THE BIOCHEMISTRY AND MEDICAL ASPECTS OF NATURALLY OCCURRING TOXINS

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PhD (Salford)

Submitted in fulfilment of the degree of

DOCTOR OF SCIENCE

In the

School of Molecular & Cellular Bio-Sciences

Faculty of Science and Agriculture

UNIVERSITY OF NATAL
PIETERMARITZBURG

Pietermaritzburg 1999

ABSTRACT

The work presented here represents research done on mycotoxins and plant toxins by the author and his postgraduate students over a period from 1964 to date. The first phase, which ends at 1980, mainly addresses the biosynthesis of the aflatoxins. The involvement of anthraquinone derivatives in this process was investigated and the role of versicolorin A and its derivatives was partially elucidated. Novel active enzymes systems were derived from protoplasts and used in these studies.

The period lasting from 1980 to 1992 concentrates on the occurrence of mycotoxins in agricultural commodities and effects on animals and their systems. Over 7000 samples were analysed using a multimycotoxin analytical method and a fungal screen. The most common mycotoxin found was aflatoxin B_1 and prevalent fungus was *Fusarium moniliforme*. Later work is indicating that fumonisin B_1 is the most commonly occurring mycotoxin. As this was only discovered in 1988, its presence was only looked from 1995 onwards. It was also found that rumen fluid could metabolise trichothecenes.

During this period (1980-1992) further work on aflatoxin metabolism was done and a novel dehydrogenase involved in aflatoxin B_1 was isolated and characterised. An Elisa assay was developed for atractyloside, a toxin found in a plant (*Callilepis laureola*) used in tradition medicine. The site of atractyloside storage was found to be in the plant vacuole.

The final period covers 1992 to the present, where the occurrence and effects of mycotoxins in human disease were studied. The major and most important finding is that fumonisin B_1 is present in the blood and tissues of many of the Black population examined in Kwazulu Natal. This includes, oesophageal cancer patients, eclamptic patients, school children and members of the rural population. A similar circumstance also appertains for the presence of aflatoxin B_1. It seems likely from these results that chronic mycotoxicoses are a common occurrence, particularly in the Black rural population and are not the sporadic rare event that is found in the first world countries.

PREFACE AND ACKNOWLEDGEMENTS

This thesis includes all my original and co-authored publications to date and some original but unpublished studies from my PhD, (1967) studies. Publications that stemmed from my Ph.D. thesis presented to the University of Salford are indicated as such and are included for the sake of continuity. Apart from these, none of them have been submitted in any form for any degree or diploma by myself to any university, apart from this submission to the University of Natal.

The chapters begin with a list of my publications relevant to each title. This is followed by a short introduction in which earlier relevant work is briefly reviewed. Commentaries on the papers are then given, which are presented in such a fashion as to key them into the current thinking.

I commenced my research work under Dr J G Heathcote during my undergraduate studies at the Royal College of Advanced Technology, Salford, United Kingdom, which was to become later the University of Salford. Although there was not a formal Department of Biochemistry, John Heathcote was the Reader in Biochemistry in the Department of Chemistry and Applied Chemistry, under the headship of Professor George Rowtree Ramage. During my undergraduate years, I gained training not only in chemistry and biochemistry but also industrial microbiology, my chosen advanced option and in bacteriology. The latter happened because I was a sandwich course student and spent one of my six months periods of training at ICI, Blackley, in their industrial bacteriology laboratory, under Dr Gordon Thomas, himself an ex-student of the great Marjorie Stephenson. I was registered for PhD studies in 1964 with Dr Heathcote as my supervisor. My chosen topic eventually became a study of the biosynthesis of the aflatoxins, which I had studied as my forth year advanced project.

I left Salford in 1967 to take the first biochemistry lecturing post at the newly formed Wolverhampton Polytechnic. The first years there were rather fallow with respect to research, as I was very busy completing the write up of my PhD, various publications and lectures and practicals for 28 hours class contact per week. My interest in mycotoxins, however, continued, for fortunately Dr Gerald Ayerst, one of the original workers on aflatoxin, was a senior lecturer in the department. In 1972, I moved to Trent Polytechnic, as a senior lecturer in biochemistry in the Department of Life Science, under the headship of Gerald Leadbeater, who was a mycologist by training. I was also very fortunate to have as a section leader in biochemistry, Dr Walter Morris, who had worked on the early commercial production of antibiotics at Boots Pure Drug Company, Nottingham, in collaboration with such great names as Florey and Chain. From that point on my work on mycotoxin and secondary metabolite biosynthesis flourished and my first, PhD student, Murray Stuart Anderson, completed his successful thesis on aflatoxin biosynthesis in 1977. I also came to know several people who were working in the Ministry of Agriculture Food and Fisheries (MAFF) on mycotoxins and these included Arthur Hacking and Tony Buckle. In addition, Regina Schoental and Maurice Moss should be mention, as highly stimulating workers in the field with whom I had contact.

In 1980 I moved to South Africa and became Senior Lecturer in Biochemistry at the University of Natal, Pietermaritzburg Campus, under the headship of Professor George Quicke. This stimulated a much wider interest in mycotoxins, as there was

nothing being done on them in Natal and it was clear that there were many mycotoxin problems. Consequently the study of their biochemistry slowly fell away, and work on their occurrence, analysis, and effects was commenced. Although George Quicke was not himself particularly interested in the field, he gave me tremendous support and encouragement in my research efforts and Mike Wallis who was then senior lecturer in Microbiology gave support from the microbiology side of things. In my earlier years at Pietermaritzburg, I was joined by Ken Westlake who had done a Masters with me at Trent and continued with his PhD at Natal. He did some excellent work on the metabolism of selected mycotoxins by the rumen and finished off at Onderstepoort working in their anaerobic laboratory on this problem.

I think it only fair to mention at this juncture, that there was a well established mycotoxin research ethos in South Africa when I arrived. Although I never worked directly with these groups, I found their presence comforting and supportive, if I needed advice and discussion. Names that are almost legendary in international mycotoxin research such as Professor Wally Marasas and Professor Pieter Steyn spring to mind. Many member of their teams such as Amelia de Jesus, Peter Gorst Allman, Rob Vleggaar, Don Trinder, Piet Thiel, Chris Rabbie and more recently Eric Sydenham, Gordon Shephard and "Blom" Gelderblom have my thanks for many hours of fruitful discussion on mycotoxins.

A major boost in my work occurred when I spent 9 months sabbatical leave at the Southern Regional Research Laboratories at the invitation of Louise Lee and Evind Lillehoj. I also spent some time at Tulane University working with Joan Bennett and these good people became my firm friends. In addition Maren Klich became a life long friend and, as she has an international reputation as a mycologist with special interest in mycotoxins, this was an extra bonus. I cannot say how important sabbatical periods are to academic researchers in developing their ideas and techniques. It seems a great shame to me that the current financial stringencies are cutting these contact periods down and almost eliminating them in some cases. I must mention that when I was I the SRRC, I often met Leo Goldblatt, a grand old man of mycotoxicology, who at 84 was still active in research.

When I returned to South Africa I took advantage of a joint scientific programme between Taiwan and South Africa and spent three months with my very good friend Dr T C Tseng (Bill) at Academic Sinica working on *Fusarium* toxins. On my return from that trip, I *was* propelled into the Headship at Pietermaritzburg due to the sudden retirement of Professor Quicke. It was a sad moment for me, not only because I lost George Quick's guiding hand but also because the headship of any department is demanding and must detract from ones research activities. However, I was very fortunate in that one of our biochemistry majors, Anil Chuturgoon became interested in mycotoxins for his honours programme. From that point on we have formed a very strong team and I know that the work I started on mycotoxins will be continued by him. Between us we took on many successful post graduate students with mycotoxin projects until he moved to the Medical Faculty as lecturer in biochemistry in 1989.

In 1990 I took another sabbatical leave for six months at Virginia Polytechnic and State University, Blacksburg with Walt Niehaus. Although Walt was not easy to work with I learnt a lot from him. I worked on fungal mannitol dehydrogenase and this

improved my knowledge and working capabilities of handling fungal enzymes. In addition I met many wonderful people in Virginia, which to me, is the best state in the Union (my friend Tate Graham, sadly passed on, would say of the Confederacy of course).

On returning from Blacksburg I immediately moved down to Durban as the Head of Physiology at the Medical Faculty. This was the commencement of my latest and probably the last phase of development in mycotoxin research into medical aspects and their effects on humans. I had already had contacts in this field when I did some work with Dr George Campbell who was at Madadeni Hospital and then later at Themba Hospital, in the then homeland of KaNgwane. Those who know George would agree that he is not the easiest of people to collaborate with. It was with much regret that our paths parted and I lost someone who had a remarkable ability to think laterally on most subjects. It was he who introduced me to the Kruger National Park and I will always recall him having a three way conversation in Afrikaans, English and Zulu in order to find our way to a kraal in the Natal Midlands on a sampling expedition.

In my latest phase I have just returned from six months at Lancaster University where I worked on grass endophyte mycotoxins with Peter Ayers' group. During this period I presented a paper at the IUPAC meeting on mycotoxins and phytotoxins where I made new contact with Professor Chris Wild, and Jean Jaques Castegnaro, who are international experts in the field of mycotoxin biomarkers, on which we are working on at the moment. I also re-made contact with Professor John Smith of Strathclyde University and John Gilbert MAFF Norwich. Both Jean Jaques Castegnaro and John Smith are entering into collaborative programmes with us and, as an extra bonus, Dr Nceba Gqaleni has joined my department as a lecturer after completing a successful PhD with John Smith on mycotoxins. It is with some pride that I can say that Nceba was a Masters student of mine at Pietermaritzburg and was a Mandela scholar during his stay at Strathclyde. He together with Anil Chuturgoon will ensure that excellence in mycotoxin research will continue at Natal.

The body of work presented in this thesis is an exposition of the attempt to understand and answer some of the following questions: how one toxin, aflatoxin B_1 is formed and biosynthesised by the fungus; to determine what mycotoxins and at what level occur in Southern African crops; to determine what these toxins do at the cellular level; to determine the exposure of human beings to mycotoxins in South Africa; and determine the role of selected mycotoxins in local diseases, e.g., aflatoxin in liver cancer. In addition a diversion is taken where a toxin found in a traditional South African medicinal plant is studied.

Additional Acknowledgements

It goes without saying that all the persons mentioned in the preceding paragraphs have my unconditional thanks and good wishes for their support, interest in the subject of mycotoxins and just plain old-fashioned friendship. Without them this thesis would not have been written nor would the work ever have been done. At the sake of becoming tedious I would also like to express my feelings of thanks to the following people:

My parents, who although never rich in a material sense, gave me riches of unstinting support and guidance through my life. My long suffering family - my wife Pauline, my sons Mark and Paul and my daughters Caroline and Angela.

Anil A Chuturgoon needs special thanks, as he in more recent years has worked very closely with me to ensure that mycotoxin research at the University of Natal has prospered. He has acted as accountant, laboratory manager, nursemaid, bench worker and many other roles too many to mention. I have greatly appreciated his advice and friendship.

All my colleagues on the Pietermaritzburg campus, Durban campus and Medical School campus, University of Natal and those at the University of Durban Westville, University of Zululand, and Natal, M L Sultan and Mangosuthu Technikons.
In addition, all the students who have worked under, and with me.

A special thanks to: Ann, Sylvia, Susan, Bob McGrath, Ken Ehrlich, Joe Neucere, Dennis Hsieh; Professors Maurice Mars, Pat Berjak, Jack Moodley and John Taylor; Janet and the three witches; and finally to my very patient administrative assistants at various times, Jenny, Nisha and especially Marie Hurley for assisting with the production of this document.

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LIST OF ABBREVIATIONS

AFB1	Aflatoxin B_1
AFB2	Aflatoxin B_2
AFB2A	Aflatoxin B_{2a}
AFB1O	Aflatoxin B_1 Epoxide
AFG1	Aflatoxin G_1
AFG2	Aflatoxin G_2
AFG2A	Aflatoxin G_{2a}
AFM1	Aflatoxin M_1
AFM2	Aflatoxin M_2
ADAM	9-AnthrylDiAzoMethane
ADP	Adenosine DiPhosphate
ATA	Alimentary Toxic Aleukia
ATP	Adenosin TriPhosphate
ATR	Atractyloside
AV	Averythrin
CMI	Commonwealth Mycological Institute
DON	Deoxynivalenol
ELEM	Equine LeucoEncephaloMalacia (horse "hole in the head syndrome")
EM	Electron Microscopy
ER	Endoplasmic Recticulum
FB1	Fumonisin B_1
GC/MS	Gas Chromatography/Mass Spectrometry
GIT	GasteroIntestinal Tract
HB	Hepatitis B
(p)HBA	p-Hydroxy Benzoic Acid
HCC	Hepatocellular carcinoma
HPLC	High performance liquid chromatography
ICC	Immunocytochemistry
KEH	King Edward VIII Hospital (Congella, Kwazulu Natal)
KR	Kwashiorkor
MAFF	Ministry of Agriculture, Fisheries and Food (UK)
mDNA	Mitochondrial DeoxyRibonucleic Acid
MT#	Masters Thesis (number)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Norsolorinic Acid
NMR	Nuclear Magnetic Resonance
NCON#	Presentations made in South Africa (number)
OC	Oesophageal Cancer
OCON#	Presentations outside South Africa (number)
OMS	*O*-Methylsterigmatocystin
PhT#	PhD Thesis (number)
RVS	Regional Veterinary Service (Natal)
SA	Sphinganine
SAX	Strong Anion Exchanger
SBS	Sick Building Syndrome
SO	Sphingosine
ST	Sterigmatocystin

TLC	Thin Layer Chromatography
TVPH	Total Vegetable Protein Hydrolysate
VA	Versicolorin A
VC	Versicolorin C
VHA	Versicolonal Hemiacetal Acetate
ZEA	Zearalenone

CHAPTER 1:

INTRODUCTION TO MYCOTOXINS

Because all animals, including man, depend either directly or indirectly on the consumption of plants for their existence, they are exposed to natural xenobiotic substances that are often toxic. These toxic metabolites derive primarily from two sources: the plant itself; or from microorganisms that have infected the plant or its products. There are a variety of terms that have been used in the scientific literature to cover naturally occurring metabolites that have toxic properties towards various types of organisms. **Phytotoxin** is often used to describe plant metabolites that are toxic towards animals but this term is ambiguous as it has been used to describe toxins produced by other organisms that kill plants. Authorities have coined specific terms, e.g., **marasmin**, a substance produced by a microorganism that affects a plant, to resolve what is meant but this specialised term is rarely used. The term **mycotoxin**, however, is well established and is used to mean metabolites from filamentous fungi toxic towards animals and man. Mycotoxin should not be used for toxic metabolites produced by the Bacidiomycetes, i.e., the well-known toadstool poisons.

Both phytotoxins and mycotoxins belong to a group of compounds called secondary metabolites. Many thousands of these compounds are know but only a percentage of them are toxic to animals, e.g., 300 fungal secondary metabolites have been identified as having toxic properties out of several thousand being described. It is a matter of strong debate, as to whether these properties are coincidental or have more deliberate evolutionary ecological significance but there is no doubt that in many cases such toxic metabolites confer an ecological advantage to the producing organism. Indeed the whole question of the function of secondary metabolism is open to speculation, as it only normally occurs during the senescence phase (called **idiophase**) of the growth cycle of the organism and does not appear to contribute to the normal growth phase (called **trophophase**) of somatic cells.

Mycotoxins can appear in the human food chain at different levels. Thus the very seed, from which the plant grows, may contain toxigenic fungal spores, which ultimately may lead to infection of the mature plant. These fungi may not be regarded by plant pathologists as true plant parasites and, indeed, some fungi that have been discovered to be systemic within plants, are normally described as saprophytes, e.g., *Aspergillus flavus* (Klich 1986). As is the case in most branches of science, idealised definitions may not fit the real world, in spite of their usefulness in rationalising our thinking. Whatever the actual plant-fungal relationship, often the presence of a fungus in, or on, a plant, results in the production of toxins both by the fungus and the plant in response to each other's presence. These interactions are studied under the rather contradictory term of chemical ecology.

In addition to the difficulties of sorting out the exact inter-action, it is also a problem to understand toxin production with respect to growth cycle. Often precise events, such as tropho- and idio-phase, cannot be easily defined in such systems and, hence to think of toxin production as an "idiophase" event is difficult. It is probably better to think of the condition where secondary metabolites appear, as being those of "stress". Hence the idiophase in pure culture becomes a stress situation, i.e., nutritional stress. Thus the production of mycotoxins in living systems is complex and currently there is little understanding of the process, although recent results from molecular biology studies do hold out hope of identifying factors involved in the control of their biosynthesis (Bennett *et al.* 1997).

Equally, the formation of mycotoxins in agricultural commodities during harvesting and storage is not fully understood, although the latter has been more fully studied. Several factors influence their production and of these moisture or water activity is the most important. However, it should be borne in mind that other factors such as: temperature; oxygen availability; type of substrate; species and strain of infecting fungi; other pests that are present, such as insects; and agricultural treatments, can dictate the type and amount of toxin formed.

Obviously without an infecting fungus there will be no mycotoxin produced, whatever else a stored commodity experiences. This inoculum is always naturally present in stored crops due to their growth taking in place in the field, which is a reservoir of inoculum. The dominance of any particular fungus at any point in the growth and subsequent harvest and storage of a crop, depends primarily on water content, provided other parameter are not extreme, e.g., temperature. This dictates a succession of fungi starting with the so-called "field" fungi and running through to those termed "storage fungi". These terms, although having an acceptance in the literature, are not accepted by all and Mycock and Berjak (1992) take the view that the division is somewhat artificial and such switches merely represent an ecological progress powered by water activity changes within the substrate.

The net result of this fungal metabolic activity, however, is the generation of secondary metabolites, which if having a detrimental action on the herbivores or omnivores that consume them, would be termed mycotoxins. Although it had been know for several centuries that such compounds could enter the food chain, e.g., in the classical case of ergot (Robbers 1979), such occurrences were regard as sporadic and at their worst a nuisance problem for humans. This view changed radically in 1961 with the outbreak of the so-called "Turkey X" disease in the United Kingdom (Blount 1961). As a result there was a burst of activity in attempting not only to understand Turkey X disease, as a mycotoxicosis of turkey poults but in the examination of many species of fungi for their production of toxic metabolites, not unlike the earlier bonanza of antibiotic screening. After all antibiotics are from the same stable as mycotoxins, it is merely a question of definition. Indeed some antibiotic are mycotoxins and vice versa. Not only were a large number of novel fungal toxic fungal metabolites discovered, some of considerable commercial importance but retrospective examination of the scientific literature showed that mycotoxins over the last hundred years at least

had been taking a heavy toll of animal and perhaps also human life (Forgacs 1962).

It might be wondered why suddenly there was this upswing in potential mycotoxicoses or was the problem always with us. Obviously fungi have had the ability to produce such metabolites since they evolved, the problem seems to be one of scale. While agriculture was practiced on a small rural scale with low populations and was labour intensive, rather than mechanised, only in times of famine were mycotoxins to play a significant role in human health. Usually only that which was needed for the coming year would be stored and any materials with frank fungal infection would be eliminated and perhaps fed to animals. Nobody likes to eat mouldy cereals and perhaps this is an evolutionary bias. Once mass agriculture commenced with huge storage systems and large scale processing, the potential for poisonings on a much larger scale was set up. The example of peanut butter is a good one. One peanut in a thousand that is heavily contaminated with aflatoxin, can increase the level of the toxin in the final product to parts per million, which is considered to be dangerous, because aflatoxin B_1 is highly carcinogenic, as well as being an acute poison.

The current answer to the problem of mycotoxin contamination is quality control supported by legislation. This in itself creates major difficulties for the following reasons:

1. There are several hundred toxic fungal metabolites already known.
2. Different crops have different attendant mycotoxin problems.
3. Different climatic regions favour the production of different toxins.
4. Different countries have different degrees of sophistication when it comes to quality control and the enforcement of law.
5. Nobody knows what mycotoxins do to human being, when consumed at a chronic level, either separately or in combination together and with the presence or absence of other nutritional factors.

It does not take much imagination to see the political, commercial and medical conflict that can arise when addressing the problem of mycotoxins. To get the whole world community to agree on the permitted levels of one toxin, i.e., aflatoxin, which had been extensively studied, has up to now almost proved extremely difficult. After several years of negotiation between Codex member States, levels have been set for AFB1 in groundnut and AFM1 in milk [(30^{th} Meeting of the Codex Alimentarius Committee on Food Additives and Contaminants March 1988, The Hague) (Rosner 1988)]. It is hoped that this will lead to further agreements on the permitted levels of mycotoxins in other products.

CHAPTER 2:

BIOCHEMICAL STUDIES ON THE AFLATOXINS

2.1: PAPERS AND STUDIES

[1]The structure, biochemical properties and origins of aflatoxins B_{2a} and G_{2a}
Paper 1 by Dutton and Heathcote

[1]O-alkyl derivatives of aflatoxins B_{2a} and G_{2a}
Paper 2 by Dutton and Heathcote

[1]New metabolites of *Aspergillus flavus*
Paper 3 by Heathcote and Dutton

[1]Some interesting relationships between the new aflatoxins B_{2a} and G_{2a} and their associated metabolites
Paper 4 by Dutton and Heathcote

Biosynthesis of aflatoxins, Part I.
Paper 6 by Heathcote, Dutton and Hibbert

Biosynthesis of aflatoxins, Part II.
Paper 7 by Heathcote, Dutton and Hibbert

The use of fungal protoplasts in the study of aflatoxin biosynthesis
Paper 8 by Dutton and Anderson

The use of cell free extracts from fungal protoplasts in the study of aflatoxin biosynthesis
Paper 9 by Anderson and Dutton

Biosynthesis of versicolorin A.
Paper 12 by Anderson and Dutton

The role of versicolorin A and its derivatives in aflatoxin biosynthesis
Paper 13 by Dutton and Anderson

The conversion of sterigmatocystin to O-methyl-sterigmatocystin and aflatoxin B_1 by a cell-free preparation
Paper 16 by Jeenah and Dutton

Biosynthetic relationship among aflatoxins B_1, B_2, M_1 and M_2
Paper 18 by Dutton, Ehrlich and Bennett

Characterisation of metabolites from a strain of *Aspergillus flavus* accumulating aflatoxin B_2
Paper 20 by Dutton

Enzymes and aflatoxin biosynthesis
Paper 25 by Dutton

The preparation of an enzyme involved in aflatoxin biosynthesis by affinity chromatography.
Paper 28 by Chuturgoon, Dutton and Berry

The appearance of an enzyme activity catalysing the conversion of norsolorinic Acid to Averantin in *Aspergillus parasiticus*
Paper 31 by Chuturgoon and Dutton

The isolation of a purified dehydrogenase involved in aflatoxin biosynthesis and its characterisation
Paper 34 by Chuturgoon and Dutton

Synthesis of sterigmatocystin derivatives and their bio-transformation to aflatoxin B_1 by a blocked mutant of *Aspergillus parasiticus*
Paper 52 by Gengan, Dutton, Chuturgoon and Mulholland

The role of O-methylsterigmatocystin in aflatoxin B_1 biosynthesis
Paper 53 by Gengan, Chuturgoon, Mulholland and Dutton

The conversion of sterigmatocystin and its derivatives aflatoxin B_1 by a partially purified enzyme system
Paper 54 by Gengan, Chuturgoon, Mudaly and Dutton

2.2 INTRODUCTION

Aflatoxins first came to the attention of the scientific community because of an outbreak of a mysterious disease amongst turkey poults, which because of its unknown aetiology at that time was termed "Turkey X" disease (Blount 1961). Subsequent work rapidly established that the cause was the presence of fungal metabolites in the feed, which originated from a cargo of groundnut imported into the United Kingdom from Brazil (Blount 1961). These toxic metabolites gave rise to acute symptoms in the birds which were principally lethargy, inappetence, staggering gait with death occurring rapidly. The metabolites were conveniently separated by thin layer chromatography (TLC) because of their strong purple and greenish blue fluorescence under long wave ultra violet light. Four such compounds were separated and termed: aflatoxin B_1 (AFB1); aflatoxin B_2 (AFB2); aflatoxin G_1 (AFG1); and aflatoxin G_2 (AFG2). These compounds were show to be produced by members of the genus *Aspergillus*, i.e., *Aspergillus parasiticus* and *Aspergillus flavus*. By 1964, thanks to work done in Europe, (Hartley *et al.* 1963; van Dorp *et al.* 1963)

the structure of the aflatoxins (Fig. 2) were elucidated. Because of their unusual structures it was clear that they were secondary metabolites but it was not obvious how they had been biosynthesised. Inspection of their molecular structure suggested several possibilities, including all the three major secondary metabolic pathways listed by Bu'Lock (1965) , i.e., polyketide, shikimate and isoprenoid pathways. Speculation was rife. Evidence and support for each pathway was forthcoming - polyketide (Biollaz *et al.* 1968) shikimate (Adye and Mateles 1964) and isoprenoid (Moody 1964).

2.3 COMMENTARY

2.3.1 Studies done at the University of Salford/Wolverhampton Polytechnic 1964-72

Some of the work report in this section was submitted for a PhD (marked [1] in the list 2.1; awarded 1969). Two main thrusts were tried in an attempt to discover the principle biosynthetic pathway for AFB1. The first was an attempt to isolate novel metabolites related to aflatoxin from cultures of *Aspergillus parasiticus* (wrongly identified as *Aspergillus flavus*) (CMI 91019B). Such metabolites isolated and characterised were: the novel aflatoxin B_{2a} (AFB2a) and aflatoxin G_{2a}, (AFG2a) as described in paper 1; their ethoxy derivatives, see paper 2; versicolorin C and averufanin, see paper 3, although at the time the significance of these compounds was unknown. The origins of AFB2a and AFG2a was shown, as described in paper 4, to be from AFB1 and AFG1 respectively by water addition across the terminal double bond of the bis dihydrofurano system under acid conditions. Their ethoxy derivatives were shown to be artifact generated in the isolation of the AFB2a and AFG2a, which are essentially hemiacetals, by the formation of the full acetals from the presence of ethanol in the chloroform used to extract the aflatoxins from culture medium. It was shown in paper 4 that substituting any alcohol in the chloroform for ethanol could generate a series of O-alkyl derivatives.

Another compound was described in paper 3, which was called aflatoxin B_3 and was isolated from cultures. This was shown to be a partly degraded AFG1. It was later isolated by other workers (Stubblefield *et al.* 1972) and called parasiticol. Logically it should arise from AFG1 by decarboxylation of the open lactone ring system, although this has never been investigated and it could well be derived from an earlier phase of the biosynthesis.

The second approach to the problem of biosynthesis of aflatoxin was done by adding labelled ^{14}C potential primary metabolic precursors such as acetate to aflatoxin producing cultures of *Aspergillus parasiticus*. It was shown in paper 6 that acetate rapidly labelled AFB1, whereas mevalonic acid only moderately labelled the molecule and shikimate related metabolites, e.g., phenylalanine and p-hydroxy benzoic acid (p-HBA) not at all. The latter observation was in contradiction to that of Adye and Mateles (1964) who had obtained quite effective labelling from added phenylalanine and tyrosine. This was explained by the fact that in my studies AFB1 was labelled by p-HBA and tyrosine simply by mixing them together. The complex

so formed was quite stable, as the label remained stuck onto the aflatoxin, even after development in three different solvent systems on TLC.

From this study it was shown clearly that AFB1 was a polyketide derived metabolite and this was in keeping with other observations made around that time (Biollaz *et al.*1968). This was proven amply later by much more powerful and elegant experiments including the total degradation of labelled AFB1 by Buchi's group (Biollaz *et al.* 1970) and ^{13}C nuclear magnetic resonance studies by Pieter Steyn's group (e.g., Steyn *et al.* 1975).

