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Detection and Partial Purification of a Monooxygenase Involved in the Second Step of Aflatoxin B_1 Biosynthesis in *Aspergillus parasiticus*

by

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The undersigned certify that they have read, and recommend to the Faculty of Gradate Studies for acceptance, a thesis entitled "Detection and Partial Purification of a Monooxygenase Involved in the Second Step of Aflatoxin B_1 Biosynthesis in *Aspergillus parasiticus*", submitted by Maria Elena Carballo in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Aflatoxins are produced by *Aspergillus parasiticus,* but limited progress has been made towards the understanding of the enzymes involved in the earlier steps of aflatoxin biosynthesis.

Nevertheless, success has been achieved for the norsolorinic anthrone monooxygenase in *A. parasiticus*. This enzyme catalyzes the second step of the aflatoxin pathway, an oxidation of norsolorinic anthrone to norsolorinic acid.

A new culture medium was described which produced cells with high levels of monooxygenase enzyme and a rapid onset of secondary metabolite (norsolorinic acid). Optimal conditions were determined for rupturing the cells and stabilizing the enzyme in crude extracts. Optimal assay conditions for the enzymatic conversion of norsolorinic anthrone to norsolorinic acid were devised. This reaction did not require any foreign electron donor, and the enzyme was found predominantly in the microsomal fraction. A preliminary purification, using salt fractionation, gel filtration chromatography, hydroxylapatite adsorption and anion exchange chromatography steps resulted in a partial purification.

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DEDICATION

Yo dedico este trabajo a mi hijo Raul Leon Carballo (Raulito) de siete años de edad en quien siempre pense y me dio fuerzas para llegar a este momento final.

I dedicate this work to my son, Raul Leon Carballo (Raulito), aged seven years, of whom I am always thinking, and who gave me the strength to reach this final moment.

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ABBREVIATIONS

ACP	acyl carrier protein
act	actinorhodin
ACV	δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine
AF(B_1,B_2,G_1,G_2)	aflatoxin
AHC	adenosylhomocysteine
cAMP	cyclic adenosine-3^1, 5^1-monophosphate
AT/MT	acetyl/malonyl/transacylase
AVh	accumulated volt hours
AVN	averantin
AVR	averufin
ºC	degree centrigrade
CHAPS	3-[(3-Cholamidopropyl) dimethyl-ammonium]-1 propane sulfonate
CHS	chalone synthetase
cm	centimeter
Co	coenzyme
DEAE	diethylamino ethyl
ddw	double distilled water
DH	dehydratase
DNA	deoxyribonucleic acid
cDNA	copy DNA
DTT	dithiothreitol
EDTA	ethylene diamine tetracetate
EGMME	ethyleneglycol monomethyl ether
FAS	fatty acid synthetase

FPEG	fresh potato extract glucose
g	gram
gra	granaticin
h	hour
HAVN	hydroxyaverantin
HPLC	high performance liquid chromatography
IDH	isoepoxydon dehydrogenase
K	kilo
Kb	kilobase
KDa	kilodalton
KR	ß-ketoreductase
KS	ß-ketoacyl synthetase
L	litre
M	molar
m	milli (10^{-3})
min	minute
4-MHA	4-methyl-3-hydroxyanthranilic acid
MSA	methylsalicylic acid
MW	molecular weight
N	normal
NA	norsolorinic acid
NADH	nicotinamide adenine dinucleotide, reduced
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
ND	not determined
nm	nanometers

nor	norsolorinic
NRRL	Northern Regional Research Laboratory
OMST	0-methyl-sterigmatocystin
PCMB	p-chloromercuribenzoate
PDA	potato dextrose agar
PEG	potato extract glucose
PHS	phenoxazinone synthetase
Pi	inorganic phosphate
PKS	polyketide synthase
PMSF	phenylmethyl sulfonyl fluoride
ppm	parts per million
Psi	pounds per square inch
PSMO	polysubstrate monooxygenase
R	oxidized cosubstrate
RH_2	reduced cosubstrate
RNA	ribonucleic acid
mRNA	messenger RNA
rpm	revolutions per minute
RVS	resveratrol synthetase
SAM	s-adenosylmethionine
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sec	seconds
SME	secondary metabolism enzymes
sp	species
STG	sterigmatocystin

tcm	tetracenomycin
TFA	trifluoroacetic acid
TRIS	tris (hydroxymethyl) aminoethane
TTI	turkey eggwhite trypsin inhibitor
$T_{\frac{1}{2}}$	half life
U	Unit
UV	ultra violet
VHA	versiconal hemiacetal acetate
Vh	volt hours
Vol	volume
v:v	volume to volume
w/v	weight to volume
x g	times the force of gravity
YES	yeast extract sucrose
%	percentage

PART I

INTRODUCTION

1. SECONDARY METABOLISM

The enzyme-mediated processes by which chemical molecules are synthesized (anabolism) and degraded (catabolism) *in vivo* are defined as Metabolism. Primary metabolism is involved with the synthesis and degradation of molecules that are essential for cellular growth and reproduction, while secondary metabolism includes compounds not essential for growth and frequently accumulate after the cessation of growth (idiophase), or in response to certain environmental signals, because during the growth phase (tropophase) the enzymes necessary for their formation are absent (Gendloff et al., 1991).

Secondary metabolites are often compounds of low molecular weight, of enormous chemical diversity and of restricted taxonomic distribution, (Turner, 1971). They are characteristic of differentiated cells and provide the raw material for most of the studies in natural product chemistry. Most secondary metabolites are produced by bacteria, actinomyces, filamentous fungi and plants. The best known secondary metabolites exhibit pharmacological activity as antibiotics, drugs and toxins, etc.

These metabolites are products of multi-step enzymatic reactions that start from intermediates of primary metabolism (Martin and Liras, 1989). The two types of metabolism are interconnected, since primary metabolism provides a number of

small molecules (i.e. amino acids, acetyl-CoA) which are utilized as building blocks for all of the important secondary metabolic pathways. Thus acetyl-CoA is used in anabolism along with its carboxylation product, malonyl-CoA, to produce fatty acids and polyketides, which are primary and secondary metabolites, respectively.

2. POLYKETIDES

The polyketides are a group of natural products which are formed as a result of the condensation of activated acyl units. Thus an acetyl-CoA chain initiator is condensed with successive 2-carboxyl-acyl-CoA chain extenders (i.e. malonyl-CoA) to yield an extended ß-ketomethylene chain which is often cyclized (Bennett et al., 1993). This chain is then released as an initial "polyketide" product, with further modifications often yielding more complex and structurally diverse molecules. The structural units of these polyketides may be residues of acetate, propionate or butyrate. These different starter molecules are the basis for the great variety of polyketides found in nature, but acetate units are the most frequently used with the main sources of acetyl-CoA being the pyruvate dehydrogenase complex and the ß-oxidation of fatty acids (Baron et al., 1987).

The origin and current understanding of how the simple polyketide chain is folded, condensed, oxidized, reduced, cleaved and rearranged is of significance for understanding

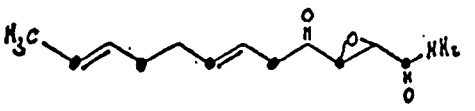
the biosynthesis of aflatoxins as well as other secondary metabolites of polyacetate origin.

These secondary metabolites are produced by prokaryotes (streptomycetes), lower eukaryotes (filamentous fungi) and higher eukaryotes (plants), and encompass a wide range of substances such as phenols, quinones, xanthones, flavonoids, and numerous mycotoxins (Table 2.1). These mycotoxins are named tetraketides, pentaketides, hexaketides and decaketides, according to the number of C_2 units that have contributed to the biosynthesis of the chain.

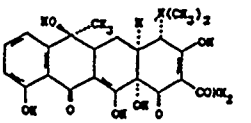
In the different types of polyketides, one class is made up of molecules composed of linear alkyl chains, for example cerulenin (Fig. 2.1) produced by *Cephalosporium caerulens* (Omura et al., 1967), a second class are polyketides in which the linear intermediate has been cyclized to form an aromatic structure (Fig. 2.1), for example tetracyclines produced by several *Streptomyces sp*, 6-methylsalicylic acid produced by *Penicillium urticae*, and aflatoxins produced by *Aspergillus parasiticus* and *Aspergillus flavus*. A third class of polyketides is the macrolides, where there are different subclasses. The first subclass generally uses methyl malonyl-CoA chain extender units and are substituted with 1 to 3 sugar moieties (Omura and Tanaka, 1983), i.e. erythromycin (Fig. 2.1) produced by *Streptomyces erythreus* and avermectin (Fig. 2.1) produced by *Streptomyces avermitilis*.

Table 2-1 MAJOR MYCOTOXINS: BIOSYNTHETIC ORIGIN, AND PRODUCING ORGANISMS

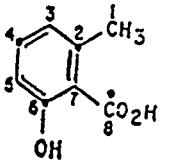
MYCOTOXIN	BIOSYNTHETIC ORIGIN	MAJOR PRODUCING ORGANISMS
Aflatoxins	Polyketide	*Aspergillus flavus, A.parasticus*
Citrinin	Polyketide	*Pencillium* sp., *Aspergillus* sp., especially *P.citrinum* and *P.viridicatum*
Citreovirdin	Polyketide	*Penicillium* sp., especially *P. citreo-viride*
Cyclopiazonic acid	Amino Acids	*P.cyclopium, A.versicolor*
Ergot alkaloids	Amino Acids	*Claviceps* sp., especially *C. purpurea* also species of *Aspergillus, Penicillium, Geotrichium, Rhizopus,Mucor* and convolvulaceous plants
Luteoskyrin	Polyketide	*P.islandicum, P.rugulosum*
Ochratoxin	Polyketide	*Aspergillus* sp.,*Penicillium* sp.,especially *A.ochraceous*
Patulin	Polyketide	*Penicillium* sp.,*Aspergillus* sp., especially *P.patulum, P. expansum*
Penicillic acid	Polyketide	*Penicillium* sp.,*Aspergillus* sp.,especially *P.puberulum, P.cyclopium*
Rubratoxin	Tricarboxylic cycle intermediates	*P.rubrum, P.purpurogenum*
Rugulosin	Polyketide	*P.rugulosin*
Sporodesmin	Amino Acids	*Pithomyces chartarum*
Sterigmatocystin	Polyketide	*Aspergillus* sp., especially *A.versicolor, Bipolaris sorokiniana*
Trichothecenes	Terpenes	*Fusarium* sp., *Myrothecium* sp., *Stachybotrys* sp.
Xanthomegnin	Polyketide	*Trichophyton* sp., *Penicillium* sp.,especially *P.viridicatum*
Zearalenone	Polyketide	*Fusarium* sp., especially *F.roseum (F.graminarium)*



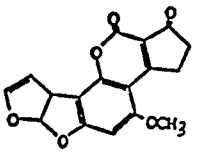
Cerulenin



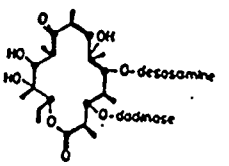
Tetracycline



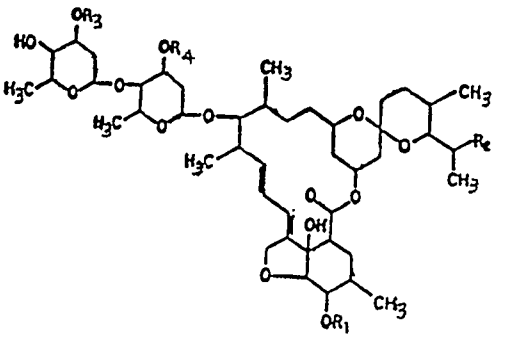
6-methylsalicylic



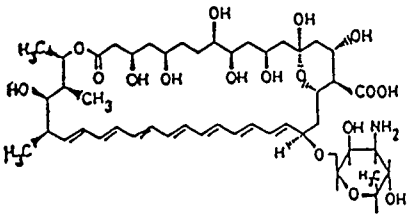
Aflatoxin B_1



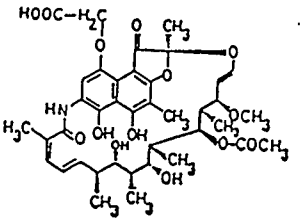
Erythromycin



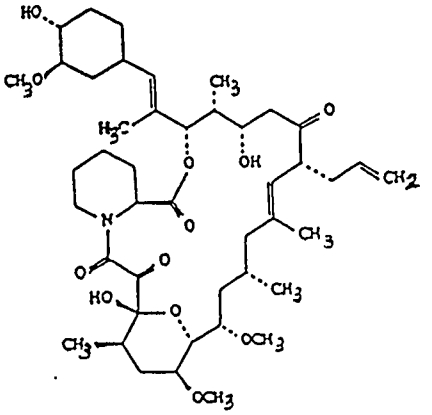
Avermectin



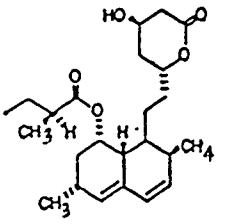
Amphotericin B



Rifamycin



FK-506



Lovastatin

Figure 2.1 Structures of some polyketides

The second subclass is composed of large macrocyclic lactones with a sequence of up to seven conjugated double bonds present in the structure (e.g. amphotericin B (Fig. 2.1) by *Streptomyces nodosus*.

The third subclass, the ansamycins, is distinguished by the presence of an aromatic chain initiator (amino benzoic acids) and because they are macrolactams as opposed to lactones, for example Rifamycin (Fig. 2.1), which when chemically modified is of considerable value as a therapeutic agent (Ghisalba, 1984).

Recently, certain polyketides have been discovered that do not fit into these groups. Noteworthy examples are FK-506 (Fig. 2.1) produced by *Streptomyces tsukubaensis* No. 9993, a 21-membered substituted macrolide, and lovastatin (Fig. 2.1) a hexahydronaphtalene lactone, produced by *Aspergillus terreus* (Alberts, 1988). These compounds are clinically valuable as an immunosuppressive and a cholesterol-lowering agent, respectively.

3. AFLATOXINS

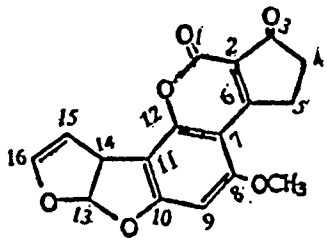
The most important representatives of the decaketides, the aflatoxins, which are derived from acetate-malonate condensation, and are therefore known as acetogenins or derivate secondary metabolites are produced by fungi (molds). The aflatoxins constitute a number of structurally related metabolites which differ considerably in their biological

effects. All these toxins contain a coumarin nucleus fused to a bisdihydrofurano moiety present in a cis fashion, and to either a five-membered lactone (for AFB_1 and AFB_2) or a six-membered lactone (for AFG_1 and AFG_2) (Palmgrend and Ciegler, 1983; Scott, 1985). These four aflatoxins are the most extensively studied; the letters B and G stand for the blue and green colours they emit upon excitation by UV light, and the subscripts $_1$ and $_2$ represent the relative position of each compound on thin layer chromatography. The structures of these aflatoxins are shown in Fig. 3.1.

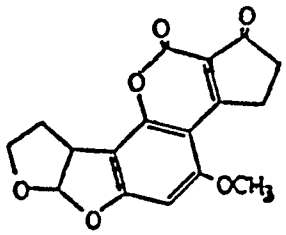
There is another aflatoxin found in cow's milk, designated aflatoxin M_1 which is a blue fluorescent compound with an Rf lower than that of aflatoxin B_1 (Allcroft and Carnaghan, 1963; De Iongh et al; 1964). Aflatoxin M_2 was isolated from the urine of sheep (Holzapfel et al., 1966). Aflatoxin GM_1 is a similarly hydroxylated derivative of aflatoxin G_1. Two additional aflatoxins have been isolated from cultures of *Aspergillus flavus* (Dutton and Heathcote, 1968) which were shown to be 2-hydroxy derivatives of aflatoxin B_1 and G_1 and were named aflatoxin B_{2a} and aflatoxin G_{2a}.

Aflatoxins are receiving increased attention from researchers, the food industry, and the general public for two main reasons:

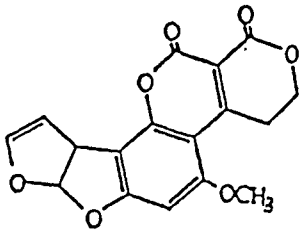
1. The metabolites (particularly aflatoxin B_1) are not only



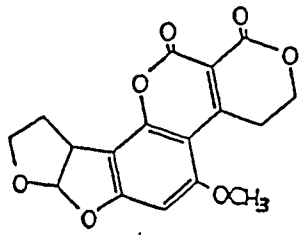
AFB_1



AFB_2



AFG_1



AFG_2

Fig. 3.1 Structures of Aflatoxins

toxic to animals as well as humans, but are also the most carcinogenic of all known natural compounds (Busby and Wogan, 1979; Groopman et al., 1981), and

2. There is a high incidence of the compounds in food and stock feed throughout the world (Jelinek et al., 1989).

3.1 BIOLOGICAL ACTIVITY OF AFLATOXINS

Aflatoxins are biologically active, and are the most toxic of the secondary metabolites produced by fungi.

Food contaminated with aflatoxins has been found to be toxic and carcinogenic to many animal species, and aflatoxins have been classified as some of the most carcinogenic natural products isolated (Baertshi et al., 1988).

Aflatoxins produce toxic effects in many species of laboratory and domestic animals, including rats, mice, rabbits, ducklings, monkeys, cattle and swine (Scott, 1985). These toxins have been shown to be mutagenic, carcinogenic, and teratogenic and they cause acute necrosis, primarily of the liver.

The relative carcinogenicity of these compounds is $AFB_1 > AFG_1 > AFB_2$, with no present evidence that AFG_2 is carcinogenic. Aflatoxin B_1 (AFB_1) has been shown to induce malignant tumors in a variety of animals at doses as low as 1μg/kg and to effect the immune system (Scott, 1985).

In vivo Aflatoxin B_1 is metabolized by different animal species to derivatives of lower toxicity. Its metabolism in relation to its biological activity has been studied and convincing evidence indicates that this toxin requires metabolic activation to exert its carcinogenic and mutagenic affect (Campbell and Hayes, 1976; Roebuck and Wogar, 1977; Swenson et al., 1977).

It has been well established that the chemical site responsible for biological activity of aflatoxins and related compounds is the C_{15}-C_{16} double bond (see AFB_1 structure, Fig. 3.1) in the dihydrofuran moiety of these molecules (Stark, 1980).

There has been a long-standing controversy about whether or not aflatoxins cause cancer in man. In certain areas of the world with a high consumption of aflatoxins, there is a very high incidence of liver cancer, primary hepato-carcinoma. These areas include Mozambique, Thailand, Swaziland, the Philippines and areas of Japan (Scott, 1985).

3.2 AFLATOXIN PRODUCING MICROORGANISMS

Aspergillus species are microorganisms which can grow on a wide variety of substrates. The large ecological distribution of the aspergilli confers upon them the ability to grow under wide extremes of temperature, humidity and other environmental factors. It also gives them a distinct survival advantage over other microorganisms.

Aspergilli have caused immense damage by causing either aspergillosis or by elaborating toxic compounds such as the aflatoxins that have been the subject of thorough investigation because of their notoriety (Subramanian, 1986).

The production of the aflatoxins is confined to certain strains of *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare (Wilson et al., 1968). *Aspergillus flavus* and *Aspergillus parasiticus* are differentiated from each other primarily by the colour of their colonies and the morphology of their conidial structures. The sterigmata of *Aspergillus flavus* are typically biseriate, whereas those of *Aspergillus parasiticus* are uniseriate (Pieter et al., 1980).

These microorganisms grow on a wide variety of crops, particularly in tropical and subtropical areas (Palmgren and Ciegler, 1983).

Wei and Jong (1986) and Klich and Pett (1988) have demonstrated physiological differences between these strains, particularly in their ability to produce aflatoxins. AFB_1, AFB_2, AFG_1 and AFG_2 are produced by *Aspergillus flavus*, while *Aspergillus parasiticus* toxin producers yield only AFB_1 and AFB_2.

3.3 CONDITIONS FOR PRODUCTION OF AFLATOXINS

Aflatoxins have been found in cocoa, fishmeal, milk, cheese, rice, coconut, cassava, corn, potatoes, peanuts,

soybeans, etc. (Christensen, 1975; Scott, 1985).

The geographical areas where aflatoxins are produced are generally warm and humid. Favourable conditions for fungal growth and aflatoxin production include high moisture content and high temperatures, optimum at 27ºC (Christensen, 1975).

In experimental conditions the amounts and relative proportions of aflatoxins B_1, B_2, G_1 and G_2 depend on the strain, balance of nutrients in the medium and culture conditions, including moisture, temperature, substrate, aeration, time of incubation, low pH and some trace metals (Detroy et al; 1971., Pait et al., 1975).

The basal medium used in most experiments with *Aspergillus parasiticus* was developed by Adyes and Mateles (1964) and is referred to as the AM medium.

Aflatoxin production is greatly affected by the identity and concentration of available carbon sources. Davis and Diener (1968) studied the growth and aflatoxin production of *Aspergillus parasiticus* on various carbon sources and concluded that, generally, compounds that are normally oxidized through both the hexose monophosphate and the glycolytic pathways supported both growth and aflatoxin production.

Shih and Marth (1974) studied aflatoxin, lipid synthesis and glucose metabolism by *Aspergillus parasiticus* during

incubation at 28°C for 15 days on glucose-salts medium or yeast extract and sucrose (YES) medium with and without agitation. Generally, more growth occurred in agitated cultures than in stationary cultures, but maximal yields of aflatoxin and total lipid were lower than in stationary cultures. These results suggest that the degree of aeration of the culture is important and that aflatoxin biosynthesis may be non-growth associated.

Engel (1975) reported the highest yield of aflatoxin production by *Aspergillus parasiticus* in yeast extract medium, with sucrose as the carbon source. Moderate yields were obtained with glucose, maltose and fructose, whereas lactose was considered unsuitable for aflatoxin formation. Similar results were reported by Honsa and Saxena (1988).

A medium that has been used for the production of aflatoxins by *Aspergillus flavus* and *Aspergillus parasiticus* is YES medium (2% yeast extract and 20% sucrose), (Smith, et al., 1992; Yabe et al., 1991, 1993). Studies by Abdollahi and Buchanan (1981) and Wiseman and Buchanan (1987) about culture media for aflatoxin production used a medium containing peptone and mineral salts with high levels of glucose.

3.4 AFLATOXIN BIOSYNTHESIS

In biosynthetic studies of secondary metabolite formation, the first and most frequently employed technique involves the testing of isotopically labelled precursors ^{2}H,

^{3}H, ^{13}C, ^{14}C, ^{15}N and ^{18}O) for specific incorporation into the metabolites. The second method employed involves the characterization of fermentation products closely related to the secondary metabolite under investigation, and the use of auxotrophic mutants in feeding experiments (Queener, 1976). By this method a substance can be established as an obligatory intermediate.

The biosynthesis of aflatoxin, via decaketide-derived intermediates is well established. The work has been greatly assisted by the use of *Aspergillus parasiticus* cultures. Using a combination of mutant strains, pathway inhibitors and feeding studies with radioactively and isotopically labelled precursors, the biosynthesis of aflatoxins has been extensively studied since their discovery in the 1960s (Sinz and Shier, 1991; Steyn, 1992). This biosynthesis is believed to begin with an enzyme-bound acetyl Coenzyme A carrier molecule which is then condensed with a variable number of malonyl Coenzyme A units, with accompanying decarboxylation of the malonate residues. Next, the long chain polyketide molecules undergo further reaction and rearrangement including aldol condensations to produce the ring structures common to many polyketide derivatives. These chemical reactions may include aromatization, oxidation, reduction, C-methylation, O-methylation, carboxylation, or decarboxylation, ring cleavage and ring expansion or contraction.

The first studies on aflatoxin biosynthesis elucidated the origin of the various carbon atoms within the aflatoxin structure using [1 - ^{14}C] and [2 - ^{14}C] acetic acid, [methyl-1 - ^{14}C] methionine and ^{14}C-labelled substrate and blocked mutants (Biollaz, et. al., 1968, 1970).

Several intermediates were isolated and proven to be precursors in the biosynthetic pathway to aflatoxin. They are the acetate-malonate condensation product, norsolorinic acid, and subsequent intermediates averantin, averufin, versiconal hemiacetal acetate, versicolorin A, sterigmatocystin and finally, the aflatoxin products. (See Fig. 3.2).

The initial steps, construction of the progenitor carbon skeleton, is believed to involve condensation of a malonyl Coenzyme A molecule with an acetyl Coenzyme A molecule, with concomitant loss of carbon dioxide and attachment to an acyl carrier protein-like molecule. The growing polyketide chain remains attached to the carrier protein until just before formation of the initial anthrone intermediate. One acetyl Coenzyme A molecule and nine malonyl Coenzyme A molecules are condensed in the pathway with the loss of nine molecules of carbon dioxide and four molecules of water to form the C-20 polyketide, anthrone intermediate (see Fig. 3.3). This anthrone is believed to undergo oxidation at the C-10 position to form the anthraquinone product norsolorinic acid, after which several reduction steps form averantin and averufin.

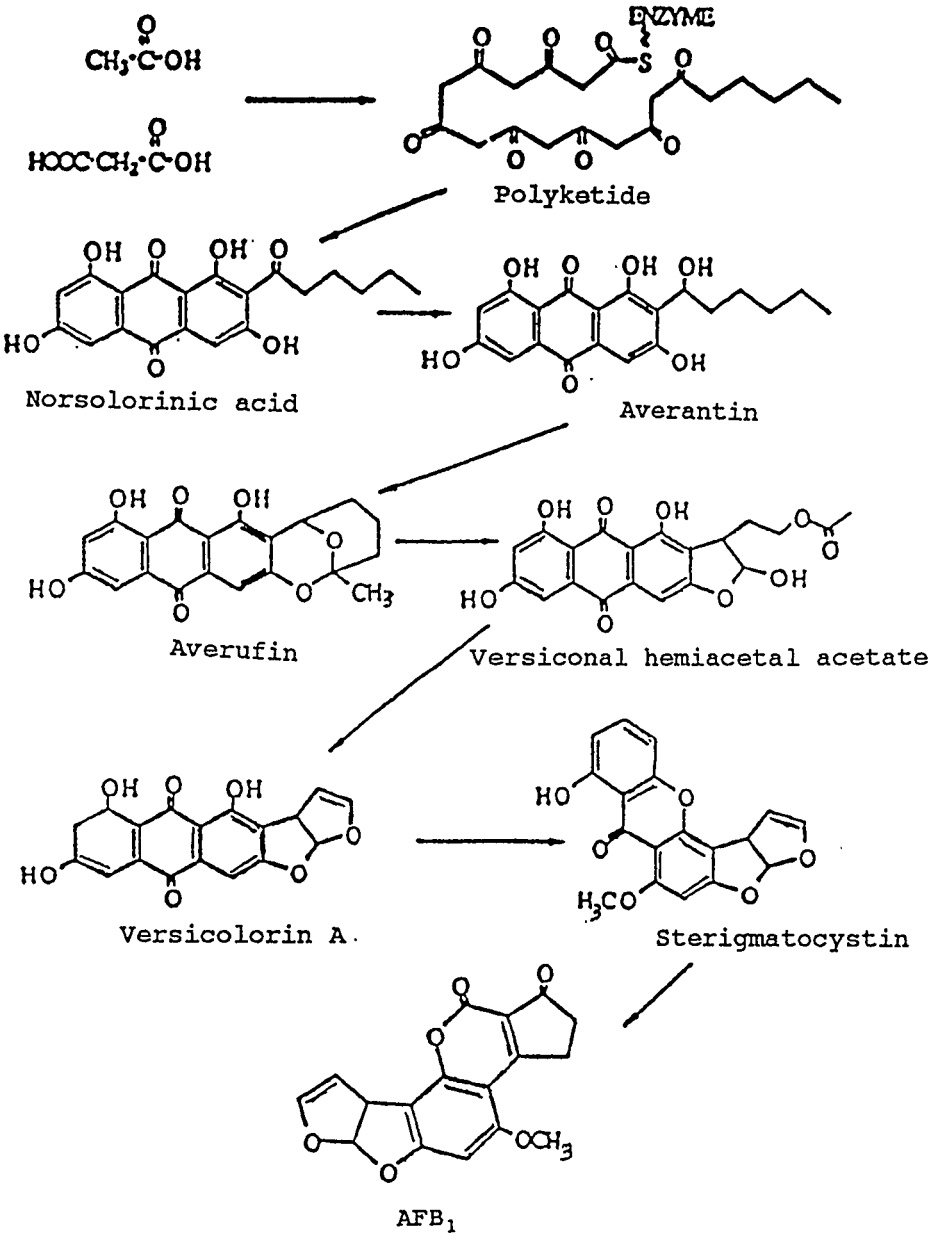
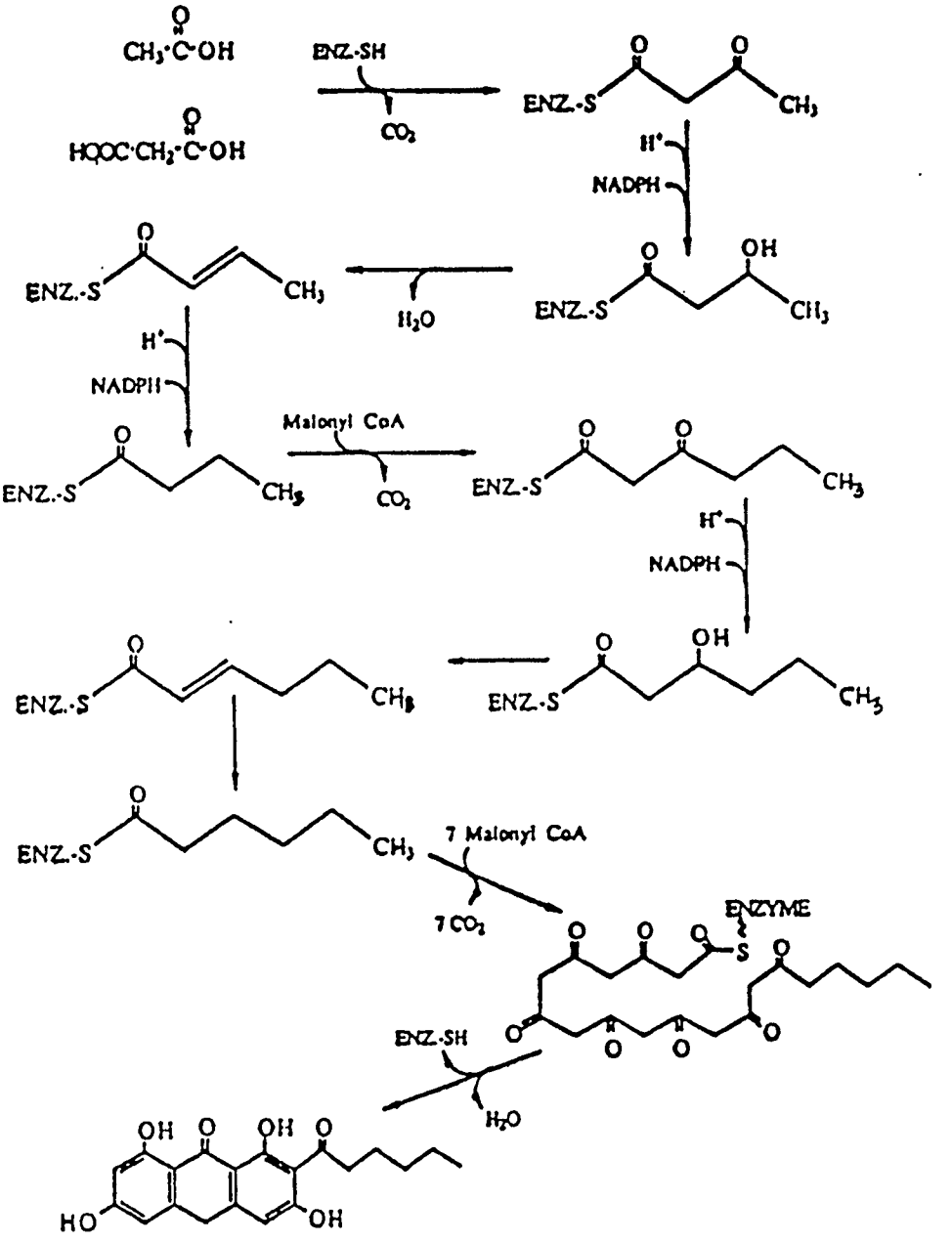


Fig. 3.2 Known intermediates in the biosynthesis of aflatoxin B_1 (Sinz and Shier, 1991)



Anthrone Intermediate

Fig. 3.3 Biosynthesis of the anthrone intermediate (Sinz and Shier, 1991)

The steps involved in the conversion of averufin to versiconal hemiacetal acetate are not yet fully understood (Sinz and Shier, 1991), although there are proposed intermediates in the conversion of averufin to versiconal hemiacetal acetate reported by Townsend and Christensen (1985) and Townsend, et. al., (1985). The next widely-accepted intermediate is versicolorin A, in which the bisfuran ring system of the aflatoxins has been formed. In the mechanism of versiconal hemiacetal acetate to versicolorin A, an oxygenase (hydroxylase) enzyme produces the unstable acylal intermediate from versiconal hemiacetal acetate, which, upon loss of acetic acid produces versiconal A hemiacetal, or by loss of water yields versicolorin A hemiacetal acetate, that can undergo transformation to sterigmatocystin (STG). The final steps in the formation of aflatoxins appear to diverge at some point near STG. The pathway to the formation of AFB_1 and AFG_1 appears to follow from STG to O-methyl-sterigmatocystin (OMST) by methylation at the C-3 hydroxyl group with S-adenosylmethionine catalyzed by a methyl transferase enzyme. AFB_1 is formed by oxidative ring cleavage of OMST, ring closure, dehydration and decarboxylation of the pentanone ring (Sinz and Shier, 1991).

