucrose concentrations plus yeast extract (54) stimulate aflatoxin production, whereas higher levels of inorganic nitrogen (136) and phosphate (156) inhibit it.

More recently, Niehaus and Dilts (142, 143) investigated both glucose-1-phosphate and mannitol dehydrogenase from a toxin-producing strain of *A. parasiticus*. Zinc is considered to favor polyketide (aflatoxin) biosynthesis rather than that of fatty acids (23) because it prevents NADPH formation by inhibition of both of these enzymes. If this is so, then it ought to be a general effect in polyketide-producing fungi.

Buchanan and co-workers (2, 34) suggested that the stimulatory effects of carbohydrates, such as glucose ("carbon catabolic induction"), be mediated through loss of NADPH generation and by repression of the tricarboxylic acid cycle enzymes (33). Other work (165) supports this, although it was concluded here that the repression of tricarboxylic acid cycle enzymes was the key. Low tricarboxylic acid cycle activity minimizes acetate oxidation, leaving it available for aflatoxin biosynthesis.

In contrast (190), aflatoxin biosynthesis has been related to high pyruvate kinase activity, which may promote the utilization of pyruvate or phosphoenolpyruvate as a source of malonyl coenzyme A (CoA). It was found that pyruvate kinase activity was high in toxigenic strains of the fungus but low in the nontoxigenic ones. Other workers (166) have also

stressed the importance of glycolytic activity together with oxygen availability.

Attention has recently been refocused on the role of nitrogen source, and in some cases the observations do not agree with earlier work. Payne and Hagler (152) found that asparagine was less stimulatory in aflatoxin production than previously reported (156). Work on NAD and NADP glutamate dehydrogenase (24) has indicated that they have a role in the generation of idiophase conditions by formation of α-ketoglutarate, which stimulates aflatoxin formation by inhibition of the tricarboxylic acid cycle as mentioned previously. A similar effect is thought to be mediated by glutamate-oxaloacetate transaminase. In another study (110) it was found that nitrate as a sole nitrogen source repressed averufin and aflatoxin synthesis, but the significance of this is not obvious.

Whether all of these effects represent a host of regulation points or whether there is a common factor, e.g., coenzyme availability, still has to be determined; possibly several events act in concert to cause the onset of the idiophase and aflatoxin production.

Biosynthetic Pathway

After 25 years of work, there is now general agreement on the identity of the intermediates involved in the biosynthesis of AFB1, which is the principal member. Evidence in support of this pathway (Fig. 4) comes from studies with putative precursors isotopically labeled with ^{14}C (25), ^{2}H, ^{13}C, or ^{18}O. The latter three isotopes were the subject of a series of elegant nuclear magnetic resonance studies by several groups (see reference 175). Most of these investigations were made with whole-cell cultures of *A. parasiticus*, usually in replacement media with blocked mutants (106, 171). Consequently, there is still much to be learned with regard to the enzymology and mechanistic details of many of the steps involved.

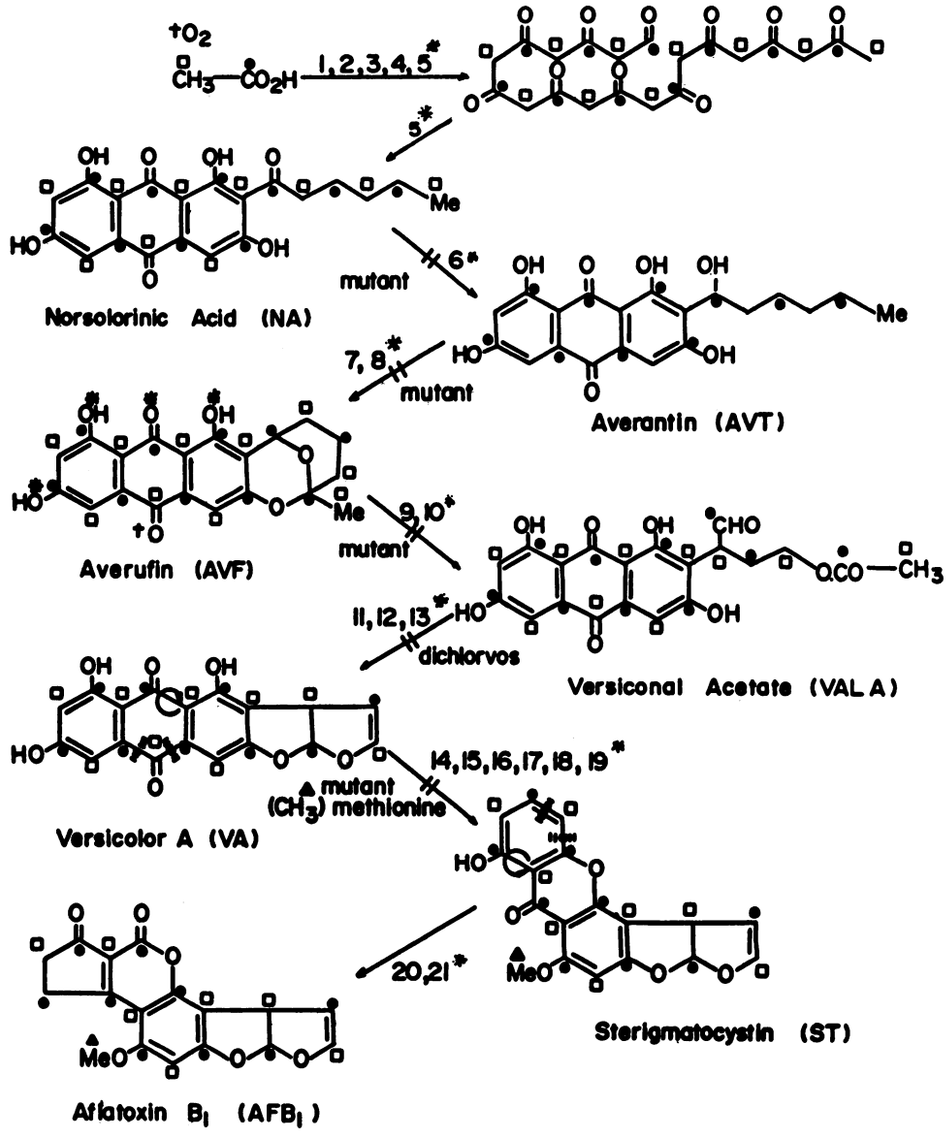

FIG. 4. Proposed biogenesis of AFB1 (189). *Minimum number of steps likely to be enzyme catalyzed. Reproduced with kind permission of the authors and Academic Press, Inc.

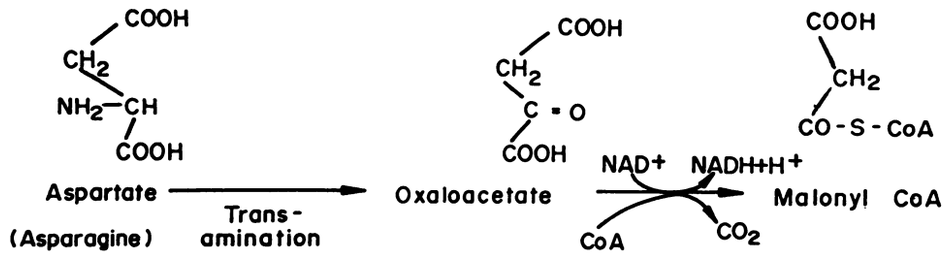

FIG. 5. Transamination of aspartate to oxaloacetate and then to malonyl CoA.

ENZYMES OF AFLATOXIN BIOGENESIS

Anthraquinone Biosynthesis

The main carbon skeleton of AFB1, although only containing 16 carbon atoms (not including the O-methyl group), has been shown by ^{13}C enrichment nuclear magnetic resonance spectroscopy to be derived from a decaketide (20 carbon atoms; Fig. 4) (147).

The polyketide pathway, as propounded by Birch (26), is analogous to fatty acid biosynthesis but without intermediate reductive steps. In the formation of polyketides a "primer" (or "starter") unit, usually an acetyl group, is transferred from acetyl CoA to a thiol group at the active center of the polyketide synthase complex, which is in the form of a flexible protein "arm" in the enzyme complex (16). The acetyl group is attached via a thioester linkage, resulting in a "high energy" conformation. Acetate units are now added sequentially, the enzyme complex utilizing malonyl CoA as the donor with a concomitant loss of carbon dioxide. Once the chain has reached the required length, it is stabilized by cyclization to an aromatic or heterocyclic ring (see Fig. 6). Stabilization in fatty acid synthesis is achieved by reducing the chain to the level of hydrocarbon with NADPH after the addition of each acetyl unit.

The source of malonyl CoA is generally taken to be acetyl CoA by carboxylation, which under idiophase conditions is most likely to be supplied by glycolysis (199). In the biosynthesis of tetracycline by *Streptomyces aureofaciens*, oxaloacetate, under the influence of oxaloacetate dehydrogenase, can give rise directly to malonyl CoA (13). It is possible that a similar scheme is extant in aflatoxin biosynthesis, as it is stimulated by asparagine and aspartate (156). In Fig. 5, aspartate is transaminated to oxaloacetate and thence to malonyl CoA.

