ENZYMATIC CONVERSION OF STERIGMATOCYSTIN TO AFLATOXIN B_1

By

MOHAMED SAYED JEENAH

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University of Natal, Pietermaritzburg.

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DECLARATION

I hereby certify that this research is a result of my own investigation

M.S. JEENAH

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CONTENTS

ABBREVIATIONS USED

Af	Aflatoxin
EDTA	Ethylenediamine tetraacetic acid
FAD	Flavine Adenine Dinucleotide
HPLC	High Performance Liquid Chromatography
I.E.F.	Isoelectric focusing
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide Reduced
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide phosphate reduced
OMS	O-Methylsterigmatocystin
PAGE	Polyacrylamide Gel Electrophoresis
TCA	Trichloroacetic acid
TEMED	N,N,N′N′-Tetramethylethylenediamine
TLC	Thin Layer Chromatography
SDS	Sodium Dodecyl Sulphate
St	Sterigmatocystin
YES	Yeast Extract Sucrose medium

ABSTRACT

The age of Aspergillus parasiticus (1-11-105Wh1) mycelium was found to have an influence on the level of enzymes, responsible for the conversion of sterigmatocystin to aflatoxin B_1 and O-methylsterigmatocystin, present. These enzymes were active over a wide range of temperature and pH.

Production of a cell free system by lyophilization yielded the highest aflatoxin B_1 synthesising activity. Three other methods of preparing the cell free system capable of synthesising aflatoxin B_1 were also studied, ie,: french press, protoplast, and grinding, but with limited success. The lyophilized preparation had narrower temperature and pH optima for the conversion than whole mycelia.

Initial purification of the aflatoxin B_1 synthesising enzyme was achieved by separating the crude cell free extract by gel filtration. The enzyme activity was located in a membrane fraction. The involvement of endoplasmic reticulum was indirectly concluded by the use of marker enzyme and chelating agents. This membrane fraction was ultracentrifuged and the released extrinsic proteins were separated by gel filtration.

A fraction containing two proteins which were capable of converting sterigmatocystin to aflatoxin B_1 was isolated and characterised by isoelectric focusing and gel

electrophoresis. The temperature and pH optima together with the cofactor requirements were studied. The Michaelis-Menten constant (Km) and the stoichiometry for the conversion of sterigmatocystin to aflatoxin B_1 was determined.

1. INTRODUCTION

1.1. MYCOTOXINS

Mycotoxins are defined as toxic substances of fungal origin (1). They belong to a family of compounds called secondary metabolites, which are products formed only under certain circumstances and have no obvious biological function. Secondary metabolites have a restricted taxonomic distribution and producing fungi in general are genotypically specific (2). Chemically they are diverse substances and are formed by a variety of pathways (3), which branch from primary metabolites at a limited number of points (4). They express the individuality of species in biochemical terms, differing from primary metabolism where the unity of living material is important.

Mycotoxins and mycotoxicoses, the diseases caused by them, were relatively obscure until the discovery of aflatoxin in the early nineteen sixty's. Since then there has been a growing interest in mycotoxins with a rapid increase in the number of related publications in the scientific literature.

1.1.1. Aflatoxin

In 1961 an extensive loss of turkey poults was reported in the United Kingdom. The loss was caused by an unknown disease that was characterised by subcutaneous haemorrhages and death. The condition was called "Turkey X" disease (5). Concurrent similar losses of partridges, pheasants, ducklings (6) pigs (7) and calves (8) were also reported. Eventually all the losses were attributed to the ingestion of aflatoxins and there is evidence to suggest that these compounds had also previously produced the disease (9).

The first mould species identified as producing aflatoxin was Aspergillus flavus (Links ex Fries). This species and the related A. parasiticus are the only aflatoxin producing moulds that have so far been isolated (10). Certain authorities considered A. parasiticus to produce both aflatoxin B and G series, whereas A. flavus produces only the aflatoxin B series. Not all isolates of these fungi produce aflatoxin (10). Aflatoxin formation in the laboratory by other mould species has been reported (11), but in no instances could the observations be repeated. Aspergillus flavus is a widespread saprophytic mould and in general the A. flavus group of fungi is a constituent of the microflora of the air and the soil throughout the world. They also contribute to the deterioration of many foodstuffs and traditionally A. flavus is classified as a storage mould but now that its occurrence has

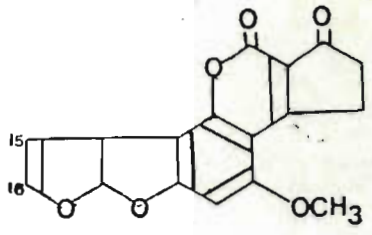
also been identified in preharvest crops, this classification has to be revised (11).

Aflatoxins have been found in the market place in sufficient amounts and incidence to warrant continued surveillance. Thus, they have been identified in samples of edible nuts (peanuts, brazil nuts and pistachio nuts) and their derived products, other oilseeds and their products, grains (corn, sorghum and millet) and figs (11). The occurrence and level vary from one geographical area to another and in general aflatoxin contamination appears to be a problem of tropical and sub-tropical areas.

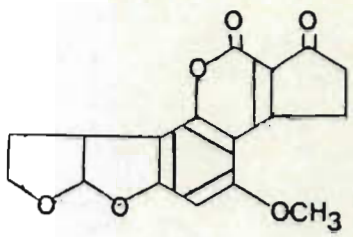
Aflatoxin is a potent toxin, a carcinogen, a teratogen and a mutagen (12) and it can impair the immune system in animals (13). It does however require metabolic activation before it can exert its carcinogenic and mutagenic effects (14). Aflatoxin B_1 is thought to be converted to a 15,16 epoxide (Fig. 1) through the action of mixed function oxygenases. It is this epoxide that is thought to be carcinogenic (15).

Four aflatoxins were originally isolated and trivially named after one of the producing moulds A. flavus. They were designated aflatoxin B_1, B_2, G_1, and G_2 after their blue and green fluorescent colours under ultra-violet light and chromatographic properties. All these toxins contain a bisdihydrofuran ring system attached to a substituted coumarin nucleus (Fig. 1). A cyclopentenone ring system (B series) or a

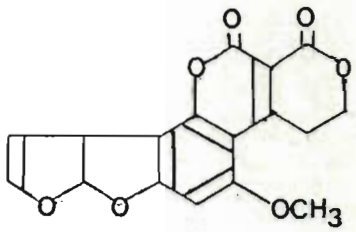
six membered lactone (G series) is attached to the coumarin nucleus. At least nine such metabolites have been isolated From A. flavus and A. parasiticus (Fig. 1). Aflatoxin B_3 was also isolated (16) and is usually referred to as parasiticol (17).



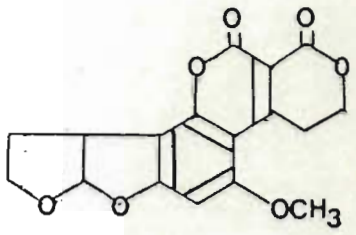
AFLATOXIN B_1



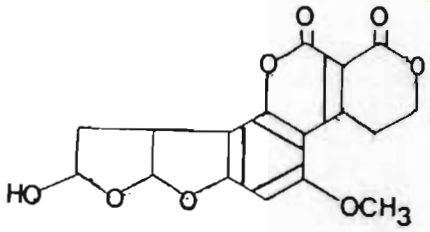
AFLATOXIN B_2



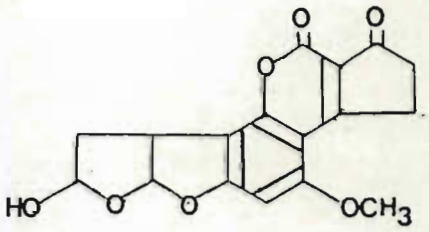
AFLATOXIN G_1



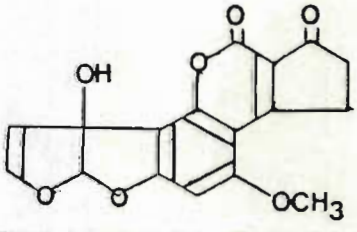
AFLATOXIN G_2



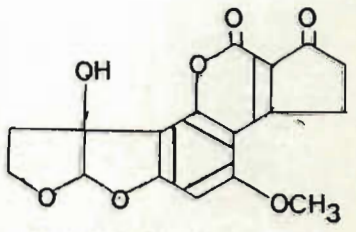
AFLATOXIN G_{2a}



AFLATOXIN B_{2a}



AFLATOXIN M_1



AFLATOXIN M_2

Figure 1: Structures of the principle aflatoxins.

1.1.2. Sterigmatocystin

Sterigmatocystin is a mycotoxin biogenetically related to aflatoxin. It was first isolated from Aspergillus versicolor (18) and has been reported from fifteen other species of fungi (19), the principal producers being A. versicolor, Aspergillus nidulans and Aspergillus ustus. It is also produced by Bipolaris sorskiniana (20).

Sterigmatocystin is carcinogenic to rats (21) and is toxic to sub-human primates. Although the toxic effects are much the same as those of aflatoxin B_1 (22), its hepatocarcinogenicity is only one-tenth that of aflatoxin B_1 in rats. Neoplastic skin lesions (23), pulmonary tumors in mice, and pathological alterations in the liver and kidney of green monkeys (22) have been attributed to sterigmatocystin.

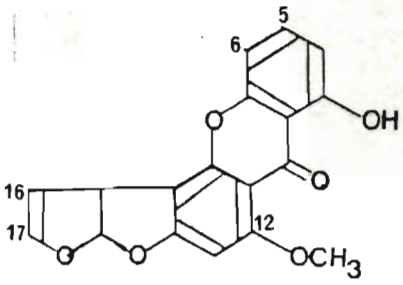
Sterigmatocystin-producing strains of fungi have been isolated from a large number of foods but sterigmatocystin as such has been found in a limited number of products, cereals and grain products (24), coffee-beans (25), pepper (26) as well as several stored and prepared foods samples. Marijhuana and in-shell pecan nuts contaminated with sterigmatocystin have also been found (27,28).

Sterigmatocystin has a xanthone moiety with an attached bisdihydrofuran ring system (Fig. 2), and,in fact, was the first

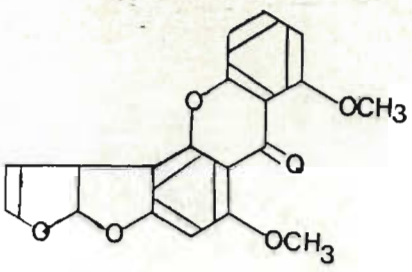
fungal metabolite found to contain the bisdihydrofuran ring system. It was isolated by Hatsuda and Kuyama (18) and Bullock (29) elucidated the structure. The absolute configuration was first suggested by Fukuyama (30) using X-ray crystallography, and the ^{13}C NMR signals of sterigmatocystin have also been assigned (31,32).

Metabolic derivatives of sterigmatocystin are also known. In 1968 O-methylsterigmatocystin (33) and aspertoxin (34) were isolated. Other metabolites belonging to this group that have been identified include (Fig. 2):

5-methoxysterigmatocystin (35),
6-methoxysterigmatocystin (36),
demethylsterigmatocystin (37),
5,6 dimethoxysterigmatocystin and
5,6 dimethoxydihydrosterigmatocystin (38).

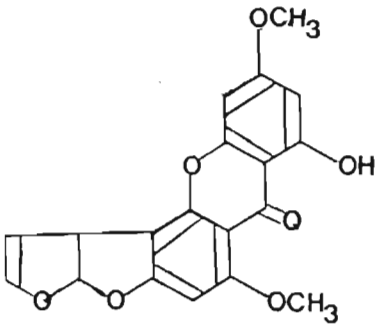


STERIGMATOCYSTIN



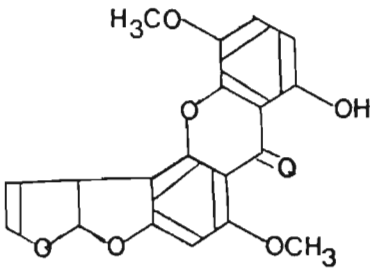
O-METHYL

STERIGMATOCYSTIN



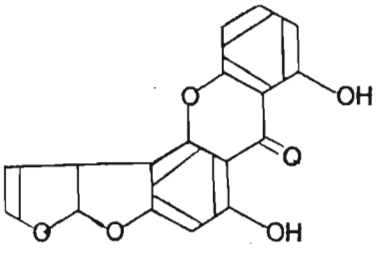
5-METHOXY-

STERIGMATOCYSTIN



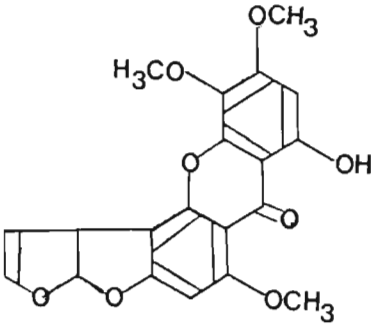
6-METHOXY-

STERIGMATOCYSTIN



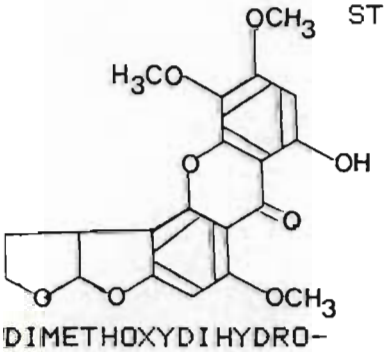
DEMETHYL-

STERIGMATOCYSTIN



5,6 DIMETHOXY-

STERIGMATOCYSTIN



5,6 DIMETHOXYDIHYDRO-

STERIGMATOCYSTIN

Figure 2: Structures of Sterigmatocystin and its related derivatives.

1.1.3. Metabolites Related to Aflatoxin

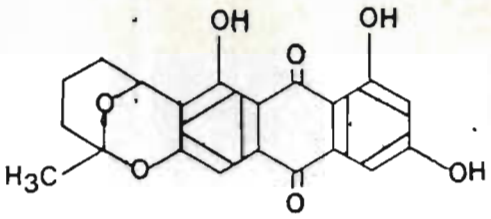
A number of compounds with a C_{20} polyketide precursor have been isolated and found to be biogenetically related to aflatoxin B_1. These metabolites are produced by genotypically similar Aspergillus species: A. versicolor, A. ustus, A. parasiticus and A. flavus. All these metabolites are anthraquinone derivatives. These anthraquinones can be separated into three classes:

(i) Those having an unbranched C_6 side chain

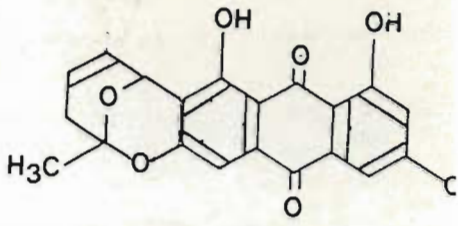
(ii) those having a branched C_6 side chain, and

(iii) those having a C_4 side chain.

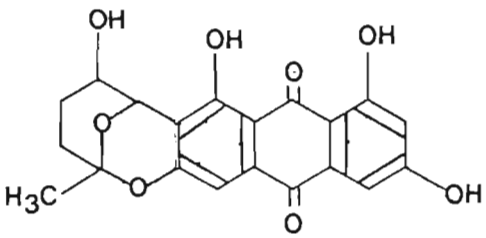
The first group includes averufin (39) (Fig. 3) and related derivatives such as deoxyaverufinone and dehydroaverufin which were characterised by Berger and Jardot (40). Other structurally similar compounds are nidurufin (41) and 6,8,O-O dimethylnidurufin (42). The latter metabolite was proposed as an intermediate in aflatoxin biosynthesis. A number of other metabolites belonging to this group that have also been isolated include: averufanin (43), averantin (44) and averythrin (45) whereas norsolorinic acid was isolated by Anderson, Thomson and Wells (46). Dehydroaverufin and norsolorinic acid were suggested as precursors of averufin by Berger (40) and Hsieh (47) respectively.



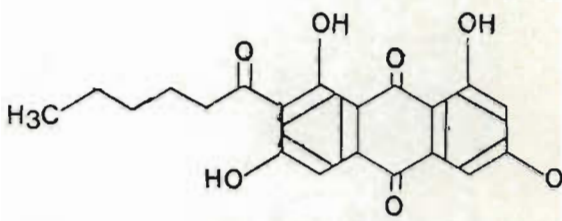
AVERUFIN



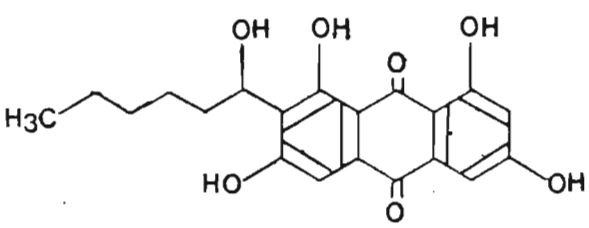
DEHYDROAVERUFIN



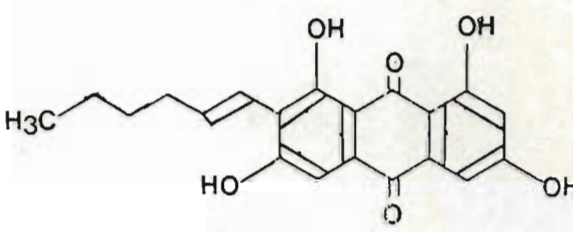
NIDURUFIN



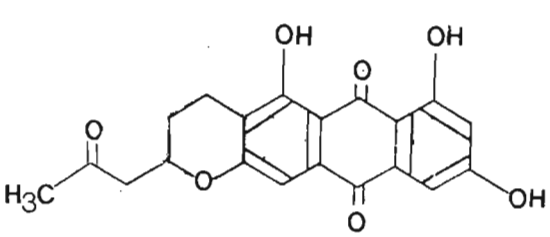
NORSOLORINIC ACID



AVERANTIN



AVERYTHRIN

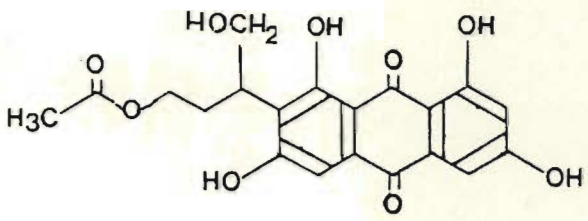


DEOXYAVERUFINONE

Figure 3: Structures of the anthraquinones with an unbranched C_6 side chain that are biogenetically related to aflatoxin.

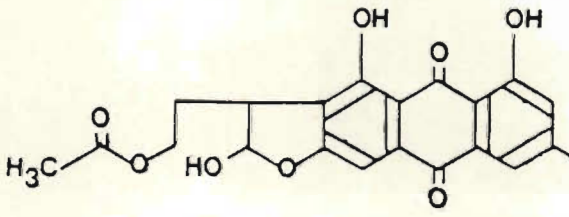
Rao and Harein found that aflatoxin biosynthesis was reduced when crops contaminated with A. flavus were treated with dichlorvos (48). Yao and Hsieh (49) observed the formation of an orange-red pigment, which they tentatively identified as versiconal acetate, when A. parasiticus was treated with dichlorvos. Versiconal acetate, which was first described by Schroeder, Cole, Grisby and Hein (50) and later renamed versiconal hemiacetal acetate by Fitzell, Singh, Hsieh and Motell (51), belongs to the second anrhraquinone group (Fig. 4). Present nomenclature has reverted to the original name of versiconal acetate (52).

The third anthraquinone group contains the versicolorin series which was found to have the bisdihydrofuran ring system found in the aflatoxins (Fig. 4). Hamasaki isolated three such metabolites: versicolorin A (53) versicolorin B (53) and its racemate, versicolorin C (54). Aversin (35) the methylated derivative of versicolorin A was isolated earlier by Bullock, Kirkaldy, Roberts and Underwood.



VERSICONOL

ACETATE

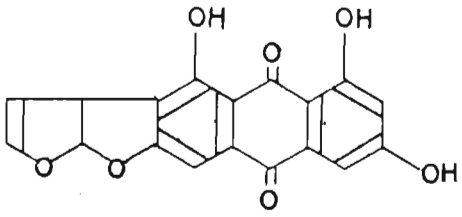


VERSICONAL

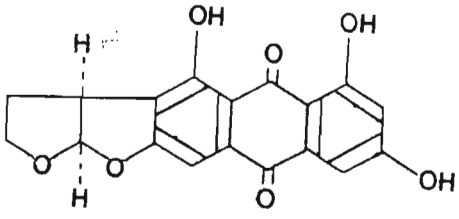
ACETATE*

Figure 4: Structures of the anthraquinones with a branched C side chain that are biogenetically related to aflatoxin.

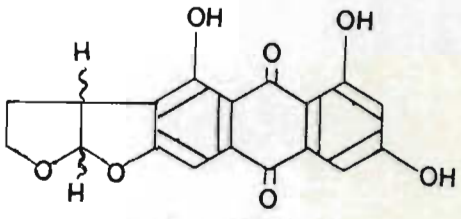
* also known as versiconal hemiacetal acetate



VERSICOLORIN A



VERSICOLORIN B



VERSICOLORIN C

Figure 5: Structures of the anthraquinones with a bisdihydrofuran ring system that are biogenetically related to aflatoxin.

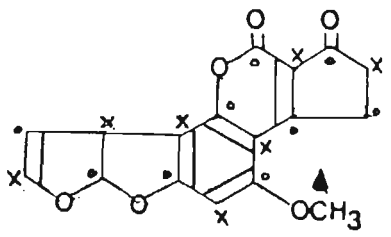
1.2. BIOSYNTHESIS OF AFLATOXIN

Initially it was thought that shikimic acid was involved in the biosynthesis of aflatoxin as the evidence indicated that phenylalanine and tyrosine were precursors (55). Moody (56) suggested that the carbon skeleton was partially derived from mevalonate. Donkersloot's group later showed that these suppositions were incorrect in that the carbon skeleton had a polyketide origin (57). Holker and Underwood (58) postulated that sterigmatocystin was a precursor of, or had a common precursor with, aflatoxin B_1 but failed to get any conversion of labelled sterigmatocystin into aflatoxin by cultures of A. flavus. From the degradation of sterigmatocystin, derived from labelled acetate, Holker and Mulheirn (59) concluded that sterigmatocystin was formed from two separate preformed polyketide units, a C_{14} and a C_4 unit. They also proposed a scheme for the conversion of sterigmatocystin to aflatoxin involving oxidative fission of an aromatic ring.

Several experiments designed to analyse the distribution of acetate carbon atoms in aflatoxin B_1 were performed using ^{14}C labelled material (60,61). Buchi's group (62) established the specific incorporation of labelled acetate. Seven labels in the aflatoxin molecule were derived from (1-^{14}C) acetate and nine from (2-^{14}C) acetate. The methoxy carbon of aflatoxin B_1 was found to be derived from the methyl

group of methionine (Fig. 6).

Biollaz, Buchi and Milne (62) proposed a hypothesis whereby a C_{18} polyhydroxynaphthacene was oxidised and then rearranged via the diradical or the zwitterion to the aldehyde. A rearrangement led to versicolorin A. Oxidative cleavage of versicolorin A, cyclization and decarboxylation formed the xanthone ring as in sterigmatocystin. This was then converted to aflatoxin B_1. This postulate was unchallenged up until the early nineteen seventy's (Fig 7) (63).



X •
CH_3COOH

▲
$CH_3SCH_2CH_2CH(NH_2)COOH$ (Methionine)

Figure 6: Labelling pattern of Aflatoxin B_1 derived from acetate and methionine..

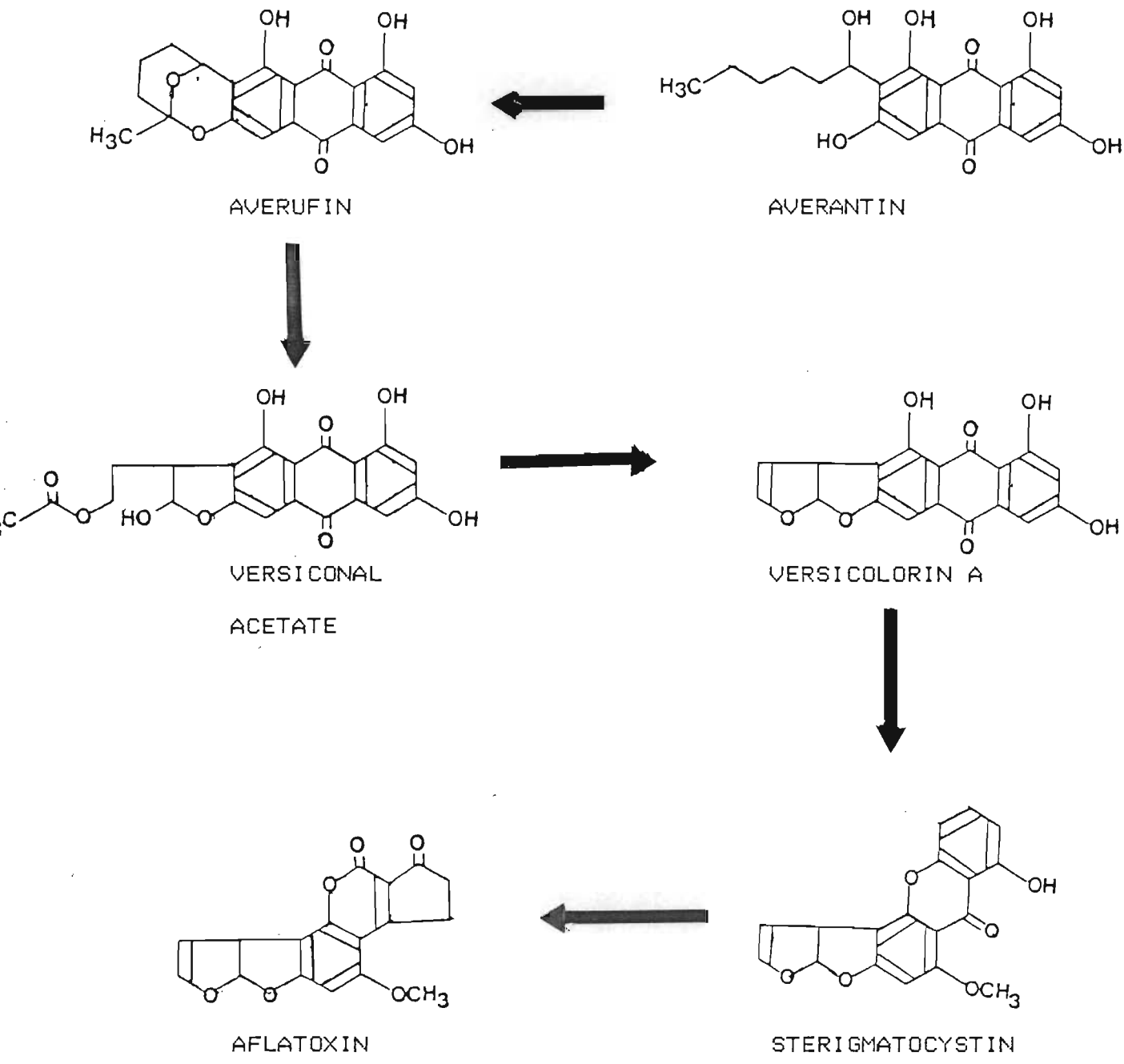


Figure 7: Proposed biosynthetic scheme of aflatoxin B_1 (63).

The biochemical events prior to the formation of aflatoxin were elucidated by the use of mutants and enzyme inhibitors on whole mycelia. The incorporation of the potential intermediates were then studied. Using this techinique Lin and Hsieh (64) showed that averufin, a C_{20} polyketide, can be converted to aflatoxin B_1 by A. parasiticus. They suggested that the starting point for aflatoxin B_1 biosynthesis was a C_{20} chain rather than a C_{18} intermediate as postulated by Biollaz et al. (62). Fitzell, Hsieh, Yao and La Mar (65) carried out ^{13}C NMR studies on averufin derived from (1-^{13}C) acetate, which confirmed the polyketide origin of the molecule. Gorst-Allman, Pachler, Steyn, Wessels and Scott (66) gave a detailed scheme of the biosynthesis of averufin and established an acetate polyketide pathway. Hsieh et al. (47) analysed aflatoxin B_1 derived from ^{13}C averufin by NMR and implied that averufin was a direct precursor of aflatoxin B_1. De Jesus, Gorst-Allman, Steyn, Vleggaar, Wessels, Wan and Hsieh (67) confirmed these findings.

Protoplasts derived from A. flavus were shown to be capable of synthesizing aflatoxin B_1 when incubated with ^{14}C acetate and ^{14}C versicolorin A (68). Tyagi, Tyagi and Venkitasubramanian used 'spheroplasts' to demonstrate the ability of A. parasiticus to incorporate labelled acetate to aflatoxin B_1 (69). They used the spheroplasts to study factors regulating aflatoxin B_1 biosynthesis and also studied the effects of sugars, inorganic acids and lipids (70).

In 1965 Thomas (71) proposed a pathway in which averufin was converted to aflatoxin via sterigmatocystin. Later as reported by Moss in 1972 thomas revised it and proposed a Baeyer-Villager oxidation on an acetylfuran intermediate (Fig. 8) (72). Kingston, Chen and Vercellotti (42) suggested that nidurufin was an intermediate, where the open chain form is altered via a pinacol type rearrangement (Fig. 9). Model studies suggest that nidurufin may be the intermediate unbranched progenitor of the branched chain aflatoxin precursors (73). Tanabe, Uramato, Hamasaki and Cary (74) proposed that a cyclopropane intermediate undergoes a Favorsky rearrangement (Fig. 10), whereas Gorst-Allman et al. (66) favoured an epoxide intermediate (Fig. 11). Fitzell et al. (51) suggested that the two terminal carbons were removed from a rearranged open chain form of averufin, followed by the addition of an acetyl group from free acetate (Fig.12). All these mechanisms result in the conversion of the C_6 side chain to acetylated C_4 branched side chain of versiconal acetate.

In a scheme suggested by Wan and Hsieh (75), versiconal acetate was converted to versicolorin A via a dehydrogenase mediated reaction (Fig. 13). Lee, Bennett, Cucullu and Ory (76) demonstrated an incorporation of 46% ^{14}C versicolorin A into aflatoxin B_1 using A. parasiticus and thus indicated that versicolorin A is an intermediate in aflatoxin biosynthesis.