An historical outline of the pathway leading to AFB_1 has been designed. (Steyn et al; 1980): polyketide precursor → norsolorinic acid (Hsieh et al., 1976) → averantin (Bennett et al., 1980) → averufanin (McCormick et al., 1987) → averufin

(Lin et al., 1973) → versiconal hemiacetal acetate (Shroeder et al., 1974) → versicolorin A (Lee et al., 1976) → sterigmatocystin (Hsieh et al., 1973) → O-methylsterigmatocystin (Bhatnagar et al., 1987) → AFB_1.

More recently, the research group of Yabe et al., have provided the experimental evidence for a more complete biosynthetic pathway between norsolorinic acid and versicolorin. The following steps were described in two papers: norsolorinic acid → averantin → 5′ hydroxyaverantin → 5′ ketonic averantin → averufin (Yabe, et al., 1993) → versiconal hemiacetal acetate ↔ versiconal acetate → versiconal → versicolorin B (Yabe and Hamasaki, 1993). It is, however, noteworthy that the earliest steps of aflatoxin biosynthesis remain unproven.

4. SECONDARY METABOLISM ENZYMES (SME)

The term SME denotes enzymes that participate directly in joining building units into more complex structures and transforming these structures to final products. They do not include enzymes synthesizing the basic building units of secondary metabolites. Secondary metabolic enzymes do have some special characteristics of their own. They are only formed at the commencement of secondary metabolism, that is during the idiophase (Behal, 1986) when normal growth has ceased and differentiation has commenced.

The diversity of structures, as well as of the bio-

synthetic pathways of secondary metabolite formation make it impossible to create a comprehensive system to classify the enzymes of secondary metabolism. They can be divided into two principal groups. First, there is the group that participates in the formation of the primary precursor or basic skeleton of the secondary product. The other group are the enzymes of secondary metabolism which modify the structure of the first committed precursor or basic skeleton, e.g. certain dehydrogenases, mono-oxygenases, methyltransferases and glycosyltransferases, which may sometimes possess a relatively low substrate specificity (Luckner, 1990).

Apart from these SMEs, tens of associated auxilliary enzymes are needed to provide energy, reduction or oxidation equivalents or the donors of the particular functional groups substituting the parent intermediate (Vanke et al., 1971).

The enzyme systems of secondary metabolism have been studied mainly for compounds of known structure and biogenesis. Polyketides, the substances formed by the condensation of acyl-CoA building units, are a relatively well-known group of secondary metabolites. A classic example of a polyketide biosynthetic pathway is the transformation of 6-methyl-salicylic acid, the parent intermediate of patulin in *Pencillium urticae*.

4.1 MULTIFUNCTIONAL ENZYMES

Multifunctional enzymes are enzymes with two or more

catalytic activities distributed along a single polypeptide chain. Only a few multifunctional enzyme systems have been discovered in secondary metabolism. One group is involved in the first step of non-ribosomal oligopeptide antibiotic biosynthesis (i.e. ACV, gramicidin, cyclosporin). Other types of multifunctional enzymes are the polyketide synthetases such as 6-methylsalicylic acid synthetase. These two groups of secondary metabolites (oligopeptides and polyketides) are assembled from completely different building blocks, but have some basic analogies in their biosynthesis. For instance, the polymerization of amino acids in the biosynthesis of the peptide antibiotics does not take place on ribosomes and is similar to the polymerization of acetate (propionate/buyrate) units in the biosynthesis of polyketides in that both the ß-ketoacyl and the aminoacyl units are bound to the enzyme complexes as thioesters. In addition, there are no detectable free intermediates and a covalently bound 4'- phospho-pantetheine group mediates the covalent transport of intermediates to the different active centres within the synthetase. Other multifunctional enzymes are either not well characterized (e.g. terpenoid synthases) or they represent single gene fusion (e.g. the expandase/hydroxylase involved in cephalosporin C biosynthesis in *Cephalosporium acremonium*.

4.2 MULTIENZYME COMPLEXES

Multienzyme complexes refers to oligomeric proteins, composed of non-covalently associated subunits with different

functions, capable of catalyzing two or more distinct reactions. There is not a clear distinction between multienzyme complexes and multifunctional enzymes since many multienzyme complexes contain both monofunctional and multifunctional chains, and some multifunctional enzymes are oligomeric proteins with multiple functions on different subunits.

Actinomycin biosynthesis has been shown to be catalyzed by a multienzyme complex. It is formed by the oxidative coupling of two 4-methyl-3-hydroxyanthranilic acid (4-MHA) pentapeptide lactones, catalyzed by a phenoxazinone synthetase isolate by Choy and Jones (1981).

4.3 MULTIFUNCTIONAL POLYKETIDE SYNTHETASES

Little is known about polyketide synthetases, due to their instability. The polyketide family of secondary metabolites include the structures assembled by the head-to-tail condensation of acetate, propionate, and butyrate units, according to the acetate "hypothesis" as derived from the classical studies of the aromatic polyketide 6-methylsalicylic acid. (Birch and Donovan, 1953). Despite the abundance of polyketides, little is known about the enzymology required for their biosynthesis. This is reflected in the fact that, although significant progress has been made in elucidating the biosynthetic origins of the various parts of many polyketides, and in determining the sequences of intermediates in their

biosynthetic pathways, these pathways have only recently begun to be confirmed by the systematic purification and characterization of the secondary enzymes involved.

4.4 MICROBIAL POLYKETIDE SYNTHETASE (PKS)

Over the past few years there has been an explosion in the amount known about polyketide biosynthesis. Much of this knowledge is based on genetic evidence from the streptomycetes, and recently from the filamentous fungi, but very little information is available about the enzymology involved.

Most of what is known about polyketide biosynthesis is inferred by analogy to fatty acid biosynthesis or from genetic evidence since very few of the enzymes involved have been purified. The problem in purifying these enzymes is that they are often present in small amounts and are highly unstable. (Dutton, 1988). To date, only one microbial polyketide synthase has been purified. That enzyme is 6-methylsalicylic acid synthetase from *Pencillium urticae* (Wang, 1991).

Although no bacterial PKS have been studied or purified to date, pioneering work on the molecular genetics of *Streptomyces* has lead to significant progress in the characterization of streptomycetes secondary genes and especially genes coding for a number of different bacterial PKS enzymes. Using the transformation of blocked mutants, the actinorhodin genes in *S. coelicolor* A_3 were the first to be

isolated. (Malpartida and Hopwood, 1984; 1986) and later the putative ß-ketoreductase gene (act III) was sequenced (Hallam et al., 1988). Using act I, the putative condensing enzyme gene and act III as heterologous probes, the PKS genes (gra) of the granaticin producer, *S. violaceoruber* were cloned and sequenced (Sherman et al., 1989). A combination of mutant complementation and hybridization with act I has identified the tetracenomycin PKS genes (tcm) of *S. glaucescens* for sequencing Bibb et al., 1989; Malpartida et al., 1987; Motamedi and Hutchinson, 1987). A similar strategy was employed to identify the oxytetracycline PKS gene (otc) from *S. rimosus* (Butler et al., 1989). The analysis of (gra) and (tcm) PKS genes (Bibb et al., 1989; Sherman et al., 1989) has provided evidence that polyketide biosynthesis in these bacteria involves a consortium of at least five (tetracenomycin) or six (granaticin) separate enzymes. Thus the bacterial PKS enzymes are not multifunctional as are the fungal enzymes.

4.5 PLANT POLYKETIDE SYNTHETASE

Only two plant PKS, a chalcone synthetase (Kreuzaler et al., 1979) and a resveratrol-forming stilbene synthetase (Schoppner and Kindl, 1984) have been purified to homogeneity.

Chalcone synthetase (CHS) catalyzes the formation of naringenin chalcone using p-coumaryl-CoA, as a chain initiator and three molecules of malonyl-CoA as chain extender. This

enzyme is a relatively small protein (84 kDa) being a dimer of identical subunits, each of about 42 kDa. Its genes have been cloned and sequenced (Niesbach-Klosgen et al., 1987). The substrate specificity has been explored and for the chain initiator it is variable. The enzyme does not have an acyl carrier protein (ACP) as do microbial PKS enzymes.

Resveratrol synthetase (RVS) (Schoppner and Kindl, 1984) is a key enzyme in the biosynthesis of stilbene-type phytoalexins, and catalyzes the formation of resveratrol, again from p-coumaryl-CoA and three molecules of malonyl-CoA. The enzyme from *Arachis hypogaea* (peanut) is very similar to the CHS. It is a dimer of identical subunits of 42 KDa. The gene (both genomic and cDNA) for the enzyme has been cloned and sequenced (Schroder et al., 1988).

4.6 MONOOXYGENASES ENZYMES

Monooxygenases (mixed function oxygenases) interact with molecular oxygen. They are defined as a group of enzymes which catalyze the incorporation of one atom of an oxygen molecule into the substrate while the second oxygen atom reacts with a reduced cosubsrate forming water. The term "mixed-function" oxygenase reflects this double function because monooxygenases may be classified on the basis of the electron donor:

1) Internal monooxygenases. These enzymes catalyze the incorporation of a single atom of molecular oxygen

concomitant with the reduction of the other oxygen atom by electrons derived from the substrate itself.

$$SH_2 + O_2 \rightarrow SO + H_2O$$

Examples of this group of enzymes are the aminoacid oxygenases.

2) External monooxygenases. These monooxygenases require various kinds of electron donors, for example: NADH, NADPH, L-ascorbic acid, tetrahydropteridines, etc., to be used as cosubstrates according to the equation:

$$S + O_2 + RH_2 \rightarrow SO + H_2O + R$$

RH_2 = reduced cosubstrate

R = oxidized cosubstrate

The hydrogen that is cleaved from the cosubstrates may be transferred in different ways to the oxygenating enzyme. In the simplest case it is taken over by a flavin cofactor which then carries out the reduction of oxygen to water. Enzymes of this type are, for instance, dehydrocyclopeptine epoxidase and cyclopenin m-hydroxylase from *Penicillium* (Luckner, 1990).

More complicated are enzymes which dehydrogenate the cosubstrate by a separate protein which itself interacts with the oxygenating enzyme. An example of this type is phenylalanine 4-monooxygenase (Luckner, 1990).

The most complex monooxygenases, however, are the membrane integrated enzyme complexes occurring, e.g. in liver and adrenocortex of mammals, which are involved in steroid

hydroxylation. These complexes possess special electron transport chains with cytochrome P450 at the terminal site. The substrate is linked to oxidized cytochrome P450. The cytochrome-substrate complex is reduced by an enzyme which has a "nonheme-iron" as prosthetic group, e.g. adrenodoxin. This protein reacts with a flavoprotein which in turn oxidizes the cosubstrate, e.g. NADPH. The reduced cytochrome substrate complex interacts with molecular oxygen and breaks down with the liberation of the oxygenated substrate and the formation of oxidized cytochrome. The most important types of reactions catalyzed by monooxygenases are as follows:

- Oxygenation of amines and thio compounds - a number of monooxygenases are capable of oxygenating amines and thio compounds to hydroxy derivatives and oxides, respectively.

- Incorporation of a single atom of molecular oxygen with the reduction of the other oxygen atom - these are internal monooxygenases.

- Hydroxylation of tetragonal carbon atoms and oxidative demethylation. Hydroxylation of tetragonal carbon atoms by monooxygenases replaces stereospecifically a hydrogen atom by a hydroxyl group. Nearby hydrogen atoms do not undergo any change in position or configuration. The monooxygenases directly attack the electrons of the C-H bond (Luckner, 1990).

Hydroxylation (oxygenase) activity has been demonstrated

in *Aspergillus* and related fungi, and is involved in oxidative modification of many secondary metabolites and this includes aflatoxins (Dutton, 1988). In the aflatoxin pathway (Fig. 4.1) the step from Averantin to 5'OH Averantin is an external monooxygenase reaction.

The biosynthesis of aflatoxins from acetate requires a series of oxidative transformations which, on general grounds, is expected to involve one or more monooxygenases which utilize p-450 membrane bound reductase (NADPH). These activities are often called polysubstrate monooxygenase (PSMO). The involvement of this enzyme system in the production of secondary metabolites has been demonstrated in several fungi, yeast and bacteria (Subramanian and Bhatnagar, 1986).

4.7 ENZYMATIC STUDIES OF AFLATOXIN BIOSYNTHESIS

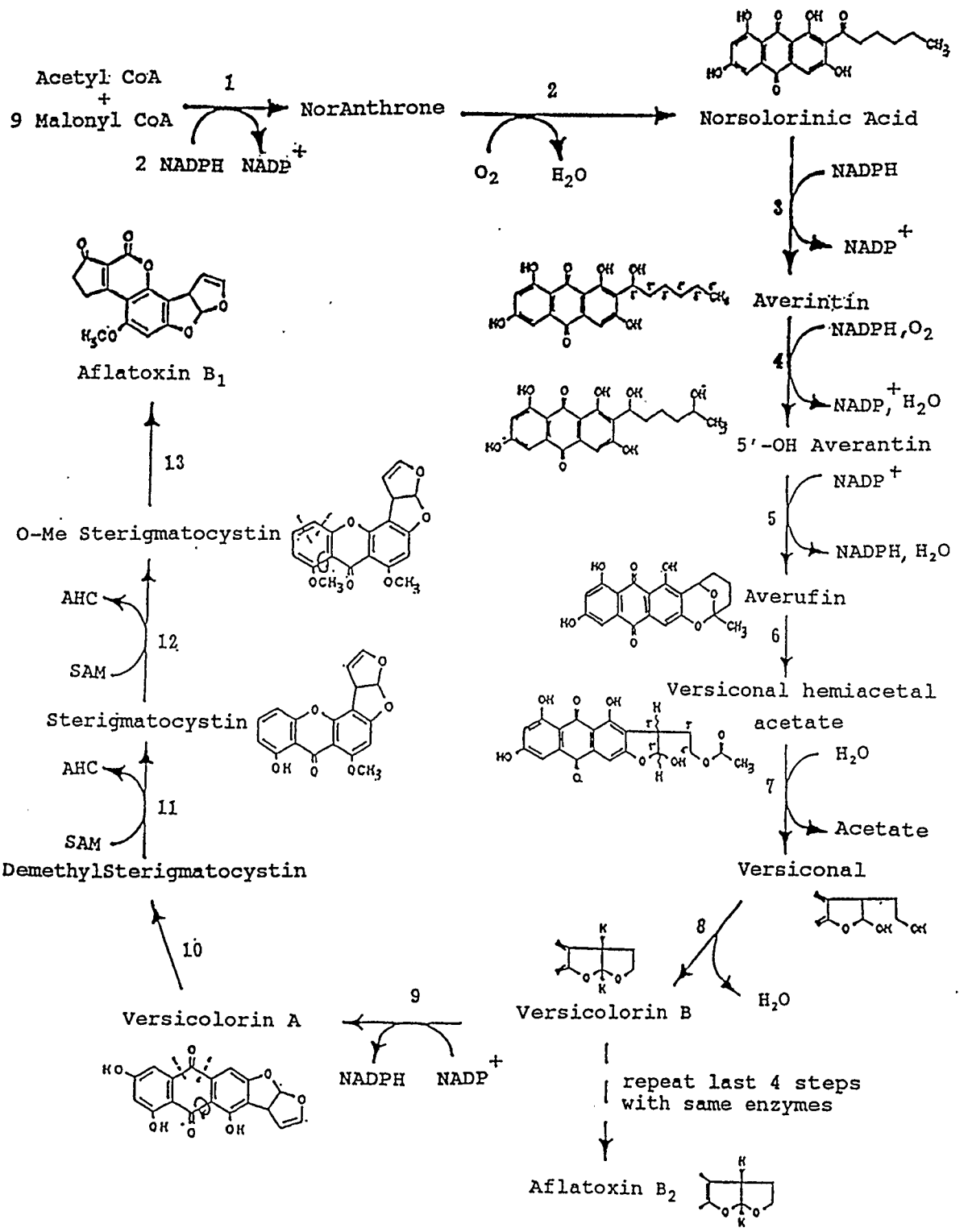
The enzymes required for the production of aflatoxins have not been characterized in as much detail as have the enzymes involved in primary metabolism. Initially enzymatic studies on aflatoxin biosynthesis were possible because the enzyme activities involved in aflatoxin biosynthesis may be divided into two main phases:

Phase 1) the enzyme activities involved with the construction of the anthraquinone precursor, catalyzed by polyketide synthetase, a multifunctional enzyme system similar to that

Fig. 4.1 Current Aflatoxin Pathway in *Aspergillus parasiticus* or *flavus*

Aflatoxin pathway enzymes that have been detected or purified in *A. parasiticus*, are as follows:

1. PKS, predicted, undetected.
2. Unknown
3. Dehydrogenase, purified (Chuturgoon et al, 1990)
4. Monooxygenase, detected (Yabe et al., 1991)
5. Dehydrogenase, detected (Yabe et al., 1991)
6. Unknown
7. Esterase, detected (Yabe et al., 1993)
8. Cyclase, purified (Lee and Anderson, 1992)
9. Desaturase, detected (Yabe et al., 1991)
10. Oxidoreductase, undetected (Yabe et al, 1988)
11. Methyl Transferase, purified (Bhatnagar et al., 1988)
12. Methyl Transferase, purified (Bhatnagar et al., 1988)
13. Oxidoreductase, detected (Cleveland and Bhatnagar, 1987)


Acetyl CoA
+
9 Malonyl CoA
1
2 NADPH NADP+
NorAnthrone
2
O2 H2O
Norsolorinic Acid
3
NADPH
NADP+
Averintin
4
NADPH,O2
NADP+, H2O
5'-OH Averantin
5
NADP+
NADPH, H2O
Averufin
6
Versiconal hemiacetal acetate
7
H2O
Acetate
Versiconal
8
H2O
Versicolorin B
repeat last 4 steps with same enzymes
Aflatoxin B2
9
NADPH NADP+
Versicolorin A
10
DemethylSterigmatocystin
AHC
SAM
11
Sterigmatocystin
AHC
SAM
12
O-Me Sterigmatocystin
13
Aflatoxin B1

involved in mammalian fatty acid biosynthesis; and

Phase 2) the enzyme activities involved with subsequent cleavage and rearrangement which take the anthraquinone to aflatoxin B_1 through versiconal acetate, versicolorin A, and sterigmatocystin. In this stage there are two conversions of sterigmatocystin (STG) to O-methylsterigmatocystin (OMST) that are catalyzed by an enzyme present in the postmicrosomal (supernatant) fraction. The second step in the conversion of OMST to AFB_1 was found to occur in the microsomal fraction (Steyn et al., 1980; Sinz and Shier, 1991).

Gatenbeck (1960) established the acetate-polymalonate origin of anthraquinones, which involves condensation of an enzyme-bound carboxylic acid derivative (acetyl) with a variable number of malonate units (nine units for the aflatoxin anthrone intermediate). The polyketide synthetase leads to enzyme-bound ß-polyketothiol esters, which undergo intramolecular condensation-aromatization and secondary transformations (Phase 1).

The isolation of secondary metabolic enzymes can often present technical difficulties because the enzyme with the desired catalytic activity is often overwhelmed by the presence of large amounts of other protein. Furthermore, during purification the small amount of enzyme present is very

sensitive to denaturation. Therefore, many secondary metabolic enzyme preparations fall far short of purification to a single protein, and often are hardly better than crude homogenates (Chuturgoon and Dutton, 1991).

To date, isolation and full characterization of the actual enzymes involved in the biosynthesis of aflatoxins has been very limited. Different works on the aflatoxin biosynthetic pathway are reviewed and will be seen to emphasize the enzyme detection and purification of Phase 2 of the pathway.

Norsolorinic acid (NA) is the first stable cyclization product detected in aflatoxin biosynthesis and an NA dehydrogenase activity converting this intermediate to averantin (AVN) has been identified and purified to homogeneity (Chuturgoon et al., 1990).

The relationship between averantin (AVN) and averufin (AVR) has been enzymatically clarified, AVN is successively converted to 5′ hydroxy averantin (HAVN) and then to AVR. Two enzymes, AVN monooxygenase and HAVN dehydrogenase, are involved in these reactions (Yabe et al., 1991, 1993).

Another enzyme that has been purified is versiconal cyclase which catalyzes the dehydration of versiconal to versicolorin B or versicolorin C. The enzyme was purified from mycelia of *Aspergillus parasiticus* by DEAE cellulose,

hydroxylapatite and Mono Q column chromatography (Lee and Anderson, 1992).

In relation to the last steps in the pathway, a methyltransferase which catalyses the conversion of sterigmatocystin (dihydrosterigmatocystin) to O-methyl-sterigmatocystin (dihydro O-methylsterigmatocystin) has been recently reported. This enzyme is produced *de novo* during the transition to idiophase in *Aspergillus parasiticus* (Cleveland and Bhatnagar, 1990) and was purified to > 90% homogeneity and the N-terminal sequence was determined (Keller et al., 1993).

The Fig. 4.1 shows the aflatoxin pathway and the enzymes that have been detected or purified.

5. SECONDARY METABOLIC GENES

Molecular biological techniques are less developed for use with the antibiotic producers than for the widely studied organisms such as bacteriophage, *E. coli* and humans. Nevertheless, considerable progress has been made with the actinomycetes because of the availability of suitable cloning vectors and efficient transformation protocols. Analysis of secondary genes isolated thus far have revealed two important features of their organization in bacteria:

1) all genes required to form secondary metabolite are clustered, covering ~20 to 70kb of contiguous DNA. (Hopwood et al., 1990); and
2) a resistance gene, usually required to protect the

producing organism from potential suicide, is closely linked to the biosynthetic genes.

Compared to their bacterial counterparts, molecular genetic studies of filamentous fungi, such as *Cephalosporium* and *Penicillium*, are still in their infancy. One of the reasons for this is the lack of an efficient transformation procedure. Recently, a number of high transformation efficiency vectors have been created for use in filamentous fungi (Austin, et al., 1990; Staben et al., 1989). These make use of phleomycin, bleomycin, or hygromycin B resistance, and in combination with a high frequency transformation procedure (for example, that of electroporation (Chakraborty and Kapoor, 1990) should lead to an explosion in the amount of knowledge available on the genetics of antibiotic biosynthesis in fungi.

5.1 POLYKETIDE SYNTHETASE GENES

As a result of the relative ease with which *Streptomyces* can be genetically manipulated, much is known about polyketide biosynthetic genes, although little is known about their enzymes. The initial genes isolated by mutant complementation were involved in actinorhodin synthesis, an acetate-derived metabolite produced by *S. coelicolor* A_3 (Malpartida and Hopwood, 1984, 1986). They were located on a 32kb stretch of DNA, within which mutations blocking actinorhodin (act) biosynthesis had been mapped.

Researchers have observed that the streptomycetes PKS

probes were unable to recognize fungal DNA homologs. As a result, knowledge of the genetics of fungal PKS genes has lagged behind those of the streptomyces.

The first fungal PKS gene, encoding 6-MSA synthetase, was isolated from *P. urticae* (Beck et al., 1990; Wang et al., 1991). The deduced amino acid sequence revealed five catalytic domains (ketoacyl synthetase (KS), acetyl/malonyl transferase (AT/MT), dehydratase (DH), ß-ketoreductase (KR), and an acyl carrier protein (ACP). These were homologous to vertebrate (i.e. rat, chicken, etc.) fatty acid synthetase (FAS) is both domain organization and amino acid sequence. In fact, this gene is more homologous to vertebrate fatty acid synthetase than to the fatty acid synthetase from *P. urticae*.

As is characteristic of secondary metabolism, the aflatoxin-biosynthetic pathway is not essential for growth (Bu'Lock, 1986). Little is known about the genetic basis of aflatoxin production versus nonproduction, largely because of the asexual nature of these *Aspergillus* species. The recent advent of molecular cloning procedures, however, has provided a means to study the mechanisms of gene expression in these fungi and to further elucidate the aflatoxin pathway.

Genes in the aflatoxin pathway can be identified by the same molecular techniques used to detect other fungal genes. Genetic complementation has been successful in detecting several genes (Ankis and Lambowitz, 1985; Kronstad et al.,

1989). For example, the *A. parasiticus trp* C gene was cloned by complementing an *E. coli* tryptophan mutant with a DNA library of the fungal genome (Horng et al., 1989). The *A. parasiticus nia* D, *pyr* G *ras*, and *ben* genes were cloned after identification with heterologous genes from other fungi (Horng et al., 1990, Skory et al., 1990). The recent isolation of the *afl*-2 and *apa*-2 genes indicates that the aflatoxin system now may also become a model for studying regulation of complex pathways in filamentous fungi (Bennett et al., 1993).

Several enzymes in the aflatoxin biosynthetic pathway have been purified with the intent to identify pathway genes (Bhatnagar and Cleveland, 1990; Keller et al., 1991).

There is evidence that some of the genes responsible for aflatoxin biosynthesis are differentially expressed under certain culture conditions related to growth and nutrition. (Cleveland and Bhatnagar, 1990; Niehaus, 1989). Hong Feng et al., (1992) reported a cloning strategy using three factors - growth phase, carbon source and temperature - which appear to induce differential gene expression prior to aflatoxin formation in *Aspergillus parasiticus* NRRL 2999. This strategy generated 19 clones enriched for genes related to alfatoxin biosynthesis.

Molecular techniques are now available for precise genetic dissection of the aflatoxin pathway. Transformation systems have been established for both toxin-producing fungi

(i.e. *A. parasiticus* and *A. flavus*). Genes have been cloned and are presently being used as probes to create a physical map of the genome in chromosome separation studies (Keller et al., 1993).

6. REGULATION OF SECONDARY METABOLISM

Regulatory mechanisms prevent metabolite overproduction and secure efficient nutrient utilization. Only extreme cultivation conditions or hereditary changes favouring the activity of secondary metabolic pathways make a major part of the metabolic activity proceed in this direction.

Secondary metabolism usually occurs when there is a decrease in the growth rate, rather than complete growth cessation. Its appearance is determined by the type of growth medium employed, and is due to a nutritional limitation which occurs after most of the cellular growth has occurred (idiophase) (Bu'Lock et al., 1965). Thus, secondary metabolite (ideolite) production is favoured while slow growth is occurring. Thus, in broad terms, growth at an optimum rate suppresses ideolite production.

The concept of the trophophase-idiophase transition was first put forth during studies on the production of patulin by Bu'Lock et al. (1965). This two phase kinetics was clearly defined in *Pencillium urticae* by Groot Wassink and Gaucher (1980).

Although the set of circumstances differs for each secondary metabolite, three nutrients have been shown to be critical: carbon, nitrogen and phosphorus. These elements can decrease secondary metabolite formation directly by targetting the enzyme involved (inhibition) or indirectly by targetting the transcription of the genes encoding those enzymes (repression).

Alternatively, positive control can affect the transcription of secondary metabolic genes. For example, a critical compound (e.g. a metal ion) may be required for the optimal transcription of a gene. This effect may act in concert with, or override, repression.

6.1 CARBON CATABOLITE REPRESSION

Carbon catabolite repression is a phenomenon referred to as the repression of biosynthesis of secondary metabolites by glucose and other simple forms of carbon, such as citrate. Thus, carbon catabolite repression cannot be considered simply as a "glucose effect". But this repression is commonly observed when glucose is used as the carbon source since it is an excellent carbon source for growth and interferes with the biosynthesis of many antibiotics. In a medium containing glucose plus a more slowly utilized carbon source, glucose is usually used first, and no antibiotics are produced. After glucose is depleted, the second carbon source may then be used for antibiotic synthesis.

The mechanism by which carbon affects gene expression is often unclear. One example is when *E. coli* is grown on glucose, the intracellular concentration of cAMP is low and a repressor is bound to the upstream region of the ß-galactosidase gene. As the concentration of glucose decreases, the level of cAMP rises, causing it to bind to its receptor. This cAMP receptor complex then binds near the promotor of the lac operon and can potentially activate gene expression.

The transcription of the lac operon does not occur until the repressor protein binding upstream of the gene is released. This only occurs in the presence of lactose, which causes allolactose (the inducer) to be formed.

It is not known whether cAMP plays a role in the carbon catabolite repression of secondary metabolite production. Studies on *S. kanamyceticus* (Saton et al., 1976) have shown that cAMP does relieve glucose repression of N-acetylkanamycin amidohydrolase, but in a majority of cases, this has not been seen. In fact, studies on *S. venezuelae* (Chatterjee and Vining, 1982), *S. griseus* (Ragan and Vining, 1978) and *P. urticae* (Lam, 1981) have suggested that an opposite effect occurs. That is, cAMP levels actually drop before secondary metabolite production begins.

Jones (1985) show that carbon catabolite repression occurs at the transcriptional level in studies on

phenoxazinone synthetase (PHS) from *S. antibioticus*. In these studies, the $_{M}$RNA for PHS seems to appear only after all of the glucose in the medium has been exhausted. In a more recent study (Espeso and Peñalua, 1992) isopenicillin M synthetase gene expression in *A. nidulans* has been shown to be controlled by carbon catabolite repression.

6.2 NITROGEN REPRESSION

Nitrogen sources such as ions can suppress the synthesis of many secondary metabolites (e.g. penicillin, cephalosporin and patulin) (Aharonowitz, 1979; Drew and Demain, 1977). A direct relationship between the levels of nitrogen nutrient and the onset of secondary metabolism has been shown in patulin biosynthesis by *P. urticae* (Groot Wassink and Gaucher, 1980).

Studies by Aharonowitz and Demain (1979), and Aharonowitz (1980), with *S. clavaligerus* have shown that the enzymes involved in ß-lactam production are regulated by the level of ammonia. Results were obtained by Sanchez et al., (1981) with *P. chrysogenum* and after using *C. acremonium* (Shen et al., 1984), where ammonium concentrations >110 mM strongly interfere with cephalosporin production, affecting expandase, but not cyclase activity. Recent studies have shown (Zhang et al., 1987) that ACV synthetase is also repressed, but not inhibited by ammonia.

In patulin biosynthesis, derepression of 6-MSA synthetase occurred when the NH_4^+ concentration was 3.5 mM and the 4th enzyme appeared ~ 2.5 h later (Newaskar, 1989). Repression of secondary messenger RNA production in *P. urticae* has also been reported (Fedeshko, 1992).

All these observations could be tied together into a working hypothesis suggesting that levels of NH_4^+ or certain amino acids repress antibiotic biosynthesis (Rollins and Gaucher, 1994). However, little is known about the molecular mechanism of nitrogen regulation at the transcriptional level.