Other experiments reported in paper 7, where various labelled aflatoxins were fed to an aflatoxin producing culture of *A. parasiticus*, indicated that aflatoxin M_1 (AFM1) was a precursor of AFB1, which is the reverse of what is now considered to be the case. Although the latter pathway does occur in lactating animals and humans, hence the 'M' for milk toxin, it is not proven for fungi and this does need further investigation.

2.3.2 Studies done at Trent Polytechnic, Nottingham 1972-1980

By 1972 the basic pathway to AFB1 had been worked out by a combination of classical techniques, including the generation of fungal mutant capable of accumulating intermediates (Lee *et al.* 1975) and back adding of such labelled putative intermediates to the wild strain fungus (Bennett *et al.* 1980). More recent methods, mainly ^{13}C NMR spectroscopy (Steyn *et al.* 1975) supported the pathway. A PhD student, Murray Anderson, and myself commenced a study in 1974 to isolate active enzymes from *A. parasiticus* that would catalyse the total or partial biosynthesis of AFB1. This was an optimistic objective, as at that time no active cell free systems for so complicated a secondary metabolite had been successfully isolated from a fungus. The obstacles were formidable. Firstly secondary metabolic enzymes are not produced in quantity, as secondary metabolism is regulated by enzyme level unlike primary metabolism, which is regulated by substrate level. Secondly secondary metabolic enzymes are only formed at a certain time during the life cycle of the organism and it is also at this time when proteolytic activity is at an optimum and when enzyme-denaturing substances, such as phenolics, are being produced. In the case of AFB1 some of its precursors are themselves phenols. If all this is not enough the fungal cell is surrounded by a tough cell wall composed of chitin and β-glucans.

Although other workers managed to isolate certain enzyme activity by mechanical disruption (e.g., Wan and Hsieh 1980) all attempts by us to achieve a fully active system came to nothing. The final break through came, when I suggested that the best way to release cellular contents was by digesting the cell walls with enzymes a technique that had been used in other studies (Peberdy 1979). Using culture fluid from *Trichoderma sp.,* we were able to produce protoplasts, which to our joy were capable of converting labelled acetate to AFB1. When these were gently lysed the cell free extract retain its capability and this system was used for further conversion studies. Both these achievements were reported in rather modest papers (889) but

in my opinion this was some of my most important work. Unfortunately the development of NMR spectroscopy and more recently, molecular biological methods have obviated the necessity of producing free active enzymes. This is not always helpful, as there are many mechanistic details of the pathway, which in my view can only be elucidated by studying the enzyme catalysed reaction. This is exemplified later in our study on the conversion of sterigmatocystin (ST) to AFB1.

The precise details of how averufin is converted to versiconal hemiacetal acetate and versicolorin A (VA) and thence via sterigmatocystin (ST) to AFB1 was unknown at the time of our studies. In order to investigate the role of VA we fed various labelled putative precursors to our system. The results reported in Papers 12 and 13 were rather perplexing, because labelled VA could not be converted to AFB1 by the enzyme system, whereas its hemiacetal and hemiacetal acetate were, even in the presence of VA. We, therefore, concluded that VA hemiacetal (or a derivative) is the true intermediate in the AFB1 pathway and that VA is a side shunt. Even now the precise mechanism of the conversion of VHA to VA and thence to AFB1 is not clear in spite of many elegant experiments.

3.2.3 Studies done at the University of Natal 1982-1991

I re-commenced work on the biosynthesis of AFB1 when I went to Natal in 1980, where I was joined by a PhD student M Jeenah. It was decide that we would look at the involvement of ST in AFB1 biosynthesis. Cell-free extracts from *A. parasiticus* were obtained by lyophalisation and extraction by buffer. A system was obtained that could convert ST to AFB1 but not to AFG1 and also another unknown metabolite with a blue fluorescence. This latter compound turned out to be O-methyl ST (MST) and its production seem to be compulsory linked to that of AFB1 (Paper 16). When I went on sabbatical leave to the Southern Regional Research Centre (SRRC), USDA, New Orleans to work with Louise Lee, I did further work on this system and the group at SRRC finally showed that MST was an intermediate between ST and AFB1 (Bhatnagar *et al.* 1987).

During my period at the SRRC, I also worked with a strain of *A. flavus* that did not produce AFB1 but only AFB2. Experiments, where labelled AFB1 hemiacetal (AFB2A) was fed to this culture amongst others, as described in Paper 18 were done. Much to my surprise the added labelled turned up exclusively in AFB2, not AFB1. As AFB2A is derived from AFB1 by water addition, see above, this provides a route for the fungal conversion of AFB1 to AFB2 via AFB2A. Whether this mechanism operates in the natural state of fungal growth is unknown but it could explain why added labelled as AFB1, sometimes partially ends up in AFB2.

The notion that AFB2 can arise independently of AFB1 was supported by the finding that this culture, in addition to forming AFB2, also accumulated versicolorin C (VC) and aflatoxin M_2 (AFM2) (Paper 20). It was evident that a metabolic grid was in operation, whereby the metabolites with the saturated bisfurano system (i.e., VC, DihydroST, AFB2, AFM2) could interchange with the unsaturated ones and their hemiacetal derivatives, provided the correct enzyme, probably a desaturase of some sort, was present (Paper 25).

When Anil Chuturgoon joined me to do his Masters, we looked at the conversion of norsolorinic acid (NA) to VA and was able to obtain an enzyme system that was capable of converting NA to averythrin (AV) using the lyophalisation method (Paper 31). This enzyme was successfully isolated using affinity chromatography (Paper 28). Norsolorinic was immobilised on to sepharose and used as the stationary phase. Unfortunately this resulted in an inactive preparation and we concluded that the phenolic properties of the anthraquinone were to blame. Treatment of the immobilised NA with diazomethane blocked off the more active groups and the resultant modified material selectively absorbed the enzyme, which was then fully characterised (Paper 34). An attempt was made with the assistance of Merck, Sharp and Dohme (USA) to determine the amino acid sequence of the protein. There were, however, difficulties not least due to the presence of N-acyl groups, in the protein, not an unusual problem in secondary metabolic enzymes.

Several other post graduate students, also investigated other aspects of AFB1 biosynthesis. Omesh Sutan used a range of modern surfactant compounds to solubilise membrane bound enzyme activities involved in oxidative steps to AFB1 biosynthesis (MT5). Shaileash Maharaj isolated several methyl esterases capable of methylating various anthraquinone precursors and also isolated some of their products from cultures of *A. parasiticus* (MT4). Some of these esterases were later describes by other workers (Yabe *et al.* 1989)

3.2.4. Studies done at the University of Natal, Medical Faculty 1992-to date

On joining the medical Faculty, University of Natal, the work on mycotoxin biosynthesis virtually ceased, as I became more interested in disease aspects of mycotoxins, which was appropriate to my new Faculty. However, I agreed to supervise Robert Gengan, a lecturer in Chemistry at M L Sultan Technikon for his PhD studies. We decided to look more closely at the biosynthetic step between ST and AFB1, which had been comprehensively studied by other workers, notably Townsend *et al.* and Bhatnager *et al.* My main reason for this was that I had never been convinced of the necessity for the step where ST is methylated to OMST. This to me seemed to include an unnecessary complication and, furthermore, resulted in a metabolically less active intermediate, an alkyl ether, as far as the action of oxygenase require for the requisite ring cleavage needed in the conversion. Gengan synthesised a range of O-alkyl ST derivatives, including ethyl, propyl and an ester, benzoyl (paper 52). These were fed to a blocked mutant of *A. parasiticus* capable of converting ST to AFB1. To our surprise all the derivatives could be converted to AFB1, including ST itself with propyl showing the most rapid conversion. On reflection I concluded that what we were measuring was a membrane effect, as propyl would in theory penetrate the fungal cellular membrane best, being the least polar.

In order to resolve this a cell free extract (CFE) of the fungus was prepared that was capable of converting ST to AFB1 (Paper 53) thus removing the effect of the cellar membrane. On adding ST, OMST and OPST to this preparation all three were

converted to AFB1. The rates of conversion of ST and OMST were indistinguishable but OPST was slower, confirming the suggested role of the membrane in its more rapid conversion in the whole cell culture. Addition of NADPH and SAM increased all rates of conversion of the precursors and the indigenous presence of these in the CFE failed to give a clear cut answer as to whether OMST was an obligatory intermediate or not. Assuming that methylation was not rate limiting, then indigenous SAM would convert ST to OMST and hence cloud the issue. In order to over come this problem the CFE was passed through a Sephadex column in order to separate the protein fraction, containing the enzyme activity, from the lower molecular weight material containing the coenzymes (Paper 54). On incubation of the precursors with this system and added combinations of coenzymes, it became apparent that ST could not be converted to AFB1 without the intermediacy of an O-alky derivative, which under natural conditions would presumably be OMST. The fact that OPST could be converted to AFB1 without the addition of SAM confirmed that OMST was not a compulsory intermediate and that most short chain alkyl groups would serve the purpose. It was concluded that the enzyme(s) responsible for the conversion of O-alkyl derivatives of ST to AFB1 exhibited relative specificity, a well-known phenomena in secondary metabolism.

PRINTED FROM CHEMISTRY AND INDUSTRY, 1968, pp. 418—421

e structure, biochemical properties and origin of the aflatoxins B_{2a} and G_{2a}

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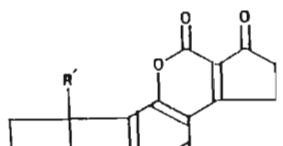
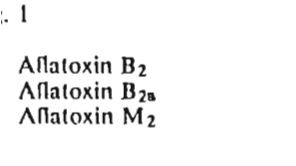
e structural elucidation of aflatoxins B_{2a} and G_{2a}

ittle while ago we reported[1] the isolation of two new lroxy aflatoxins, which we designated as B_{2a} (Fig. 1, II) l G_{2a} (Fig. 2, VII), because they were structural deriva- :s of the corresponding aflatoxins B_2 (Fig. 1, I) and Fig. 2, VI). The hydroxyl group present in these com- inds was substituted at position 2 of the terminal furan g in contrast with the structures of aflatoxins M_1 (Fig. 3 X) l M_2 (Fig. 1, III).[2] A more detailed account of the ntity and properties of aflatoxins B_{2a} and G_{2a} is given e in order to distinguish them more readily from the ıtoxins M_1 and M_2.



. 1

	R	*R'*
Aflatoxin B_2	H	H
Aflatoxin B_{2a}	OH	H
Aflatoxin M_2	H	OH
Acetyl derivative of Aflatoxin B_{2a} (also† 2, Acetoxy †3, hydro addition product of B_1)	CH_3CO-	H
†2, Methoxy †3, hydro addition product of aflatoxin B_1	CH_3O-	H



. 2

	R
Aflatoxin G_2	H
Aflatoxin G_{2a}	OH
Acetyl derivative of Aflatoxin G_{2a} (also †2, Acetoxy †3, hydro addition product of aflatoxin G_1)	CH_3CO-

oduction and isolation of the hydroxy aflatoxins

toxin-producing strain of *Aspergillus flavus* (C.M.I.)19b) was cultured on potato-dextrose-agar in medicine ttles at 25° until prolific growth had occurred. Sufficient rile water was then added to the bottles in order to tain a heavy suspension of the spores and mycelium of : mould. This suspension was then used to inoculate 400 ml. samples of sterile medium which had been dis pensed into conical flasks (1 l.). The medium used had th following composition: dipotassium hydrogen phosphate 3 g; potassium dihydrogen phosphate, 1 g; magnesiu sulphate, 1 g; ammonium sulphate, 8 g; glucose, 20 g; an zinc sulphate, 5 mg made to 1 l. with distilled water. Th flasks were then incubated at 25° until a maximum concen tration of aflatoxin was produced in the culture fluid. Thi point was ascertained by extracting portions (2 ml. each) o the culture fluid, after various periods of incubation, wit 20 ml. of chloroform, and measuring the optical density o this extract at 363mμ. This reading was taken as a direc measure of the total aflatoxins present in the culture fluid. When a maximum of aflatoxin had been reached in th culture fluid, the mycelia were harvested, the culture fluid were bulked together, and evaporated to about a fifth o their original volume in vacuo, at a temperature not ex ceeding 30°. This concentrate was then extracted repeatedl with an equal volume of chloroform until all the aflatoxin were extracted. The chloroform extracts were combined an evaporated in vacuo at room temperature to a volume o about 2 ml., which was then chromatographed on a colum of silica-gel (Merck 0·05; 0·2 mm), using methanol (2 pe cent v/v) in chloroform as the eluant. (The chloroform used in this, as in all the other chromatographic procedures was of Analar grade and thus contained at least 1 pe cent (v/v) of ethanol.) The resulting aflatoxin fractions wer further resolved into their components by thin-layer chroma tography employing silica-gel G (Merck nach Stahl), an methanol (2 per cent v/v) in chloroform as the developin solvent. The individual aflatoxins which separated wer scraped from the chromatoplates, eluted from the silic with either chloroform or chloroform/methanol mixture and obtained as pure crystalline solids by evaporation of th solvent under nitrogen, and recrystallisation from a suitabl solvent.

Preparation of aflatoxin derivatives

The acetyl derivative of the hydroxy aflatoxin B_{2a} (Fig. IV) was prepared by dissolving 20 to 30 mg. of the con pound in pyridine (0·1 ml.), adding acetic acid anhydrid (1 ml.), and allowing the mixture to stand overnight room temperature. The excess solvents were then remove in vacuo, and the acetyl derivative was separated from th residue by thin-layer chromatography, using the sam system and method as for the isolation of the paren aflatoxin. The pure crystalline derivative was finally ob tained by recrystallisation from methanol. The acet derivative of G_{2a} (Fig. 2, VIII) was prepared in a simil manner.

The trimethyl silyl ether of aflatoxin B_{2a} was prepared for mass spectrometry by treating a little of the compound with an excess of 'Tri-Sil'* (proprietary silylating agent), and allowing the mixture to stand for a few minutes before examination in the mass spectrometer.

The 2,† acetoxy 3,† hydro addition products of aflatoxins B_1 (Fig. I, IV), and G_1 (Fig. 2, VIII), were prepared by treating the aflatoxin (30 mg of each) with an excess of glacial acetic acid; a few drops of thionyl chloride were added as a catalyst.[4] The pure derivatives were obtained in a similar manner to that used for the isolation of the acetyl derivatives.

The 2,† methoxy 3,† hydro addition product of aflatoxin B_1 (Fig. 1, V), was prepared in a manner analogous to that used for the preparation of the acetoxy derivative except that methanol was used in the place of acetic acid.

Aflatoxins B_{2a} and G_{2a} and the various derivatives of the aflatoxins were examined by a number of spectroscopic techniques, these included: ultraviolet and visible absorption spectroscopy on a Perkin-Elmer 137 spectrometer using spectrosol methanol as a solvent; infrared absorption spectroscopy using a Perkin-Elmer 337 spectrometer, and nujol, and hexachlorobutadiene as mulling agents; Proton Magnetic Resonance absorption spectroscopy was carried out using a varian A60, or HA 100, instrument according to availability, and either deuterio chloroform or deuterio pyridine as the solvent, signals being measured in 'τ' values using tetramethylsilane as internal standard.

For mass spectrometry an 'MS 9' (A.E.I.) instrument was used.

Results

The maximum concentration of total aflatoxins in the culture fluid, expressed in terms of aflatoxin B_1, was usually about 80 mg/l. This concentration was generally achieved after incubation for a period of 8 to 10 days.

From the column chromatography of the culture fluid extract two fractions were obtained which fluoresced in ultraviolet light. The first fraction eluted from the column yielded, on thin-layer chromatography, the four known aflatoxins, B_1, B_2, G_1, and G_2. The R_F values of these compounds were in order, 0·6, 0·56, 0·54 and 0·5.

The second fraction from the column was found (by t.l.c.) to contain two major, and three minor, components. Of the major components, one fluoresced blue under ultraviolet light and had an R_F value of about 0·13, while the other fluoresced green at an R_F value of about 0·10. Both these components were unstable on the silica-gel thin-layers and, in the presence of alkalis, gave yellow decomposition products. Nevertheless, pure compounds were finally obtained as pale yellow plates by recrystallising twice from a mixture of chloroform and methanol. The chief component of the three minor ones isolated, fluoresced blue under ultraviolet light and had an R_F value a little higher than the blue fluorescent major component. This metabolite was isolated in similar manner to the others previously mentioned, and was shown to be identical by chromatography and mass spectrometry, with a sample of aflatoxin M_1 (Fig. 3, X) kindly supplied by Dr C. W. Holzapfel.

* Marketed by the Pierce Chemical Co., Rockford, Illinois

† Refers to the position in the terminal furan ring.

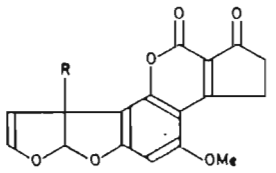


Fig. 3

	R
IX Aflatoxin B_1	H
X Aflatoxin M_1	OH

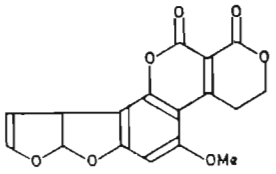


Fig. 4
XI Aflatoxin G_1

The infrared spectra of both the major compounds were similar to those of aflatoxins B_2 and G_2, except for an additional band at 3620 cm^{-1}. There were no bands due to the presence of a vinyl-ether system such as occurs in the spectra of aflatoxins B_1 (Fig. 3, IX), and G_1 (Fig. 4, XI). Further physical characteristics of these compounds, their derivatives, and useful derivatives of the known aflatoxins, are summarised below:

Blue fluorescent compound (aflatoxin B_{2a})
mp 240° (decomp.)
Light absorption max. in methanol 228mμ, 256mμ and 363mμ (ε_M in order 17,600; 10,300; and 20,400).
Molecular weight, 330.
Molecular formula, $C_{17}H_{14}O_7$.

Acetyl derivative of B_{2a}
mp 225°
Light absorption max. in methanol 221mμ, 226mμ and 363mμ (ε_M in order 19,140; 14,880; and 23,400).
Molecular weight, 372.
Molecular formula, $C_{19}H_{16}O_8$.

Trimethyl silyl derivative of B_{2a}
Molecular weight, 402.
Molecular formula, $C_{20}H_{22}O_7Si$

Green fluorescent compound (aflatoxin G_{2a})
mp 190° (decomp.).
Light absorption max. in methanol 223mμ, 242mμ, 262mμ and 365mμ (ε_M in order 18,600; 10,100; 8,700; and 18,000)
Molecular weight, 346.
Molecular formula, $C_{17}H_{14}O_8$.

Acetyl derivative of G_{2a}
mp 207° (decomp.).
Light absorption max. in methanol 215mμ, 243mμ, 266mμ and 364mμ.
Molecular weight, 388.
Molecular formula, $C_{19}H_{16}O_9$.

2, † *Methoxy* 3†, *hydro addition product of aflatoxin B_1*
mp 240°.
Light absorption max. in methanol 224mμ, 265mμ and 362mμ.
Molecular weight, 344.
Molecular formula, $C_{18}H_{16}O_7$.

All molecular weights and molecular formulae in this summary were determined by mass spectrometry

2,† *Acetoxy* 3,† *hydro addition product of aflatoxin* B_1.
mp 225°, mp on admixture with the acetyl derivative of aflatoxin B_{2a} 223°.
Light absorption max. in methanol 221mμ, 266mμ, and 326mμ (ε_M in order 19,000; 14,870; and 23,400).
Molecular weight, 372.
Molecular formula $C_{19}H_{16}O_8$.

2,† *Acetoxy* 3,† *hydro addition product of aflatoxin* G_1
mp 204° (decomp.), mp on admixture with acetyl derivative of aflatoxin G_{2a} 202° (decomp.).
Light absorption max. in methanol 217mμ, 245mμ, 266 mμ and 363mμ.
Molecular weight, 388.
Molecular formula, $C_{19}H_{16}O_9$.

Proton Magnetic Resonance Spectra
All of these were determined in deuterio chloroform (Table I)

Table I
Proton magnetic resonance absorption data for the new hydroxy aflatoxins and related derivatives in deuterio chloroform

Position of Proton(s) (Figure 5)	*No. of Protons involved*	τ values ‡ A60		*Acetyl Derivative of*		†2, *Acetoxy* †3, *Hydro addition product of*		†2, *Methoxy* †3, *hydro addition product of*
		B_{2a}	G_{2a}	B_{2a}	G_{2a}	B_1	G_1	B_1
'a'	1	3·42(D)	3·57(D)	3·45(D)	3·44(D)	3·45(D)	3·45(D)	3·48(D)
'b'	1	5·80(M)	5·80(M)	5·82(M)	5·85(M)	5·80(M)	5·82(M)	5·85(M)
'c'	2	7·50(M)	7·60(M)	7·55(M)	7·52(M)	7·55(M)	7·53(M)	7·62(M)
'd'	1	4·23(M)	4·20(M)	3·62(M)	3·58(M)	3·62(M)	3·54(M)	4·77(M)
'e'	1	3·65(S)	3·65(S)	3·68(S)	3·65(S)	3·68(S)	3·66(S)	3·66(S)
'f'	3	6·04(S)	6·09(S)	6·05(S)	6·09(S)	6·10(S)	6·10(S)	6·04(S)
'g'	2	6·60(T)	6·58(T)	6·62(T)	6·56(T)	6·62(T)	6·58(T)	6·63(T)
'h'	2	7·53(M)	5·60(T)	7·55(M)	5·61(T)	7·55(M)	5·63(T)	7·45(M)
'd'								
Acetoxy group	3	—	—	8·32(S)	8·30(S)	8·25(S)	8·30(S)	—
Methoxy group	3	—	—	—	—	—	—	6·83(S)

Instrument used: Varian HA 100 except where indicated ‡
(S) = singlet, (D) = doublet, (T) = triplet, (M) = multiplet

but, because the signals were not very strong in the case of B_{2a}, the spectra of B_{2a} and G_{2a} were also determined in deuterio pyridine (Table II).

Table II
Proton magnetic resonance absorption data for hydroxy aflatoxins B_{2a} *and* G_{2a} *in Deuterio Pyridine*

Position of Protons (Figure 5)	*No. of Protons involved (by Integration)*	τ Values (A60) ‡ B_{2a}	G_{2a}
a	1	3·21(D) (J=6)	3·22(D) (J=6)
b	1	5·89(M)	5·64(M)
c	2	7·43(M)	7·52(M)
d	1	4·0(M)	3·95(M)
e	1	3·49(S)	3·47(S)
f	3	6·25(S)	6·24(S)
g	2	6·82(T) (J=6)	6·78(T) (J=6)
h	2	7·43(M)	5·64(M)

Instrument used: Varian HA 100 except where indicated ‡
Signals due to the protons at positions 'c' and 'h' in aflatoxin B_{2a} are superimposed on each other. Similarly signals from positions 'b' and 'h' in aflatoxin G_{2a} are superimposed

Discussion

The spectra properties of the two new compounds, showed that they were similar to the known aflatoxins in structure, although their chromatographic behaviour indicated that they were more polar than the aflatoxins. The infrare spectra of the two compounds showed that they wer structurally derived from aflatoxins B_2 and G_2. The blu fluorescent compound was accordingly named aflatoxi B_{2a}, and the green fluorescent one, aflatoxin G_{2a}. The ban in the infrared spectra of both compounds at 3,620 cm^{-1} indicated that both compounds were hydroxy aflatoxins, an this was supported by the mass spectral results. The presenc of a hydroxyl group in these compounds was finally prove by the preparation of their acetyl derivatives, and th trimethyl silyl ether of aflatoxin B_{2a}, the spectral propertie of these derivatives being in agreement with those expected It followed from this evidence, that aflatoxins B_{2a} and G_2 are the hydroxyl derivatives of aflatoxins B_2 and G respectively, and the possibility arose that the position c substitution of the hydroxyl group might be the same as i the aflatoxins M_1 and M_2.[2]

The proton magnetic resonance spectra of both aflatoxi B_{2a} and G_{2a} were consistent with the conclusion that the were the respective hydroxyl derivatives of aflatoxins E and G_2, for the following reasons:

From the molecular formulae of aflatoxins B_{2a} and G_2 14 protons had to be accounted for, and eight of these wer obviously assigned by proton magnetic resonance absorp tion spectroscopy (Tables I and II) to positions e, f, g an h in the aflatoxin molecule (Fig. 5). See for example, th work of Büchi and his collaborators on aflatoxins B_1 an G_1.[5] The other six protons were assigned to the bis-tetra hydrofuran moiety as found in aflatoxins B_2 and G_2 exce that a proton in position 'd' was substituted by a hydrox group. This was deduced from the observation that th signal due to the proton at position 'a' was a doublet τ=3·21 (J=6 c/s) (Table II) for both aflatoxins B_{2a} an G_{2a}, indicating that a coupled proton was occupyin position 'b' (cf. aflatoxin M_1 where the proton at positio 'a' gives rise to a singlet, due to the occupation of positio 'b' by the hydroxyl group).[2] The signal due to the proton position 'b' was observed as a multiplet at τ=5·89/5·6 (Table II).

The two protons at position 'c' in the aflatoxin B_{2a} molecule gave rise to signals at $\tau=7{\cdot}43$, which were complicated by the superimposition of those from the protons at position 'h'. However, in the spectrum of aflatoxin G_{2a} the signals due to the protons at position 'h' were displaced to a lower τ value where they were superimposed on the signals from position 'b', but left a broad multiplet at $\tau=7{\cdot}52$ associated with the protons at position 'c'.

The validity of these assignments is further supported by similarity of the proton magnetic absorption spectra to that observed for other compounds containing the bis-tetrahydrofuran system. (See Table III.)

Table III

Proton magnetic resonance absorption data of the bis-tetrahydro furan system in various known compounds

Compound	*Solvent*	*τ Values of Signals Corresponding to Proton(s) at various positions Δ Position of Proton(s)*			
		H'a'	*H'b'*	*H_2'c'*	*H_2'd'*
o-Methyl aversin[6]	Methylene Dichloride	3·78(D) (J=5·5)	6·0	7·74	6·40
Aflatoxin B_2[7]	Deuterio chloroform	3·54(D)	5·76	7·74	5·76
Aflatoxin G_2[7]	Deuterio chloroform	3·52(D)	5·80	7·75	5·80

Δ Positions as in Fig. 5.

The positions for the protons in the aflatoxins B_2 and G_2 have been assigned by the present authors, as the values were published before the structures of the compounds had been elucidated. As Hartley et al.[7] did not observe a separate signal which could be assigned to protons at position 'd,' it is assumed that the signal is superimposed on that assigned to position 'b'.

The signal from the remaining proton at position 'd' was observed at $\tau=4{\cdot}0$ as a multiplet. This lowering of the τ value compared with those of the compounds in Table III, is due to the proximity of the hydroxyl group on the same carbon atom. The proton on this hydroxyl group was found as a very broad signal *c.* $\tau=3{\cdot}0$ to $\tau=4{\cdot}0$, by integration of the spectrum before, and after treatment of the sample with deuterium oxide.

Additional evidence for the structures which have been proposed for aflatoxins B_{2a} and G_{2a} was obtained by showing that their acetyl derivatives were identical with the 2,† acetoxy 3,† hydro addition products of aflatoxins B_1 and G_1, respectively. This showed conclusively that in aflatoxins B_{2a} and G_{2a} the hydroxyl group is at position 'd'. In aflatoxins M_1 and M_2, however, the hydroxyl group is at position 'b' (Fig. 5).

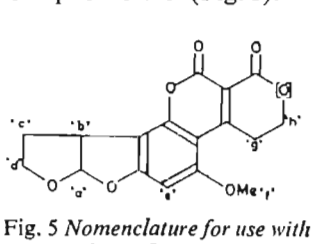


Fig. 5 *Nomenclature for use with the proton magnetic resonance studies of the aflatoxins*

Biochemistry and Toxicology

In view of the high toxicity of the known aflatoxins, B_1, G_1, B_2 and G_2 (LD. 50 values for one day-old ducklings are, in order, 18·2 μg, 84·8 μg, 39·2 μg and 172·5 μg)[8], it was decided to test our new hydroxy aflatoxins for these properties.