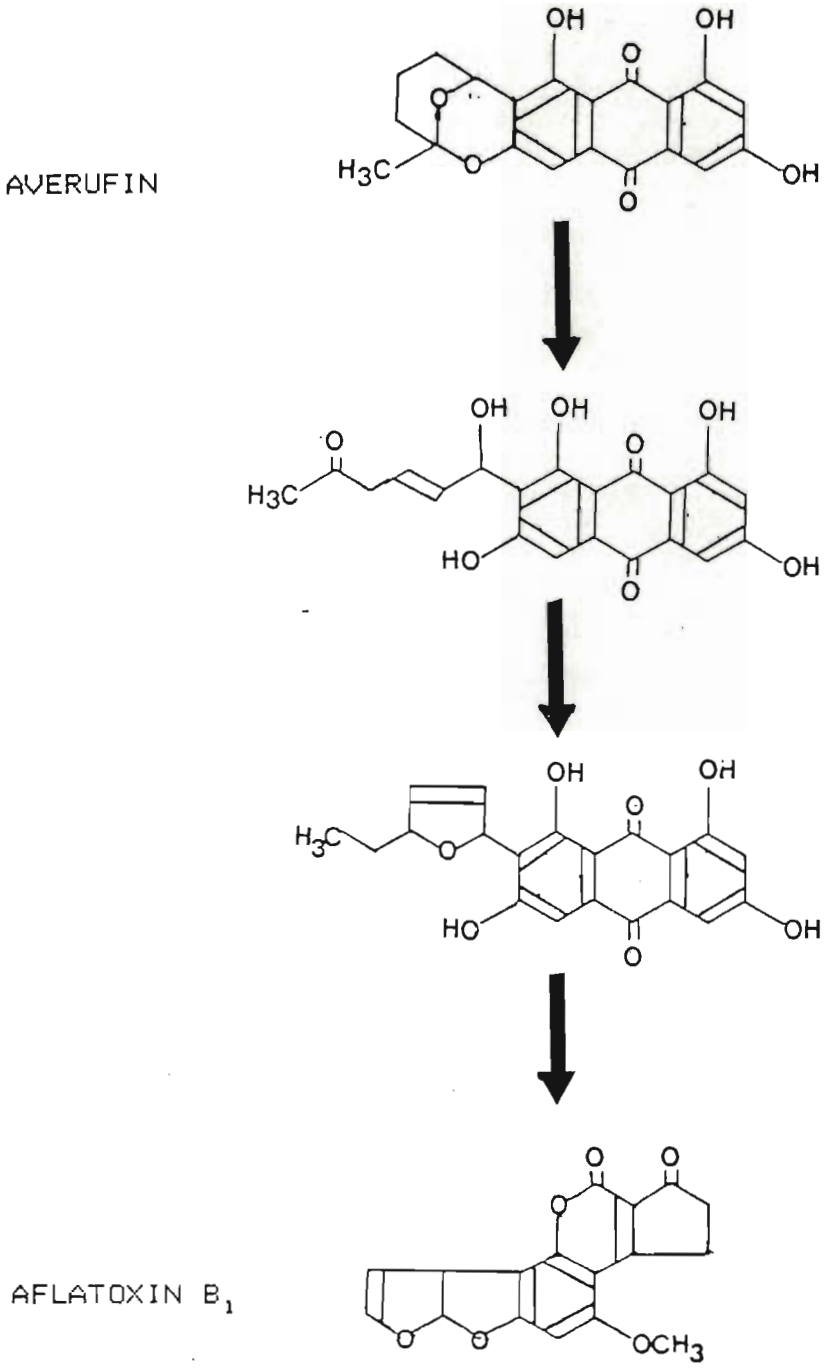


Figure 8: Mechanism for the conversion of the C_6 side chain of averufin to the bisdihydrofuran ring (after Moss (72)).

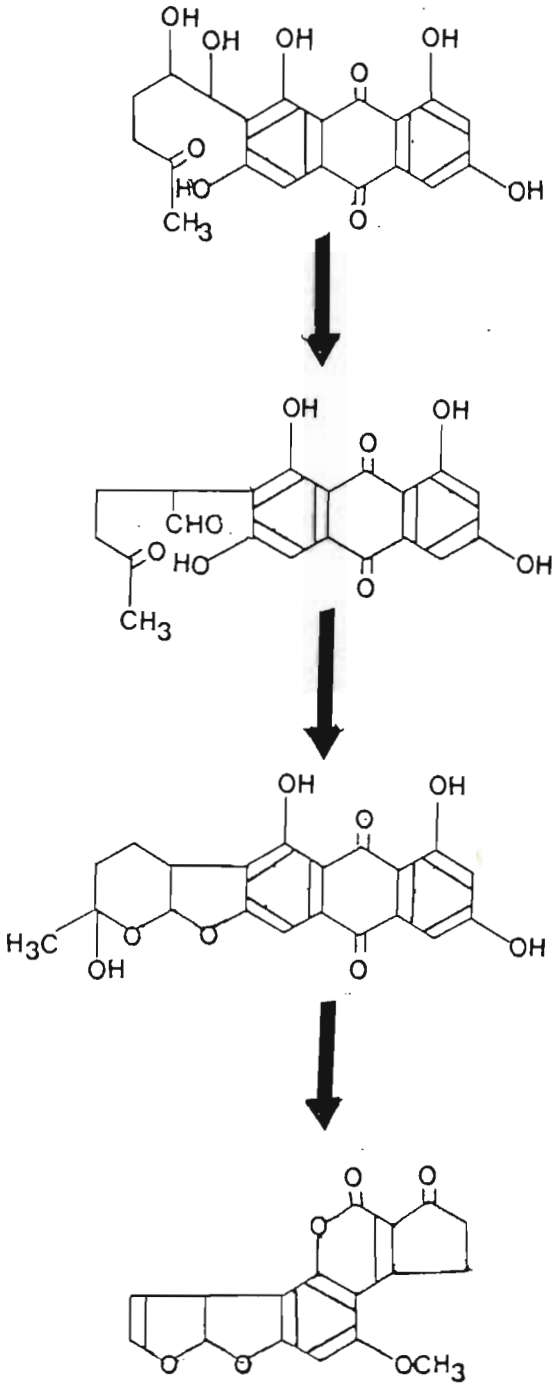


Figure 9: Formation of aflatoxin B_1 from nidurufin (after Kingston et al (42)).

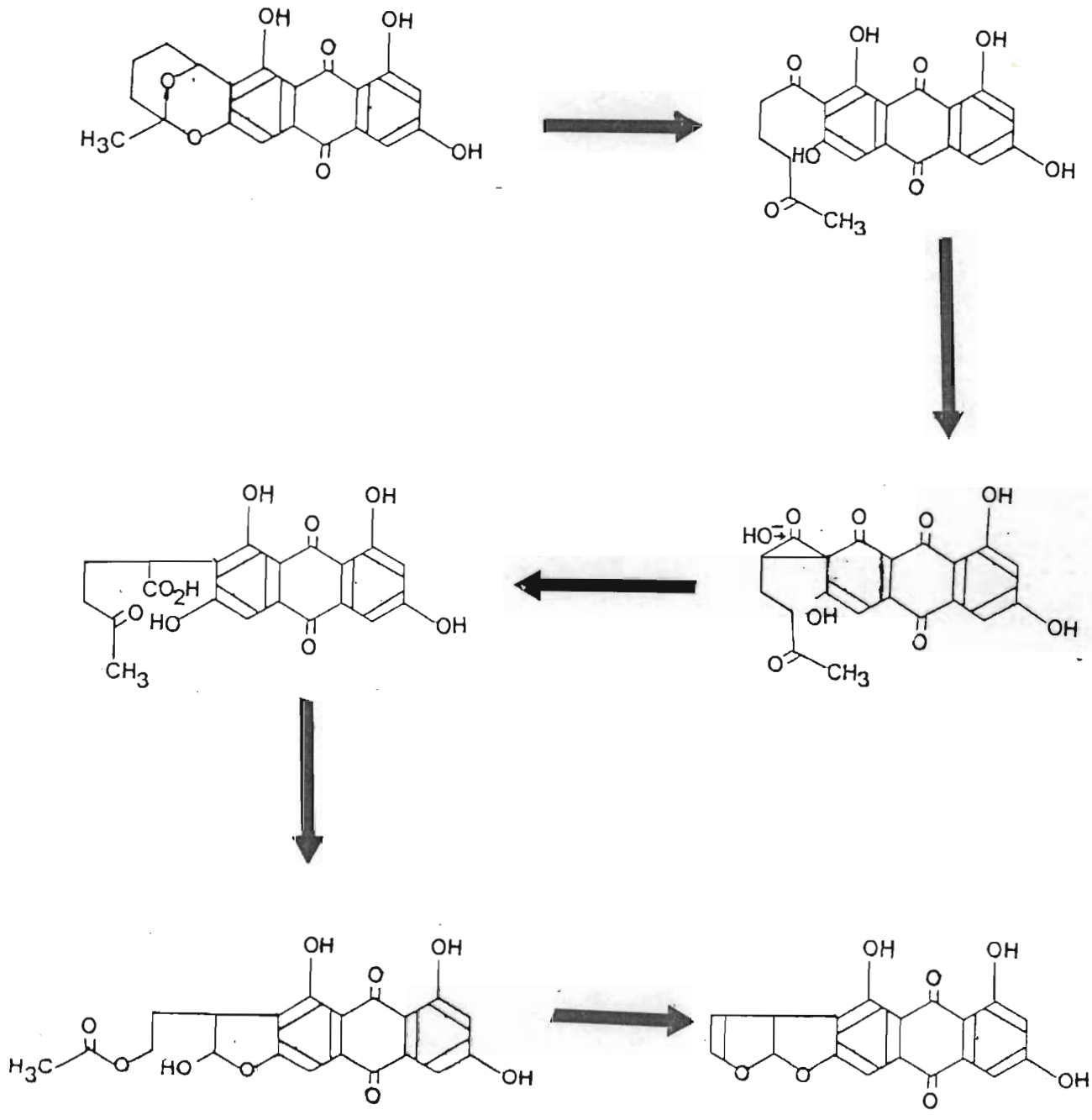


Figure 10: Formation of versicolorin A from averufin (af
Tanabe (74)).

AVERUFIN

RING OPENING HYDRATION

DEHYDRATION

EPOXIDATION

BAEYER-VILLIGER OXIDATION

EPOXIDE REARRANGEMENT

VERSICONAL ACETATE

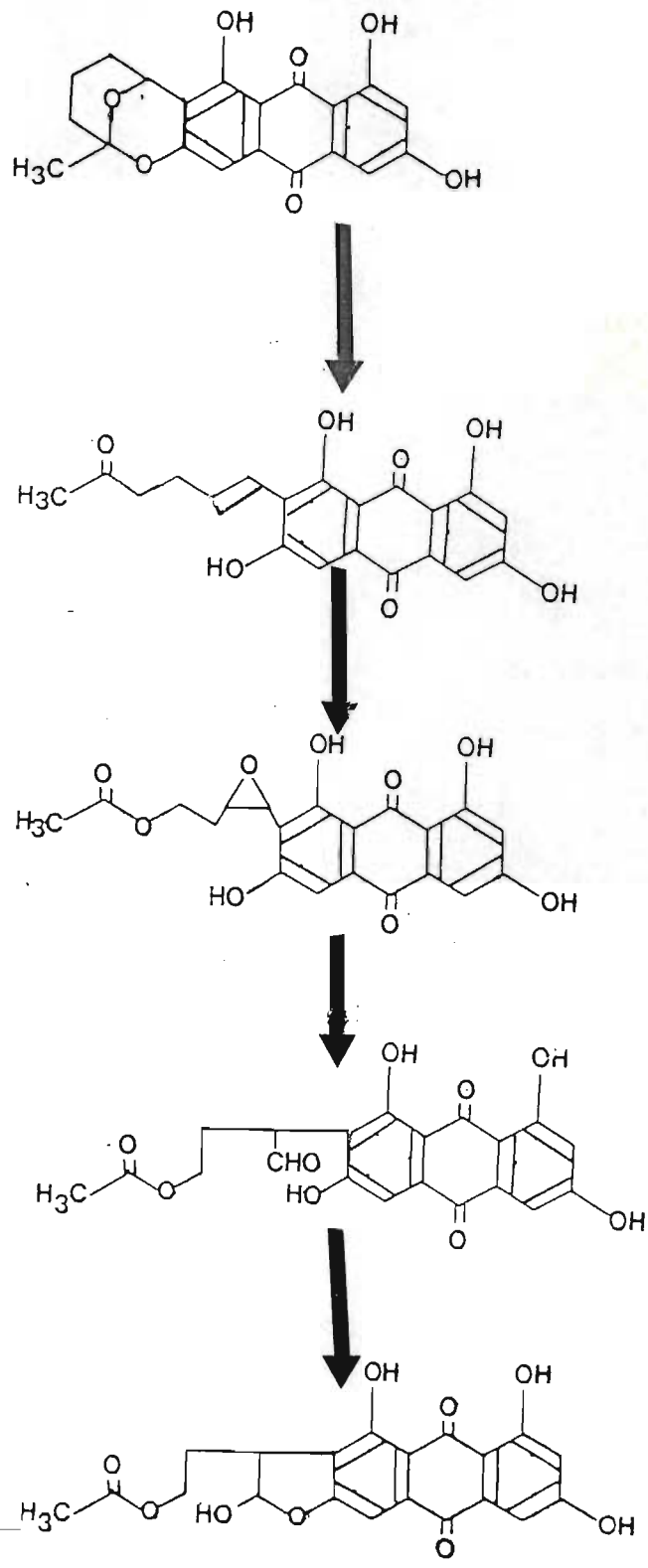


Figure 11: Pathway for formation of versiconal acetate from averufin (after Gorst-Allman (66)).

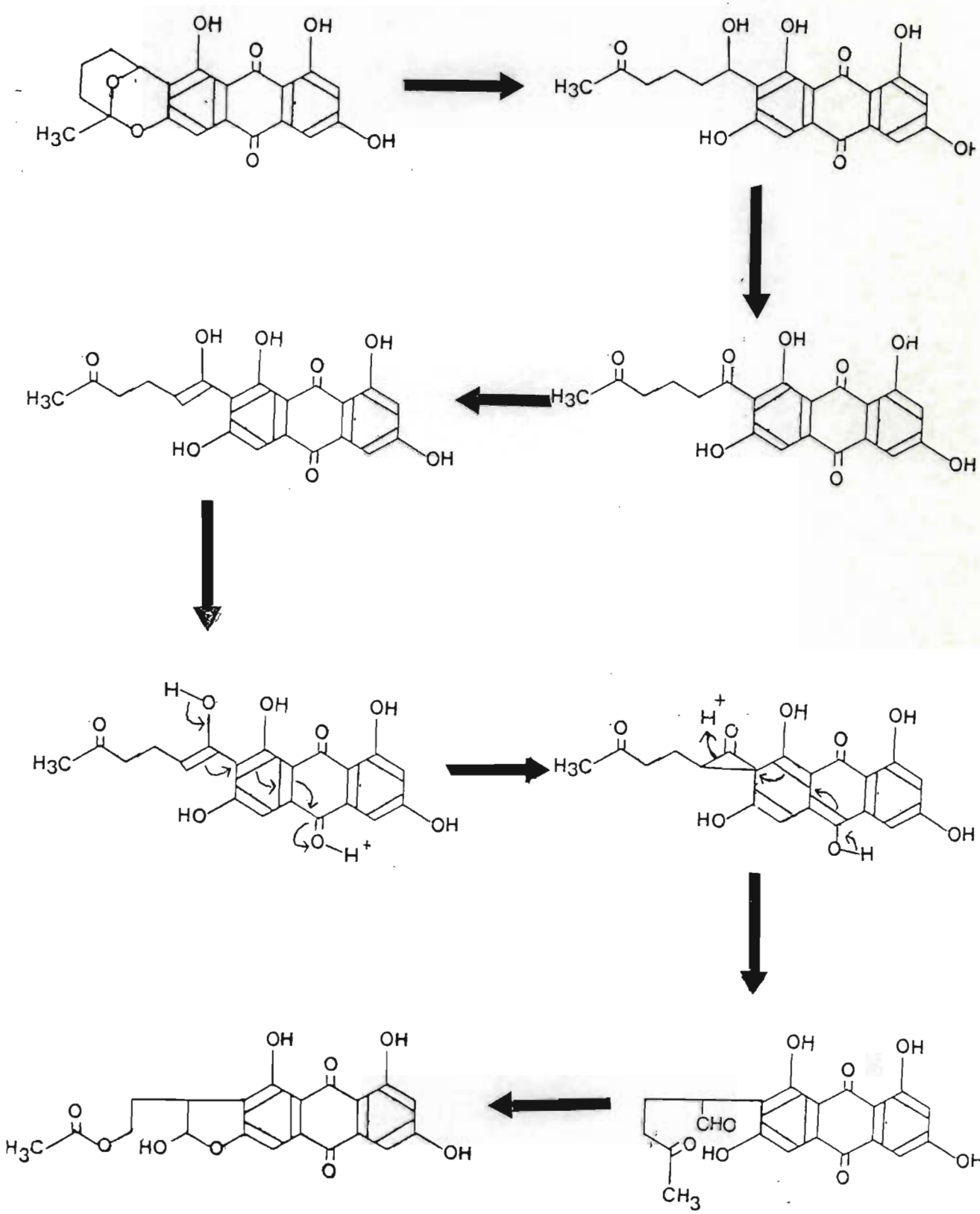


Figure 12: Formation of versiconal acetate from averufin (after Fitzell (51)).

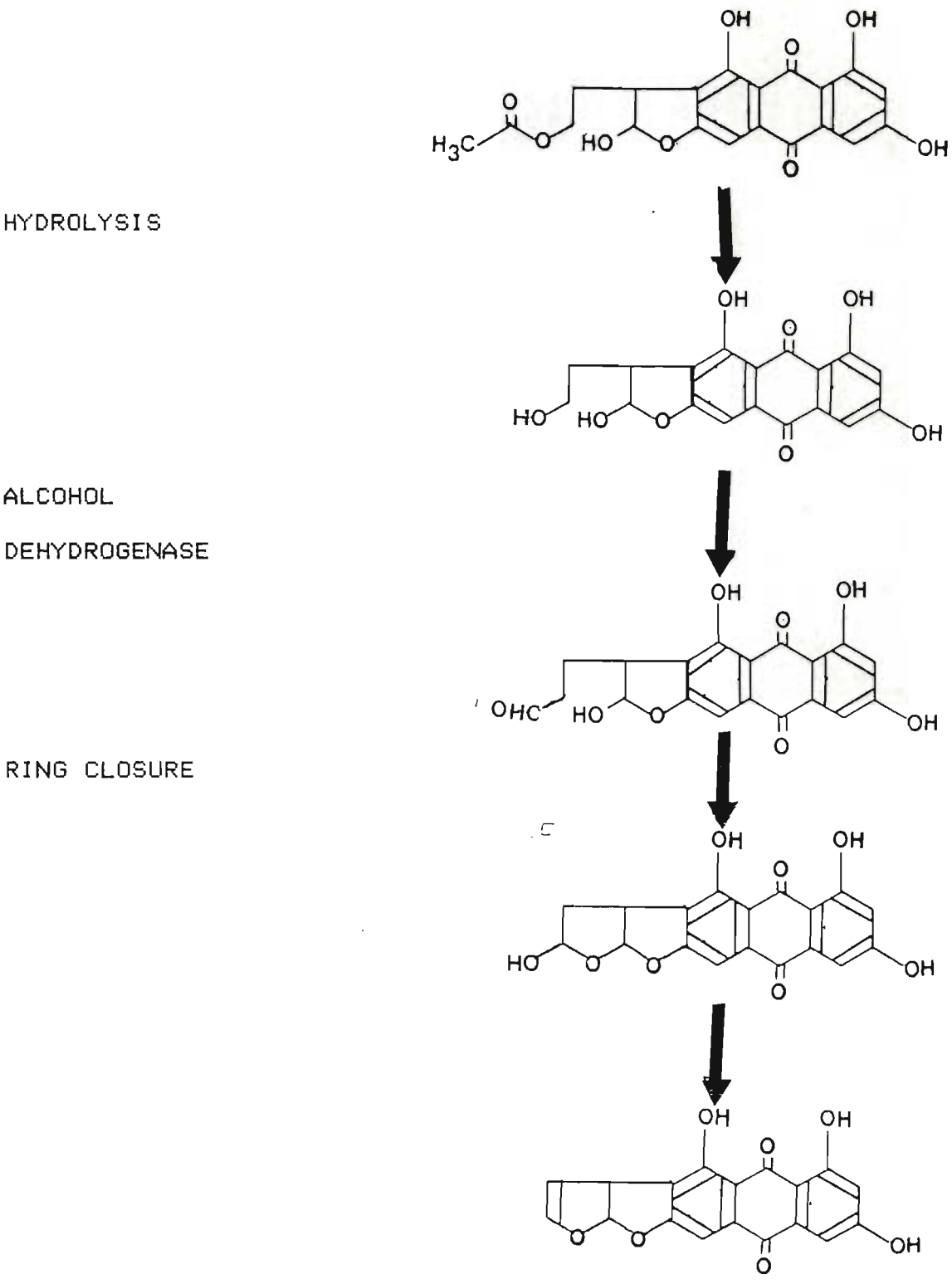


Figure 13: Conversion of versiconal acetate to versicolorin A (after Wan (75)).

Dutton and Anderson (77) found that in cell free systems versicolorin A is not converted to aflatoxin and have proposed an alternative pathway from versiconal acetate involving an oxygenase (Fig. 14). They proposed this result can be explained if it is assumed that versicolorin A is not in the direct pathway to aflatoxin but is closely related to an intermediate, eg., versicolorin A hemiacetal.

Anthraquinones have been reported to be transformed by fungal enzymes into xanthones (78). From ^{13}C studies it has been proposed that versicolorin A is converted into sterigmatocystin by cleavage, oxidative decarboxylation resulting in an elimination of a carbon atom derived from the C_2 carbon of acetate (79). However, very little is known of the biochemical steps involved in its conversion. Holker and Kagel (36) proposed a pathway, similar to that in Fig. 15, which implied that 6-hydroxysterigmatocystin and not sterigmatocystin lies on the direct pathway to aflatoxin. This was supported by a mechanism, proposed by Simpson and Stenzel (79), which involved 6-hydroxysterigmatocystin (Fig. 16).

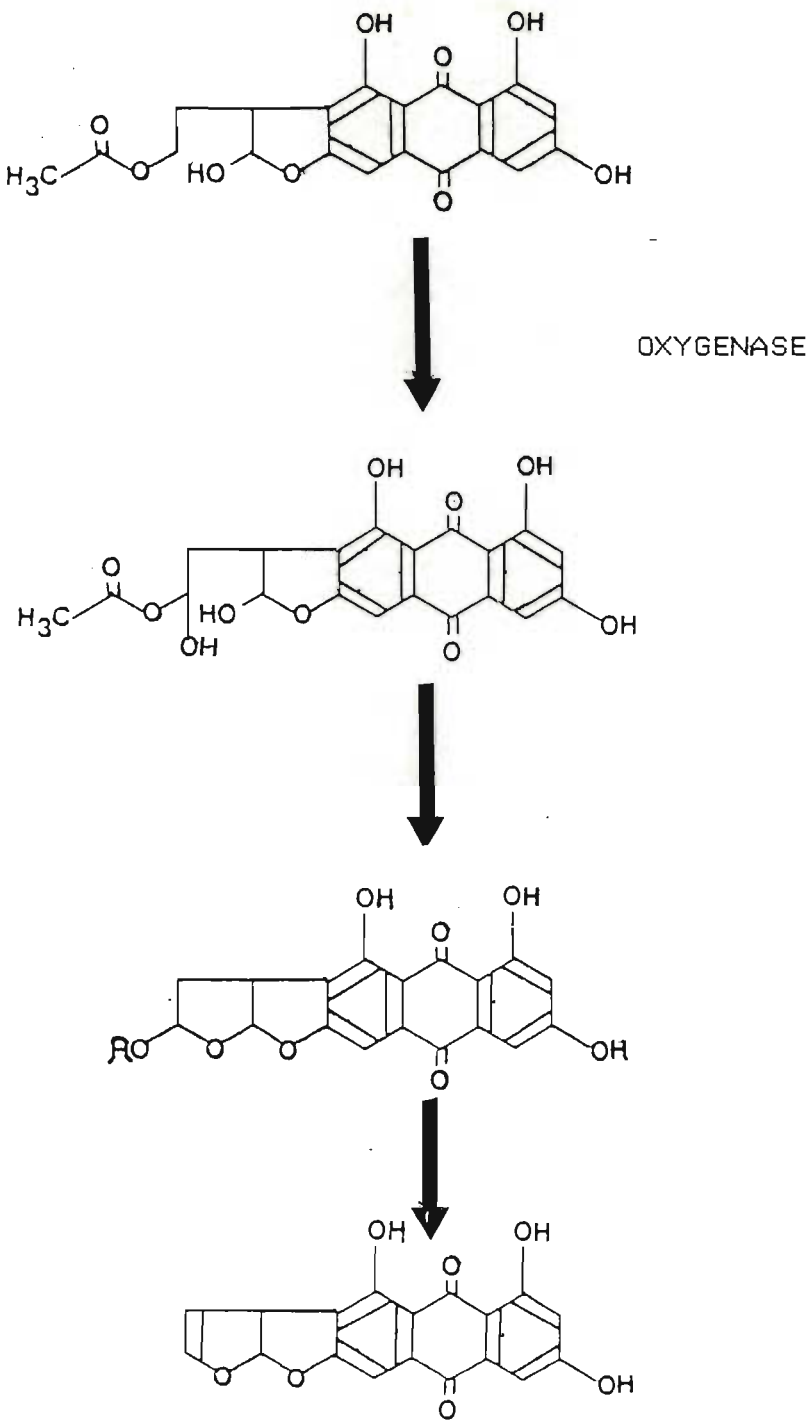
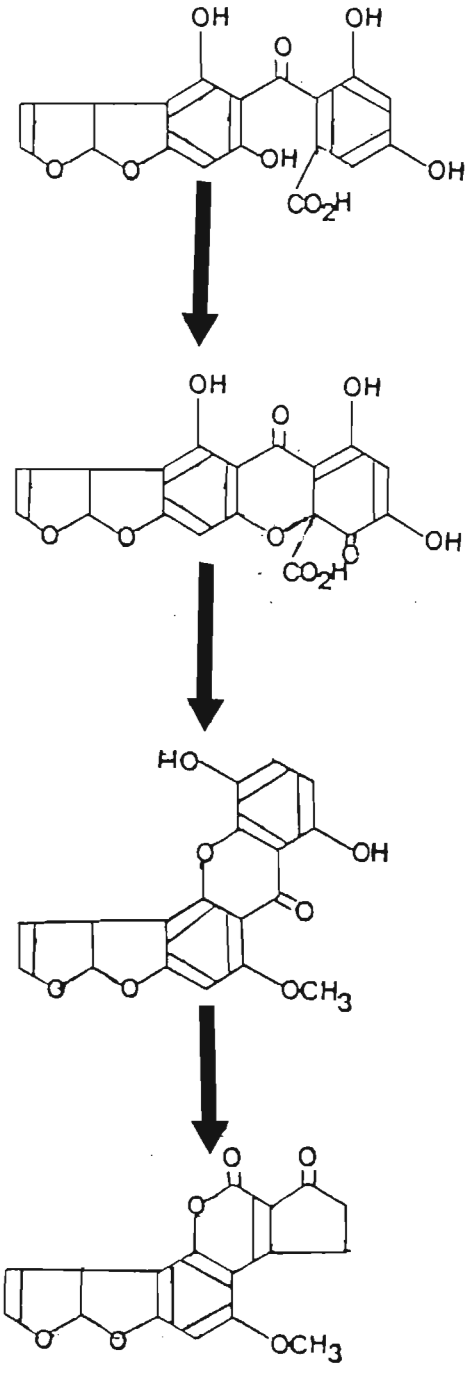


Figure 14: Proposed pathway for the biosynthesis of aflatoxin B_1 (after Dutton and Anderson (77)).

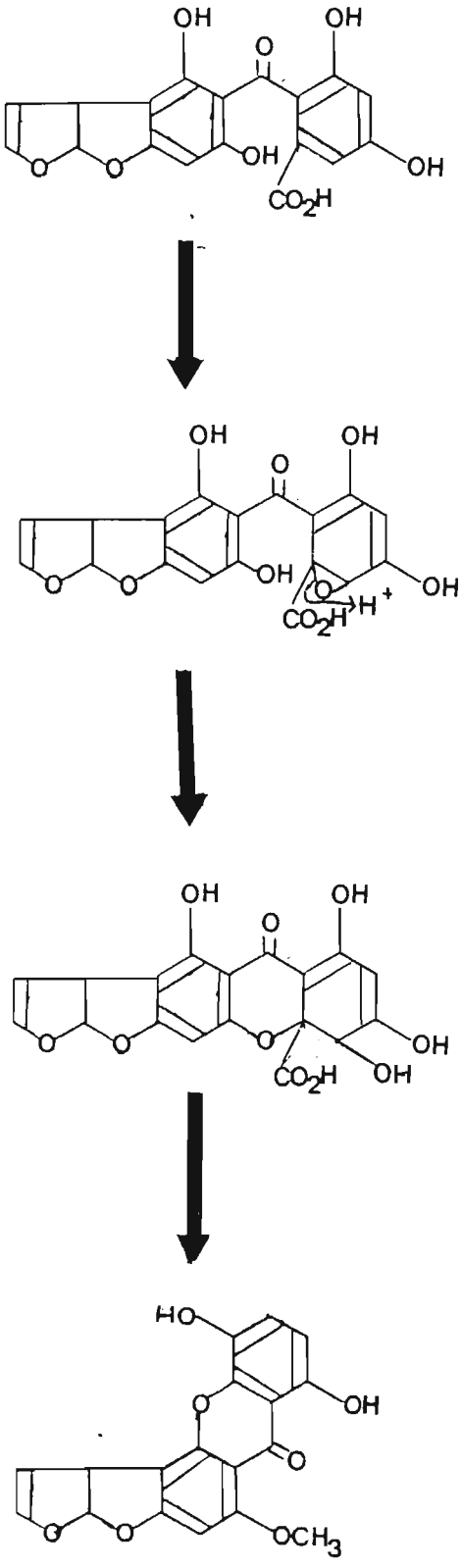


6-HYDROXY-

STERIGMATOCYSTIN

AFLATOXIN B_1

Figure 15: Aflatoxin biosynthetic pathway involving 6-hydroxysterigmatocystin (36).



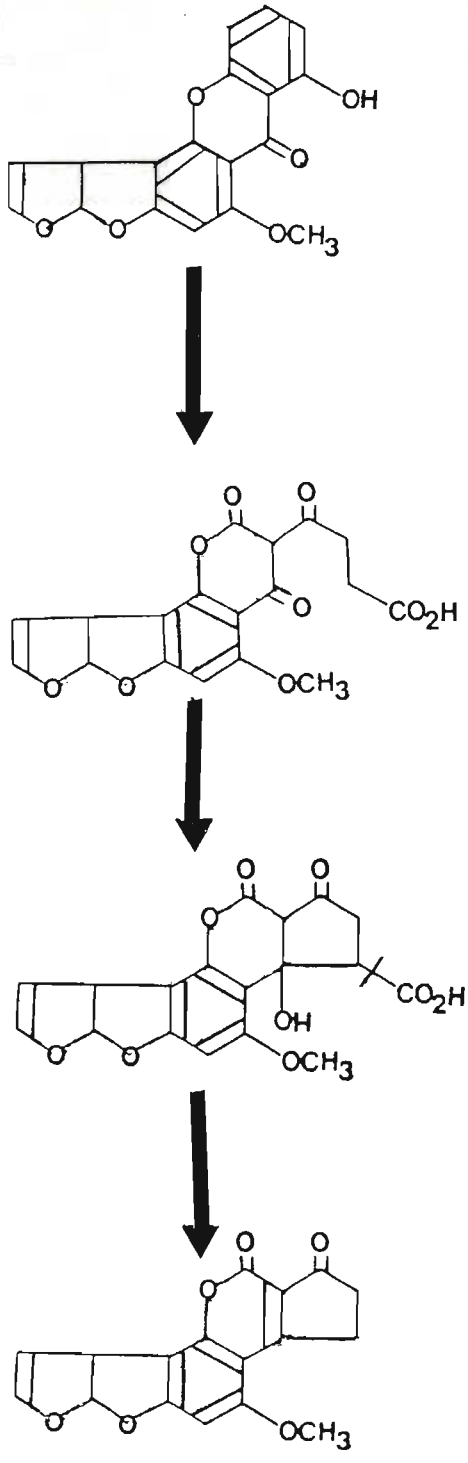
6-HYDROXYSTERIGMATOCYSTIN

Figure 16: Possible mechanism for xanthone ring formation involving 6-hydroxysterigmatocystin (79).