6.3 PHOSPHATE REGULATION

Inorganic phosphate (Pi) is involved in the regulation of secondary metabolism. Generally, the synthesis of secondary metabolites and of structures required for cellular differentiation has a much narrower range of tolerance for environmental concentrations of Pi and of specific trace metals than does vegetative growth of the producer cells (Weinberg, 1982).

The formation of a number of secondary metabolites is inhibited by the presence of phosphate ions in the medium even at concentrations that are suboptimal for the growth of the production microorganism.

This type of regulation is a well known phenomenon in the biosynthesis of several compounds (e.g. tetracyclines,

macrolides, aminoglycosides and ansamycins). This regulation can effect different enzymes as shown in Table 6.1 (Liras et al., 1990).

There is a large body of experimental evidence that Pi favours the metabolic pathways of primary metabolism, namely the catabolism of carbohydrates, thus limiting the synthesis of the inducers or precursors of secondary biosynthesis.

6.4 POSITIVE REGULATION (INDUCTION)

Enzyme induction is a regulatory mechanism involved in controlling secondary metabolite biosynthesis. One of the best characterized examples of induction in fungi is quinic acid utilization in *Neurospora* (Geever et al., 1989). The presence of quinic acid causes the transcription of the genes required for its utilization.

The stimulation of idiolite biosynthesis is possible by the addition of a small molecule which is either a precursor/intermediate of the pathway, such as: methionine stimulation of cephalosporin production by *C. acremonium* (Sawada et al., 1988) and 6-MSA and m-hydroxy benzyl alcohol induction of all but the first of the patulin pathway enzymes in *P. urticae* (Gaucher et al., 1981).

Weinberg (1970) has reported that secondary metabolic processes require a unique quantity of a single trace element ("Key" metal ion) at a concentration that will neither

Table 6.1 Enzymes regulated by phosphate that are involved in antibiotic biosynthesis (Liras et al., 1990)

Antibiotic	Producing Organism	Target Enzyme	Mechanism of regulation[a]
Candicidin	*Streptomyce griseus*	p-Aminobenzoate synthetase	R
Cephalosporin	*Acremonium chrysogenum*	Deacetoxycephalosporin C synthetase[b]	D
Cephamycin	*Streptomyces clavuligerus*	α-Aminoadipyl-cysteinyl-valine synthetase	D
		Deacetoxycephalosporin C synthetase[b]	I
		Isopenicillin N synthetase[b]	I
Cephamycin	*Norcardia lactamdurans*	Deacetoxycephalosporin C synthetase[b]	I
Gramicidin S	*Bacillus brevis*	Gramicidin S synthetase	D
Neomycin	*Streptomyces fradiae*	Neomycin phosphate phosphotransferase	R
Streptomycin	*Streptomyces griseus*	Streptomycin-6-phosphate phosphotransferase	R
Tetracycline	*Streptomyces aureaufaciens*	Anhydrotetracycline oxygenase	R
Tylosin	*Streptomyces fradiae*	valine dehydrogenase	D
		Methylmalonyl-CoA:pyruvate transcarboxylase	D
		Propionyl-CoA carboxylase	D
		Protylonolide synthetase[c]	D
	Streptomyces T59-235	dTDP-D-glucose-4, 6-dehydratase	R
		dTDP-mycarose synthetase	R
		Macrocin O-methyltransferase	R

a R, repression; I, inhibition; D, depression of the enzyme occurs but the evidence does not prove unequivocally the existence of a repression mechanism.

b Enzymes involved in cephamycin and cephalosporin biosynthesis are less sensitive to phosphate control than other antibiotic biosynthetic enzymes.

c This is the only polyketide synthetase in this list of secondary metabolic enzymes.

stimulate nor inhibit vegetative growth. However, only recently has the requirement of a single metal ion for the expression of secondary metabolic genes been confirmed (Brown et al.,1990; Scott et al., 1986).

An important role in the induction of secondary metabolism can be played by endogenous inducers such as the "A-factor" in the synthesis of streptomycin (Khokhlov et al., 1967). The A-factor is excreted by cells of *Streptomyces griseus* and *Streptomyces bikinniensis*, and induces the synthesis of the streptomycin biosynthesis enzyme apparatus (Khokhlov and Tovorova, 1972) and at the same time affects development and morphological differentiation in these fungi.

6.5 REGULATION OF AFLATOXIN BIOSYNTHESIS

Investigations of the regulation of aflatoxin production have been focused on nutritional factors that enhanced or suppressed aflatoxin biosynthesis (see Section 3.3). Sources of carbohydrates are particularly important since carbohydrates provide the two carbon precursors (i.e. acetate) for toxin synthesis (Abdollahi and Buchanan, 1981; Neihaus, 1989).

Therefore, abundant aflatoxin is generally associated with substrates containing elevated levels of specific carbohydrates (Applebaum and Buchanan, 1979; Shih and Marth, 1974). Media containing certain carbohydrates, e.g. maltose, allowed induction of aflatoxin, but media using peptone as the sole carbon source did not allow this induction. In these

studies it was reported that one or more of the enzymes needed for aflatoxin synthesis were apparently not produced when *Aspergillus parasiticus* was cultured in a peptone-mineral salts medium, but transferring the cultures to a glucose-mineral salts medium resulted in aflatoxin production. Glucose appeared to not only serve as the carbon source for aflatoxin synthesis, but also played a role in regulating the induction of the enzymatic pathway responsible for the biosynthesis of the mycotoxins. (Abdollahi and Buchanan, 1981). However, the minimum amount of glucose needed to achieve this effect was not determined.

The type and concentration of the nitrogen source in the medium can have major effects on the production of aflatoxin. In general, organic nitrogen has been reported to stimulate aflatoxin synthesis. Kleinkauf and von Dohren, (1983) reported that nitrate is the main repressive nitrogen source of aflatoxin production in *Aspergillus parasiticus*.

Reddy et al., (1971) reported that aflatoxin production by *Aspergillus parasiticus* growing in a sucrose and asparagine medium was drastically reduced by increasing KH_2PO_4 concentration from 0.75 to 10 gL^{-1}.

In the biosynthesis of aflatoxins, trace metal ions play an important role and that is not completely understood, but it is known that the trace elements K^+, Fe^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, and Ca^{2+} are essential for fungal growth and most of them

are involved as prosthetic groups of enzymes (Fe^{2+}, Cu^{2+}, Zn^{2+}) or as enzyme activators (Mn^{2+}, Zn^{2+}, Co^{3+}, Fe^{2+}, Ca^{2+}). Maggon et al., 1977, reported that it is probable that Cu^{2+} and Fe^{2+} are essential for the cyclization of the polyketide progenitors of the aflatoxins. The stimulatory effect of Zn^{2+} on aflatoxin production is well documented. Lee et al., (1966) reported maximal aflatoxin production by *Aspergillus flavus* in a medium containing o.8 mg of zinc per litre. The minimum requirement of zinc for aflatoxin production was found to be o.4 μg mL- (Metales and Adye, 1965). Angelov et al., (1979) concluded that zinc probably participates in some of the enzyme systems which are responsible for the initial condensation of the acetate units.

Bennett has studied the effects of trace elements, nitrogen source, and white light on versicolorin production. (Bennett, 1979, Bennett et al.,1981).

In the regulation of aflatoxins many compounds, including a number of botanical substances and food preservatives, inhibit aflatoxin biosynthesis in *Aspergillus flavus* and *Aspergillus parasiticus*. (Bhatnagar and McCormick, 1988; Zaika and Buchanan, 1987). These compounds, however, are usually required in very high concentrations to inhibit fungal growth or aflatoxin biosynthesis at an early site in the aflatoxin pathway. A number of insecticides, e.g. dichlorvos, carbaryl, trimethacarb and pyrethrum also inhibit the

aflatoxin pathway. (Draughon and Ayres, 1981; Hsieh, 1973). Dichlorvos, an organophosphate that is a strong aflatoxin inhibitor, blocks aflatoxin synthesis at a point near the end of the pathway between versiconal hemiacetal acetate (VHA) and sterigmatocystin, causing the accumulation of VHA. (Bennett et al., 1976).

7. OBJECTIVES OF THE PRESENT STUDY

The long term research objective of this laboratory has been to obtain a complete molecular understanding of fungal polyketide biosynthesis. Initial attention was focussed on the biosynthesis of the classic aromatic tetraketide 6-MSA, an intermediate in patulin biosynthesis.

Recently we have been working with the intention of expanding our knowledge of fungal PKS enzymes, using other model polyketide pathways such as aflatoxin biosynthesis. The biosynthesis of these secondary metabolites involves the synthesis of polyketide intermediates by large PKS complexes which contain all the enzyme activities required for chain elongation, carbonyl reduction, and ring formation.

Aflatoxins are potent carcinogens and are economically important. For these reasons fundamental studies of the biosynthesis and molecular genetics of aflatoxin production may lead to a solution to the aflatoxin problem. But limited progress has been made towards an understanding of the enzymes

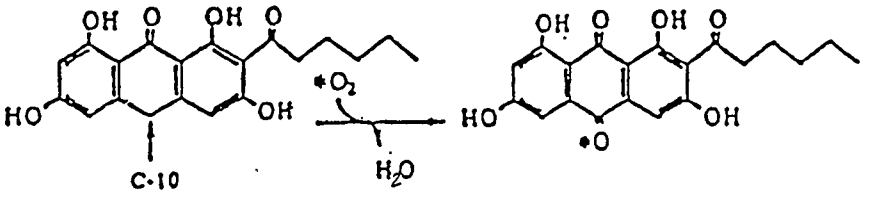
involved in aflatoxin biosynthesis and their regulation. To date very few enzymes involved in the biosynthesis of the aflatoxins have been isolated and fully characterized, and the enzymes responsible for the earliest steps in the pathway have received even less attention. In this pathway a condensation product, an anthrone, is first oxidized to an anthraquinone, norsolorinic acid (NOR) but no intermediate anthrone has ever been isolated and nothing is known about the requisite oxygenase. Thus, the purpose of the present investigation was to carry out a preliminary study of the production and purification of a new enzyme at the beginning of the aflatoxin pathway, norsolorinic anthrone monooxygenase. This secondary enzyme catalyses the formation of norsolorinic acid from norsolorinic anthrone, (Fig. 7.1), and is thus the second step of the pathway.

The specific objectives of this study were:

1. to determine culture conditions which would improve the production of norsolorinic acid and the enzyme that produces it;
2. to develop and optimize the assay for the monooxygenase;
3. to determine the culture dynamics of enzyme appearance in order to obtain cells with maximum secondary enzyme content;
4. to optimize the stability of the enzyme in cell extracts in anticipation of the purification;
5. to maximize the solubilization of the enzyme if membrane

bound;

6. to carry out a preliminary purification of the enzyme.



Anthrone Intermediate Norsolorinic Acid

Fig. 7.1 Transformation of the anthrone intermediate to Norsolorinic acid (Sinz and Shier, 1991)

PART II

EXPERIMENTAL PLAN

8. CULTIVATION METHODS

All procedures requiring aseptic manipulation were performed in a sterile laminar flow hood (Labgard Laminar Flow Biological Safety Cabinet, Model NV-408 FM600, Nu Aire Inc.). The work surface was wiped with 95% ethanol before use. Sterile technique was carefully observed.

Sterilization of solutions and equipment was carried out at 121°C and 20 psi in automatic autoclaves (General Purpose and Vacmatic "A" models, AMSCO) for 20-45 minutes using liquid or dry cycles as appropriate.

8.1 FUNGAL STRAIN

A norsolorinic acid accumulating mutant, *Aspergillus parasiticus* ATCC 24690/SRRC 162A was used as the source of cells for production studies and purification of the nor-anthrone monooxygenase enzyme.

8.2 STRAIN MAINTENANCE

Preservation of the fungal strain was carried out by two methods:

8.2.1 Filter paper strip preservation: This method was used for long term storage (>1 year). Single colony isolates from Petri dish culture were grown on Potato Dextrose agar slants for 8 days at 28°C. A suspension of conidia was prepared by adding 5 mL of detergent solution (Aerosol OT, 450 ppm in double de-ionized water, Fisher) and then mixing (30 s,

top speed, Vortex-Genie, Fisher). Sterile Pasteur pipettes were used to transfer 2-3 drops of this spore suspension to filter paper strips (2 X 10 mm, 3 MM, Whatman) in cotton wool plugged, neutral glass ampoules (Gold Band Cryule, Fisher) that had been previously sterilized. Ampoules were then lyophilized (24 h, Freezemobile 12, Virtus), evaporated using an electric vacuum pump, sealed using a Bunsen flame and stored at -70°C. These paper strips were in turn used to inoculate agar slants for the subsequent preparation of silica gel stock cultures.

8.2.2 Silica gel/lyophilization techniques: This method was used for shorter time intervals (1 month to 1 year). Glass specimen bottles (29.6 mL, Fisher) were filled to a vertical depth of 2 cm with a non-fluorescent silica gel (Silica Gel 60, 70-230 mesh, Merck), topped with cotton plugs, lightly capped and dry sterilized (20 minutes). Since silica gel releases heat upon addition of water, sterilized bottles containing silica gel were cooled (4°C) prior to the addition of the spore suspension. This suspension was prepared from 2 to 3 agar slant cultures by the successive tranfer of a single 5 mL aliquot of detergent solution as described above. This spore suspension was slurried with the silica gel, and then lightly vortexed to ensure even distribution of spores prior to lyophilization (24 h). All subsequent inocula were taken from these silica gel stocks, which were stored tightly capped at 4°C.

8.3 POTATO DEXTROSE AGAR CULTURES

Agar surface cultures were used for strain preservation and as a source of conidial inocula for submerged cultures. The agar solution was prepared by heating and stirring 39 g of Potato Dextrose Agar (PDA) (Difco) with 20 g Bacto Agar (Difco) in 1 L of double de-ionized water until a homogeneous solution was achieved. Aliquots (10 mL) of this solution were pipetted into glass specimen bottles (29.6 mL, Fisher), lightly capped and autoclaved (20 min). Following autoclaving the caps were tightened and the agar was allowed to solidify after placing the bottles horizontally, supported by a 10 mL pipette beneath their capped ends. Spores from silica gel stocks (8.2.2) were inoculated onto the surface of these PDA slants by use of a moistened, sterile platinum wire loop. Inoculated slants were loosely capped and incubated at 28°C for 8 days. Spore suspensions were obtained by the addition of detergent (Aerosol OT, 5mL/slant) and vortexing as above (8.2.1).

8.4 LIQUID CULTURE MEDIUM

Potato Extract Glucose (PEG) medium: this was developed for the cultivation of *Aspergillus parasiticus* SRRC 162A in submerged culture in our laboratory (Wong,1993, personal communication). This medium consists of:

	Final Concentration (g/L)
Potato Extract (Difco)	4
Glucose	40

KH_2PO_4	13.7
NH_4Cl	1.6 (30 mM)
Trace Elements	10 mL/L

Pre-sterilization pH adjusted to 5.6 with 1N HCl.

Fresh Potato Extract Glucose (FPEG) medium: this was developed for the cultivation of *Aspergillus parasiticus* SRRC 162A in submerged culture in our laboratory, because the dry powdered potato extract from Difco was discontinued and the other medium components were the result of the study of medium conditions. This medium consists of:

	Final Concentration (g/L)
Fresh Potato Extract (800mL)	from 300g potato
Glucose	40
KH_2PO_4	13.7
NH_4Cl	0.8 (15 mM)
Trace elements	10 mL/L

Pre-sterilization pH adjusted to 4.0 with 1N HCl.

In both media the glucose was made up as a 20% solution (40g/200mL) and autoclaved separately to avoid caramelization. The remaining components were dissolved in double de-ionized water (ddw) for PEG medium and for FPEG in fresh potato extract in a final volume of 800 mL.

The fresh "home made" potato extract was prepared by using 300g of white unpeeled fresh potatoes washed and cut into approximately 1.5 inch squares. The pieces were then

boiled in 1L of ddw for about 30 minutes. Once the potatoes were cooked they were filtered through a double layer of cheesecloth to obtain the potato extract.

The trace metals solution was prepared according to Yamamoto and Segel (1966) and was composed of the following salts:

	g/L	Metal (μM)
Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	50	2460
Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	5	ND
Manganous chloride ($MnCl_2 \cdot 4H_2O$)	3	152
Ferric chloride ($FeCl_3 \cdot 6H_2O$)	2	74
Zinc chloride ($ZnCl_2$)	2	174
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.75	51
Cobaltous chloride ($CoCl_2 \cdot 6H_2O$)	0.2	8.4
Cupric chloride ($CuCl_2.2H_2O$)	0.2	11.7
Molybdenum pentachloride ($MoCl_5$)	0.1	3.7
Sodium Borate ($Na_2B_4O_7.10H_2O$)	0.1	ND

Chloroform (2 mL/L) was added to this trace metal solution as a preservative, and the solution was stored in dark bottles at 4°C.

8.5 STUDY OF THE CULTURE MEDIUM CONDITIONS FOR IMPROVED NORSOLORINIC ACID AND ENZYME PRODUCTION

For a good growth of A. *parasiticus* and a good production of norsolorinic acid, so as to increase the levels of the enzymes responsible for norsolorinic acid synthesis, the

effect of the following were studied:

1) Nutritional factors - The effect of changing the concentration of different medium components individually with respect to the composition of the original medium (8.4) was studied as follows: lower glucose (30 g/L) and NH_4Cl 0.53 g/L (10 mM), 0.8 g/L (15 mM) were examined. Then additional variations were also examined such as: NH_4NO_3 0.8 g/L (10 mM) and 1.2 g/L (15 mM) and fresh "home made" potato extract at different concentrations (600, 450 and 300 g potatoes/L of medium). The glucose concentration experiment was carried out using fresh potato extract (300g potatoes/L of medium). All these experiments were carried out in shake flasks using an inoculum concentration of 10^6 spore/mL.

2) pH - The effect of initial culture pH on norsolorinic acid production was determined in shake flask cultures. Erlenmeyer flasks (500 mL) containing 50 mL of Fresh Potato Extract Glucose medium (see 8.4) adjusted to various pH's from 3.0 to 5.6, were inoculated with a suspension of *A. parasiticus* spores (10^6 spores/mL) and incubated at 28°C and 280 rpm.

3) Inoculum concentration - Different inoculum concentrations of 10^5 and 10^6 spores from a suspension of conidia (8.2.1) were obtained by spore counting using a Neubauer haemocytometer and Jenaval light microscope. These concentrations were used to inoculate Erlenmeyer flasks (500 mL) containing 50 mL of Fresh Potato Extract Glucose medium

(see 8.4).

8.6 SHAKE FLASK CULTURE

Shake flask cultures were grown in 500 mL Erlenmeyer flasks containing 50 mL of medium. Thus aliquots (40 mL) of glucose deficient PEG medium prepared with different variations (see 8.5) were dispensed into flasks and were plugged with cotton wool/linen plugs and autoclaved for 20 minutes. The sterile glucose solution (10 mL) was then added to each flask prior to inoculation. In order to increase culture homogeneity and minimize wall growth, considerable care was taken in the preparation of these flasks. Flasks were soaked in 10-20% Extran 300 detergent solution (BDH) for at least 24 h and then thoroughly scrubbed, washed, rinsed, dried and coated before use. Coating was achieved by rinsing the flasks with a 10% solution of the water repellant dimethyldichlorosilane (Dri-film 5C-87, Pierce) in chloroform, after which they were drained and heated in an oven at 100°C for 1 hour.

After inoculation, flasks and their contents were incubated at 28°C on a New Brunswick Gyrotory Shaker (Model G10) operating at 280 rpm, with a stroke of 2.5 cm.

8.7 FERMENTOR CULTURE

The fermentor culture was grown in a 14 L fermentor jar (Micro Ferm Fermentor, New Brunswick Scientific) containing 8L of Potato Extract Glucose (PEG) medium or Fresh Potato Extract

Glucose (FPEG) medium (8.4). The latter was a medium developed from shake flask culture experiments (8.5). Thus, 6.4 L of each medium solution was prepared directly in a 14 L fermentor jar, and a glucose solution (1.6 L) was made up in a Fernbach flask separately, and both were autoclaved for 45 min. Before medium preparation, coating of the interior of the clean fermentor jar was achieved by wiping with Kimwipes previously impregnated with a 10% solution of dimethyldichlorosilane in chloroform, and air-dried overnight. After inoculating PEG medium with spores (1 x 10^6 spore/mL), or Fresh PEG medium with spores (1 X 10^5 spore/mL), the fungus was grown at 28°C with an aeration rate of 8L per min. The agitation was maintained at 300 rpm using two Rushton impellers positioned 8cm apart on the impeller shaft. Antifoam A (100 mL of a 3% emulsion, Sigma) autoclaved was added to each 8L used to prevent foaming.

8.8 EFFECT OF NH_4Cl CONCENTRATION IN FERMENTOR AND REPLACEMENT CULTURES

The effect of NH_4Cl concentration (15 mM) was studied in a fermentor with Fresh PEG medium, (see 8.4) and with an inoculum concentration of 10^6 spores/mL. In a separate experiment, 50 mL aliquots from a 24 h old 8 L fermentor culture grown on 15 mM NH_4Cl, were transferred to eight 500 mL Erlenmeyer flasks. Two of the flasks were untreated (keeping the same medium composition as the fermentor) while the remaining flasks were supplemented with ammonium chloride to

give final concentrations of 30 mM, 50 mM and 75 mM in duplicate flasks. After an additional 48 h of incubation at 28°C and 280 rpm, norsolorinic acid production was determined.

8.9 SAMPLING OF CULTURES

Sampling of shake flask cultures was carried out by removing two flasks from the shaker at 24, 30, 48, and 54 h. Sampling from fermentor cultures were made by syphoning about 300 mL aliquots out of the fermentor at different times. Culture parameters such as dry weight, pH, norsolorinic acid concentration and enzyme assays were studied with these samples.

9. CULTURE PARAMETERS/ASSAYS

9.1 DRY WEIGHT MYCELIUM

Culture growth was monitored by determining the total dry weight of mycelium (biomass) per L of culture. This parameter was most often used as a measure of the observed growth of the organism. The mycelium was harvested by vacuum filtration through Kimtuff towels (Kimberley-Clark, Toronto), washed with distilled water, the biomass was dried at 80°C for 24 h in FISHER, isotemp oven, 200 Series, and weighed immediately after cooling for 5 min to room temperature.

9.2 CULTURE pH

The pH of shake flask cultures and fermentor cultures were monitored by inserting a pH probe (Corning pH/Ion Meter 135) into the medium containing mycelium. After a 5 minute,

equilibration period with stirring, the pH was recorded.

9.3 NORSOLORINIC ACID CONCENTRATION

Norsolorinic acid concentration was determined by treating an aliquot (25mL) of the whole cell culture with 10M NaOH to give a final concentration of 0.2M (pH 11.0). The resultant mixture was stirred, using a Corning hot plate/stirrer PC 351, for 20 minutes at room temperature and the absorbancy read at 560 nm after centrifugation at 15,000 rpm (Eppendorf Centrifuge 5414). The norsolorinic acid concentration was determined from a standard plot prepared using norsolorinic acid synthesized and purified in our laboratory (Wong, 1993, personal communication).

9.4 PREPARATION OF CELL-FREE EXTRACTS

Cell-free extracts containing nor-anthrone monooxygenase activity were prepared from 200 mL of culture (equivalent to approximately 3g of dry cells) harvested at 24, 30, 48 and 54 h from shake flasks and from the fermentor cultures at 24 to 78 h. The cells were collected by vacuum filtration through a double layer of Kimtuff tissues (Kimberley-Clark, Toronto) and washed with de-ionized water. The washed cells were then resuspended in 100 mL of breakage buffer (100 mM phosphate [pH 6.5], 10% glycerol and 10 mM dithiothreitol, DTT) for cell breakage. The cells were ruptured by one pass through a Microfluidizer (Model 110T, Microfluidic, Newton, MA) with an inlet pressure of 90-95 psi (pressure was obtained by using a

pressurized nitrogen cylinder). This breakage was carried out in the cold room (4°C) with the entire base of the microfluidizer, including the interaction chamber, immersed in an ice water bath. A clarified supernatant of this crude extract (~ 100 mL) was prepared by centrifugation at 15,000 rpm (Eppendorf Centrifuge 5414) at room temperature for 10 minutes. Both the crude extract and the clarified supernatant were used for enzyme assays.

9.5 ENZYME APPEARANCE STUDIES

To ascertain the trophophase-idiophase transition time, time course studies of dry weight accumulation and appearance of nor-anthrone monooxygenase were conducted. Fermentor cultivation was carried out using Fresh PEG medium as described previously (8.4). Then the first samples were taken 22 hours after inoculation, and at different time intervals up to 68 hours after inoculation. The pH, dry weight, norsolorinic acid concentration and enzyme activity of these samples were determined (see 9.1, 9.2, 9.3, and 9.4).

10. DEVELOPMENT OF AN ENZYME ASSAY

This assay is based on the measurement of the formation of norsolorinic acid from nor-anthrone by a monooxygenase enzyme. The initial monooxygenase assays were carried out in a total assay volume of 500 μL as follows:

1M phosphate buffer, pH 7.0	33 μL
H_2O	84 μL
Ethylene glycol monomethyl ether (EGMME)	233 μL
Nor-anthrone* (1.0 mM in EGMME)	50 μL
Enzyme (crude extract or supernatant)	100 μL (20% of Total Vol.)
	500 μL

[* Final assay concentration of substrate = 0.1 mM]

The assays for monooxygenase activity were compared to boiled (30 min) enzyme to account for non-enzymatic oxidation of noranthrone. For the normal assay and the boiled enzyme assay, blanks were prepared with all of the above components except the anthrone substrate. All of the assay reactions were incubated at 10°C for 10 minutes and then stored at -70°C until analysis by HPLC.

10.1 NORSOLORINIC ACID QUANTIFICATION

Norsolorinic acid was quantitated directly by HPLC using a Shimadzu (SCL-6B) HPLC system equipped with dual LC-94 pumps (SPD-6AV), UV-VIS spectrophotometric detector, and a CR501 data processing unit with a Supelcosil LC-18 25.0 cm X 4.6 mm column. Enzyme reaction mixtures were centrifuged at 15,000 rpm for 2 minutes and an aliquot (20 μL) of the supernatant was injected onto the column. Elution was carried out isocratically at 1.5 mL/min with 1.2:0.3 (v:v) of solvent A: solvent B (A = 0.01% trifluoroacetic acid (TFA), in water, and

acetonitrile, 80:20, v:v; B = water: 0.05% TFA in acetonitrile, 15:85, v:v) and monitored spectrophotometrically at 238 nm. NA was identified in reaction mixtures based on its retention time compared to standard samples. Then the area of each norsolorinic acid peak from the HPLC analysis was converted to an amount of norsolorinic acid (nMol):

μg NA in 20 μL of assay mixture

$$= \frac{\text{area count-intersect with Yaxis}}{\text{slope of curve}}$$

to convert μg NA to nMoles of NA/assay

$$= \mu\text{g NA} \times \frac{\text{assay volume (500 } \mu\text{L)}}{\text{sample size (20}\mu\text{L)}} \times \frac{1}{\text{MW of NA (370)}} \times 10^3$$

$$= \mu\text{gNA} \times 67.57$$

The enzyme containing crude extract or supernatant already contained some norsolorinic acid and there is non-enzymatic production so the real activity was calculated as follows:

$$\frac{\text{Enzyme Activity}}{\text{(nMol NA/assay)}} = [NA_e - NA_{eb}] - \{NA_{ne} - NA_{neb}\}$$

Where: NA_e = NA from enzymatic reaction

NA_{eb} = NA in enzymatic blank (No substrate)

NA_{ne} = NA from non-enzymatic reaction (boiled enzyme plus substrate)

NA_{neb} = NA in non-enzymatic blank (boiled enzyme, no substrate)

$[NA_e - NA_{eb}]$ = NA from enzymatic and non-enzymatic reactions minus *in situ* NA

$\{NA_{ne} - NA_{neb}$ = NA from non-enzymatic reactions minus *in situ* NA

Then monooxygenase activity = nMol NA/assay and this unit of enzyme activity was used for all development work since assay times, temperatures, etc. were changed. Later when the assay was optimized or finalized a milliunit (mU) of norsolorinic anthrone monooxygenase was defined as the amount of enzyme catalyzing the formation of 1 nMole of nor-acid/min., under the now standardized conditions. To convert nMol nor-acid/assay to nMol nor-acid/min (mU) values were multiplied by two because this fixed time assay was carried out for 30 sec. See 10.2 for the description of the final optimized assay.

10.2 OPTIMIZATION OF THE MONOOXYGENASE ACTIVITY

Accurate culture dynamics require the development of the most sensitive, and accurate enzyme assay. Thus, to confirm if the initial assay conditions (10) were optimum, the following variables were studied:

1) Reaction time - incubation times of the enzyme assay were varied (0.25, 0.5, 1.0, 2.0, 5.0, 10, 20 and 30 minutes). The assay conditions for the incubation times of 2 to 30 min. were as described in section 10. For the earlier incubation times (0.25-2.0 min) conditions were: pH 7.5, temperature 10°C, enzyme concentration 100μL and substrate concentration 0.6 mM, and the reactions were terminated by rapid freezing with dry ice.

2) Temperature - the effect of incubation

temperature was determined by performing assays at 30°C, 20°C, 10°C, 0°C, -10°C and -15°C. The assay conditions were: pH 7.5, enzyme volume 100 μL, substrate concentration 0.6 mM, and a reaction time of 30 secs.

3) pH - the enzyme was assayed with 1M phosphate buffer using different pH values (6.0, 6.5, 7.0, 7.5), 1M citrate buffer (pH 4.0, 5.0, 5.5) and 1M TRIS at pH 8.0 and 8.5. The assay conditions were: temperature 0ºC, substrate concentration 0.6 mM, enzyme volume 100 μL, reaction time 30 sec.

4) Substrate concentration - to ensure that substrate concentration was not limiting, assays were performed at various concentrations (0.1 mM, 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, and 1.0 mM) of nor-anthrone as final concentrations in the assay. The assay conditions were: pH 7.5, enzyme volume 100 μL, temperature 0ºC, reaction time 30 sec.

5) Enzyme concentration - various enzyme volumes were used (0, 2, 4, 8, 10, 16 and 20% of the total volume of the assay). The assay conditions were: pH 7.5, temperature 0ºC, substrate concentration 0.6 mM, reaction time 30 sec.

All subsequent enzyme assays, except for experiment (12.1) and the first part of the experiment (12.2) were performed using the optimum conditions for the nor-anthrone

monooxygenase, which were:

1M phosphate buffer, pH 7.5	33 μL
H_2O	84 μL
EGMME	233 μL
Nor-anthrone	50 μL (0.6 mM final assay concentration)
Enzyme (crude extract or supernatant)	100 μL (20% of total vol.)
	500 μL
Incubation temperature:	0ºC
Reaction time:	30 sec. terminated with a freeze quench (dry ice)

10.3 INACTIVATION OF THE MONOOXYGENASE AND THE NON-ENZYMATIC LOSS OF THE ANTHRONE SUBSTRATE

To verify if 30 minutes of boiling was sufficient for enzyme inactivation, the enzyme (i.e. crude extract) was boiled for 20, 30 and 40 minutes, then assayed for activity. A 4.5 mL aliquot of the crude cell-free extract (enzyme) was also treated with proteinase K (0.5 mL of a 10 mg/mL solution) for 1 h at room temperature. Untreated crude extract was treated in an identical manner and assayed at the same time as the proteinase K treated sample.

The lability of the substrate (nor-anthrone) was investigated in the following conditions, H_2O (184 μL), EGMME (233 μL), nor-anthrone (0.6 mM, 50 μL), 1M phosphate buffer (33 μL) at pH 6.0 and 7.0 for 5 and 30 minute incubations at each pH, in the absence of any cell-free extract. The samples

were stored at -70°C until HPLC analysis.