AFB1 is derived from nine acetyl units added in seretia to the acetyl primer, resulting in a chain that has seven carbonyl groups, two having been reduced during chain elongation

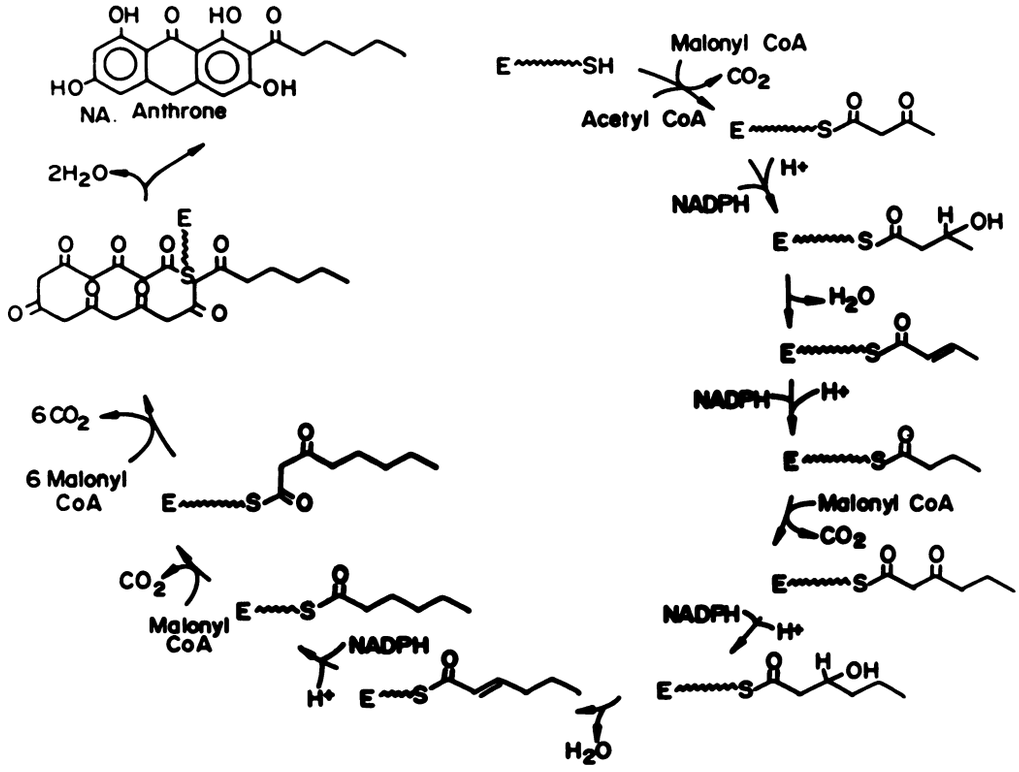

FIG. 6. Hypothetical scheme for the assembly of anthraquinones by a "polypeptide synthase" enzyme complex in *Aspergillus* species (16). Reproduced with kind permission of the authors and Academic Press, Inc.

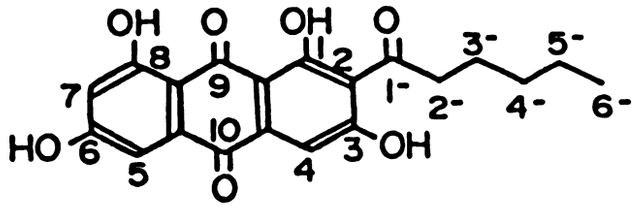

FIG. 7. Structure of NA.

(Fig. 6). The method by which this chain is stabilized during its extension is unknown, but it is probable that noncovalent bonding to the enzyme surface is involved (36). Once the chain has reached the required length, it is stabilized by cyclization as an aromatic or heterocyclic product. The mechanism is obscure, as similar chains can give rise to different folding arrangements depending on the organism (99). Biomimetic studies (92) have shown that the cyclization process can be varied by the experimental condition. Presumably, in vivo the active site in the enzyme provides the directing influence.

Polyketide synthases probably evolved by gene duplication (16), resulting in two sets of genes for fatty acid synthase. The duplicate could mutate and "evolve" into a polyketide synthase. The main changes are the loss of the intermediate reducing steps and the gain of polyketide chain folding and condensing ability. The nature of the evolutionary drive behind these changes is a matter of controversy, but polyketides are metabolically unlike fatty acids and hence could act as regulatory metabolic shunt metabolites, a role suggested for secondary metabolites by at least one authority (39).

The theoretical product in aflatoxin polyketide biosynthesis is an anthrone derivative which has never been isolated, presumably because of its rapid oxidation to the more stable anthraquinone, norsolorinic acid (NA) (Fig. 7), a conclusion supported by ^{18}O-labeling studies (193).

Polyketide biosynthesis at the enzyme level has not been well studied; an exception is the formation of 6-methylsalicylic acid (6-MSA; Fig. 8) (206), which is a simple system, derived from a tetraketide. The polyketide synthase was found to be similar in character to fatty acid synthase, from

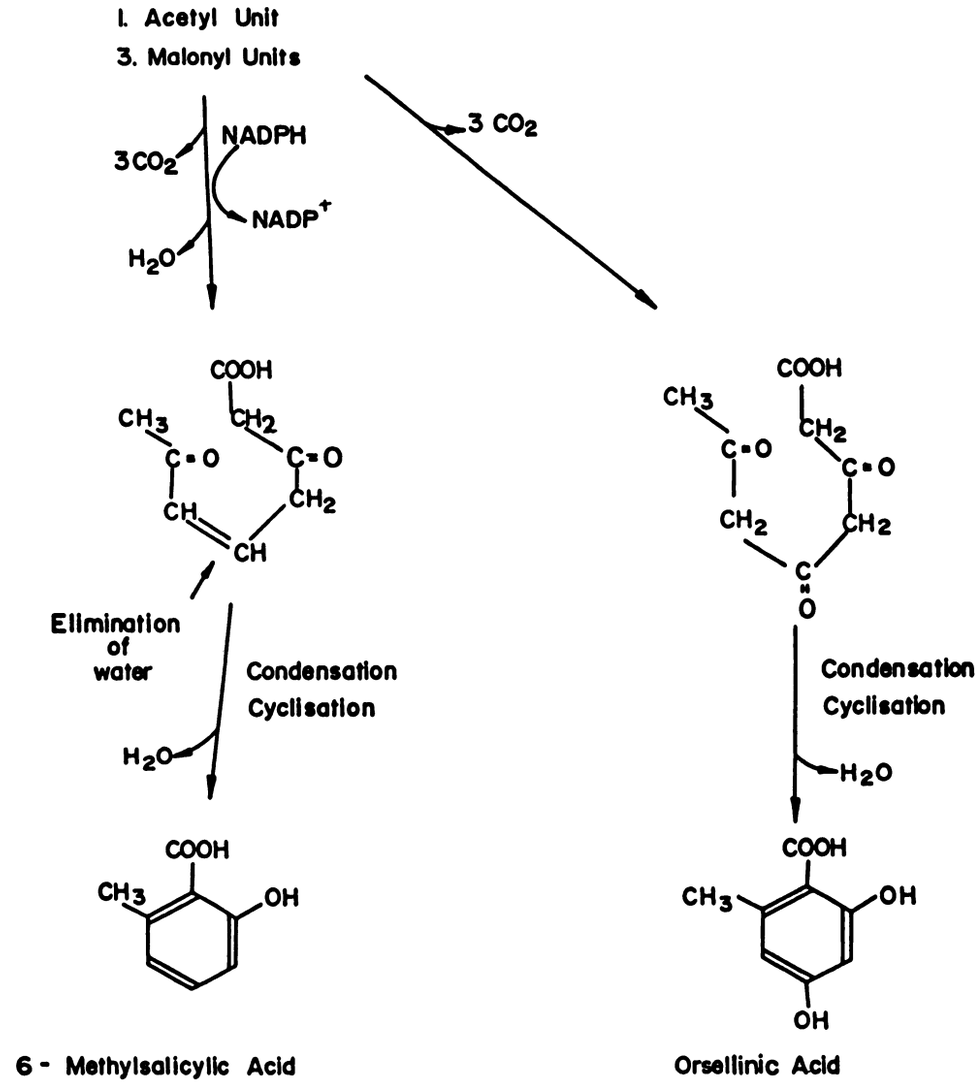

FIG. 8. Formation of a tetraketide and its conversion to 6-MSA or orsellinic acid.