The conversion of sterigmatocystin into aflatoxin involves the loss of another carbon atom derived from acetate (43). Singh and Hsieh demonstrated that A. parasiticus was able to convert ^{14}C sterigmatocystin to aflatoxin (80), which supported Thomas's earlier (71) proposal of oxidative cleavage of sterigmatocystin followed by an aldol condensation to yield a substituted cyclopentanone carboxylic acid. Decarboxylation and dehydration of this product would give aflatoxin B_1 (Fig. 17). Later, Simpson proposed a similar scheme but with a slight modification (81) in that a quinone is formed as an intermediate (Fig. 18).

Thus the currently accepted scheme for the biosynthesis of aflatoxin B_1 takes into account norsolorinic acid, averantin, averufin, versiconal acetate, versicolorin A and sterigmatocystin. Singh and Hsieh using blocked mutants and the specific inhibitor dichlorvos have found evidence to support this scheme (Fig. 19) (82).

STERIGMATOCYSTIN



AFLATOXIN B_1

Figure 17: Conversion of sterigmatocystin to aflatoxin B_1 as proposed by Thomas (71).

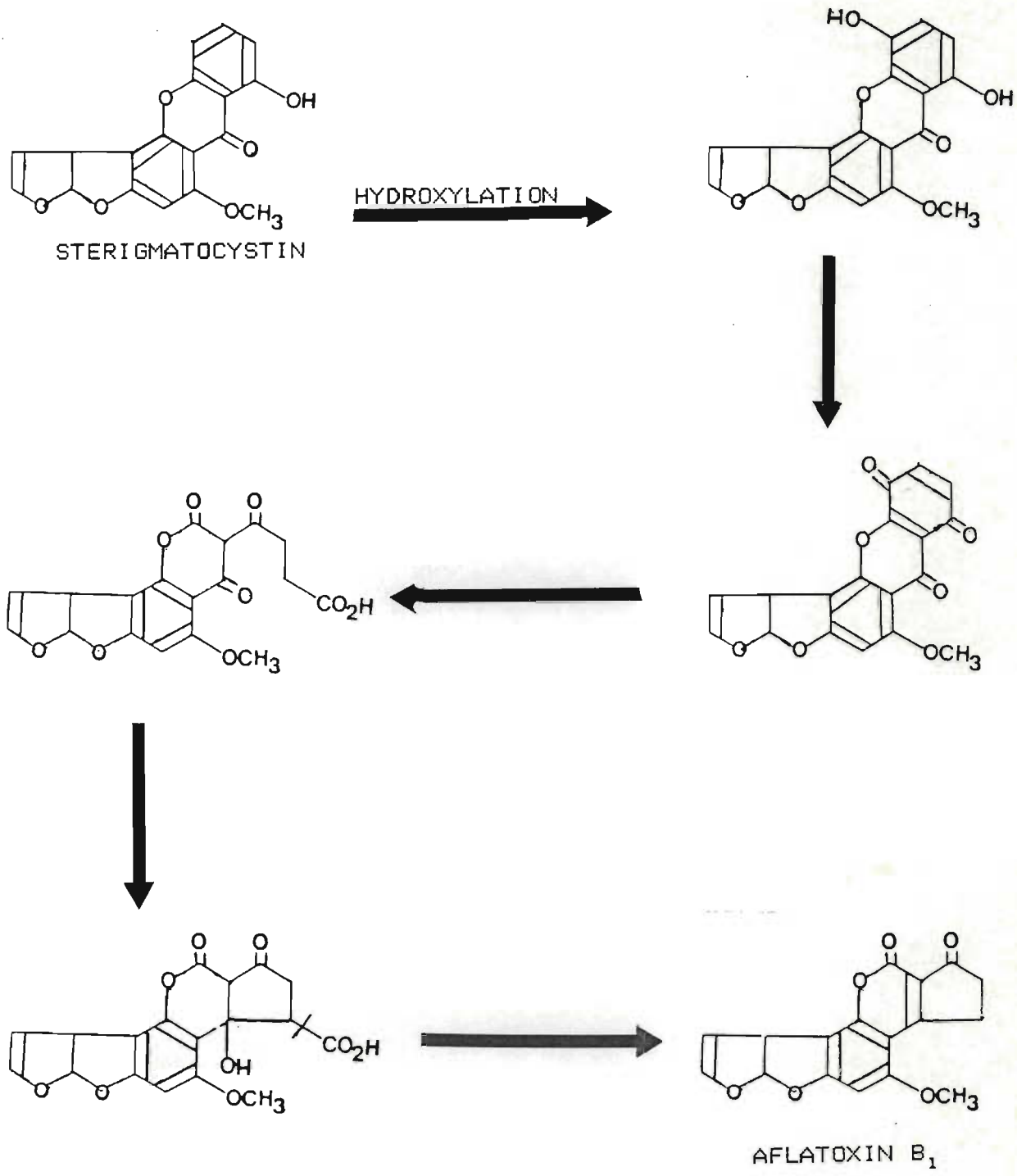


Figure 18: Conversion of sterigmatocystin to aflatoxin B_1 as proposed by Simpson (81).

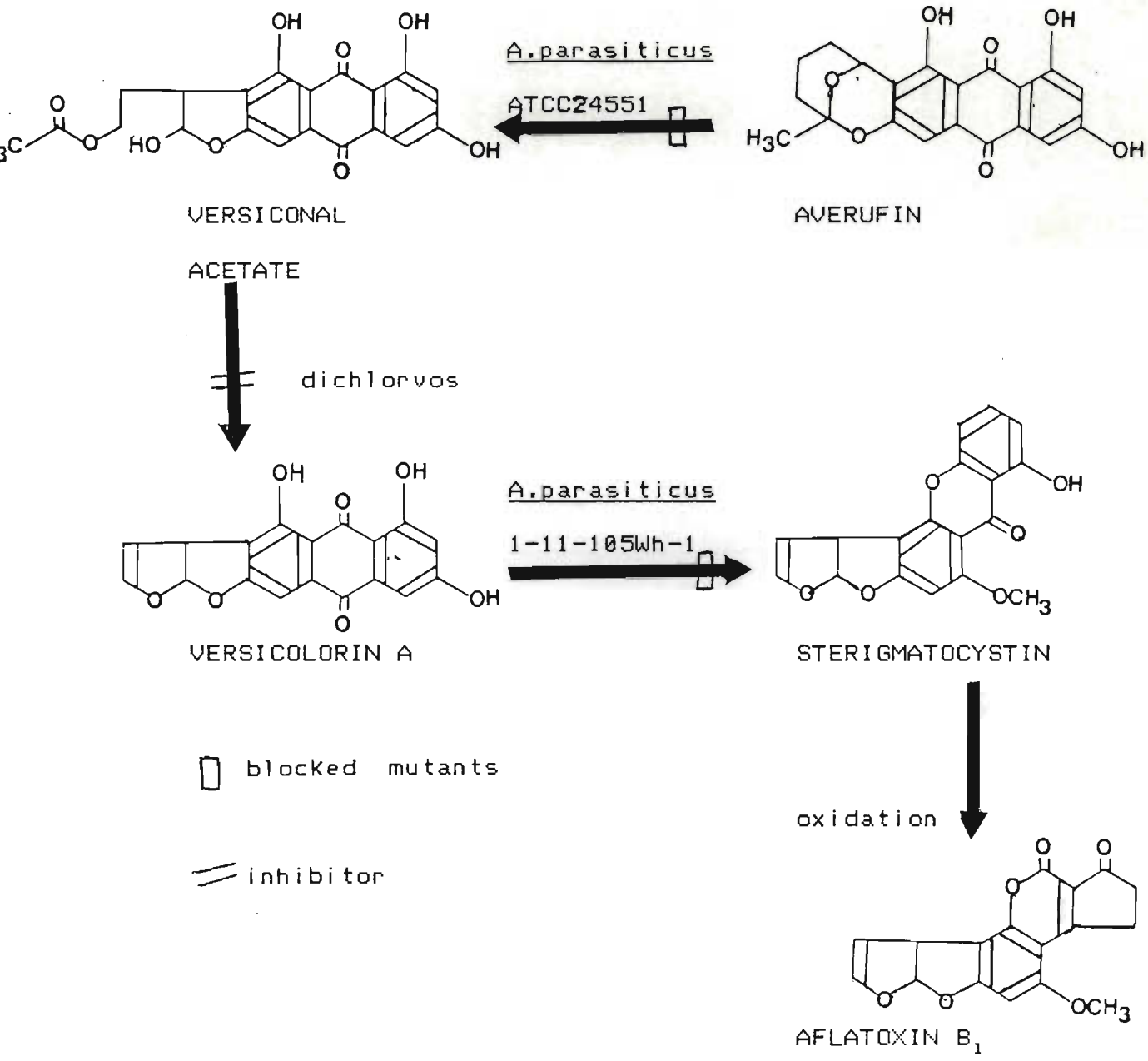


Figure 19: Aflatoxin biosynthetic pathway as deduced by the use of blocked mutants and specific inhibitors after Singh and Hsieh (82).

1.3. CELL FREE BIOSYNTHESIS OF AFLATOXIN

Nearly all of the evidence in support of the biosynthetic pathway of aflatoxin suggested above (Fig. 19) has been obtained from isotopic and chemical analysis with whole mycelia or crude cell free preparation. The individual steps, in the pathway and their mechanisms can only be conclusively proved by isolating and studying the enzymes responsible.

In the study of primary metabolism many cell free systems and pure enzyme preparations have been used successfully. These techniques however have not been greatly employed in the study of secondary metabolism, mainly because of the low levels of enzymes involved and the difficulty in purifying these secondary metabolic enzymes (83).

Of the secondary metabolic enzymes studied those involved in the biosynthesis of patulin have had a considerable amount of work centred on them. Basset and Tanenbaum (83) obtained the first cell free system capable of converting glucose, acetyl CoA and 6-methylsalicylate to patulin. Lynen and Tada (84) obtained a similar system which required the presence of NADPH. Lynen (85) speculated that a multi-enzyme complex, similar to that involved in fatty acid synthesis was involved. In this system the polyketide intermediate would be bound by thioester linkages to the enzyme complex. A number of other enzymes involved in the biosynthesis of patulin have been isolated: a hydroxylase

which converts hydroxy benzyl alcohol to gentisyl alcohol (86), a dehydrogenase and a dioxygenase, which converts gentisyl alcohol to patulin (87).

Other examples of fungal cell free systems of note are those involved in the transformation of steroids by Penicillium lilanum, (88,89), the conversion of precursors to ergot alkaloids (90,91), the synthesis of trichodiene (92), the synthesis of cyclopiazonic acid (93), and the formation of orsellinic acid, alternariol and phenols from acetate (94, 95).

The first cell free biosynthesis of aflatoxin was claimed by Raj et al. (96). These workers found that labelled acetate, mevalonate and leucine were incorporated into aflatoxin B_1. These findings are not in keeping with a polyketide scheme of biosynthesis. Yao and Hsieh (49), using a cell free system, suggested that the enzymes responsible for aflatoxin B_1 biosynthesis were located in the mitochondrial fraction, whilst Singh and Hsieh (80) proposed that the conversion of sterigmatocystin to aflatoxin B_1 was carried out in the cytoplasm by an oxygenase, although no attempt was made to isolate the enzyme(s) or intermediate(s) involved.

Anderson and Dutton (97) found that a cell free system derived from A. flavus was capable of converting sterigmatocystin and versiconal acetate but not versicolorin A to aflatoxin B_1. They suggested that versicolorin A is not in the direct pathway to aflatoxin B_1 but is closely related to

an intermediate such as versicolorin A hemiacetal. Wan and Hsieh (75) isolated a relatively stable enzyme system from A. parasiticus that converted versiconal hemiacetal acetate to versicolorin A. The system was incubated at pH 7.5. Dutton and Jeenah (98) have shown that the conversion of versicolorin A to aflatoxin B_1 was pH dependent and supported the view that under the right pH conditions versicolorin A is converted to versicolorin A hemiacetal.

Electrophoretic comparisons of mycelial enzymes from aflatoxin B_1 producing and non-producing strains have been carried out (99). No distinct differences in enzyme patterns between the two groups were detected.

1.4. REGULATION OF SECONDARY METABOLIC ENZYMES

During the rapid mycelial growth in the trophophase, (the phase during which cell mass increases exponentially), all nutrients are balanced and few if any intermediates accumulate. When an essential growth factor is depleted, growth stops and idiophase (the period in which secondary metabolites appear) begins (100). Depletion of phosphate in the case of Claviceps sp. stops growth and causes the induction of the enzymes responsible for secondary metabolism (101). This termination of balanced growth leads to an accumulation of a variety of primary metabolic intermediates, which could lead to the induction of the secondary metabolic enzymes responsible for the transformation of the intermediates into other compounds or would lead to the excretion of the product (102). In the biosynthesis of 6-methylsalicylate an accumulation of acetyl-CoA or more probably malonyl-CoA could be the trigger (103)

Thus 6-methylsalicylate synthase is somehow 'activated' or induced when the idiophase begins. In contrast the ability to form patulin is established 'adaptively' needing continued protein synthesis in the idiophase (104). In ergot alkaloid synthesis however, it was necessary for co-factors (eg. NADPH) to be produced in the trophophase (101).

Detroy and Hesseltine studied the effect of cycloheximide on the metabolism of aflatoxin (105). They found that when added to the culture fluid it prevented aflatoxin from being synthesised but when the antiobiotic was removed, aflatoxin biosynthesis was recommenced. They concluded that some or all of the enzymes were formed in the idiophase.

1.5. RING CLEAVAGE ENZYMES

Singh and Hsieh (80) had found that NADPH was required for the conversion of sterigmatocystin to aflatoxin B_1 implicating the involvement of an oxygenase. In general oxygenases are divided into two classes- monooxygenases and dioxygenases (106).

Monooxygenases, sometimes referred to as mixed function oxygenases (107), are responsible for the incorporation of a single atom of oxygen into the substrate (108) (Fig 20), whereas dioxygenases catalyse the incorporation of two atoms of oxygen into a molecule of substrate (Fig 20). Monooxygenases may be classified on the basis of the electron donor involved: internal monooxygenases in which the reducing agent is internally supplied i.e. the substrate, but the more common types of monooxygenases, the external monooxygenases require various kinds of external electron donors eg. NADPH, $FADH_2$ and reduced iron-sulphur proteins. Many monooxygenases catalyse hydroxylation of aromatic and aliphatic compounds but they also catalyse a seemingly diverse group of reactions including epoxide formation, dealkylation and deamination, but the primary chemical event is identical. All the processes are initiated by the addition of one atom of oxygen into the substrate.

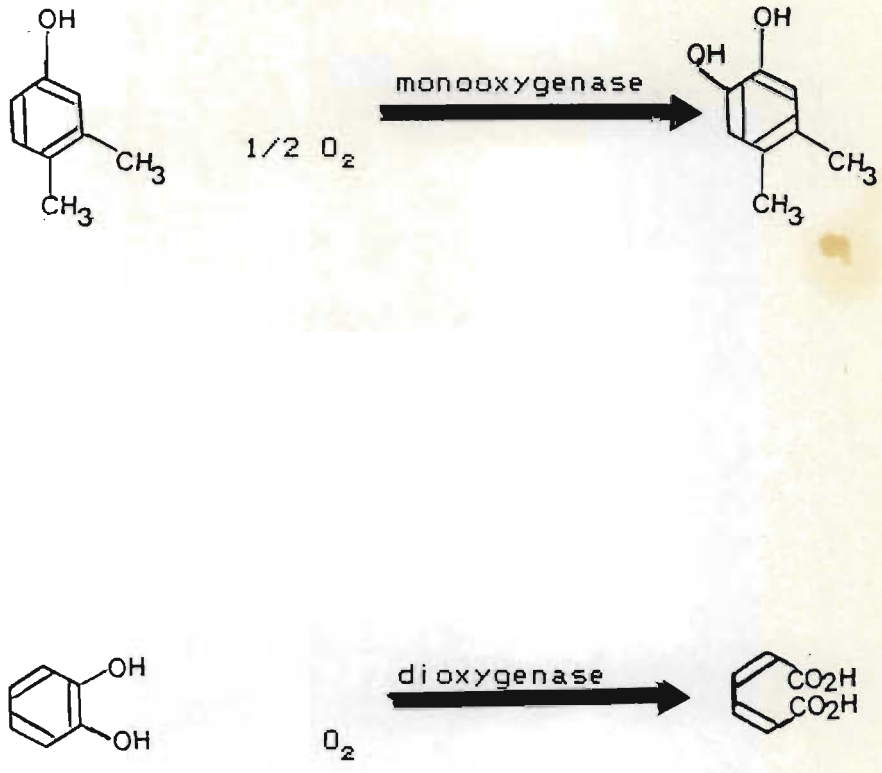


Figure 20: Incorporation of oxygen by oxygenases.

After the initial monooxygenase reaction the compounds become more soluble in water or more biologically reactive in the sense that they are susceptible to the action of other enzymes, eg. dioxygenases (106).The dioxygenase cleaves the aromatic double bond either between two hydroxylated carbon atoms ('ortho cleavage') or adjacent to a hydroxylated carbon atom ('meta cleavage').

Some dioxygenases such as trytophan 2,3-dioxygenase contain haem as the prosthetic group (109) while others such as pyrocatechase contain non-haem iron (110), while enzymes such as quercetine dioxygenase contain copper as the cofactor (111). All of the phenolic dioxygenases (those that cleave the phenol or catechol ring) that have been purified contain non-haem iron as the sole cofactor. It is assumed that iron plays an active role in activating the oxygen as well as the substrate (112). These enzymes have also been found to be inhibited by iron chelating agents (110). The reaction mechanism for dioxygenase was postulated as follows:- the enzyme containing ferrous ion, combines with an organic substrate, resulting in the reduction of the iron, which then reacts with the oxygen to form a ternary complex (112). The question whether the enzyme binds first to the substrate or the oxygen has not been resolved (106).

Monooxygenases are also responsible for inserting an oxygen atom directly into the ring. This type of reaction is found in the formation of a lactone from cycloalkanones thus emulating the Baeyer-Villager type of reaction (114). This reaction is an

oxidation step in which a ketone is converted to an ester or lactone with a peracid by migration of an O from the peracid. The key step is the heterolytic dissociation of the O-O bond of an adduct that is formed (115). Cyclohexanol is metabolised by Nocardia globerlae (114) to cyclohexanone and thence to the lactone.

1.6. CELLULAR LOCATION OF OXYGENASES

Membrane bound proteins are classified into two catogeries: peripheral (extrinsic) and integral (intrinsic) (116). Peripheral membrane proteins are loosely attached and can be removed from the membrane by mild treatments such as changing the ionic concentration of the medium or by treating with EDTA (117). Cytochrome C and spectrin are examples of this type of protein. Integral proteins on the other hand can only be isolated by more drastic treatments with detergents, bile salts and organic solvents because of the close association with the membrane lipids. Cytochrome B and glycophorin are examples of this group.

Peripheral proteins are found on both the inner as well as the outer sides of the membrane. In bacteria, mild treatments such as sonic vibration release the outer protein. High speed centrifugation of cell homogenates releases the peripheral protein in to the supernatant where cytoplasmic enzymes are usually found (118). Mitchell (119) coined the term periplasmic for this type of enzyme. Periplasmic proteins have primarily been studied in bacteria where they are present as degradative enzymes (118).

Oxygenases are known to occur in the liver microsomes of many vertebrates (120), and OKamato and coworkers have shown that kynurenine-3-hydroxylase is localised in the outer membrane

of rat liver mitchondria (121). However very little is known of the localisation of oxygenases in fungi. Monooxygenases in eukaroyotic organisms are most often found in the particulate fraction whilst the dioxygenases are soluble proteins (122). Hsieh and Mateles (123) had predicted that the enzymes responsible for the biosynthesis of aflatoxin occurred extra mitochondrially. Wan found that all the activity for aflatoxin biosynthesis was present in the supernatant fraction when centrifuged at 105,000xg for 2 hours (124) thus indicating that the enzyme was present in the cytoplasm.

1.7. AIMS OF THE PROJECT

The prime objective of the project was to isolate and purify the enzyme(s) involved in the conversion of sterigmatocystin to aflatoxin B_1. The characterisation of a pure enzyme system and the mechanism of the reactions they catalyse would elucidate the role of sterigmatocystin in aflatoxin biosynthsis.

Such information would contribute to the understanding of aflatoxin biosynthesis and would enable a more effective control of aflatoxin production in the field to be formulated. It would also contribute greatly to our general knowledge of fungal enzymology.

2. MATERIALS AND METHODS

2.1. ORGANISMS

The species, strains and mutants of the genus Aspergillus that were used in this study are listed in Table 1.

Trichoderma viride (CBS 354-33), supplied by Dr. J. Peberdy (Nottingham Univ. U.K.) and Oerskovia xanthineolytica, supplied by Dr. Broek (Agricultural Univ. Wageningen, Netherlands), were used for the production of digestive enzymes.

All the cultures were maintained on potato dextrose agar (Difco laboratories, Detroit, Michigan, U.S.A.) except Oerskovia xanthineolytica which was maintained on nutrient agar (Difco).

TABLE 1

Strains of *Aspergillus* species used in the study

FUNGAL STRAIN	SOURCE	PRODUCT
A. parasiticus[a] N1X	CMI[b]	Aflatoxins B_1, B_2, G_1, G_2
A. flavus W49	Dr. J Donkersloot[c]	Averufin
A. parasiticus 1-11-105Wh1	Dr. J Bennett[d]	Versicolorin A
A. quadrilinitus	Dr. C Rabie[e]	Sterigmatocystin

[a] derived from CMI91019b

[b] Commonwealth Mycological Institute, Kew, London.

[c] National Institute of Health, Maryland, U.S.A.

[d] Tulane University, Louisiana U.S.A.

[e] M.R.C., Tygerberg, S.A.

2.2. GROWTH

2.2.1. Growth media

Three different growth media were used for the production of mycelium and metabolites by *Aspergillus* species, Reddy's (125) (Appendix 1), YES (126) (Appendix 1) and resting culture (127) medium (Appendix 1). *Trichoderma viride* and *O. xanthineolytica* were grown in liquid media (128, 129) (Appendix 1).

All media were sterilised by autoclaving for 15 minutes at 15 psi and 120°C.

2.2.2 General Growth Conditions

2.2.2.1. Production of Mycelium

A. parasiticus 1-11-105Wh1 was grown at 25°C in shake cultures (150 r.p.m.) for 5 days in 250 ml flasks containing 100 ml Reddy's medium (Appendix 1), which were inoculated with a spore suspension containing approximately 1 million spores. Spore suspensions were obtained by adding 10 ml sterile sodium dodecyl sulphate (0.1% w/v) to a sporulated culture in either medical flats or petri dishes. To harvest the mycelium it was filtered through cheese cloth and freeze dried.

2.2.2.2. Production of Metabolites

One litre flasks containing 400 ml YES medium were inoculated with a spore suspension of the required fungal strain (Table 1) containing approximately 1 million spores and grown at 25°C in shake cultures (150 r.p.m.) for 8 days. The mycelium and culture fluid were then extracted (see section 2.3.1).

2.2.2.3. Production of Digestive Enzymes

Trichoderma viride and Oerskovia xyantilitica were grown in their respective liquid media in both 1l flasks, and in a fermenter (10l) (New Brunswick Magnaferm). The medium in the shake flasks (400 ml) (100 r.p.m.) was inoculated with a 2 day old seed inoculum (40 ml) and maintained at 25°C for 10 days.

The medium in the fermenter (10l) was inoculated with a 2 day old seed inoculum (300 ml) and maintained at 25°C, 80% oxygen saturation and pH 6.6 for 10 days. The filtered culture fluid was used as the digestive enzyme.

2.3. EXTRACTION AND PURIFICATION OF METABOLITES

2.3.1. Extraction

Mycelium (500g) (section 2.2.2.2) was filtered, freeze dried and extracted in a soxhlet apparatus with ethyl acetate (500 ml)[a]. The culture fluid (or incubation mixture) was extracted three times with 1 volume each of chloroform:ethyl acetate (1:1). The organic solvents were dried over anhydrous sodium sulphate for 10 minutes, which was then removed by filtration through Whatman Number 1 filter paper. The combined dried solvent fraction was concentrated in a rotary evaporator at 60°C to a volume of 20 ml. For conversion studies the solvent was evaporated to dryness under nitrogen. The extracted aqueous fraction was discarded.

2.3.2 Purification

The concentrate (5 ml) was streaked on to three, 40 x 20 cm Kieselgel 'G' (Merck, Darmstadt, West Germany) chromatoplate (0.3 mm thick, which was adequate for the amount of material purified). The line of the origin was approximately 2cm from the bottom edge of the plate. The loaded chromatoplate was developed in chloroform:acetone (9:1 v/v). When the solvent front reached the top of the plate it was removed and air dried.

[a] all reagents used were of analytical reagent grade unless otherwise stated.

The resultant chromatographs were viewed under UV light (253 and 365 nm), any observed spots marked and compared with known standards.

The area of silica gel containing the required metabolite (aflatoxin B_1 sterigmatocystin or versicolorin A) was scraped off and the product was eluted from the gel with acetone. The acetone fraction was concentrated to dryness in a rotary evaporator and rechromatographed using toluene:ethyl acetate:acetone:acetic acid (60:25:15:1) as the solvent. The fractions were recovered and rechromatographed as before. The fraction with the desired metabolite was rechromatographed in the aforementioned solvent systems until a single band was obtained. The metabolite was tested chromatographically for purity by correlating the RF with the that of a pure standard (kindly supplied by Dr. M.F. Dutton) on a two dimensional chromatograph (section 2.4.1.1.).

2.4. ASSAY METHODS FOR PRODUCTS OF ENZYME CATALYSED REACTIONS

2.4.1. Identification

2.4.1.1. Thin Layer Chromatography (TLC)

The extracted metabolite (section 2.3.1) was dissolved in methanol (100µl) and then spotted on to two dimensional chromatographs consisting of aluminium-backed silica gel 60 TLC plates (10 cm x10 cm) (0.2 mm thick) (Merck). The plates were run in chloroform:acetone (9:1) in the first dimension and in toluene:ethyl acetate:acetone (60:25:15) in the second dimension. The resultant chromatographs were viewed under UV light (253 and 365 nm), any observed spots marked and compared with known standards.

2.4.1.2. Mass Spectra

Mass spectra of the metabolites produced by the enzyme system were obtained on a Varian Mat model CH7 single focusing instrument. Electron-impact spectra were run at 70eV with an acceleration voltage of 3000 volts and a source temperature of 100° to 125°C. Criteria for identification of samples were based on TLC analyses and correlation of the mass spectral data with those reported in the literature (130).

2.4.2. Quantitative

2.4.2.1. Thin Layer Chromatography

When analysing extracts derived from conversion studies the metabolites were dissolved in methanol (100µl). The sample (40µl) was spotted on to Kieselgel 'G' TLC plates (10 x 10 cm) (0.3 mm thick). The plates where run in two dimensions, using chloroform:acetone (9:1) in the first dimension and toluene:ethyl acetate: acetone (60:25:15) in the second dimension. The plates were viewed under UV light, and the silica containing the aflatoxin sterigmatocystin and O-methylsterigmatocystin spots was scraped off.

Each metabolite was eluted from the silicia with acetone (5 ml), evaporated to dryness and the product was taken up in methanol (2 ml). An Hitachi model 220 spectrophotometer was used to measure the absorbance of the sample at the relevant λmax (Appendix 2) (130).

The concentration of the product was calculated from the absorbance by using its molar extinction coefficient.

$$mg/ml = (A \times MWt)/(\epsilon \times P)$$

A = absorbance

MWt = molecular weight

ϵ = molar extinction coefficient

P = path length

2.4.2.2 High Pressure Liquid Chromatography

A Waters Associates C_{18} column connected to a Varian Model 5000 liquid chrotomatograph, equipped with Varichrom (325nm) and Fluorochrom detectors, was used for the separation and detection of aflatoxin, sterigmatocystin and O-methylsterigmatocystin. A Hewlett-Packard HP3390A integrator was used to calculate peak areas and concentrations of the separated components. Acetonitrile (spectroscopy grade, Merck) and distilled water (55:45) were used to separate aflatoxin, sterigmatocystin and O-methylsterigmatocystin. The solvent used to separate the aflatoxins B_1, B_2, G_1 and G_2 was an isocratic mixture of acetonitrile (spectroscopy grade), methanol (spectroscopy grade) and water (3:2:5).

In order to test the effectiveness of the separation, standard samples of aflatoxin, sterigmatocystin and O-methylsterigmatocystin were chromatographed together.

When analysing extracts derived from incorporation studies the metabolites were dissolved in methanol (spectroscopic grade) (30 µl) and the sample (10 µl) was injected on to the column.

2.5. PROTEIN ASSAY

The Biuret method (131) was used to estimate the protein content of various samples.

SOLUTIONS.

1. Aqueous Bovine Serum Albumin Solution (5 mg/ml) (BSA)
2. Biuret Reagent. Three grams copper sulphate pentahydrate and 9g sodium potassium tartrate were dissolved in 500 ml, 0.2M sodium hydroxide. Potassium iodide (5 g) was added, and the resulting solution was made up to 1l with 0.2 M sodium hydroxide solution.

Biuret reagent (3 ml) was added to protein solution (2 ml), mixed, heated at 37°C for 10 min and cooled. The protein concentration was estimated from a standard curve constructed in the range 0 to 5 mg of BSA/ ml by measuring the absorbance at 540nm.

2.6. MARKER ENZYME AND LIPID TEST

The presence of glucose-6-phosphatase was used as the marker enzyme to indicate the presence of a microsomal fraction containing material from the endoplasmic reticulum.