11. REQUIREMENT FOR COFACTORS IN MONOOXYGENASE ACTIVITY

Monooxygenase activity was determined using the optimum assay conditions (10.2), and adding to the reaction mixture the following compounds dissolved in the reaction buffer (1.0M phosphate, pH 7.5):

Individual Cofactors	Final Concentration (mM)
NADPH	9
DTT	3.3
$CoCl_2$	5
EDTA	5
FAD	5
FMN	5
$FeSO_4$	5
$CuSO_4$	5
$MgSO_4$	5
$ZnSO_4$	5
Ascorbic acid	2.9
$CdCl_2$	5
α-ketoglutarate	0.825
Mixture #1: Ascorbic acid	2.9
α-ketoglutarate	0.825
$MgSO_4$	5
$FeSO_4$	5
DTT	3.3
Mixture #2: Ascorbic acid	2.9
α-ketoglutarate	0.825
$MgSO_4$	5
DTT	3.3

The assay was also performed in Eppendorf tubes in the

absence of O_2 by bubbling N_2 through the reaction mixture (minus substrate) for 25 min, then sealing the tube with parafilm after adding substrate to start the reaction. All the assays were incubated at 0°C for 30 s and kept at -70°C until HPLC analysis.

12. PRELIMINARY PURIFICATION OF NOR-ANTHRONE MONOOXYGENASE ENZYME

12.1 COMPARISON OF CELL BREAKAGE METHODS

Cells from a 42 h old 8L fermentor culture were harvested by vacuum filtration using Kimtuff as a filter. After washing with ddw, the mycelial pad was resuspended in the desired amount of cold breakage buffer (100 mM phosphate buffer, pH 6.5, 10% glycerol, and 10 mM DTT) in preparation for cell disruption.

Microfluidizer - this method was carried out as described in 9.4.

Braun cell homogenizer - for the ballistic disruption of mycelia with glass beads, 12 mL of a cell-buffer suspension (i.e. 0.4 g of moist cells in 12 mL of buffer) were transferred to prechilled Braun homogenizing bottles (50 mL size) each containing 20 g of glass beads (0.45 - 0.50 mm, Braun). The Braun bottles and their contents were shaken for 2 min using a Braun cell homogenizer (400 rpm, Model MSK, Braun Instruments) and the temperature was kept below 5°C with 1 second bursts of liquid carbon dioxide every 15 seconds.

The homogenates obtained from the Microfluidizer and the

Braun cell homogenizer were used for enzyme assays. The assay was done using a temperature of 10ºC (before optimization of this parameter to a temperature of 0ºC.)

12.2 OPTIMIZATION OF MONOOXYGENASE STABILITY IN CRUDE CELL-FREE EXTRACTS

The cell-free extracts used for *in vitro* stability studies were prepared from a fermentor culture at the optimum culture age (time) and were broken in 0.1M phosphate buffer at different pH values (6.0, 6.5, 7.0, 7.5) with 10% glycerol and 10 mM DTT. The assay in this experiment was done using the initial conditions (10). The effects of several additives at the optimal pH (6.5) were studied. A proteinase inhibitor (turkey egg white trypsin inhibitor, TTI, 1 mg/mL) was added separately or in combination with 10% glycerol and either 0 mM, 5 mM, 10 mM, or 15 mM DTT in cold (4°C) cell breakage buffer.

The stability of nor-anthrone monooxygenase in cell-free extracts, in the presence or absence of the desired additives, was monitored at 4°C and 100 μL aliquots were removed at different times (0, 6, 18, 28, 44, 82, 120 hours) in each case and assayed for monooxygenase activity.

12.3 ENZYME SOLUBILIZATION

12.3.1 Centrifugation studies

The crude cell extract was used directly or subjected to fractionation by high speed centrifugation (105,000 X g) for

60 minutes or low speed centrifugation (10,000 x g) for 30 minutes, to determine whether the monooxygenase is soluble or particulate. The cytosol fraction was obtained from the supernatant while the microsome fraction were prepared by resuspending the pellet from each centrifugation in cell breakage buffer (0.1M phosphate buffer, pH 6.5, 10% glycerol, 10 mM DTT). Crude cell extracts and these fractions were examined for enzyme activity.

12.3.2 Detergent studies

These studies were carried out by using three detergents: Triton X-100, CHAPS and Octylglucoside at the following final concentrations (0.01, 0.1 and 1.0%). The different detergents were added to 100 mL of crude enzyme extracts obtained from cells with monooxygenase activity and the extracts were gently stirred, using a Corning hot plate/stirrer PC 351, at 4°C. Samples were taken after 1 h of treatment and centrifuged at 105,000 X g for 60 minutes to yield a supernatant, which was then assayed to determine any increase in the soluble enzyme.

The effect of different solubilization times was studied with 0.1% Triton X-100 in 2000 mL of cell-free extract. Samples (100 mL) were taken at different times and the pellet and supernatant fractions were assayed after 10,000 X g centrifugation for 30 minutes at 4°C.

12.4 REMOVAL OF DETERGENT

A batch method was used to remove detergent. The crude

extract containing detergent was mixed with a hydrophobic resin (Amberlite XAD-2) for 45 minutes at 4°C with gentle stirring, then filtered through gauze. The resin was washed with distilled water before mixing with the extract. The amount of solid resin needed was calculated for the amount of detergent to be removed as follows:

$$\text{Amount of resin (gm)} = \frac{\text{amount of detergent (mg)}}{\text{adsorption capacity (mg/gm)}}$$

For Triton X-100 the adsorption capacity is 157mg Triton X-100/gm of resin

12.5 SALT FRACTIONATION

A culture sample (4 L) from the fermentor was harvested after 42 hrs in a normal manner (9.4), resuspended in 2 L cold (4°C) cell breakage buffer (0.1 M phosphate buffer, pH 6.5, containing 10 mM DTT and 10% glycerol) and subjected to disruption by one pass through the Microfluidizer. The crude extract was then treated with detergent (Triton X-100 0.1%) for 10 hrs with gentle stirring at 4°C, then the detergent was removed with Amberlite XAD-2 (12.4) After centrifugation at 10,000 g for 30 min at 4°C, approximately 1400 mL of this extract was used for the salt precipitation with ammonium sulfate to 35 and 65% saturation. Dissolution of salt was carried out at 4°C for about 30 minutes with gentle stirring using a Corning hot plate/stirrer, PC 351. At the same time, the pH was kept at 7.0 by adding 10M sodium hydroxide dropwise

with a Pasteur pipette and monitoring with a pH electrode. Following centrifugation at 10,000 X g (Sorval SS34 rotor) at 4°C for 30 min, the supernatants from 35 and 65% saturation were assayed for enzyme activity, and the pellets from 65% saturation were stored at -70°C for further use when needed. Note that at 35% ammonium sulfate there was no precipitate formed and therefore no pellet to assay.

12.5.1 Stability of the enzyme in the ammonium sulfate precipitate

After the precipitation with ammonium sulfate, 2 g of the 65% pellet was divided into 4 equal fractions (0.5 g) for studying the stability of the enzyme over time, in "dry" form or resuspended in 2 mL of purification buffer (same as cell breakage buffer), using two storage temperatures (-20°C and -70°C). After 2 and 4 weeks the pellets were thawed and the "dry" pellets were resuspended in the 2 mL of the same buffer. The monooxygenase activity was then determined, and compared to the activity before storage (i.e. 0 h after precipitation).

12.6 GENERAL COLUMN CHROMATOGRAPHY PROCEDURES

12.6.1 Gel filtration chromatography

Ammonium sulfate pellets (~ 16g from 1400 mL of detergent treated supernatant), stored at -70°C (dry form) were resuspended in approximately 60 mL of purification buffer (0.1 M phosphate buffer, pH 6.5, 10% glycerol, and 10 mM DTT) by homogenization using a 20 mL Potter-Elvehjen tissue grinder. This solution was clarified by centrifugation at 10,000 X g

(Sorvall, GSA rotor) for 10 min at 4°C and the resulting supernatant (15 mL) was applied to a Bio-Gel A 1.5M (agarose gel, fractionation range, 10,000 to 1.5 X 10^6) column (2.5 X 40 cm, 150 mL) previously equilibrated with two column volumes of purification buffer. Elutions from this and all other columns were done using purification buffer and were monitored continuously at 280 nm using a UV-2 dual path monitor and a chart recorder (Pharmacia). After sample loading, the column was eluted at 1.0 mL/min with purification buffer (300 mL, 2 volume), and 6 fractions (72 mL) were collected. The monooxygenase activity in the fractions was assayed.

12.6.2 Hydroxylapatite adsorption chromatography

Fractions from gel filtration containing the greatest monooxygenase activity were pooled (72 mL), then loaded onto an 18 mL (1.6 X 9 cm) hydroxylapatite (Fast flow, Calbiochem) column previously equilibrated with purification buffer. This column was run at 1.0 mL/min, using step gradient elution with 0.025 to 0.25M phosphate in the purification buffer (400 mL). The monooxygenase activity in the 23 fractions collected with 17 mL fraction was assayed.

12.6.3 DEAE Sephadex column chromatography

Active fractions collected from the hydroxylapatite adsorption were applied to a DEAE-Sephadex A-25 (particle size: 40-120 μm column (1.6 X 9 cm, 18 mL) previously equilibrated with purification buffer. This column was washed

at 1 mL/min with 36 mL of the same buffer, then elution of the column was carried out with a step gradient elution from 0 to 1M KCl in purification buffer (360 mL). The monooxygenase activity in each of 45 fractions 8 mL each was assayed.

12.7 PHAST SYSTEM SEPARATION TECHNIQUE (SDS-PAGE)

A Phast (Pharmacia) Polyacrylamide Gel Gradient 8-25, that has a continuous 8 to 25% gradient gel zone with 2% crosslinking, was used. The buffer system in the gel contained 0.112M acetate and 0.112M TRIS, pH 6.4. The buffer system in Phast Gel SDS Buffer Strips contained 0.2M TRIS-HCl, 0.2M Tricine and 2% SDS, pH 8.1. The buffer strips were made of 2% agarose.

Samples were prepared by mixing 5 μL of each sample with 5 μL of SDS buffer, then heating at 100°C for 5 min. Insoluble material was removed by centrifugation to prevent streaking patterns in the developed gel. Samples included the crude extract, supernatant, ammonium sulfate precipitate, and fractions with enzyme activity from the different chromatography columns (gel filtration, Hydroxylapatite, and DEAE Sephadex).

The protein standards kit used contained:

Protein	μg/vial	MW
Myosin	25	212,000
α, 2-Macroglobin	100	170,000

ß-Galactosidase	16	116,000
Transferrin	17	76,000
Glutamine dehydrogenase	18	53,000

The separations took approximately 30 min with the following programmed conditions: 250V, 10mA, 3.0W, 15°C to 95 Vh.

12.8 PROTEIN ASSAYS

For cell-free extracts, pellet or purified fractions, the protein concentration was measured by the Bradford method (1976), which utilizes an absorbance shift from 465 to 595 nm when Coomassie Blue G-250 binds to protein in an acidic solution. The assay used consisted of mixing 4 mL of Protein Assay Reagent (Pierce Chemical Co.) with 0.1 mL of sample containing an appropriate amount of protein. After incubation for 5 min, the absorbance of the solution was measured at 595 nm against a blank (4 mL of the Protein Assay Reagent and 0.1 mL of the appropriate breakage buffer). To determine the protein concentration, a standard curve was prepared using bovine serum albumin (2 mg/mL in ddw) as the standard. This produced a linear plot that could be used to estimate protein concentration in the samples.

PART III

RESULTS AND DISCUSSION

13. DEVELOPMENT OF AN IMPROVED GROWTH MEDIUM FOR *A. parasiticus* AND FOR ENZYME PRODUCTION

Studies on the effect of nutrition on aflatoxin production started soon after its discovery (Diener and Davis, 1969). The production of aflatoxin is influenced by different factors such as the strain, environmental factors and the availability of substrates (Bu'Lock, 1975; Bennett and Christensen, 1983).

Further improvements to the cultivation conditions for *A. parasiticus* have been made with the objective of maximizing aflatoxin pathway enzymology in fermentor grown cells.

Studies in our laboratory, using *A. parasiticus* ATCC 24690/SRRC 162A, a mutant, blocked immediately after nor-acid in the biosynthetic pathway (see Fig. 4.1), indicated that with the growth conditions for submerged cultures found in the literature (Smith et al., 1992 and Yabe et al., 1991, 1993), the mold had a relatively slow growth with pellet formation, and the appearance of norsolorinic acid was very gradual. Thus a new culture medium (PEG medium) was designed [8.4], (Wong, 1993, personal communication).

The characteristics of 8L fermentor cultures grown in this medium are shown in Fig. 13.1. The increase in the dry weight was calculated and it is evident that the maximum increase in dry weight occurred by 48 h and the maximum biomass achieved was about 14 g/L. Subsequently no increase

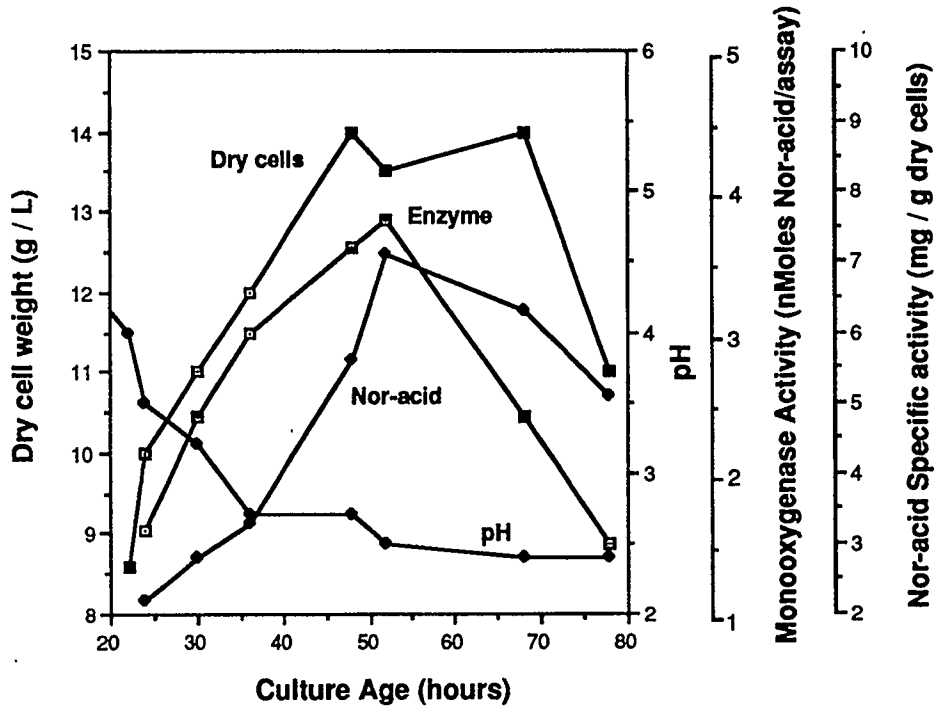


Figure 13.1 *A. parasiticus* fermentor (8 L) culture dynamics

PEG medium composition:		
Potato extract (Difco)	4.0	g / L
Glucose	40.0	g / L
KH_2PO_4	13.7	g / L
NH_4Cl	1.6	g / L (30mM)
Trace elements	10.0	mL / L
pH	5.6	
Spore concentration	10^6	Spores/mL

in biomass was obtained between 48 and 68 h. The pH dropped quickly from an initial value of 5.6 to 2.7 during the rapid growth phase (22-48 h), but after it was almost constant (48 -78 h). The earliest nor-acid production started around 24 h as shown by a rapid production of orange pigment (characteristic of this anthraquinone) that became more intense with time. Maximum norsolorinic acid production occurred at 52 h (7.1 mg nor-acid/g dry cells) in response to high monooxygenase levels which also reached a maximum at a culture age of about 52 hours. The results demonstrated a stimulatory effect on fungal growth and on the level of secondary metabolism enzymes with respect to the results obtained using culture media from the literature (Wong, 1993, personal communication) (data not shown).

In the literature the effect of trace metals is not completely understood. There are different metals such as the Mn^{2+}, that could be involved in cell wall synthesis as well as in nucleic acid synthesis to favor cellular growth (Luchese and Harrigan, 1993). Also studied was the effect of zinc on the stimulation of aflatoxin production or of any intermediate in this pathway (Niehaus, 1983; Niehause and Dilts, 1982, 1984). Angelov et al., (1979) concluded that Zn^{+2} probably participates in some of the enzyme systems which are responsible for the initial condensation of acetate units. On the other hand organic nitrogen has been reported to stimulate aflatoxin synthesis. Mateles and Adye (1965) found

that ammonia was a good nitrogen source for the production of high levels of aflatoxin. Another important component in the culture medium was KH_2PO_4, because inorganic phosphate is involved in many enzymatic reactions in the fungal cell, as well as in the regulation of secondary metabolism; phosphate was reported to stimulate aflatoxin production (Gupta et al., 1975).

Although our medium supported mycelial growth, Fig. 13.1 shows a gradual increase in both dry weight and nor-acid production. This suggests a non-homogeneity in the culture, where growth and secondary metabolism are taking place at the same time.

To achieve both a metabolic homogeneity for the cell population and a synchronous switch to secondary metabolism we studied the effect of different characteristics of the culture medium. This was done because we wished to maximize the level of aflatoxin pathway enzymes in all of the cells in order to facilitate the isolation of the nor-anthrone monooxygenase.

13.1 STUDY OF THE CULTURE MEDIUM CONDITIONS

13.1.1. Nutritional Factors

13.1.1.1 Fresh Potato Extract

We discovered that substituting fresh liquid potato extract (the extract from 600 g of pieces of potato/L of medium) for the powdered Difco potato extract, resulted in a 1.5 fold increase in biomass to give 21g dry cells/L.

Secondary metabolism, as indicated by production of the orange nor-acid was not negatively affected by this increase in biomass.

To confirm the optimum potato extract concentration other concentrations were selected and studied in shake flask cultures. The results (Fig. 13.2) showed a clear inverse relationship between growth and specific production of nor-acid. Since the growth of *A. parasiticus* increased with higher potato concentrations, this caused a high culture viscosity that may have affected the oxygenation of the culture. This is an important consideration forof aflatoxin biosynthesis. The best concentration was the extract from 300g of potatoes/L of medium. This gave 16 g/L of dry cells and a nor-acid specific activity of 15.4 mg/g dry cells at a culture age of 48 hours. This is a significant improvement over the 7.1 mg nor-acid/g dry cells reported in Fig. 13.1.

13.1.1.2 Effect of Glucose Concentration

Abundant aflatoxin production is generally associated with substrates containing elevated levels of specific carbohydrates (Applebaum and Buchanan, 1979; Davis et al., 1966 and 1967; Davis and Diener, 1968; Mateles and Adye, 1965).

The effect of glucose concentration on the growth of *A. parasiticus* and on nor-acid production is depicted in Fig. 13.3; with 30g/L of glucose the growth and nor-acid production

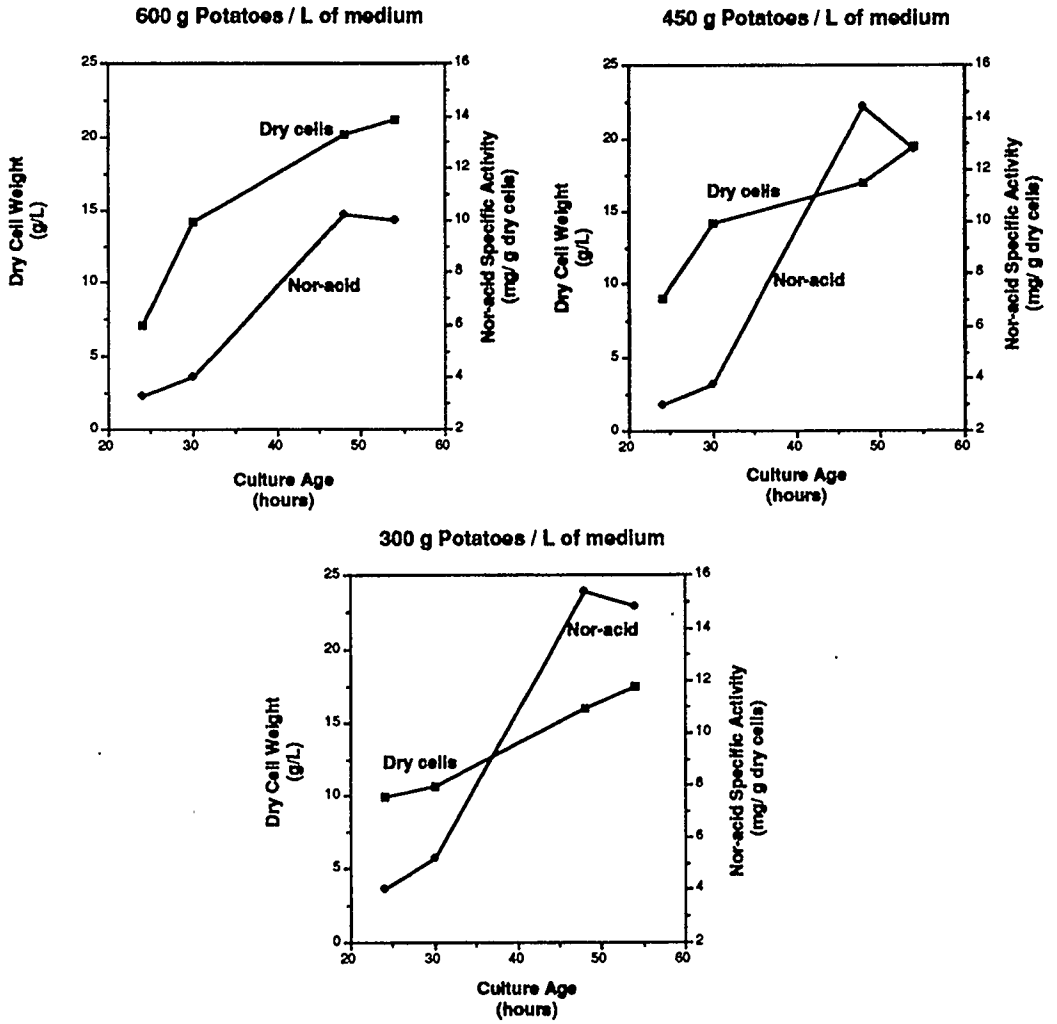


Figure 13.2 Effect of freshly prepared potato extract concentration on *A.parasiticus* growth and Nor-acid production in 50 mL shake flask cultures.

Medium composition :	
Glucose	40.0 g / L
KH_2PO_4	13.7 g / L
NH_4Cl	1.6 g / L (30mM)
Trace elements	10.0 mL / L
pH	5.6
Spore concentration	10^6 Spores / mL

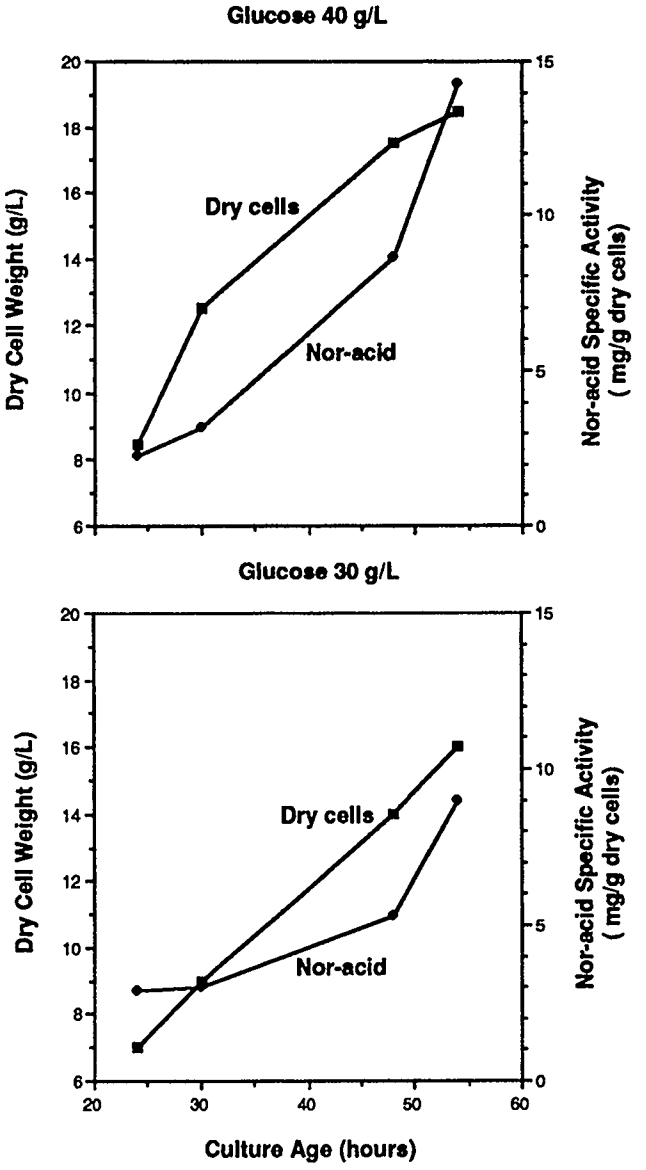


Figure 13.3 Effect of glucose concentration on *A.parasiticus* growth and Nor-acid production in 50 mL shake flask cultures.

Medium composition:		
Fresh potato extract	300.0	g Potatoes / L
KH_2PO_4	13.7	g / L
NH_4Cl	1.6	g / L (30mM)
Trace elements	10.0	mL / L
pH	5.6	
Spore concentration	10^6	Spores / mL

were lower than with 40g/L, which gave a specific nor-acid production of 14.3 mg/g dry weight at 52 h. This resulted in a 1.6 fold increase with respect to 30 g/L of glucose. This clearly indicated that maximal nor-acid production is dependent on the availability of elevated concentrations of glucose. This carbon source may influence the production of the precursors used for nor-acid biosynthesis in *A. parasiticus*.

It is reported in the literature that commercial sources of carbohydrates are particularly important because these compounds provide the two-carbon precursors for toxin syntehsis. For this reason a number of investigators have studied sugar and related carbo-hydrates that support fungal growth and aflatoxin production (Buchanan and Stahl, 1984; Prasad, 1983).

Shih and Marth (1974) reported maximal yield of aflatoxin to occur with 30g of glucose/L, whereas maximal growth was obtained when the medium contained 10g of glucose/L. They have demostrated that glucose can be catabolized to acetyl-CoA, which then acts as the building block for the carbon skeleton of aflatoxin. Also, studies by Applebaum and Buchanan (1979) on glucose catabolism in *A. parasiticus* indicated that either glucose or a product of its metabolism may also have a regulatory role, acting as an inducer of one or more of the enzymes needed for aflatoxin synthesis.

Abdollahi and Buchanan (1981) observed that a wide variety of carbohydrates could induce aflatoxin synthesis and suggested that the utilization of a readily metabolizable carbohydrate results in an elevated energy status.

This experiment also confirmed the positive effect of the fresh potato extract, because the composition of the medium for 40 g/L of glucose was the same to the medium in fig. 13.2, with 300g potatoes/L of medium and in both results the production of nor-acid were very similar.

13.1.1.3 Effect of Different Types and Concentrations of Nitrogen Sources.

Studies by Rollins and Gaucher (1994) have shown that the addition of 30 mM NH_4Cl during synthesis of m-hydroxybenzyl alcohol dehydrogenase causes an abrupt repression and subsequent loss of this enzyme which is involved in polyketide biosynthesis (Groot Wassink and Gaucher, 1980). Further regulatory studies have shown the negative effect of NH_4^+ on patulin biosynthesis using cultures grown continously in a cyclone bioreactor (Newaskar, 1989). The negative effect of NH_4^+ has also been observed in many other secondary metabolite fermentations, e.g. rifamycin (Jiao et al., 1979), chloramphenicol (Westlake et al., 1968), actinomycin (Katz, 1980) and leucomycin (Omura et al., 1980).

For all of these reasons, the objective of the present experiment was to determine whether a culture medium with

lower concentrations of nitrogen than used in the initial medium (30 mM of NH_4Cl) supported the production of norsolorinic acid and could stimulate the production of this metabolite. The effect of different types and concentrations of nitrogen sources on nor-acid production (Fig. 13.4) showed that the growth of *A. parasiticus* with ammonium chloride (10 mM, 15 mM) and with ammonium nitrate (10 mM, 15 mM) was not very different, yielding around 18.5 g dry cells/L of culture at 45 h. However, higher levels of nor-acid (11 mg/g dry cells) were observed in cultures grown on ammonium chloride (15mM).

An examination of these data indicates that the presence of two nitrogen sources, each at 10 mM, results in a decrease in nor-acid specific activity which is considerably more than what would be expected if the results with 15 mM NH_4Cl are considered. This suggests that nitrate may repress the synthesis of nor-acid more than an equivalent concentration of ammonium ion. Thaper (1988) reported higher yields of aflatoxin with certain nitrogen sources (especially casamino acids and NH_4NO_3) and significantly lower yields with NH_4Cl. The difference between these results and ours suggest that the exact response to individual nitrogen sources can be influenced by the strain of fungus and the culture conditions.

NH_4Cl (15 mM) was selected to analyze the production of nor-acid in an 8L fermentor culture. The results (Fig. 13.5)

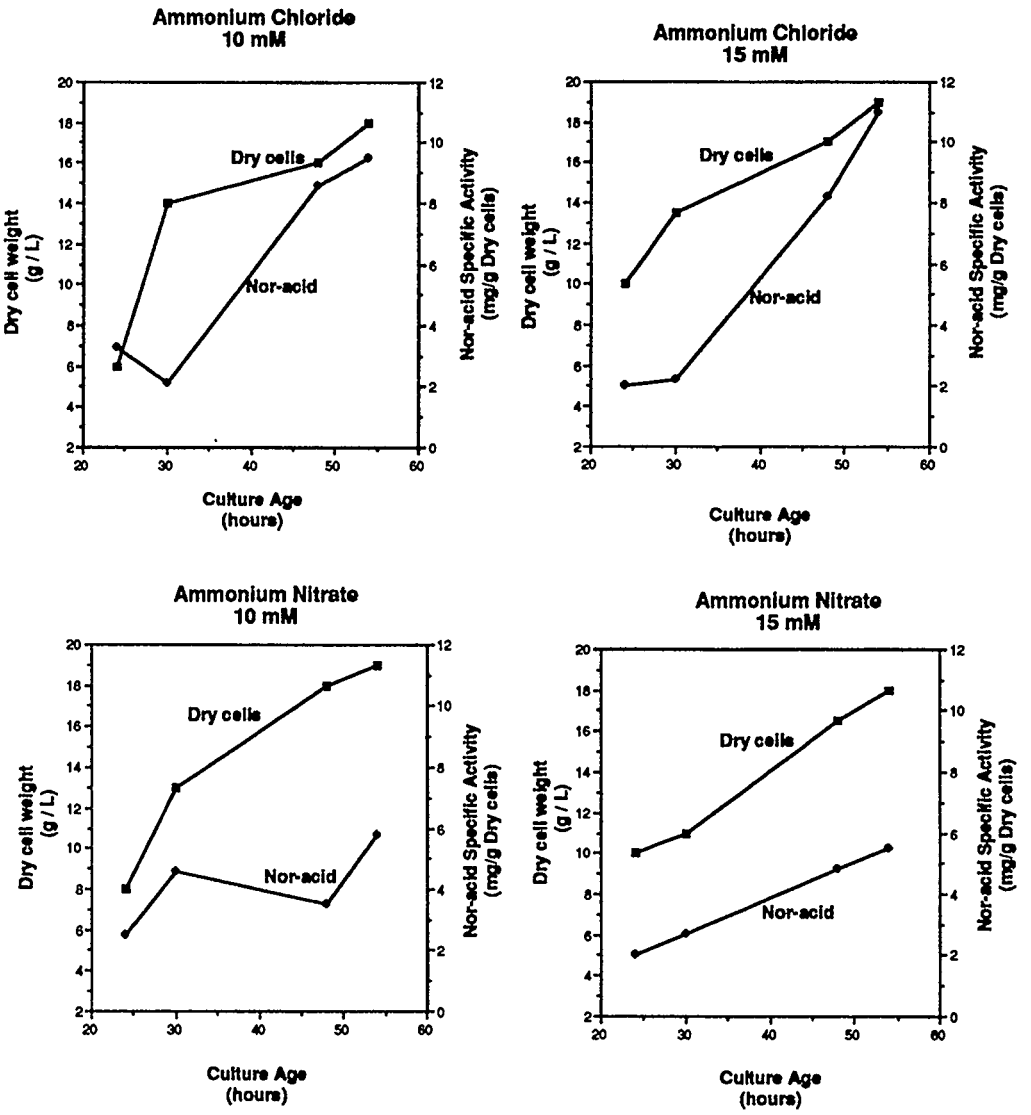


Figure 13.4 Effect of nitrogen source and concentration on *A.parasiticus* growth and Nor-acid production in 50 mL shake flask cultures.