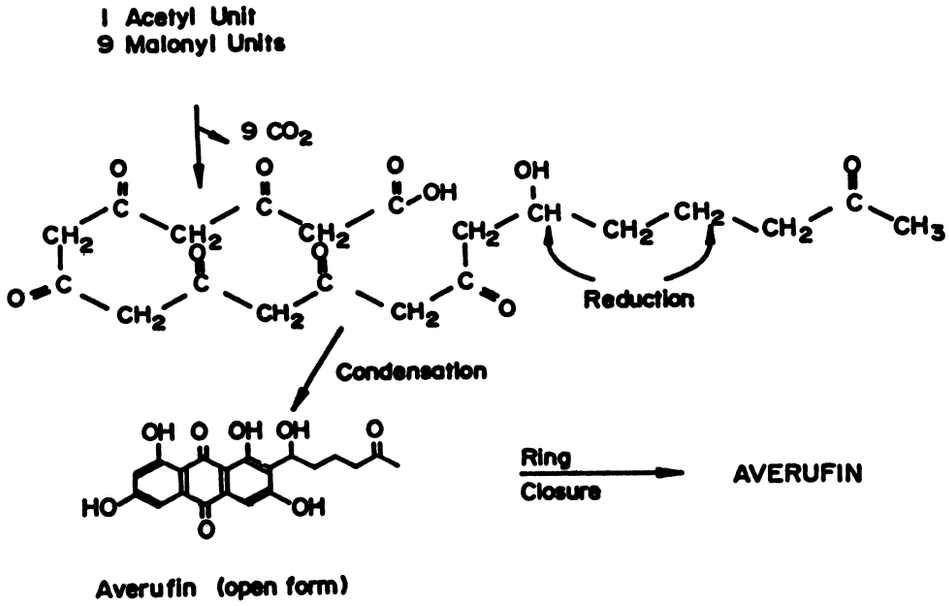

FIG. 9. AVF from the polyketide chain by reduction of 1′ and 3′ carbonyls.

which it could be separated by density gradient centrifugation (124). It has an absolute requirement for NADPH in contrast to classical polyketide systems, in which the nascent β-ketide chain is formed without reduction, although this may occur after stabilization. The necessity for NADPH is a step occurring after addition of the second acetate unit. The carbonyl function is reduced to a hydroxyl, which undergoes an elimination reaction to generate a double bond (Fig. 8), as confirmed by Scott et al. (162). The reducing step is possibly an echo of its origins from fatty acid synthase, for other polyketide synthases have no reducing action, e.g., in the related unreduced tetraketide orsellinic acid (Fig. 8) (80). Manganese is also limiting in patulin biosynthesis (formed from 6-MSA), the effect being exerted during transcription rather than at the enzyme level (163). The time of appearance, concentration, and stability of patulin biosynthetic enzymes have been studied (79). Ideally, this approach should be applied to secondary metabolism in general and to aflatoxin biosynthesis in particular.

The biosynthesis of 6-MSA is relevant to that of NA, for in its side chain the two end acetate units are reduced to hydrocarbon. These units could arise in an analogous manner to that for 6-MSA, i.e., during chain elongation. In fact, averufin (AVF; Fig. 9) could arise directly from the polyketide chain by reduction of the 1′ and 3′ carbonyls to hydroxyl and methylene, respectively.

Studies by Townsend et al. (188) provide an alternative scheme, whereby hexanoic acid (caproic acid) is incorporated intact into the side chain of NA. Chandler and Simpson (45), however, showed that AVF does have a normal acetate starter unit and suggested that added hexanoate could exchange with that bound to the polyketide synthase (Fig. 10).

Most organisms will rapidly beta-oxidize such a fatty acid and utilize it as a carbon source. Possibly hexanoate escapes this fate, because at the commencement of the idiophase, fatty acid anabolism, not catabolism, is operative. This conclusion is supported by the following: some randomization of the label was observed during hexanoate incorporation but with other labeled fatty acids the randomization was complete, indicating a lack of uptake as an intact unit. This phenomenon should be investigated further with cell-free preparations of NA synthase, as polyketide synthases may be relatively specific; e.g., 6-MSA synthase utilizes propionyl Co-A at 13% of the rate of acetyl CoA (59).

To summarize, NA arises by the sequential addition of nine acetate units to an acetate primer unit via a transitionary anthrone, and free hexanoate can exchange with enzyme-bound hexanoate (Fig. 10).

Generation of the Bisdihydrofuran System

Most of the subsequent steps in aflatoxin biosynthesis are oxidative; first are those modifying the side chain of NA to form a novel four-carbon bisfuran moiety found in the aflatoxins (Fig. 3).

The first step is the reduction of the carbonyl in the side chain of NA to a hydroxyl (Fig. 4), resulting in averantin (AVT; Fig. 11). This was isolated from a mutant of *A. parasiticus* impaired in aflatoxin production and shown to be a precursor of AFB1 by tracer studies (17). It has a chiral center (S) at the 1′ position (185), and studies in our laboratory indicate that the enzyme responsible for its formation is a dehydrogenase. An enzyme preparation was made from lyophilized mycelium (M. F. Dutton and A. Chuturgoon, Abstr. First Joint Cong. S. A. Biochem. Genet. Microbiol. Soc. 1986, P265) that can promote the reaction in both directions in the presence of either NAD or NADP. It has no effect on simple alcohols, etc., and therefore is distinct from common alcohol dehydrogenase.

Formation of AVF (Fig. 12) which like AVT is the S isomer (115), requires that the penultimate carbon atom (5′) of the chain be oxidized to a ketone, which is masked in AVF as a stable internal ketal derivative. Introduction of the β carbonyl into the chain probably occurs via hydroxyl group, introduced by a monooxygenase (see references 4 and 181) which is then oxidized to the ketone by means of a dehydrogenase (Fig. 13). The enzyme may be the same as the NA dehydrogenase.

The hydroxy intermediate has not been isolated, although a ring closed product isolated from *A. parasiticus*, averufa-

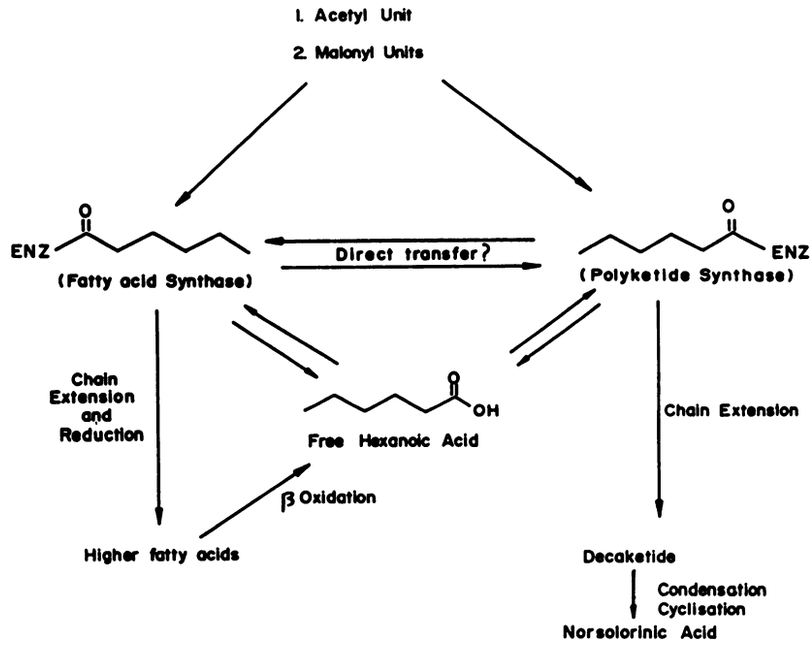

FIG. 10. Role of hexanoic acid in NA biosynthesis.

nin (96), is probably derived from it by dehydration. Recent experiments (137) have implicated averufanin as an intermediate, but it is possible that it acts as a side shunt at the 5'-hydroxy-AVT level.

Versiconal acetate (VAL A; Fig. 14), also known as versiconal hemiacetal acetate [72], is the first intermediate with the branched side chain characteristic of the aflatoxins. Other examples of fungal metabolites with branched structures are asperone (168) and gibberellin (61). The bisfuran system is not formed in VAL A because closure of its terminal furan ring is prevented due to the esterified acetate group.

The pathway immediately prior to VAL A was obscure, because several schemes had been proposed (82, 112, 179, 186). Work by Townsend and Christiansen (184) has ruled out the intermediacy of the 3'-hydroxy derivative, nidurufin, although this role may be filled by the unsaturated equivalent, dehydroaverufin (19). Until the correct mechanism is identified, the enzymology must be based on the fact that an oxidative step is followed by a rearrangement.

The esterified acetate unit in VAL A is derived from the two terminal carbons of the six-carbon side chain (176) and is generated by a BV reaction (187). Similar reactions are known in the degradation of long-chain ketones by fungi (4, 181).

Acid hydrolysis of the ester group causes rapid ring closure to versicolorin C (VC) (175) (Fig. 15), a known metabolite of both *A. versicolor* (88) and *A. parasiticus* (96). Thus, a plausible suggestion is that the next step is catalyzed by an esterase, and in fact the addition of dichlorvos, an inhibitor of acetyl choline esterase, to fungal cultures causes the accumulation of VAL A (204). Certain other organophosphorus compounds show a similar effect (64), and both these and dichlorvos do inhibit aryl esterases in *A. parasiticus* (9). Other compounds also promote the accumulation of pigments in aflatoxigenic strains of *Aspergillus*, e.g., benzoate (191), although this work was not repeatable by others (192).