Solution for assay of glucose-6-phosphatase

1. Sodium cacodylate buffer (0.1M, pH 6.5). Sodium cacodylate (15.9. g dissolved in 50 ml) was added to HCl (29 ml, .2M) and made up to 100 ml with distilled water.
2. Ethylene diamine tetraacetic acid (10 mM in cacodylate buffer adjusted to pH 6.5) (EDTA)
3. Glucose-6-phosphate 50 mM in 0.1M cacodylate buffer
4. Trichloracetic acid solution (10%, in distilled water) (TCA)
5. Ammonium molybdate solution (5%, in distilled water)
6. Copper acetate buffer (pH 4.0, 0.1M). Copper sulphate (2.5 g) and sodium acetate (46 g) was dissolved in 1 l acetic acid (2M).
7. Metol-sodium sulphite solution. Metol (4-methyl-aminophenolsulphate) (2 g) was dissolved in a 10% (m/v) solution of sodium sulphite solution and made up to 100 ml.

Test sample (0.1 ml) (fraction 2 from gel filtration) was added to a mixture of cacodylate buffer (0.6 ml), EDTA (0.1 ml) and glucose-6-phosphate (0.1 ml) and incubated for 15 minutes. Blanks consisting of cacodylate buffer in place of

the test samples were included. The reaction was stopped by the addition of ice cold TCA (1 ml). The solution was centrifuged and the supernatant (1 ml) was added to a mixture of acetate buffer (3 ml), ammonium molybdate (0.5 ml) and metol-sulphite solution (0.5 ml). The reaction mixture was allowed to stand for 10 minutes and the absorbance was read at 880nm. The sample (fraction 2 from gel filtration) was also tested for lipids by comparing the solubility in water and a chloroform-methanol (1:1) mixture.

2.7. ENZYME ASSAY

Enzyme activity was monitored by incubating the sample (section 2.10) in sodium phosphate buffer (0.1M, pH 7) with sterigmatocystin (10 µg dissolved in 10 µl dimethylformamide) at 25°C for 5 hours. In all experiments 10 µg sterigmatocystin was used, unless otherwise stated. Controls, consisting of phosphate buffer (0.1M, pH 7) or boiled (5 min) sample in place of the test sample, were incubated similtaneously. The mixture was extracted (section 2.3.1) and aflatoxin, O-methylsterigmatocystin and sterigmatocystin were assayed by HPLC (section 2.4.2.2) and TLC (section 2.4.1.1). The enzyme system was considered active if aflatoxin was produced.

2.8. PREPARATION OF CELL FREE SYSTEMS

2.8.1 Protoplasts

Four different digestive enzyme preparations were used to obtain protoplasts from 5 day old mycelium of *A. parasiticus* 1-11-105Wh1.

The liquid media of the *Trichoderma viride* cultures in shake flasks and the fermenter, as well as the media from the *Oerskovia xanthineolytica* cultures were lyophilized, and 100 mg each of the lyophilized enzymes were dissolved in phosphate buffer (0.1M, pH 7) (500 ml). These solutions were used as a digestive enzyme system, separately and in a mixture.

Chitinase (*Streptomyces griseus*) (Sigma), pronase (*Streptomyces griseus*) (Sigma) and lysing enzyme (*Rhizoctonia solani*) (Sigma) (50 mg each) were dissolved in phosphate buffer (0.1M, pH 7, 500 ml) and used as a commercial digestive enzyme system.

The digestive enzyme (50 ml) was incubated with whole mycelium (20 g wet weight, 5 day old) in a flask (250 ml) at 28°C on a rotary shaker (100 rpm) for 3 hours in phosphate buffer (50 ml, 0.1M, pH 5.8, containing 0.4 M magnesium sulphate). The magnesium salt was used as a protoplast stabiliser. The resulting slurry was filtered through glass wool. The filtrate containing the protoplasts was then centrifuged (500xg for 10 min). Sedimented protoplasts were washed with buffer-stabilizer and recentrifuged (500xg for 10 min). The clean protoplasts were utilised for conversion

min). The clean protoplasts were utilised for conversion studies. The protoplasts were lysed by rapidly freezing with liquid nitrogen and then allowing the solid to thaw. The mixture was centrifuged (1000xg for 10 min) and the supernatant was used as a cell free system.

2.8.2. French Press

Whole mycelium (A. parasiticus 1-11-105 Wh1, 5 day old) was passed through a french press (Apex, London) (20000 psi). The resulting slurry was passed through the press for the second time with the pore size reduced (pore size not calibrated). The slurry was centrifuged (1000xg for 10 min) and the supernatant was used as the cell free preparation.

2.8.3 Grinding

Whole mycelium (A. parasiticus 1-11-105Wh1, 5 day old) was mixed with acid-washed sand and then ground with a pestle and mortar. The mixture was suspended in phosphate buffer (0.1M, pH7, 5°C) and centrifuged (1000xg for 10 min). The supernatant was used as the cell free preparation.

2.8.4 Lyophilization

Whole mycelium (A. parasiticus 1-11-105 Wh1, 5 day old) was washed several times with distilled water (20°C) and then freeze-dried. The dried mycelia were powdered using a dry mortar and pestle. The powder (5 g) was suspended in cold phosphate buffer (10 ml, 0.1M, pH 7, 5°C) and centrifuged (10000xg for 20 min). The supernatant was used as the cell free preparation.

2.9. ENZYME PURIFICATION

2.9.1. Ultrafiltration

The supernatant obtained from the lyophilization preparation (section 2.8.4) (200 ml, 50 mg protein/ml) was ultrafiltered (Amicon Model 202, 47 mm diameter), using an Amicon PM10 membrane (10000 M.Wt. cut off). The filtration was carried out at 25 psi of nitrogen and at 5°C until the supernatant was reduced to 10% of its original volume.

2.9.2. Ultracentrifugation

The supernatant obtained from the lyophilization preparation (section 2.8.4) (50 mg protein/ml) was centrifuged under the following conditions:

RELATIVE G FORCE	TIME (HOURS)
10000 xg	.5
40000 xg	1
105000 xg	2

The various supernatant fractions (10 ml) and pellets were tested for enzyme activity (section 2.7).

2.9.3 Gel Filtration

2.9.3.1 Enzyme Separation

Sephadex G-200 (Pharmacia Fine Chemicals) was used for the purification of the enzyme. The gel was soaked in phosphate buffer (0.2M, pH 7.5), heated in a boiling water bath for 1 hour and left for 24 hours at room temperature. A glass column (25x200 mm) was packed with the swollen gel and equilibrated at a flow rate of 25 ml/ hour with 500 ml of phosphate buffer before being used. The supernatant obtained from the lyophilization preparation (section 2.8.4) (300mg in 10 ml) was loaded on to the column, which was run at 4°C and at a flow rate of 18 ml/hour. Blue dextran (BDH) was used as a marker for void volume. An LKB Uvicord was used to monitor the protein content of the column eluant at 285nm. The eluant from the column was collected on a fraction collector (LKB Redirac). The fractions (1 ml) were pooled as shown below and analysed for enzyme activity (section 2.7) and protein concentration (section 2.5). The cell free system was also gel filtered through Sepharose 6B under identical conditions to the gel filtration with Sephadex G-200. A second gel filtration through Sephadex G-200 was carried out with a cell free system in phosphate buffer (0.1M, pH 7.5), treated with EDTA (final concentration 2mM) for 6 hours.

The fractions were split as follows:

FRACTION	ELUTION VOL. (ml)
1	0-20 (void volume)
2	21-38
3	38-91
4	92-120

Enzyme activity (section 2.7) was tested using the following combinations of the above fractions.

FRACTION	EXPERIMENT NUMBER					
	1	2	3	4	5	6
1	*	*	*	-	-	-
2	*	*	-	*	*	-
3	*	*	*	*	-	*
4	*	-	*	*	*	*

* fraction added to the incubation mixture

- fraction not added to the mixture.

Fraction 2 was concentrated, using ultrafiltration (10000 M.Wt. cut off), to 10 ml and

(i) Rechromatographed on the Sephadex G-200 column. The fractions were collected and analysed as indicated above

(ii) Ultracentrifuged at 105000xg for 2 hours. The supernatant (10 ml) and the pellet were tested for enzyme activity (section 2.7). The supernatant was reloaded on to the Sephadex G-200 column (25x200 mm) and the eluant collected.

Four fractions were collected:

FRACTION	ELUTION VOL. (ml)
2.1	21-38
2.2	39-71
2.3	72-91
2.4	92-120

Enzyme activity (section 2.7) was tested using the following combinations of the above fractions together with NADPH and fraction 4 from the first gel filtration as cofactors.

FRACTION	EXPERIMENT NUMBER					
	1	2	3	4	5	6
2.1	*	*	*	-	-	-
2.2	*	*	-	*	*	-
2.3	*	*	*	*	-	*
2.4	*	-	*	*	*	*

* Fractions added to incubation mixture

- Fractions not added to the incubation mixture

Reference to fraction 2, 2.2 and 4 in the rest of the text relates to the fractions obtained from gel filtration, on Sephadex G-200, of the untreated cell free system.

2.9.3.2 Column Calibration

The column was calibrated for molecular weight by eluting a mixture (5 ml) of 5 mg/ml each of

SAMPLE	M.Wt.
myoglobin	17000
ovalbumin	45000
bovine serum albumin	66000
phosphorylase B	97400

The M.Wt. of unknown proteins were calculated from a standard curve constructed from the elution volumes of samples with known M.Wt. A graph of elution volume against Log M.Wt was plotted. An LKB Uvicord was used to monitor the protein content of the column eluant at 285nm.

2.9.4 Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) and PAGE in the presence of sodium dodecyl sulphate (SDS) were carried out on the crude enzyme preparation (section 2.8.4) as well as fractions 2, 3 and 2.2 from gel filtration. Horizontal flat bed gels were used, according to the manufacture's instructions for use with the LKB 2117 Multiphor apparatus.

Reagents

1. Stock buffer

Phosphate buffer (0.1M,pH 7.1), 10 g SDS (BDH. Chemicals) in 5l distilled water.

2. Acrylamide solution

22.2 g acrylamide (BDH Chemicals), 0.6 g N,N'-methylene-bis-acrylamide (BIS) (BDH Chemicals) in 100 ml distilled water

3. Ammonium persulphate solution

150 mg Ammonium persulphate (BDH Chemicals) in 10 ml distilled water.

4. Electrode Buffer

1 part stock buffer plus 1 part distilled water

5. Bromophenol blue 0.25% (w/v)

25 mg Bromophenol blue (Merck) in 10 ml stock buffer.

6. Fixing solution

57 g Trichloroacetic acid (Merck), 17 g Sulphosalicylic acid (Merck), dissolved in a mixture of 150 ml methanol and 350 ml of distilled water.

7. Staining solution

1.25 g Coomassie blue (Sigma) dissolved in a mixture of 227 ml methanol, 227 ml distilled water and 46 ml of acetic acid.

8. Destaining solution

1.5l ethanol and 500 ml acetic acid was made up to 5l with distilled water.

9. Tris-glycine stock buffer

Glycine 75.1 g (Merck) and sodium azide 2.5 g were dissolved in 3l distilled water, titrated with Tris to pH 8.9 and made up to 5l.

10. N,N,N′,N′-tetramethylethylenediamine (TEMED)

The polyacrylamide gel (3.5% polyacrylamide) was made up by mixing the components in the proportions given below:

SOLUTION (AS DESCRIBED)	VOLUME (ml)
distilled water	14.9
stock buffer	33*
acrylamide solution	14.8
ammonium persulphate	3.2
TEMED	0.1

* For SDS-PAGE, solution 1 (phosphate stock buffer) was used and for PAGE, solution 9 (tris-glycine stock buffer) was used.

SDS-PAGE was conducted at:

temperature	5°C
current	80 mA (constant)
field strength	6 V/cm
time	1.5 hours

PAGE was conducted at:

temperature	5°C
current	50 mA (current)
field strength	15 V/cm
time	1.5 hours

After the run, the plate was fixed, stained with coomassie blue and then destained.

The SDS gel was calibrated for molecular weight by electrophoresis of cross-linked haemoglobin and haemocyanin (Sigma) under the same electrophoretic conditions. The M.Wt. of the protein bands in fraction 2.2 was calculated from a standard curve constructed from the migration of proteins of known molecular weights relative to bromophenol blue against Log M.Wt.

2.9.5 ELECTROFOCUSING

2.9.5.1. Analytical Electrofocusing

Analysis of the protein components in different preparations (crude cell free, fraction 2, 3 and 2.2 from gel filtration) was carried out using isoelectric focusing. The samples, originally in 0.1M phosphate buffer, were transferred to 0.01M phosphate buffer pH7 and then applied to Ampholine PAGplates, pH range 3.5-9.5 and focused, according to manufacture's instructions (LKB instructions, LKB Ampholine PAGplates for Analytical Electrofocusing on Polyacrylamide Gels). The experiments was run under the following conditions:

anode	1M H_3PO_4
cathode	1M NaOH
power	7.5 W
voltage	1500 V max.
current	50 mA start
time	1.5 hours
temperature	8°C

At 1.5 hours equilibrium was reached. The plate was fixed, stained and destained as in the case of electrophoresis (section 2.9.4).

2.9.5.2. Preparative Electrofocusing

Fraction 2.2 was subjected to preparative electrofocusing in a granulated gel using an LKB 2117 Multiphor apparatus in the pH range 3-10 in accordance with the manufacture's instructions. The gel bed (Sephadex IEF, Pharmacia Fine Chemicals) containing ampholytes (LKB Ampholine, pH 3-10, 2% m/v) was cast in the glass trough of the apparatus, and evaporated to the required consistancy. A section of the gel was removed and suspended in sample (3 ml dissolved in 0.01M phosphate buffer) and reapplied to the gel bed. Focusing was achieved at a constant power of 4W, at 8°C for 16 hours. A paper print of the gel was taken by placing a Whatman number 1 filter paper over the gel for 1 minutes. The paper was fixed, stained with comassie blue and then destained (section 2.9.4). The bed was then sectioned into 30 fractions and the enzyme activity of each fraction was assayed (section 2.7).

2.10. CONVERSION STUDIES

The following systems were used to study the conversion of sterigmatocystin to O-methylsterigmatocystin and aflatoxin:

(i) whole mycelia (section 2.2.2.1) (2 g wet wt. in 100 ml 0.1M phosphate buffer)

(ii) crude enzyme preparation (section 2.8.4) (500 mg. of protein in 10 ml of 0.1M buffer)

(iii) fraction 2 plus fraction 4 (section 2.9.3.1) (80 mg. of protein in 10 ml of 0.1M phosphate buffer),

(iv) fraction 2.2 and original fraction 4 from gel filtration (section 2.9.3.1) (3.5 mg. of protein in 10 ml of 0.1M phosphate buffer).

NADPH (10mM) was routinely added to the to all the incubation mixtures, except in the case of whole mycelial studies and where otherwise stated.

Controls, consisting of phosphate buffer(0.1M, pH 7) or boiled enzyme (5 min) inb place of test sample, were incubated similtaneously.

Phosphate buffer 0.1M and pH7 was used unless otherwise stated. Citrate buffer 0.1M was used for pH values of 3, 4 and 5. Fraction 4 from gel filtration was added to the partially purified and the purified enzyme systems as a source of cofactors unless otherwise stated.

The effect of the following parameters on the conversion of sterigmatocystin were studied:

(a) Age of mycelia - 3 to 7 days

(b) Temperature - 15°C to 40°C

(c) pH - 3.0 to 8.0

(d) Time course study - 20°C, pH 7 over 5 hours

(e) NADPH (10 mM)

(f) Cofactors (10mM)
- NAD, NADH, FAD, Fe^{++}, Fe^{+++},
- NAD + FAD + Fe^{++} + Fe^{+++},
- Fe^{++} + Fe^{+++}
- S-adenosylmethionone

(g) Ethionine (10mM)

These cofactors were used instead of fraction 4.

2.11. KINETIC STUDIES ON PURIFIED ENZYME

Sterigmatocystin (10µg) was incubated with different concentrations of enzymes (1, 2, 3, 5 and 10 mg in 8 ml reaction mixture) in the presence of Fe^{++} (10mM) and NADPH (10mM) for 1 hour at 20°C and pH 7.2. The products were extracted (section 2.3.1.) and quantified (section 2.4.2.2).

The purified enzyme system (fraction 2.2 section 2.9.3.1) (3.5 mg protein/ml) was incubated with different concentrations of sterigmatocystin (0.5, 0.6, 0.75, 1, 2 and 3 µg in 8 ml reaction mixture) in the presence of Fe^{++} (10mM) and NADPH (10mM), for 1 hour at 20°C, pH 7.2. The products were extracted (section 2.3.1) and quantified (section 2.4.2.2).

The stoichiometry of the reaction was measured by monitoring the conversion of NADPH (10µM) to NADP, amount of sterigmatocystin (10 µg) used and the amount of product formed in 1 hour by the purified enzyme system (10 mg protein in 10 ml reaction mixture). The amount of NADPH converted was monitored by measuring the decrease in absorbance at 340 nm on a Hitachi model 220 spectrophotometer.

The specificity of the enzyme was investigated with the use of the following substrates (Kindly supplied by Dr M.F. Dutton):

dihydrosterigmatocystin,

hydroxysterigmatocystin and

desmethoxysterigmatocystin.

These substrates were dissolved in dimethylformamide (10 µg in 10 µl) and used in place of sterigmatocystin in the standard assay (section 2.7) with fraction 2.2 (section 2.10.).

3 RESULTS

3.1. Analytical

3.1.1. Thin Layer Chromatography and Mass Spectrometry

Sterigmatocystin and its conversion products (product 1 and 2) from the different conversion studies (section 2.10) were identified by TLC and mass spectroscopy (Tables 2 and 3)

Products 1 and 2 had identical R_F values to aflatoxin B_1 and O-methylsterigmatocystin respectively. They were therefore tentatively identified as such. On spraying the plates with 20% ethanolic aluminium chloride and then heating them, the standard and product 2 gave a characteristic yellow-green fluoresence thus supporting the suggested identification of product 2 as O-methylsterigmatocystin. The identity of products 1 and 2 was confirmed as aflatoxin B_1 and O-methylsterigmatocystin respectively, by comparing the mass spectra with spectral data in the literature (130). Small quantites precluded confirmation by other physical measurements.

Sterigmatocystin, aflatoxins B_1, B_2, G_1 and G_2 and O-methylsterigmatocystin were separated and quantified by HPLC (Tables 4 and 5)

TABLE 2

R_F values of sterigmatocystin (St) aflatoxin B_1 (AFB_1) and O-Methylsterigmatocystin (OMS), standards in different solvent systems.

Solvent System	R_F of Metabolites[a] AFB_1	OMS	St
chloroform:acetone 9:1(v/v)	0.52±.03	0.51±03	0.95±.04
toluene:ethyl acetate: acetone 60:25:15 (v/v/v)	0.36±.02	0.27±.01	0.9±.05

[a] Results are mean values for five observations

TABLE 3

Mass spectroscopy data of the conversion products (1 and 2) of sterigmatocystin..

Product 1 m/e	Relative Intensity	Product 2 m/e	Relative Intensity
312	100	338	100
284	28	323	35
269	16	309	47
256	25	292	18
241	22	279	13
227	30	265	15
199	12	249	20

TABLE 4

Retention times of sterigmatocystin (St) aflatoxin B_1 (AFB_1) and O-Methylsterigmatocystin (OMS), standards separated by HPLC using acetonitrile:water (55:45) solvent mixture.

Metabolite	Retention times (min.)[a]
St	3.74±.03
OMS	2.84±.03
AFB_1	2.12±.02

[a] Results are mean values for five observations

TABLE 5

Retention times of aflatoxins B_1, B_2, G_1 and G_2 standards, separated by HPLC using acetonitrile:methanol:water (3:2:5) solvent mixture.

Aflatoxin	Retention time (min)[a]
G_2	2.59±.03
G_1	2.77±.02
B_2	3.03±.02
B_1	3.28±.04

[a] Results are mean values for five observations

3.2. ENZYME PURIFICATION

3.2.1. Cell Free System

The effectiveness of different digestive enzymes in producing protoplasts from *A. parasiticus* mycelium was investigated. The results are presented in Tables 6 and 7.

The commercial enzyme was found to be the most effective in producing protoplasts (Table 6). Four day old mycelium was least resistant to digestive enzyme action and produced the most number of protoplasts (Table 7), but 5 day old mycelium was used to prepare a cell free system as it had the highest sterigmatocystin to aflatoxin B_1 biosynthetic activity (Table 21).

TABLE 6

Release of protoplasts from 5 day old *A. parasiticus* mycelium[a] by different diggestive enzymes at 25°C, pH 6 and over 3 hours[b].

Enzyme System	No. of Protoplasts/ml
T. viride (flask)	$(8.0\pm.3)\times10^6$
T. viride (fermentor)	$(1.3\pm.2)\times10^7$
O. xanthylitica	$(6.5\pm.25)\times10^6$
T. viride plus *O. xanthylitica*	$(2.0\pm.1)\times10^7$
commercial enzyme	$(2.0\pm.15)\times10^8$

[a] 20g wet weight in 100 ml reaction mixture

[b] Results are mean values for five observations

TABLE 7

The resistance of different ages of mycelium[a] to a digestive enzyme (produced by *T. viride*) at 25°C, pH 6 and over 3 hours[b].

Age (days)	No. of protoplasts/ml
3	$(5.6\pm.25)\times10^6$
4	$(2.0\pm.11)\times10^7$
5	$(8.9\pm.31)\times10^6$

[a] 20 g wet wt in 100 ml reaction mixture

[b] Results are mean values for five observations

Four different methods (lysed protoplast, french press, grinding and lyophilization) were used to prepare cell free systems (section 2.8) and the effectiveness of the different systems in converting sterigmatocystin to aflatoxin was investigated.

Lyophilization proved to be the most efficient method in producing a cell free system that could convert sterigmatocystin to aflatoxin (Table 8).

TABLE 8

The conversion of sterigmatocystin[a] to aflatoxin B_1 (AFB_1) and O-methylsterigmatocystin (OMS) by four cell free systems[b] produced by different methods.[c]

Method of Producing The System	Metabolite Produced (µg) AFB_1	OMS	Enzyme activity (µg aflatoxin B_1/ g of protein)
Lysed protoplasts	4.0±.4	1.1±.2	8.0 ±.8
French press	0.0	1.5±.3	0.0
Grinding	0.0	0.0	0.0
Lyophilization	8.2±.1	1.7±.1	16.4±.2

[a] 10 µg in 10 ml reaction mixture

[b] 500 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

3.2.2. Partially Purified Enzyme System

Three methods were used to purify the crude cell free systems obtained by lyophilization (enzyme activity 16.4 µg aflatoxin/g of protein, Table 8). They were ultrafiltration, ultracentrifugation and gel filtration. The results are summarised in Tables 9, 10, 13, 14, and 15.

3.2.2.1 Ultrafiltration

Ultrafiltration was carried out on the cell free system (section 2.9.1.) (Table 9).

Ultrafiltration resulted in no increase in enzyme activity but was useful as a concentration step.

TABLE 9

Conversion of sterigmatocystin[a] to aflatoxin B_1 (AFB_1) and O-methylsterigmatocystin (OMS) by retentate[b] and filtrate[c] at 25°C, pH 7 and over 5 Hours[d]

Fraction	Metabolite Produced (µg) AFB_1	OMS	Enzyme activity (µg aflatoxin B_1 / g of protein)
Retentate	7.9±.1	1.7±.1	15.8±.2
Filtrate	0.0	0.0	
Both fractions combined	7.8±.1	1.7±.1	14.1±.2

[a] 10 µg in 10 ml reaction mixture

[b] 500 mg protein in 10 ml reaction mixture

[c] 50 mg protein in 10 ml reaction mixture

[d] Results are mean values for five observations

3.2.2.2. Ultracentrifugation

Ultracentrifugation at different relative centrifugal forces was carried out on the cell free system (section 2.9.2.) (Table 10).

Ultracentrifugation results suggest that the aflatoxin B_1 and O-methylsterigmatocystin synthesising enzymes are located in the cytosol.

TABLE 10

Conversion of sterigmatocystin[a] to aflatoxin B_1 (AFB_1) and O-methylsterigmatocystin (OMS) by the supernatant and pellet[b] after centrifugation of the crude cell free preparation at different relative centrifugal forces (RCF) at 25°C, pH 7 and over 5 hours[c]

RCF AND Derived Fractions	Metabolite Produced (µg) AFB_1	OMS	Enzyme activity (µg aflatoxin B_1/ g of protein)
10000xg[d]			
Supernatant	8.2±.1	1.6±.1	23.5±.3
Pellet	0.5±.2	0.5±.1	3.6±.5
40000xg[e]			
Supernatant	8.0±.1	1.5±.1	22.9±.3
Pellet	0.3±.2	0.9±.1	2.1±.5
105000xg[f]			
Supernatant	7.3±.1	1.4±.1	20.5±.3
Pellet	0.4±.1	1.3±.1	2.9±.5

[a] 10 µg in 10 ml reaction mixture

[b] 350 and 150 mg protein respectively in 10 ml reaction mixture

[c] Results are mean values for five observations

[d] Centrifuged for 0.5 hours

[e] Centrifuged for 1 hour

[f] Centrifuged for 2 hours

3.2.2.3. Gel filtration

Gel filtration on Sephadex G-200 was carried out, on the crude cell free system and cell free system treated with EDTA. Gel filtration of the cell free system was also carried out on Sepharose 6B (section 2.9.3).

Results from gel filtration of cell free system on Sephadex G-200 suggest that the enzymes had a molecular weight >350000 since it was eluted in the void volume (Table 13 and Fig. 21). The cell free system was also gel filtered using Sepharose 6B and these results suggest that the active fraction had a molecular weight $>3 \times 10^{6}$ (Table 15 and Fig. 23).

To check the possibility that fraction 2 was a membrane fraction a marker enzyme for endoplasmic reticulum, glucose-6-phosphatase was used. A positive test indicated that fraction 2 was a membrane fraction. To investigate the possibility that the enzyme was membrane associated, the cell free system was subjected to treatment with buffered EDTA which is responsible for removing peripheral proteins from membranes. This treatment resulted in the active fraction being shifted from fraction 2 to 3 (Table 14). The elution profile was also altered by treating the cell free system with EDTA (Fig. 22).

TABLE 11

Elution volumes of fractions obtained from gel filtration of a cell free system[a] by Sephadex G-200[b].

Fraction	Elution vol. (ml)
1	1-20
2	21-38
3	39-91
4	92-120

[a] Two cell free systems were used, one treated with buffered EDTA and one not treated with EDTA

[b] Results are mean values for five observations.

TABLE 12

Elution volumes of fractions obtained from gel filtration of a cell free system on Sepharose 6B.

Fraction	Elution vol. (ml)
1	0-21
2	21-38
3	39-91
4	92-120

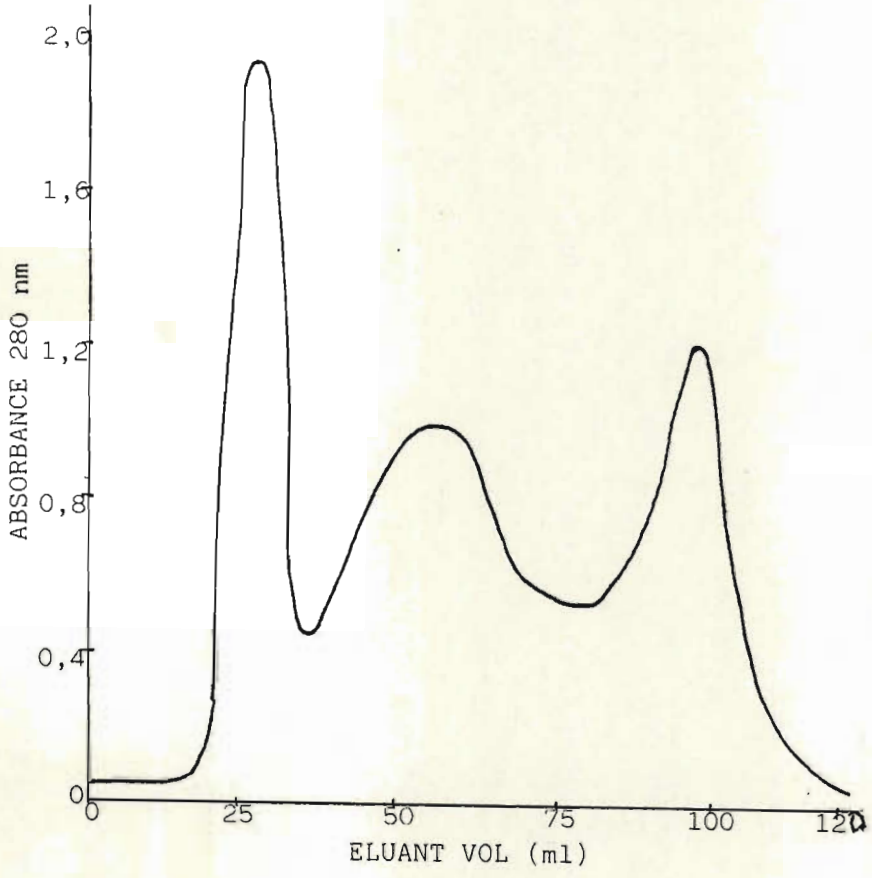


Figure 21: Protein elution profile of cell free preparation when separated on Sephadex G-200.

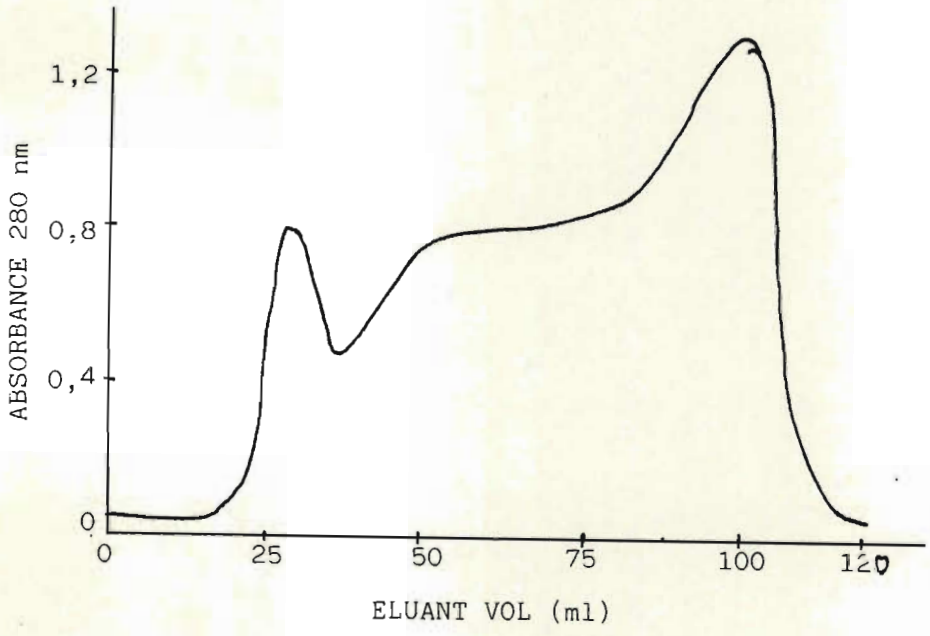


Figure 22: Elution profile obtained from gel filtration, of a cell free system pretreated with EDTA, on a Sephadex G-200 column.