Medium composition:		
Potato extract (Difco)	4.0	g / L
Glucose	40.0	g / L
KH_2PO_4	13.7	g / L
Trace elements	10.0	mL / L
pH	5.6	
Spore concentration	10^6	Spores / mL

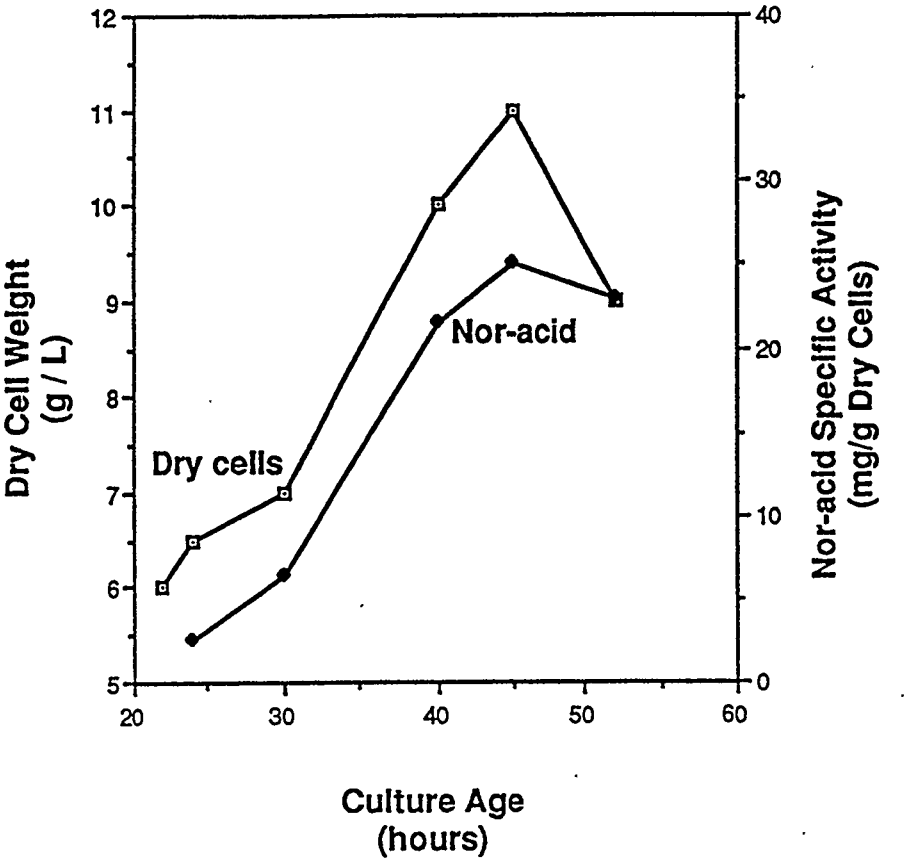


Figure 13.5 Effect of ammonium chloride (15mM) on Nor-acid production and growth of *A.parasiticus* in an 8 L fermentor culture.

Medium composition :		
Fresh potato extract	300.0	g Potatoes / L
Glucose	40.0	g / L
KH_2PO_4	13.7	g / L
NH_4Cl	0.8	g / L (15mM)
Trace elements	10.0	mL / L
pH	4.0	
Spore concentration	10^6	Spores / mL

showed that the production of nor-acid was very good and reached its maximum (25mg/g dry weight) by 45 h when the culture was in idiophase. This is a 3.5 fold increase in comparison with the use of 30 mM NH_4Cl in the earlier 8L fermentor culture (Fig. 13.1). However, this fermentor culture medium also differed in the potato extract and initial pH, two factors that also have a significant effect on nor-acid specific activity. The data suggests that a high concentration of NH_4Cl caused a significant decrease in the levels of the enzyme involved in this metabolic pathway.

The effect of high NH_4Cl concentration on nor-acid production in replacement cultures of *A. parasiticus* was studied in a separate experiment. After an additional 48 h of incubation, nor-acid levels were determined to be (Fig. 13.6) 0.36, 0.27, 0.19, and 0.14 g/L for the 15 mM, 30 mM, 50 mM and 75 mM treatments, respectively. These results indicated that 15 mM ammonium chloride was optimal for nor-acid production. Thus higher concentrations of this nitrogen source did not appear to improve the onset or the level of enzyme activity responsible for nor-acid formation.

13.1.2 Effect of the Initial Culture pH

Among the conditions that influence fungal growth and the yield of secondary metabolites are the initial pH of the culture growth medium. The effects of initial culture pH on the growth of *A. parasiticus*, and on norsolorinic acid

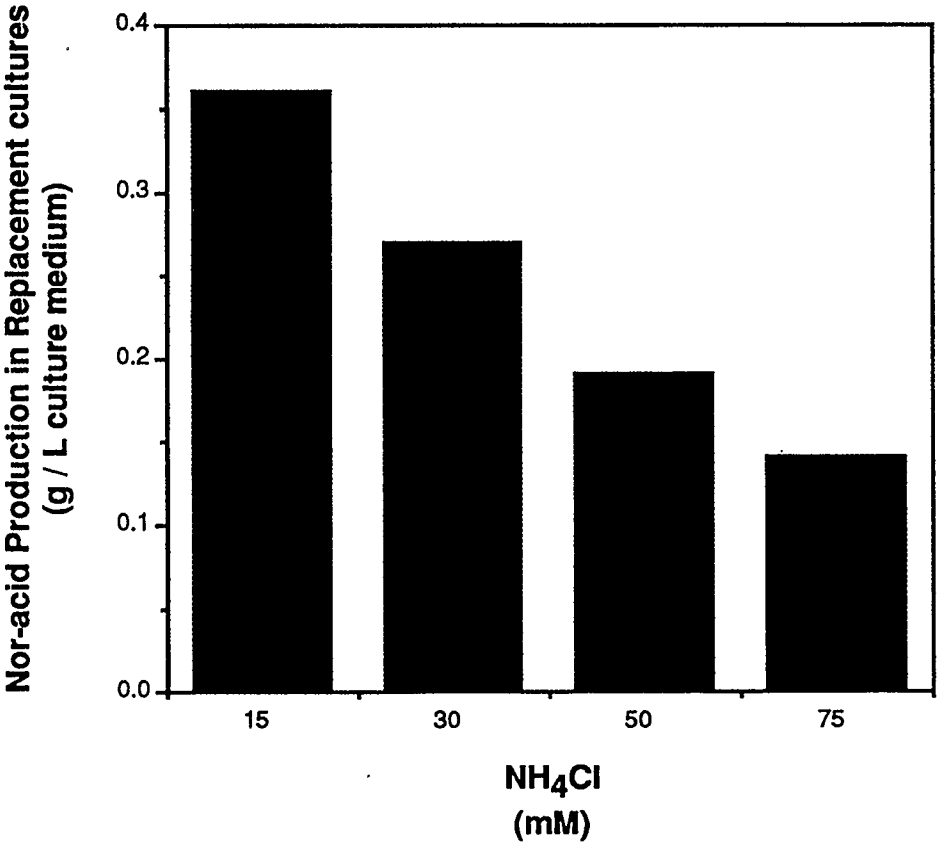


Figure 13.6 Effect of NH_4Cl concentration on Norsolorinic acid production in replacement cultures.

As described in section [8.8], the cells from 50 mL portions of a 24 hour old 8L fermentor culture were transferred to 500 mL Erlenmeyers containing fresh medium with various concentrations of NH_4Cl. The Nor-acid production was determined after 48 hours of cultivation in the fresh medium.

production, and the changes in the pH of the culture medium are shown in Fig. 13.7. These results indicate a direct relationship between pH and growth. At low pH the growth is less, with a pH of 5.6 for optimal fungal growth. This relationship is not true in the case of nor-acid production, which is favored by a low pH. The best specific activity was at pH 4.0. Because of this relationship pH 4.0 was chosen as the optimum initial culture pH for nor-acid production. Buchanan and Ayres (1976) also reported that the yield of aflatoxin and its intermediates are a function of the initial pH. In all tested media, the pH was shown to drop until growth slowed and the production of nor-acid began at about 24 h. The pH remained almost constant thereafter.

13.1.3 Inoculum Concentration

The effect of inoculum concentration on nor-acid production is shown in Fig. 13.8. The growth is less in cultures initiated with the lower spore concentration (10^5 spores/mL of culture). Inoculum concentration has been recognized to be important in the morphology of mold growth. The production of secondary metabolites very frequently depends on this. Sharma and Padwal-Desai (1985) reported that with *A. parasiticus*, spore number in the inoculum influences the fungus morphology, depending on the culture conditions. There is a relationship between spore number, morphology and the yield of aflatoxin, at low spore concentrations (10^2 - 10^4 spore/mL). Pellet formation was observed and at the lower

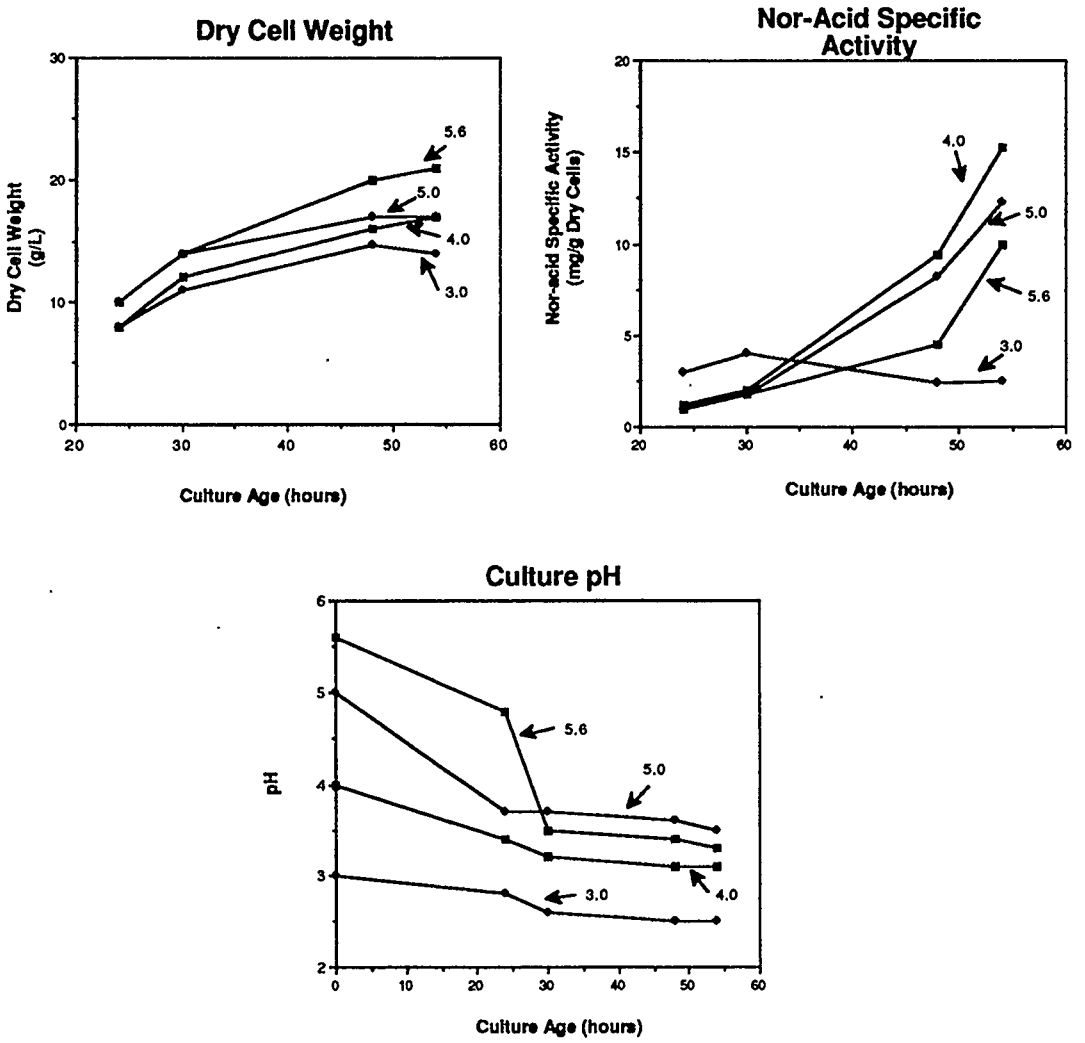


Figure 13.7 Effect of initial culture pH on *A. parasiticus* growth and Nor-Acid production in shake flask cultures. Note the initial pH values are those before medium sterilization

Media composition:

Fresh potato extract	300.0	g Potatoes/ L
Glucose	40.0	g/L
KH_2PO_4	13.7	g/L
NH_4Cl	0.8	g/L (15mM)
Trace elements	10.0	mL/L
Spore concentration	10^6	Spore/mL

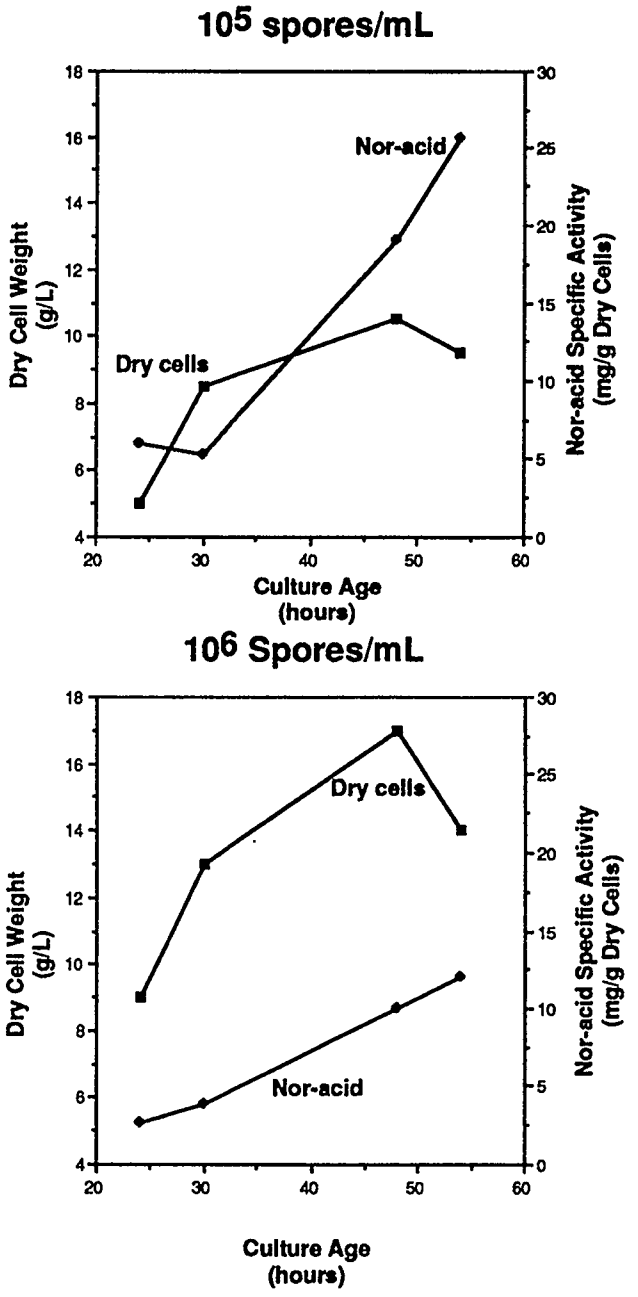


Figure 13.8 Effect of the initial spore/inoculum concentration on *A.parasiticus* growth and Nor-acid production in shake flask cultures.

Medium composition:		
Fresh potato extract	300.0	g Potatoes / L
Glucose	40.0	g / L
KH_2PO_4	13.7	g / L
NH_4Cl	0.8	g / L (15mM)
Trace elements	10.0	mL / L
pH	4.0	

concentration (i.e. 10^2) the size of the pellet was bigger. This was not favorable for aflatoxin biogenesis because of an oxygen-depleted center.

In our study with two concentrations of inoculum, the growth was in a freely disperse mycelial form and not in pellets. An inverse relationship between the spore concentration and nor-acid production was found to exist. The maximal nor-acid level was 25.7 mg/g dry cells and 12.1 mg/g dry cells for inoculums of 10^5 and 10^6 spore/mL, respectively. This indicated that with less viscosity (i.e. less mycelia) there were no limiting oxygen levels. Proper oxygenation promoted the biogenesis of nor-acid by guaranteeing suitable levels of the substrate (oxygen) that the monooxygenase uses to convert nor-anthrone into nor-acid. The much higher growth yield with 10^6 spore/mL also may negatively affect the specific activity of nor-acid.

13.2 FERMENTOR CULTURE DYNAMICS AND THE APPEARANCE OF THE ENZYME CATALYZING THE CONVERSION OF NOR-ANTHRONE TO NOR-ACID

Following the above results, we developed new growth conditions for the cultivation of *A. parasiticus* in submerged culture (8L fermentor). The objective was a high-yielding production of the enzymes of norsolorinic acid biosynthesis. The growth medium consisted of fresh potato extract (300g/L), glucose (40g/L), ammonium chloride (15 mM), KH_2PO_4(13.7 g/L) and trace elements (10 mL/L), adjusted to a pre-sterilization

pH of 4.0. The inoculum concentration was 10^5 spores/mL. The culture dynamics of *A. parasiticus* and the appearance time of the nor-anthrone monooxygenase under these conditions is shown in Fig. 13.9. Growth continued up to 42 h, the amount of dry cells remained constant from 42-58 h, and then decreased due to cell lysis. Norsolorinic acid was produced by *A. parasiticus* in detectable amounts by ~ 24 h but a rapid increase in nor-acid per g dry cells only began as growth ceased at about 42 h. The exhaustion of an essential nutrient led to this cessation of growth.

In comparison with the initial fermentor conditions (Fig. 13.1) these optimized conditions (Fig. 13.9) produced much more nor-acid product per given amount of cells. Thus, at the end of each stationery growth phase (i.e. at 68 h and 58 h respectively) the specific activity was 6.4 and 22mg nor-acid/gm dry cells, respectively. This is a 3.4 fold improvement. It is also noteworthy that different initial medium pH values (i.e. 5.6 and 4.0) gave quite different values at 35 h (i.e. 2.7 vs. 3.25, respectively).

The biosynthesis of norsolorinic acid from acetate requires an oxidative transformation, involving the monooxygenase. The involvement of a monooxygenase enzyme in the production of secondary metabolites has been demonstrated in several fungi, yeasts and bacteria (Bu'Lock and Winstanley, 1971). A rapid appearance of monooxygenase enzyme was

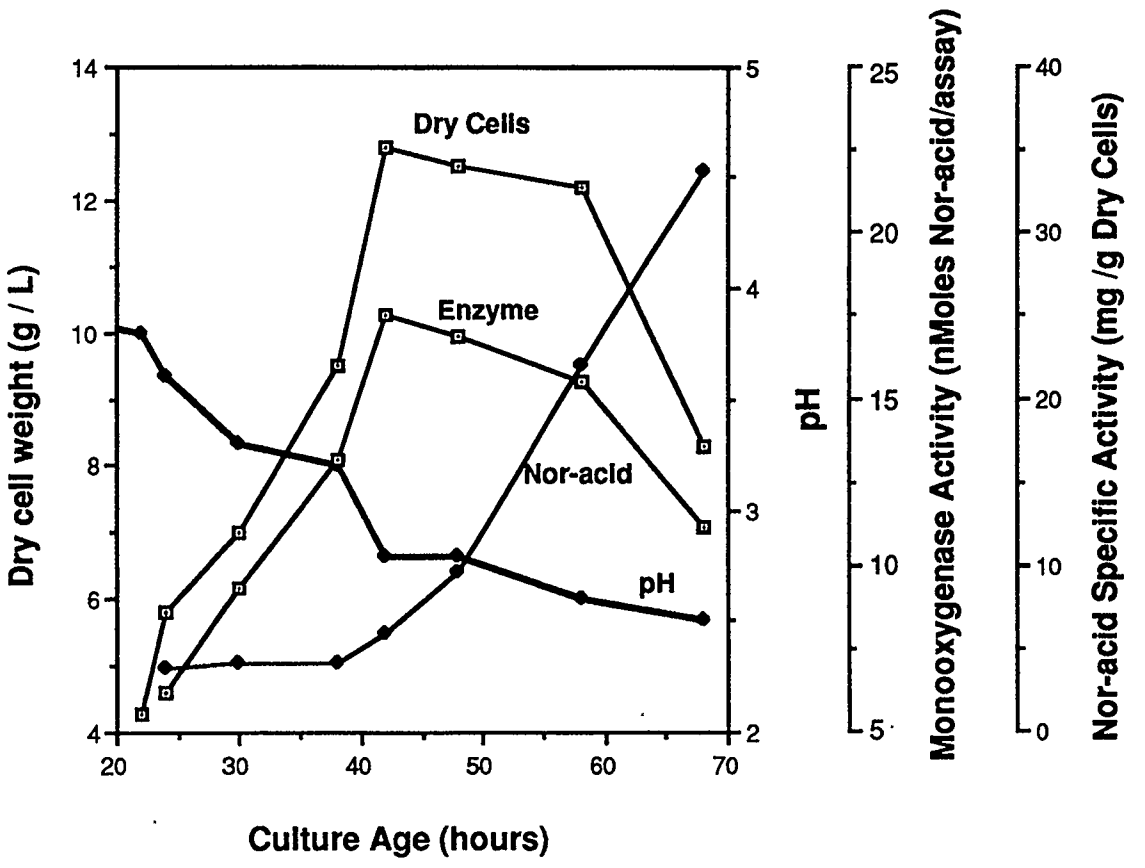


Figure 13.9 *A. parasiticus* fermentor culture dynamics.

Media Composition:		
Fresh potato extract	300.0	g Potatoes/L
Glucose	40.0	g/L
KH_2PO_4	13.7	g/L
NH_4Cl	0.8	g/L (15mM)
Trace elements	10.0	mL/L
pH	4.0	
Spore concentration	10^5	Spores/mL

observed after about 24 h, and the enzyme level was maximal at the beginning of stationary growth in both fermentor cultures (i.e. at ~ 52 h and 42 h; see Figs. 13.1 and 13.9). This peak of enzyme activity preceded the intense norsolorinic acid synthesis period only in the optimized fermentation (Fig. 13.9). This appearance time of the monooxygenase enzyme was reproducible from run to run in our different experiments.

After 42 h the activity associated with the monooxygenase declined (stationary growth phase). This transient activity is characteristic of secondary enzymes whose encoding genes are expressed only during a limited period in the cell growth cycle and in response to environmental stimuli (Wang, 1991). It is also possible that levels of intracellular proteinases may increase early in the stationary phase, as has been reported by Gaucher et al., (1981). A temporal relationship exists between the intracellular proteinase activity and the enzymology associated with patulin production. The relationship between 6-MSA synthetase and intracellular proteinase was more recently reported in the purification of this enzyme (Wang, 1991). Fedeshko (1992) also showed that the peak of IDH activity coincided with a high level of intracellular proteinase in the culture.

The rapid appearance of nor-anthrone monooxygenase enzyme suggests that a metabolically homogeneous population of cells is engaged in a relatively synchronous initiation of a

secondary metabolism. Both homogeneity and synchrony are essential prerequisites for studying the events leading to the initiation of secondary metabolisms (Groot Wassink and Gaucher, 1980) and are also very useful for enzymological studies. These results suggest that the monooxygenase catalyzing nor-acid synthesis is produced *de novo* during the transition between the exponential growth phase (prior to ~ 24 h) and the stationary phase (42 - 58 h).

Earlier work using submerged *A. parasiticus* cultures suggested that aflatoxin biosynthesis required *de novo* enzyme synthesis (Cleveland et al., 1987; Drew and Demain, 1977). More recently Bhatnagar et al., (1988) showed that aflatoxin synthesis occurred in the late growth phase. Other experiments with a wild-type fungal isolate showed AFB_1 accumulation at high levels after 48 h of mycelial development (Pathre and Mirocha, 1977). These results also suggested that the proteins catalyzing AFB_1 synthesis were produced *de novo* during the transition between the exponential growth phase and the stationary phase.

The improvements achieved by our development of optimum cultivation conditions gave a high yield of nor-acid and high levels of monooxygenase. This is summarized in Table 13.1. These data clearly indicate that a careful selection of inoculum, culture medium constituents and initial medium pH for *A. parasiticus* dramatically improved the production of

norsolorinic acid. For the two fermentor cultures (Fig. 13.1 and 13.9) the specific productivity of nor-acid can be estimated to be: 7.1 mg/g dry cell/28 h (i.e. 24-52 h) = 0.25 mg/g dry cell/h, and 33.7 mg/g dry cell/30 h (i.e. 38-68 h) = 1.12 mg/g dry cell/h. This represents a 4.48 fold increase in the specific productivity of nor-acid.

A higher nor-acid level can be obtained using a 14L fermentor containing 8L of medium, where all the requirements necessary for the production of secondary metabolites are present: [1] the physical presence of the mold capable of synthetizing the metabolite; [2] a suitable substrate for the growth of the mold; and [3] a suitable environment for the growth of the mold (all the factors that collectively can stimulate secondary metabolite formation). In addition, the peak activity of the monooxygenase appeared 10 h earlier than that obtained in the fermentor with the initial culture medium (Fig. 13.1) and reached a maximum activity which was 4.6 fold higher (Fig. 13.9). The best production of nor-acid or any other aflatoxin pathway product reported in the literature is for Nor-acid (0.024 μg/mg dry cells) (Detroy et al., 1973) and for Sterigmatocystin (7.6 μg/mg fresh weight of mycelia), O-Methylsterigmatocystin (0.17 μg/mg fresh weight of mycelia) and Averantin (0.74 μg/mg fresh weight of mycelia) (Fanelli, et al., 1989). Assuming a four fold increase if per gm dry cells these specific activities would be 30.4, 0.68, 2.96 mg/gm dry cells.

Table 13.1 Summary of Nor-acid Production and Monooxygenase Activity Under Different culture Conditons in Fermentor and Shake Flasks Incubated at 28°C

	Norosiorinic Acid Specific Activity	Monooxygenase Activity(nMoles nor-acid/assay)
Initial fermentor[a] Conditions (Fig. 13.1)	7.1 (Max at 52 h)	3.7 (Max at 52 h)
300 g Potatoes/L (Fig. 13.2)	15.4	ND
40 g/L glucose (Fig. 13.3)	14.3	ND
15 mM NH_4Cl (Fig. 13.4)	11.0	ND
15 mM NH_4Cl[b] (Fig. 13.5)	25.0	ND
pH 4.0 (Fig. 13.7)	15.3	ND
10^5 spore/mL (Fig. 13.8)	25.7	ND
Improved fermentor[c] Conditions (Fig. 13.9)	33.7 (Max at 68 h)	17 (Max at 42 h)

ND = not determined

[a] Fermentor medium was: Potato extract (Difco) 4.0 g/L, glucose 40.0 g/L, KH_2PO_4 13.7 g/L, NH_4Cl 30 mM, Trace elements 10 mL/L, pH 5.6, spore concentration 10^6 spores/mL.

[b] Fermentor medium was: Fresh potato extract 300g Potatoes/L, glucose 40.0 g/L, KH_2PO_4 13.7 g/L, NH_4Cl 15 mM, pH 4.0, spore concentration 10^6 spores/mL.

[c] Fermentor medium was: Fresh potato extract 300 g Potatoes/L, glucose 40 g/L, KH_2PO_4 13.7 g/L, NH_4Cl 15 mM, trace elements 10 mL/L, pH 4.0, spore concentration 10^5 spores/mL.

14. OPTIMIZATION OF THE NOR-ANTHRONE MONOOXYGENASE ASSAY

The first step towards the purification and isolation of an enzyme is to develop an appropriate enzyme assay. Optimal assay conditions such as substrate concentration, pH, temperature, reaction time and enzyme concentration must be tested. All of these parameters were studied in order to obtain enough sensitivity and accuracy. However, this assay proved to have numerous inherent problems which were unexpected and unusual. These problems were a poor substrate and product solubility in water which necessitated the use of a water miscible solvent (EGMME; ethyleneglycol monomethyl ether). Another problem was the overlapping spectra of substrate and product which dictated the use of a fixed time rather than a kinetic assay. Two additional problems were the non-enzymatic conversions of the anthrone substrate to the anthraquinone product and of this product to unknown side products. The resolution of the latter two problems is discussed below.

14.1 REACTION TIME OF THE MONOOXYGENASE ASSAY

In preliminary studies the production of nor-acid in assays of crude cell extracts at 10°C and pH 7.0 for assay times of 2, 5, 10, 20 and 30 min. was maximal at about 2 min. and decreased by at least 22% by 5 min (data not shown). After this, as described in section 10.2, changes in the assay (i.e. pH 7.5, and a substrate concentration of 0.6 mM)

required another assessment of the best assay time. Thus, another experiment was designed to determine if the optimal time of incubation for the nor-anthrone monooxygenase assay, using pH 7.5 and a nor-anthrone assay concentration of 0.6 mM could be less than 2 minutes. The results (Fig. 14.1) showed that the activity reached a maximum at about 15 sec.

This conversion of norsolorinic anthrone to norsolorinic acid was very rapid and in daily work it was very difficult to work with such a short time of reaction (15 sec.) Thus 30 sec. was chosen as the reaction time, since there was no significant difference in the activity with respect to 15 sec.