Hydrolysis of VAL A, however, results in VC, which is at the wrong oxidation level (i.e., it lacks a double bond) to be directly converted to AFB1; in addition, it is a racemate (75), versicolorin B (Fig. 16) being the natural stereoisomer (18, 89) and having the same conformation as AFB1 depicted in Fig. 3. It is significant that the unsaturated analog, versicolorin A (VA; Fig. 17) (122), has both the correct oxidation level and stereo structure (R1', S2') (82) of the bisfuran moiety to be a direct precursor of AFB1 (121).

The question of the stereochemistry of the putative anthraquinone precursors has been addressed by Townsend (182) as this can influence reaction mechanisms. Stereospecificity is the hallmark of enzyme action, and it is not surprising that when there is the choice between several diastereoisomers one should be preferentially formed.

Both substrate conformation (183) and enzyme action (175) have been evoked as factors in generating the stereo

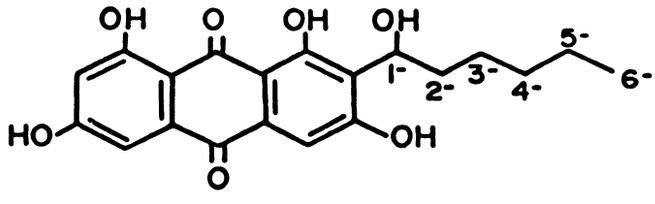

FIG. 11. Structure of AVT.

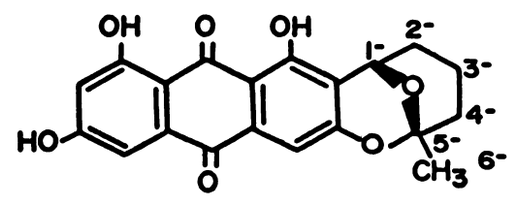

FIG. 12. Structure of AVF.

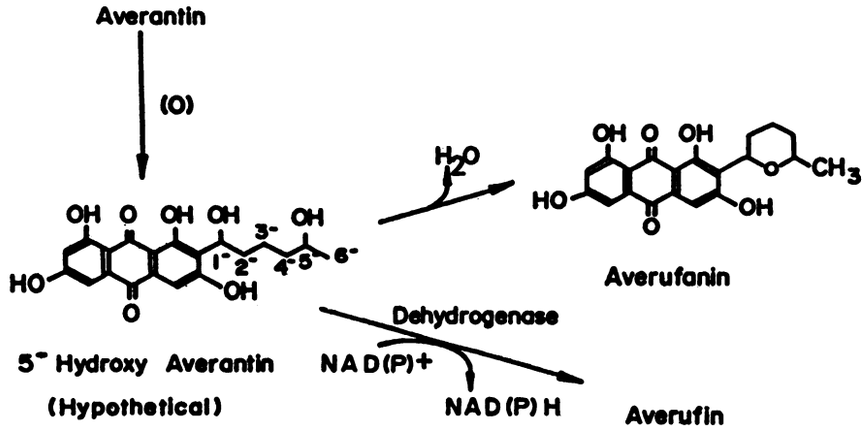

FIG. 13. AVT to AVF via oxidation of a monooxygenase to a ketone by means of a dehydrogenase.

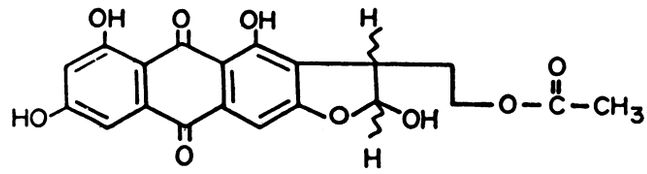

FIG. 14. Structure of VAL A.

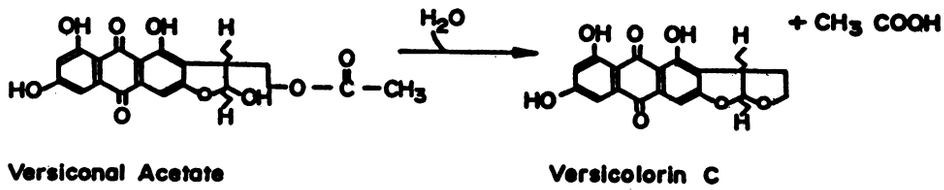

FIG. 15. Rapid ring closure to VC caused by acid hydrolysis of ester group.

structure of the bisfuran system. As the substrates AVT and AVF are chiral whereas VAL A (the result of enzyme inhibition) is racemic, it would seem that these factors act in concert. The key arrangement, in the bisfuran system, is at the 2'-carbon atom; the conformation at 1' could be either enantiomer, being in the form of a hemiacetal (Fig. 18).

Because of the existence of VC and VAL A as racemates, the mechanism giving rise to the 2'-carbon atom in them can produce either an S or an R arrangement in spite of substrate chirality, i.e., of AVF. The inhibitor dichlorvos must block a part of the active site of the enzyme synthase, so that the reaction becomes nondirected with regard to stereo arrangement, allowing the product to disassociate prematurely, possibly as the hypothetical intermediate versicoloronal (named in accordance with versicolorone [20]) (Fig. 19).

The enzyme, versicoloronal synthase, is possibly membrane bound to facilitate substrate solubility and must serve at least two functions: (i) to generate an electron-deficient center at position 2' of AVF, and (ii) to direct the reaction so that an S conformation occurs at position 2' in the bisfurano system. One model is given in Fig. 20. As hydroxylation is inappropriate in generating the electron-deficient center (184), an alternative is the removal of hydride, this being accepted by an oxidized coenzyme such as $NADP^+$. The bonding electrons from the adjacent carbon (1') of AVF now attack this position, leaving this carbon (1') electron deficient and detached from the aromatic ring. The carbon is now stabilized both electronically and spatially by a nucleo-

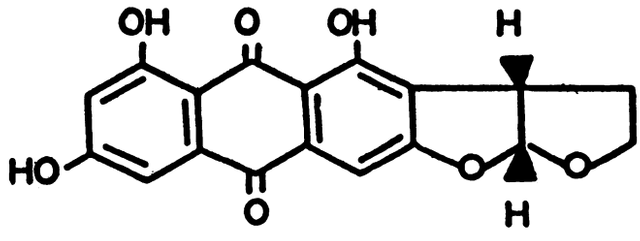

FIG. 16. Structure of versicolorin B.

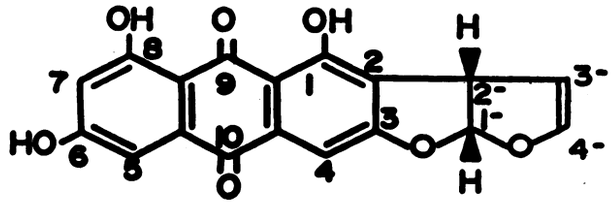

FIG. 17. Structure of VA.

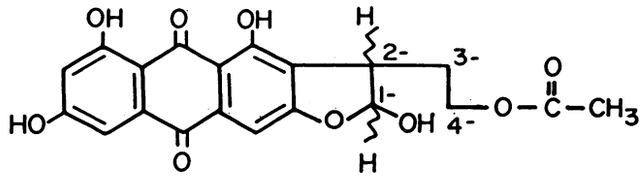

FIG. 18. Nomenclature of bisfuran system depicted as part of VAL A, the naturally occurring stereo arrangement at C-2′ being for VA, etc.

philic attack from an adjacent serinyl hydroxyl (or equivalent) in the active site. This ensures that the rearrangement takes place in such a way that the new tertiary carbon has the S conformation as required.

Dichlorvos blocks the stabilization step by phosphorylating the serinyl hydroxyl of the active site, which is its known mode of action (94), the extruded carbon (C-1′) being stabilized by a random nucleophilic attack of hydroxyl. The reaction thus occurs in a nonchiral fashion, resulting, after a BV reaction, in VAL A (Fig. 21). Note that hydroxyl may compete with serinyl under natural conditions, which would account for the accumulation of VC as a side shunt metab-

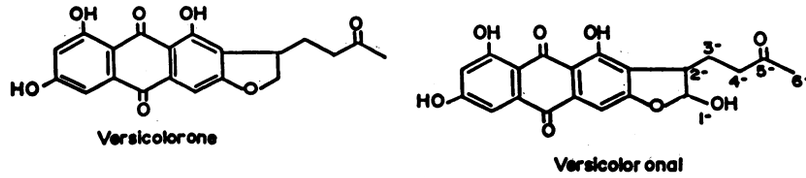

FIG. 19. Structures of versicolorone and versicoloronal.