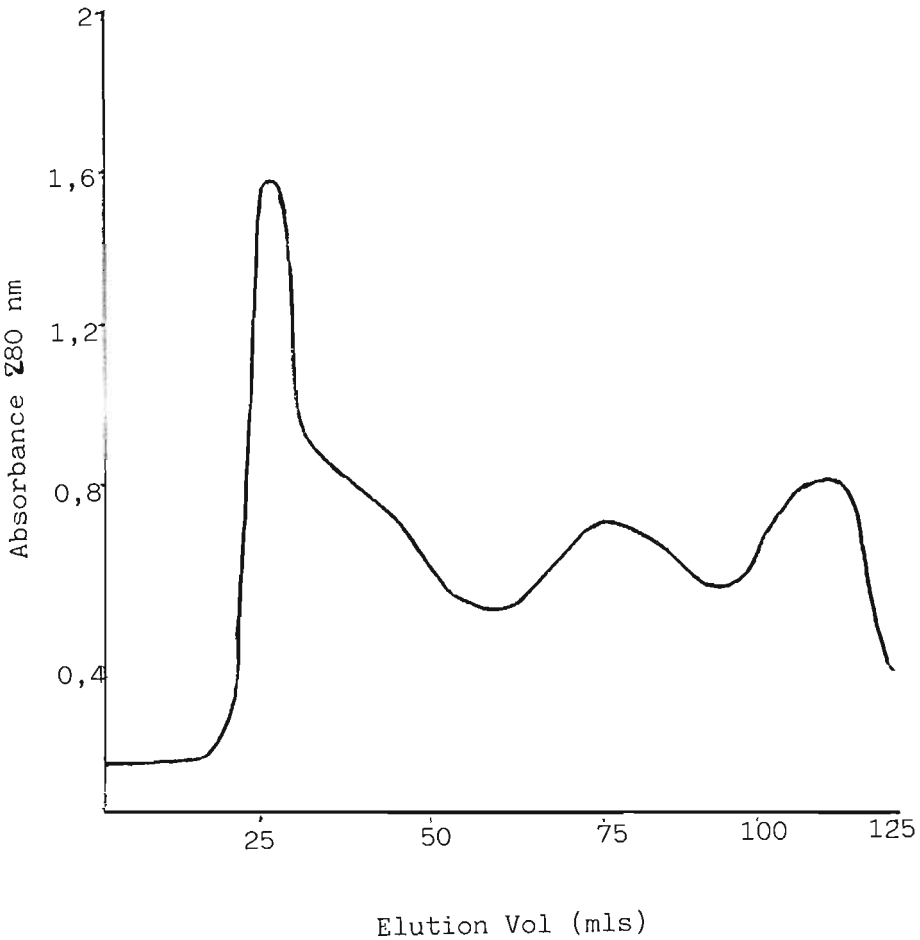


Figure 23: Protein elution profile of cell free preparation when separated on Sepharose 6B.

TABLE 13

Conversion of sterigmatocystin[a] to aflatoxin B_1 (AFB_1) and O-methylsterigmatocystin (OMS) by four fractions[b] obtained from gel filtration of the cell free system on a Sephadex G-200 column[c]

Fractions.	Metabolite Produced (µg) AFB_1	OMS	Enzyme activity (µg aflatoxin B_1 / g of protein)
1+2+3+4	8.1±.1	1.7±.1	27.0±.3
1+2+3	0.0	0.0	
1+3+4	0.0	1.7±.1	
2+3+4	8.1±.1	1.7±.1	27.0±.3
3+4	0.0	1.8±.1	
2+4	7.9±.1	0.0	98.8±1.5

[a] 10 µg in 10 ml reaction mixture

[b] Fractions 2 and 3 were protein fractions with 80 mg and 220 mg of total protein respectively. Fractions 1 and 4 had no protein. The volume of the reaction mixture was 10 ml

[c] Results are mean values for five observations

TABLE 14

Conversion of sterigmatocystin[a] to aflatoxin B_1 by four fractions[b] obtained after the cell free system was treated with EDTA and then separated by gel filtration on Sephadex G-200 column [c].

Fractions.	Aflatoxin B_1 Produced (µg)	Enzyme activity (µg aflatoxin B_1 / g of protein)
1+2+3+4	7.3±.2	24.3±.5
1+2+3	0.0	
1+3+4	7.5±.1	31.3±.3
2+3+4	7.2±.3	24.0±.1
2+4	0.0	
3+4	7.1±.1	29.5±.3

[a] 10µg in 10 ml reaction mixture

[b] Fractions 2 and 3 were protein fractions with 60 & 240 mg of total protein respectively. Fraction 4 had no protein. The volume of the reaction mixture was 10 ml.

[c] Results are mean values for five observations.

TABLE 15

Conversion of sterigmatocystin[a] to aflatoxin B_1 (AFB_1) and o-methylsterigmatocystin (OMS) by four fractions[b] obtained from gel filtration of the cell free system on a Sepharose 6B column[c]

Fractions.	Metabolite Produced (µg) AFB_1	OMS	Enzyme activity (µg aflatoxin B_1 / g of protein)
1+2+3+4	7.9±.1	1.5±.1	26.3±.3
1+2+3	0.0	0.0	
1+3+4	0.0	1.7±.1	
2+3+4	8.2±.1	1.4±.1	27.3±.3
3+4	0.0	1.8±.1	
2+4	8.0±.1	0.0	160.1±1.5

[a] 10 µg in 10 ml reaction mixture

[b] Fractions 2, 3 and 4 were protein fractions with 50 mg and 150 mg 100 mg of total protein respectively. Fractions 1 and 4 had no protein. The volume of the reaction mixture was 10 ml

[c] Results are mean values for five observations

3.2.3. Purified enzyme system

3.2.3.1. Purification of fraction 2

The partially purified enzyme (fraction 2) was subjected ultrafiltration followed by ultracentrifugation. The supernatant was loaded on to a Sephadex G-200 column (section 2.9.4.). The results are summarised in Tables 38, 39, 40 and Fig 35.

Fraction 2, from gel filtration of cell free system not treated with EDTA, was centrifuged (105000xg for 2 hours) and the active fraction was found to be present in the supernatant (Table 16). The pellet gave a positive test for lipids. The elution profile of the supernatant after gel filtration was different from that of fraction 2 and the active fraction was located in fraction 2.2 (elution volume 39-71) (Tables 17 and 18 and Fig.24). From the elution volume and comparison with a calibration curve (section 2.9.3.2.), the molecular weight was estimated as 105,000.

TABLE 16

Conversion of Sterigmatocystin[a] to Aflatoxin B_1 by Supernatant[b] and Pellet[c,d], after centrifugation of fraction 2, at 25°C, pH 7 and over 5 Hours[e]

Fraction	Aflatoxin B_1 Produced (µg)	Enzyme activity (µg aflatoxin B_1 / g of protein)
Supernatant	4.2±.3	247.6±17.6
Pellet	0.0	0.0
Both fractions	7.89±.1	102.4±5.8

[a] 10µg in 10 ml reaction mixture

[b] 17 mg protein in 10 ml reaction mixture

[c] 60 mg protein in 10 ml reaction mixture

[d] fraction 2 from gel filtration was ultracentrifuged at 105000xg for two hours

[e] Results are mean values for five observations

TABLE 17

Elution volumes of fractions obtained after fraction 2 was ultracentrifuged[a] and then separated by gel filtration on a Sephadex G-200 column[b]

Fraction	Elution vol. (ml)
2.1	21-38
2.2	39-71
2.3	72-91
2.4	92-120

[a] Ultracentrifuged at 105000 xg for 2 hours

[a] Results are mean values for five observations

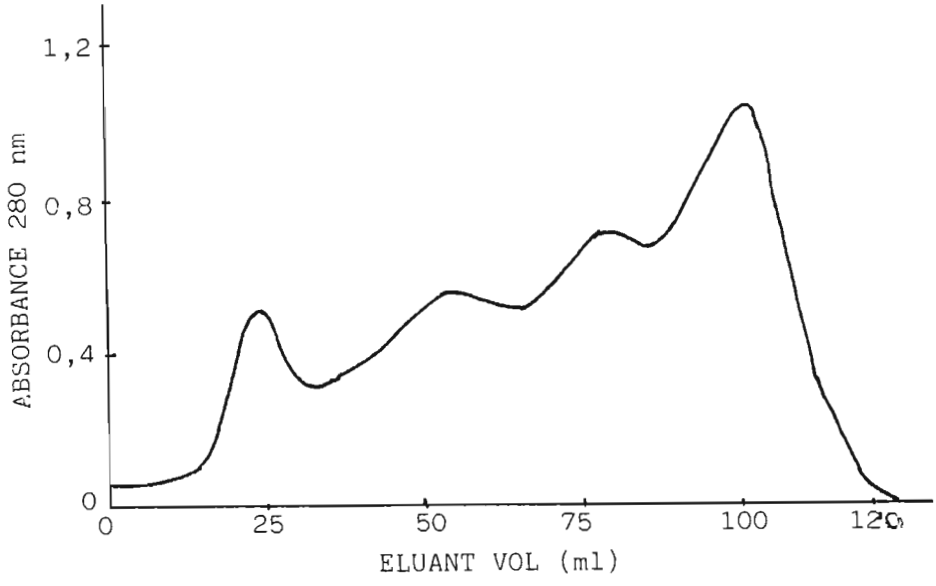


Figure 24: Elution profile of fractions obtained after fraction 2 was ultracentrifuged (105000xg for 2 hours) and then separated by gel filtration on a Sephadex G-200 column

TABLE 18

Conversion[a] of sterigmatocystin[b] to aflatoxin B_1 by four fractions[c] obtained from gel filtration[d] on Sephadex G-200 column[e]

Fractions.	Aflatoxin B_1 Produced (µg)	Enzyme activity (µg aflatoxin B_1 / g of protein)
2.1+2.2+2.3+2.4	4.21±.22	246.1±12.9
2.1+2.2+2.3	4.24±.22	247.9±12.9
2.1+2.3+2.4	0.0	
2.2+2.3+2.4	4.13±.1	458.8±12.5
2.3+2.4	0.0	
2.2+2.4	4.31±.08	1231.4±20.4

[a] No activity was recorded unless fraction 4 of the original gel filtration was added

[b] 10µg in 10 ml reaction mixture

[c] Fractions 2.1, 2.2 and 2.3 were protein fractions with 8.1, 3.5 and 5.5mg of total protein respectively. Fraction 2.4 had no protein. The volume of the reaction mixture was 10 ml

[d] Fraction 2 was ultrafiltered, ultracentrifuged (105000 xg for 2 hours) and the supernatant was gel filtered

[e] Results are mean values for five observations

3.2.3.2 Rechromatography

Fraction 2 from the original gel filtration of the cell free system was ultrafiltered and rechromatographed (section 2.9.3) (Table 19 and Fig. 25).

The active peak remained at fraction 2.

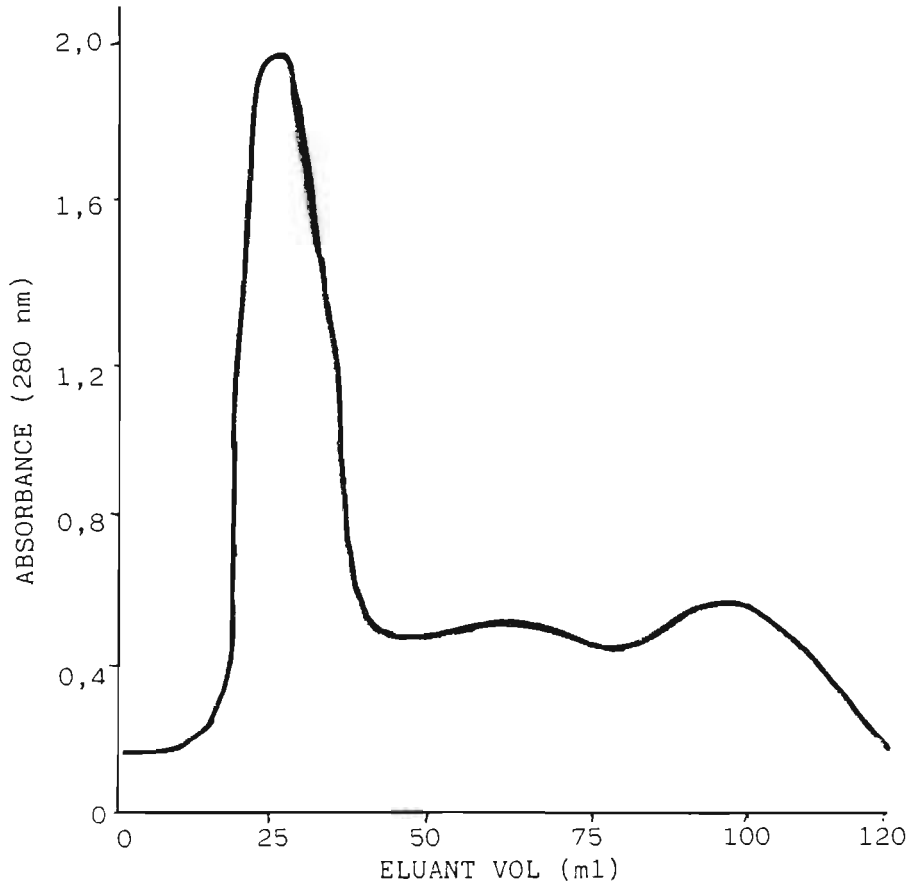


Figure 25: Protein elution profile of fraction 2, from gel filtration rechromatographed on Sephadex G-200.

TABLE 19

Conversion[a] of sterigmatocystin[b] to aflatoxin B_1 (AFB_1) by four fractions[c] obtained from gel filtration of fraction 2 rechromatographed on a Sephadex G-200 column[d]

Fractions.	Aflatoxin B_1 Produced (µg)	Enzyme activity (µg aflatoxin B_1 / g of protein)
1+2+3+4	8.0±.1	100.0±1.25
1+2+3	0.0	
1+3+4	0.0	
2+3+4	8.1±.1	101.2±1.25
3+4	0.0	
2+4	7.8±.1	104.0±1.5

[a] No activity was recorded unless fraction 4 of the original filtration was added

[b] 10 µg in 10 ml reaction mixture

[c] Fractions 2 and 3 were protein fractions with 75 mg and 5 mg of total protein respectively. Fractions 1 and 4 had no protein. The volume of the reaction mixture was 10 ml

[d] Results are mean values for five observations

3.2.4. Enzyme Purity

The purification of the enzyme responsible for the conversion of sterigmatocystin to aflatoxin B_1 was followed by isoelectric focusing. The results are shown in Fig. 26

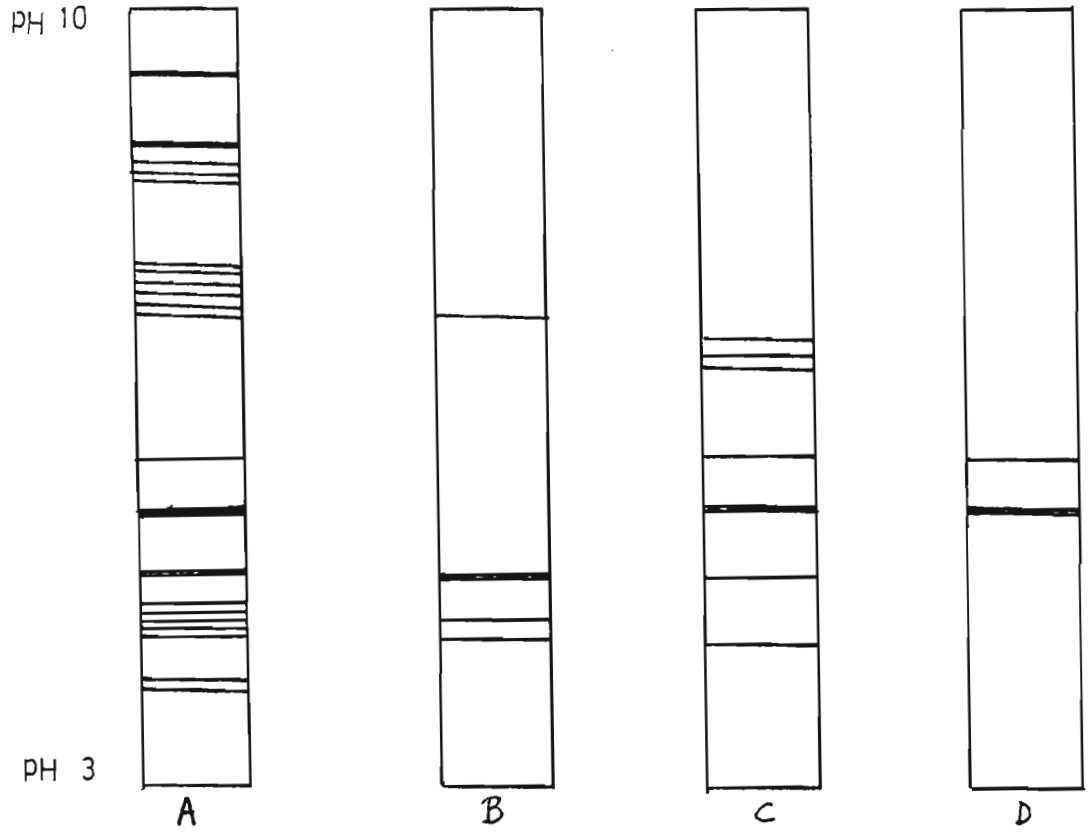


Figure 26: Isoelectric focusing of the aflatoxin B_1-synthesising enzyme(s) during purification (anode at the bottom). A, crude cell free system; B, fraction 2 from gel filtration on a Sephadex G-200 column; C, supernatant after centrifugation of fraction 2 at 105000xg; D, fraction 2.2 from gel filtration on Sephadex G-200.

The two proteins from fraction 2.2 were separated by preparative isoelectric focusing and had no detectable (HPLC) independent activity with respect to converting sterigmatocystin to aflatoxin B_1.

A summary of the increase in enzyme activity with purification is given in the table below.

TABLE 20

Purification of the enzyme responsible for the conversion of sterigmatocystin to aflatoxin B_1.

Fraction	Total Protein (mg)	Specific Activity (µg/g)[a]	Increase in purification
Cell Free Extract	500	16.3	-
Fraction 2	80	98.8	6.0
Fraction 2.2	3.5	1231	75

[a] µg aflatoxin B_1 formed from 10µg sterigmatocystin/g protein.

The final enzyme system was purified 75 fold.

The purity and physicochemical properities of fraction 2.2 was determined by the use of 3 different techniques; PAGE, SDS-PAGE and isoelectric focusing.

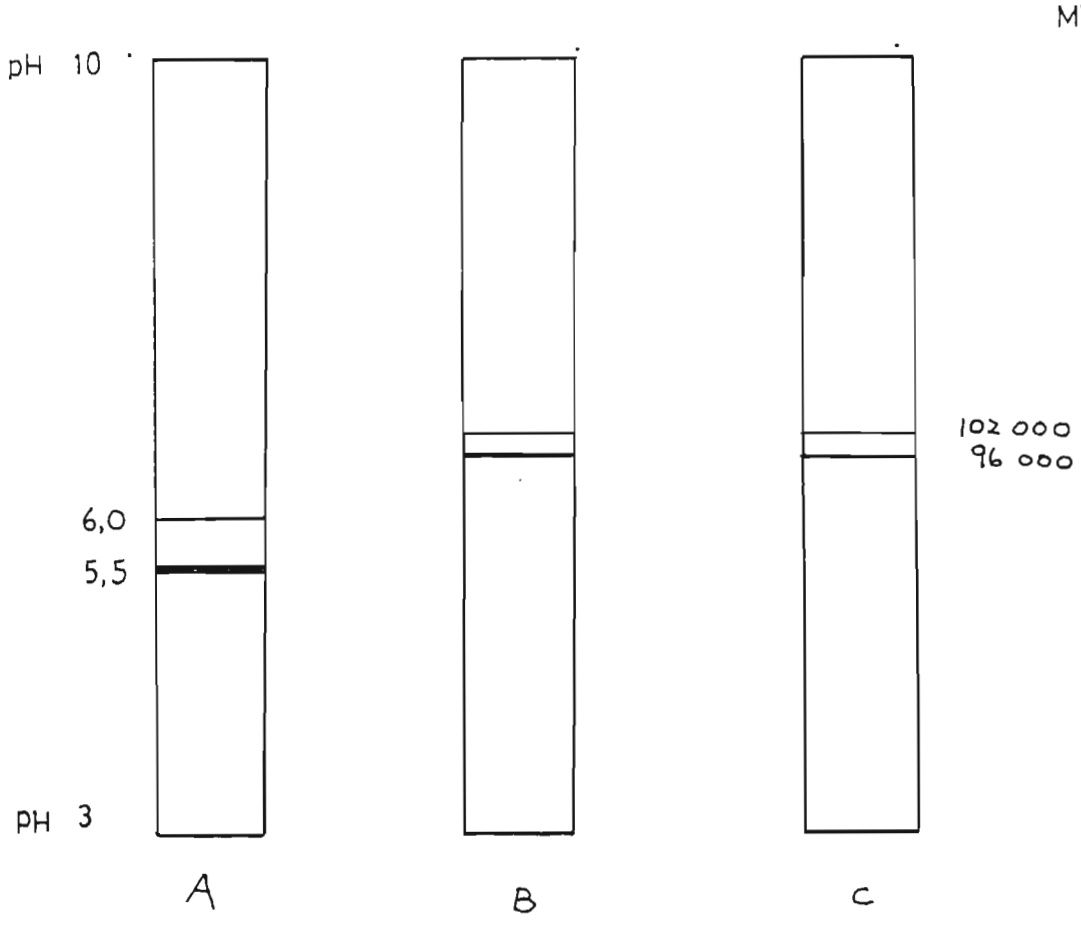


Figure 27: Protein patterns of fraction 2.2 (anode at bottom). A, Isoelectric focusing; B, PAGE ; C, SDS-PAGE ;

3.3. CONVERSION STUDIES

3.3.1. The Effect of Mycelial Age

The effect of age of mycelia on the conversion of sterigmatocystin into aflatoxin B_1 and O-methyl-sterigmatocystin was determined (section 2.10) (Table 21 and Fig 28).

The complete conversion of sterigmatocystin to aflatoxin B_1 and O-methylsterigmatocystin was achieved by 5 day old mycelium.

TABLE 21

Conversion of sterigmatocystin[a] to aflatoxin B_1 (AFB_1) and O-methylsterigmatocystin (OMS) by whole A. parasiticus Wh1 mycelium[b] of different ages at 25°C, pH 7 and for 5 hours.

Age (days)	Metabolite Produced(µg)[c]	
	AFB_1	OMS
3	5.2±.2	1.2±.1
4	6.4±.2	1.4±.1
5	8.2±.2	1.7±.2
6	7.4±.1	1.5±.1
7	6.0±.1	1.4±.1

[a] 10 µg in 100 ml reaction mixture

[b] 2 g wet wt in 100 ml reaction mixture

[c] Results are mean values for five observations

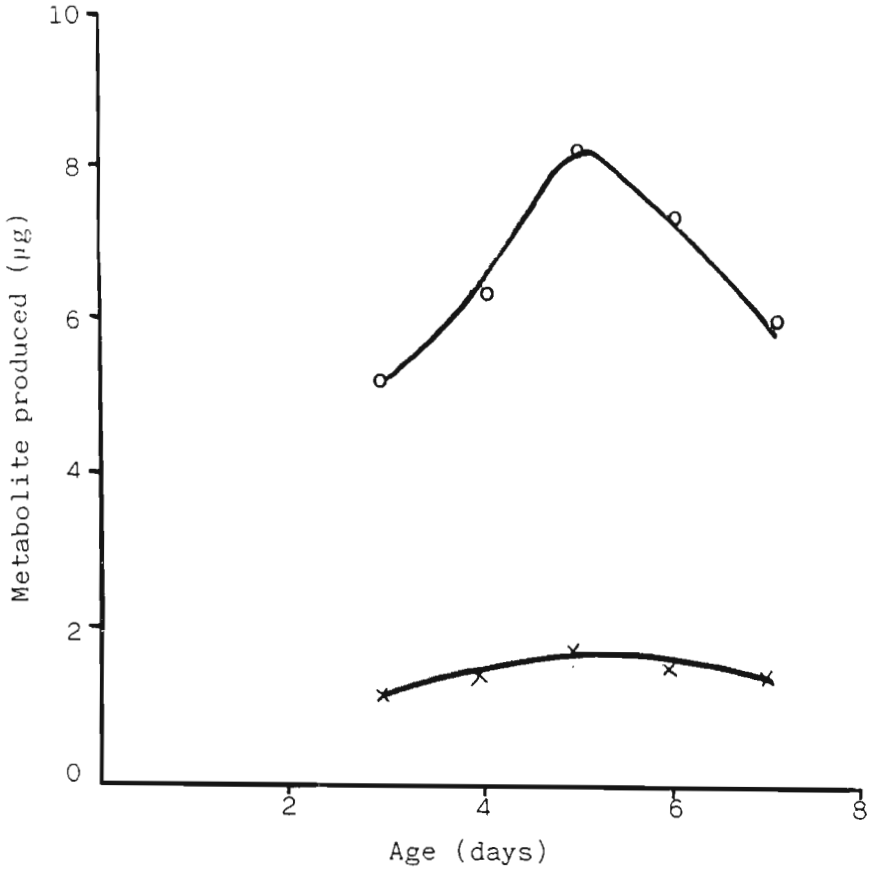


Figure 28: Conversion of sterigmatocystin[a] to aflatoxin B_1 (0) and O-methylsterigmatocystin (x) by whole *A. parasiticus* Wh1 mycelium[b] of different ages at 25°C, pH 7 and for 5 hours[c].

[a] 10 µg in 100 ml reaction mixture

[b] 2 g wet wt in 100 ml reaction mixture

[c] Results are mean values for five observations

3.3.2. The Effect of Temperature

The temperature profiles for the conversion of sterigmatocystin into aflatoxin B_1 and O-methylsterigmatocystin by whole mycelium, crude cell free system, partially purified enzyme and purified enzyme system were determined at pH 7 and over 5 hours (section 2.10) (Tables 22-25 and Figs 29-32), and the optimum temperature was found to be:

	AFB_1	OMS
whole mycelia	25°C	30°C
crude cell free system	25°C	30°C
partially purified enzyme	20°C	-
purified enzyme system	20°C	-

It was noted that the optimum temperature decreased as the enzyme was purified and that O-methylsterigmatocystin was not produced by the latter two systems.

TABLE 22

Conversion of sterigmatocystin[a] to aflatoxin B_1 (AFB_1) and O-methylsterigmatocystin (OMS) by whole 5 day old *A. parasiticus* mycelium[b] at pH 7, over 5 hours and at different temperatures[c]

Temperature (°C)	Metabolite Produced (µg)	
	AFB_1	OMS
5	4.0±.1	0.0
10	4.9±.1	0.0
15	6.2±.1	0.8±.1
20	7.0±.1	1.1±.1
25	8.1±.2	1.8±.1
30	7.8±.2	2.1±.2
40	2.7±.2	2.1±.1

[a] 10 µg in 100 ml reaction mixture

[b] 2 g wet wt in 100 ml reaction mixture

[c] Results are mean values for five observations

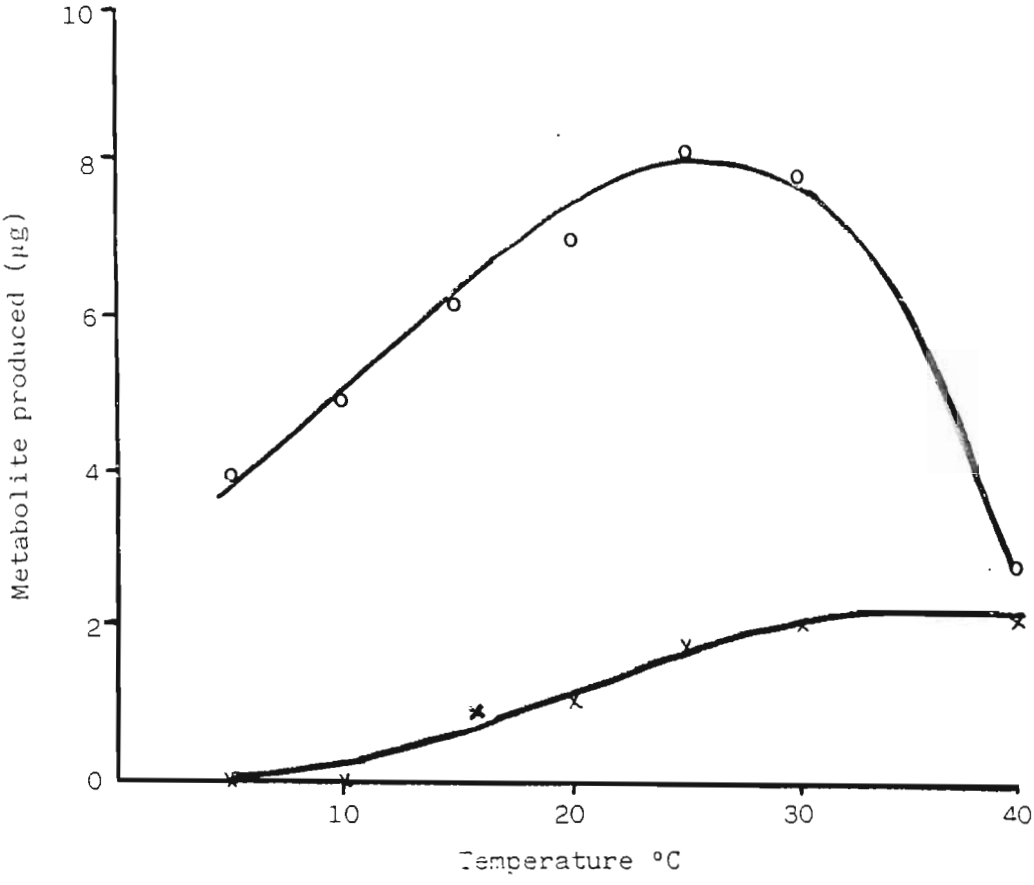


Figure 29: Conversion of sterigmatocystin[a] to aflatoxin B_1 (o) and O-methylsterigmatocystin (x) by whole 5 day old A. parasiticus mycelium[b] at pH 7, over 5 hours and at different temperatures[c]

[a] 10 µg in 100 ml reaction mixture

[b] 2 g wet wt in 100 ml reaction mixture

[c] Results are mean values for five observations

TABLE 23

Conversion of sterigmatocystin[a] to aflatoxin B_1 (AFB_1) and O-methylsterigmatocystin (OMS) by the cell free system[b] at pH7, over 5 hours and at different temperatures.