The toxicity of aflatoxins B_{2a} and G_{2a} was tested on day-old Khaki Campbell ducklings at the following dose rates:—

Aflatoxin	*μg*
B_{2a}	300; 600; 900; 1,200
G_{2a}	400; 800; 1,200; 1,600

There was no significant difference in growth between control and dosed birds nor were there any of the characteristic liver lesions associated with aflatoxin poisoning.

These results indicate that B_{2a} and G_{2a} are much less toxic than B_1, B_2, G_1 and G_2.

We have shown (unpublished experiments) that treatment with cold dilute aqueous mineral acid produces aflatoxin B_{2a}; and likewise that aflatoxin G_{2a} arises from G_1 under the same conditions. Büchi, Foulkes, Kurono and Mitchell (1966)[9] also have demonstrated this addition of water to aflatoxin B_1, their catalyst being trifluoroacetic acid.

From consideration of the above findings, it would seem that an acid treatment of aflatoxin-contaminated feedstuff could be of value in lowering their toxicity substantially especially as B_1 and G_1 are by far the major components produced by the mould.

During incubation in liquid medium, the culture fluid becomes increasingly acidic and it is possible, therefore, that a proportion of the aflatoxins B_{2a} and G_{2a} arises by this mechanism. However, the possibility that aflatoxins B_2 and G_{2a} can give rise to aflatoxins B_1 and G_1 by dehydration cannot be entirely excluded.

We wish to acknowledge the kind help of Dr R. Allcroft and Dr W. H. Butler in carrying out the toxicity tests on ducklings.

We are grateful to Dr M. Barber and Mr A. Wolstenholme of A.E.I. (Trafford Park) for the mass spectra determinations and to the S.R.C. for a grant to M.F.D.

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19 July 1969 Chemistry and Industry

O-Alkyl derivatives of aflatoxins B_{2a} and G_{2a}

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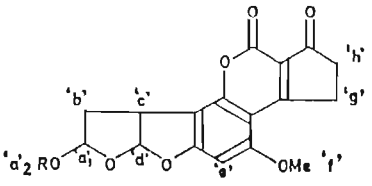
Since the discovery of the aflatoxins and the elucidation of their structures by Büchi and co-workers,[1] these compounds have been the subject of intensive study particularly with regard to the methods of assay. Such assays are of considerable importance because of the high toxicity of the compounds towards animals and it has been established that at least one of them (aflatoxin B_1) is carcinogenic. In the past we have reported[2] the existence of two other aflatoxins, B_{2a} and G_{2a}, which can easily arise from aflatoxins B_1 and G_1. An account is given in the present paper of other derivatives which may arise in cultures of *A. flavus* and during the extraction of the aflatoxins. It will also be shown how these compounds may interfere with procedures for the assay of the aflatoxins.

Production and isolation of the new aflatoxin derivatives

A toxin-producing strain of *A. flavus* (CMI 91019b) was cultivated on a simple salts medium, and the aflatoxins were extracted as described previously.[2] The aflatoxin-containing extract was subjected to chromatography on a column of silica gel, whereupon two aflatoxin-containing fractions were obtained. The first fraction eluted from the column contained aflatoxins B_1, B_2, G_1 and G_2, which were separated initially into four fractions by t.l.c. using kieselgel 'G'/methanol-chloroform (1:49 v/v). The second fraction from the column contained the aflatoxins, M_1, B_{2a} and G_{2a}, which have been investigated previously.[2] The four fractions containing the individual aflatoxins, B_1, B_2, G_1 and G_2 were next chromatographed on t.l.c. using a benzene-formamide system, which was based on that described by Adye and Mateles.[3] The thin-layer plates were prepared by slurrying cellulose powder† (MN 300 HR, 15 g), with 70 ml. of formamide-acetone (1:9 v/v) and spreading five 20 cm × 20 cm chromatoplates with this slurry. The chromatoplates were ready for use after allowing them to dry in air for 15 min. Each aflatoxin-containing concentrate was applied to several of these chromatoplates as bands, and then developed with benzene saturated with formamide. The aflatoxin-containing bands were scraped from the plate after location under ultraviolet light, and eluted from the cellulose with chloroform in a sintered-glass funnel (porosity 3). The relatively pure compounds, obtained after evaporation of the solvent, were recrystallised from a suitable solvent, usually chloroform, and then samples were rechromatographed in both t.l.c. systems in order to ascertain their purity.

Preparation of aflatoxin derivatives

The 2*-methoxy, 3*-hydro addition product of aflatoxin B_1 (Fig. 1, I) was prepared by treating aflatoxin B_1 (50 mg)



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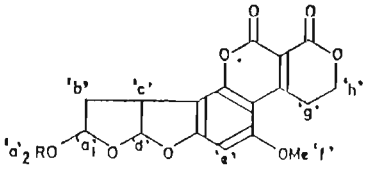
I. CH_3-2*-methoxy, 3*-hydro addition compound of aflatoxin B_1 (Ex_2 B_1)

II. C_2H_5-2*-ethoxy, 3*-hydro addition compound of aflatoxin B_1 (Ex B_2)



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III. C_2H_5-2*-ethoxy, 3*-hydro addition compound of aflatoxin G_1 (Ex G)

Fig. 1

with an excess of methanol; a few drops of thionyl chloride were added to act as a catalyst,[4] and the mixture was allowed to stand overnight at room temperature. The excess solvent was then removed *in vacuo*, and the derivative was isolated by t.l.c. using the keiselgel 'G'/methanol-chloroform (1:49, v/v) system. The pure, crystalline material was obtained by recrystallisation from chloroform.

The 2*-ethoxy, 3*-hydro addition products of aflatoxins B_1 (Fig. 1, II) and G_1 (Fig. 1, III), were prepared from the appropriate aflatoxin, in the same manner as was the 2*-methoxy, 3*-hydro addition product of B_1, except that ethanol was used in place of methanol.

Preparation of compounds resulting from the treatment of aflatoxins B_{2a} and G_{2a} with acid

Aflatoxins B_{2a}, and G_{2a}, (30 mg. of each) were individually treated with an excess of cold 4N hydrochloric acid and allowed to stand overnight. Each reaction mixture was extracted three times with an equal volume of chloroform, and the combined extracts were concentrated *in vacuo* to a suitable volume (about 1 ml.). The two concentrates were then examined by t.l.c., using the keiselgel system, and the reaction products were isolated.

The various compounds and derivatives prepared above were examined by a number of spectroscopic techniques, these included: ultraviolet and visible absorption spectroscopy on a Perkin-Elmer 137 spectrometer, using spectrosol

† (Supplied by Camlab).

* Refers to the position in the terminal furan ring.

methanol as the solvent; where sufficient material was available, proton magnetic resonance absorption spectroscopy was carried out using a Varian HA 100 instrument, and deuterio chloroform as the solvent, signals being measured in τ values using tetramethyl-silane as internal standard. For mass spectrometry an MS 9 (AEI) instrument was used.

Results

On chromatography using the benzene-formamide system, each aflatoxin-containing fraction yielded further fluorescent compounds at higher R_F values than the known aflatoxins (see Fig. 2). The aflatoxin B_1 fraction yielded two blue

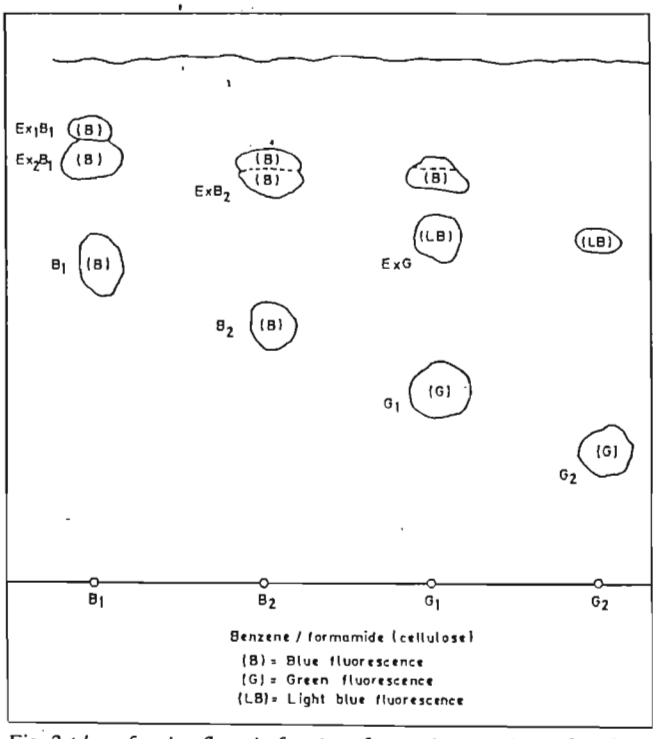

Fig. 2 *t.l.c. of main aflatoxin fractions from culture medium of A. flavus*

fluorescent compounds, one named compound Ex_1B_1, at an R_F value of 0·86, and the other, compound Ex_2B_1 at an R_F value of 0·81. The aflatoxin B_2 fraction yielded two blue fluorescent compounds, one corresponding to compound Ex_2B_1 and another, named compound ExB_2 with an R_F value of 0·77. Both the aflatoxin G_1 and G_2 fractions gave rise to a green fluorescent compound (named ExG) with an R_F value of 0·66. After isolation, these compounds were shown to be pure by further t.l.c., and it was found that, on using the keiselgel/methanol/chloroform system, they had R_F values very close to those of the known parent aflatoxins.

The new compounds had the following properties:

Compound Ex_1B_1
mp 83°C
Mol. wt.** 440
Mol. formula** $C_{29}H_{60}O_2$

Compound Ex_2B_1
mp 236°C
mp on admixture with 2*-methoxy-3*-hydro addition compound of aflatoxin B_1, 237°C
Mol. wt.** 344
Mol. formula** $C_{18}H_{16}O_7$
Light absorption max. in methanol 224 mμ, 265 mμ and 362 mμ

Compound ExB_2
mp 245°C
mp on admixture with the 2*-ethoxy-3*-hydro addition compound of aflatoxin B_1 242°C
Mol. wt.** 358
Mol. formula** $C_{19}H_{18}O_7$
Light absorption max. in methanol 223 mμ, 266 mμ and 364 mμ

Compound ExG
mp 200°C
mp on admixture with the 2*-ethoxy-3*-hydro addition compound of aflatoxin G_1 202°C
Mol. wt.** 374
Mol. formula** $C_{19}H_{18}O_8$
Light absorption max. in methanol 223 mμ, 245 mμ, 266 mμ and 366 mμ

The addition compounds derived from aflatoxins B_1 and G_1 had the following physical characteristics:

2-Methoxy, 3*-Hydro addition product of aflatoxin B_1*
mp 240°C
Mol. wt.** 344
Mol. formula** $C_{18}H_{16}O_7$
Light absorption max. in methanol 224 mμ, 265 mμ and 362 mμ

2-Ethoxy, 3*-Hydro addition product of aflatoxin B_1*
mp 247°C
Mol. wt.** 358
Mol. formula $C_{19}H_{18}O_7$
Light absorption max. in methanol 226 mμ, 266 mμ and 364 mμ
(ε_M in order 14,640, 12,140 and 12,580)

2-Ethoxy, 3*-Hydro addition product of aflatoxin G_1*
mp 203°C
Mol. wt.** 374
Mol. formula** $C_{19}H_{18}O_8$
Light absorption max. in methanol 223 mμ, 244 mμ, 266 mμ and 366 mμ
(ε_M in order 17,140, 12,840, 11,700 and 19,500)

For the proton magnetic resonance data of the above three compounds see Table I.

Products resulting from the treatment of aflatoxins B_{2a} and G_{2a} with acid

Two compounds were isolated in about 70 per cent yield, each with a higher R_F value (using the keiselgel/methanol/chloroform system) than its respective parent aflatoxin. One was isolated from the aflatoxin B_{2a} reaction mixture which was found to be identical with the 2*-ethoxy, 3*-hydro addition compound of aflatoxin B_1, and the other from the aflatoxin G_{2a} reaction mixture which was identical with the 2*-ethoxy, 3*-hydro addition derivative of aflatoxin G_2. This was confirmed by t.l.c., mass spectrometry, proton magnetic resonance spectroscopy, and by mixed melting point determinations.

**All molecular weights and molecular formulae in this summary were determined by mass spectrometry.

Table I

Proton magnetic resonance absorption data for various addition compounds of aflatoxins B_1 and G_1

Position of proton(s) (Fig. 1)	Additional group	No. of protons involved	Values of following aflatoxin addition compounds: 2*-methoxy 3*-hydro B_1	2*-ethoxy 3*-hydro B_1	2*-ethoxy 3*-hydro G_1
a_1	—	1	4·77 (Mt)	4·65 (Mt)	4·70 (Mt)
a_2	Methoxy	3	6·83 (St)	—	—
a_2	Ethoxy				
a_2	(Methyl)	3	—	9·06 (T)	9·04 (T)
	(Methylene)	2	—	6·49 (Mt)	6·48 (Mt)
b	—	2	7·62 (Mt)	7·63 (Mt)	7·62 (Mt)
c	—	1	5·85 (Mt)	5·89 (Mt)	5·86 (Mt)
d	—	1	3·54 (D)	3·46 (D)	3·42 (D)
e	—	1	3·73 (St)	3·68 (St)	3·69 (St)
f	—	3	6·04 (St)	6·04 (St)	6·04 (St)
g	—	2	6·63 (T)	6·57 (T)	6·84 (T)
h	—	2	7·45 (T)	7·40 (T)	5·73 (T)

St=singlet; D=doublet; T=triplet; Mt=multiplet.

Discussion

Three of the four metabolites, isolated by means of the benzene-formamide system and named Ex_2B_1, ExB_2 and ExG, were found to be identical, in order, with the 2*-methoxy, 3*-hydro addition compound of aflatoxin B_1, the 2*-ethoxy, 3*-hydro addition compound of aflatoxin B_1 and the 2*-ethoxy, 3*-hydro addition compound of G_1. The remaining metabolite, compound Ex_1B_1 was found to be unconnected with the aflatoxins and, from mass spectrometry, it appeared to be a long chain dihydric alcohol.

Although the structure of the new aflatoxin derivatives had been resolved, the problem still remained as to how they arose in the aflatoxin-containing extracts. That they did not arise during chromatography was shown by repeated chromatography in both the benzene-formamide and keiselgel/methanol/chloroform systems, whereupon no additional compounds were noted either from the new derivatives themselves, or from the known aflatoxins B_1,B_2, G_1 or G_2. The problem was, however, explained from the observation that extracts of aflatoxins B_{2a} and G_{2a} from acidic mixtures, gave rise to reaction products on t.l.c., which proved to be identical with the 2*-ethoxy, 3*-hydro addition compounds of aflatoxins B_1 and G_1††. It was difficult, initially, to explain how these compounds arose, as there seemed to be no possible source of ethyl groups in the reaction mixtures. This point was resolved when it was realised that the chloroform used to extract the reaction mixtures contained about 2 per cent (v/v) ethanol as a preservative. Hence it became apparent that aflatoxins B_{2a} and G_{2a} were reacting with this ethanol under acidic conditions to yield their ethoxy derivatives (*cf.* glycoside formation in sugars). This could also explain how compounds ExB_2, and ExG appeared in the main aflatoxin-containing fraction.

In order to test this hypothesis, acidified solutions of aflatoxin B_{2a} were extracted (a) with alcohol-free chloroform and (b) with pure chloroform to which various alcohols had been added (*e.g.* methanol, ethanol, propan-2-ol, N-butanol, and benzyl alcohol). The alcohol-free chloroform was prepared by treatment with activated molecular seive. On t.l.c., each extract gave a blue fluorescent spot at a higher R_F value than the original aflatoxin B_{2a}. Mass spectrometry of the new products showed that they were O-alkyl derivatives of aflatoxin B_{2a}, each corresponding to the particular alcohol added. Thus chloroform containing N-butanol gave O-N-butyl aflatoxin B_{2a} (this is synonomous with the 2*-N-butyl, 3*-hydro addition compound of aflatoxin B_1). Hence it was shown that O-alkyl derivatives of aflatoxins B_{2a} and G_{2a} could arise by extracting the parent aflatoxin (*i.e.*, B_{2a} and G_{2a}) with impure chloroform. Furthermore, these O-alkyl derivatives could be formed from aflatoxins B_1, and G_1, under these conditions, as aflatoxins B_{2a}, and G_{2a} are readily formed from aflatoxins B_1, and G_1 in acid solution,[2] (Fig. 3). Compounds Ex_2B_1, ExB_2 and ExG did

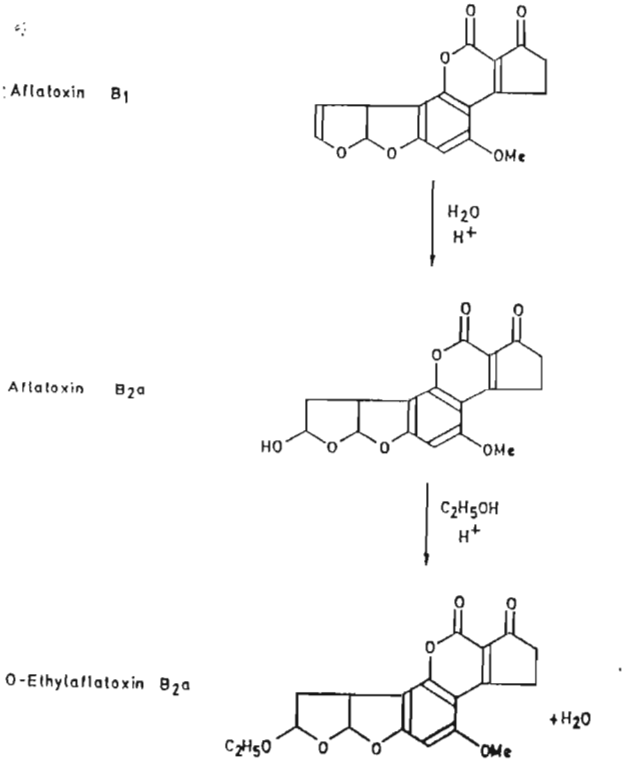

Fig. 3 *Formation of O-ethylaflatoxin B_{2a} from aflatoxin B_1 in acidic media*

††These compounds are synonomous with the corresponding O-alkyl derivatives of aflatoxins B_{2a} and G_{2a}.

not arise exactly in this way, as they were still found in culture fluids extracted with pure chloroform. In this case the alcohol presumably occurs in the culture fluid itself due to fermentation reactions and then proceeds to react as suggested.

It is important to note that the formation of aflatoxins B_{2a}, G_{2a} and their O-alkyl derivatives as outlined, may complicate the assay of individual aflatoxins from acidic cultures. For example, a substantial quantity of the aflatoxins B_1, and G_1 may be present as aflatoxins B_{2a} and G_{2a}, and the extraction of such cultures with ordinary chloroform would give rise to the corresponding O-ethyl derivatives. These have similar R_F values to aflatoxins B_1, B_2, G_1 and G_2 in the t.l.c. system using keiselgel/methanol/chloroform and would confuse the assay.

In order to overcome these difficulties culture fluids should be neutralised prior to extraction, the latter being carried out preferably with freshly prepared, alcohol-free chloroform. In addition to these safeguards extracts from such cultures should be investigated using the benzene-formamide system, which would reveal the presence of any O-alkyl derivatives of aflatoxins B_{2a} and G_{2a}.

Thanks are due to Drs M. Barber and A. Wolstenholme, and Mr T. Kemp of AEI Manchester for the mass spectrometry; and to the SRC for a research grant to MFD.

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Margarine is 100 years old

This year, the centenary of margarine is being celebrated in Britain and many other countries across the world and tribute is paid to the French food chemist, Hippolyte Mège Mouriès, who invented it in 1869.

Two unrelated events in Europe acted as the spur for the development of a new food which could be used instead of butter. In 1867, the Victualling Department of the French Navy were worried at growing complaints from its crews that the butter carried aboard ship was often rancid by the time it was served. They commissioned Mouriès to find a butter replacement. About the same time, Europe was threatened by a severe food shortage due to a rapid increase in population, and there was also a general shift of population from the country areas to the growing industrial centres. Butter became scarce and very costly. In January 1869, Napoleon III, recognising a wider need than the Navy's to keep people properly fed, offered a prize to the person 'who discovers a suitable substance to replace butter.'

By the spring of 1869 Mouriès triumphantly announced his new product which was named 'margarine' after the Greek word *margarites* meaning pearl. His margarine, basically an emulsion of beef fat and milk, was patented in Britain on 17 July and in France on 15 July that same year and won him the Emperor's prize.

Today, margarine is a household word and the world consumes more than 4·5m ton/year. The manufacturing techniques and the raw materials used, have changed radically since Mouriès equipped the first margarine factory at Poissy. Beef fat has been superseded by vegetable oils from coconuts, sunflowers and other oil-bearing nuts and seeds, and more recently still from highly refined marine oils.

Mr F. B. Kitchen is the president of the UK Margarine & Shortening Manufacturers' Association, which is sponsoring the centenary programme in this country.

P.H.

Royal Institution Australian science scholars

Five British sixth-formers have now been awarded Royal Institution Australian Science Scholarships for 1969. These scholars will attend the Twelfth International Science School for High School Students in Sydney, Australia, from 25 August to 5 September this year.

The International Science School, sponsored by the Science Foundation for Physics within the University of Sydney, is under the Directorship of Professor H. Messel. Its programme will include 22 lectures by distinguished scientists from Australia, Great Britain and the United States of America, on the general theme 'Nuclear energy today and tomorrow.'

The five British scholars will attend a series of receptions during July. On the 14 August they will be joined in London by the Japanese group and after a few days in London the combined party will fly to Washington where, together with the American group, they will meet Vice-President Agnew. After three days in Washington, the twenty scholars will leave for Australia *via* Honolulu. In Sydney the overseas scholars will attend the 1969 Science School in the company of more than one hundred boys and girls from high schools in Australia and New Zealand. On their return journey the British party will make stops at Bangkok, Delhi and Rome.

The five British sixth-formers were selected by a joint committee of the Royal Institution of Great Britain and the Association for Science Education. The main criteria for selection were: (a) academic performance and promise, (b) width of interests, and (c) personality and likelihood of being good 'ambassadors.' The national short-list, from which the final selections were made, was prepared by the Association for Science Education using its regional organisation. The five British scholars selected were R. H. Bricknell (Easingwold, Yorkshire), R. P. Hobson (West Wickham, Kent), Miss M. Jones (Neath, Glamorganshire), Miss J. A. Price (Cleethorpes, Lincolnshire) and D. A. V. Stow (Linslade, Bedfordshire).

Tetrahedron. Vol 25, pp 1497 to 1500 Pergamon Press 1969 Printed in Great Britain

NEW METABOLITES OF *ASPERGILLUS FLAVUS*

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(*Received in the UK 10 September 1968; accepted for publication 30 October 1968*)

Abstract—Two pigments have been isolated from a yeast culture of *A. flavus*. One of these is identical with a tetrahydroxy-anthraquinone previously isolated from *A. versicolor*, the other being versicolorin C. The culture medium also yielded small amounts of aflatoxins M_1 and GM_1 and also a new compound, designated aflatoxin B_3, and for which a tentative structure is put forward.

DURING our investigation of the various aflatoxins produced by a strain of *A. flavus*[1] (C.M.I. 91019b), several coloured metabolites were also observed in extracts from a culture of the mould. In order to investigate these metabolites further, the mould was cultured on an aqueous medium containing yeast extract and glucose. The mycelium obtained from this culture was carefully dried, ground to a powder, and soxhlet-extracted with chloroform acetone (9:1 v/v) until the extracting solvent was colourless. The extract was evaporated to a suitable volume *in vacuo*, and subjected to column chromatography on kieselgel.

New pigments

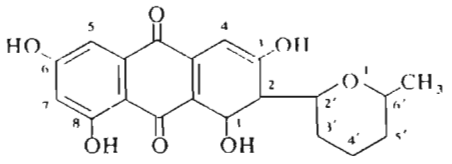
The pigments were separated from the aflatoxins by elution with diethyl ether, the fraction so obtained was evaporated to a small volume, and then examined by TLC using kieselgel G and chloroform as the solvent. A number of orange spots was observed on the chromatoplate, the main one occurring at an R_f value of about 0·34, (this was at a position a little lower than aflatoxin G_2 in the fraction containing the aflatoxins). This orange spot was resolved into two compounds Or_1 and Or_2, at R_f values of 0·46 and 0·39 respectively, by preparative TLC using kieselgel G and ethyl acetate–benzene (1:9 v/v) as the solvent.

Compound Or_1 (from chloroform) had m.p. 271°, λ_{max} (MeOH) 223 mμ, 263 mμ, 292 mμ, 312 mμ and 452 mμ, (ε in order 20,180; 14,500; 17,880; 9940 and 8650), and was shown by mass spectrometry to have a mol wt of 370, and a mol formula of $C_{20}H_{18}O_7$. On full methylation it yielded a tetramethoxy derivative. The properties of Or_1 indicated that it was a derivative of a tetrahydroxy-anthraquinone and this was further confirmed by the NMR spectrum* of its tetramethoxy derivative. The spectrum was, in fact, consistent with that of a 1,3,6,8-tetrahydroxy-2,2′-(6′-methyl-tetrahydropyran) anthraquinone (I) and a compound with this structure was recently isolated from a culture of *A. versicolor* by Holker *et al.*[2] That structure I was the correct one for our compound was verified by comparing its properties with those of an authentic sample of material obtained from Dr. J. S. E. Holker of Liverpool University. The two compounds were also shown to have identical NMR spectra.

Versicolorin C

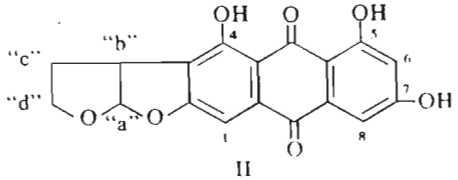
Compound Or_2 (from $CHCl_3$) had m.p. 350°, $[\alpha]_D = 0$, ($c = 0{\cdot}1$ in $CHCl_3$), λ_{max}

* Carried out on a Varian HA-100 instrument and using deuterochloroform as the solvent.



I

(MeOH) 222 mμ, 265 mμ, 290 mμ, 312 mμ and 450 mμ, (ε in order 28,100; 16,600; 22,900; 11,120 and 6920), and was shown by mass spectrometry to have a mol wt of 340, and a mol formula of $C_{18}H_{12}O_7$. On full methylation it yielded a trimethoxyl derivative the properties of which indicated that Or_2 was a trihydroxy-anthraquinone. The NMR spectrum* of Or_2 was identical with that published by Hamasaki *et al.*[3] for dihydroversicolorin A, which is also the same for the natural products, Versicolorins B and C (II), which the same authors isolated from a culture of *A. versicolor*.[3] Our compound seems to correspond to Versicolorin C, which was claimed to be the racemic form of Versicolorin B[3], as it has no optical properties and its other properties were identical with those of an authentic sample of Versicolorin C kindly donated by Dr. Y. Hatsuda, Tottori University.