14.2 EFFECT OF TEMPERATURE ON MONOOXYGENASE ASSAY

As with any reaction this enzyme assay should increase with temperature until enzyme inactivation begins to occur. However, in this assay, the product (nor-acid) is lost in a non-enzymatic reaction, especially at temperatures above 10°C. Thus the optimum assay temperature is affected by the degradation as well as the formation of the product. The effect of incubation temperature for the fixed time assay (30 sec) was determined by performing assays at -15°C, -10°C 0°C, 10°C, 20°C and 30°C. The nor-anthrone monooxygenase mediated production of nor-acid was found to be maximum at 0°C (Fig. 14.2). The activity decreased with further increases in incubation temperature. This loss of activity with the increase of temperature appeared to be due to a loss of the

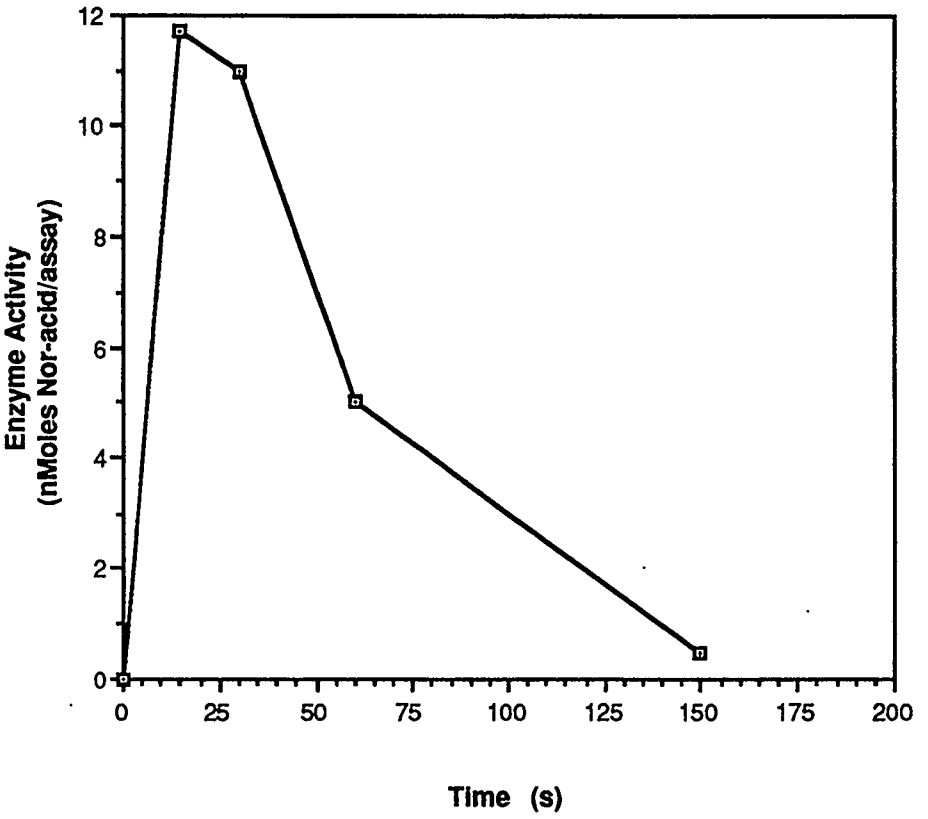


Figure 14.1 Effect of time on nor-anthrone monooxygenase assay of unpurified cell-free extracts from *A. parasiticus*

Assay Conditions:	
Buffer	phosphate, 1M pH 7.5
Substrate concentration	0.6mM
Enzyme concentation	100µL
Temperature	10 °C

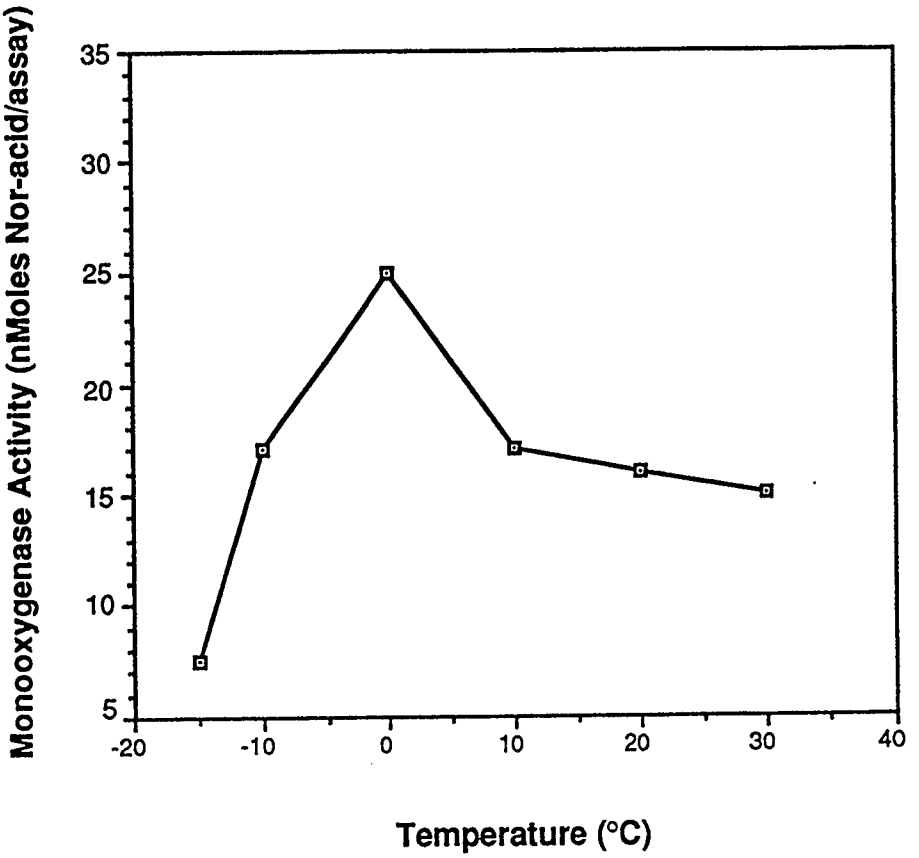


Figure 14.2 Effect of Temperature on the nor-anthrone monooxygenase assay of unpurified cell-free extracts from *A. parasiticus*.

Assay Conditions:	
Buffer	phosphate, 1 M pH 7.5
Substrate concentration	0.6 mM
Enzyme concentration	100 mL
Reaction time	30 sec

product by promoting chemical reactions which have not been characterized. An assay temperature of 0ºC was adopted for the standard monooxygenase assay, along with an assay time of 30 sec.

14.3 EFFECT OF pH ON THE MONOOXYGENASE ASSAY

The necessity of maintaining a stable pH when assaying enzymes is well established. Biochemical processes can be severely affected by minutes changes in proton concentrations.

The enzyme activity was tested over a pH range of 4 to 8.5. A pH of 7.5 corresponded to the highest activity of the monooxygenase (Fig. 14.3). A similar result was obtained in an earlier experiment using different pH values and a 2 min reaction time (data not shown). There was a significant decline in activity below pH 7.0 and above pH 7.5. Our results are in agreement with the pH optimum reported for other fungal oxygenases such as Questin oxygenase (pH optimum = 7.5), a novel anthraquinone enzyme from *A. terreus* (Fujii et al., 1988). A standard assay pH of 7.5 was chosen even though this pH promotes degradation of both substrate and product as discussed in Section 14.6. The detrimental effects of pH 7.5 were offset by using a temperature of 0ºC and a fixed time of only 30 sec.

14.4 EFFECT OF SUBSTRATE CONCENTRATION ON THE MONOOXYGENASE ASSAY

To ensure that substrate was not limiting the reaction,

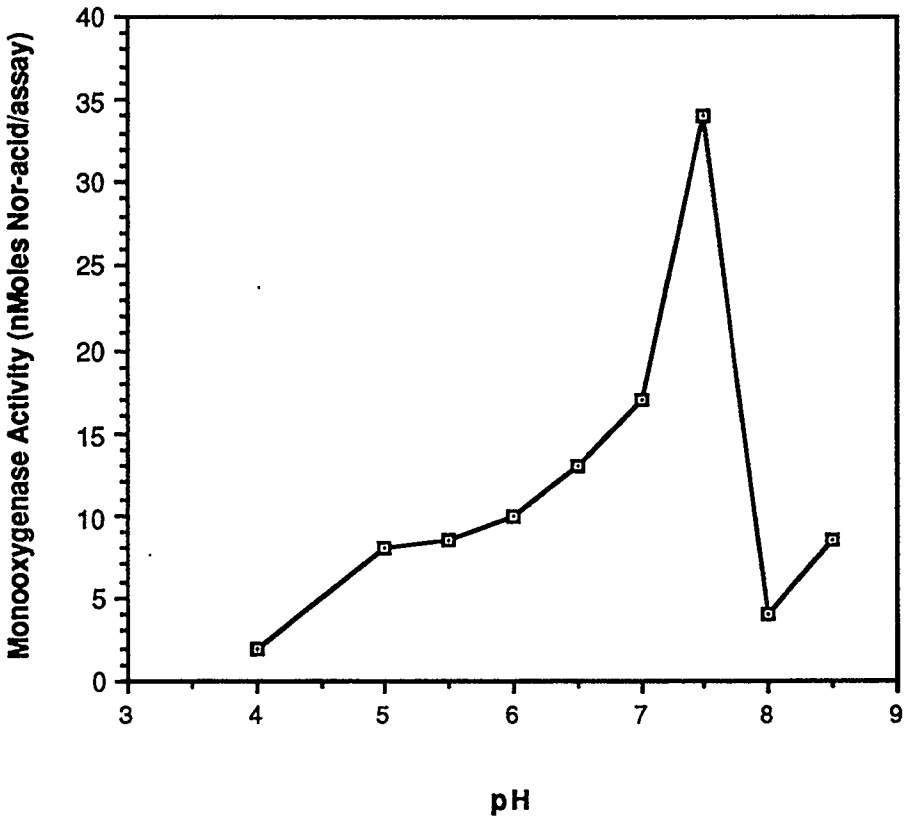


Figure 14.3 Effect of pH on the nor-anthrone monooxygenase assay of unpurified cell-free extracts from *A. parasiticus.*

Assay conditions:	
Buffer (see section 10.2)	Tris, phosphate, citrate, 1M
Substrate concentration	0.6mM
Enzyme concentration	100µL
Reaction time	30 sec
Temperature	0°C

assays were performed at various substrate concentrations (from 0.1 mM to 1.0 mM). The activity increased with the substrate concentration (Fig. 14.4), reaching a maximum for a substrate concentration of 0.6 mM. Thus we chose to run assays with 0.6 mM of nor-anthrone. See Section 14.6 for a discussion of why higher substrate concentrations could lead to higher rates of non-enzymatic losses of substrate.

14.5 EFFECT OF ENZYME CONCENTRATION ON THE MONOOXYGENASE ASSAY

To determine the concentration range of the enzyme where the proportionality between enzyme concentration and velocity of reaction is maintained, different cell extract volumes were studied with respect to the total volume of the assay mixture (i.e. o.5 ml). There was (Fig. 14.5) a linear response with increasing amounts of extract (enzyme). We chose the cell extract concentration of 20% (corresponding to 100μL) in the assay because this amount of enzyme showed the higher activity in the linear zone of the curve. Thus a high sensitivity was assured.

14.6 NON-ENZYMATIC OXIDATION/DEGRADATION OF ANTHRONES AND ANTHRAQUINONES

14.6.1 Anthrone Instability

The involvement of nor-anthrone as an intermediate in the aflatoxin biosynthetic pathway and its non-enzymatic oxidation to give norsolorinic acid has been proposed but never proven (Bhatnagar et al., 1992; Sinz and Shier, 1991). Anthrones are known to undergo air oxidation to give anthraquinones. In the

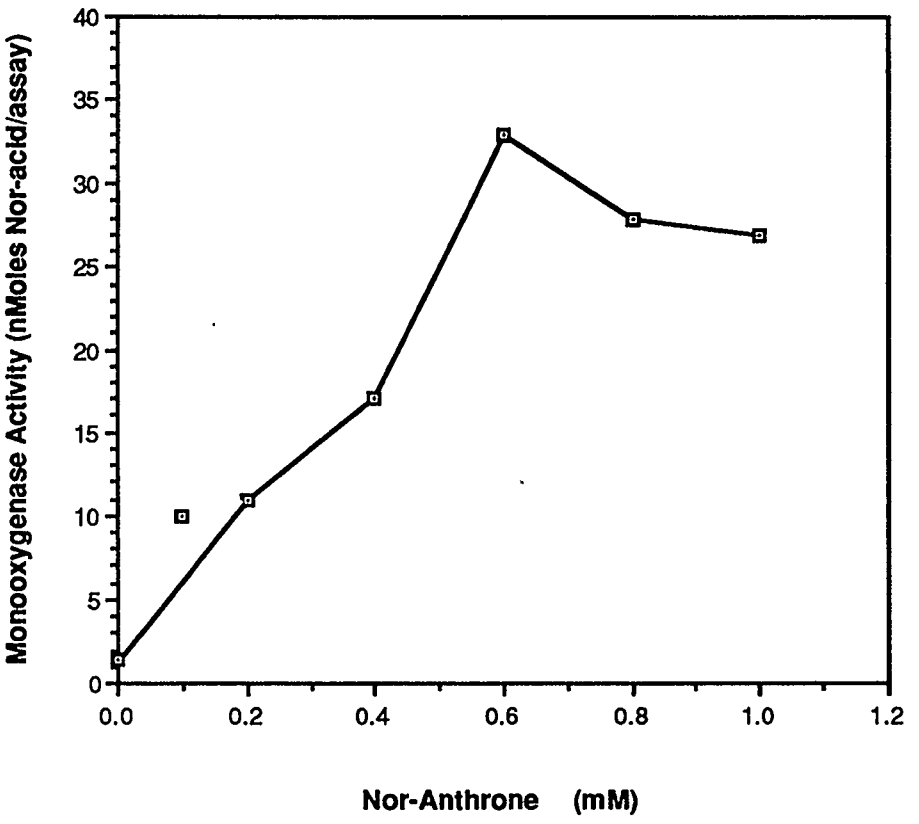


Figure 14.4 Effect of substrate concentration on the nor-anthrone monooxygenase assay of unpurified cell-free extracts from *A. parasiticus*.

Assay conditions:	
Buffer	phosphate, 1M pH 7.5
Enzyme concentration	100µLTemp. 0°C
Reaction time	30 sec
Temperature	0°C

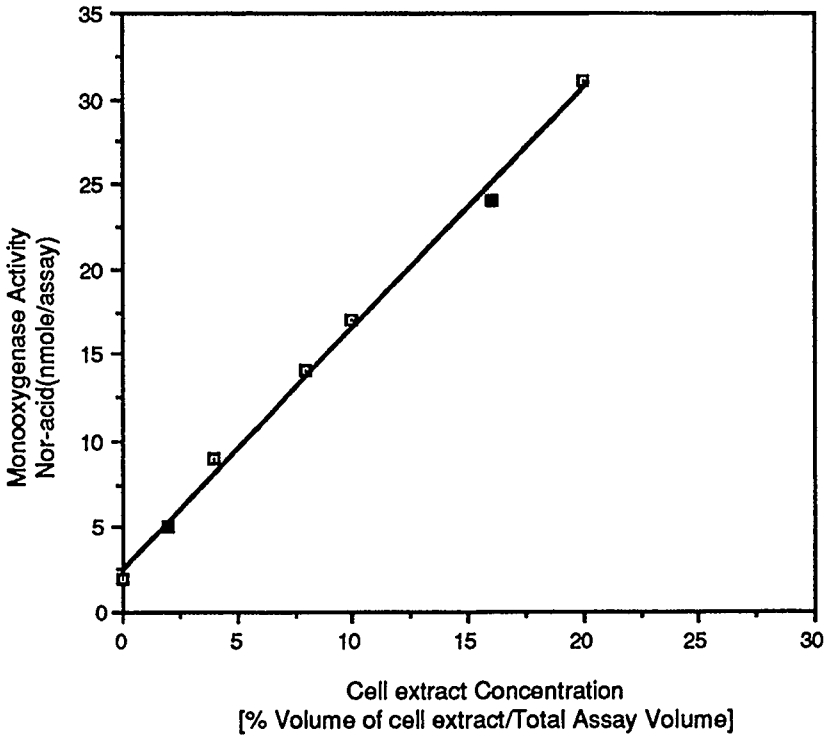


Figure 14.5 Effect of cell extract concentration on the nor-anthrone monooxygenase assay using unpurified cell-free extracts from *A. parasiticus.*

Assay conditions:	
Buffer	phosphate, 1M pH 7.5
Substrate concentration	6.mM
Reaction time	30sec
Temperature	0°C

determination of whether air oxidation of nor-anthrone was a significant route to nor-acid we found (Table 14.1) that while the anthrone rapidly disappeared from the assay mixture at pH 6.0 (95% gone after 30 min.) and pH 7.0 (100% gone after 30 min.) very little nor-acid was formed as a result. This is illustrated by the chromatograms shown in Fig. 14.6 Since we specifically assayed for nor-acid, the loss of anthrone substrate does not interfere with the product assay. This suggested that non-enzymatic oxidation was not an important route to nor-acid.

The assay sensitivity was later improved and as a result we observed some non-enzymatic oxidation of nor-anthrone. These assays were performed in parallel with a blank (boiled enzyme) and the difference between the two is an accurate measure of the monooxygenase activity. Fig. 14.7 shows a time course of the nor-acid production by a crude cell-free extract (enzymatic synthesis) and by a boiled cell-free extract (blank). Nor-acid synthesis was very rapid and reached a maximum by 30 sec., followed by a decline in the case of the cell-free extract. Non-enzymatic generation of nor-acid accounted for 33% of the total nor-acid production. This showed that cell extracts produced much more nor-acid (67%), confirming the conversion of nor-anthrone to nor-acid through an enzymatic reaction mediated by a monooxygenase.

Table 14.1 Non-Enzymatic Degradation of Norsolorinic Anthrone in Assay Mixture at 30°C

TIME (MIN)	ANTHRONE REMAINING AT TWO pH VALUES	
	6.0	7.0
5	72%	37%
30	5%	0%
60	0%	0%

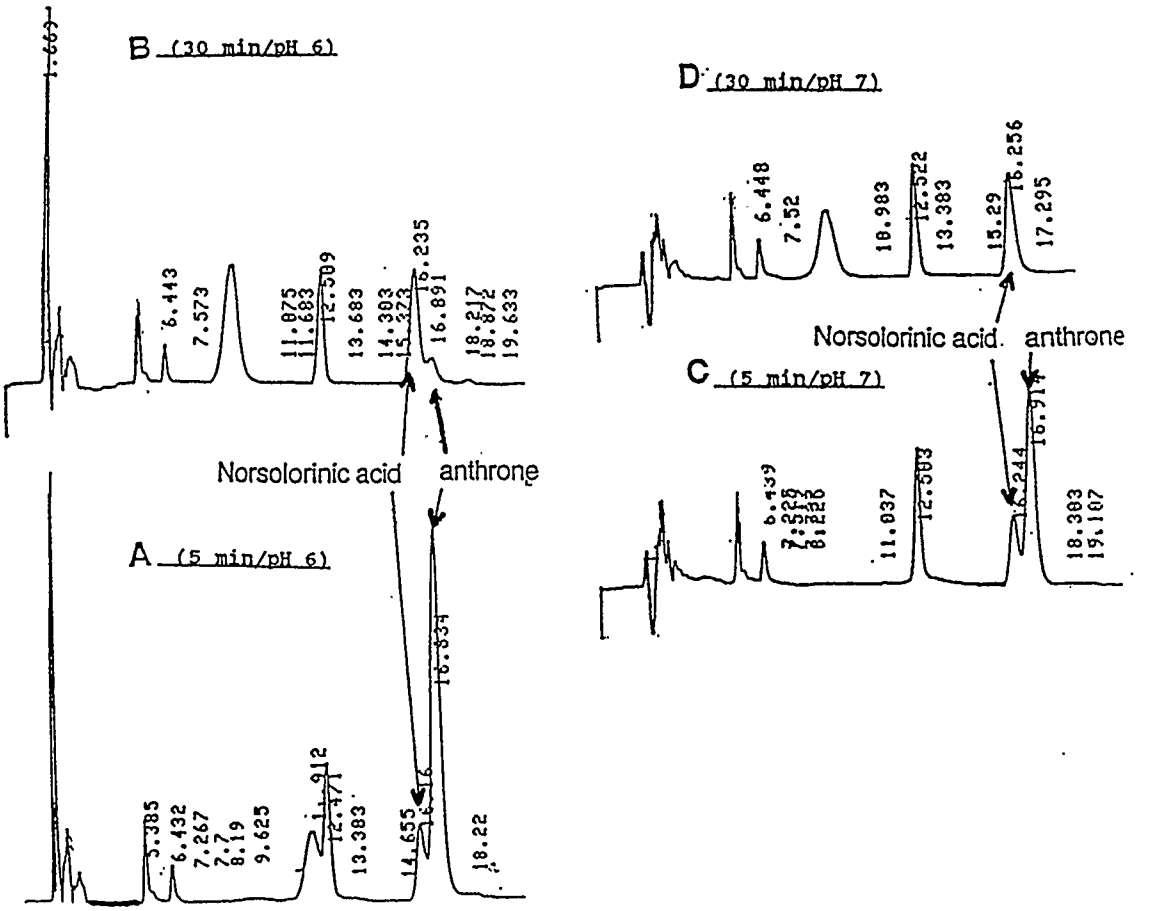


Fig. 14.6 Non-enzymatic degradation of norsolorinic anthrone in a standard assay mixture at 30°C, a pH of 6 or 7 and an incubation time of 5 or 30 min. The usual 100μL of cell extract was replaced with phosphate buffer and the concentration of anthrone was 0.6 mM.

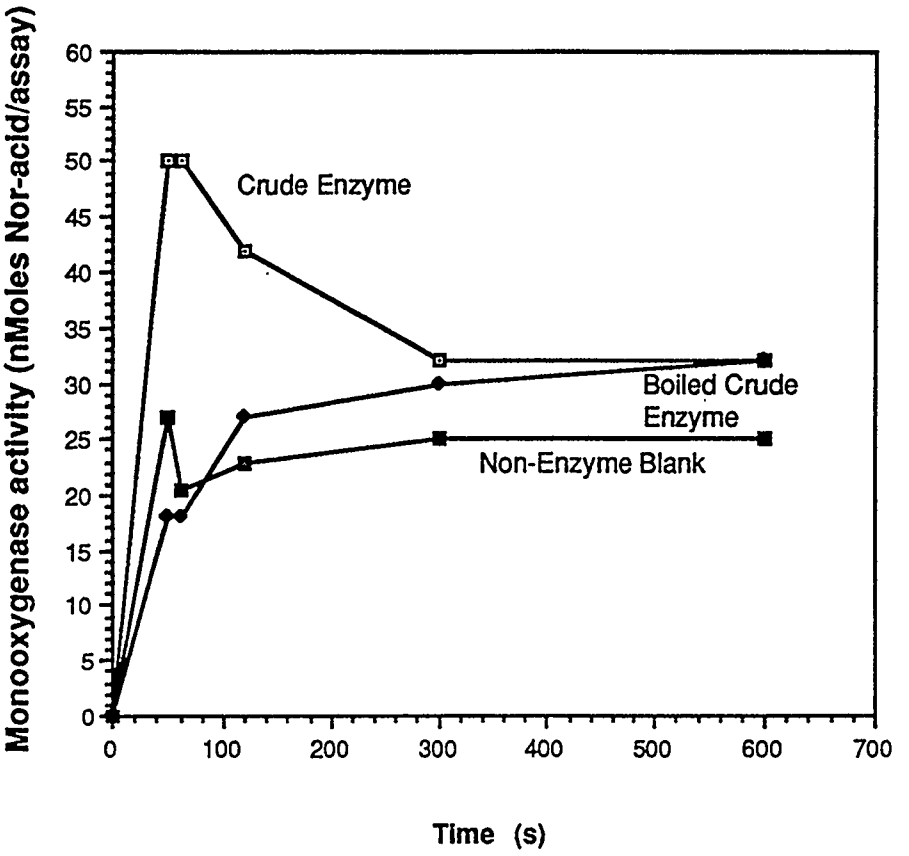


Figure 14.7 Enzymatic and non-enzymatic production of norsolorinic acid

Assay conditions:

Buffer	phosphate, 1M pH7.5
Substrate concentration	0.6mM
Enzyme concentration	100µL
Temp.	0°C

Crude enzyme was boiled at 100°C, for 30 minutes before use.

The results of Fig. 14.8 show the effect of boiling time and proteinase K treatment on enzyme inactivation, in order to determine the non-enzymatic production of nor-acid under optimum assay conditions (See 10.2). There was only a slight difference between boiling the cell extract for 20 min., 30 min., or 40 min. The proteinase K treated sample was more active than the boiled control, suggesting an incomplete proteolysis, but in all treatments there was an inactivation of the enzyme. This experiment confirmed that about 64% of the nor-acid conversion was enzymatic. Thus a 30 min. boiled enzyme blank was subtracted from all assays as described in 10.2.

14.6.2 Anthraquinone Instability

As described above under sections 14.1 and 14.2 (see Figs. 14.1 and 14.2) the norsolorinic acid produced in the assay of the monooxygenase is not stable. Thus initial high product concentrations are not maintained with time and this loss of product is much greater at temperatures above 10°C. Although the exact degradation products of norsolorinic acid were not determined, its degradation was minimized by the choice of an assay temperature of 0°C, and a total assay time of 30 sec.

15. COFACTORS REQUIRED FOR MONOOXYGENASE ACTIVITY

The unpurified monooxygenase was used to study its requires for different components in the reaction mixture.

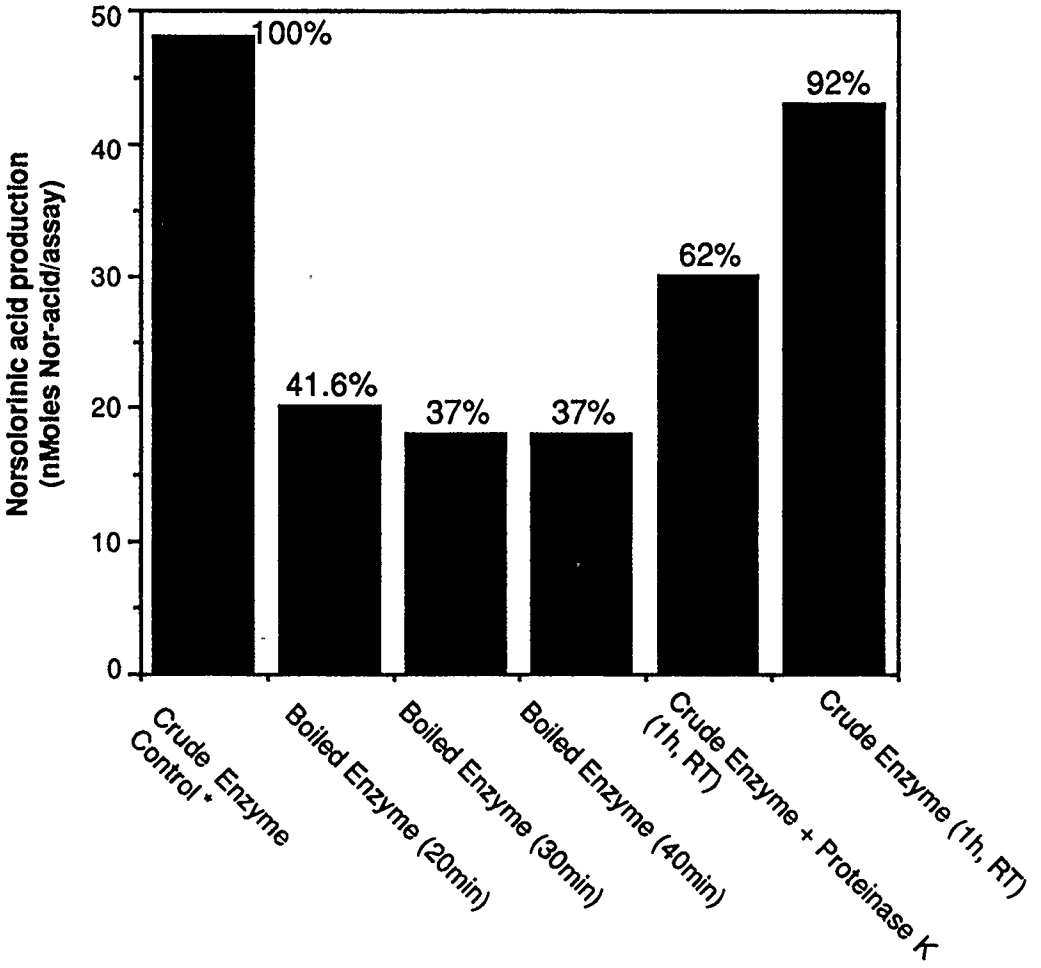


Figure 14.8 Determination of the extent of the production of Nor-acid after enzyme inactivation.

Assay conditions:	
Buffer	phosphate, 1M pH 7.5
Substrate concentration	0.6mM
Enzyme concentration	100µL
Reaction time	30 sec
Temp.	0°C

* This crude enzyme control was assayed immediately without a 1h incubation at room temperature (RT).

Table 15.1 shows the effect of these components on the activity of the enzyme. The addition of NADPH did not produce activation or inactivation of the monooxygenase. The rest of the additives were inactive as cofactors, but the degree of inhibition was different depending on the component; DTT, $CoCl_2$, and EDTA showed a slight inhibition, followed by FAD and FMN; the different divalent cations produced a significant inhibition, especially in the case of $CdCl_2$ which is known to react with sulfhydryl groups. Ascorbic acid and especially α-ketoglutarate also had a detrimental effect on activity. The enzyme was also inhibited by the absence of O_2 as was expected. All these results suggested that the reaction did not require any foreign electron donor, which would be indicative of an internal monooxygenase. Similar results were reported in the identification of emodinanthrone oxygenase, which catalyzes the oxidation of emodinanthrone to emodin, in the fungus *Aspergillus terreus* (Fujii et al., 1991).

Other enzymes used in the biosynthesis of aflatoxin (e.g. versiconal cyclase) in *A. parasiticus* were reported to have no necessary cofactors for enzyme activity (Lin and Anderson, 1992).

The "inhibitory effect" of a lack of O_2 in the assay suggests that the enzyme requires molecular oxygen as a substrate, for maximal activity. Thus, our enzyme may be tentatively designated as an internal monooxygenase.

Table 15.1 Effect of Different Cofactors on Monooxygenase Activity in a Broken Cell Suspension

Additions	monooxygenase activity (%)
Control[a]	100
NADPH (9)[b]	100
O_2 deficiency[c]	70
FAD (5)	88
FMN (5)	81
DTT (3.3)	95
Ascorbic Acid (2.9)	51
α-ketoglutarate (0.825)	25
EDTA (5)	90
Fe_2SO_4 (5)	81
$CoSO_4$ (5)	69
$CoCl_2$ (5)	93
$MgSO_4$ (5)	62
$ZnSO_4$ (5)	65
$CdCl_2$ (5)	28
Mix 1 (Ascorbic acid (2.9)), α-ketoglutarate (0.825), $MgSO_4$ (5), $FeSO_4$ (5), DTT (3.3)	47
Mix 2 (Ascorbic acid (2.9)), α-ketoglutarate (0.825), $MgSO_4$ (5), DTT (3.3)	42

[a] Control: phosphate buffer 1M pH 7.5 (33 μL), EGMM (283 μL), H_2O (84 μL), crude cell-free extract (100 μL), 0.6 mM substrate (nor-anthrone) (50 μL)

[b] Final assay concentrations in mM of each additive are given in brackets

[c] Oxygen was removed from the assay mixture by bubbling N_2 as described in section 11 with no additives

16. PRELIMINARY PURIFICATION OF THE NOR-ANTHRONE MONOOXYGENASE

6.1 CELL BREAKAGE METHOD

For complete disintegration of the cells, high protein yields and, most importantly, enhanced enzyme survival, there are different cell disruption methods that allow the release of intracellular proteins. Secondary metabolic enzymes, whose levels are usually lower in the cell than those of primary metabolic enzymes, are sensitive to environmental changes, such as temperature, pH, and the presence of proteolytic enzymes. Liquid shear forces generated by the cell disruption method may also be detrimental to enzyme survival. Thus, it is very important to choose a cell breakage method which will offer good temperature control and enzyme survival

Several techniques have been used to disrupt *A. parasiticus* mycelia and to obtain active cell-free preparations (Dutton, 1988). Cane (1986), in a study of the purification of enzymes involved in the late stages of aflatoxin biosynthesis, described cell-free extracts prepared by grinding the mycelia under liquid nitrogen. This procedure was found by Bhatnagar et al., (1988) to be the most efficient method for the recovery of enzyme activity in cell-free preparations, as compared to other procedures, such as sand grinding, glass bead grinding and lyophilization.

Given the lengthy experience of this laboratory only two cell breakage methods were compared for their efficiency in

the survival of nor-anthrone monooxygenase activity: the Braun homogenizer, which ruptures the cells by a ballistic mechanism and is used for small volumes (e.g. 12 ml) and the microfluidizer which uses liquid shear forces and is used for medium and large sample volumes (e.g. 100-2000 ml).

The results (Fig. 16.1) indicated that, although both methods offered acceptable temperature control for the broken cell suspensions (4ºC and 3ºC, respectively), the microfluidizer, with only one pass, gave the cell-free extract with the highest monooxygenase activity. Similar results were reported by Wang (1991) and Fedeshko (1992). In our case we could, moreover, observe that the microfluidizer produced more homogeneous broken cell samples than the Braun homogenizer. The microfluidizer was thus chosen to prepare cell free extracts with higher enzymatic activities.

16.2 *IN VITRO* STABILIZATION OF THE NOR-ANTHRONE MONO-OXYGENASE ENZYME

In vitro stability studies are important because of the unstable nature of most secondary enzymes. Loss of activity in crude cell extracts is the main problem affecting enzyme isolation and purification. However, if enzyme stabilization in crude extracts is successfully achieved, purification may then be attempted.