Temperature (°C)	Metabolite Produced (µg) AFB_1	OMS	Enzyme activity (µg aflatoxin B_1 / g of protein)
5	3.9±.2	0.0	7.8±.3
10	4.8±.1	0.0	9.6±.2
15	5.9±.2	0.3±.1	11.8±.3
20	6.5±.1	1.1±.1	13.0±.1
25	8.2±.2	1.8±.2	16.4±.3
30	7.0±.1	2.5±.4	14.0±.2
40	0.2±.1	2.2±.1	0.4 ±.2

[a] 10 µg in 10 ml reaction mixture

[b] 500 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

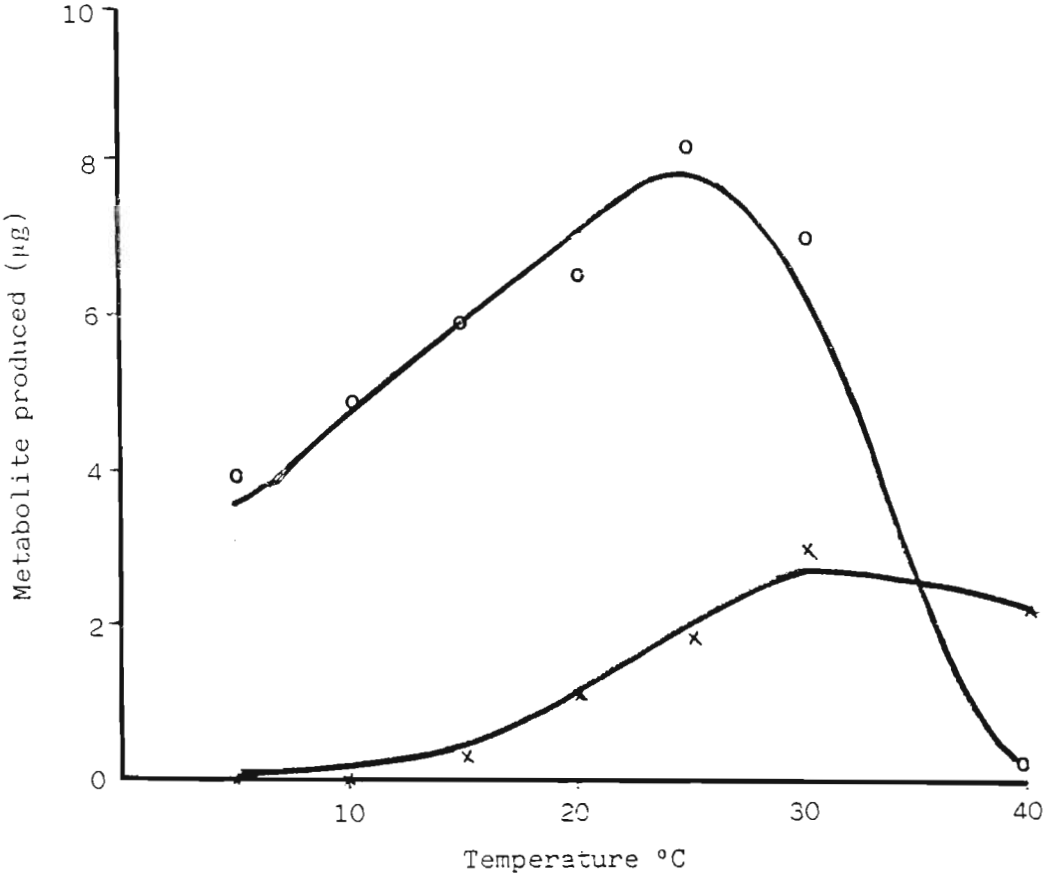


Figure 30: Conversion of sterigmatocystin[a] to aflatoxin B_1 (o) and O-methylsterigmatocystin (x) by the cell free system[b] at pH7, over 5 hours and at different temperatures[c].

[a] 10 µg in 10 ml reaction mixture

[b] 500 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

TABLE 24

Conversion of sterigmatocystin[a] to aflatoxin B_1 by partially purified enzyme[b] and fraction 4 from gel filtration at pH7, over 5 hours and at different Temperatures[c]

Temperature (°C)	Aflatoxin B_1 Produced (µg)	Enzyme activity (µg aflatoxin B_1 / g of protein)
5	3.3±.2	41.2±2
10	3.8±.4	47.5±4
15	5.3±.3	66.2±3
20	7.1±.2	88.5±2
25	6.8±.3	85.5±1
30	4.9±.2	61.2±3
40	0.0	0.0

[a] 10µg in 10 ml reaction mixture

[b] 80 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

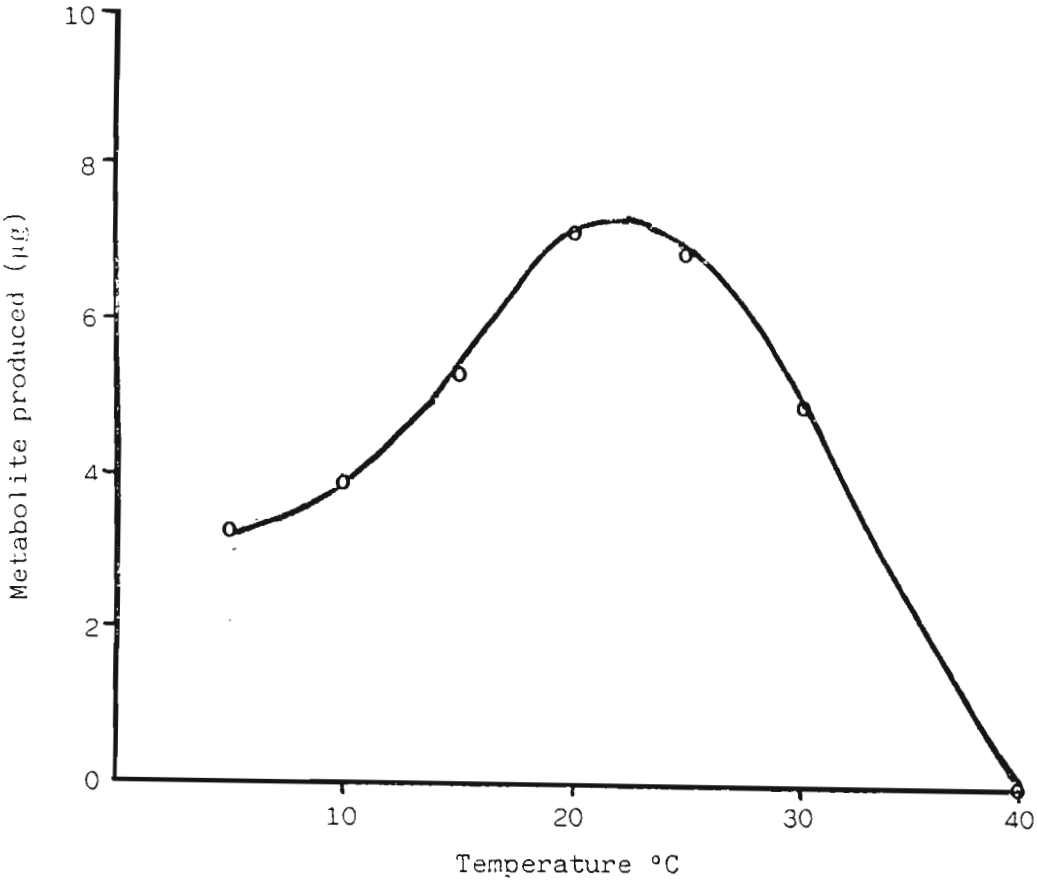


Figure 31: Conversion of sterigmatocystin[a] to aflatoxin B_1 by partially purified enzyme and fraction 4 from gel filtration[b] at pH 7, over 5 hours and at different Temperatures[c].

[a] 10 µg in 10 ml reaction mixture

[b] 80 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

TABLE 25

Conversion of sterigmatocystin[a] to aflatoxin B_1 by purified enzyme system and fraction 4 from gel filtration[b] at pH 7, over 5 hours and at different Temperatures[c]

Temperature (°C)	Aflatoxin B_1 Produced (µg)	Enzyme activity (µg aflatoxin B_1 / g of protein)
5	1.7±.2	485.7 ±62
10	2.4±.2	685.7 ±65
15	4.0±.2	1128.5±42
20	5.0±.4	1414.2±111
25	4.2±.2	1202.8±65
30	2.1±.2	600 ±48
40	0.0	0.0

[a] 10µg in 10 ml reaction mixture

[b] 3.5 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

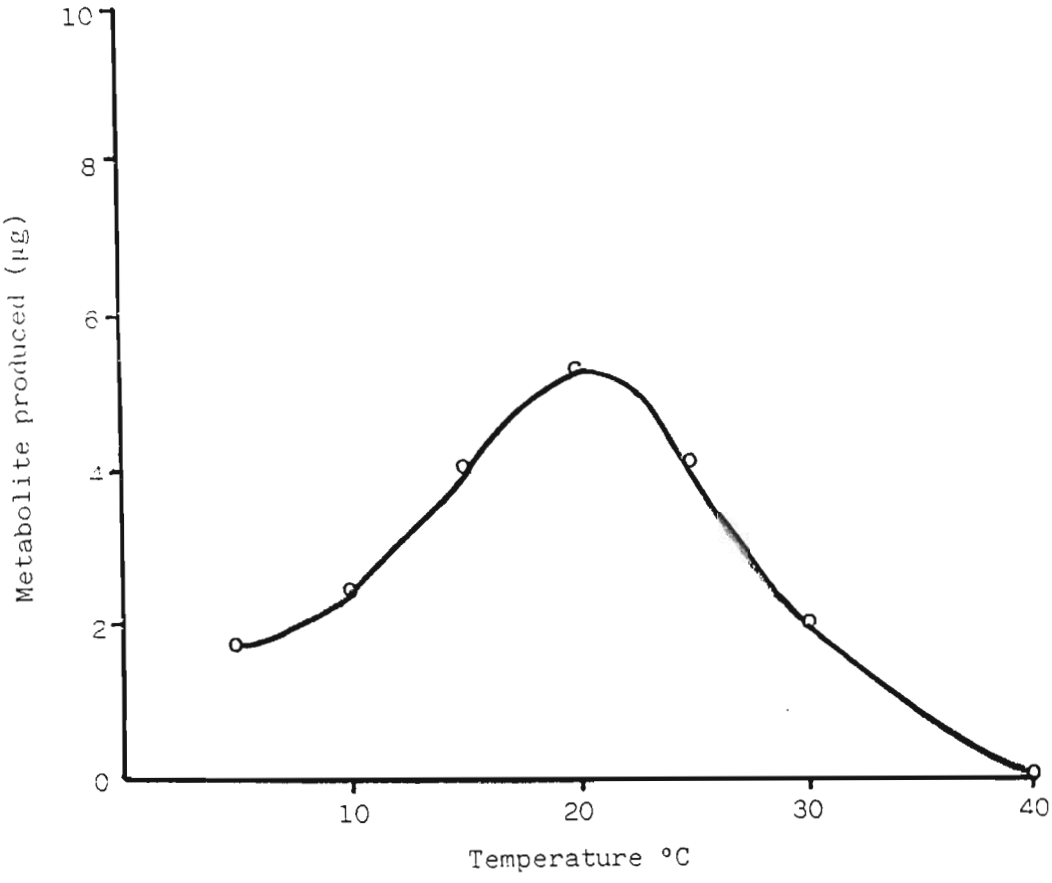


Figure 32: Conversion of sterigmatocystin[a] to aflatoxin B_1 by purified enzyme system[b] and fraction 4 from gel filtration at pH 7, over 5 hours and at different Temperatures[c]

[a] 10 µg in 10 ml reaction mixture

[b] 3.5 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

3.3.3. The Effect of pH

The pH profiles for the conversion of sterigmatocystin into aflatoxin B_1 and O-methylsterigmatocystin by whole mycelium, crude cell free system, partially purified enzyme and purified enzyme system were determined at 25°C and over 5 hours (section 2.10) (Tables 26-29 and Figs. 33-36). The optimum pH value was found to be:

	AFB_1	OMS
whole mycelia	7	7
crude cell free system	7	7
partially purified enzyme	7	-
purified enzyme system	7.2	-

The pH optimum for the different systems was fairly constant.

TABLE 26

Conversion of sterigmatocystin[a] into aflatoxin B_1 (AFB_1) and O-methylsterigmatocystin (OMS) by whole 5 day old *A. parasiticus* mycelium[b] at 25°C, over 5 hours and different pH values[c].

pH values	Metabolite Produced (µg)	
	AFB_1	OMS
2	0.0	0.0
3	0.0	0.0
4	0.0	0.4±.1
5	2.1±.1	0.8±.1
6	3.8±.1	1.1±.1
6.5	6.9±.1	1.5±.1
7	8.2±.1	1.8±.1
7.5	7.5±.1	2.2±.1
8	5.0±.1	2.0±.1

[a] 10µg in 100 ml reaction mixture

[b] 2 g wet wt in 100 ml reaction mixture

[c] Results are mean values for five observations

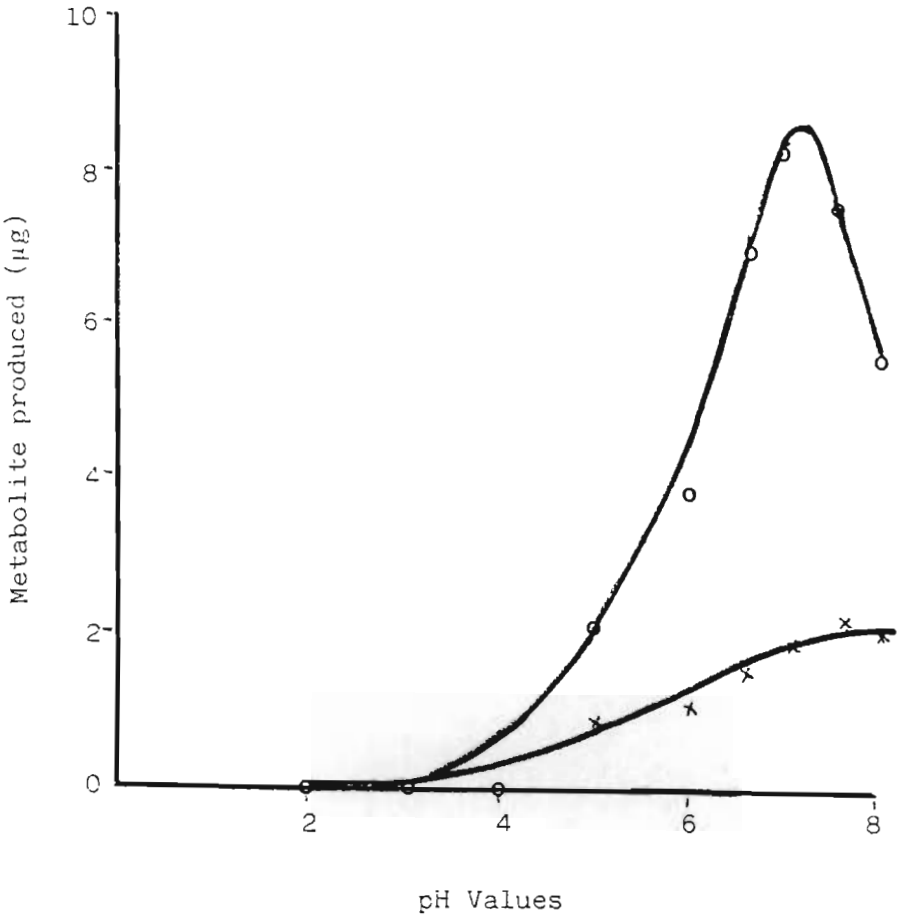


Figure 33: Conversion of sterigmatocystin[a] into aflatoxin B_1 (o) and O-methylsterigmatocystin (x) by whole 5 day old A. parasiticus mycelium[b] at 25°C, over 5 hours and different pH values[c].

[a] 10µg in 100 ml reaction mixture

[b] 2 g wet wt in 100 ml reaction mixture

[c] Results are mean values for five observations

TABLE 27

Conversion of sterigmatocystin[a] to aflatoxin B_1 (AFB_1) and O-methylsterigmatocystin (OMS) by a cell free system[b] at 25°C over 5 hours and at different pH values[c].

pH values	Metabolite Produced (µg) AFB_1	OMS	Enzyme activity (µg aflatoxin B_1 / g of protein)
3	0.0	0.0	0.0
4	0.0	0.0	0.0
5	0.0	0.0	0.0
6	5.3±.3	0.9±.1	10.6±.5
6.5	6.7±.1	1.5±.1	13.4±.1
7	8.2±.1	1.7±.1	16.3±.2
7.5	7.0±.1	1.6±.1	14.1±.1
8	3.0±.3	1.7±.1	6.0 ±.7

[a] 10µg in 10 ml reaction mixture

[b] 500 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

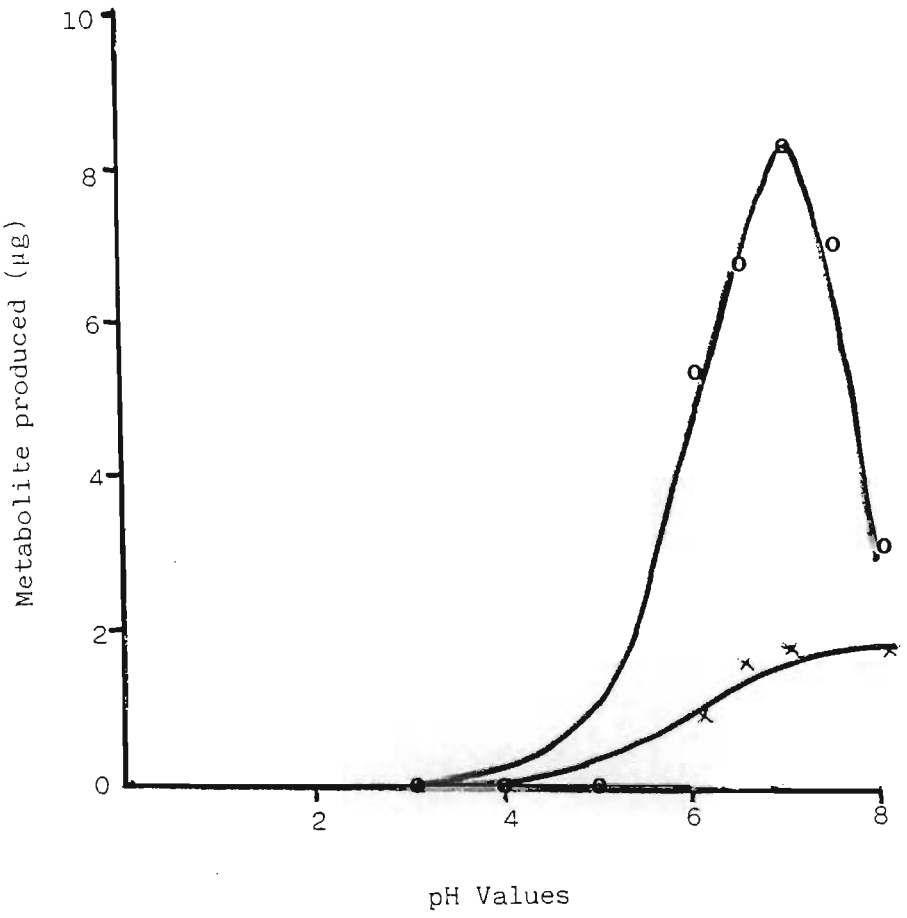


Figure 34: Conversion of sterigmatocystin[a] to aflatoxin B_1 (o) and O-methylsterigmatocystin (x) by a cell free system[b] at 25°C over 5 hours and at different pH values[c].

[a] 10µg in 10 ml reaction mixture

[b] 500 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

TABLE 28

Conversion of sterigmatocystin[a] to aflatoxin B_1 by a partially purified enzyme[b] and fraction 4 at 20°C, over 5 hours and at different pH values[c]

pH Values	Aflatoxin B_1 Produced (µg)	Enzyme activity (µg aflatoxin B_1 / g of protein)
3	0.0	0.0
4	0.0	0.0
5	0.2±.1	2.5 ±1.2
6	0.4±.1	5.0 ±1.7
6.5	3.3±.2	41.3±3
7	7.3±.3	91.3±3.7
7.5	3.4 ±.3	42.5±3.3
8	0.0	0.0

[a] 10µg in 10 ml reaction mixture

[b] 80 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

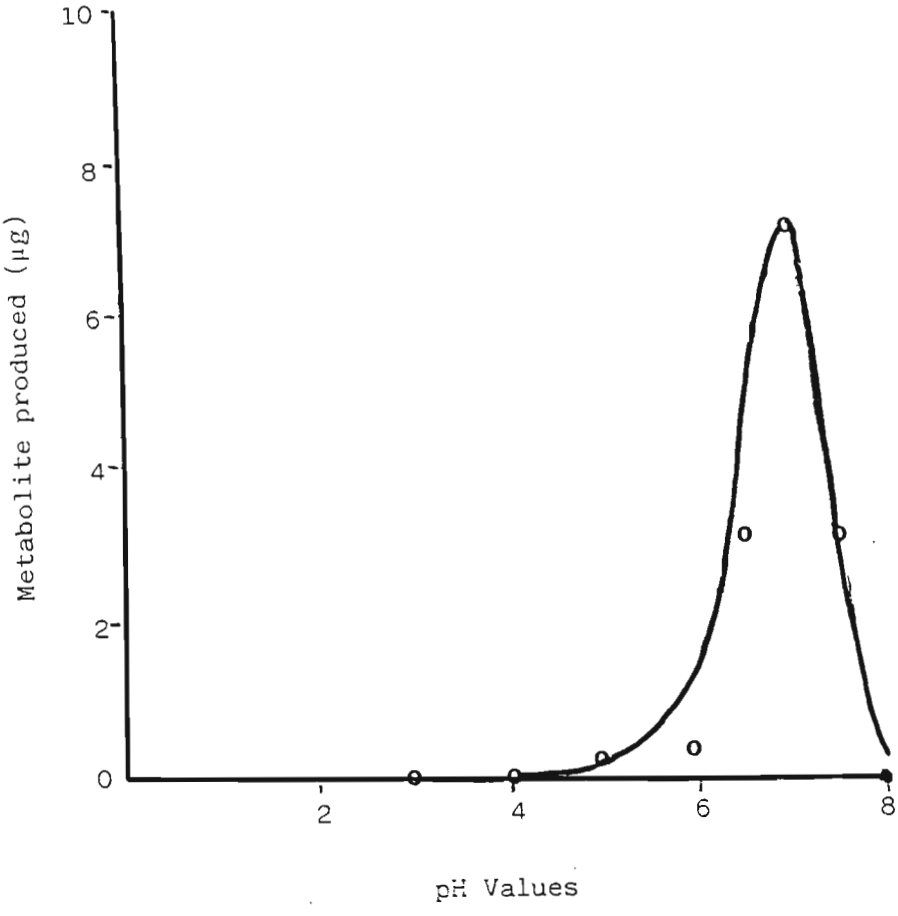


Figure 35: Conversion of sterigmatocystin[a] to aflatoxin B_1 by a partially purified enzyme and fraction 4[b] at 20°C, over 5 hours and at different pH values[c]

[a] 10µg in 10 ml reaction mixture

[b] 80 mg in 10 ml reaction mixture

[c] Results are mean values for five observations

TABLE 29

Conversion of sterigmatocystin[a] to aflatoxin B_1 by the purified enzyme system and fraction 4[b] at 20°C, over 5 hours and at different pH values[c]

pH values	Aflatoxin B_1 Produced (µg)	Enzyme activity (µg aflatoxin B_1 / g of protein)
5	0.0	0.0
6	0.0	0.0
6.5	1.2±.1	342.5 ±28
6.8	4.5±.3	1285.2±85
7.0	5.0±.2	1428.3±65
7.2	5.1±.1	1457.3±28
7.4	2.1±.2	600.0 ±65
8	0.0	0.0

[a] 10µg in 10 ml reaction mixture

[b] 3.5 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

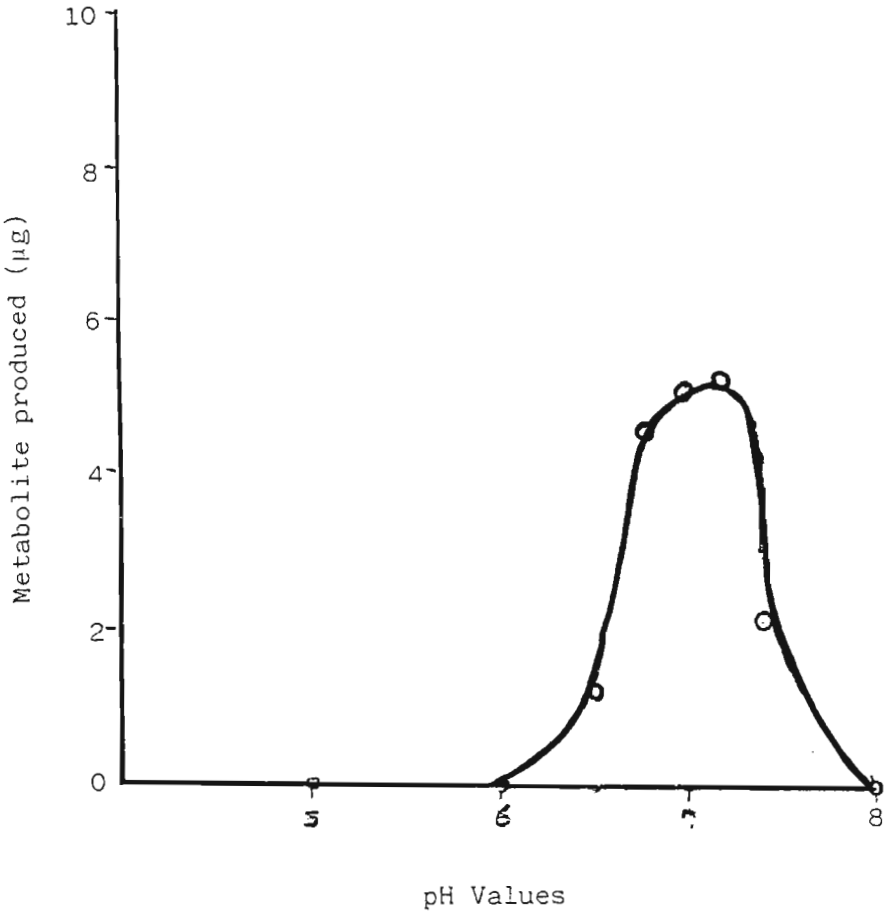


Figure 36: Conversion of sterigmatocystin[a] to aflatoxin B_1 by the purified enzyme system[b] and Fraction 4 at 20°C, over 5 hours and at different pH values[c]

[a] 10µg in 10 ml reaction mixture

[b] 3.5 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

3.3.4. The Effect of Time

The time course for the conversion of sterigmatocystin into aflatoxin B_1 and O-methylsterigmatocystin by whole mycelium, crude cell free system, partially purified enzyme and purified enzyme system were determined at 25°C, pH and over 5 hours (section 2.10) (Tables 30-33 and Figs. 37-40), and maximum conversion was found to be within:

	AFB_1	OMS
whole mycelia	3 hrs.	2 hrs.
crude cell free system	5 hrs.	3 hrs.
partially purified enzyme	4 hrs.	-
purified enzyme system	4 hrs.	-

TABLE 30

Conversion of sterigmatocystin[a] to aflatoxin B_1 (AFB_1) and O-methylsterigmatocystin (OMS) by whole 5 day old A. parasiticus mycelium[b] at 25°C pH 7 and over 5 hours[c].

time (hours)	Metabolite Produced (µg) AFB_1	OMS
0	0.0	0.0
.3	0.0	0.0
.5	3.2±.2	0.9±.1
1	5.8±.1	1.4±.1
2	7.2±.1	1.8±.1
3	8.1±.1	1.8±.1
4	8.1±.1	1.8±.1
5	8.1±.1	1.8±.1

[a] 10µg in 100 ml reaction mixture

[b] 2 g wet wt in 100 ml reaction mixture

[c] Results are mean values for five observations

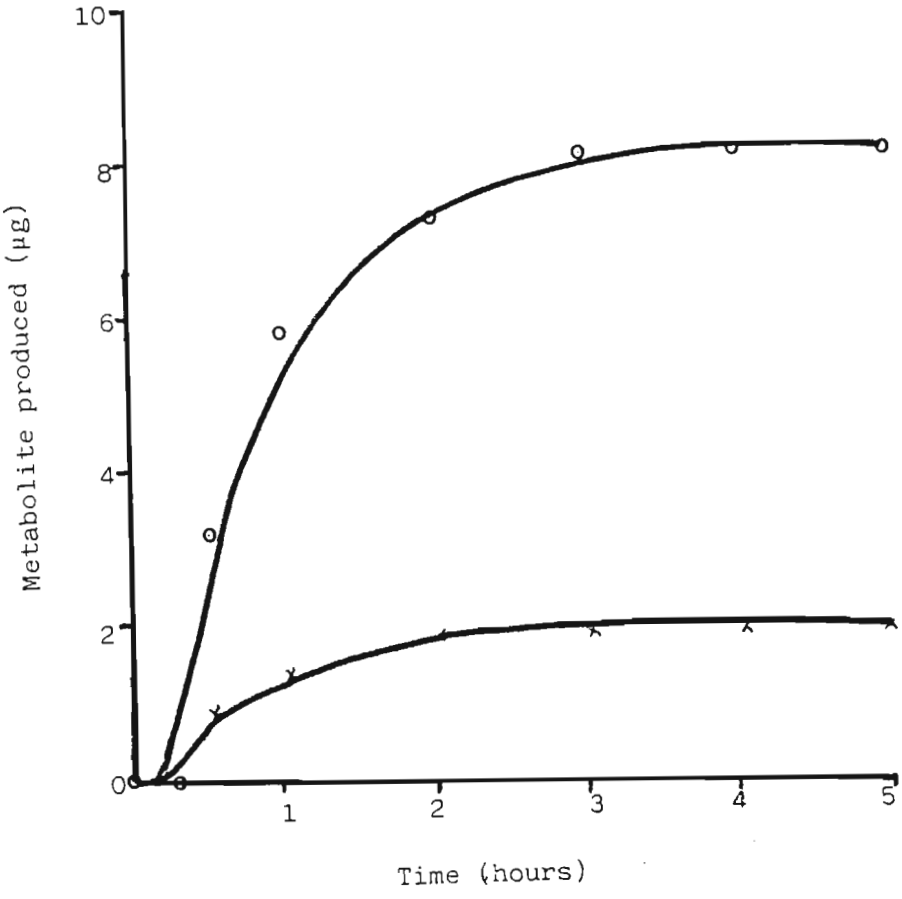


Figure 37: Conversion of sterigmatocystin[a] to aflatoxin B_1 (o) and O-methylsterigmatocystin (x) by whole 5 day old *A. parasiticus* mycelium[b] at 25°C pH 7 and over 5 hours[c].