II

A discrepancy noted in the NMR spectrum was the assignment by Hamasaki *et al.* of the signal at 5·95 τ to the protons at "d" and at 6·37 τ to the proton at "b". In our spectrum the multiplet at 5·95 τ was shown to be coupled to the doublet at 3·62 τ, (proton associated with this signal assigned to position "a") by irradiation of the multiplet at 2572 cycles/sec, thus the multiplet at 5·95 τ must be assigned to the proton at position "b", and hence it follows that the signal at 6·37 τ must be assigned to the proton at position "d". Our assignments seem to agree with those in the literature for similar systems, e.g. Aversin.[4]

The isolation of these two pigments from a toxin-producing strain of *A. flavus* shows the close biogenetic link between this species and *A. versicolor*, a point made by other workers[5] and thus adds further weight to the view that the aflatoxins and sterigmatocystin are derived from the same or similar precursors.[6] The isolation of Versicolorin C from both species would indicate that this compound is a common precursor as suggested by Thomas.[7]

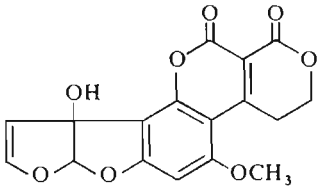
New aflatoxin metabolites

Two aflatoxin-containing fractions were also obtained from these culture media by extraction with chloroform and column chromatography as described previously.[1] The first fraction from the column contained the aflatoxins B_1, B_2, G_1 and G_2, and the second, three metabolites, which were separated by preparative TLC using Kieselgel G, and chloroform as the solvent. Two of these compounds behaved

* Obtained on a Varian HA-100 instrument using deuterochloroform as the solvent.

similarly (on TLC) to aflatoxins B_{2a} and G_{2a}, one of R_f value 0·18 fluorescing blue under UV light, and the other of R_f value, 0·15 fluorescing green. The third compound gave a blue fluorescent spot with an R_f value of 0·28, i.e. somewhere between the positions of aflatoxins M_1 and G_2 in the same TLC system. On further examination of the compounds thought to be aflatoxins B_{2a} and G_{2a}, it became apparent that they were in fact aflatoxin M_1, (by comparison with an authentic sample of this compound kindly donated by Dr. I. F. M. Purchase, N.N.R.I. South Africa), and a compound which appeared to be the G analogue of aflatoxin M_1 (designated as aflatoxin GM_1). This compound, which has the structure (III), has been tentatively reported elsewhere (Nabney *et al.*,[8] Purchase *et al.*[9]).

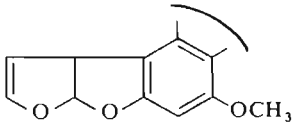
It has (from $CHCl_3$) m.p. 276°, λ_{max} (MeOH) 235 mμ, 262 mμ and 358 mμ (ε in order 21,200; 16,300 and 12,000). Mol wt, 344,* mol formula, $C_{17}H_{12}O_8$.* It forms a mono-acetyl derivative (m.p. 280°) on treatment with acetic anhydride/pyridine. Due to the lack of material its structure could not be definitely assigned, but the properties so far determined were consistent with the tentative structure. (III).



III

Aflatoxin B_3

The third compound isolated was named aflatoxin B_3 because of its blue fluorescence, although it became apparent later that it was closer in structure to the aflatoxin G series. It has (from $CHCl_3$): m.p. 217°, λ_{max} (MeOH) 229 mμ, 253 mμ, 262 mμ and 326 mμ; ε in order 10,000; 7300, 7550 and 9350; Mol wt, 302,* Mol formula, $C_{16}H_{14}O_6$;* IR spectrum (main bands) 3400, 3040, 2900, 1725, 1622 and 1070 cm^{-1}. Negative reactions were obtained with Gibbs reagent, neutral ferric chloride and 2,4-dinitrophenyl hydrazine reagent. A mono-acetyl derivative was formed on treatment with acetic anhydride/pyridine. The NMR spectrum† of aflatoxin B_3 showed that the bisdihydrofuran system, which is found in aflatoxins B_1 and G_1, was present in the molecule together with the same aromatic nucleus and OMe group, accounting for eight protons, see IV: i.e. Triplets at 3·40 τ (1 proton); and 4·58 τ (1 proton), J = 1·5 c/s; Multiplet at 5·32 τ (1 proton); doublet at 3·14 τ (1 proton), J = 3·0; singlets at 6·40 τ (3 protons), 3·54 τ (1 proton) cf. the spectra of aflatoxins B_1 and G_1.[10]



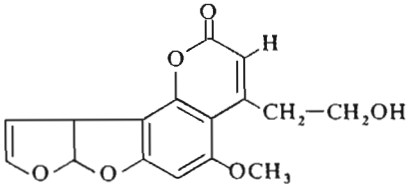
IV

* Determined by mass spectrometry.

† Determined on a Varian HA-100 instrument using deuteropyridine.

Of the remaining 6 protons, four were observed as a pair of coupled triplets at 6·75 τ, and 5·97 τ, $J = 3{\cdot}0$, (cf. aflatoxin G_1 triplets at 6·84 τ and 5·73 τ, $J = 3{\cdot}0$), and another as a singlet at 3·73 τ. The remaining proton was not detected even after treatment with deuterium oxide and was, therefore, assumed to be present at an OH group giving rise to a very broad signal. The presence of an alcohol group was supported by the band in the IR spectrum at 3400 cm^{-1}, by the formation of a mono-acetyl derivative, and the absence of phenolic-type reactions. In spite of the similarities of the NMR spectra of aflatoxin G_1 and B_3, the IR spectrum of B_3 showed that the dilactone system was lacking in this compound (G_1 giving rise to bands at 1770 cm^{-1}, and 1670 cm^{-1})[4] although a strong band at 1725 cm^{-1} indicated the presence of a lactone group.

All these facts can be reconciled in structure V, the olefinic proton giving rise to the singlet at 3·73 τ, and the 4 protons on the aliphatic side-chain producing the coupled pair of triplets at 6·75 τ and 5·97 τ.



V

It can be seen from the above structure that aflatoxin B_3 can easily be obtained from aflatoxin G_1 by a simple hydrolytic process followed by decarboxylation (another point in favour of Structure III). This process occurs in the mould culture itself, as no aflatoxin B_1 was obtained when quantities of pure aflatoxin B_1 or G_1 were subjected to the same extraction procedures.

This decarboxylation process may very well be the first step in the microbial breakdown and disappearance of the aflatoxins from mould cultures, observed by many workers, including ourselves and, e.g. Ciegler *et al.*[11]

Acknowledgements—We thank the S.R.C. for a grant to M.F.D.; also Drs. M. Barber, A. Wolstenholme and Mr. T. Kemp of A.E.I. for mass spectra.

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14. SOME INTERESTING RELATIONSHIPS BETWEEN THE NEW AFLATOXINS AND THEIR ASSOCIATED METABOLITES

By M. F. DUTTON and J. G. HEATHCOTE

During our investigations on the metabolites of *A. flavus* we have isolated a number of compounds, many of which are related to the aflatoxins, and others which, though having no direct relationship to the aflatoxins, have shed some light on the problem of their biogenesis, e.g. versicolorin C (Heathcote and Dutton 1969).

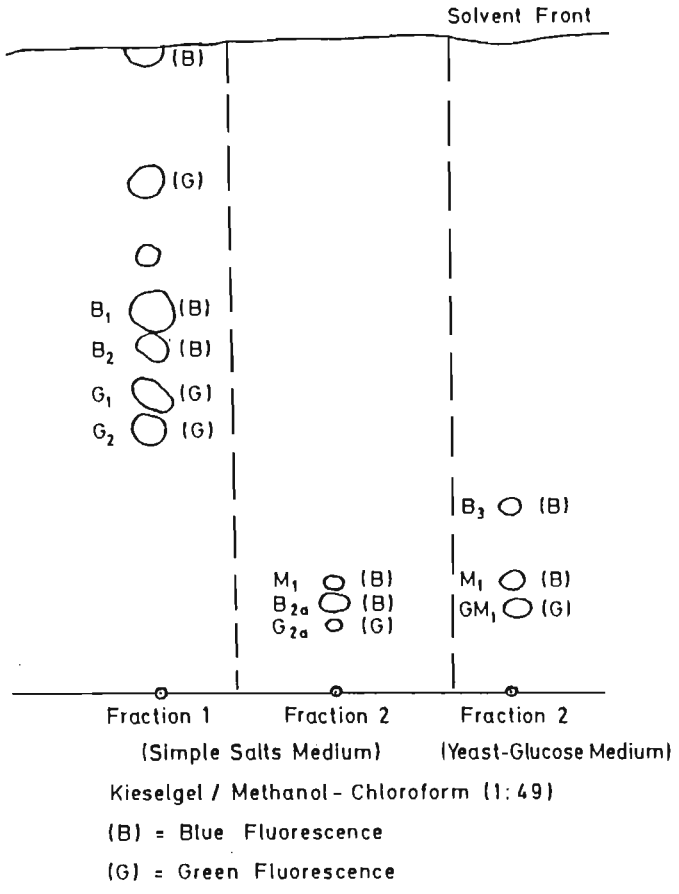
Aflatoxins M_1, B_{2a} and G_{2a}:

Two metabolites included in the former group are those designated by ourselves as aflatoxins B_{2a} and G_{2a} (Dutton and Heathcote 1966 and 1968). These compounds were isolated from a culture of the mould *A. flavus* (C.M.I. 91019b.) cultivated on a simple salts medium of the following composition: dipotassium hydrogen phosphate, 3 g.; potassium dihydrogen phosphate, 1 g.; magnesium sulphate, 1 g.; ammonium sulphate, 8 g.; glucose, 20 g., and zinc sulphate, 5 mg., all made to 1 litre with distilled water. The aflatoxins were obtained by repeated extraction with chloroform and the combined extracts were concentrated *in vacuo*. The concentrate (about 2 ml.) was then applied to a column of silica gel and chromatographed, using methanol (2 per cent., v/v) in chloroform as eluant. Thin-layer chromatography (TLC) was then applied to the various fractions obtained from the column. The first fraction eluted from the column (Fraction I, Fig. 1) contained the known aflatoxins, viz.: B_1 (I), B_2 (II), G_1 (III) and G_2 (IV), (Figs. 2 and 3) while the next fraction contained three metabolites, two of which fluoresced blue under ultra-violet light, and a third which fluoresced green. The blue fluorescent compound which had the highest R_F value on TLC, (kieselgel G/methanol-chloroform 1 : 49) (Fig. 1, Fraction 2) proved to be aflatoxin M_1 (V) (Fig. 4) (Holzapfel, Steyn and Purchase, 1966) as was shown by comparison with an authentic sample, kindly presented to the authors by Dr. Purchase (N.N.R.I. Pretoria). The other blue fluorescent metabolite had an R_F value on TLC similar to that of aflatoxin M_2 (VI) (Fig. 4) but, although it had the same molecular weight and formula, it was shown to be a different and hitherto unknown, isomer and was named aflatoxin B_{2a} (VII) (Fig. 5). The green fluorescent metabolite was found to be the G analogue of aflatoxin B_{2a} and was therefore named aflatoxin G_{2a} (VIII) (Fig. 5). Their structures and relationships with the known aflatoxins were proved unequivocally by proton magnetic resonance, absorption spectroscopy studies and by the preparation of derivatives. An example is given in the scheme of Fig. 6, where it is seen that O-acetyl aflatoxin B_{2a} (IX) and 2*-acetoxy, 3*-hydro-aflatoxin B_1 possesses a common structure.

* This refers to the position in the terminal furan ring.

Fig. 1

TLC of Silica Gel-Column Fractions from Culture Media of A. flavus

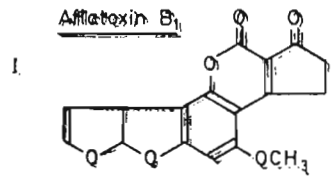


The new aflatoxins had the following properties:†

Aflatoxin B_{2a} (Blue fluorescent compound):
m.p., 240° (dec.)
Molecular weight, 330
Molecular formula, $C_{17}H_{14}O_7$
Light absorption max. in methanol, 228 mμ, 256 mμ, 363 mμ
(ϵ_M in order, 17,600, 10,300, 20,400)

† All molecular weights and molecular formulae in this summary were determined by mass spectrometry.

Fig. 2



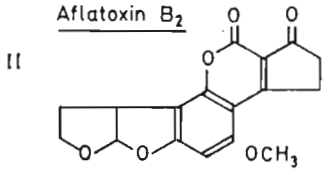
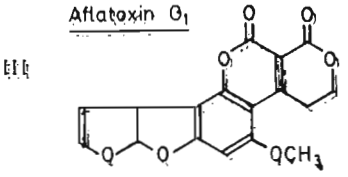


Fig. 3



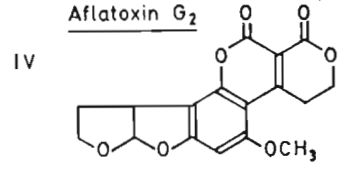
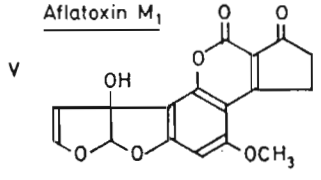


Fig. 4



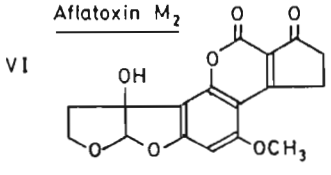
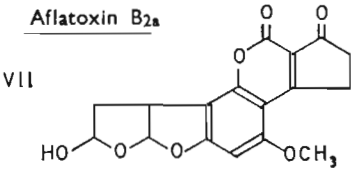
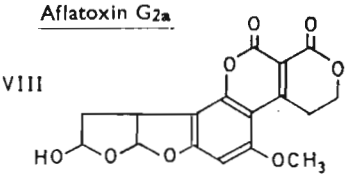


Fig. 5





Aflatoxin G_{2a} (Green fluorescent compound):

m.p., 190° (dec.)

Molecular weight, 346

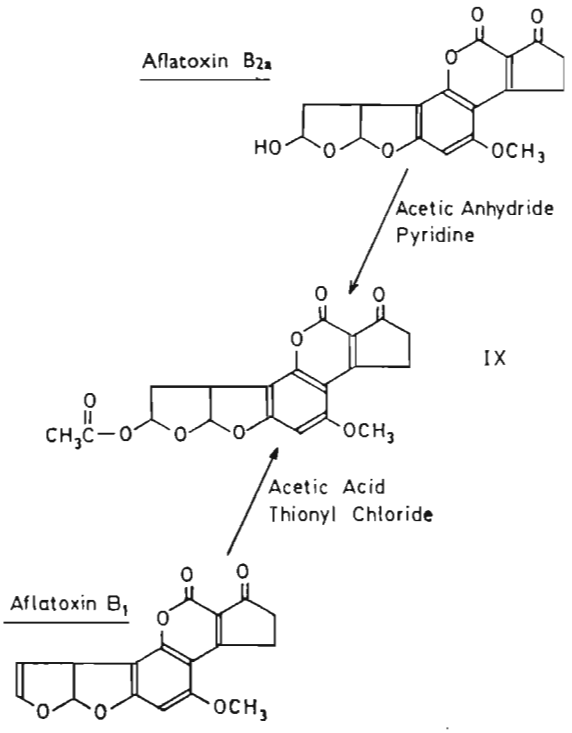
Molecular formula, $C_{17}H_{14}O_8$

Light absorption max. in methanol, 223 mμ, 242 mμ, 262 mμ and 365 mμ. (ϵ_M in order, 18,600, 10,100, 8,700 and 18,000).

Fig. 6

Scheme 1

A Common Derivative of Aflatoxins B_1 and B_{2a}



These compounds were considered to arise from aflatoxins B_1 and G_1, respectively, by the addition of water across the isolated double bond in the terminal furan ring, in the presence of acid (see Fig. 7). This was shown by the preparation of aflatoxins B_{2a} and G_{2a} from B_1 and G_1 by treatment with cold dilute hydrochloric acid, and confirmed by cultivating the mould on neutralized media, whereupon no aflatoxin B_{2a}, or G_{2a}' was produced.

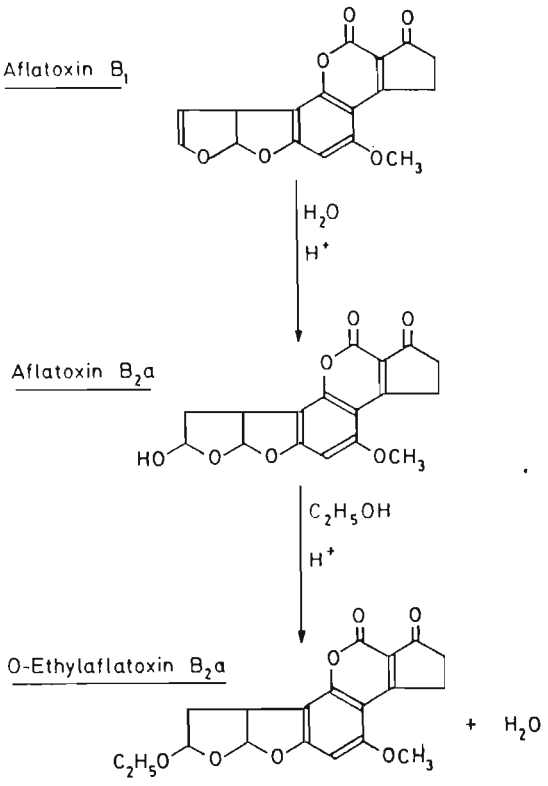
An important property of the aflatoxins B_{2a} and G_{2a} is their non-toxicity towards ducklings, and hence, presumably, towards other animals. The toxicological studies were kindly carried out by Dr. Ruth Allcroft at the Central Veterinary Research Laboratories, Weybridge, and Dr. W. H. Butler at the M.R.C. Toxicological Research Unit, Carshalton, Surrey. One-day Khaki Campbell ducklings were dosed with aflatoxins B_{2a} and G_{2a} at the following rates:

Aflatoxin	μg.
B_{2a}	300: 600: 900: 1,200
G_{2a}	400: 800: 1,200: 1,600

Fig. 7

Formation of O-Ethylaflatoxin B_{2a} from Aflatoxin B 1

in Acidic Media



There was no significant difference in growth between control and dosed birds, nor were there any of the characteristic liver lesions associated with aflatoxin poisoning.

The results of these tests on ducklings, together with the fact that aflatoxins B_1 and G_1 are easily converted to aflatoxins B_{2a} and G_{2a}, would seem to indicate that this might be a practical method for the detoxification of feedstuffs especially as aflatoxins B_1, and G_1, are by far the more commonly occurring toxic aflatoxins. Treatment of contaminated material with dilute mineral acid over a number of hours should be sufficient to lower the toxicity substantially.

After our studies had been completed on the fraction containing aflatoxins M_1, B_{2a} and G_{2a}, we turned our attention to the first fraction eluted from the column which contained the known aflatoxins.

Examination of aflatoxins B_1 and B_2:

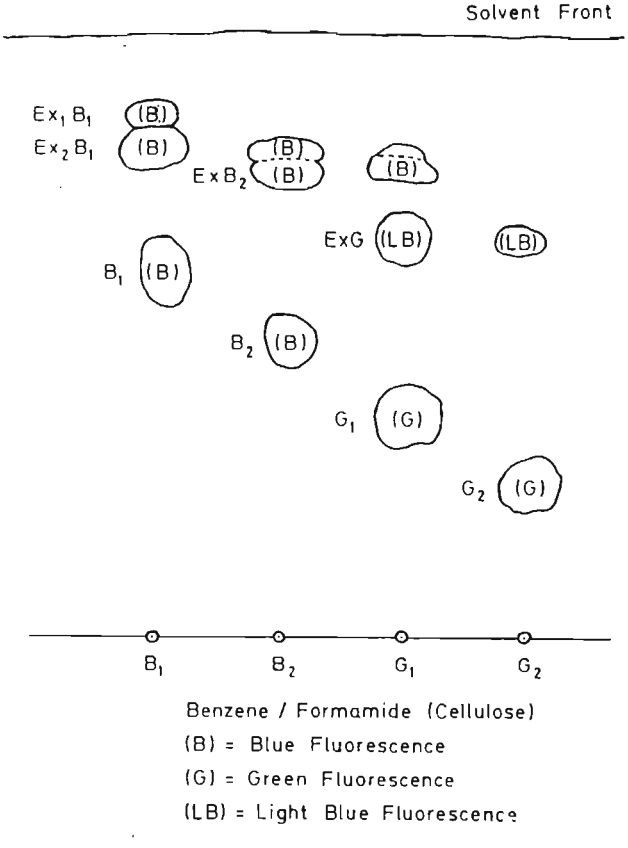
It had been reported by Adye and Mateles (1964) that chromatography of the aflatoxins on kieselguhr saturated with formamide and developed with benzene, gave a better separation than that using the usual keiselgel-methanol-chloroform system. We

therefore decided to apply this system to the known aflatoxins we had obtained in our first fraction. Thin-layer chromatoplates were prepared by slurrying cellulose powder [MN300 HR (supplied by Camlab) 15 g.] with 70 ml. of formamide-acetone (1 : 9 v/v), and spreading five 20 cm. × 20 cm. chromatoplates with this slurry. The plates were ready for use after drying in the air for five minutes.

The various aflatoxins obtained as single fractions after chromatography on columns of keiselgel G, using methanol-chloroform (1 : 49 v/v) as the solvent system, were applied as bands to the thin-layers of cellulose impregnated with formamide and developed with formamide-saturated benzene. From each aflatoxin-containing fraction further fluorescent compounds were obtained at higher R_F values than the known starting aflatoxin. Thus the *aflatoxin B_1 fraction* yielded two blue fluorescent metabolites, one named compound Ex_1B_1 (having an R_F value of 0·86), and the other labelled Ex_2B_1, (with an R_F value of 0·81) (Fig. 8). The *aflatoxin B_2 fraction* yielded

Fig. 8

TLC of Main Aflatoxin Fractions from Culture Medium of A. flavus



two blue fluorescent metabolites close together, one corresponding to Ex_2B_1 and another which was named ExB_2 and which had an R_F value of 0·77. Both the *aflatoxin fractions G_1 and G_2* gave rise to the same light blue fluorescent metabolite with an R_F value of 0·66 which was named ExG (Fig. 8).

That the new fluorescent compounds were not produced as artefacts during chromatography of the known starting aflatoxin was shown by isolating small amounts of them and of the known starting aflatoxins using the benzene-formamide system. The samples isolated were then re-chromatographed in this system, and also in the kieselgel-methanol-chloroform system, followed by the benzene-formamide system again. No further quantities of the new compounds were formed in either case, and the new compounds were found to have R_F values on the kieselgel G system similar to those of their respective parent aflatoxin fractions, e.g. Ex_2B_1 had the same R_F value as aflatoxin B_1, etc. The new compounds had the following properties:

Compound Ex_1B_1
m.p., 83°
Molecular weight, 440
Molecular formula, $C_{29}H_{60}O_2$

Compound Ex_2B_1
m.p., 236°
Molecular weight, 344
Molecular formula, $C_{18}H_{16}O_7$
Light absorption max. in methanol, 224 mμ, 265 mμ, 362 mμ.

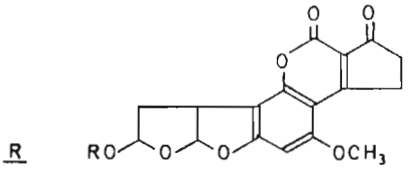
Compound ExB_2:
m.p., 245°
Molecular weight, 358
Molecular formula, $C_{19}H_{18}O_7$
Light absorption max. in methanol, 223 mμ, 266 mμ and 364 mμ.

Compound ExG:
m.p., 200°
Molecular weight, 374
Molecular formula, $C_{19}H_{18}O_8$
Light absorption max. in methanol, 223 mμ, 245 mμ, 266 mμ and 366 mμ.

Unfortunately, insufficient material was available for full characterisation of the above compounds, but the following was deduced from the information obtained: From mass spectrometry, compound Ex_1B_1 was probably a long-chain alcohol quite unconnected with the aflatoxins. Compounds Ex_2B_1, ExB_2 and ExG were evidently aflatoxin derivatives as they had ultra-violet, and mass spectra which were characteristic of the aflatoxins, e.g. light absorption maximum at about 363 mμ, loss of m/e of 29 on mass spectrometry. From their molecular formulae it seemed that Ex_2B_1 was a methyl derivative of aflatoxin B_{2a}, that ExB_2 was an ethyl derivative of aflatoxin B_{2a}

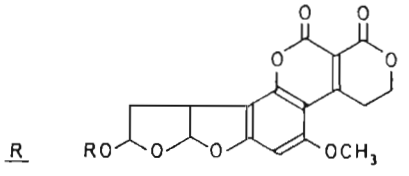
and that compound ExG was an ethyl derivative of aflatoxin G_{2a}. This possibility was investigated by preparing the 2*-methoxy, 3*-hydro-addition compound of aflatoxin B_1 (X), Fig. 9 and the 2*-ethoxy, 3*-hydro-addition compounds of aflatoxins B_1 (XI) and G_1 (XII). (These compounds, it will be noted, are synonomous with the equivalent

Fig. 9



X CH_3- 2*-Methoxy. 3*-hydro addition compound of aflatoxin B_1 (Ex_2B_1)

XI C_2H_5- 2*-Ethoxy, 3*-hydro addition compound of aflatoxin B_1 (Ex B_2)



XII C_2H_5-2*-Ethoxy, 3*-hydro addition compound of aflatoxin G_1 (Ex G)

O-alkyl derivatives of aflatoxins B_{2a} and G_{2a}.) The compounds were prepared by treating the appropriate aflatoxin with either methanol or ethanol in the presence of a few drops of thionyl chloride, to act as an acid catalyst (Andrellos and Reid 1964), and separating the products by TLC. Thus by a direct comparison of properties, compound Ex_2B_1 was shown to be 2*-methoxy, 3*-hydro-aflatoxin B_1; substance ExB_2 was 2*-ethoxy, 3*-hydro-aflatoxin B_1; and compound ExG became identified with 2*-ethoxy, 3*-hydro-aflatoxin G_1.

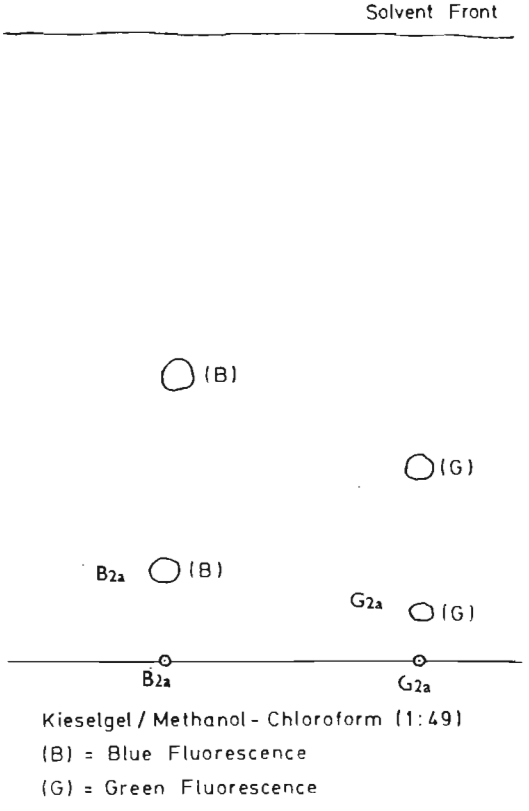
Although the structures of these compounds had now been settled, the problem still remained as to how they arose in the aflatoxin-containing extracts. However, the problem was solved in a most unexpected way. It had been noted that, after treatment with formic acid and extraction with chloroform, aflatoxins B_{2a} and G_{2a} gave rise to new fluorescent products on TLC (kieselgel G-methanol-chloroform), which had R_F values very similar to those of the known aflatoxins B_1 and G_1. Aflatoxin B_{2a} gave rise to a blue fluorescent product, and aflatoxin G_{2a} to a green fluorescent one (Fig. 10). On

* Refers to the position in the terminal furan ring.

Fig. 10

TLC of Products from Formic Acid Treatment of

Aflatoxins B_{2a} and G_{2a}



Kieselgel / Methanol - Chloroform (1:49)

(B) = Blue Fluorescence

(G) = Green Fluorescence

examination by mass spectrometry, ultra-violet spectroscopy and proton magnetic resonance absorption spectroscopy, they were found to be identical with the 2*-ethoxy, 3*-hydro-addition compounds of aflatoxins B_1 and G_1. Initially there was some mystery as to how these compounds arose, as there was no apparent source of ethyl groups in the reagents with which the compounds had been treated. The matter was clarified when it was realized that the chloroform used to extract the reaction mixture contained ethanol (about 2%, v/v) as a stabilizer. It thus became apparent that aflatoxins B_{2a} and G_{2a} were reacting with this ethanol under acid conditions, in a manner similar to that found in glycoside formation in sugars. This could also explain how the compounds, Ex_2B_1, ExB_2, and ExG came to appear as contaminants of the principal aflatoxins, B_1, B_2, G_1 and G_2.