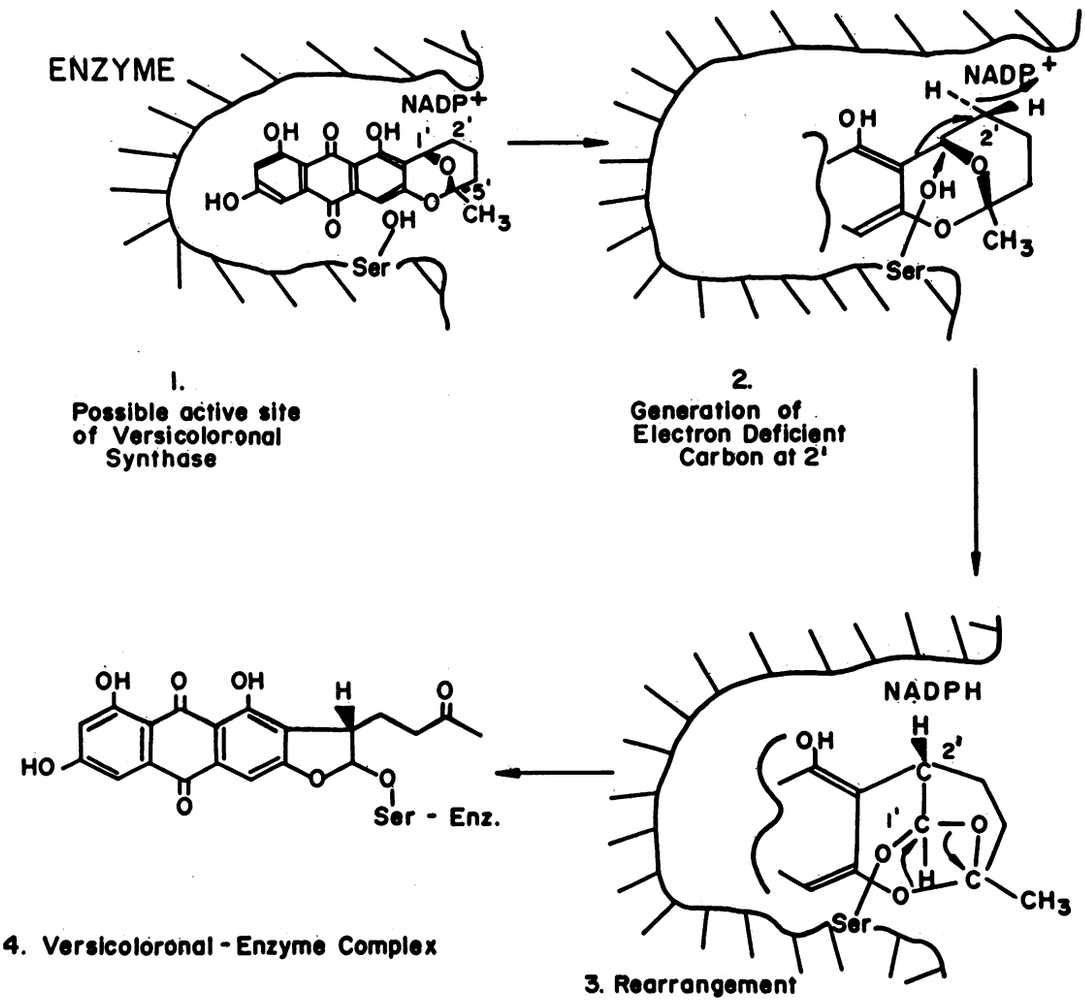

FIG. 20. Suggested mechanism for enzymatic conversion of AVF to versicoloronal.

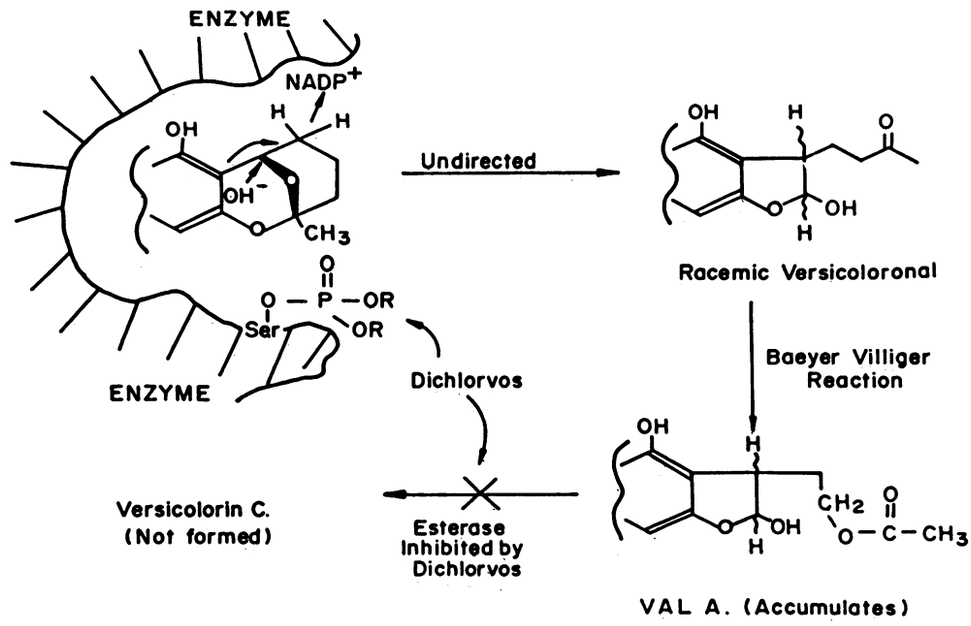

FIG. 21. Inhibition of versicoloronal synthase and VAL A esterase by dichlorvos.

olite by chemical or enzymatic hydrolysis of the resultant VAL A.

Summarizing, AVF is rearranged to VAL A by an enzyme that can oxidize the 2′ carbon atom and stabilize the resultant carbonium ion at the extruded C-1′ by binding to an enzyme residue such as serine. Further modifications, such as a BV reaction, can take place while the versicoloronal is bound to the enzyme. Dichlorvos has its action by binding to the serine residue and inhibiting esterase activity so that VAL A accumulates rather than VC (Fig. 21).

Wan and Hsieh (198) and Anderson and Dutton (9) have isolated an oxygen-requiring cell-free system that converts VAL A to VA. This system promotes at least two reactions: a hydrolysis and oxidation. The former authors suggest that a primary alcohol group derived from the open form of the terminal furan ring is oxidized to an aldehyde. This ring closes to a hemiacetal, which loses water to form the stable vinyl ether system of VA (see Fig. 22, substituting H for Ser-Enz).

Little comment is made on the nature of the enzyme system, and it is difficult to believe that the ring open form of versicolorin B exists long enough to be oxidized to aldehyde, unless it is stabilized by attachment to an enzyme such as depicted in Fig. 22. Presumably ring closure of the aldehyde is enzyme directed because of the R arrangement of C-1′ in VA. Conversion of VAL A is accommodated by recombination with enzyme; the reaction must be independent of chirality, as an almost complete conversion was observed (198), although the stereochemistry of the final product was not investigated. An alternative is that a desaturase such as is found in fatty acid metabolism effects the oxidation (202), and this could operate either at the VAL A level or on a ring closed system.

An AFB2-accumulating strain of *A. flavus* (SRRC 141) was also found to produce VC (66). Following on from the above discussion, it is possible that there is a malfunction in the versicoloronal synthase, resulting in a nonstereoselective mechanism (i.e., attack by OH^-) and the release of VAL A, which is converted by an esterase to VC and then to AFB2 via part of a metabolic grid. If the subsequent enzyme(s) in the pathway exhibits relative specificity, the route to AFB2 becomes rate limiting and excess VC accumulates.

Anthraquinone Modification

Sterigmatocystin (ST; Fig. 23) was originally isolated from *Aspergillus versicolor* and was the metabolite reported to contain the bisdihydrofuran system (35). It has one less skeletal carbon atom than VA, forming a xanthone nucleus (147), but has gained another by O-methylation.

It is likely that the 6-hydroxyl group of VA is removed prior to xanthone formation to form 6-deoxyversicolorin A, a known metabolite (68). Little is known with respect to this event, but recently Anderson (6) has observed the conversion of emodin to chrysophanol in a cell-free preparation derived from *Pyrenochaeta terrestris*, which can be regarded as an analogous reaction. Maximum conversion was obtained under anaerobic conditions in the presence of adenosine 5′-triphosphate, NADPH, mercaptoethanol, and ferrous iron. The result is somewhat nullified by the behavior of a cell-free extract from *A. parasiticus* towards emodin, whereby it was methylated to physcion (7). Whether this reflects potential enzyme activity or is due to isolation methodology remains to be determined.

The first step is cleavage of the anthraquinone moiety adjacent to carbonyl indicated in Fig. 24. The reaction is a BV oxidation resulting in a hypothetical lactone; precedents are sulochrin (51), secalonic acid (119), and ravenelin (27). Cleavage may take place on either side of the carbonyl, but subsequent reactions can lead to the same benzophenone derivative after oxidative decarboxylation.