[a] 10µg in 100 ml reaction mixture

[b] 2 g wet wt in 100 ml reaction mixture

[c] Results are mean values for five observations

TABLE 31

Conversion of sterigmatocystin[a] to aflatoxin B_1 (AFB_1) and O-methylsterigmatocystin (OMS) by a a cell free system[b] at 25°C and pH 7 over 5 hours[c]

Time (hours)	Metabolite Produced (µg) AFB_1	OMS	Enzyme activity (µg aflatoxin B_1 / g of protein)
0	0.0	0.0	0.0
.5	2.8±.1	0.7±.1	5.6±.1
1	5.1±.1	1.4±.1	10.2±.2
2	6.6±.2	1.8±.1	13.1±.3
3	7.1±.1	1.8±.1	14.2±.2
4	8.2±.1	1.8±.1	16.4±.2
5	8.2±.1	1.8±.1	16.4±.2

[a] 10µg in 10 ml reaction mixture

[b] 500 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

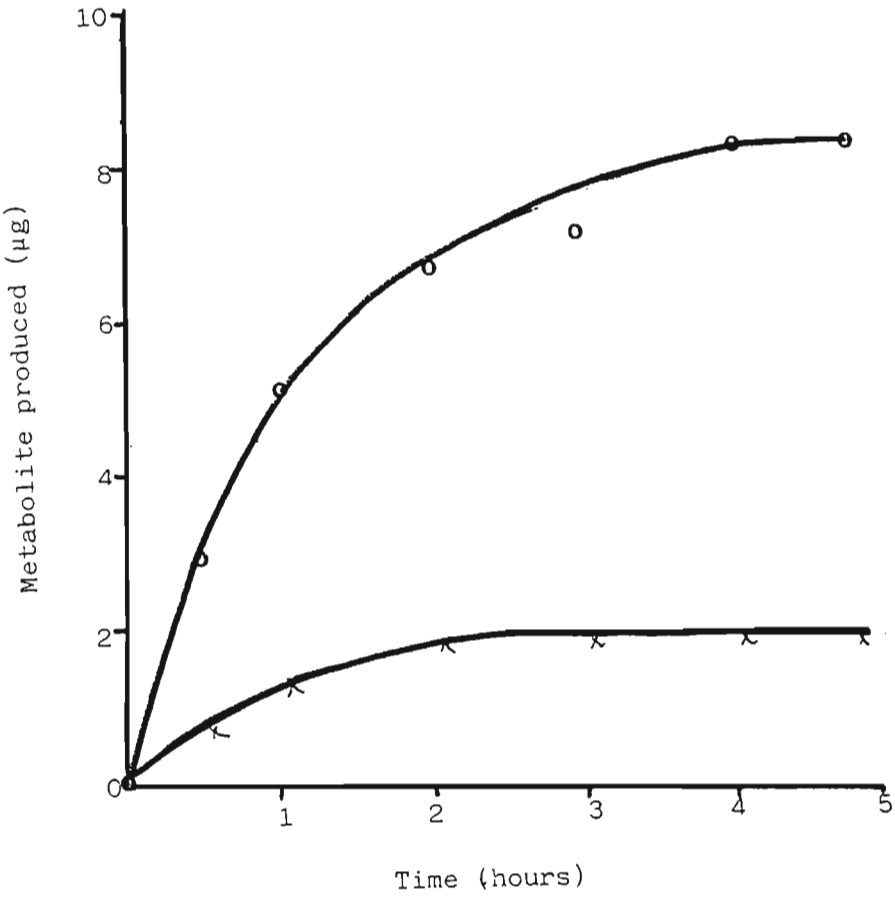


Figure 38: Conversion of sterigmatocystin[a] to aflatoxin B_1 (o) and O-methylsterigmatocystin (x) by a a cell free system[b] at 25°C and pH 7 over 5 hours[c]

[a] 10µg in 10 ml reaction mixture

[b] 500 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

TABLE 32

Conversion of sterigmatocystin[a] to aflatoxin B_1 by partially purified enzyme[b] and fraction 4 from gel filtration at 20°C and pH 7 over 5 hours[c]

Time (hours)	Aflatoxin B_1 Produced (µg)	Enzyme activity (µg aflatoxin B_1 / g of protein)
0	0.0	0.0
.25	0.0	0.0
.5	2.0±.2	25.2±3
1	4.2±.2	52.2±2.3
2	5.7±.1	71.7±1.6
3	6.3±.2	78.7±2.3
4	7.2±.1	90.0±1.5
5	7.3±.1	91.3±1.5

[a] 10µg in 10 ml reaction mixture

[b] 80 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

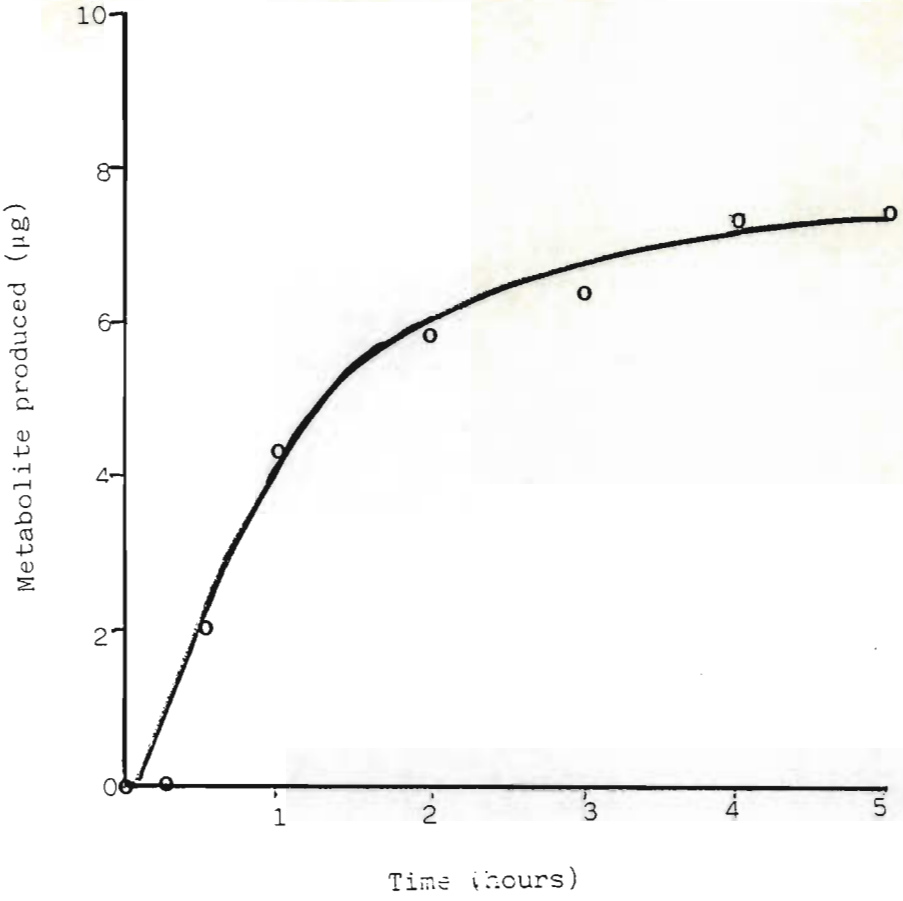


Figure 39: Conversion of sterigmatocystin[a] to aflatoxin B_1 by partially purified enzyme [b] and fraction 4 from gel filtration at 20°C and pH 7 over 5 hours[c]

[a] 10µg in 10 ml reaction mixture

[b] 80 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

TABLE 33

Conversion of sterigmatocystin[a] to aflatoxin B_1 by a purified enzyme system[b] at 20°C and pH 7 over 5 hours[c]

Time (hours)	Aflatoxin B_1 Produced (µg)	Enzyme activity (µg aflatoxin B_1 / g of protein)
0	0.0	0.0
.25	0.6±.1	171.8 ±22
.5	1.2±.1	342.8 ±28
1	2.9±.2	828.4 ±65
2	4.2±.2	1200.6±68
3	4.5±.2	1285.2±68
4	4.9±.3	1400.4±91
5	5.0±.3	1428.3±94

[a] 10µg in 10 ml reaction mixture

[b] 3.5 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

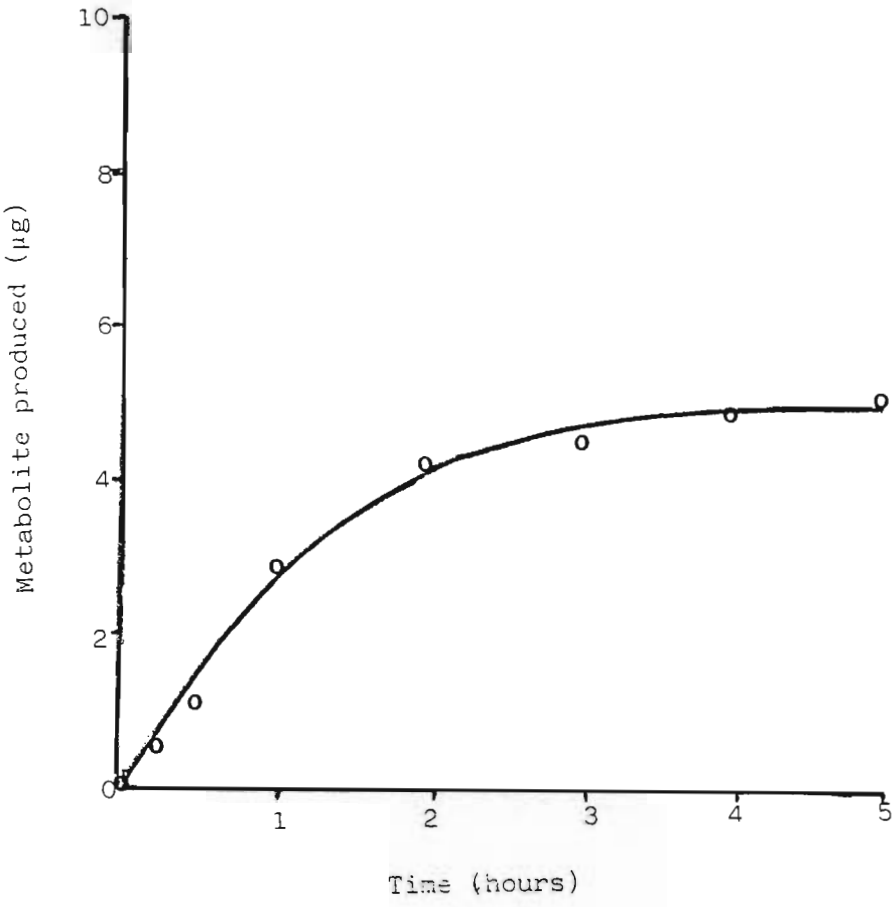


Figure 40: Conversion of sterigmatocystin[a] to aflatoxin B_1 by a purified enzyme system[b] at 20°C and pH 7 over 5 hours[c]

[a] 10µg in 10 ml reaction mixture

[b] 3.5 mg protein in 10 ml reaction mixture

[c] Results are mean values for five observations

3.3.5. NADPH Requirement

The conversion of sterigmatocystin into aflatoxin B_1 and O-methylsterigmatocystin crude cell free system, partially purified enzyme and purified enzyme system was determined in the presence and absence of NADPH at 25°C and over 5 hours (section 2.10) (Tables 34).

All three systems were dependent on the avalibility of exogenous NADPH for the production of aflatoxin B_1 from sterigmatocystin, but the production of O-methylsterigmatocystin was independent of NADPH. O-Methylsterigmatocystin is however not produced by the partially purified and purified enzyme systems.

TABLE 34

Conversion of sterigmatocystin[a] to aflatoxin B_1 (AFB_1) and O-methylsterigmatocystin (OMS) by different enzyme systems at 25°C, pH 7 over 5 hours in the presence and absence of NADPH[b].

Enzyme System	NADPH	Metabolite Produced (µg)		Enzyme activity (µg aflatoxin B_1 / g of protein)
		AFB_1	OMS	
Cell free[c]	+	8.2±.1	1.6±	16.4±.3
	-	0.0	1.6±.3	0.0
Partially Purified[d]	+	7.2±.1		90.0±1.3
	-	0.0		0.0
Purified[e]	+	4.8±.1		1371±28
	-	0.0		0.0

[a] 10µg in 10 ml reaction mixture

[b] Results are mean values for five observations

[c] 500 mg protein in 10 ml reaction mixture

[d] 80 mg protein in 10 ml reaction mixture

[e] 3.5 mg protein in 10 ml reaction mixture

3.4. KINETIC STUDIES

3.4.1. Enzyme and Substrate Concentration

The effect of enzyme and substrate concentration on the conversion of sterigmatocystin to aflatoxin B_1 was investigated. A K_m value for the system was derived from these results (Tables 35 and 36 and Figs. 41-43)

The conversion of sterigmatocystin to aflatoxin B_1 was dependent on the enzyme concentration. The amount of product formed increased with increasing enzyme concentration (Table 35 and Fig. 40).

At low substrate concentrations the amount of product formed is proportional to the substrate concentration. The shape of the curve shown in Fig 41 is typical of Michaelis-Menten kinetics (Table 36 and Fig.41). The double reciprocal Lineweaver-Burk plot was drawn to obtain the K_m and V_{max}.

K_m (calculated) 0.38μM of sterigmatocystin

V_{max} (calculated) 876 μg aflatoxinB_1/g of protein/hour (Fig.42).

TABLE 35

Conversion of sterigmatocystin[a] into aflatoxin B_1 by different concentrations of the purified enzyme system[b] at 20°C, over 1 hour and at pH 7.2[c].

Enzyme Concentration mg/ml	Aflatoxin B_1 Produced (µg)	Enzyme activity (µg aflatoxin B_1 / g of protein)
.125	0.8±.1	800±30
.25	1.5±.1	750±50
.375	2.3±.1	766±33
.625	4.1±.3	820±60
1.25	7.3±.2	730±23

[a] 10µg in 8 ml reaction mixture

[b] 8 ml reaction mixture

[c] Results are mean of five observations

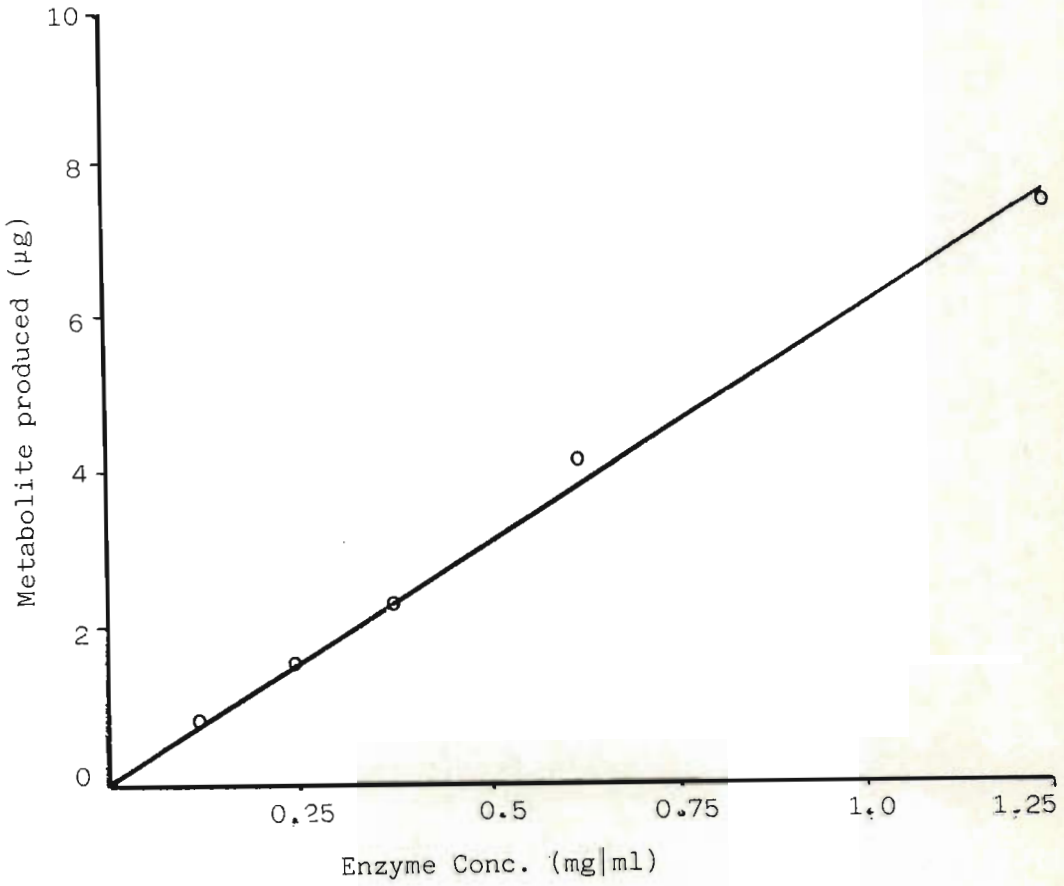


Figure 41: Conversion of sterigmatocystin[a] into aflatoxin B_1 by different concentrations of the purified enzyme system[b] at 20°C, pH 7.2 and over 1 hour[c].

[a] 10µg in 8 ml reaction mixture

[b] 8 ml reaction mixture

[c] Results are mean of five observations

TABLE 36

Conversion of different concentrations of sterigmatocystin into aflatoxin B_1 by purified enzyme system[a] at 20°C, pH 7.2 and over 1 hour[b].

Amount Substrate µg	Substrate Conc. $Mx10^{-7}$	Aflatoxin B_1 Produced $M \times 10^{-7}$	Enzyme activity $M \times 10^{-7}$ aflatoxin B_1 /g of protein
0.5	1.92	1.0±.1	285±14
0.6	2.31	1.1±.2	314±57
0.75	2.89	1.3±.1	371±28
1	3.85	1.7±.1	485±28
2	7.70	2.0±.2	571±57
3	11.25	2.3±.1	657±28

[a] 3.5 mg of protein in 8 ml reaction mixture

[b] Results are mean of five observations

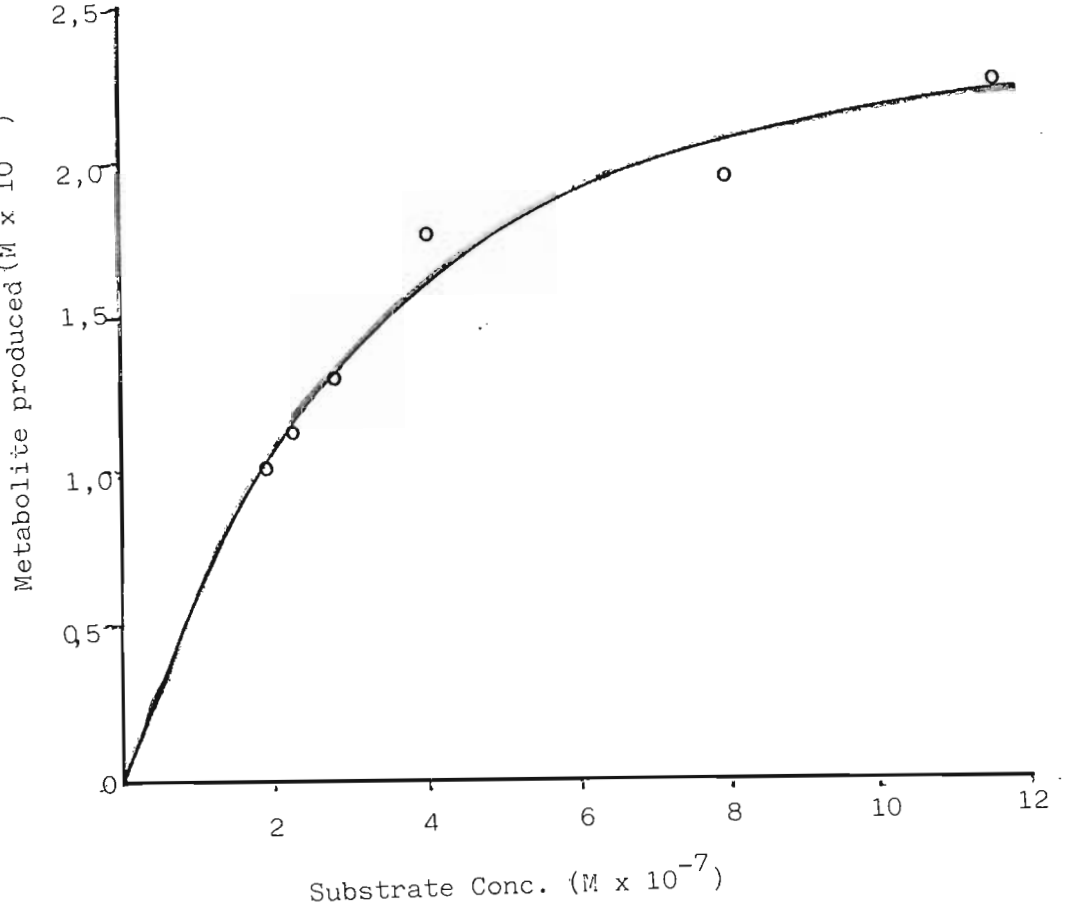


Figure 42: Conversion of different concentrations of sterigmatocystin into aflatoxin B_1 by purified enzyme system at 20°C, over 1 hour and at pH 7.2

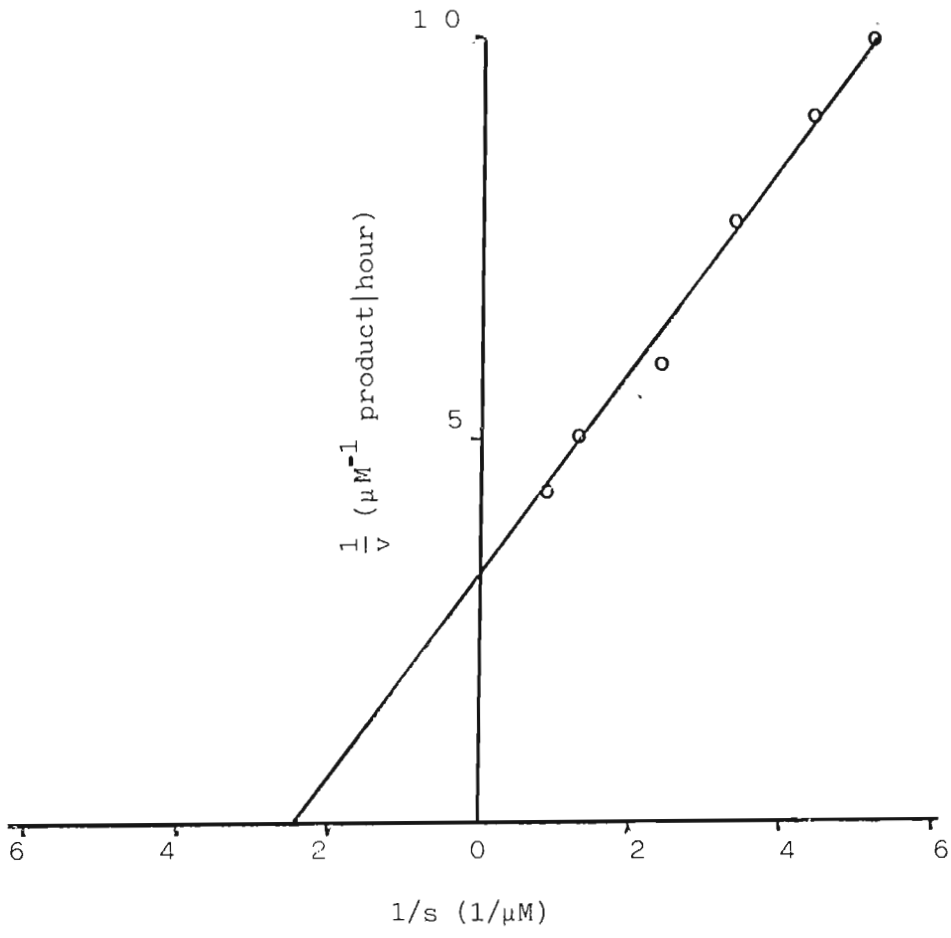


Figure 43: Lineweaver-Burk plot of the conversion of sterigmatocystin into aflatoxin B_1 by purified enzyme system[a] at 20°C, pH 7.2 and over 1 hour[b].

[a] 3.5 mg of protein in 8 ml reaction mixture

[b] Results are mean of five observations

3.4.2. Substrate Specificity

A number of substrates were substituted for sterigmatocystin and the relative conversion of these substrates were measured and expressed as a percentage of the conversion of sterigmatocystin (Table 48).

TABLE 37

Substrate specificity of the purified enzyme system[a] at 20°C, over 1 hour and at pH 7.2.

Substrate[b]	conversion product	relative conversion (%)[c]
Hydroxysterigmatocystin	aflatoxin B_{2a}	65
Dihydrosterigmatocystin	aflatoxin B_2	47
desmethoxysterigmatocystin	no product	

[a] 3.5 mg protein in 10 ml reaction mixture

[b] 10µg in 10 ml reaction mixture

[c] Results are expressed as a percentage of the maximum conversion of sterigmatocystin.

The enzyme system converted dihydrosterigmatocystin to aflatoxin B_2 and hydroxysterigmatocystin to aflatoxin B_{2a}, but did not act on desmethoxysterigmatocystin. Aflatoxin B_2 and B_{2a} were identified by TLC and were found to have R_F values of 0.3 and 0.1 respectively in chloroform:acetone (9:1) solvent. The R_F's were similar to those of standards.

3.4.3. Stoichiometry

The consumption of NADPH during the conversion of sterigmatocystin to aflatoxin B_1 was measured.

TABLE 38

Stoichiometry of the conversion of sterigmatocystin to aflatoxin B_1 and utilization of NADPH by the purified enzyme system[a] within 1 hour at 20°C and at pH 7.2.

Time hours	NADPH µM	Sterigmatocystin µg	Sterigmatocystin µM	Aflatoxin B_1 µg	Aflatoxin B_1 µM
0	10	10	3.08	0	0
1	7.7	2.4	0.74	7.18	2.27

[a] 10 mg protein was used in 10 ml reaction mixture

2,3 µM NADPH was used in the conversion of 2.4µM of sterigmatocystin therefore a 1:1 relation between NADPH utilisation and sterigmatocysin conversion exist.

3.4.5. Cofactor Requirement

A number of cofactors were substituted for fraction 4 and the amount of aflatoxin B_1 produced was measured (Table 39).

Ferrous ions were was found to be essential for the conversion of sterigmatocystin to aflatoxin B_1. Ferric ions had a slight effect on the enzyme system, but the other cofactors, apart from NADPH, were not required (Table 39).

The effect of ethionine (Et) and S-adenosylmethionine (SAM) on the conversion of sterigmatocystin to O-methyl-sterigmatocystin and aflatoxin B_1 by the crude cell free preparation in the presence of NADPH was also investigated (Table 40).

Ethionine inhibits the production of O-methyl-sterigmatocystin and promotes that of aflatoxin B_1, whilst the reverse was true with S-adenosylmethionine (Table 40).

TABLE 39

Effect of cofactors on the conversion of sterigmatocystin[a] to aflatoxin B_1 by a purified enzyme system[b] at 20°C, over 1 hour and at pH 7.2 in the presence of NADPH[d]

.

Cofactor Added[c]	Aflatoxin B_1 produced µg
NAD	0
NADH	0
FAD	0
Fe^{++}	1.9
Fe^{+++}	.4
Fe^{++} + Fe^{+++}	1.9
NAD + FAD Fe^{++} + Fe^{+++}	2.0

[a] 10µg in 10 ml reaction mixture

[b] 3.5 mg in 10 ml reaction mixture

[c] 10mM of each cofactor was added

[d] Results are a mean of five observations.

TABLE 40

Effect of ethionine (Et) and S-adenosylmethionine (SAM) on the conversion of sterigmatocystin[a] to aflatoxin B_1 (AFB_1) and O-methylsterigmatocystin (OMS) by the crude cell free system at 25°C, over 5 hours and at pH 7.

Reagent[c]	Metabolite produced (µg)	
	AFB_1	OMS
Et	8.8	0.8
SAM	2.3	4.8
No factor added	7.4	2.4

[a] 10µg in 10 ml reaction mixture

[b] 500 mg in 10 ml reaction mixture

[c] 10mM in reaction mixture

4. DISCUSSION

The primary objective of this study was to isolate, purify and characterise the enzyme system responsible for the conversion of sterigmatocystin to aflatoxin B_1, the final step in the proposed synthesis of aflatoxin B_1 (80). Most of the evidence in support of the currently accepted biosynthetic pathway of aflatoxin B_1 has been obtained from isotopic and chemical analysis with whole mycelium or crude cell free preparations, the individual steps and their mechanisms can only be conclusively proved by isolating and studying the enzymes responsible.

4.1. CHOICE OF ORGANISM

The mutant strain *A. parasiticus* 1-11-105Wh1, used to produced the cell free system in this study has an enzyme block at the versicolorin A to sterigmatocystin step and therefore accumulates versicolorin A (Fig. 5, pg. 15). It does not produce any aflatoxin B_1, although it has been reported that whole mycelium converts sterigmatocystin to aflatoxin B_1 (132). This organism therefore provides an ideal system in which the conversion of sterigmatocystin to aflatoxin B_1 could be investigated without the interference of any endogenous aflatoxin B_1. Thus, any aflatoxin B_1 produced will have to originate from sterigmatocystin added to the system and, with suitable controls, eliminates the necessity of using radiolabelled compounds.

The initiation of secondary metabolism is probably affected in many cases by the induction of appropriate enzymes (101) at a certain point in the growth cycle. Thus in order to determine whether the enzyme responsible for a secondary metabolic pathway was present, the efffect of age of mycelium on the ability of the organism to convert sterigmatocystin to aflatoxin was investigated.

4.2. EFFECT OF MYCELIAL AGE

It was found that the ability of A. parasiticus 1-11-105Wh1 to convert sterigmatocystin to aflatoxin B_1 and O-methylsterigmatocystin was dependent on the age of the mycelium. Five day old mycelium had the most activity in this respect (Fig 28 pg. 107). The end of the trophophase, which in the case of A. parasiticus 1-11-105Wh1 is indicated by the start of versicolorin A production, coincides with the onset of secondary metabolism. The secondary metabolic enzymes are thought to be at a maximum concentration at the start of idiophase and then lose activity as time progresses (87). The results given in Table 21 (pg. 106) show that there is an increase in enzyme activity (as measured by the conversion of sterigmatocystin to aflatoxin B_1 and O-methylsterigmatocystin) from the third day until the fifth day of culture, after which the activity decreases. Consequently, it can be concluded that by the third day the fungus begins to switch over towards idiophase but, because of the non-synchronous nature of the

culture, there will also be a certain amount of primary metabolism occurring. By the fifth day, the change to secondary metabolism is complete and it can be assumed that enzyme synthesis is slowing and there will be a general decline in enzyme activity. Hence change over from primary metabolism to secondary metabolism is a gradual rather than precipitate transition. The fact that both enzyme systems have a similar dependance on the age of culture suggests that both their metabolic enzymes are produced at the same time and that possibly a single trigger mechanism is responsible for their appearance.

4.3. ENZYME PURIFICATION

Having established that 5 day old mycelium had the highest aflatoxin B_1 synthesising activity (Table 21, pg. 106), mycelium harvested at this time was used to produce a cell free system.