In order to test this possibility, acidified solutions of aflatoxin B_{2a} were extracted either with alcohol-free chloroform, or with pure chloroform to which one of the following solvents, methanol, ethanol, propan-2-ol, butan-1-ol or benzyl alcohol, had

been added. (The alcohol-free chloroform was prepared by treatment with activated molecular sieve 5A.) On TLC, each extract gave a blue fluorescent spot at a higher R_F value than the original aflatoxin B_{2a}, and on mass spectrometry of these products it was found that they corresponded to the appropriate O-alkyl derivatives of aflatoxin B_{2a}, e.g. the chloroform containing butanol gave O-n-butyl aflatoxin B_{2a}. In this way it was shown that O-alkyl derivatives of aflatoxin B_{2a}, could arise by extracting acidic solutions of either aflatoxin B_1 or B_{2a} with impure chloroform (see Fig. 7). Similar O-alkyl derivatives of G_{2a} could arise by the extraction of acidic solutions of G_1 or G_{2a}. Compounds Ex_2B_1, ExB_2 and ExG did not arise exactly in this way, as they still arose on TLC of culture fluids extracted with alcohol-free chloroform. In this case the alcohol presumably occurs in the culture due to fermentation reactions and then proceeds to react as outlined.

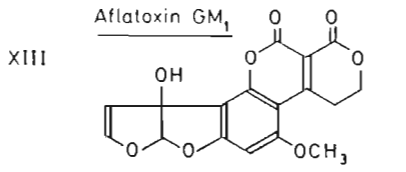
It is important to note that the formation of aflatoxins B_{2a} and G_{2a} and also of their O-alkyl derivatives as outlined above, may complicate the individual assay of aflatoxins from acidic culture fluids. For example, a substantial quantity of the aflatoxins B_1 and G_1 in any preparation may actually be present as aflatoxins B_{2a} and G_{2a} and the extraction of such cultures with ordinary chloroform would give rise to their O-ethyl derivatives; the latter, having similar R_F values to the more common aflatoxins B_1, B_2, G_1 and G_2 in the TLC systems using keiselgel, would falsify the assay result.

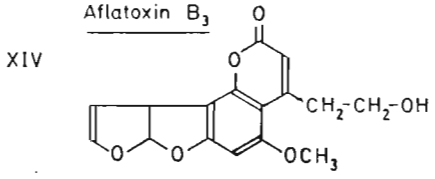
In order to overcome these difficulties, culture fluids should be neutralized prior to extraction, which should be carried out, preferably, with freshly prepared alcohol-free chloroform. In addition to these safeguards, extracts from such cultures should be investigated, using the benzene-formamide system, which would reveal the presence of any O-alkyl derivatives of aflatoxins B_{2a} and G_{2a}.

Aflatoxins GM_1 and B_3:

In addition to being cultured on simple salts media as in the previous work, *A. flavus* was also grown on a yeast extract-glucose medium (based on that described by Davis, Diener, and Eldridge, 1966). This medium was used for the quantitative production of the aflatoxins as it was known to produce them in high yield (Davis *et al.*, 1966). On column chromatography of the aflatoxin-containing extract from this culture, two fractions were obtained in the same manner as was described previously for the simple salts medium. The first fraction contained the known aflatoxins, while the second was found to contain two metaboites, which fluoresced blue, and one which fluoresced green when examined by thin-layer chromatography in the kieselgel/methanol-chloroform system. The compound with the highest R_F value, i.e. with a value halfway between the positions of aflatoxins G_2 and M_1 (Fig. 1), proved to be a new compound which was named aflatoxin B_3 on account of its blue fluorescence. The other two compounds, of lower R_F values, appeared in positions analogous to those of aflatoxins B_{2a} and G_{2a}, i.e. the blue fluorescent spot was a little higher on the chromatoplate than the green fluorescence. However, on further examination it was found that the blue fluorescent metabolite was identical with aflatoxin M_1, and that the green

Fig. 11





fluorescent compound had properties which were in accordance with its being the G analogue of aflatoxin M_1 XIII (Fig. 11). This latter compound had been tentatively reported by other workers, e.g. Purchase and Theron (1967), who had named it aflatoxin GM (private communication). We have more specifically designated it aflatoxin GM_1 in keeping with the other aflatoxin nomenclature. The properties of aflatoxins GM_1 and B_3 are as follows:

Aflatoxin GM_1:
m.p., 276°
Molecular weight, 344
Molecular formula, $C_{17}H_{12}O_8$
Light absorption max. in methanol, 235 mμ, 262 mμ and 358 mμ
(ϵ_M in order, 21,200, 16,300 and 12,000).

Aflatoxin B_3:
m.p., 217°
Molecular weight, 302
Molecular formula, $C_{16}H_{14}O_6$
Light absorption max. in methanol, 229 mμ, 253 mμ, 262 mμ and 326 mμ
(ϵ_M in order, 10,000, 7,300, 7,500 and 9,350)

Both compounds formed mono-acetyl derivatives on acetylation with acetic anhydride in pyridine.

The proton magnetic resonance spectrum of aflatoxin B_3 and its other properties are in keeping with its having the structure XIV (Fig. 11) (Heathcote and Dutton, 1969). From this structure it can be seen that aflatoxin B_3 probably arises in the culture fluids of *A. flavus* from a degradation of aflatoxin G_1 by a decarboxylation reaction, involving either the coumarin or δ-lactone system present in this compound. As an analogous compound corresponding to the degradation product of aflatoxin B_1 has not been observed, (i.e. with an intact cyclopentenone ring system), it seems very probable that the δ-lactone system is hydrolysed and then decarboxylated. This process presumably occurs during incubation of the culture and not as a result of the extraction procedure, as aflatoxin B_3 did not arise when relatively large amounts of pure aflatoxin B_1 or G_1 were incubated in un-inoculated culture fluid and passed through the extraction process. It may well be that aflatoxin B_3, and like compounds, represent steps in a degradative process, occurring in cultures of the mould, which lead to the disappearance of the aflatoxins from cultures of *A. flavus*, a process which has been observed by ourselves and other workers, e.g. Ciegler, Peterson, Lagoda and Hall (1966).

Thanks are due to: Dr. I. F. M. Purchase and colleagues of the N.N.R.I. Pretoria, for donation of samples and help given; Dr. Ruth Allcroft of the Central Veterinary Laboratories, Weybridge, Surrey and Dr. H. M. Barnes and Dr. W. H. Butler of the M.R.C. Toxicology Research Unit, Carshalton, Surrey for carrying out toxicological studies on animals; Drs. M. Barber and A. Wolstenholme, and Mr. T. Kemp of A.E.I., Manchester, for mass spectrometry; and the British S.R.C. for a research grant to one of us, M.F.D.

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Biosynthesis of aflatoxins

John G Heathcote, Michael F Dutton and John R Hibbert

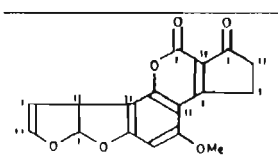
Despite the interest which has been aroused by the discovery of the aflatoxins and the work which has been carried out on them[1–3] surprisingly little is known about their biogenesis.

A number of hypothetical biosynthetic routes were advanced by Moody,[4] who suggested that the aflatoxin molecule could arise more easily from isoprenoid units rather than from acetate and he put forward various schemes for their biosynthesis from isoprenoid units.

An attractive suggestion was put forward by Holker and Underwood.[5] Because of the presence of the *bis*-dihydrofuran moiety in both aflatoxins and sterigmatocystin (a metabolite of *A. versicolor*), they suggested that sterigmatocystin is probably a precursor of the aflatoxins or has a precursor in common with them, and they put forward a possible route for the conversion of sterigmatocystin to aflatoxin B_1. Experimental support for this theory was lacking, as cultures of *A. flavus* and *A. parasiticus* did not convert labelled sterigmatocystin to aflatoxin, and no sterigmatocystin could be detected in cultures of *A. flavus* cultivated on labelled acetate.

Thomas[6] also took the view that the aflatoxins are produced via sterigmatocystin, and suggested that sterigmatocystin itself arises from versicolorin A, another metabolite of *A. versicolor*. His overall scheme however, differed from that put forward by Holker and Underwood.[5]

It has now been shown, by labelling and degradative studies, by Biollaz, Buchi and Milne[7a,b], that the aflatoxins are acetate derived and that the acetate units are distributed



(I)

Fig 1 Distribution of acetate units in aflatoxin B. (Derived from $\overset{*}{C}H_3\overset{*}{C}OOH$, Biollaz *et al*)

through the molecule as indicated in Fig 1. This has also been confirmed by Raj *et al*[8] who found that labelled acetate, leucine and mevalonate, were incorporated into aflatoxin molecules in the presence of a cell-free enzyme system prepared from a toxin-producing strain of *A. flavus*.

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Dr Dutton and Dr Hibbert are at Trent Polytechnic and Manchester Polytechnic, respectively

Recently further credence has been given to the proposals of Holker *et al*[5] and Thomas[6] following the isolation of other metabolites related to sterigmatocystin, *viz*. *O*-methylsterigmatocystin – Burkhardt and Forgacs[9]; Aspertoxin – Rodricks, Lustig, Campbell, Stoloff and Henery-Logan[10] and Versicolorin C – Heathcote and Dutton[11]; all from aflatoxin producing cultures of *A. flavus*. In view of these discoveries, a biosynthetic scheme for aflatoxin starting from acetate and involving a versicolorin type precursor becomes feasible, as the versicolorin molecule has an anthraquinone nucleus typical of those synthesised in accordance with the acetate theory, e.g. as suggested for averythrin by Roberts and Roffey.[12]

In order to investigate the biosynthesis of the aflatoxins by this latter route, the incorporation of various possible precursors into the aflatoxins produced by *A. flavus* has been studied. This is described in the experiments which follow.

Experimental

Cultivation of the mould on labelled substrates

Cultures of *A. flavus* (CMI 91019b) were prepared in 250cm

Table 1 **Labelled compounds* (counts/min.) added to cultures of A. flavus**

	Compound added*	Specific activity μc/μ mole	Counts /min added
($^{14}C_1$)	Sodium acetate	44·4	14,100
($^{14}C_2$)	Sodium acetate	38·0	20,400
($^{14}C_1$)	Sodium malonate	14·5	111,000
($^{14}C_2$)	Sodium malonate	18·3	111,000
($^{14}C_4$)	Ethyl acetoacetate	3·7	40,000
($^{14}C_2$)	DL Mevalonic acid lactone	4·8	111,000
(U-^{14}C)	Glucose	194·0	12,727
($^{14}C_1$, $^{14}C_4$)	Fumaric acid	194·0	44,000
($^{14}C_2$, $^{14}C_3$)	Fumaric acid	2·2	44,000
($^{14}C_1$, $^{14}C_4$)	Maleic anhydride	3·1	111,000
(U-^{14}C)	L-Malic acid	34·7	111,000
($^{14}C_1$, $^{14}C_4$)	Succinic acid	8·8	11,100
(G-^{14}C)	Kojic acid**	$1·8 \times 10^{-8}$	13,950
(U-^{14}C)	L-Aspartic acid	217·0	11,100
(U-^{14}C)	L-Isoleucine	290·0	111,000
(G-^{3}H)	L-Phenylalanine	750·0	111,000
(G-^{3}H)	*p*-Aminobenzoic acid	205·0	160,000
(U-^{14}C)	L-Glutamic acid	130·0	111,000
($^{14}C_2$)	inside chain DL-Tyrosine	15·7	99,57
($^{3}H_1$, $^{3}H_5$)	in nucleus DL-Tyrosine	500·0	105,78

* Unless otherwise stated, all compounds were obtained from Radiochemical Centre, Amersham

** Obtained from a culture of *A. flavus* grown on (U-^{14}C) glucose a uncorrected for quenching and efficiency of instrument

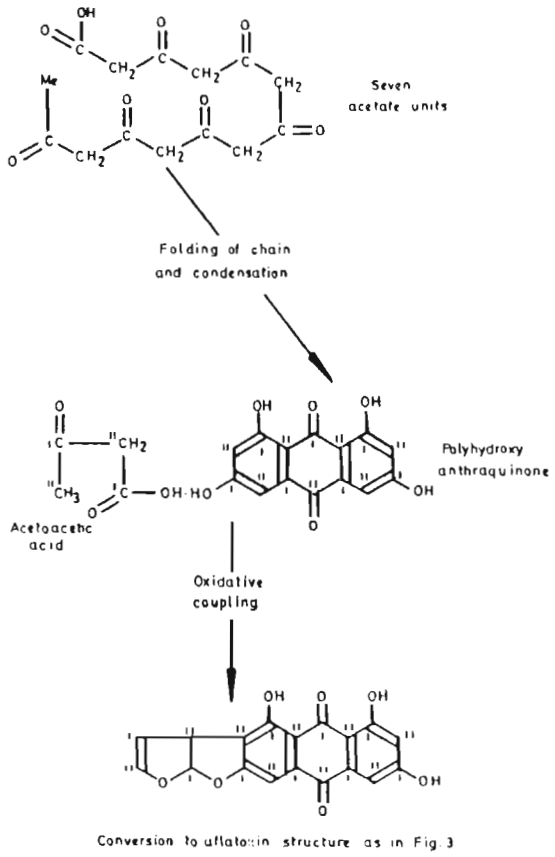

Fig 4 Possible route for the biosynthesis of aflatoxin via the acetate pathway and oxidative coupling of acetoacetate[15]

More recently further experiments with certain mutant strains of *A. flavus* have been carried out in this laboratory, the results of which are now being prepared for publication. These lend support to the above scheme and indicate that a biosynthetic system which involves a common precursor for the versicolorins, sterigmatocystins and the aflatoxins is quite likely.

M. F. Dutton would thank the SRC for a research grant.

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findings agree with the work of Donkersloot, Hsieh and Mateles,[13] who showed that labelling from these compounds does not enter the aflatoxin-containing fraction. The earlier reports by Adye and Mateles[14] that labelling from both these compounds entered the aflatoxin-containing fraction may be explained from the observation that the aflatoxins form complexes with added labelled amino acids which are very hard to break down. For example, at least three developments on t.l.c., using two different solvent systems, were required to free the aflatoxin-containing fraction from tyrosine, while labelled *p*-aminobenzoic acid was never separated from the aflatoxins.

From the evidence of our results, it seems that the aflatoxins are derived purely by the 'acetate' route, an observation which is in agreement with the findings of Biollaz *et al*[7a,b]. It would seem clear to us that the aflatoxins must arise by the secondary transformation of some preformed compound. If it is assumed that this precursor is an anthraquinone, e.g. versicolorin A (III) Fig 3, a major difficulty still remains as to how the bis-dihydrofuran moiety in the molecule is formed. It has been suggested (e.g. by Dr J. S. E. Holker of Liverpool University) in a private communication that this system might arise from a 'C4 unit' e.g. oxaloacetate, combining with the preformed benzenoid part of the molecule, as shown in Fig 2. In spite of the feasibility of this scheme, it

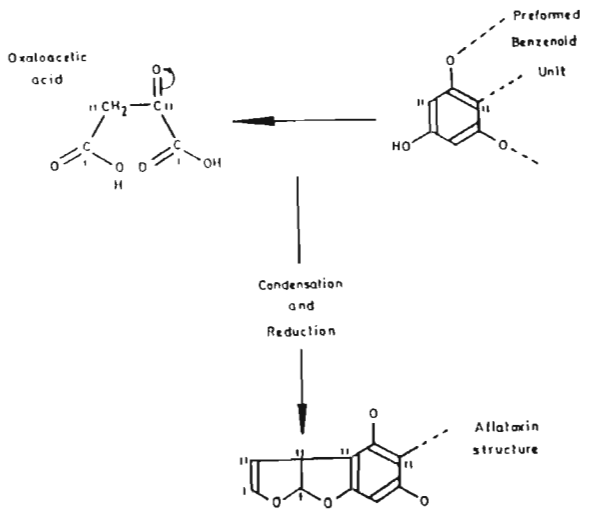

Fig 2 Possible route for the biosynthesis of the bis-dihydrofuran system in aflatoxin

is not supported by the experimental work reported here. All the 'C4' units examined, *viz* malate, fumarate, succinate, maleate and aspartate, (apart from acetoacetate, which is involved in the acetate pathway), showed little or no uptake into the aflatoxin-containing fraction.

Support for isoleucine being the precursor of the bis-dihydrofuran system in the aflatoxins was forthcoming in that reasonably rapid uptake into the aflatoxin-containing fraction occurred when labelled isoleucine was added to cultures of *A. flavus*. However, this result is complicated by the fact that mevalonic acid (a compound with the same carbon skeleton as isoleucine) shows a rate of uptake into the aflatoxin-containing fraction which is similar to that shown by isoleucine. As there is no known biosynthetic link between these two compounds, the question arose as to which one, if either,

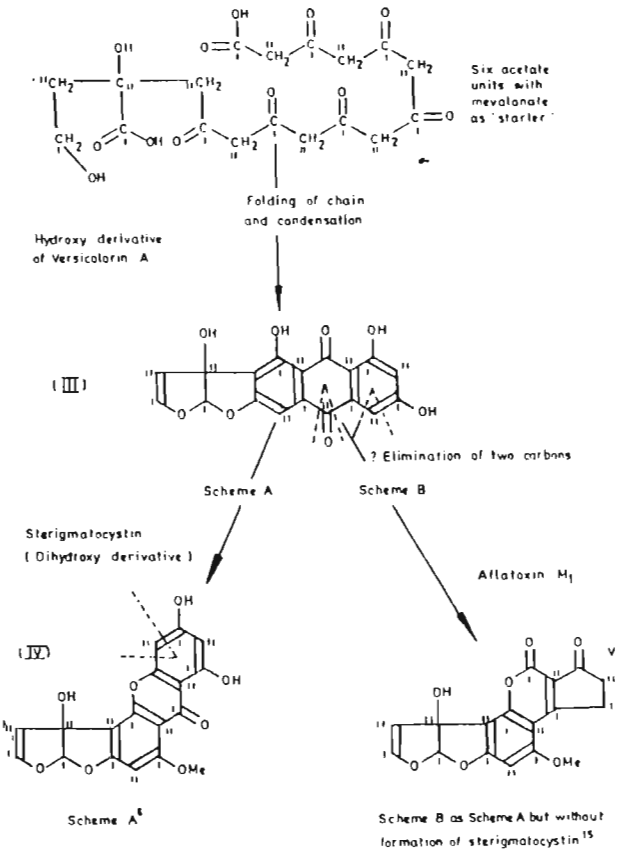

Fig 3 Possible route for the biosynthesis of aflatoxin via acetate pathway with mevalonate as a starter unit

was actively involved in the biosynthesis of the bis-dihydro furan system. As mevalonic acid is often found as the starte unit in acetate-derived compounds, and is already in a highl oxidised state, we felt, at the time, that this compoun seemed the more likely of the two, and hence the scheme i Fig 3 was suggested for the total biogenesis of aflatoxin B_1 starting from a mevalonate 'starter', and acetate units. Th recent work of Biollaz *et al*[7a] has invalidated this scheme, fo although the resultant labelling pattern which would b obtained from labelled acetate is in keeping with that found b Biollaz *et al*[7a] for the main part of the molecule, that pro duced in the bis-dihydrofuran moiety is not (Figs 1 and 3 If isoleucine is substituted for mevalonate in the scheme (Fi 3) the labelling pattern is still ambiguous, because isoleucin can arise from acetate by more than one route, However, th possibility of some charge transfer or other complex bein formed in the case of isoleucine should not be entirel overlooked.

Thus, from the results, the problem of the biosynthet pathway to the aflatoxins still remains unsolved, although o the basis of data derived from selective degradation of labelle aflatoxins, Biollaz *et al*[7b] have suggested a scheme startin from either a polyhydroxynaphthacene or benzanthracen However, there is no experimental evidence at the moment favour of it, especially as compounds of about this molecul size isolated from the *Aspergilli* are often substituted anthr quinones, e.g. endocrocin and averantin. We should lik to propose an alternative possibility, where acetoacetate oxidatively coupled to a preformed anthraquinone molecu (cf. phenol oxidative coupling) to give the desired labellin pattern (Fig 4).

conical flasks on a simple salts medium (50cm³) of the following composition: dipotassium hydrogen phosphate (3g); potassium dihydrogen phosphate (1g); magnesium sulphate (1g); ammonium sulphate (8g) and glucose (40g) all made to 1 litre with distilled water. The inoculation was carried out by cutting a disc from an actively growing colony of *A. flavus* on potato dextrose agar, with a sterile cork borer and adding this to the medium. After incubating the culture at 25° for about two days, and just on the appearance of the aflatoxins in the culture medium, a sterile sample of the test substrate labelled with radioactivity (either ^{14}C or ^{3}H) was added (Table 1).

Method of counting activity

Counting of the β-emissions from the disintegration of the ^{14}C and ^{3}H isotopes was carried out by liquid scintillation counting, using the Nuclear Chicago 'Liquid Scintillation Spectrometer System Model 724'. The instrument was set up for counting separate samples of ^{3}H and ^{14}C consecutively, and its counting efficiency was determined.

The scintillation solution used had the composition; P.P.O. (4g) and P.O.P.O.P. (0·4g) made to 1 litre with toluene. The count was corrected for the efficiency of the instrument, the background, and the quenching caused by the aflatoxin present.

Preparation of sample for counting

Immediately after the addition of the labelled substrate, a sample of culture fluid (5cm³) was withdrawn from the flask by means of a sterile pipette. This sample was then neutralised by shaking with a little solid sodium bicarbonate in a separating funnel. The neutralised sample was then extracted three times with chloroform (10cm³). The combined extracts were dried over anhydrous sodium sulphate and filtered, the residue of sodium sulphate being washed with a little dry chloroform. The chloroform extracts and washings were evaporated to dryness and the residue was made up to 10cm³ with chloroform in a graduated flask. The optical density of this solution was read at 363nm using either a 1cm or a 2cm silica cell depending upon the concentration of aflatoxin present. From this reading, the aflatoxin concentration present in the sample was calculated in terms of μ mole of aflatoxin B_1 (E=20,150). The total aflatoxin-containing chloroform solution, including washings from the optical cells, was evaporated to dryness *in vacuo*, and the residue was taken up in toluene (4cm³). This solution was then transferred to a scintillation bottle, the residual solution being washed into the bottle with toluene (1cm³). To this solution, 5cm³ of scintillation solution were added. The scintillation bottle was placed in the dark for one hour, and then counted. After correction of the count, the result was expressed in counts/min/μ mol of solution. This gave the zero-time reading. If the reading contained appreciable activity, it was assumed that added tracer was being carried over into the aflatoxin fraction and it was necessary to purify this fraction further by t.l.c. on 'Keiselgel' G using methanol-chloroform (1:49) as the solvent system. The purified aflatoxin-containing fraction so obtained was re-assayed and re-counted. The t.l.c. process was repeated several times more, using other systems, if necessary, until the activity was reduced to zero. Where, in certain cases, a constant residual activity still remained in the zero-time fraction, its activity was subtracted from the activity of samples subsequently taken. In practice nearly all the zero-time samples were purified by t.l.c. to minimise this interference. Subsequent samples of culture fluid were taken after appropriate time intervals and treated in the same manner.

In order to check whether a general labelling of all metabolites was occurring, when substantial amounts of radioactivity were passing into the aflatoxin-containing fraction, a sample of pure kojic acid was isolated from the culture fluid, and its activity was determined. It was not possible to find the specific activity of the kojic acid, due to the quenching effect of the material. However, the technique used was adequate for ascertaining whether moderate amounts of activity were passing into the kojic acid.

Results

Both acetate and malonate were taken into the aflatoxin-containing fraction rapidly as was aceto-acetate, activity appearing in the aflatoxins within six hours of the substrate being added. Mevalonate and isoleucine showed a slightly slower uptake but were beginning to be incorporated within a day.

Of the substrates involved in shikimate metabolism, none showed rapid uptake. Activity from the tyrosine did not appear in the aflatoxin-containing fraction until after four days and then in small amounts. Activity from phenylalanine had not appeared in the aflatoxin-containing fraction after six days. It was difficult to draw any conclusion in the experiment with *p*-aminobenzoic acid as it was impossible to reduce the activity of the zero-time sample, even after five purifications on t.l.c. The above results are detailed in Table 2.

The incorporation of radioactivity was negligible when any of the following labelled materials were added to cultures of *A. flavus*: fumaric acid, malic acid, maleic anhydride, glutamic acid, succinic acid, kojic acid and glucose.

Discussion

That the aflatoxins are not 'shikimate-derived' is supported by the result that neither labelled tyrosine nor phenylalanine is rapidly incorporated into the aflatoxin molecule. These

Table 2 **Incorporation of labelled compounds into the aflatoxin fraction of cultures of A. Flavus. Specific activity (count/min/μ mole) of aflatoxin fraction**

	Days incubation after addition							
	0·25	0·5	1	2	3	4	5	6
($^{14}C_1$) Sodium acetate	3593	8387	7582	9978	4474	3184	2552	
($^{14}C_2$) Sodium acetate	13,362	62,821	72,894	17,695	11,433	6350	8348	
($^{14}C_1$) Sodium malonate	26,804	50,098	29,192		19,429	46,459	18,565	
($^{14}C_2$) Sodium malonate	9277	20,450	59,273		40,433	27,081	22,717	
($^{14}C_3$) Ethyl acetoacetate	9961	59,540	129,175	22,071	30,102			29,343
($^{14}C_1$) DL-Mevalonic lactone	0	308	174	396	219	981	269	
(U-^{14}C) Isoleucine	0	54	1301	4184	4479	3497	2042	
($^{14}C_1$) (side chain) DL-Tyrosine	0	0	0	0	0	90	144	381
($^{3}H_3$: $^{3}H_3$) (in nucleus) DL-Tyrosine	0	0	0	0	0	0	83	359
(G-^{3}H) L-Phenylalanine	0	0	0	0	0	0	0	0
(G-^{3}H) *p*-Aminobenzoic acid		413,043	207,478	119,687	990,370	99,765	107,296	13,933

Biosynthesis of aflatoxins

Part II

John G Heathcote, Michael F Dutton and John R Hibbert

It is now generally accepted that acetate and malonate are the most likely starter blocks for the biosynthesis of the aflatoxins but, as yet, little is known of the intermediate metabolites on the pathway. In a previous paper[1] we considered several possible routes to the formation of the aflatoxin molecule on the basis of the incorporation of labelled compounds into the aflatoxins produced by *Aspergillus flavus* cultures. As a result of these findings, we suggested a scheme embodying a versicolorin type molecule as an intermediate in the biogenesis of the aflatoxins. Because of a dearth of suitable mutants possessing enzymically blocked pathways to aflatoxin biosynthesis, workers have been forced in the past to rely on indirect methods in approaching the problem of biosynthesis.

On the basis of evidence obtained from *in vitro* degradation studies[2] on labelled aflatoxins, Biollaz *et al*[3] suggested a biosynthetic scheme starting from either a polyhydroxynaphthacene or benzanthracene. These workers came to the conclusion that aflatoxin B_1 is the precursor of aflatoxin M_1. Recently, however, Steyn *et al*[4] fed [2—^{13}C]-and [1,2-^{13}C]-acetate to a culture of *A. flavus* and established that the arrangement of intact acetate units differed from that postulated by Biollaz.[5] Their results removed a previous ambiguity by demonstrating that a C_{18}-naphthacene precursor is no longer tenable. Their findings were in complete agreement with the ^{14}C-labelling pattern reported originally by Holker and Mulheirn,[6] and Steyn *et al*[4] suggest that it now seems more likely that biosynthesis takes place by the addition of a C_4- unit to a preformed anthraquinone molecule, as postulated by Heathcote *et al*.[1] This hypothesis has also received support from the findings of Hsieh *et al*[7] that sterigmatocystin could be converted into aflatoxin B_1 by cultures of *Aspergillus parasiticus*, and, even more recently,[8] that the C_{20}-polyhydroxyanthraquinone averufin can be converted into aflatoxins B_1, B_2 and G_1.