At least three other events occur: (i) hydrolysis of the lactone either spontaneously or by the action of a lactonase; (ii) loss of the exposed carboxyl of the substituted 2,6,2′-trihydroxybenzophenone-6′-carboxylic acid by oxidative de-

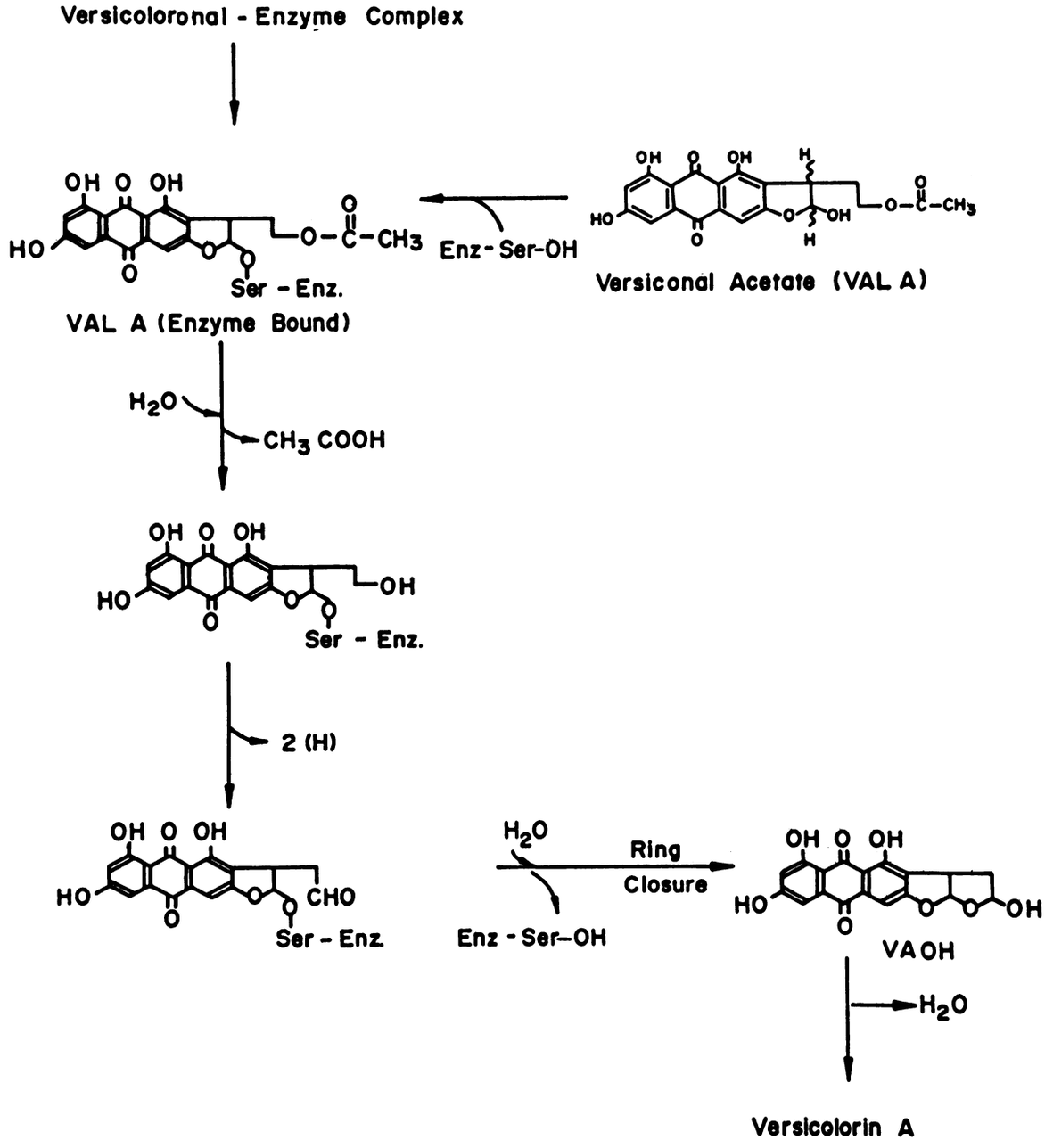

FIG. 22. System showing conversion of VAL A to VA.

carboxylation to form a substituted 2,6,2',6'-tetrahydroxybenzophenone derivative (Fig. 25); (iii) methylation of the hydroxyl at position 6, forming a substituted 2,2',6'-trihydroxy,6-methoxybenzophenone or the alternative 2,2'-dihydroxy,6-methoxy-6'-carboxylic acid (DHMBCA), depending on the order of events (Fig. 25).

The molecule now swivels about the carbonyl bridge, and ring closure occurs to yield ST (Fig. 26).

The nonlinear arrangement may be due to the methylation, which effectively blocks ring closure at the 6 position. Examples of linear xanthones are known, e.g., sterigmatin from *A. versicolor* (90) and austocystins from *A. ustus* (174) (Fig. 27). The xanthone ring oxygen originates from acetate (141), suggesting that a phenol hydroxyl is involved in an addition-elimination reaction with the loss of atoms derived from molecular oxygen. Other studies (169), however, do not support the symmetrical dihydroxy specie 2,6,2',6'-tetrahydroxybenzophenone (Fig. 25) as an intermediate, an alternative being an epoxide.

In Fig. 25, it is assumed that carbon dioxide is eliminated but other cleavage reactions involving the loss of carbon monoxide are known, e.g., flavonoid degradation by *A. flavus* (167); this could be investigated in future studies.

A purified methyltransferase which may be responsible for the O-methylation has been isolated in our laboratories (R. K. Berry, A. Chuturgoon, and M. F. Dutton, Proc. XIV

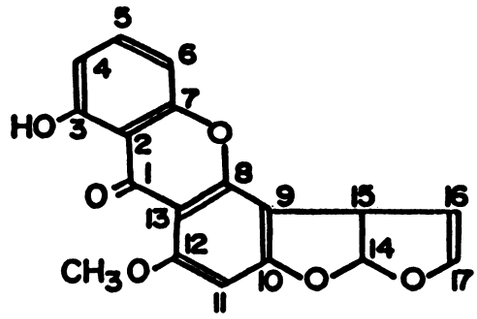

FIG. 23. Structure of ST.

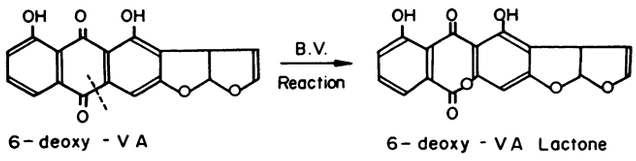

FIG. 24. Conversion of 6-deoxy-VA to 6-deoxy-VA lactone.

Bot. Cong., Berlin, 1987, 3-29-3, p. 174). It is not possible to prove conclusively that this is the enzyme involved, as the natural substrate is not available, but it cannot convert emodin to physcion (see reference 7). It methylates ST to *O*-methylsterigmatocystin (Fig. 28), a known metabolite of *A. flavus* (40), in the presence of *S*-adenosylmethionine, this being the means of assay. Other work (47) on this methyltransferase implicates it in the conversion of ST to AFB1.

Tracer experiments with replacement cultures have shown that ST can act as a precursor to AFB1 (105). Later experiments, however, using a microcolony technique (207), cast doubt on this and it was concluded that ST may be a side shunt metabolite. Other work by Holker and co-workers (68) showed that 6-hydroxydihydrosterigmatocystin could be converted to AFB2; an extrapolation of this result infers that 6-hydroxysterigmatocystin (6-OHST) is intermediate between ST and AFB1, arising at the substituted benzophenone level by hydroxylation (Fig. 26); ST would, therefore, occur as a result of the intermediate not undergoing hydroxylation.

A cell-free extract isolated from *A. parasiticus* (170) converted ST to AFB1, although a purified enzyme(s) was not isolated. The enzyme system was located in the cytosol and required NADPH for activity, implying the presence of a monooxygenase. Other work (109) confirmed these findings and showed that this system is composed of at least two enzymes, one of which is likely to be a monooxygenase requiring NADPH and the other a dioxygenase requiring the presence of ferrous ions (M. S. Jeenah, Ph.D. thesis, University of Natal, Pietermaritzburg, Natal, South Africa). The two purified proteins, however, when recombined plus cofactors could not carry out the conversion, nor could they independently convert ST to 6-OHST, which should be an obligatory intermediate. This may be resolved if the missing factor is the methyltransferase mentioned above (47).

If an ortho cleavage (Fig. 1a) operates, then the ring-cleaved product has an excess of one double bond equivalent to form AFB1 (Fig. 29), a point not very well accommodated in many published pathways. Possibly there is some other reaction mechanism or enzyme that has been overlooked in this conversion.