A previous report on the effect of breakage buffer composition on the recovery of 6-MSA synthetase activity

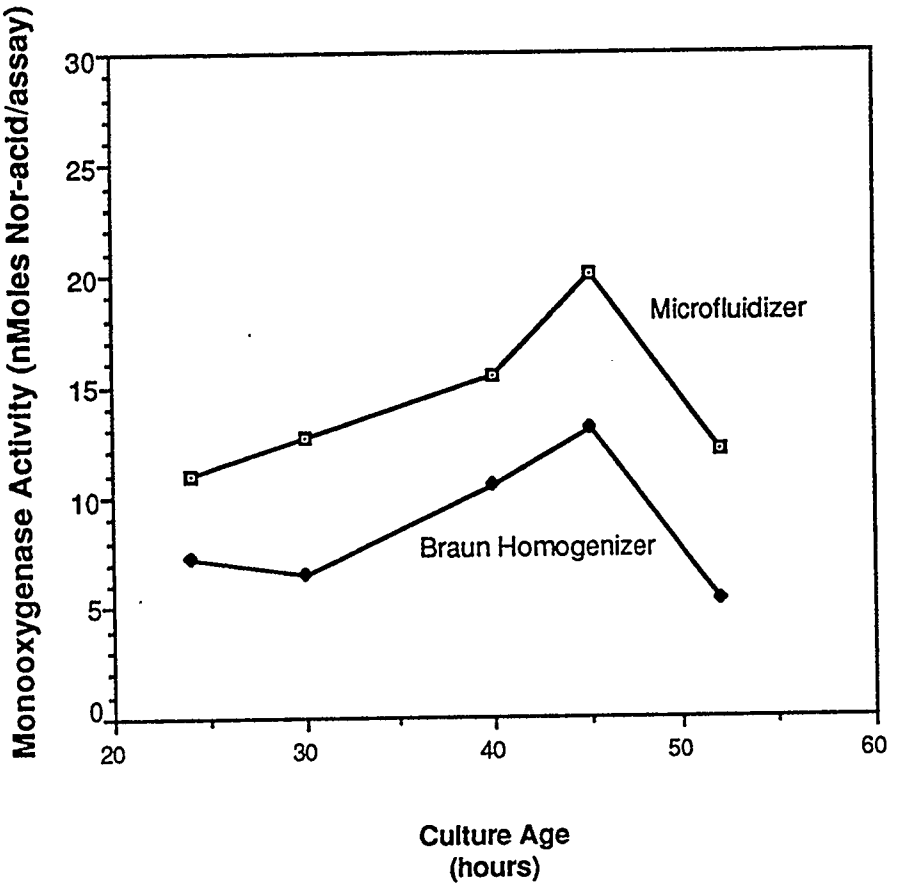


Figure 16.1 A comparison of nor-anthrone monooxygenase activity in cell-free extracts from standard fermentor grown cells using two different cell breakage methods.

A fresh cell suspension (12 and 100 mL, respectively) were used in the Braun Homogenizer and Microfluidizer methods. The assay used is described in the beginning of section 10.2.

(Scott et al., 1974) indicated that a concentration of 10% glycerol in the buffer significantly increased extractable synthetase activity over lower (0% and 5%) concentrations.

Other studies by Wang (1991) and Fedeshko (1992) reported an increase of the half-life of 6-MSA synthase and the dehydrogenase IDH, using 100 mM phosphate buffer (pH 7.6), containing 15% glycerol, DTT (3mM), and turkey egg white trypsin inhibitor (TTI) (1mg/mL).

A preliminary investigation of the effect of the breakage buffer pH on monooxygenase activity was done, using 0.1M phosphate (pH 6, 6.5, 7.0, 7.5), glycerol (10%) and DTT (10 mM) at 4ºC. Phosphate was chosen instead of Tris, since it was shown by Scott et al., (1986) to increase the stability of m-hydroxybenzyl alcohol dehydrogenase.

The objective was to establish the effect of the pH of the extraction buffer on enzyme activity. Figure 16.2 shows that the activity was higher at pH 6, 6.5 and 7, as opposed to pH 7.5 (crude extracts). Lower activity was obtained for supernatant extracts at all pH values. We chose pH 6.5 for our next experiments and decided to work with unclarified crude extracts since the lower supernatant activity suggested that the monooxygenase might be partially membrane bound.

At a constant pH of 6.5, the effects of DTT (5 mM, 10 mM, 15 mM) glycerol (10%) and TTI (1mg/mL) were tested on the

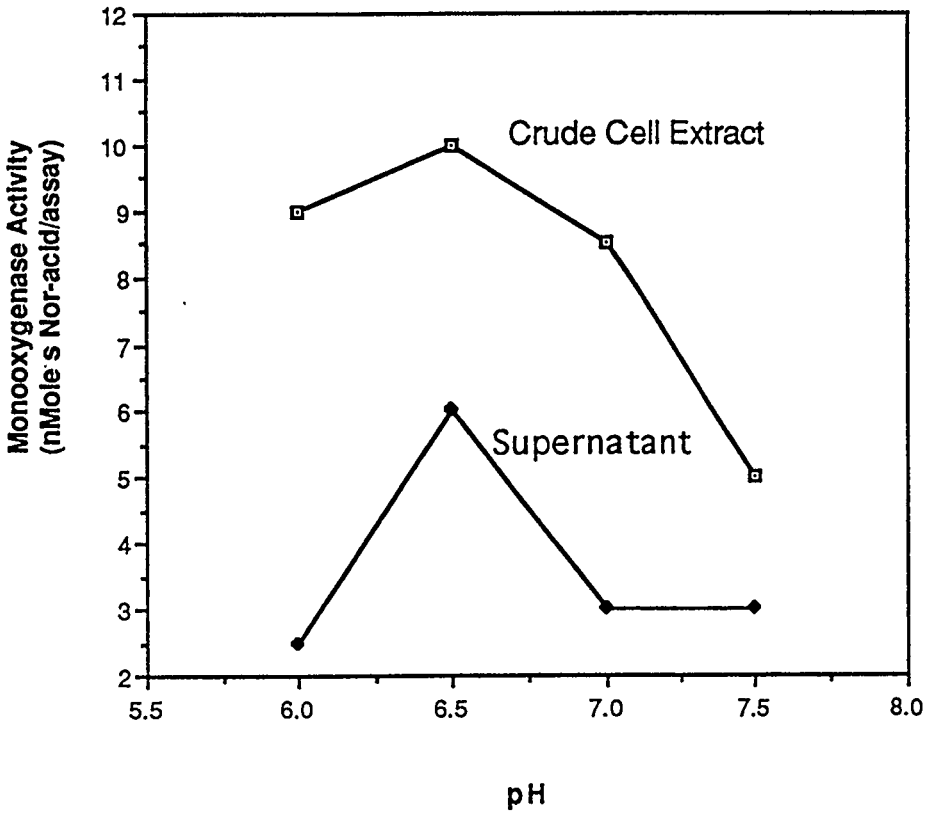


Figure 16.2 Effect of the cell breakage buffer pH on monooxygenase activity in cell free extracts from 42h-old fermentor grown cells. All cell samples (100 mL) were broken using the microfluidizer and the resulting crude extract was assayed at pH 7.5 without any prior clarification. A supernatant prepared by centrifugation (10,000xg) was also assayed for enzyme activity.

stability of the monooxygenase. For this purpose, the half-life (the time necessary to lose fifty percent of the initial activity at 4°C) was used as the criterion for enzyme stability.

As shown in Table 16.1, inclusion of dithiothreitol (DTT) alone increased the monooxygenase half-life from 7 h to 28 h, 32 h and 40 h when increasing its concentration to 5 mM, 10 mM and 15 mM, respectively. The presence of glycerol 10% also increased the half-life four fold over the control. A combination of 10 mM DTT and (10%) glycerol extended the *in vitro* half-life of the monooxygenase to about 91 h (thirteen fold increase over the control). Adding the proteinase inhibitor (TTI) [glycerol 10%, TTI 1mg/mL] and DTT (10 mM) and TTI (1mg/mL) did not increase the half-life as compared to DTT (10 mM) and glycerol (10%) alone. As expected, the combination of DTT (10 mM), glycerol (10%) and TTI (1mg/mL) in the breakage buffer did not further improve the longevity of the enzyme.

All these results suggested that the most important components for the stability of the monooxygenase enzyme and the increase in its longevity were the addition to the buffer of DTT and of glycerol. DTT alone improved the stability of the enzyme, indicating that inactivation of the enzyme could be promoted by oxygen and may involve oxidation and inactivation of some of the enzyme's sulfhydryl groups. Glycerol interacts with water, so that protein-water

Table 16.1 The Effect of Buffer Additives on the Stability of Nor-anthrone Monooxygenase Activity in Cell-free Extracts of 42 h-old *A. parasiticus* Cells.

Cell Breakage Buffer Components	$t_{\frac{1}{2}}$ (hrs) at 4°C	Fold Increase in Enzyme Half-Life
100 mM Phosphate Control (pH 6.5, no additives)	7	1.0
100 mM Phosphate 5 mM DTT	28	4.0
100 mM Phosphate 10 mM DTT	32	4.5
100 mM Phosphate 15 mM DTT	40	5.7
100 mM Phosphate 10% glycerol	28	4.0
100 mM Phosphate 10 mM DTT/10% glycerol	91	13
100 mM Phosphate 10 mM DTT/1 mg/mL TTI	28	4.0
100 mM Phosphate 10% glycerol/1 mg/mL TTI	24	3.4
100 mM Phosphate/10 mM DTT 10% glycerol/1 mg/mL TTI	26	3.7

All Assays Were Carried Out in Duplicate.

interactions are decreased (Scopes, 1987); thus glycerol acts as a stabilizer of protein conformation to make them less susceptible to proteinases. The ineffectiveness of TTI could be due in part to the absence of high levels of proteinase in the crude extract, or to the early harvest time of the cells from the culture (40-42 h), which would avoid proteolytic attack from idiophase-specific proteinases. The effect of some proteinases could also be minimized by the effect of glycerol. This suggested that the age at which cultures were harvested could have a marked effect on the *in vitro* half-life of the nor-anthrone monooxygenase. Similar results were obtained for 6-MSA synthase (Wong, 1991) and IDH (Fedeshko, 1992). The breakage buffer with DTT, glycerol and TTI showed that the turkey egg white trypsin inhibitor (TTI) had a negative effect on the activity of the enzyme; the enzyme may be sensitive to TTI, because TTI never increased the half-life of the enzyme as did DTT and glycerol. Previous studies (Rollins and Gaucher, 1994; Scott, et al., 1986c) on *Penicillium urticae* have shown that serine and cysteine proteinases predominate; these proteinases are inhibited by PCMB, chymostatin and, to a lesser extent, by PMSF. However, because of the sensitivity of IDH to PCMB (Sekiguchi and Gaucher, 1979) it was not used as a proteinase inhibitor. In this study proteinases inhibited by TTI may not be effective in degrading the monooxygenase. In fact, because of its membrane bound nature, this enzyme may not be very susceptible

to most proteinases.

In conclusion, a cell breakage buffer was developed that consisted of the following: 0.1M phosphate (pH 6.5 at 4ºC) containing 10 mM DTT and 10% glycerol. This protects the noranthrone monooxygenase enzyme against inactivation (t = 91 hours at 4ºC) in crude cell extracts from 40 - 42 h-old cells of *A. parasiticus* and increased the *in vitro* half-life of the monooxygenase by thirteen fold. This buffer was used routinely for cell breakage and enzyme purification. It was also possible to avoid the use of the proteinase inhibitor TTI which would be prohibitively expensive if used in all buffers during the purification. Finally, although higher levels of DTT (i.e. 15 mM) might provide a little more stability the extra cost of using a higher concentration throughout the purification was not deemed necessary since a half-life of about 91 h was sufficient.

16.3 ENZYME SOLUBILIZATION

The results obtained in the study of the *in vitro* stabilization of the enzyme (Fig. 16.2) indicated that our enzyme was possibly a membrane-bound protein. Eukaryotic enzymes involved in oxidation processes often exist as membrane components (Fujii et al., 1988). It was important to determine to what extent this enzyme was soluble, and to maximize its solubility before purification was begun.

Table 16.2 shows the effect of centrifugation on

Table 16.2 Effect of Centrifugation on the Distribution of Monooxygenase Between Soluble and Insoluble Fractions

	Total	% Activity	
	Activity (mU)	Pellet	Supernatant
Low Speed Centrifugation (10,000 x g)	34.8	74	26
	25.4	76	24
High Speed Centrifugation (105,000 x g)	202	62	38
	132	76	24

In four different experiments an unclarified broken cell suspension was centrifuged and the activity in the pellet and the supernatant was determined. In each case cells were broken using the Microfluidizer as described in section 9.4.

monooxygenase enzyme solubilization, as a criterion for distinguishing between soluble and insoluble membrane proteins. There was some activity in the soluble fractions but most of the monooxygenase activity was associated with the particulate fractions. This suggested that the enzyme catalyzing the conversion of nor-anthrone to nor-acid was found predominantly (> 70%) in the microsomal fraction.

The use of low or high speed centrifugation made little difference to the distribution between pellet and supernatant. This suggests that this enzyme is associated with relatively large particles that are sufficiently sedimented at only 10,000 x g.

Fujii et al., (1991) reported the same localization for emodinanthrone to emodin. This is a very similar, in fact, identical reaction in *A. terreus*.

Detergents have gained an important status in protein biochemistry, because they are essential for the isolation and study of membrane-bound proteins. Nor-anthrone monooxygenase also required detergent solubilization before its purification could be attempted. Fig. 16.3 illustrates the effect of different detergents (Triton X-100, Octylglucoside and 3-[(3-Cholamidopropyl) dimethyl-ammonium]-1 propanesulfonate (CHAPS) at different concentrations, on the release of enzyme activity into a high-speed supernatant fraction. Triton X-100 was clearly the most effective detergent producing a three fold increase in soluble monooxygenase activity when 1% detergent

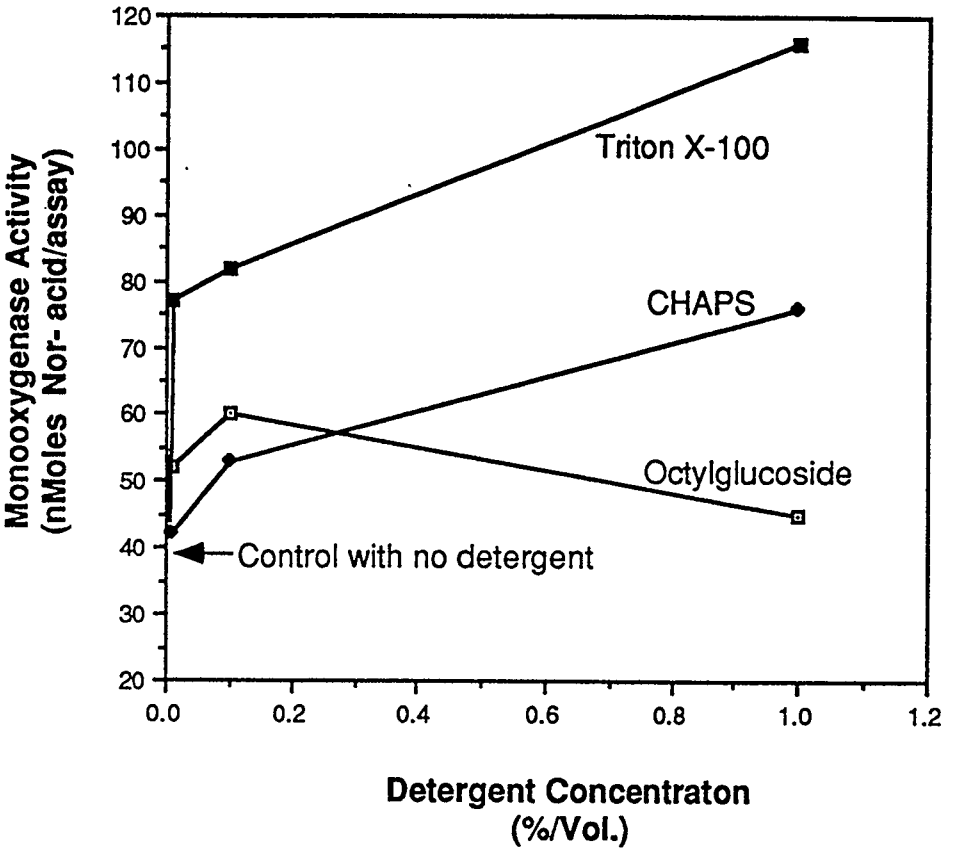


Figure 16.3 Effect of a one hour detergent treatment of crude extract (100 mL) on the release of enzyme activity into a high-speed supernatant fraction prepared by centrifugation at 105,000xg.

was used. However, 0.1% Triton X-100 was used for the purification of the enzyme because high concentrations of Triton can interfere with the different chromatographic steps.

The effectiveness of a detergent, in addition to the above factors, can depend on the sample volume and on the time of contact with the enzymatic extract. Small samples (50 mL) required less time for a major effect of 0.1% Triton X-100 on enzyme solubilization (data not shown).

Fig. 16.4 shows the enzyme solubilizing effect of treating a large (2000 mL) unclarified crude cell extract prepared from 40 h-old fermentor grown cells with 0.1% Triton X-100 at 4°C with magnetic stirring. Samples (100 mL) were taken from this detergent-treated suspension over a 24 h period and were centrifuged (10,000 x g) to yield a pellet and a supernatant which were then assayed for monooxygenase activity. Enzyme assays of pellet and supernatant indicated that the best time for maximal solubilization was about 10 h. At this time the monooxygenase activity in the supernatant had increased by 3.7 fold. Longer times resulted in a loss of activity in both the pellet and the supernatant, probably due to denaturation of the enzyme.

16.4 DETERGENT REMOVAL

Excess detergent should be removed because that usually improves the quality of subsequent chromatographic steps and because the detergent may interfere with the enzyme assay.

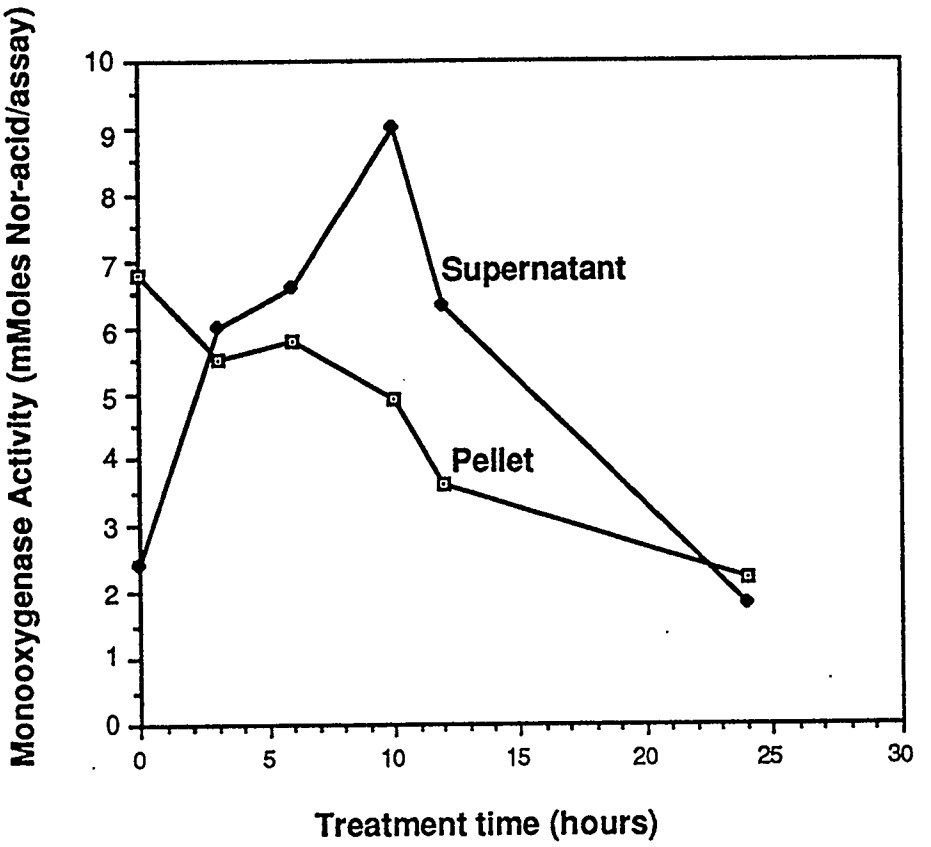


Figure 16.4 Effect of different detergent (Triton X-100, 0.1%) treatment times on the solubilization of monooxygenase in a large volume (2 L) of crude extract at 4°C. At various treatment times a 100 mL sample was centrifuged (10,000xg) and the pellet and supernatant were assayed for monooxygenase activity.

Table 16.3 shows the effect of Amberlite XAD-2 on detergent removal. First, these results indicate that the detergent had a positive effect on the detection of monooxygenase because its activity (30 mU) is higher (1.9 fold) as opposed to the control (16 mU). This is probably similar to the effect obtained by Fujii et al., (1988) who reported that non-ionic detergents such as Triton X-100 and Tween 80 at concentrations of 0.05-0.1% (w/v) showed a remarkable stabilizing effect on questin oxygenase activity. Hyelmeland and Chramback (1990) report that if a detergent can induce higher activity in a supernatant as opposed to the crude extract, then a detergent with this property should be chosen for the solubilization of the enzyme.

The activity in the supernatant after Triton X-100 treatment, showed that there was a 65% solubilization compared with a 23% natural solubilization of the enzyme. When the detergent was removed, there was a small but definite increase in the detection of soluble enzyme (69% vs. 65% obtained before Amberlite XAD-2 treatment). This increase in activity may be due to a negative effect of excess detergent on the enzyme assay. It also indicated that this excess of detergent was removed by Amberlite XAD-2, although Triton is an unusually difficult detergent to remove, because it has a low critical micelle concentration and a high micelle molecular weight. With a higher concentration of Amberlite there was a loss of activity (to 44%), which could

Table 16.3 Effect of Detergent on Solubilization of Nor-Anthrone Monooxygenase Activity

ENZYME SOURCE	ENZYME ACTIVITY (m Units)	% SOLUBLE ENZYME
Crude Broken Cell Suspension	16	-
10,000 x g pellet	14	-
and supernatant	4.2	23% (4.2/18.2)
Supernatant + Triton X-100	30[a]	-
10,000 x g pellet	10	-
and supernatant	18.2	65% (18.2/28.2)
Triton X-100 supernatant + Amberlite XAD-2 (6 g/L)	22[b]	69% (22/32)
or + Amberlite XAD-2 (24 g/L)	14[c]	44% (14/32)

[a] broken cell suspension was treated with 0.1% (v/v) Triton X-100 (10 h at 4°C)

[b] excess Triton X-100 was removed by a batch adsorption (45 min. at 4°C) to decrease inhibitory effect of detergent in the enzyme assay.

[c] greater removal of Triton X-100 decreased the amount of soluble enzyme.

be due to nonspecific binding of the enzyme to the resin or to a loss in soluble/detectable enzyme.

16.5 SALT FRACTIONATION AND STORAGE OF CRUDE MONOOXYGENASE AS AN AMMONIUM SULFATE PRECIPITATE

Large volumes of cell extract (i.e. ~1500 mL) need to be reduced to a more suitable volume for column chromatography. The most commonly used technique is to concentrate a dilute protein solution by ammonium sulphate precipitation. In an early purification attempt precipitation of the enzyme present in a 10,000 x g supernatant obtained from crude extract after Triton X-100 treatment (10 h), detergent removal with resin (45 min), and centrifugation (10,000 x g), was carried out using a salt fraction range (35% - 65% of saturating ammonium sulfate) as described in previous work (Wang, 1991).

Table 16.4 shows the results of detergent treatment to solubilize the monooxygenase, followed by $(NH_4)_2SO_4$ precipitation. Enzyme recovery was poorest for the detergent treatment (i.e. 48% loss), was relatively good for the salt precipitation (i.e. only a 22% loss) and was quite small for the clarification of the resuspended pellet (i.e. only a 10% loss). Overall these preliminary steps gave an enzyme recovery of 36% and a two fold purification. Although a considerable loss of enzyme occurred (i.e. 64%), the detergent treatment was needed to solubilize more of the enzyme and the salt precipitation was crucial for enzyme concentration,

Table 16.4 Preliminary Steps in the Purification of Nor-Anthrone Monooxygenase

	Total Volume (mL)	Total Protein (mg)	Total Activity (mUnits)	Specific Activity (mU/mg)	Yield Overall (%)	Fold Purification Overall
Crude Broken Cell Suspension	1500	24,165	76,200	3.1	100	1
Triton X-100 Treatment (10 h) detergent removal (45 min) and centrifugation (10,000 x g) to yield supernatant	1400	11,497	39,760	3.4	52	1.1
65% $(NH_4)_2SO_4$ pellet resuspended in purification buffer	60	7,073	31,104	4.4	41	1.4
Clarified resuspension (centrifugation 10,000 x g)	60	4,436	27,960	6.3	36.1	2.0

reducing the volume of the crude broken cell suspension from 1500 mL to 60 mL which is more suitable for column chromatography. Both treatments can certainly be further improved to give a better yield of monooxygenase.

In a separate experiment, the effect of different storage conditions on the monooxygenase activity in the ammonium sulfate precipitate was examined. The results (Fig. 16.5) showed that in dry form (pellet without purification buffer) there was more loss of activity after two and four weeks of storage at -20°C (14% and 20%, respectively) than at -70°C (5% and 7%, respectively). Even at -70°C, storage of a resuspended pellet (~ 0.5 gm) in 2 mL of purification buffer gave losses of 42-48%.

Ammonium sulfate precipitated pellets containing 6-MSA synthetase activity could be stored at -70°C for more than one year without loss of the activity (Wang, 1991). Also large amounts of IDH could be stored in a stable form at -70°C after ammonium sulfate precipitation (Fedeshko, 1992). Monsan and Cowbes (1984) reported that enzyme stability increased in concentrated media when water activity decreased. In previous studies, it was found that enzymes remain very stable when stored as a dried powder (Schmid, 1979). The increased protection (see Fig. 16.5) could be due to the elimination of water, because water participates either directly or indirectly in all covalent interactions that affect the native

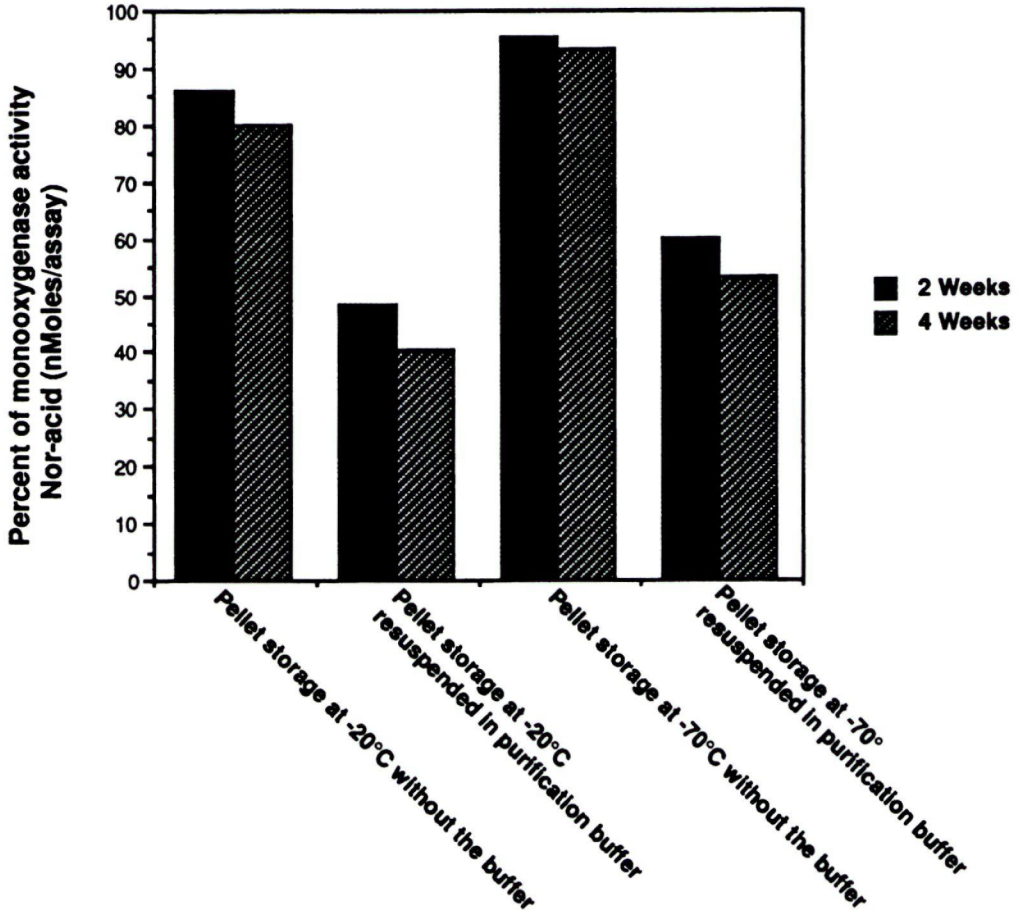


Figure 16.5 Stability of the monooxygenase in 35-65% Ammonium sulfate precipitates (i.e. pellets). The purification buffer used was 100 mM Phosphate (pH-6.5) + 10 mM DTT + 10% Glycerol. The pellet was resuspended by mixing 0.5 g of pellet in 2 mL of purification buffer and then frozen.

active conformation of the enzyme. Removal of water could drastically distort the conformation of the enzyme, but only a monolayer of water around the enzyme is actually needed. The catalytic activity of an enzyme is directly related to a precise three-dimensional conformation and any modification in this structure can result in a loss of catalytic activity. The three-dimensional conformation is stabilized by many low-energy bonds that are broken mainly as a result of an increase in vibration energy and in collisions with water molecules, favouring the denaturation (Dobbins, 1973).

In conclusion, reducing the amounts of water by storing the enzyme as an ammonium sulfate precipitate results in an increased protection. Ammonium sulfate can therefore be used as an efficient concentration step. The precipitate can also be effectively stored providing a constant supply of active crude monooxygenase enzyme for purification studies. The early steps finally decided upon are summarized in detail in Fig. 16.6.

16.6 CHROMATOGRAPHIC PURIFICATION STEPS

16.6.1 Gel Filtration Chromatography

Following dissolution of the ammonium sulfate pellet (~ 16 gm) in purification buffer (60 mL), a desalting step was necessary. The gel filtration offers two advantages:

1) it makes the selection of further column chromatography steps more flexible, and

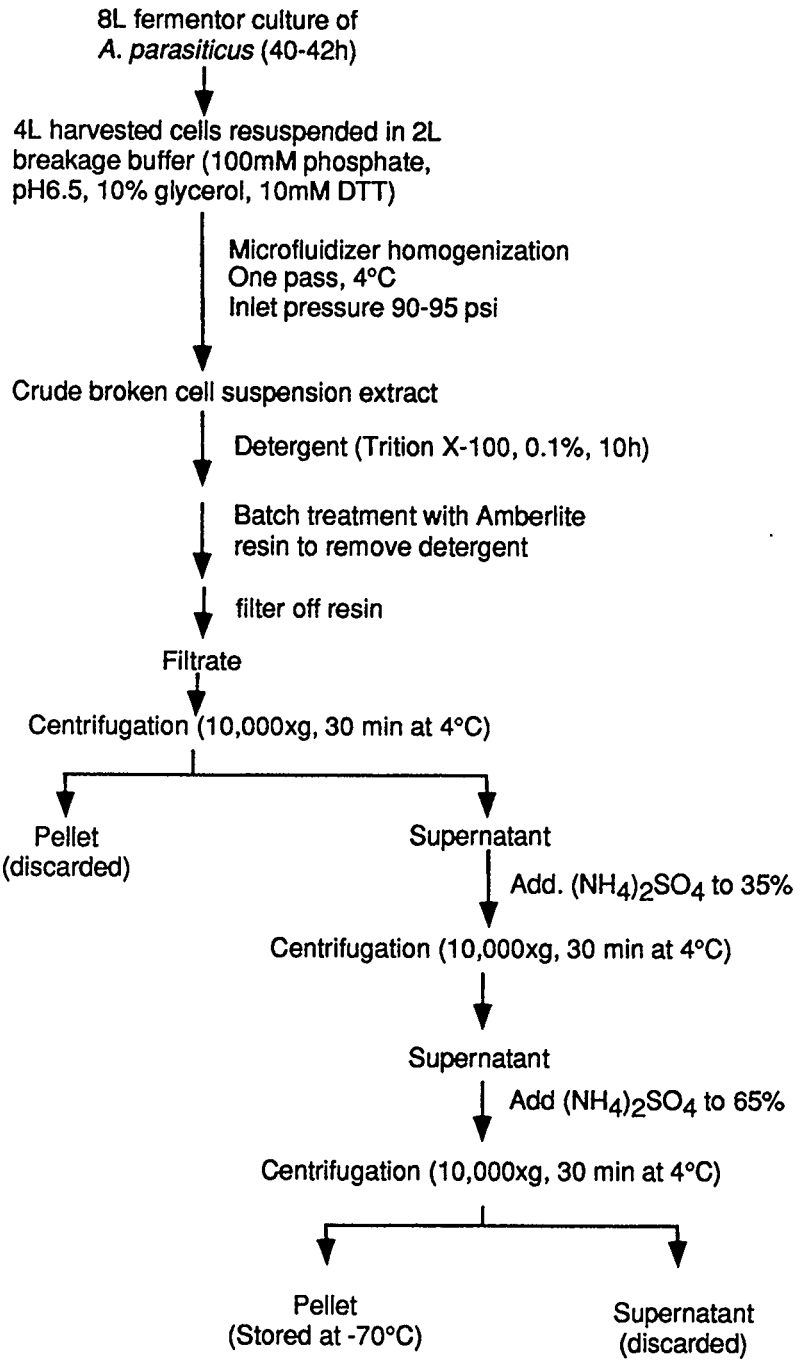


Figure 16.6 Pre-chromatography steps in the purification of nor-anthrone monooxygenase from an 8L fermentor culture to an ammonium sulfate precipitate.