Another method of ascertaining the later stages of biosynthesis of the aflatoxins is to add various labelled aflatoxins to cultures of *A. flavus* and to ascertain the extent to which they are interconvertible. In this way, it is possible to ascertain whether an individual aflatoxin acts as a precursor to any of the other aflatoxins. Using this technique several years ag Dutton[9] obtained results in this laboratory which indicat that the sequence of aflatoxin production was probably the order of $M_1 \rightarrow B_1 \rightarrow G_1$. These experiments ha not previously been reported and since they were carried o we have greatly improved the chromatographic techniqu necessary for the isolation and purification of pure af toxins,[10] and the earlier experiments have been confirm and extended.

In the present paper the order of natural biosynthesis h been re-examined by feeding labelled aflatoxins and oth related metabolites to actively growing cultures of *A. flav* CMI 91019b. In addition to the parent strain of the organis two mutant strains[11] were also utilised in these studies. Th was partly because one of these mutants (CMI 91019b produced both versicolorin and sterigmatocystin in mu greater quantities than the parent strain on all med Furthermore, electron microscopy had shown that the c wall structure of the mutants differed from that of the pare particularly in having thinner walls and larger pyci intuckings of the plasmalemma.[12] It was hoped that th differences in cell structure might enhance our knowledge the mechanism by which the secondary metabolites transported across the mycelial wall.

Experimental

Preparation of ^{14}C-labelled metabolites

The labelled aflatoxins and other secondary metabolites w prepared for the feeding experiments by growing cultures *A. flavus* 91019b either on a simple salts medium supp mented with glucose (Heathcote *et al*[1]) or, in the later periments with mutants (Table 3), on the medium of Br *et al*[13] in which the ammonium nitrate had been replaced ammonium sulphate (GAS medium). The radioact aflatoxins and other metabolites were isolated, individu purified and assayed as described in the feeding experim below.

Feeding experiments

Actively growing cultures of CMI 91019b and its muta 91019b$_I$ and 91019b$_{II}$

In the initial experiments reported in Table 1 the medi used for the actively growing culture was the same sim salts medium referred to above supplemented with gluc

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extent, into aflatoxin B_2. The distribution of incorporated radioactivity between B_1 and B_2 indicates that the latter may well arise from the B_1 first produced directly from M_1. It would seem from the fact that this conversion of aflatoxin M_1 to B_1 and B_2 is by no means quantitative, that it is probably dependent upon its permeability into the fungal cell from the culture fluid.

In contrast to the above finding, none of the aflatoxins of the B or G series was converted into aflatoxin M_1 in any appreciable quantity. The radioactivity appearing in the aflatoxin M_1 when aflatoxin B_1 was added to the culture was small and could have arisen from contamination with aflatoxin B_{2a}. The latter was highly radioactive in these experiments and appreciable difficulty is encountered in separating M_1 from B_{2a} by t.l.c.

There is little doubt that aflatoxin B_1 may be converted into most of the other aflatoxins such as B_2, B_{2a}, G_1, G_2 and G_{2a} (the latter two aflatoxins being derived, presumably, via G_1). Aflatoxin B_2, though apparently convertible to B_1, is not converted to G_2. The latter was not readily converted to any of the other aflatoxins. Neither B_{2a} nor G_{2a} appeared to be convertible to other aflatoxins.

The results obtained by adding labelled aflatoxin B_1 to resting cultures agreed with those obtained from the actively growing cultures. It would appear from the results below that the radioactivity of B_1 was incorporated into all other aflatoxins except M_1.

From these results it would appear, therefore, that aflatoxin B_1 is not a precursor of aflatoxin M_1, as suggested by Biollaz *et al*,[2] but rather that the biosynthetic route may well be from $M_1 \rightarrow B_1 \rightarrow G_1$.

Similar experiments were carried out after an interval of about two years, during which time we had improved the t.l.c. separation techniques. These techniques[10] enabled us to separate kojic acid from the relatively polar hydroxyaflatoxins and to measure the degree of incorporation of radioactivity into the kojic acid produced by the mould. It was found, in practice, that none of the kojic acid contained any appreciable activity. This suggested that the aflatoxin tracers were not themselves metabolised as general carbon sources by the mould.

The new t.l.c. systems also enabled us to prepare a variety of U-^{14}C-labelled metabolites in pure form for feeding to the mould and its mutants. The results of these feeding experiments are summarised in Table 3. They show that, except for some variations, the redistribution of labelling that occurred when aflatoxin B_1 was added to cultures of the white and brown mutants was similar to that shown by the parent strain (CMI 91019b). Thus, aflatoxin B_1 appears to be acting as a precursor of B_2, G_1 and B_{2a} - a finding in agreement with the earlier results.

The high level of labelling in aflatoxin GM_1 when radioactive M_1 was added to cultures is in complete contrast to the absence of significant labelling in M_1 when ^{14}C-GM_1 was added. This indicates that aflatoxin GM_1 is probably derived from aflatoxin M_1 in the same way as aflatoxin G_1 is derived from aflatoxin B_1.

Likewise, the incorporation of significant labelling into aflatoxin M_{2a} occurred only when labelled aflatoxin M_1 was added to cultures of the mould growing in acid medium. This indicates that this dihydroxy aflatoxin, M_{2a}, is derived from aflatoxin M_1 and does not arise from aflatoxin B_1 via aflatoxin B_{2a}.

These experiments confirmed the earlier observation that the radioactivity in ^{14}C-aflatoxin B_{2a} was not incorporated into any of the other aflatoxins. On the other hand, the labelling in M_1 and GM_1 was incorporated into other aflatoxins. This seems to suggest that some selection process is at work in the re-entry of the labelled aflatoxins into the mycelium. Thus, the presence of a hydroxyl group on the C-2 atom appears to prevent re-entry into the mycelium, whereas its presence on the C-4 atom does not appear to have this effect.

The results obtained when aflatoxin ^{14}C-M_1 was added to the cultures support the deduction from the earlier experiments that M_1 acts as a precursor of both B_1 and G_1. Aflatoxin GM_1 however, appears to act as a precursor of G_1 only.

The absence of any significant incorporation of radioactivity into versicolorin C or sterigmatocystin, when labelled aflatoxin was added to cultures of the mutant strains, strongly suggests that the aflatoxins do not act as precursors to either of these metabolites, even in cultures of moulds that produce all three compounds.

The rapid incorporation of labelling into sterigmatocystin when ^{14}C-labelled asperfoxin was added to cultures of mutant strains indicates that asperfoxin can act as a precursor of sterigmatocystin.

None of the activity contained in versicolorin C was incorporated into the aflatoxins or other metabolites, and it would appear that versicolorin C does not act as a precursor to sterigmatocystin or the aflatoxins. However, this does not preclude the possibility that versicolorin A may act as a precursor of the aflatoxins, and recent reports on the presence of hydroxyanthraquinones in *A. parasiticus*[14,15] appear to hold promise for further investigations in this field.

Summary

The technique of feeding labelled aflatoxins and related metabolites to cultures of aflatoxin-producing moulds has thrown fresh light on the relationships between these compounds.

Aflatoxin B_1 may be converted into most of the other aflatoxins such as B_2, B_{2a}, G_1, G_2 and G_{2a}. However, none of the aflatoxins of the B or G series appears to be converted to aflatoxin M_1.

The results presented in this paper show that the later stages of biosynthesis of the aflatoxins most probably follow the general order: Aflatoxin $M_1 \rightarrow$ Aflatoxin $B_1 \rightarrow$ Aflatoxin G_1.

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Table 1 Interconversion of aflatoxins by cultures of *A. flavus* (Strain 91019b). Specific activity (count/min/μmol) of aflatoxins recovered

U-^{14}C aflatoxin (count/min added)	From culture fluid							From mycelium (total fraction)
	B_1	B_2	G_1	G_2	M_1	B_{2a}	G_{2a}	
B_1 163000	3763 ± 3	2992 ± 8	4093 ± 5	2871 ± 8	559	4344	1197	3071
B_2 331000	1572	2035	210	0	0	310	69	2135
G_1 15800	919	563	5236	1290	291	312	8052	1916
G_2 14030	50	0	308	28191	0	0	856	2536
M_1 7746	1819	101	17	0	3717	41	17	1461
B_{2a} 37080	0	0	0	0	120	3407	79	91
G_{2a} 43000	0	0	0	0	247	82	41375	0

(1 per cent w/v), and was dispensed in 50cm³ portions into 250cm³ conical flasks.

A known quantity of the U-^{14}C-labelled aflatoxin under test was added to each of these flasks, the amount added depending on the specific activity of the compound. The flasks were then incubated with discs cut from a colony of *A. flavus* CMI 91019b growing actively on potato-dextrose-agar, until it was judged that a maximum concentration of aflatoxins had been produced. In the later experiments involving mutants (Table 3), a spore suspension was used to inoculate the GAS medium, the latter being dispensed in 60cm³ portions in the conical flasks.

The culture fluid was decanted from the mycelium, passed through a glass filter pad and neutralised with sodium bicarbonate to prevent the acid-catalysed production of O-alkyl derivatives of the aflatoxins. It was then extracted three times with an equal volume of alcohol-free chloroform. The combined extracts were taken to dryness *in vacuo* and purified by preparative t.l.c. In the early experiments (Tables 1 and 2), Kieselgel-G, Merck, was used as the thin layer support medium and chloroform-methanol (49:1, v/v) was the solvent system. In the later experiments described (Table 3), Mallincrodt TLC-7G was the support medium and chloroform-methanol was the solvent in the proportions of 49:1, v/v; 97:3, v/v; and 93:7, v/v.

Table 2 **Incorporation of radioactively-labelled aflatoxin B_1 into resting cultures of *A. flavus*. Specific activity (count/min/μmol) of aflatoxins recovered**

U-^{14}C aflatoxin B_1 (Count/min added)	B_1	B_2	G_1	G_2	M_1	B_{2a}	G_{2a}
163,000	5321	787	502	962	0	4980	543

Each individual mycelial mat was washed free of medium. It was then either dried in a vacuum desiccator over P_2O_5 or, in the later experiments,[10] freeze-dried. The dried mycelia were ground to a fine powder and extracted with warm chloroform (30°) under reduced pressure as described elsewhere.[10] Extracts were either evaporated to a small volume (1 cm³) or freeze-dried.

Aflatoxins and pigments were separated by preparative t.l.c. on Mallincrodt TLC-7G using chloroform-methanol and toluene-ethylacetate solvent systems. In these experiments the kojic acid in the culture fluid was also isolated in order to check whether the aflatoxins were being broken down into smaller units which could act as general carbon sources. The aflatoxins and other radioactive metabolites were assayed and their activity was determined by liquid scintillation counting (Heathcote *et al*[1]).

Replacement cultures

Two cultures of *A. flavus* CMI 91019b were prepared as described above and, after six days of incubation, just before maximum production of mycelium, the culture fluid was decanted. The mycelial mats were washed with sterile distilled water. A sample (50 cm³) of sterile (Seitz-filtered) resting culture medium containing U-^{14}C labelled aflatoxin B_1 was then carefully added, so that the mycelial mat floated on the surface of the medium. The composition of the resting medium was the same as that of the salts and glucose medium used for the actively growing cultures but the nitrogen source (ammonium sulphate) was omitted. The replacement cultures were incubated for three days and then harvested. Before extraction of the aflatoxins a suitable volume of a concentrate containing all the aflatoxins was added as a carrier to the replacement culture. The aflatoxins so obtained were then assayed and counted as before.

Results and discussion

The addition of labelled aflatoxins to cultures of *A. flavus* and the subsequent assessment of radioactivity produced in other aflatoxins would seem to be a useful method of studying their interconvertibility. Judging from the radioactivity found in the mycelium after the addition of such labelled aflatoxins, some of these compounds are able to re-enter the fungal cells from the medium and to expose themselves to any modifying processes occurring there as well as to those present in the culture fluid.

It will be seen from Table 1 that when aflatoxin M_1 was added to actively growing cultures it was converted predominantly by the mould into aflatoxin B_1 and, to a smaller

Table 3 **Conversion of ^{14}C-Labelled metabolites into related metabolites by cultures of *A. flavus*. Specific activity (count/min/μmol) of metabolites recovered**

U-^{14}C Metabolite Added	*A. flavus* strain	Count/min added	From culture fluid									From mycelium	
			Aflatoxin										
			B_1	B_2	G_1	M_1	GM_1	B_{2a}	M_{2a}	Aspertoxin	Sterigmatocystin	Total	Versicolorin C
Aflatoxin B_1	(91019b)	109300	4571	3857	2000	437	452	7305	—	—	0	463	57
Aflatoxin B_1	(91019b$_I$)	109300	4101	3404	4115	744	1666	6851	—	0	370	601	113
Aflatoxin B_1	(91019b$_{II}$)	109300	4767	2010	2770	362	948	6001	—	180	461	606	44
Aflatoxin M_1	,,	42400	1268	—	107	51990	58882	—	36000	1148	—	1937	37
Aflatoxin GM_1	,,	41680	13	—	1294	654	29500	—	130	1000	—	764	11
Aflatoxin B_{2a}	,,	48400	0	0	0	8	57	7211	0	0	—	25	—
Aspertoxin	,,	19000	41	—	23	142	56	—	—	14117	43973	814	63
Versicolorin C	,,	104800	0	0	0	0	0	0	0	0	0	755	806

Separatum EXPERIENTIA 34, 22 (1978)
Birkhäuser Verlag, Basel (Schweiz)

The use of fungal protoplasts in the study of aflatoxin biosynthesis

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Summary. Protoplasts derived from *Aspergillus flavus* are shown to be capable of synthesizing aflatoxins when incubated in a chemically defined medium. ^{14}C-Acetate and ^{14}C-Versicolorin A, added to protoplasts from 3-day-old mycelium, are incorporated into aflatoxin B_1.

One great difficulty in the study of fungal metabolism is the presence of a tough cell wall which prevents easy access to the cytoplasmic content and hence to the enzymes that it contains. However, some success using mechanical disruption of mycelium has been achieved in enzymatic studies of fungal secondary metabolism[1,2]. During our studies on aflatoxin biosynthesis we have attempted to produce active enzyme preparations from *Aspergillus flavus*, using a variety of disruptive methods, with varying degrees of success. Several negative results which we have obtained have been attributed to denaturing effects during the disruptive procedure. However, one method which does not depend on mechanical disruption is the removal of the cell wall by enzymatic digestion, resulting in the formation of protoplasts[3,4]. These are much more easily disrupted than intact mycelium and hence are more likely to yield active enzyme preparations. Protoplasts are currently being used to investigate fungal organelles[5], cell wall synthesis[6] and antibiotic production[7]. Thus we have studied the potential of protoplasts, derived from toxin-producing strains of *A. flavus*, for converting possible intermediates to the aflatoxins as a prerequisite to the study of isolated cell-free enzymes.

Table 1. Production of aflatoxin in Reddy's chemically defined medium by protoplasts from mycelium of *Aspergillus flavus* N1 of different ages

Age of mycelium (days)	Aflatoxin (μg) formed per 2 ml suspension after: 0 h	1 h	2 h	3 h	18 h
1	4.96	5.46	5.55	5.10	8.08
2	9.92	11.84	14.53	14.89	14.96
3	5.60	5.60	8.08	9.50	13.75
4	3.20	2.00	0.50	0.50	2.70
5	7.10	5.30	1.80	1.20	2.10

Materials and methods. The lytic enzyme was prepared from *Trichoderma viride* CBS 354-33 (kindly supplied by Dr J. Peberdy, University of Nottingham) using the method of Peberdy and Issacs[8]. The growth medium contained 5.0 g *Aspergillus flavus* mycelium dry weight per litre in place of the polysaccharide used by Peberdy and Issacs. *Trichoderma viride* was maintained on the growth medium containing 2% agar.

Aspergillus flavus N1 (a single spore isolate of CMI 91019b) was maintained on potato dextrose agar. A spore suspension was prepared in 0.1% sodium lauryl sulphate from a 14-day-old culture. A 250 ml conical flask containing 50 ml Reddy's[9] chemically defined medium was inoculated with 1 ml of spore suspension (12×10^6 spores) and incubated at 25°C for 3 days on a rotary incubator (100 rev/min). The mycelium was harvested and washed with buffer-stabilizer (0.4 M $MgSO_4$ in 0.2 M phosphate

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Table 2. Incorporation of U-^{14}C-acetate and G-^{14}C-versicolorin A into aflatoxin B_1 by protoplasts derived from 3-day-old mycelium, suspended in Reddy's chemically defined medium minus sucrose incubated for 3 h

Precursor Amount added			Product aflatoxin B_1 Amount formed		
	μCi	Specific activity (mCi/mole)	μCi	Specific activity (mCi/mole)	Percent conversion*
(U)-^{14}C-Acetate	0.21	234.2	0.0006	34.7	0.28
(G)-^{14}C-Versicolorin A	0.0084	28.6	0.0013	21.2	15.5

* $\frac{\mu Ci\ product}{\mu Ci\ precursor} \times 100$.

buffer, pH 5.8) then suspended in the lytic enzyme preparation (200 mg mycelium/ml lytic enzyme) and an equal volume of buffer-stabilizer was then added. The digest was incubated for 3 h at 25 °C in a rotary incubator (100 rev/min). Protoplasts were then isolated using the method of Peberdy[4]. Versicolorin A was isolated from *Aspergillus parasiticus* 1-11-105 Whl (kindly supplied by Dr J. Bennett, Tulane University, Louisiana) using the method described by Lee et al.[10]. ^{14}C-Versicolorin A was prepared using the method of Lee et al.[11]. 'Versiconal Acetate' and Versicolorin C were obtained from cultures of *A. flavus* N1 treated with dichlorvos[12]. Sterigmatocystin was kindly supplied by Dr J. S. Holker of the University of Liverpool.

All potential substrates were dissolved in N,N, dimethylformamide (Analar) and added as solutions (total of 100 µg substrate) to suspensions of protoplasts in buffer-stabilizer. When incubated in Reddy's medium, 1 ml of protoplast suspension (680 nm; OD = 0.26 = 32 mg protoplasts) was added to 9 ml of Reddy's medium. All experiments were incubated at 25 °C, 2 ml portions of the suspension were removed at various time intervals. To act as a control experiment, protoplasts were prepared from *A. flavus* mycelium and placed in buffer-stabilizer without the addition of a carbon source. The aflatoxins were extracted from the suspension with chloroform and estimated spectrophotometrically by measuring their extinction at 363 nm (Nabney and Nesbitt[13]). The aflatoxins were then estimated visually on thin layer chromatograms using toluene: ethyl acetate:acetone: glacial acetic acid (60:25:15:2, v/v) as the solvent system. ^{14}C-Versicolorin A and ^{14}C-Aflatoxin B_1 were re-chromatographed until their specific activity was constant before counting the samples in a liquid scintillation counter (Packard, Tricarb Model 3300).

Table 3. Conversion of added compounds to aflatoxins using protoplasts derived from 3-day-old mycelium, suspended in buffer-stabilizer

Precursor added (100 µg/10 ml)	Aflatoxin (µg) formed per 2 ml suspension after: 1 h	18 h
Versicolorin A	3.60	13.00
Sterigmatocystin	5.67	9.57
Versicolorin C	4.00	—*
'Versiconal acetate'	3.70	—*
No precursor	< 0.05	< 0.05

* No reading taken.

Results and discussion. Reddy's medium has been shown to stimulate aflatoxin biosynthesis in *A. flavus* N1 cultures, therefore, it was used as a suitable chemically defined medium in the investigation of aflatoxin biosynthesis by protoplasts. In order to optimize aflatoxin formation, protoplasts from mycelium of different ages were investigated (table 1).

Protoplasts from 1-day-old mycelium seem to have little aflatoxin producing capability over the first few h but they do acquire the ability to form aflatoxins during the 18 h period. Protoplasts from 4- and 5-day-old mycelium appear to degrade aflatoxin over the initial 2 h period of incubation; this result has been observed on a number of occasions with cultures of *A. flavus*[14], which show a decrease in aflatoxin concentration with increasing age of mycelium. It was noted that aflatoxins were already present in the protoplast preparations showing that they had been synthesized by the mycelial stage and not all of them secreted before and during protoplast formation.

One difficulty with interpreting these results is that as protoplasts are 'naked' mycelium, deviod of a cell wall, they will probably revert to primary metabolism or resynthesize cell wall material when placed in Reddy's medium. This seems to be reflected in the results of the 2- and 3-day-old material; where aflatoxin biosynthesis occurs most rapidly over the first few h but slows down during the subsequent 18-h-period, certainly, after 18-h mycelial cell walls are discernable in all protoplast preparations.

Another difficulty is that the aflatoxin biosynthesis observed is the result of a number of enzyme activities. These activities may be divided into 2 main phases, a) presumably the formation of an anthraquinone precursor via a polyketide synthetase and b) a cleavage phase involving at least 4 ring-cleaving steps involving enzymes having different substrate specificities[15]. It seems likely that the latter enzymes are induced in response to the appearance of an anthraquinone precursor which in the light of current evidence is probably averufin[16], or a closely related compound[17].

It is, however, clear that protoplasts derived from mycelium of different ages are capable of de novo aflatoxin biosynthesis, this being confirmed by the conversion of labelled ^{14}C acetate to aflatoxin (table 2) and it therefore follows that they must contain the total complement of enzymes required for aflatoxin biosynthesis.

In order to investigate some of these enzyme activities, a number of proven and possible intermediates in aflatoxin biosynthsis were added to the protoplast preparations. Several of the intermediates were converted to aflatoxin while controls containing no added compound did not produce aflatoxin during the same period (table 3). It was observed that protoplasts rapidly take up anthraquinone from the stabilizer-buffer solution as they became stained a yellow-orange colour in the presence of anthraquinone precursors with a corresponding loss of colour from the solution. It was shown that penetration of the membrane had occurred by adding versicolorin A to a suspension of protoplasts which were then centrifuged. The resultant protoplast pellet was washed with buffer-stabilizer, lysed by freezing and thawing in buffer (pH 5.8), and the membrane fraction was centrifuged down; 35% of the versicolorin A that had been added was present in the supernatant fraction indicating that it may pass into the protoplasm.

Work in this laboratory is currently being carried out with lysed protoplasts and preliminary experiments show that they are suitable for preparing cell-free extracts of *A. flavus* capable of carrying out several of the steps involved in aflatoxin biosynthesis.

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Separatum EXPERIENTIA 35, 21 (1979)
Birkhäuser Verlag, Basel (Schweiz)

The use of cell free extracts derived from fungal protoplasts in the study of aflatoxin biosynthesis

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Summary. A supernatant fraction derived from protoplasts of *Aspergillus flavus* was shown to be capable of converting both sterigmatocystin and versiconal hemiacetal acetate to aflatoxin B_1. Versicolorin A was not converted under the same conditions.

The isolation and characterization of the individual enzymes involved in the biosynthesis of aflatoxins is a difficult undertaking as the liberation of these labile enzymes by mechanical means can result in their denaturization[1,2]. In order to overcome this difficulty the technique of digesting the cell wall has been employed, using lytic enzymes derived from *Trichoderma viride*, resulting in the formation of fungal protoplasts[3]. These protoplasts have already been shown to be capable of synthesizing aflatoxins[4], hence the results reported here describe the isolation of a cell-free extract from lysed *Aspergillus flavus* protoplasts capable of converting ^{14}C versiconal hemiacetal acetate to aflatoxin B_1.

Materials and methods. Protoplasts were isolated from 3- and 4-day-old *Aspergillus flavus* mycelium as previously described[4] and collected by centrifugation at 500 × g for 10 min. The pellet was shaken with 5 ml 0.1 M phosphate buffer, pH 8.0 and frozen for 30 min at 0 °C. The resulting slurry was then thawed and centrifuged (10,000 × g for 30 min) to yield a supernatant fraction which was utilized as the cell-free extract, and a residue fraction. Protein was estimated using the Biuret method (1 ml of extract). The remaining extract (4 ml) was added to a cofactor medium[6] (1 ml) to give a final concentration of FAD (10^{-6} M), EDTA (10^{-3} M), methionine (10^{-3} M), dithiothreitol (10^{-3} M), NADPH (1 µmole), NADH (1 µmole) and the labelled substrate dissolved in NN-dimethylformamide. Labelled substrates were prepared after the method of Yao and Hsieh[7]. The 'cell-free' extract was incubated in a standard Warburg flask at 30 °C and shaken constantly. At

Conversion of added compounds to aflatoxin B_1 by a supernatant fraction isolated from lysed protoplasts of *Aspergillus flavus**

	Substrate added**	Incubation period (h)	Aflatoxin B_1 formed µCi recovered	Specific activity (mCi/mole)	Percent conversion**
A	(G)^{14}C Versiconal hemiacetal acetate	1	0.0000072	0.9	3.6
		18	0.0000192	2.0	9.6
B	(G)^{14}C Versiconal hemiacetal acetate	18	0.0000002	0.02	0.1
C	(G)^{14}C Versiconal hemiacetal acetate	1	0.0000153	1.9	7.7
	(G)^{3}H Sterigmatocystin	1	0.000056	ND	28.0
	(G)^{14}C Versicolorin A	1	zero	-	-
		18	zero	-	-

* All results are an average of essentially reproducible duplicate experiments. ** 0.0002 µCi of sp. act. 3.8 mCi/mole were added in eac experiment. *** $\frac{\mu Ci \text{ product formed}}{\mu Ci \text{ precursor added}} \times 100$. A, Fraction derived from protoplasts of 3-day-old mycelium; B, residue fraction derive from protoplasts of 3-day-old mycelium; C, fraction derived from protoplasts of 4-day-old mycelium. ND, not determined.

various time intervals portions of the incubation mixture were removed and the aflatoxin B_1 was extracted and estimated[8] and counted as described[4]. The added precursor was also isolated and measured in a like manner. Aflatoxin B_1 was isolated from zero time samples in order to ascertain its initial concentration and activity. These results were subtracted from subsequent values in order to allow for physical association of substrate with product and for aflatoxin B_1 not synthesized de novo.

Results and discussion. The table shows that the supernatant fraction of lysed protoplasts plus added cofactors is capable of converting versiconal hemiacetal acetate and sterigmatocystin to aflatoxin B_1. This result was supported by a commensurate loss of precursor. The much higher conversion of sterigmatocystin clearly reflects its close proximity to aflatoxin B_1 in the metabolic pathway.

As the residue fraction, which contains mitochondria and cell membranes, was not capable of such conversions it would seem that the enzymes involved in this section of the biosynthesis pathway are present in the microsomal fraction isolated from the lysed protoplasts. Hsieh and Matales[5] have shown that aflatoxins are acetate derived and probably synthesized extramitochondrially. Singh and Hsieh[1] demonstrated the conversion of ^{14}C sterigmatocystin to ^{14}C aflatoxin B_1 in the post-mitochondrial fraction of a cell-free extract derived from *A. parasiticus* ATCC 15517, they also suggest the involvement of an oxygenase in the conversion of sterigmatocystin to aflatoxin B_1. In our preparation, removal of the FAD from the incubation mixture results in an essentially zero incorporation of ^{14}C into aflatoxin B_1 indicating the presence of oxygenase(s).

It is possible that low conversion rates observed in our experiments may be due to the absence of an NADPH/NADH regenerating system. 'Cell-free' extracts of protoplasts derived from 4-day-old mycelium appear to be able to convert more substrate to aflatoxin B_1 than the 'cell-free' extracts of 3-day-old mycelium protoplasts. This may indicate a greater abundance of enzymes involved in substrate conversion in the 4-day-old material.