Other Aflatoxins

The metabolic relationship between aflatoxins is unclear, and several schemes have been proposed (e.g., references 97 and 130). In general, it has been assumed that they all arise from AFB1 with the proviso that there may be a metabolic grid (37) in operation (Fig. 30). This belief is well founded, for AFG1 could arise from AFB1 by means of a BV reaction, AFB2 and AFG2 being formed by reduction of the terminal

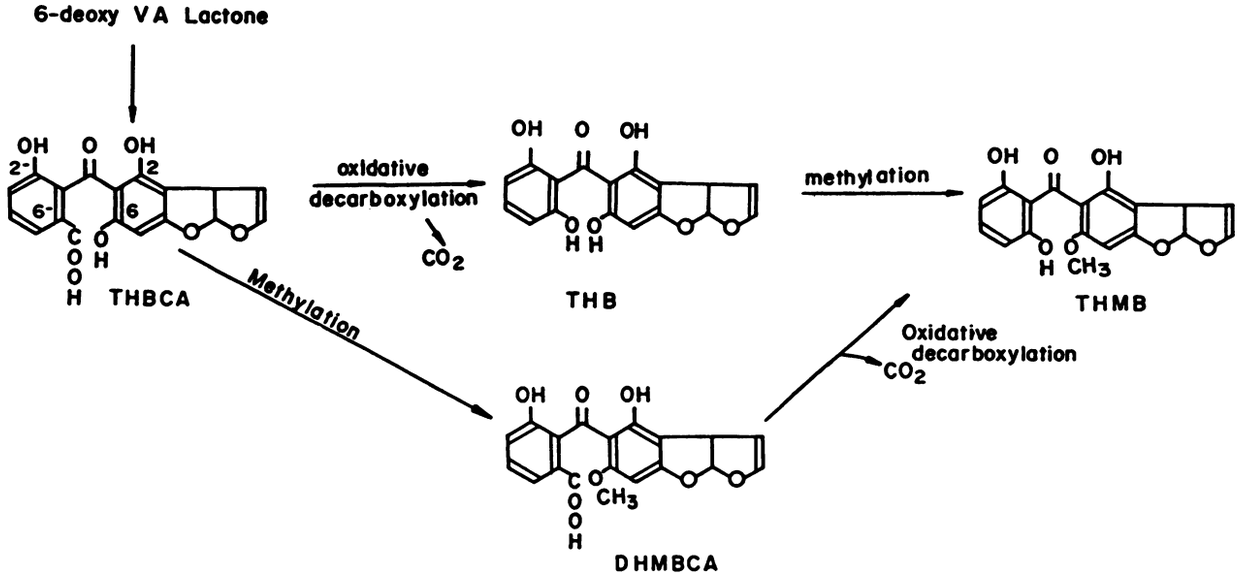

FIG. 25. Formation of 2,6,2',6'-tetrahydroxybenzophenone derivative (THB) and substituted 2,2',6'-trihydroxy,6-methoxy-benzophenone (THMB) or 2,2'-dihydroxy, 6-methoxy-6'-carboxylic acid (DHMBCA) from THBCA (substituted 2,6,2'-trihydroxybenzo-phenone-6'-carboxylic acid).

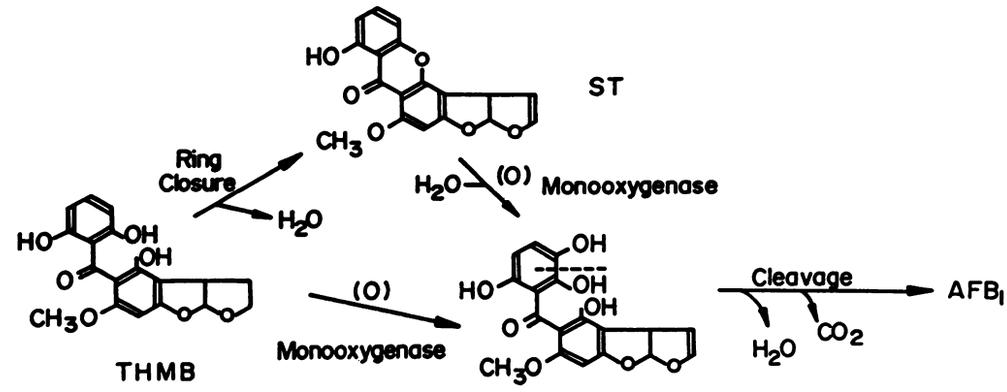

FIG. 26. ST from substituted 2,2',6'-trihydroxy,6-methoxy-benzophenone (THMB) via ring closure.

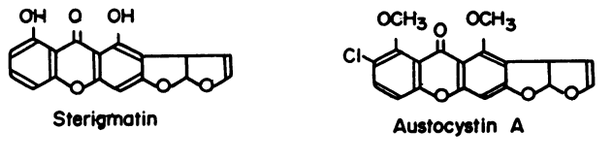

FIG. 27. Examples of linear xanthones.

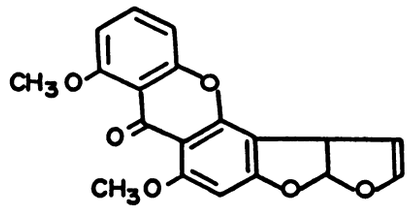

FIG. 28. Structure of *O*-methylsterigmatocystin.

double bond in AFB1 and AFG1, respectively (Fig. 31). The aflatoxin M and GM series may be formed by hydroxylation of the tertiary carbon of the bisfuran system in the relevant precursor aflatoxin. The time of appearance and amounts of the various aflatoxins in cultures of *A. flavus* and *A. parasiticus* (130) gives support to this proposal. Studies by Floyd and Bennett (73), however, indicated that AFB1, AFB2, AFG1, and AFG2 can arise independently of each other. Furthermore, several strains of *A. flavus* are known to produce AFB2 alone (148). One of these, *A. flavus* SRRC 141, produced AFB1 when presented with exogenous ST (66), demonstrating that some of the enzymes for the production of AFB1 were present; it was concluded that a metabolic grid existed whereby AFB1 and AFB2 could be biosynthesized independently of each other.

The same investigation provided evidence for AFM1 and AFM2 arose from the analogous B toxins. Presumably, a monooxygenase is responsible for both conversions, as hydroxylation of tertiary carbon atoms by filamentous fungi is known to occur (111).

Further studies (M. F. Dutton, 1985 Abstr. Proc. Sixth Int. Symp. Mycotoxins Phycotoxins [IUPAC], P9) have shown that a strain of *A. flavus* could convert AFB2a to AFB2; the responsible enzyme may be an alcohol dehydrogenase or involved in the formation of VA (198). AFB2a arises in cultures of *A. parasiticus* by the addition of water to the terminal double bond in AFB1 (67) under conditions of low pH and is considered to be an artifact (Fig. 32).

This casts a new role for AFB2a and its xanthone, ST hemiacetal, and anthraquinone, VA hemiacetal, analogs (46) as they could act as intermediates between the dihydro- and tetrahydrobisfuran series. Possibly the metabolic grid depicted in Fig. 30 is more complicated and should include the pathway presented in Fig. 33.

A related metabolite, aflatoxicol, is formed in certain microbial cultures exposed to AFB1 (56) by the reduction of the cyclopentenone system to cyclopentenol, which can exist in two enantiomeric forms, aflatoxicols A and B (48). A dehydrogenase is involved, the reaction being very similar in character to several reported in microbial steroid conversions (111). Such systems have also been isolated from liver homogenates when the enzyme has stereospecificity in that the product is aflatoxicol A (151).

GENERAL COMMENTS

Of the identifiable enzyme-catalyzed reactions in aflatoxin biosynthesis (Table 3), at least six are mediated by oxygen-

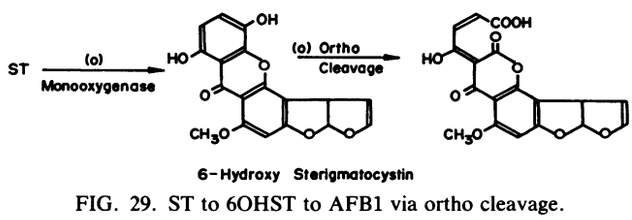

FIG. 29. ST to 6OHST to AFB1 via ortho cleavage.

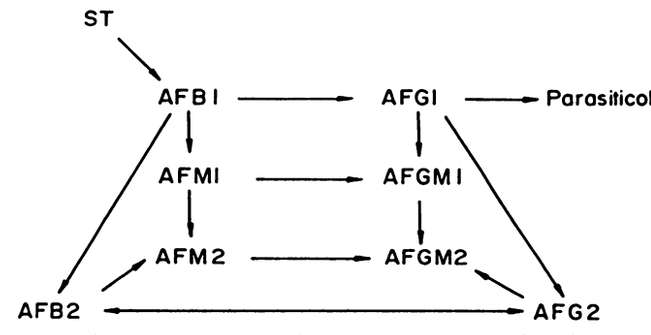

FIG. 30. Metabolic grid interconnecting major aflatoxins.