2) it achieves some degree of purification by removing protein contaminants of different molecular weight.

Bio-gel A-1.5M (agarose gel, fractionation range 10,000 to 1.5 x 10^6 was chosen (Fig. 16.7)) Table 16.5 shows the Bio-gel A 1.5M gel filtration step, using 15 mL of the resuspended pellet, resulted in essentially no purification and a poor yield of about 41% over the activity present in the sample $(NH_4)_2SO_4$ pellet resuspended in buffer and clarified by centrifugation (10,000 x g). Whether contaminating proteins observed in the ammonium sulfate precipitate were removed after gel filtration as revealed by SDS - PAGE analysis (see Fig. 16.10) is uncertain. The early appearance of activity (Fig. 16.7) suggests that the enzyme has a high molecular weight which could be due to the absence of Triton in this step, and as this enzyme is a membrane bound enzyme, possibly aggregation could increase the apparent molecular weight. This is probable since the denatured polypeptide appears to have a molecular weight of less than 50,000 (Fig. 16.10).

16.6.2 Hydroxylapatite Adsorption

Hydroxylapatite adsorbs proteins on the surface of calcium phosphate particles and inside crevices and cracks within the crystalline particles (Scopes, 1987). The pooled gel filtration fractions (72 mL) were applied to a hydroxylapatite column (1.6 x 9 gm, 18 mL). A step gradient using 0.025-0.25 M phosphate (pH 6.5) revealed that mono-

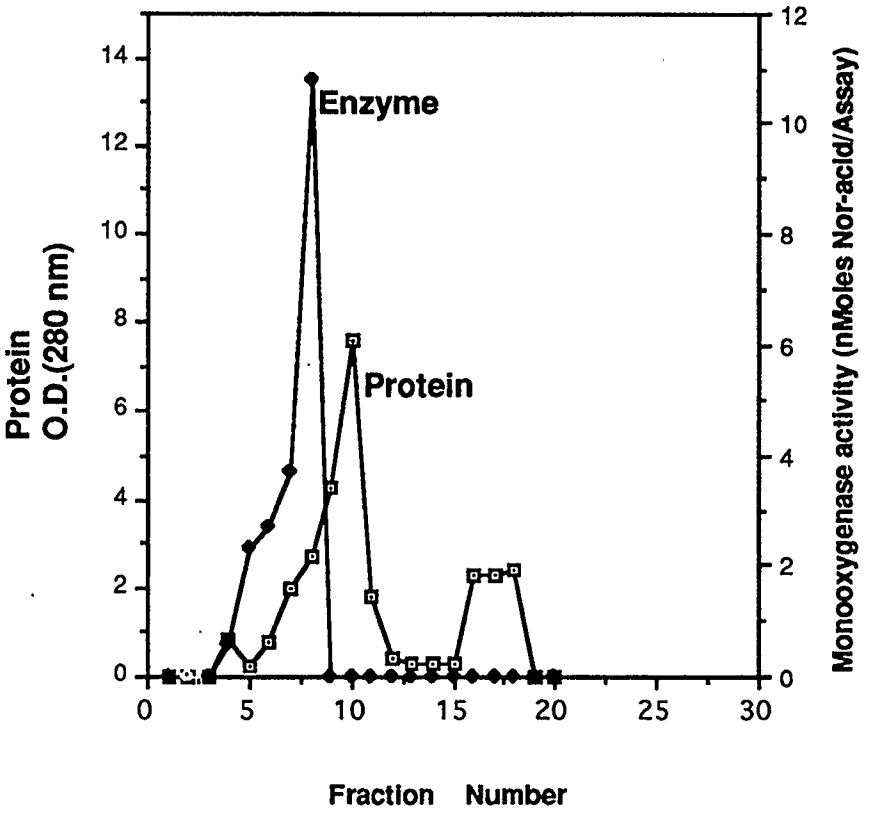


Figure 16.7 Distribution of protein and nor-anthrone monooxygenase activity after gel filtration (150 mL Bio-Gel A 1.5M column) of a solubilized ammonium sulfate precipitate. Purification buffer (100mM phosphate, pH6.5, 10% glycerol, 10mM DTT) was used for elution at a flow rate of 1mL/min. Each fraction volume was 12mL, and fractions #4 to #9 were pooled. A 100 mL aliquote of each fraction was assayed for enzyme activity using the optimized assay described in section 10.2.

Table 16.5 Column Chromatography Steps Used in a Preliminary Purification of Nor-anthrone Monooxygenase

	Total Volume (mL)	Total Protein (mg)	Total Activity (mUnits)	Specific Activity (mU/mg)	Yield Per Step (%)	Fold Purification Per Step
Two Fold Purified Preparation[a]	15	1109	6,990	6.3	-	-
Gel Filtration[b] (Biogel A-1.5 M)	72	638	2,894	4.5	41.4	0.7
Storage at 4°C[c]	72	300[d]	2,469	8.2	85.3	1.8
Adsorption[b] (Hydroxylapatite)	187	599[e]	8,901[e]	14.9	360.5[e]	1.8
Storage at 4°C[c]	187	186[d]	1,496	8.0	16.8[e]	0.54
Ion Exchange[b] (DEAE-Sephadex)	24	57	573	10.1	38.3	1.3
Storage at 4°C	24	13[d]	458	35.2	79.9	3.5

[a] One quarter (i.e. 15 mL) of a clarified resuspension of an ammonium sulphate precipitate (See Table 16.3)

[b] The values for the individual fractions to be pooled were added together from the respective Figs.16.7, 16.8 and 16.9 and are reported here.

[c] The time consuming nature of the HPLC enzyme assay necessitated the storage of individual fractions at 4°C for 48-72 h until those containing enzyme could be identified and pooled.

[d] In each case storage at 4°C for 48-72 h results in a significantly lower protein value, probably because of precipitation or settling.

[e] This unexpected increase in both protein and enzyme activity is probably due to some experiment error.

oxygenase activity could be desorbed from 0.05 M to 0.2 M phosphate; thus a further purification of the monooxygenase was achieved as shown in Fig. 16.8. The active fractions collected from the flow through provided good material for further chromatography using anion exchange. The proteins exhibited a banding pattern similar to that seen after gel filtration. An SDS-PAGE (see Fig. 16.10) gel showed decreased levels of almost all the contaminating proteins. Thus, this step is believed to have resulted in some purification (Fig. 16.8). However, as seen in Table 16.5, an error in the determination of the enzyme activity values in fractions 7-17 make it impossible to accurately comment on the degree of purification achieved. If the total enzyme activity after storage at 4ºC (Table 16.5) is the more accurate value, then an enzyme recovery of greater than 61% is probable.

16.6.3 Anion Exchange Chromatography on DEAE-Sephadex

Ion exchange chromatography has been recognized as a technique that can provide good results with complex mixtures (Scopes, 1987). Pooled fractions (187 mL) from the hydroxylapatite chromatography were run on a DEAE-Sephadex column (Fig. 16.9). Much of the protein contamination could be eluted without KCl or with a low concentration of KCl (0.1-0.4 M KCl) in the purification buffer, with no monooxygenase activity being detected in the eluent. Further elution with 0.5 M KCl removed almost all the monooxygenase activity from

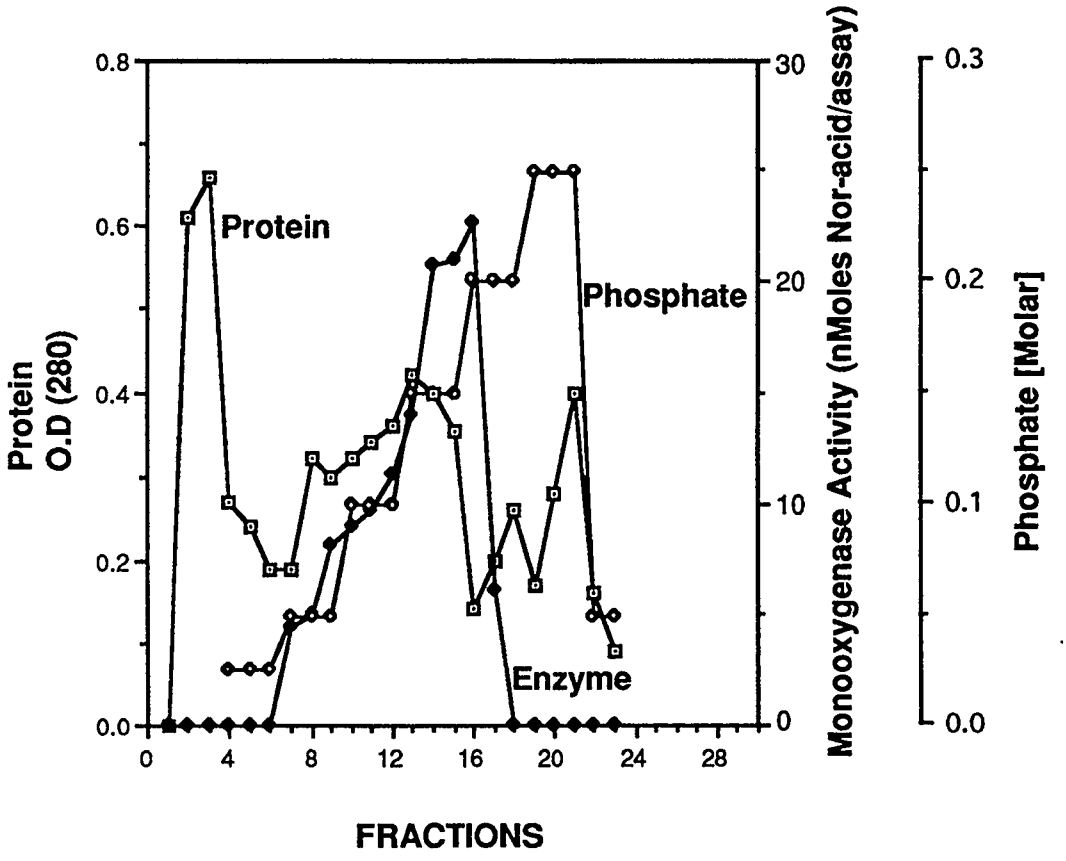


Figure 16.8 Hydroxylapatite adsorption chromatography of gel filtration recovered activity.
Monooxygenase activity was eluted using a 1 mL/min step gradient of phosphate (0.025-0.25M) in the purification buffer. Fractions (17 mL) were collected and fractions 7-17 were pooled for use in the next step.

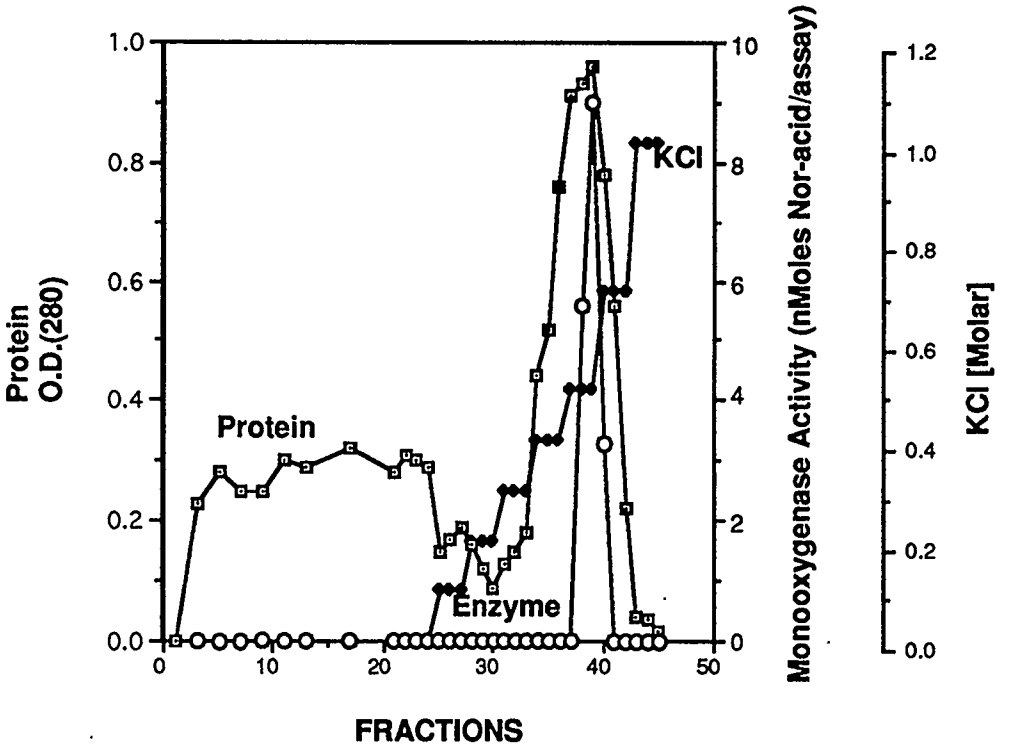


Figure 16.9 DEAE-Sephadex Ion Exchange chromatography of nor-anthrone monooxygenase enzyme from the hydroxylaptite chromatography step. The monooxygenase activity was eluted using a step gradient of KCl (0-1.0M) in purification buffer (100 mM Phosphate buffer, pH-6.5; 10 mM DTT; 10 % Glycerol). The enzyme began to elute at approximately 0.5M KCl. Three fractions (#38-40) of 8 mL each were pooled (24 mL) for SDS-PAGE assessment.

the column. The remaining activity was eluted with 0.7 M KCl. The yield for the DEAE-Sephadex chromatography step was only about 38% and gave only a little purification (1.3 fold increase, see Table 16.5). At this point there was so little protein left that the monooxygenase level was not visible on the SDS-Page gel (Fig. 16.10).

A condensed version of Table 16.5 is presented in Table 16.6 and shows an overall purification of 5.6 fold and an enzyme recovery of 6.6%. When combined with the data in Table 16.4, this admittedly imperfect purification from a crude broken cell suspension suggests that an overall purification of more than 11.2 fold (i.e. 2 x 5.6) may be possible with an overall yield of 2% (i.e. 36% x 6.6%) or better.

An SDS-PAGE gel (Fig. 16.10) examination of the entire purification shows little purification from the crude cell-free extract to the ammonium sulfate pellet. The Bio-gel A 1.5 gel filtration may remove some contaminants but the purification is minimal. A significant number of contaminants are removed by the hydroxylapatite column (the only band that can be seen is associated with the monooxygenase activity). As noted above the purification achieved using DEAE-Sephadex chromatography was not evident on the SDS-PAGE gel due to a lack of protein. A flow chart of this latter part of the purification is presented in Fig. 16.11.

Table 16.6 The Continued Purification of Nor-Anthrone Monooxygenase via Chromatography

	Total Volume (mL)	Total Protein (mg)	Total Activity (mUnits)	Specific Activity (mU/mg)	Yield Overall (%)	Fold Purification Overall
Two Fold Purified Preparation[a]	15	1109	6,990	6.3	100	1
Gel Filtration plus storage (4°C)[b]	72	300	2,469	8.2	35	1.3
Adsorption plus storage (4°C)[b]	187	186	1,496	8.0	21	1.3
Ion-Exchange plus storage (4°C)[b]	24	13	458	35.2	6.6	5.6

[a] One quarter (i.e. 15 mL) of a clarified resuspension of an ammonium sulfate precipitate (See Table 16.3)

[b] The deleterious impact of these storage times are indicated in Table 16.4.

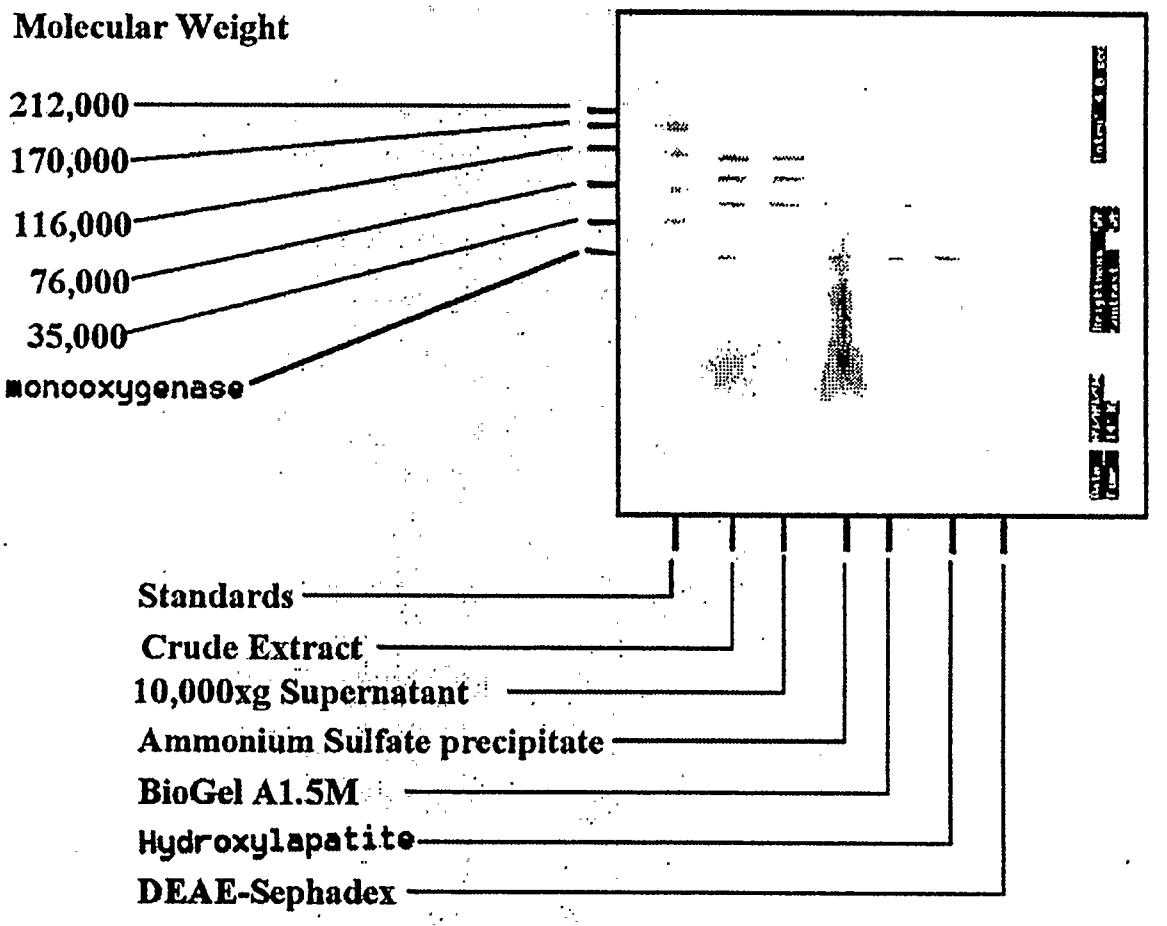


Fig. 16.10 Progressive Purification of Nor-anthrone Mono-oxygenase Visualized by SDS-PAGE Using a Phast Gel Gradient of 8-25.
For identity of Standard Proteins see (Fig. 11.7)

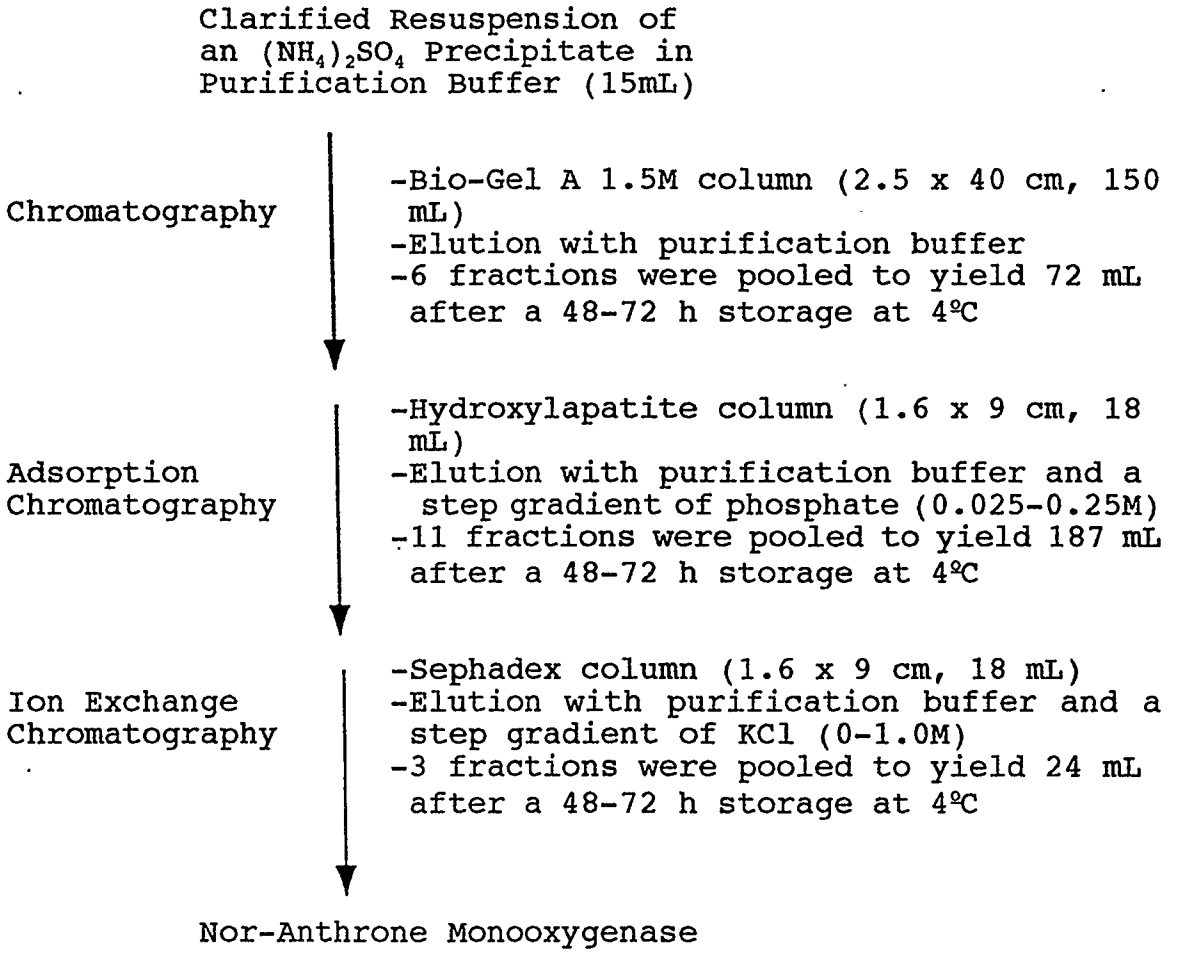


Fig. 16.11 Summary of the Final Three Chromatography Steps Used in the Preliminary Purification of Nor-Anthrone Monooxygenase

PART IV

GENERAL DISCUSSION

17. GENERAL DISCUSSION

Secondary metabolites are natural products of incredible diversity, which are now generally recognized as helping organisms to successfully compete with others in a particular environmental niche. Hence their diversity and contrast to primary nutrients and metabolites which are needed for simple growth and replication. Indeed, they are normally synthesized after active growth has stopped. Idiolite is a synonym that emphasizes the non-growth associated nature of secondary metabolites.

The mycotoxins form a subclass of this group of naturally occurring products. Mycotoxins are produced by a few genera of molds, namely *Aspergillus, Penicillium* and *Fusarium*. The majority of mycotoxins are produced through the polyketide pathway.

Although much is now known about polyketide biosynthesis, most of this knowledge is based on comparative analysis with fatty acid biosynthesis, because the main difference is the absence of intermediate reductive steps in the polyketide pathway.

An important group of mycotoxins are the aflatoxins. Investigations have led to the elucidation of their biosynthetic pathway (Bennett and Christensen, 1983), but much work is still needed in order to understand the enzymology of this pathway.

Studies on the enzymology of fungal secondary metabolism have had a slow start mostly because it is difficult to obtain active cell-free preparations. The first step is to determine the best moment (in the growth cycle) to harvest and extract the cells. This is not difficult to ascertain but investigations are needed to find when the trophophase (i.e. growth phase) switches into the idiophase and what triggers this event. The trigger can be quite complex, because it is often controlled by catabolite repression (e.g. a source of carbon and/or nitrogen). Other concerns are the transient appearance of the secondary metabolic enzyme, its inherent instability (often due to proteinases), and its low cellular concentration. Thus, the key to being able to detect and purify these enzymes is to obtain a cell sample in which essentially all of the cells contain a maximum level of the enzyme of interest. The next step is to choose an appropriate method of cell disruption to obtain active enzyme.

All these concerns have limited our understanding of polyketide biosynthesis at the enzyme level.

The major intermediates in the biosynthetic pathway leading to the aflatoxins have now been identified. Preliminary characterizations of some of the enzymes involved have been carried out. Initially, enzymatic studies on aflatoxin biosynthesis were limited to what were believed to be the early and the final steps of the pathway. The earliest

steps are assumed to be catalyzed by an enzyme system similar to that involved in fatty acid biosynthesis, but this polyketide synthase has never been detected in cell extracts until very recently (Wong and Gaucher, personal communication).

As described in Fig. 4.1, the first committed intermediate of this pathway has been identified as norsolorinic acid. However, the predicted product of the aflatoxin polyketide synthase is not the anthraquinone, norsolorinic acid, but the less oxidized norsolorinic anthrone. Thus, until this study the first two steps of the aflatoxin pathway remained uninvestigated. Although many workers have tried to detect these early enzymes there is no such report in the literature.

A preliminary study of the enzyme catalyzing this second step was the objective of this work. To enhance our chances of detecting the early enzymes of the aflatoxin pathway in fermentor grown cells of *Aspergillus parasiticus* (i.e. a mutant blocked after norsolorinic acid), we developed new culture conditions that provided:

1) pellet-free growth;

2) a rapid onset of secondary metabolism (e.g. at a culture age of 24 h), and

3) a high production of norsolorinic acid per given amount of cells.

Given a reproducible supply of cells containing high

norsolorinic acid producing activity, the preparation of an active cell extract was achieved simply by using our laboratory's proven large scale cell breakage apparatus, the microfluidizer. This included a careful selection of a cell breakage buffer which would stabilize the specific enzyme in the broken cell suspension.

At this point a maximally sensitive and reproducible assay for the enzymatic conversion of norsolorinic anthrone to norsolorinic acid was devised. The substrate anthrone was prepared synthetically from purified norsolorinic acid by a colleague (J. Wong). While it could be predicted that this monooxygenase would be membrane associated, other complications such as substrate and product insolubilities and instabilities were discovered during the development of the enzyme assay. Thus, we optimized different parameters (pH, temperature, enzyme concentration, substrate concentration and reaction time) to determine how to obtain the maximal monooxygenase activity with a minimal non-enzymatic contribution (due to instability of the substrate and of the product). The result after many difficulties was a reproducible assay which demonstrated that an enzyme mediated conversion of nor-anthrone to nor-acid only occurred in cells actively engaged in nor-acid production. Our preliminary characterization of the monooxygenase assay was as follows: pH optimium 7.5; linear response to the amount of extract (enzyme); assay time of 30 sec. at 0°C; substrate

concentration of 0.6 mM; labile to boiling and to treatment with proteinase K. No cofactors were found to be required, perhaps because they are firmly bound to the enzyme or its aggregate. However, as expected, a determined attempt to exclude oxygen showed that this oxidant was required. Since *in vitro* non-enzymatic conversion can also occur, the question of whether the *in vivo* conversion is solely enzyme mediated remains to be determined. At this point, it was possible to work with this monooxygenase activity and to attempt a preliminary characterization and purification.

In vitro stability studies indicated that the enzyme is stablized at pH 6.5 after adding 10% glycerol and 10mM DTT, which indicates that the monooxygenase is sensitive to O_2 (the DTT offers protection of the sulfhydryl groups).

The enzyme is associated with the particulate fraction, since only ~ 23% of the activity is soluble. However, addition of 0.1% of the non-ionic detergent Triton X-100 to the crude broke cell suspension yielded a 1.9 fold increase in activity after 10h. Thus, an additional 42% solubilization (10,000 x g supernatant) was added to the natural solubilization of 23%. Total solubilization detected was thus about 65%). An enhancement of soluble activity was obtained when the excess detergent was removed by batch adsorption with Amberlite XAD-2.

When a large amount of stable and soluble monooxygenase could be produced, an appropriate means of concentrating the enzyme before chromatography had to be found. Ammonium sulfate precipitation is an effective technique to do this, yielding a final pellet that can be stored at -70ºC with no significant loss in activity. Since this salt fractionation was not optimized at all, it is probable that this step can be significantly improved.

A pre-purification and a preliminary purification scheme were then developed that utilized a minimal number of steps and could be quickly carried out.

The presence of a high salt concentration is not compatible with some types of chromatography. Thus, after resuspension of ~ 16g of a 65% $(NH_4)_2SO_4$ pellet in 60 ml of purification buffer (100 mM phosphate, pH 6.5, 10% glycerol, 10 mM DTT), gel filtration chromatography was used to desalt the protein solution. The results obtained and discussed in the three chromatographic columns suggest that there was an imperfect purification and clearly there is still much work to be done in order to obtain a good purification of this difficult enzyme.

SDS-PAGE was used to follow the progress of these preliminary purification steps.

17.1 CONCLUSION

Studies on nor-anthrone monooxygenase have provided us with valuable information regarding an unknown early step in the biosynthesis of aflatoxin, an anthraquinone polyketide, mycotoxin. This study indicates that nor-anthrone is the immediate product of the PKS enzyme and that the second step of the aflatoxin pathway appears to be a monooxygenase-mediated conversion of nor-anthrone to nor-acid.

17.2 FUTURE EXPERIMENTS

With the knowledge from the results of this work, further research could be carried out to determine the optimum medium conditions with the objective of reaching the best levels of secondary enzymes and also to improve the solubilization of the enzyme and the ammonium sulfate precipitation, obtaining a good yield of the enzyme.

Another experiment that should be carried out is to develop a complete purification and to obtain purified enzyme for characterization. Some peptide sequences of this enzyme are of particular interest, because this represents an important step toward the isolation of this monooxygenase gene which could then be cloned and sequenced. The reason for these experiments is to provide valuable new information about the important polyketide biosynthetic pathway which produces aflatoxin, the most potent naturally occuring carcinogen known.

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