It was not possible to demonstrate the conversion of versicolorin A to aflatoxin B_1 even though the experiment was repeated a number of times. This result was unexpected as versicolorin A is a well-documented precursor of aflatoxin B_1[9] and because intact protoplast are capable of its conversion to aflatoxin B_1[4] it seems likely that some activating factor, present in the residue fraction, is required for its conversion. Work is currently in progress in order to clarify this point.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1980, p. 706-709 Vol. 40, No. 4
0099-2240/80/10-0706/04$02.00/0

Biosynthesis of Versicolorin A

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The incorporation of various potential intermediates into versicolorin A by a versicolorin A-accumulating mutant of *Aspergillus parasiticus* was studied. Both whole mycelium and cell-free extracts of this mutant were able to convert ^{14}C-labeled versiconal hemiacetal acetate to versicolorin A. By the use of a labeled double substrate technique it was shown that two other compounds, versicolorin A hemiacetal and its acetate derivative, were also converted to versicolorin A. It is concluded that one or both of these compounds are intermediates in the biosynthesis of versicolorin A and therefore may possibly be involved in the biogenesis of the aflatoxins.

The role of versicolorin A (VA) as a key intermediate in the biosynthesis of aflatoxin B_1 (compound III) is now generally accepted. There is a sufficient body of evidence to support this conclusion, including the conversion of VA to aflatoxin B_1 by whole mycelium (8), protoplasts (3), and nuclear magnetic resonance studies of ^{13}C-enriched material (6). The accumulation of VA in the mutant *Aspergillus parasiticus* (1-11-105 Wh1) is also suggestive of its role in aflatoxin biosynthesis (9).

However, one result which is not in agreement with this large volume of evidence is that obtained by utilizing cell-free preparations derived from protoplasts of *Aspergillus flavus* (1). Although the closely related anthraquinone versiconal hemiacetal acetate (VHA) was converted to aflatoxin B_1 (16% conversion), no incorporation of VA was observed. Thus, this particular area in aflatoxin B_1 biosynthesis is rather obscure, although it has been demonstrated that VHA is a precursor of VA (14). The work reported here investigates the role of VHA and attempts to identify other related intermediates involved in the biosynthesis of VA.

MATERIALS AND METHODS

Preparation of labeled metabolites. Labeled precursors were prepared as per the reference cited. Purity of the material was ensured by repeated preparative chromatography, which involved the use of two different solvent systems. This was followed by a recrystallization step. The amount of material present was determined by weighing, and this was checked from the extinction coefficient at a suitable absorbance maximum. The activity of the material was found by dissolving a known quantity in scintillation fluid in a scintillation vial and counting as outlined below. Identity of the material was established by comparison with authentic material both by mass spectrometry and thin-layer chromatography.

VHA (I). [^3H]- and [^{14}C]VHA were prepared from sodium [^3H]- and [^{14}C]acetate utilizing a culture of *A. flavus* (N1) treated with dichlorvos (15). The solvent systems used were toluene-ethyl acetate-acetone-acetic acid (50:35:15:2) and chloroform-acetone (9:1) on Silica Gel G60 layers. R_f values were 0.73 and 0.22, respectively; λ_{max} was 450 nm; Σ was 7,400.

VA (II). [^3H]- and [^{14}C]VA were prepared from sodium [^3H]- and [^{14}C]acetate with *A. parasiticus* (1-11-105 Wh1) (8). The solvent systems used were toluene-ethyl acetate (7:1) and chloroform-acetone, both with Silica Gel G60 layers and R_f values of 0.70 in both cases; λ_{max} was 453 nm; Σ was 8.166.

VC. ^{14}C-labeled versicolorin C (VC) was conveniently prepared by acid hydrolysis of [^{14}C]VHA (12). [^{14}C]VHA was treated with an excess of 2 M sulfuric acid containing acetone (20%, vol/vol). The mixture was refluxed for 8 h, cooled, and extracted with portions of ethyl acetate until no further pigment passed into the organic layer. The ethyl acetate was removed under reduced pressure, and the [^{14}C]VC was separated chromatographically by three runs in the toluene-ethyl acetate system (R_f 0.68) and one run in the chloroform-acetone system (R_f 0.64). The product was recrystallized from acetone; λ_{max} was 456; Σ was 6,100.

VAOH (IVA). ^3H- and ^{14}C-labeled versicolorin A hemiacetal (VAOH) were prepared from [^3H]- and [^{14}C]VA by treating with an excess of cold 2 M sulfuric acid containing acetone (20%, vol/vol) with stirring for 18 h. The reaction mixture was extracted with ethyl acetate and separated until chromatographically pure by three runs in the toluene-ethyl acetate system (R_f 0.60) and one run in the chloroform-acetone system (R_f 0.45). The chromatographic properties of this derivative were exactly the same as those of a nonactive sample prepared in the same manner, which had UV and mass spectral properties identical to those reported by Chen et al. for this compound (2); λ_{max} was 456; Σ was 6,100.

VAAC (IVB). ^3H- and ^{14}C-labeled versicolorin A hemiacetal acetate (VAAC) were prepared from [^3H]- and [^{14}C]VA by adding excess glacial acetic acid and a few drops of thionyl chloride and allowing to stand at room temperature for 24 h. The reaction mixture was extracted with ethyl acetate and separated chromatographically until pure by the same

systems as used for VAOH, with R_f values of 0.65 and 0.49 for the toluene-ethyl acetate and chloroform-acetone systems, respectively; λ_{max} was 459; Σ was 7,100. The reaction for the preparation of this derivative is analogous to that for preparing a similar acetoxy derivative of aflatoxin B_1 (5). The mass spectrum of this compound was consistent with its proposed structure (IVB).

Whole-cell experiments. *A. parasiticus* (1-11-105 Wh1) mycelium, incubated in Reddy synthetic low-salts medium (11) for 48 h, was filtered and suspended in replacement medium (70 ml) (7). Labeled substrate was added as an acetone solution (0.4 ml) and incubation was continued. At suitable intervals samples were removed and assayed.

Cell-free extracts. Protoplasts of *A. parasiticus* (1-11-105 Wh1) were prepared as previously described for *A. flavus* N1 (3). Protoplast suspensions in 0.2 M phosphate buffer containing 0.4 M $MgSO_4$, pH 5.8, were pelleted by centrifugation (500 × *g* for 10 min) and lysed by suspending the pellet in 5 ml of lysing buffer (0.1 M phosphate buffer, pH 8.0, at 4°C). This suspension was then homogenized in a hand-held ground glass homogenizer (3 min at 4°C). The lysate was centrifuged (10,000 × *g* for 30 min), and the supernatant was utilized as a cell-free extract. Protein was measured by the biuret method (protein concentration of 3 mg/ml). Cofactors were added to give a final concentration of flavin adenine dinucleotide (10^{-6} M), EDTA (10^{-3} M), dithiothreitol (10^{-3} M), NADPH (1 μmol/4 ml), NADH (1 μmol/4 ml) and substrate (1 μmol/4 ml). Radioactive substrates were added as solutions in *N,N*-dimethyl formamide. Cell-free extracts were incubated in standard Warburg flasks with KOH papers as a CO_2 trap at 30°C with constant shaking. Samples were removed at zero time; after 1 h of incubation, metabolites were extracted. Zero time counts (disintegrations per minute) of all metabolites were subtracted from 1-h counts to give actual counts recorded.

Assay of metabolites. The metabolites were extracted from samples (15 ml) of whole cultures by filtering off the mycelium and washing it in sequence with acetone (5 ml), chloroform (20 ml), and ethyl acetate (5 ml). The washings were shaken with the filtrate, and the organic layer was separated and dried over anhydrous sodium sulfate. The filtrate was extracted with a portion of ethyl acetate (10 ml), which was also dried and added to the washings. The solvent was removed under reduced pressure, and the extract was chromatographed on Silica Gel G60 with toluene-ethyl acetate-acetic acid (60:30:1) as the solvent system. R_f values for this system were: VA, 0.72; VC, 0.70; VHA, 0.52; VAOH, 0.55; and VAAC, 0.61. The thin-layer chromatograph plate was subjected to autoradiography (Kodirex X-ray plate), and the active spots were identified by developing the plate after 10 days of exposure. The silica containing the active metabolites was scraped from the plate, and the compound was washed off with acetone. The compounds were rechromatographed until a single band was observed with two to three runs of the toluene-ethyl acetate-acetic acid solvent system and one run with chloroform-acetone-acetic acid (85:15:1). The R_f values in the latter system were: VA, 0.72; VC, 0.70; VHA, 0.28; VAOH, 0.48; and VAAC, 0.55. Samples of the whole-cell cultures were taken at zero time and treated as above to act as controls for activity appearing in the various fractions which was not due to metabolic activity. The absorption spectra of the metabolites were recorded on a Pye Unicam SP 1800. The activities of the metabolites were determined on a Packard Tricarb 330 liquid scintillation counter; counts were corrected for background, efficiency, and quenching.

Samples (2 ml) from the cell-free systems were extracted with ethyl acetate (2 × 4 ml); the combined extract was dried over anhydrous sodium sulfate and then treated in a manner similar to that described above for the whole cell extracts. When ^{14}C and ^{3}H were counted in the presence of each other, background, efficiency, and quenching were estimated with an external standard.

Effect of dichlorvos on cell-free extracts. (i) Esterase activity. The esterase activity of cell-free extracts was determined by the method of Rahim and Sih (10). The assay was repeated in the presence of various concentrations of dichlorvos (5 to 50 μg/ml); the results are expressed as micromoles of *p*-nitrophenol released per milligram per minute.

(ii) Oxygen uptake. Cell-free extracts (4 ml) containing cofactors were placed in a Warburg flask containing KOH paper as a CO_2 trap. A solution of dichlorvos (0.2 ml) was placed in the side arm, giving a final concentration of 20 μg/ml when added to the main chamber. The flask was equilibrated at 25°C, and oxygen uptake was followed for 1 h. The dichlorvos solution was then added, and oxygen uptake was followed for another hour.

(iii) Inhibition of conversion of substrates. A cell-free extract containing the substrate under test was set up in a Warburg flask as described above, except that the mixture contained 20 μg of dichlorvos per ml. This mixture was incubated for 1 h, and the metabolites were extracted and counted as described above.

Incubation of VC with alcohol dehydrogenase. A solution of alcohol dehydrogenase (Sigma Chemical Co.) was prepared in phosphate buffer (0.1 M, pH 6.0) together with NAD^+ (0.2 μmol/ml). VC was added in *N,N*-dimethyl formamide (0.1 ml) to give a final concentration of 0.5 mg/ml. The mixture (4 ml) was incubated at 25°C for 10 h and then extracted and examined for various metabolites.

RESULTS AND DISCUSSION

The conversion of [^{14}C]VHA to VA by whole cells (Table 1) and cell-free extracts (Table 2) of *A. parasiticus* confirms the results of other workers (13) (Fig. 1). It is evident from the structures of these compounds that the conversion is not a single step, as treatment of VHA with dilute acid (12) has been shown to yield VC; thus, an oxidative step is required to produce VA. As the conversion rates of [^{14}C]VC and [^{14}C]VHA by cell-free preparations were similar, it is possible that VC is a metabolic intermediate between VHA and VA and that the step VC to VA is rate limiting. A possible mechanism for

TABLE 1. *Incorporation of ^{14}C-labeled VHA, VAOH, and VAAC into VA by whole mycelium of the VA-accumulating mutant A. parasiticus (1-11-105 Wh1)*

^{14}C-labeled precursor[a]	Incubation period (h)	Isolated VA	
		Radioactivity (dpm)	Incorporation efficiency (%)[b]
VHA	6	585	11.7
	24	755	15.1
	48	705	14.1
VAOH	48	950	19.0
VAAC	48	940	18.8

[a] Concentration and activity of ^{14}C-labeled precursors: VHA, 4.08 μmol, 5,000 dpm; VAOH, 3.4 μmol, 5,000 dpm; VAAC, 1.3 μmol, 5,000 dpm.

[b] (Microcuries of product)/(microcuries of precursor) × 100.

TABLE 2. *Incorporation of ^{14}C-labeled VHA, VC, VAOH, and [^{3}H]VAAC into VA by a cell-free extract derived from the VA-accumulating mutant A. parasiticus (1-11-105 Wh1)*

Labeled precursor[a]	Precursor activity (added dpm)	Isolated VA[b]	
		Radioactivity (dpm)	Incorporation efficiency (%)[c]
[^{14}C]VHA	5,500	340	6
[^{14}C]VC	4,500	270	6
[^{14}C]VAOH[d]	5,000	765	15
[^{3}H]VAAC[d]	8,500	1,310	13
[^{14}C]VHA[e]	5,500	0	0
[^{14}C]VAOH[e]	7,800	1,248	16
[^{3}H]VAAC[e]	6,900	1,242	18

[a] Concentration of labeled precursor added, 1 μmol/4 ml.

[b] Incubation period, 1 h in each case.

[c] (Microcuries of product)/(microcuries of precursor) × 100.

[d] Labeled substrates added in the presence of each other.

[e] Dichlorvos (20 μg/ml) added to the incubation mixture.

such a reaction is that the ring open form of VC behaves like an alcohol and is converted to an aldehyde by the action of an alcohol dehydrogenase "type" enzyme. An alternative possibility is that the terminal section of the bisdihydrofuran ring system is hydroxylated in some manner by means of an oxygenase. To verify which mechanism occurs, if either, several experiments were carried out.

When VC was incubated with alcohol dehydrogenase there was no observable action; however, this may be due to other factors, such as enzyme specificity. Experiments have shown that dichlorvos inhibits aryl esterase activity (Table 3) which may suggest that the inhibitor blocks VA biosynthesis by preventing hydrolysis of the ester VHA. However, dichlorvos also prevents oxygen uptake by cell-free extracts, the rate being reduced from 0.75 to 0.15 μl of O_2/min, suggesting the inhibition of an oxygenase. Thus, it is possible that an oxygenase which may be involved in the conversion of VHA to VA is being inhibited.

A possible scheme is suggested in Fig. 2. An oxygenase introduces an hydroxyl adjacent to the ester oxygen atom, causing the formation of an unstable acylal intermediate (V) which can either eliminate water to form VAAC (IVB) or eliminate acetic acid to form VAOH (IVA). These could then eliminate acetic acid or water, respectively, to produce VA (II). This system may be regarded as a metabolic grid if the oxygenase is relatively specific and causes, by an

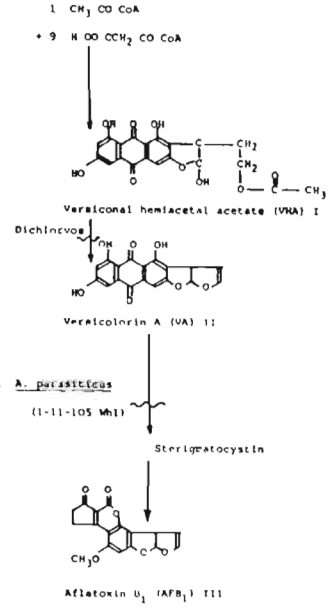

FIG. 1. *Partial scheme for the biosynthesis of aflatoxin B_1 (after Singh and Hsieh [13]).*

TABLE 3. *Effect of increasing concentrations of dichlorvos on the aryl esterase activity of cell-free extracts derived from A. parasiticus (1-11-105 Wh1)*[a]

Concn of dichlorvos added (μg ml^{-1})	*p*-Nitrophenol liberated (μmol mg of protein^{-1} min^{-1})
0.0 (control)	0.024
5.0	0.020
10.0	0.012
20.0	0.003
50.0	0.001

[a] Measured by the method of Rahim and Sih ().

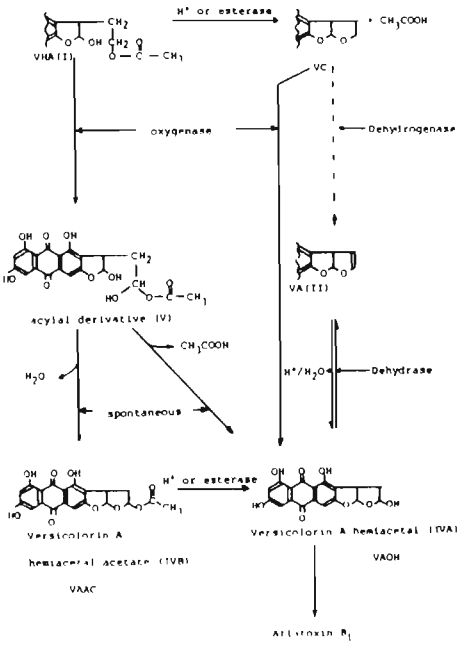

FIG. 2. *Proposed pathway for the conversion of VHA to VA.*

analogous reaction, the conversion of VC to VAOH, while esterase activity causes the hydrolysis of VAAC to VAOH.

For investigation of this possibility, both [^{3}H]VAAC and [^{14}C]VAOH were added to a cell-free extract, in which they were rapidly converted to VA at similar rates (Table 2); comparable results were also found for this conversion in whole mycelium (Table 1). The inability of dichlorvos to inhibit the conversion of [^{3}H]-VAAC and [^{14}C]VAOH to VA in cell-free extracts (Table 2) supports the view that both of these compounds can act as precursors to VA and that they are closer in this capacity than VHA or VC, although this does not necessarily mean that they are intermediary between VHA and VA. The lack of incorporation of VA into aflatoxins found in previous work with cell-free extracts (1) may have been due to difference in water solubility of VHA and VA. However, a scheme is suggested in Fig. 2 whereby VA becomes a side shunt metabolite and VAOH (or VAAC) becomes a direct precursor of VA and is intermediate in aflatoxin biosynthesis. However, VA is metabolically related in such a way as to account for the ^{13}C studies and may enter the mainstream again by addition to water to give VAOH, a process which readily occurs at the acid pH normally found in fungal cultures. Thus, the lack of incorporation of VA into aflatoxins in cell-free preparations (1) may be explained by their alkaline pH. Evidence for VAOH as an intermediate is supported by the observation that it accumulates in cultures of *A. parasiticus* (1-11-105 Wh1) even though the culture pH may be alkaline; hence, it probably arises enzymatically (4).

To clarify the proposed metabolic scheme outlined in Fig. 2, work in this laboratory is currently proceeding with cell-free extracts of an aflatoxin-producing strain of *A. flavus*. It is intended that study of the enzymes involved in VHA conversion will clarify the oxygenase or dehydrogenase (14) theory.

ACKNOWLEDGMENTS

We thank J. Bennett (Tulane University, New Orleans, La.) for a culture of *A. parasiticus* (1-11-105) and S. Allsop for technical assistance.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1982, p. 548–551 Vol. 43, No. 3
0099-2240/82/030548-04$02.00/0

Role of Versicolorin A and Its Derivatives in Aflatoxin Biosynthesis

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Received 12 May 1981/Accepted 29 September 1981

The involvement of various anthraquinone metabolites in the biosynthesis of aflatoxin B_1 was investigated by using a labeled double-substrate technique in a cell-free system. The results showed that both versicolorin A hemiacetal and versicolorin A hemiacetal acetate were converted to aflatoxin B_1, whereas versicolorin A was not, even though it was added to the same cell-free system. Thus, versicolorin A hemiacetal, versicolorin A hemiacetal acetate, or both were implicated as key intermediates, whereas versicolorin A and C became side shunt metabolites. These latter compounds reentered the pathway depending on the availability of the appropriate enzymes and suitability of conditions. Dichlorvos, a specific inhibitor of aflatoxin biosynthesis, is considered to have its primary action on either an oxygenase or dehydrogenase involved in the pathway and to act in a secondary capacity as an inhibitor of an esterase which may also be involved in the pathway.

The biosynthesis of the aflatoxins by *Aspergillus* species has been intensively studied over the last decade by techniques such as replacement cultures (3, 16) and cell-free extracts (15). The conversion of radioactively labeled precursors by these systems to aflatoxin B_1 (AFB_1) (VII) has provided evidence for the involvement of averufin, versiconal hemiacetal acetate (VHA) (I), versicolorin A (VA) (IV) (12), and sterigmatocystin (St) (VI) (10) in the biosynthesis of this important mycotoxin.

These observations are supported in part by the use of fungal protoplasts which have been shown to be capable of converting VHA and VA to AFB_1 (5). Further studies showed that an enzyme preparation, derived from lysed protoplasts, was capable of converting VHA and St to AFB_1, providing that the coenzyme flavin adenine dinucleotide was present (1). However, an unexpected result from this work was the lack of incorporation of VA into AFB_1 in spite of repeated efforts to effect this conversion under a variety of conditions. Recent studies have implicated versicolorin A hemiacetal (VAOH) (IIIA) and versicolorin A hemiacetal acetate (VAAC) (IIIB) as direct precursors of VA (2). It was concluded that either one or both of these compounds could be intermediates of aflatoxin biosynthesis, and thus VA becomes a side shunt metabolite. The work reported here attempts to resolve the role of VA in aflatoxin biosynthesis.

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MATERIALS AND METHODS

Preparation of labeled metabolites. The following radioactively labeled substrates were prepared and their activities were determined as previously described (2). [^{3}H]- and [^{14}C]VHA were prepared from cultures of *Aspergillus flavus* (NI) containing uniformly labeled sodium acetate and treated with dichlorvos. [^{3}H]- and [^{14}C]VA were prepared from cultures of *A. parasiticus* (1-11-105 Wh 1) containing uniformly labeled sodium acetate. [^{14}C]versicolorin C (VC) (II) was prepared by acid hydrolysis of [^{14}C]VHA. [^{3}H]- and [^{14}C]VAOH and -VAAC were prepared from labeled VA by treatment with 2 M sulfuric acid (for VAOH) and thionyl chloride-acetic acid (for VAAC).

[^{3}H]St was obtained from an St-producing strain of *A. nidulans* (M56). A solid medium, consisting of shredded wheat (10 g) wetted with distilled water (5 ml) dispensed into 250-ml conical flasks and sterilized, was inoculated with spores of *A. nidulans* (M56) and incubated as a static culture at 25°C. Once fungal growth was visible (2 to 3 days), the culture was sprayed with a sterile solution of [^{3}H]sodium acetate and incubated for a further 10 days. The culture was then washed into a Buchner funnel with warm acetone, and the acetone extract was filtered off by suction and evaporated to dryness in a rotary film evaporator. The residue was subjected to preparative thin-layer chromatography until a single band was obtained which fluoresced dark red under UV light. Two solvent systems were used: (i) chloroform, R_f = 0.7; and (ii) carbon tetrachloride-methanol (98:2), R_f = 0.45. The prepared St was found to cochromatograph with an authentic sample of St and to give a UV absorption spectrum identical with that of St.

Cell extracts. Protoplasts from *A. flavus* (NI) were prepared as previously described (5). The protoplasts were lysed, homogenized with a hand-held homogenizer, and centrifuged to yield a cell-free extract, the residue containing mitochondria, membranes, and oth-

er cellular debris (1). The cell-free extract was then incubated with various cofactors and radioactively labeled substrates in standard Warburg flasks; samples were removed at zero time and at suitable periods during incubation, after which metabolites were extracted and assayed (1).

Assay of metabolites. Samples from the cell-free system (2 ml) were extracted with ethyl acetate (2 × 4 ml), the combined extracts being dried over anhydrous sodium sulfate, evaporated, and subjected to thin-layer chromatography. Radioactive compounds were located by autoradiography and scraped from the thin-layer plate, and their specific activity was determined as previously described (2). The absorption spectra of the metabolites were recorded on a Pye Unicam S.P. 1800, and the activities were determined on a Packard Tri-Carb 3300 liquid scintillation counter. Counts were corrected for background efficiency and quenching by the standard channels ratio method; where ^{3}H and ^{14}C were counted in the presence of each other, an external standard was used. Zero time counts (disintegrations per minute) of all metabolites were subtracted from the results obtained for incubation times to give actual counts recorded.

Incubation of [^{14}C]VA with reconstituted total cell homogenate. The residue fraction, obtained by centrifugation of the protoplast homogenate, was partially solubilized by treatment with 5 ml of phosphate buffer (0.1 M, pH 8.0) containing 0.1% Triton X-100. This was then incubated with cofactors and [^{14}C]VA, plus an equal volume of the supernatant fraction. Samples were removed at zero time and after 3 h of incubation in a Warburg flask, after which they were extracted and assayed for metabolites.

RESULTS AND DISCUSSION

The results confirmed that protoplasts are a useful source of cell-free extracts capable of carrying out several steps in the biosynthesis of the aflatoxins. The disruption of protoplasts with a hand-held homogenizer produced a greater yield of active enzymes than the freeze-thaw method (1), and conversion rates compared favorably with those obtained by other workers (15), although the protein release (3 mg/ml) was not as high. Another advantage of this method is that it did not disrupt organelles, thus confirming the cytoplasmic origin of aflatoxin biosynthesis.

It was apparent that VA was not a direct intermediate involved in AFB_1 biosynthesis, as there was no appreciable incorporation of either ^{14}C- or ^{3}H-labeled VA into AFB_1. To prove that this lack of incorporation was not due to lack of enzyme activity, experiments utilizing labeled double substrates were carried out. In the first instance, [^{3}H]VHA was used as a reference substrate, as it has already been shown to be converted to AFB_1 (18) and also because it is produced as a result of the specific inhibition of a single step in AFB_1 biosynthesis (14). The result of the double-substrate experiment (Table 1) revealed that VA was not converted to AFB_1, but VHA underwent a conversion rate of 14% over a period of 1 h. The possibility of spurious results being obtained due to ^{3}H exchange was eliminated by reversing the labels on the substrates, whereupon similar conversion rates were found. On incubating [^{14}C]VA with the partially solubilized membrane fraction, with or without the addition of the supernatant fraction, there was no conversion of VA to AFB_1. This result indicated that the lack of conversion of VA to AFB_1 was not due to the absence of enzymes required for AFB_1 biosynthesis in the cell-free preparation, which are normally present in the membrane of other organelles, although it is possible that the solubilizing technique may not have released the active enzyme(s).

These results show that VA is not directly involved in AFB_1 biosynthesis, so an explanation has to be sought to accommodate the strong evidence which is contrary to this conclusion. The two views may be reconciled if it is assumed that VA is not in the direct pathway to AFB_1 biosynthesis but is closely related to an intermediate which is. Some credible possibilities for the identity of this hypothetical compound are: (i) an intermediate with the bisdihydrofurano system in an open-ring configuration, as typified by VHA, which would allow a specific binding point, e.g., an aldehyde group, to which the enzyme might attach itself; (ii) an anthraquinone related to VA, e.g., VAOH or VAAC; and (iii) a derivative of VA with a modification in the anthraquinone nucleus, e.g., aversin (4).

VAOH satisfies conditions (ii) and also (i), as hemiacetals of the bisdihydrofuran system under certain conditions can exist in an open-ring form (13). Furthermore, as both it and VAAC have already been implicated as precursors in VA biosynthesis (2), they were added in a labeled form to cell-free extracts. The results (Table 1) indicate that they are converted to AFB_1 at a rate marginally greater than that for VHA. This suggests that VAOH and VAAC may occur in the metabolic sequence between VHA and AFB_1.

To accommodate this conclusion, a number of schemes may be postulated. Evidence has been found, by implication, for the involvement of an alcohol dehydrogenase in the conversion of VC to VA (17). VHA is first hydrolyzed, presumably under the influence of an esterase, to VC, which in the open-ring form is dehydrogenated to an aldehyde. Ring closure of this product yields VAOH, which can dehydrate to VA (Fig. 1). A difficulty with this scheme is that at physiological pH the open-ring form of VC is likely to be only transient and therefore not very available for the dehydrogenase, although it seems reasonable to suppose that this reaction occurs in the conversion of versiconal to VC (8).