Four different methods of producing a cell free system were used, with varying degrees of success (Table 8, pg. 82). The method that holds favour with some research groups is the production of protoplasts (also referred to as spheroplasts) which are then lysed (97). This method is reported to result in minimum enzyme denaturation. However only limited success was achieved in producing an extract with the required enzyme activity, i.e., conversion of sterigmatocystin to aflatoxin B_1 (Table 8, pg. 82). The lack of activity could possibly be

due to the level of solubility of the sterigmatocystin to aflatoxin B_1 synthesising enzyme being low.

The fungal cell wall was variable in susceptibility to the digestive action of the different enzyme systems used (Table 6, pg. 81). Digestive enzymes were prepared from T. viride and O. xanthineolytica culture filtrates which had previously been reported to have lytic activity (129,133). T. viride was grown in shake flasks as well as in a fermenter. In these preparations the enzymes present would have been a combination of mixed glucanases, chitinase and protease, because the organisms were grown on media that stimulated the production of these enzymes. An increased activity of the enzymes produced by T. viride (as monitoired by the release of protoplasts from the mycelium) was obtained in a fementer as compared to those grown in shake flasks (Table 6, pg. 81). This may be due to the greater control of dissolved oxygen and pH levels in the fementer resulting in a more balanced fermentation. A higher yield of protoplasts was obtained when T. viride (flask) and O. xanthineolytica enzyme preparations were mixed together as compared to the enzymes being used separately (Table 6, pg. 81). However the most effective digestive enzyme system was a mixture of three commercially available enzymes. The commercial preparation was a mixture of lysing enzyme (containing glucanase, protease and 'lytic activity'), chitinase and pronase.

The higher activity of the commercial preparation is probably due to the higher specific activity (with respect to release of protoplasts) and concentration of the digestive enzymes. It is also possible that a different combination of enzymes was present, or that the conditions with respect to cofactors, pH and ionic concentration might not have been optimum for lytic activity.

The age of the mycelium was also considered in the production of protoplasts since the ability of the mycelium to convert sterigmatocystin to aflatoxin B_1 and it's susceptibility to enzyme digestion is dependant on its age (131). A T. viride enzyme preparation was used for this study so that the results of mycelial resistance to age could be compared with similar results published in the literature (127). The number of protoplasts released from the mycelium of different ages were slightly higher than values reported in the literature. The optimum age of mycelium that was used to produce protoplasts was found to be 4 days (Table 7, pg. 81). This age yielded the highest number of protoplasts per wet weight of mycelium. Five day old mycelium were less susceptible to digestion because the cell wall becomes more chitinous with age and this provides for greater rigidity of the wall (134). The variation in the number of protoplasts released could also be a function of the ratio of cell wall to cytosol. If the percentage of cell wall to cytosol is high, fewer protoplasts would be produced. Since the best conversion of sterigmatocystin to aflatoxin B_1 was obtained with 5 day old mycelium (Table 21

pg. 106) this was used in preference to 4 day old material.

The french press system proved unsuccessful in producing a cell free system that could convert sterigmatocystin to aflatoxin. On passing the whole mycelium through the french press the resultant cell free system had lost its ability to form aflatoxin B_1 but did retain O-methylsterigmatocystin biosynthetic activity (Table 8, pg. 82). The french press treatment is extremely disruptive as the mycelium is passed through a tiny pore under very high pressure (20000 psi). Hence it was concluded that this action, which generates heat, destroys the enzyme activity. The loss in activity could also be due to the high shear forces that would be acting on the enzymes.

Further studies showed that a cell free system obtained by lyophilization followed by powdering and re-suspension, gave a better conversion of sterigmatocystin to aflatoxin B_1 than lysed protoplasts or the french press preparations (Table 8, pg. 82). It was found that the mycelium had to be totally dry, as the slightest trace of moisture reduced the efficience of the powdering procedure and also produced heat with loss of enzyme activity.

A fourth method investigated was the grinding of wet mycelium with sand in a mortar and pestle. This method destroyed the activity of both the O-methylsterigmatocystin and aflatoxin B_1 enzyme systems probably because of the heat and shear forces generated by this prosedure.

Cell free systems produced by lyophilization were used in the rest of the study because of the simplicity of the method and the optimum yields of enzyme activity (Table 8, pg. 82).

The localisation of the enzymes responsible for the conversion of sterigmatocystin to aflatoxin B_1 was determined by ultracentrifugation. The enzyme activity resided in the supernatant after centrifugation at 105000xg indicating that the enzymes were soluble proteins in the cytosol, and not associated with organelles (Table 10, pg. 86). Singh and Hsieh (80) suggested that the enzymes responsible for the conversion of sterigmatocystin to aflatoxin B_1 were localised in the cytosol, on the basis of the enzyme being soluble in the supernatant after ultracentrifugation at 105000xg for 2 hours.

Gel filtration was carried out in the first instance with a Sephadex G-200 column (Fig. 21, pg. 89), the enzyme activity (sterigmatocystin to aflatoxin B_1) was located in fraction 2 and O-methylsterigmatocystin synthesising activity in fraction 3 (Table 13, pg. 92). Fraction 2 appears at, or close to void volume, indicating large molecules with molecular weights greater than 350000. This fraction was turbid, suggesting that the particles were extremly large possibly because of conglomeration. Sepharose 6B was also used as the column packing material, and fraction 2 (the active fraction) remained at, or near to void volume (exclusion limit of M.Wt. 3×10^6) (Tables

12 and 15 and Fig. 23, pgs. 88, 94 and 91). Due to the extremely large apparent molecular size of fraction 2, a possible explanation of this observation could be that the enzyme(s) was bound to a membrane.

The ultracentrifugation results suggest that the enzymes were soluble in the cytosol whilst a possible explanation for the gel filtration result was that the enzyme was membrane bound. To resolve this conflict the cell free system was treated with EDTA. It has been reported that some membrane bound proteins are removed from the membrane by EDTA (117). These proteins are referred to as peripheral proteins. EDTA treatement of the cell free system, followed by gel filtration altered the elution profile when compared to the untreated cell free system (Fig. 21 and Fig. 22 pgs. 89 and 90). There was a decrease in the peak area corresponding to fraction 2 and an increase in the peak areas corresponding to fractions 3 and 4. These results indicate that the EDTA removed some of the proteins from the membrane. The enzyme activity for the conversion of sterigmatocystin to aflatoxin B_1 was found at a lower apparent molecular weight (fraction 3) (Table 14, pg. 93). There is a decrease in the specific activity of the enzyme, (based on protein concentration), after EDTA treatment followed by gel filtration, but this decrease is due to the presence of other proteins in fraction 3.

In animal tissue, the majority of glucose-6-phosphatase activity is associated with the endoplasmic reticulum and the

nuclear envelope (135). Gierow and Jergil (136) have described glucose-6-phosphatase as being located mainly in the endoplasmic reticulum and it is commonly used as a marker for the presence of endoplasmic reticulum.

The localisation of glucose-6-phosphatase activity has been determined in different fungi (137). The major sites of glucose-6-phosphatase activity are endoplasmic reticulum and the forming face of the golgi bodies in Achyla bisexuals. In Mucor mucedo, maximum glucose-6-phosphatase activity was observed in endoplasmic reticulum and in the nuclear envelope. The localisation of glucose-6-phosphatase in fungi seems to be similar to its localisation in animal tissues. The presence of glucose-6-phosphatase in fraction 2 (section 3.2.2.3. pg.) would strongly suggest that the fraction was a membrane fraction internal to the fungal cell. The initial cell free fraction was centrifuged at 10,000 xg for 20 min (section2.8.4.). This procedure would remove the nucleus and the golgi bodies. Thus the presence of glucose-6-phosphatase activity in fraction 2 after gel filtration indicates the presence of endoplasmic reticulum membrane.

The filtrate after ultrafiltration showed no enzyme activity as expected but the retentate was active in converting sterigmatocystin to aflatoxin B_1 and O-methylsterigmatocystin (Table 9, pg. 84). No cofactor was added to the retantate since ultrafiltration would not remove all the cofactors. This method was used to concentrate fraction 2 prior to rechromatograpy and

ultracentrifugation.

Fraction 2 was centrifuged at 105000xg for 2 hours and the active supernatant (Table 16, pg. 96) was reloaded on to the Sephadex G-200 column. the activity of the supernatant (expressed as µg product/g of protein) after this centrifugation step was higher than the activity of the supernatant after the cell free system was centrifuged under the same conditions (Tables 10 and 16 pgs. 86 and 96). This increase in activity was due to the removal of non-active protein (approximately 70%) by gel filtration.

Four new peaks were obtained whose appearance would suggest that these proteins were released during centrifugation (Table 17 and Fig. 24, pgs. 97 and 98) . It was concluded that they were initially physically bound, perhaps to the membrane. This view is supported by reports in the literature (118) of peripheral proteins being released from membranes by ultracentrifugation. This gave extra evidence in support of the notion that the enzyme(s) is a peripheral protein. A further indication that fraction 2 was a membrane fraction was that the pellet was partially organic solvent soluble indicating, the presence of lipid material (section 3.2.3.1).

Peak 2.1 (Table 17 and Fig. 24) appears to be the same as the original fraction 2 but with very little enzyme activity (Table 18, pg. 99). This activity was now located in fraction 2.2 which concides with the elution volume of fraction 3 from

the previous run. Thus, passing the cell free system through the gel filtration column before ultracentrifugation was useful as a clean-up step, in that,fraction 3 proteins were removed prior to the separation of fraction 2.2 (the active fraction).

Fraction 2 was also rechromatographed, and the activity (sterigmatocystin to aflatoxin) was also in the new fraction 2 (Table 19 and Fig. 25, pgs. 101 and 100). It was concluded that gel filtration on its own did not cause the shift in the elution volume of the active proteins that was noted after fraction 2 was ultracentrifuged and then gel filtered (Table 17, pg. 97)

The purification of the enzyme was followed by isoelectric focusing (I.E.F.) (Fig. 26, pg. 102). Isoelectric focusing of Fraction 2 did not reveal any proteins at pI's of 5.5 and 6. Proteins with these pI's were present in fraction 2.2 (the purified enzyme system). A possible explanation for this observation could be that the interaction between the membrane and the proteins had effected the pI or the solubility of the proteins.

The I.E.F. patterns of lipoproteins (such as low density lipoproteins) is complicated by multiple components arising through differences in bound lipids, apoproteins or in associated carbohydrates (138). Apoproteins of lipoproteins behave similarly to peripheral proteins. They are stripped, to an extent, from the core of the molecule by ultracentrifugation in potassium bromide density gradiants (139). To assign pI's to

EDTA extractable proteins (peripheral) from erythrocyte membranes the proteins are extracted before being separated by I.E.F. (140). It would appear that I.E.F. per se would not necessarily separate out peripheral proteins from membranes.

The degree of purification with respect to specific enzyme activity (sterigmatocystin to aflatoxin) was calculated. The enzyme activity of the purified enzyme system was 75 times greater than the activity of the cell free system (lyophilized preparation (Table 20, pg. 103).

Three different methods were used to check the purity and physicochemical properties of fraction 2.2: PAGE, SDS-PAGE and isoelectric focusing. Fraction 2.2 was shown to consist of two proteins with molecular weights of 91,000 and 102,000 and pI's of 5.5 and 6 (Fig. 27, pg. 104). The molecular weight of fraction 2.2 was estimated to be 105000 by gel filtration (section 3.2.3.1.). SDS-PAGE also indicated that the two enzymes were single protein units and not sub-units.

4.4. FACTORS AFFECTING CONVERSION OF STERIGMATOCYSTIN

4.4.1. Effect of Temperature

There was a broad temperature range over which the four systems investigated, whole fungal mycelium, cell free system, fraction 2 (partially purified enzyme) and fraction 2.2 (purified enzyme system), were able to convert sterigmatocystin to aflatoxin B_1. In fungal mycelium, the conversion to aflatoxin B_1 was obtained between 10°C and 40°C (Table 22, Fig.29, pgs. 109 and 110), with optimum conversion at 25°C. At 40°C there was only a small quantity of aflatoxin B_1 formed indicating that the conversion enzyme(s) was denatured to a large extent at this temperature. The aflatoxin B_1 synthesising enzyme in the cell free system had an optimum temperature of 25°C with only trace amounts of aflatoxin B_1 being produced at 40°C (Table 23 and Fig. 30, pgs. 111 and 112) while with fraction 2 and fraction 2.2 no aflatoxin B_1 was produced at 40°C. The optimum temperature for fraction 2 and fraction 2.2 was 20°C which is lower than the optimum for the other two systems (Tables 24 and 25 and Figs. 31 and 32, pgs. 113-116). At each purification step the trend was towards a narrowing of the temperature range. It would appear that the internal enviroment has a stabilising effect on the enzyme. As the enzyme is purified it becomes more temperature labile. For fraction 2 and fraction 2.2 there is a sharp decrease in activity between 25°C and 30°C while with the mycelium the drop was not as great.

The temperature profile of the enzyme responsible for the production of O-methylsterigmatocystin, in whole mycelium and the cell free system, was different from that for aflatoxin B_1 production, maximum synthesis of O-methylsterigmatocystin occurred at 30°C and that at 40°C the decrease in O-methylsterigmatocystin production was not as great as in the case of aflatoxin B_1. It can be concluded that the O-methylsterigmatocystin producing enzyme might be less heat labile than the aflatoxin B_1 producing enzyme and that the former also has a higher thermal stability. At the lower temperatures of 5°C and 10°C only trace amounts of O-methylsterigmatocystin was produced. The reaction rate is negligable at temperatures below 15°C.

4.4.2. Effect of pH

A broad pH range for enzyme activity, from pH 5 to pH 8, was observed in whole mycelium. The optimum activity was found to be at about pH 7 (Table 26, Fig.33, pgs. 118 and 119). This wide range is probably due to the cell wall and membrane providing a protective layer and minimizing changes in the internal environment. The neutral to slightly alkaline optimum pH value agreed with results obtained for other secondary metabolic enzymes, the pH optimum for the conversion of versiconal acetate to versicolorin A was 7.2 to 7.8 (124) and the optimum pH for the patulin producing enzymes was also within the neutral to slightly basic range (87). Neither aflatoxin

B_1 nor O-methylsterigmatocystin activity was present at pH values 3 and 4 but both enzymes were still active at pH 8. The lack of activity at the low pH values shows that the insulating effect of the membrane and the internal buffering capacity of the cytoplasm was not sufficient to protect the enzymes at extreme pH's. Severe changes in pH cause a disruption in the conformation of the enzymes.

Both aflatoxin B_1 and O-methylsterigmatocystin producing enzymes in the cell free preparation were more susceptible to pH changes than in intact mycelium. The optimum pH was about pH 7 (Table 27 and Fig 34, pgs.120 and 121). No conversion of sterigmatocystin took place at pH values below 6 and at pH 8, there was a large decrease in activity.

The enzymes in the cell free system are more susceptible to changes in temperature and pH than in the whole mycelium because of the removal of the protective cell wall. This system provided a more accurate assessment of temperature and pH optima than whole mycelium studies.

Similar results were obtained for the pH optimum of the aflatoxin B_1 synthesising enzymes in fraction 2 but with a much narrower active pH range (Table 28, Fig. 35, pgs. 122 and 123), optimum conversion being achieved at about pH 7. The enzyme is only slightly active at pH 6.5 and 7.5 with no activity at other pH values. The limited range is due to the enzyme being in an unprotected environment and therefore more

susceptible to denaturation. With a slight change in pH and the lack of the buffering capacity of the enzyme enviroment the charge of the bonding groups on the protein would be altered resulting in an alteration of the conformation of the molecule.

The pH optimum for the enzymes in fraction 2.2 was about 7.2 with little activity 0.4 pH units on either side of the optimum (Table 29, Fig. 36, pgs. 124 and 125).

4.4.3. Time Course Study

The rate of conversion of sterigmatocystin to aflatoxin B_1 was studied over a 5 hour period. Sterigmatocystin was totally converted to aflatoxin B_1 and O-methylsterigmatocystin by whole mycelium within 3 hours (Table 30, Fig 37, pgs.127 and 128). This result also showed that there was a lag phase which lasted for fifteen minutes. This is probably due to the limiting effect of diffusion through the membrane.

On incubation of sterigmatocystin with the cell free preparation, both aflatoxin B_1 and O-methylsterigmatocystin were detected within 0.5 hours. The concentration of O-methylsterigmatocystin was greater than aflatoxin B_1 and reached a maximum after 2 hours, while aflatoxin B_1 production only reached its maximum at 4 hours.(Table 31, Fig. 38, pgs. 129 and 130). The loss of O-methylsterigmatocystin production after 2 hours could possibly be due to product inhibition once O-methyl- sterigmatocystin had reached a certain level or the

exhaustion of the co-factor S-adenosylmethionine. These possibilities were not investigated further. However it was found that the S-adenosylmethionine stimulated the production of O-methyl sterigmatocystin at the expense of aflatoxin B_1 while ethionine had the opposite effect (Table 40 pg. 147). These results can be explained if it is assumed that a methyl transferase enzyme is involved in the formation of O-methylsterigmatocystin and that ethionine inhibits the methylation reaction and the S-adenosylmethionine promotes it.

The time course for the synthesis of aflatoxin B_1 by fraction 2 was similar to the mycelium and cell free system, maximum conversion occurred within 4 hours of adding the substrate (Table 32 and Fig 39, pgs. 131 and 132). However all of the sterigmatocystin is not converted by 5 hours. Time course studies of the conversion of sterigmatocystin to aflatoxin B_1 by the cell free system indicated that the substrate was totally utilised within 4 hours and that substrate concentration was not limiting under the conditions studied. It is therefore likely that substrate concentration would not be the limiting factor in this instance. The conditions used were also similar to those used for the cell free system.. The cofactor NADPH could also be ruled out since it was added in excess (10mM). The incomplete conversion could possibly be due to enzyme denaturation. A similar time profile was obtained for the enzymes in fraction 2.2 (Table 33, Fig 40, pgs. 133 and 134)

K_m for the purified enzyme was found to be 0.38 µM of

sterigmatocystin, indicating that the enzyme system had a high affinity for sterigmatocystin (Table 36 and Figs. 42 and 43, pgs.140, 141 and 142).

The conversion of sterigmatocystin to aflatoxin B_1 is dependant on the concentration of the enzyme (Table 35, Fig. 41, pgs. 138 and 139). With an increase in enzyme concentration a greater percentage of sterigmatocystin is converted to aflatoxin B_1. This lends support to the view that the all the sterigmatocystin is not used up because of enzyme denaturation.

4.4.4. Effect of NADPH

Singh and Hsieh (80) postulated that an oxygenase is involved in the conversion of sterigmatocystin to aflatoxin B_1. The effect of NADPH was therefore studied, since it is a cofactor for oxygenase activity.

NADPH was required for the conversion of sterigmatocystin to aflatoxin B_1 but not to O-methylsterigmatocystin (Table 34 pg. 136). NADPH was therefore routinely added to the incubation mixtures (section 2.10). Thus the requirement of NADPH for the formation of aflatoxin B_1 suggested that the conversion of sterigmatocystin to aflatoxin B_1 is mediated by a NADPH-dependent oxygenase. Oxygenases are regarded as being the principal means by which biological ring cleavage occurs (106).

Fraction 2 and fraction 2.2 only exhibited enzyme activity when fraction 4 from gel filtration were added (Table 34, pg. 136), the latter containing material with molecular weight lower than 1000. Evidently certain low molecular weight cofactors were required. The investigation of cofactor requirements revealed that ferrous iron was the other cofactor required for the conversion of sterigmatocystin to aflatoxin B_1 (Table 39 pg. 146).

An equal number of moles of NADPH and sterigmatocystin were used in the formation of aflatoxin B_1 by the purified enzyme system (Table 38, pg. 144). This result suggest that the NADPH is used in only one of the enzyme mediated reactions involved in the conversion.

4.4.5 Specificity

The enzyme activity is not affected by substitution at positions 1 and 2 in sterigmatocystin (Fig. 2 pg. 10). This is shown by the conversion of dihydro- sterigmatocystin and hydroxysterigmatocystin to aflatoxin B_2 and aflatoxin B_{2a} respectively by the purified enzyme. Both dihydrosterigmatocystin and hydroxysterigmatocystin are substituted at positions 16 and 17. The methyl group at position 12 on sterigmatocystin has an effect on the activity of the enzyme in that it does not act on desmethoxysterigmatocystin which has the methyl group replaced by hydrogen.

4.5. MECHANISM OF CONVERSION

Based on the available information, the conversion of sterigmatocystin to aflatoxin B_1 involves two enzymes (Fig. 44, pg 169). The formation of aflatoxin B_1 from sterigmatocystin requires a ring cleavage. The first step involves the hydroxylation of sterigmatocystin at position 6. Monooxygenase is a typical enzyme responsible for this type of reaction (106). The enzyme is responsible for the insertion of one atom of oxygen into the substrate (sterigmatocystin) and would also require a reducing agent eg. NADPH (106). Stoichiometric stydies show that a 1:1 relationship exists between sterigmatocystin converted and NADPH utilised during the formation of aflatoxin B_1 by a purified enzyme system. It is possible that the NADPH which is required for the conversion would act as the reducing agent for the monooxygenase.

The hydroxylation step would make the compound susceptible to the action of dioxygenases (106). Dioxygenase enzymes are responsible for the cleavage of the aromatic bond adjacent to a hydroxylated carbon atom. It is therfore likely that the hydroxylated sterigmatocystin would be cleaved by a dioxygenase. Phenolic dioxygenases require non-haem iron as a cofactor. Ferrous iron plays a role in activating the oxygen as well as the substrate (112). Cofactor requirement analysis show that Fe^{++} is necessary for the formation of aflatoxin B_1 . It is possible that Fe^{++} is involved in the second step (Fig. 44). The cleavage provides an open ring structure. The open ring form

is an unstable structure which would undergo spontaneous decarboxylation followed by ring closure to the stable aflatoxin B_1 structure. It is therefore possible that the enzymes isolated are a monooxygenase and a dioxygenase.

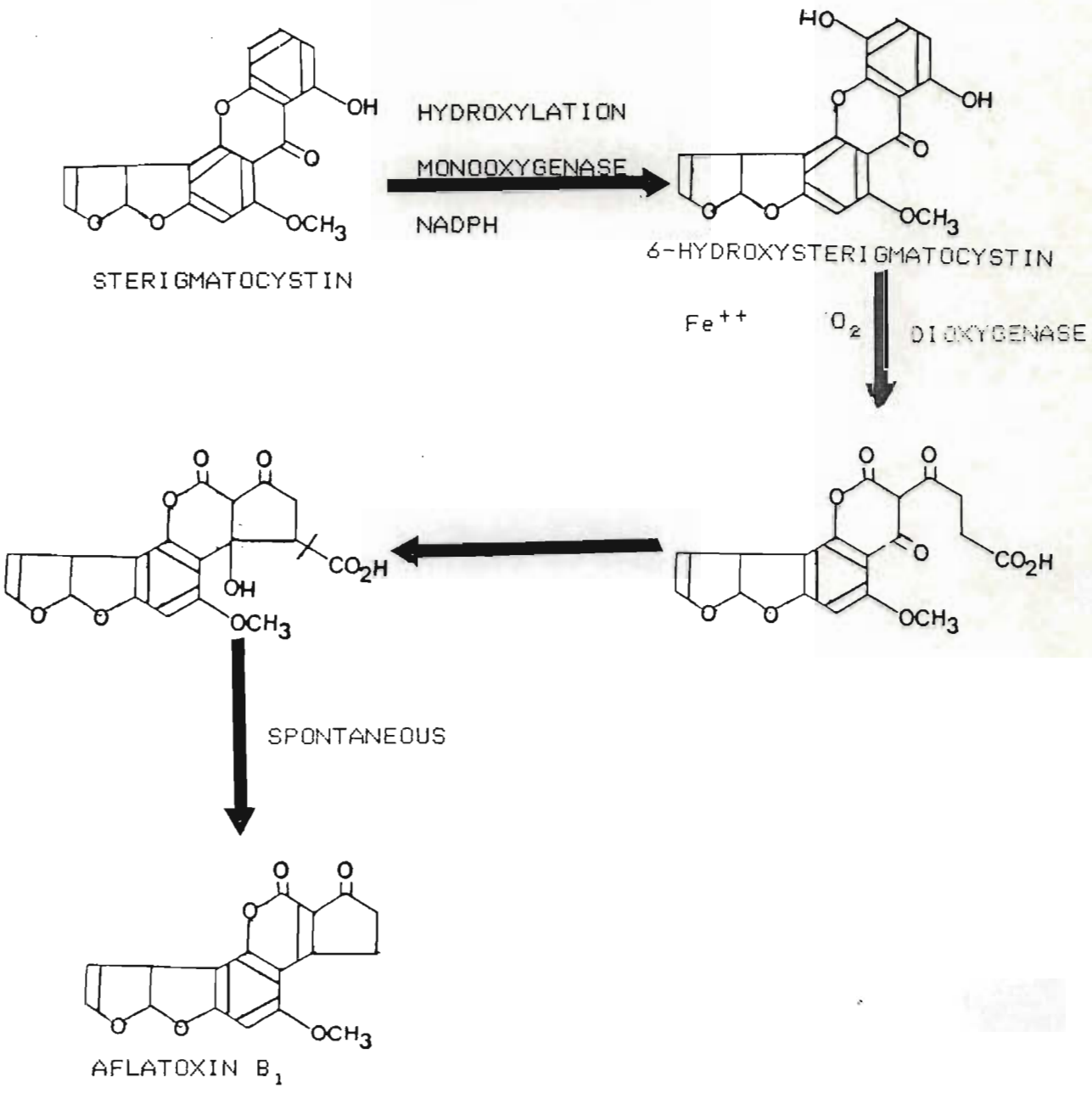


Figure 44: Proposed scheme for the conversion of sterigmatocystin to aflatoxin B_1 involving a mono- and a dioxygenase.

Both the proteins (shown by I.E.F. Fig.27 pg. 104) were required for the conversion of sterigmatocystin to aflatoxin B_1. The activity was lost when the isolated bands were incubated with sterigmatocystin separately. This suggests that the conversion of sterigmatocystin to aflatoxin B_1 involves two enzyme mediated reactions. The reason why the intermediate product between sterigmatocystin and aflatoxin was not detected could be due to two factors:

(a) a very high affinity of the second enzyme for this product and an effective conversion to aflatoxin i.e. the first step is rate limiting and

(b) an effective product inhibition on the first enzyme by this intermediate product resulting in a very low production of the intermediate itself. With only the first enzyme present no detectable (<1% by HPLC) conversion of sterigmatocystin occurred.

Due to the high affinity of the second enzyme for this "product" its inhibitory effect is not felt in the presence of the second enzyme. There is no reference in the literature of the likely intermediate product, i.e., (6-hydroxy-sterigmatocystin) (Fig. 44 pg. 169) in the conversion of sterigmatocystin to aflatoxin being isolated. However its methoxy equivalent 6-methoxysterigmatocystin (Fig. 2, pg. 10) has been isolated (36). This could be due to the fact that if the second enzyme were to be blocked the organism would methylate the intermediate product.

This scheme is similar to the one proposed by Thomas (Fig. 17, pg. 33) (71) who based his mechanism upon theoretical considerations. The likely intermediate has also been proposed by two other groups (36, 79) (Figs. 15 and 16 pgs. 30 and 31). The isolation of the enzymes has enabled us to give further support for this type of mechanism for the conversion of sterigmatocystin to aflatoxin B_1. This conversion also proves that sterigmatocystin is a true intermediate in the aflatoxin B_1 biosynthetic pathway. Another important finding of this work is that the enzyme is bound to the endoplasmic reticulum and not free within the cytosol as previously proposed (123, 124). The fact that the enzyme is membrane bound would help explain the solubility of the anthraquinone intermediates in the cell, since they have very low solubilities in water.

These findings call for a re-examination of the localisation of other secondary metabolic enzymes since if the enzyme was present in the supernatant after ultracentrifugation, then it was concluded that the enzyme was in the cytosol(85). Observations in this project indicate that the enzymes are not necessarily in the cytosol but may be extrinsic proteins that are removed from the membrane by ultracentrifugation.

The isolated enzyme system consisted of two enzymes with molecular weights of 96000 and 102000 (Fig 27 pg.104), pI's of 5.5 and 6 (Fig. 27) and a K_m of 0.38 µM sterigmatocystin.

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APPENDIX 1

Composition of Reddy's Medium (125)

Potassium dihydrogen orthophosphate	750 mg
Magnesium sulphate septahydrate	350 mg
Calcium chloride dihydrate	75 mg
Sucrose	85 g
L-Asparagine	10 g
Ammonium sulphate	3.5 g
Zinc sulphate septahydrate	10 mg
Manganous chloride quadrahydrate	5 mg
Ammonium molybdate	2 mg
Disodium tetraborate decahydrate	2 mg
Ferrous sulphate septahydrate	2 mg
Distilled water to	1l

Composition of YES Medium (126)

Yeast extract	20 g
Sucrose	100 g
Agar	0.1 g
Distilled water to	1l

Composition of Resting Culture Medium (127)

Potassium dihydrogen orthophosphate	5 g
Magnesium sulphate septahydrate	0.5 g
Zinc sulphate septahydrate	10 mg
Potassium chloride	0.3 mg
Manganous chloride quadrahydrate	0.11 mg
Ammonium molybdate	5 mg
Disodium tetraborate decahydrate	0.7mg
Ferrous sulphate septahydrate	10 mg
Copper sulphate quinhydrate	0.3 mg
Distilled water to	1l

Composition of Growth Medium used for the culturing of *T. viride* (128)

Glucose	3.0 g
Hyphal walls	5.0 g *
Bactopeptone	1.0 g
Potassium dihydrogen orthophosphate	2.0 g
Magnesium sulphate septahydrate	0.3 g
Calcium chloride dihydrate	0.3 g
Ammonium sulphate	1.4 g
Zinc sulphate septahydrate	10 mg
Manganous chloride quadrahydrate	5 mg
Ammonium molybdate	2 mg
Disodium tetraborate decahydrate	2 mg
Ferrous sulphate septahydrate	2 mg
Distilled water to	1l

* Hyphal walls were obtained by growing any *Aspergillus* species on Reddy's medium and extracting the mycelium with solvents.

Composition of Growth Medium for the culturing of *O. xanthineolytica* (129)

Hyphal wall	5.0 g
Magnesium sulphate septahydrate	0.5 g
Calcium chloride dihydrate	0.1 g
Ammonium sulphate	5.0 g
Sodium chloride	0.1 g
Phosphate buffer (0.2M, pH7) to	1l

APPENDIX 2

Metabolite absorbance maxima (130)

METABOLITE	WAVELENGTH (nm)	MOLAR EXTINCTION(ε)
aflatoxin	363	21,800
sterigmatocystin	249	27,500
O-methyl-sterigmatocystin	310	16,500
averufin	294	30,800
versicolorin A	321	12,118

Mass spectroscopy data of aflatoxin B_1 and O-methyl-sterigmatocystin -literature values (130)

Product 1		Product 2	
m/e	Relative Intensity	m/e	Relative Intensity
312	100	338	100
284	31	323	39
269	17	309	51
256	29	292	20
241	20	279	12
227	32	265	17
199	10	249	20