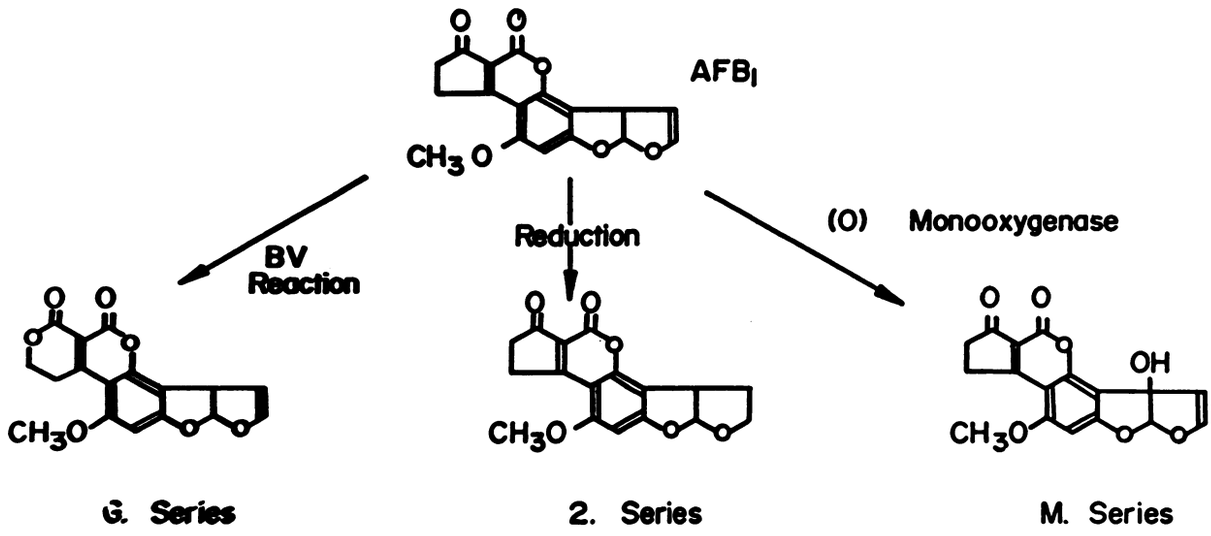

FIG. 31. Scheme showing that AFG1 could arise from AFB1 via a BV reaction and possible formation of AFB2 and AFG2.

ases. Some of these may be of the cytochrome P-450 type, as carbon monoxide has been found to inhibit aflatoxin biosynthesis (32) and flavin adenine dinucleotide is required to convert VA to AFB1 (65). As oxygenases are primarily involved in the detoxification of xenobiotics (28, 123), it may be that the organism reacts to its secondary metabolite, i.e., NA, as if it were a xenobiotic and metabolizes it.

Paradoxically, in the case of aflatoxin, the response of the "detoxification" is generation of a metabolite that is more toxic than the parent (63), although the resultant metabolite is more water soluble, a property that aids elimination. Certain authorities (e.g., reference 127) may have no difficulty with this concept, as they group xenobiotic with secondary metabolism.

Aflatoxin biosynthesis is inducible (22, 149) possibly through lipoperoxide production (150); inducibility, as well as enzyme relative specificity (28), is characteristic of detoxification. Consequently, studies are necessary to resolve the role of enzymes responsible for the latter part of the pathway.

A final point concerning enzyme specificity stems from the fact that the biosynthetic pathway follows an orderly sequence of events, with first the anthraquinone side chain being modified and then the anthraquinone nucleus itself. Is this sequence obligatory; e.g., is there a xanthone analog of AVF? No such analog has been isolated so an ordered sequence is favored. This may be due to true enzyme specificity, the recalcitrant nature of the intermediates, their solubilities, or a cellular compartmentalization effect (128); this also can only be resolved by further enzyme studies.

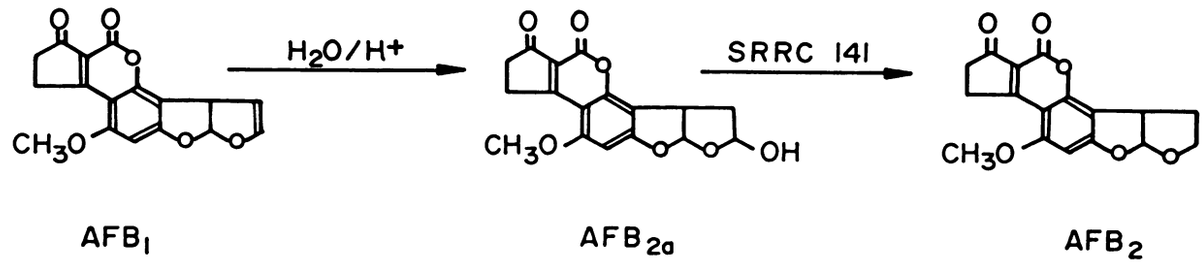

FIG. 32. AFB2a from AFB1 after addition of water.

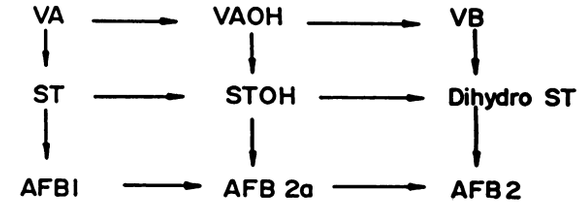

FIG. 33. Partial metabolic grid accommodating hemiacetal derivatives.

TABLE 3. Resume of enzymes required for biosynthesis of AFB1 from acetyl CoA[a]

Enzyme[b]	No.[c]	Substrate	Cofactor[d]	Product
Polyketide synthesis				
Acetyl CoA (K) carboxylase	1	Acetyl CoA, CO_2	Mg^{2+}, ATP	Malonyl CoA
Acetyltransferase (K)	2	Acetyl CoA		Primed synthase
Malonyltransferase (K)	3	Malonyl CoA		Primed synthase
Hexanoyltransferase (I)	4	Hexanoyl CoA		Primed synthase
N Acid synthase complex (I)	5	Acetyl CoA? Hexanoyl CoA? Malonyl CoA	NADPH?	NA anthrone
Dehydrogenases				
NA (K)	6	NA	NADPH, Zn^{2+}	AVT
5'-Hydroxy-AVT (I)	8	5'-Hydroxy-AVT	$NADP^+$?	AVF
Oxygenases				
AVT-5'-hydroxylase (H)	7	AVT, O_2	NADPH	5'-Hydroxy-AVT
Versicoloronal (BV) oxygenase (H)	10	Versicoloronal, O_2	NADPH?	VAL A (SR)
VA (BV) oxygenase (H)	15	VA, O_2	NADPH?	VA lactone
ST-6-hydroxylase (K)	20	ST, O_2	NADPH	6-OHST
6-OHST dioxygenase (K)	21	6-Hydroxy-ST	Fe^{2+}	AFB1
Hydrolases				
VAL A esterase (K)	11	VAL A, H_2O		Versicolorin B, acetic acid
VA hemiacetal dehydratase (H)	13	VAOH		VA
Lactonase (I)	16	VA lactone		THBCA
Xanthone cyclase (I)	19	THMB		ST, H_2O
Miscellaneous				
Versicoloronal synthase (H)	9	Averufin	NADPH?	Versicoloronal
Versicolorin B oxidase (H)	12	Versicolorin B, O_2	$NADP^+$	VA hemiacetal
VA reductase (H)	14	VA	?	6-Deoxy-VA
THBCA decarboxylase (I)[e]	17	THBCA	?	THB, CO_2
Methyltransferase (I)[e]	18	THB	SAM	THMB

[a] ATP, Adenosine 5'-triphosphate; THBCA, substituted (3,4) 2,6,2'-trihydroxybenzophenone-6'-carboxylic acid; THMB, substituted (3,4) 2,2',6'-trihydroxy-6-methoxy-benzophenone; THB, substituted (3,4) 2,6,2',6'-tetrahydroxybenzophenone; SAM, *S*-adenosylmethionine.

[b] Trivial enzyme names; letters in parentheses: K, known or studied enzyme; I, by inference from similar system; H, hypothetical based on proposed reaction. Note: Synthase now replaces synthetase and is used throughout this review (Nomenclature Committee of the International Union of Biochemistry, 1984).

[c] Placement number in proposed pathway (Fig. 4).

[d] Known required coenzyme and metal ions. ? = Unknown/unsure of situation.

[e] Sequence of reactions may be different.

CONCLUDING REMARKS

It is clear that there is some way to go before the biogenesis of aflatoxin can be described in terms of the enzymes that promote the individual reactions. What has been uncovered does pose a whole series of questions that have long since been answered for primary metabolism. If this review has defined the questions that must be addressed and also generated interest in the answers, then I will be well satisfied. New horizons in enzymology and molecular biology should make it possible to conclude what the natural-product chemists began a long time ago and realize the statement made in 1963 by W. B. Whalley: "The next advances must surely consist in the exploration of biosynthetic processes by cell free, enzymic extracts, the definition of the role of the unusual metabolites of the economy of the microorganisms and the elucidation of the sequence of the various reactions by which primary precursors are converted into the ultimate metabolites" (203).

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