TABLE 1. Incorporation of radioactivity into AFB_1 from substrates added to cell-free extracts

Experiment[a]	Substrate and amt (dpm) added	AFB_1 recovered (dpm) at time:		% Incorporation[b]
		0	1 h	
1	[^{3}H]St, 5,000	70	1,470	28
2[c]	[^{3}H]VHA, 9,000	70	1,320	14
	[^{14}C]VA, 5,500	30	40	0.2
3[c]	[^{14}C]VHA, 5,500	50	900	15.5
	[^{3}H]VA, 8,000	70	80	0.2
4[c]	[^{3}H]VHA, 9,000	70	1,330	14
	[^{14}C]VC, 4,500	30	165	3
5[c,d]	[^{3}H]VAAC, 8,500	70	1,260	14
	[^{14}C]VHA, 5,500	20	570	10
6[c,e]	[^{14}C]VAAC, 5,000	30	930	18
	[^{3}H]VHA, 9,000	70	1,330	14
7[c,f]	[^{3}H]VAOH, 8,000	60	1,340	16
	[^{14}C]VHA, 5,500	30	800	14
8[c]	[^{3}H]VAAC, 8,500	70	1,430	16
	[^{14}C]VA, 5,500	30	43	0.2

[a] All results are an average of two experiments and never varied more than 1.5% from the conversion stated.
[b] Calculated: (microcuries of product/microcuries of precursor) × 100.
[c] Substrates added in the presence of each other.
[d] Conversion of VHA to VAAC/VAOH = 17%.
[e] Conversion of VHA to VAAC/VAOH = 12%.
[f] Conversion of VHA to VC = 10%.

A similar system is observed in the conversion of AFB_2 to AFB_1 (9), albeit in this case the hemiacetal, AFB_{2a}, is not implicated in the process, and it may well be that in both systems the dehydrogenase is of the alkyl type, which generates a double bond directly in the terminal furano ring system (Fig. 1). The pathway involving the alcohol dehydrogenase-type enzyme, however, fits much of the data derived from cell-free studies if the side shunt role of VA is accepted, it reentering the biosynthetic route to AFB_1 by conversion back to VAOH, a process occurring readily at acid pH values (Fig. 1).

A stumbling block to this solution is the observation that VC was not converted at the same rate as VHA to AFB_1 (Table 1). To overcome this difficulty, an alternative to the above-described route has been proposed (2) whereby an oxygenase (hydroxylase) generates an unstable acylal derivative (V) (Fig. 2). The acylal derivative can spontaneously form either VAOH or VAAC, depending on whether acetic acid or water is eliminated (Fig. 2). Although the precise hydroxylation placement is unprecedented, oxygenase activity has been demonstrated many times in the *Aspergillus* genus and related fungi (7). The observed slower conversion of VC to AFB_1 may be explained on the basis of a lower relative specificity of the oxygenase for VC compared with that of VHA.

It is certain that dichlorvos owes its action in blocking AFB_1 biosyntheses to its property of enzyme inhibition, which appears to be specific (6). It inhibits esterase activity in *A. flavus*, and there is also evidence that the oxygenase (2) or the (implied) dehydrogenase activities are blocked (17). Esterase inhibition may be the reason for the accumulation of VHA which

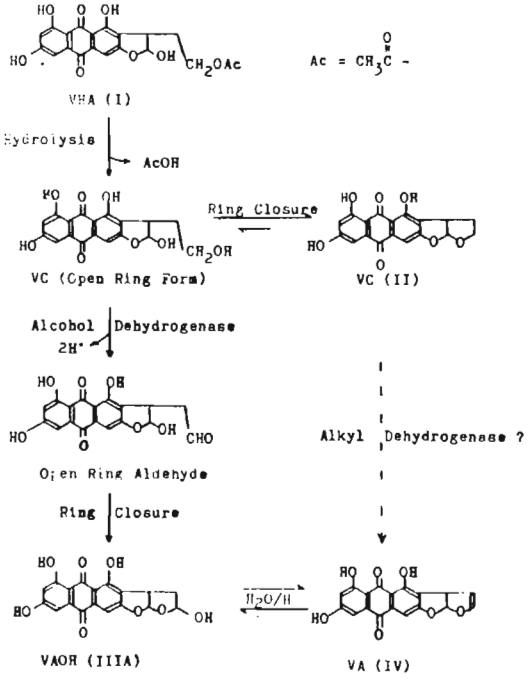

FIG. 1. Dehydrogenase-mediated conversion of VHA to VA (after Wan and Hsieh 1980 [17], →; alternative, - - - - - →).

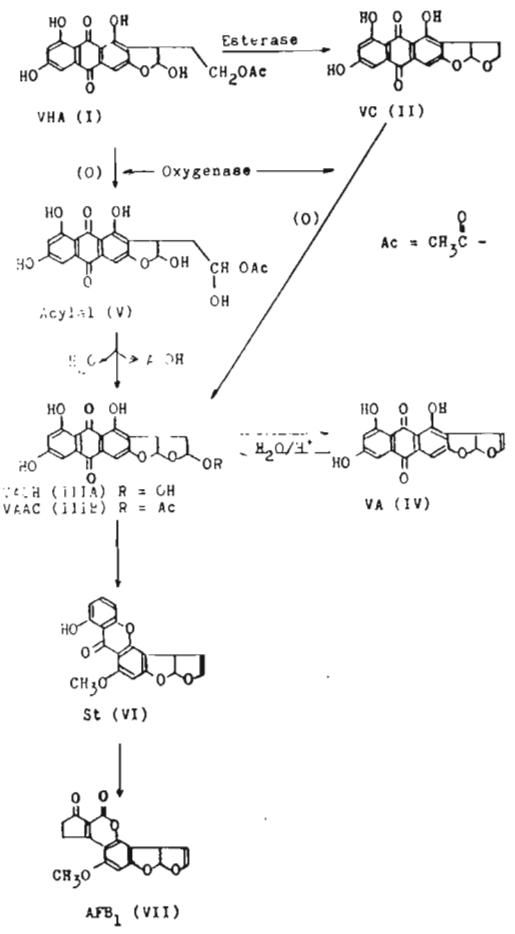

FIG. 2. Proposed pathway for the biosynthesis of AFB_1 involving an oxygenase.

prevents its hydrolysis to VC, although this does not seem to be the case, as VC does accumulate in dichlorvos-treated cultures at levels similar to those in untreated controls (3). These latter results do not clarify the situation any further, for in the oxygenase scheme both VHA and VC would tend to accumulate, assuming that the esterase is only partially inhibited, and in the alternative pathway it must be the dehydrogenase itself which is strongly inhibited with secondary inhibition of esterase activity to explain VHA accumulation.

It is quite clear from these deliberations that further investigations are required to discover the correct pathway and equally clear that this will be achieved only by enzymatic studies.

ACKNOWLEDGMENTS

We thank J. Bennett (Tulane University, New Orleans) for a culture of *A. parasiticus* (1-11-105 Whl), J. S. E. Holker (University of Liverpool) for a sample of sterigmatocystin, and A. Buckle (ADAS, M.A.F.F., Shardlow) for a culture of sterigmatocystin-producing strain of *A. nidulans* (M56).

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Vol. 116, No. 3, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 15, 1983 Pages 1114-1118

THE CONVERSION OF STERIGMATOCYSTIN TO O-METHYLSTERIGMATOCYSTIN AND AFLATOXIN B1 BY A CELL-FREE PREPARATION

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Received October 3, 1983

Summary: A cell-free system derived from a versicolorin A-accumulating mutant of Aspergillus parasiticus was found to convert sterigmatocystin to both O-methylsterigmatocystin and aflatoxin B1. It is suggested that the similarity in the chromatographic properties of these two metabolites has caused erroneous conclusions to be made with regards to the biosynthesis of aflatoxin B1.

Most of the intermediates in the biosynthetic pathway to aflatoxin B1 have been identified by the use of labelled putative precursors and whole mycelium of aflatoxin producing-strains of Aspergillus flavus and parasiticus (1). Some of the details of this pathway are still unclear and in other parts there is conflicting evidence (2) concerning the exact route. We think that the only way to resolve these difficulties is to demonstrate the in vitro enyzymatic promotion of each step, a criterion which was applied successfully to primary metabolic pathways.

Some progress has already been made with this approach and cell-free preparation have been used to investigate steps leading to versicolorin A (3) and from sterigmatocystin to aflatoxin B1 (4).

The role of sterigmatocystin in aflatoxin B1 biosynthesis is now uncertain, as it has been shown by a pulse labelling technique that aflatoxin B1 is produced prior to sterigmatocystin in the order of appearance of labelled products (5). It was concluded from this observation

Abbreviations:

CA-chloroform:acetone
TEA-toluene:ethyl acetate:acetone
CAP-chloroform:acetone:propan-2-ol
BA-benzene:acetic acid
BCA-benzene:cyclohexane:acetone
St-sterigmatocystin
Af-aflatoxin
Oms-O-methylsterigmatocystin

0006-291X/83 $1.50

that sterigmatocystin is not a true intermediate in aflatoxin biosynthesis but a side shunt metabolite.

In this report we present evidence from cell-free studies which show sterigmatocystin can be converted to both O-methylsterigmatocystin, which is a side shunt metabolite (6) and aflatoxin B1 and that this may give rise to confusion in biosynthetic studies made on aflatoxin.

Methods

An aflatoxin blocked mutant of Aspergillus parasiticus (1-11-105 Wh 1), which accumulated versicolorin A was maintained on potato dextrose agar. It was cultivated on Reddy's medium (7),70ml per 250ml conical flask, at 25°C in shake culture (150rpm). After 120hours the mycelial pellets were collected in cheese cloth and these were then washed well with 0,2M phosphate buffer, pH 7.

The washed mycelia was freeze dried over 48hours and then fragmented by gently grinding in a dry chilled pestle and mortar at 4°C. The powdered mycelium was suspended in 0.2M phosphate buffer pH7 (20ml/g mycelium) and the homogenate was centrifuged at 10000xg for 20 minutes at 4°C. The supernatant was removed, freeze dried and used as the cell-free preparation. Protein was determined by the Biuret method.

A reaction mixture was prepared by dissolving 50mg of the cell-free preparation in 20ml 0.2M phosphate buffer (2.5mg protein/ml), together with 10mMoles NADPH. Sterigmatocystin (10ug) dissolved in 10ul N,N dimethyl formamide was added as a substrate and the total reaction mixture was incubated for 18 hours at 20°C. The reaction was stopped by adding 20ml of chloroform-ethyl acetate 1:1v/v, which also extracted unused substrate and products from the reaction mixture. The extraction was repeated twice more with further 20ml aliquots of solvent. The total extract was then dried over anhydrous sodium sulphate, and evaporated to dryness with a stream of nitrogen and gentle heat. The residue was then dissolved in 100ul methanol.

Similar experiments were conducted containing the above reaction mixture with the following additions:

(i) ethionine 2mg,
(ii) S-adenosyl methionine 2mg,
(iii) boiled enzyme
(iv) without sterigmatocystin

All these were treated as for the original reaction mixture.

The extract (10ul) was spotted onto the origin of an aluminium backed silica gel G chromatoplate (Merck) 10 x 10cm and developed in the following solvent systems:

(CA) 9:1
(TEA) 60:25:15
(CAP) 85:15:2.5
(BA) 95:5
(BCA) 88:7:5

Aflatoxin was visualised under ultra-violet light. Sterimatocystin and its derivatives were was sprayed with aluminium chloride in ethanol (20% w/v), heated for a few minutes at 120°C and then viewed under ultra-violet light (8).

The quantification of the metabolites was achieved by h.p.l.c.. A sample of extract (10ul) was applied to a reverse phase C18 column and the constituent metabolites were separated using acetonitrile and water

TABLE 1. The Conversion of Sterigmatocystin to Aflatoxin B1 and O-methylsterigmatocystin by a Cell-free System Derived from a Versicolorin A-Accumulating Mutant of Apergillus parasiticus

Treatment	Metabolites Isolated		
	St	Af	Oms
No sterigmatocystin	- (Ø)	- (Ø)	-(Ø)
Boiled enzyme	+++(1ØØ)	-(Ø)	-(Ø)
Untreated	+(21)	++(54)	+(25)
Plus ethionine	-(Ø)	+++(8Ø)	+(8)
Plus S-adenosylmethionine(a)	+(29)	+(23)	++(48)

(a) = 1Øug St was added to the reaction mixture
(b) = -,+,++,+++ scoring for intensity on thin-layer chromatograph, (solvents: first system CA; second TEA). Figures in parenthesis mol % based on original concentration (1ØØ %) of Sterigmatocystin added as determined by h.p.l.c.

(55:45). The metabolites were detected and quantified at 325 nm. The products of the enzyme action were isolated by preparative t.l.c. using the CA and TEA systems until a single spot was obtained on two dimensional chromatography of the isolated material. This was subjected to mass spectrometry.

Results and Discussion

The results in TABLE 1 show that sterigmatocystin can be converted to two different products by the cell-free extract, i.e. aflatoxin B1 and O-Methylsterigmatocystin. The identity of these products was established by t.l.c. against standards (TABLE 2) and mass spectrometry (TABLE 3); the results from the mass spectra agreeing with literature values (9). That these products arise via enzyme action and were derived from the added sterigmatocystin was proved by the lack of their appearance in the boiled enzyme and nil-sterigmatocystin controls (TABLE 1). An interesting point

TABLE 2. Mass Spectral Data of the Isolated Products Derived from the Cell-Free System

Oms m/e	Af B1 m/e
338	312
323	284
3Ø9	269
292	256
279	241
265	227
249	199

TABLE 3. A Comparision of the Chromatographic Properties of Aflatoxin B1 and O-Methylsterigmatocystin

Solvent System	Rf values	
	Af B1	Oms
CA	0.52	0.51
TEA	0.36	0.27
CAP	0.88	0.88
BA	0.03	0.03
BCA	0.04	0.04

which arises from this is that O-methylsterigmatocystin has not been previously reported as arising in cell-free systems, although it has been isolated from whole cultures of Aspergillus flavus (6).

The addition of ethionine enhances the production of aflatoxin B1 and decreases that of O-methylsterigmatocystin (TABLE 1), whilst the reverse is true if S-adenosyl methionine is added. These results can be explained if it is assumed that there are two enzyme systems which compete for the substrate, sterigmatocystin. One is a cleavage enzyme, which results in aflatoxin B1 and the other is a methyl transferase giving rise to O-methylsterigmatocystin. Ethionine inhibits the methylation reaction, whereas S-adenosyl methionine promotes it.

As aflatoxin B1 and O-methylsterigmatocystin have similar fluorescence and chromatographic properties (TABLE 3) it seems highly likely that these two products have been confused in the past, particularly as sterigmatocystin is rapidly converted to O-methylsterigmatocystin both in this cell free system and by whole mycelium of wild-type Aspergillus flavus (10). This in turn could explain the discrepancy in the literature with regards to the involvement of sterigmatocystin and other precursors of aflatoxin B1 biosynthesis, whereby O-methylsterigmatocystin is mistaken for aflatoxin B1.

In order to avoid ambiguity, we recommend that thin layer chromatographs be be treated with the aluminium chloride spray reagents which give a bright yellow-green fluorescences with O-methylsterigmatocystin and other xanthones but not with the aflatoxins.

Acknowledgements

We are grateful to the University of Natal and the C.S.I.R South Africa for grants supporting M.S.Jeenah. We also wish to thank Dr. J. W. Bennett, Tulane University for the gift of a culture of the mutant Aspergillus parasiticus (1-11-105 Whl), Mr M. Watson for the mass spectrometry and Mr R.K. Berry for editing the manuscript.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1985, p. 1392–1395 Vol. 49, No. 6
0099-2240/85/061392-04$02.00/0

Biosynthetic Relationship among Aflatoxins B_1, B_2, M_1, and M_2

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Received 10 December 1984/Accepted 14 March 1985

Aflatoxins are a family of toxic, acetate-derived decaketides that arise biosynthetically through polyhydroxyanthraquinone intermediates. Most studies have assumed that aflatoxin B_1 is the biosynthetic precursor of the other aflatoxins. We used a strain of *Aspergillus flavus* which accumulates aflatoxin B_2 to investigate the later stages of aflatoxin biosynthesis. This strain produced aflatoxins B_2 and M_2 but no detectable aflatoxin B_1 when grown over 12 days in a low-salt, defined growth medium containing asparagine. Addition of dichlorvos to this growth medium inhibited aflatoxin production with concomitant accumulation of versiconal hemiacetal acetate. When mycelial pellets were grown for 24, 48, and 72 h in growth medium and then transferred to a replacement medium, only aflatoxins B_2 and M_2 were recovered after 96 h of incubation. Addition of sterigmatocystin to the replacement medium led to the recovery of higher levels of aflatoxins B_2 and M_2 than were detected in control cultures, as well as to the formation of aflatoxins B_1 and M_1 and *O*-methylsterigmatocystin. These results support the hypothesis that aflatoxins B_1 and B_2 can arise independently via a branched pathway.

The aflatoxins are a family of structurally related, highly toxic, and carcinogenic mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* (11, 23). Biosynthetically, the aflatoxins are acetate-derived decaketides that arise through polyhydroxyanthraquinone intermediates (2, 11, 18, 19). The major cyclopentenone ring-containing aflatoxins are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin M_1 (AFM_1), aflatoxin M_2 (AFM_2) (structures shown in Fig. 1). Of these, AFB_1 is the most toxic and is usually produced in the greatest quantity. Most biosynthetic studies have emphasized AFB_1 and assumed that the other aflatoxins are metabolically related to it by a direct interconversion process (2). Several workers have reported the conversion of labeled AFB_1 into AFB_2 and the G aflatoxins (10, 14), but more recent studies with blocked mutants of *A. parasiticus* suggest that the aflatoxins arise independently via a branched pathway (7; J. C. Floyd, unpublished data). The facts that certain strains of *A. flavus* can accumulate AFB_2 in excess of AFB_1 (9, 15, 17, 21) and that a derivative of dihydrosterigmatocystin (Fig. 1), rather than sterigmatocystin (ST; Fig. 1), has been shown to be a precursor of AFB_2 (6) support this view.

In this report, the relationship between the cyclopentenone ring-containing aflatoxins (AFB_1, AFB_2, AFM_1, and AFM_2) was studied by monitoring the metabolism of ST, AFB_1, and AFM_1 in an AFB_2-accumulating strain (SRRC 141) of *A. flavus*.

MATERIALS AND METHODS

Organisms and growth conditions. The AFB_2-accumulating *A. flavus* strain (ATCC 24109), originally isolated by Schroeder and Carlton (17), was provided by M. Klich, Southern Regional Research Center, New Orleans, La., where it is maintained as SRRC 141. Stock cultures were grown on potato glucose agar. The growth medium (GM) was developed by Reddy et al. (16), and the replacement medium (RM) was developed by Adye and Mateles (1). In most experiments, cultures were incubated in 70 ml of GM or RM in 250-ml Erlenmeyer flasks on a shaking incubator at 27°C and 150 rpm. For bulk extraction of metabolites, cultures were similarly incubated in 500 ml of GM in 2.5-liter Fernbach flasks for 7 days. Mycelia from GM were harvested by vacuum filtration and washed with sterile distilled water. In some experiments, 140 μl of dichlorvos (Shell Oil Co., Modesto, Calif.) in 0.4 ml of acetone was added to 70 ml of GM. Dry weights of mycelia were obtained by drying washed triplicate samples of mycelia at 50°C until constant weight was obtained.

In biosynthetic studies, 70 ml of RM was supplemented with ST (1 mg), AFB_1 (0.1 mg), or AFM_1 (0.1 mg) in 0.4 ml of acetone; RM with 0.4 ml of acetone alone was used for controls. Washed, harvested mycelial pellets (4 g [wet weight]) were obtained after 24, 48, and 72 h of growth in GM and then incubated an additional 1 to 96 h in supplemented or unsupplemented RM in duplicate.

Extractions. Mycelia from 7-day-old cultures in GM were harvested by vacuum filtration and washed with acetone until the washings became colorless. A 0.5 volume of water was added to the extract, and the aqueous acetone was extracted successively with two 0.5 volumes of hexane and then chloroform until the aqueous portion became colorless. The pooled chloroform extracts were dried over anhydrous sodium sulfate and reduced to about 25 ml on a rotary evaporator. The culture filtrate was extracted with 2 equal volumes of methylene chloride-acetone (9:1, vol/vol), and then the pooled extracts were dried over anhydrous sodium sulfate and reduced to about 5 ml in a rotary evaporator. Reduced extracts were purified by preparative thin-layer chromatography.

Mycelia from RM were harvested by vacuum filtration after appropriate intervals of incubation and washed with acetone (10 ml), chloroform (25 ml), and then acetone (5 ml) again. All washings were combined with the culture filtrate and then transferred to a separatory funnel. The organic layer was passed through a bed of anhydrous sodium sulfate into a rotary evaporation flask. The remaining aqueous layer was further extracted with two portions of chloroform (25 ml each), and these were run through the sodium sulfate bed. The total extract was evaporated over a rotary vacuum evaporator, and the residue was transferred in a small

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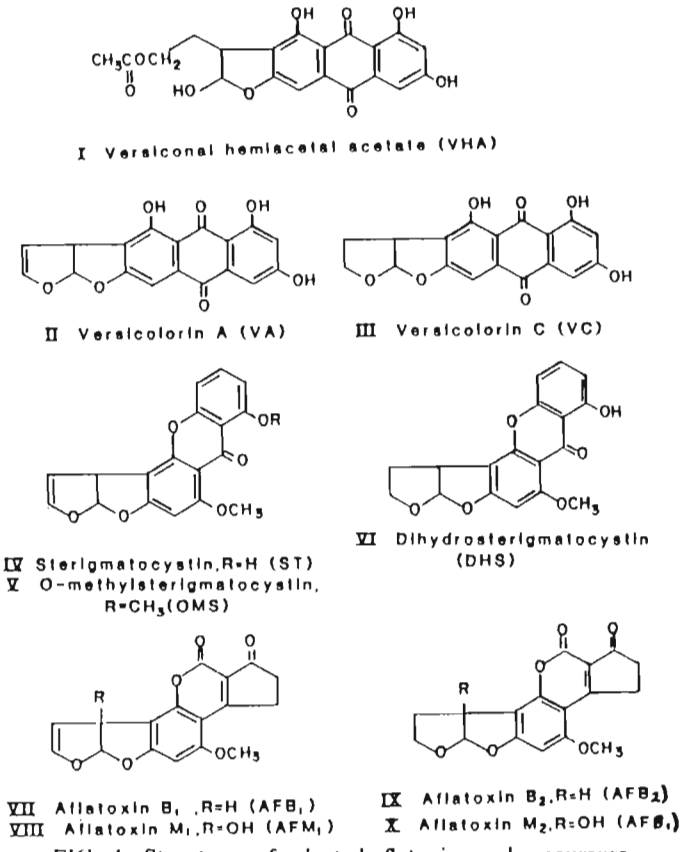

FIG. 1. Structures of selected aflatoxins and precursors.

amount of acetone to a 3.7-ml vial. The acetone was then evaporated under a stream of nitrogen, and the final residue was dissolved in 200 μl of a 1:1 chloroform-acetone mixture.

Qualitative assay of metabolites. Identities of metabolites were established by chromatographic behavior on thin-layer chromatographs and by mass spectrometry with a Finnegan 4000 instrument. When feasible, the metabolites were completely characterized by nuclear magnetic resonance spectroscopy (Varian CFT 20 spectroscope).

Aflatoxins, STs, and anthraquinones were separated by two-dimensional thin-layer chromatography. The extract (5 to 20 μl) was spotted onto aluminum-backed Silica Gel 60 plates (10 by 10 cm) (Merck 5388; Merck & Co., Inc., Rahway, N.J.). The solvent used in the first dimension was chloroform-acetone (85:15, vol/vol). For separation of aflatoxins and Sts, the second solvent was diethyl ether-methanol-water (96:3:1, vol/vol/vol). For the separation of anthraquinone pigments, the second solvent was toluene-ethyl acetate-acetone-acetic acid (60:25:15:2, vol/vol/vol/vol). After development, the plates were inspected under long-wavelength UV light, and the various fluorescent spots were marked. ST and its derivatives were detected by spraying the plates with 20% aluminum chloride in ethanol (vol/vol) and heating them for a few minutes in an oven at 120°C.

Quantitative assay of metabolites. After thin-layer chromatography in the appropriate solvent system, a visual estimate of the amount of metabolite was made, and this was used to estimate the appropriate amount of extract to spot for one-dimensional chromatography on prescored 250-μm-thick Silica Gel G plates (20 by 20 cm) (Analtech, Newark, Del.) for densitometry readings. For estimation of aflatoxins and *O*-methylstergmatocystin (OMS, Fig. 1), plates were developed in the diethyl ether-methanol-water system; for estimation of ST, they were developed in ethanol-carbon tetrachloride (2:98, vol/vol) and then sprayed with aluminum chloride (20). Plates were scanned for fluorescent materials by using a Schoeffel SD 3000 recording densitometer (Schoeffel Instruments, Westwood, N.J.) and excitation at 360 nm. The quantities of aflatoxins, ST, and OMS were calculated based on comparisons of areas of standards run on the same plate.

RESULTS

Under the culture conditions used, the high-AFB_2-producing strain of *A. flavus* produced no detectable AFB_1 or G aflatoxins over the course of 12 days in GM (Table 1). AFB_2 was the major aflatoxin produced. AFM_2 was also formed after 3 days of incubation, together with several orange pigments. The principal pigments detected were versicolorin C, averufin, and versiconol. When dichlorvos was added to GM at the beginning of the incubation period, versiconal hemiacetal acetate (VHA; Fig. 1) accumulated with concomitant blockage of aflatoxin production.

Data on aflatoxin production by cultures grown in GM for 24, 48, or 72 h and then transferred to RM with and without ST are shown in Table 2. Control cultures produced largely

TABLE 1. Mycelial dry weight and aflatoxin production by a high-AFB_2-accumulating strain of *A. flavus* grown on a low-salt–asparagine growth medium over 12 days

Days of incubation	Mycelial dry wt (g)	AFB_2 (nmol)		AFM_2 (nmol)	
		Mycelium	Culture fluid	Mycelium	Culture fluid
1	0.5	Tr[a]	Tr	ND[b]	ND
2	0.8	Tr	3	ND	ND
3	1.5	1	3	ND	Tr
4	2.0	2	9	Tr	Tr
5	1.2	5	40	Tr	1
6	1.2	76	81	Tr	2
12	1.3	98	116	1	4

[a] Tr, Less than 0.5 nmol/70 ml of medium.
[b] ND, None detected.

TABLE 2. Production of AFB_2 and AFM_2 by 24-, 48-, and 72-h-old mycelia of an AFB_2-accumulating strain of *A. flavus* over 96 h with and without ST

Aflatoxins	Mycelial age (h)	With or without ST (1 mg/70 ml)	Aflatoxins (nmol) recovered after incubation for the following time (h) in RM							
			1	2	4	6	8	24	48	96
AFB_2	24	Control		Tr[a]	Tr		Tr	1	2	1
		+ST	Tr		1	2		2		4
	48	Control	1	1	1	1	4	3	15	1
		+ST	2	Tr	Tr	2	3	2	100	220
	72	Control		5		2		8	340	17
		+ST	18	4	3	14	1	265	130	3
AFM_2	24	Control		ND[b]	ND		ND	Tr	Tr	Tr
		+ST	ND		ND	1		Tr		
	48	Control	ND	ND	ND	ND	ND	Tr	Tr	Tr
		+ST	ND	ND	ND	Tr	1	Tr	3	1
	72	Control		ND		Tr		Tr	1	
		+ST	ND	ND	ND	Tr	Tr	5	7	1

[a] Tr, Less than 0.5 nmol/70 ml of medium.
[b] ND, None detected.

TABLE 3. Production of AFB_1, AFM_1, and OMS by and recovery of ST from an AFB_2-accumulating strain of *A. flavus* incubated w ST in RM

Compound	Mycelial age (h)	Aflatoxins (nmol) recovered after incubation for the following time (h) in RM:							
		1	2	4	6	8	24	48	96
AFB_1	24	Tr[a]		Tr	5		6		38
	48	3	9	31	19	80	202	1,394	1,600
	72	21	7	5	62	7	750	1,001	1,027
AFM_1	24	ND[b]		ND	ND		ND		ND
	48	ND	ND	ND	Tr	1	1	5	12
	72	ND	ND	ND	3	Tr	4	6	2
OMS	24	ND		ND	672		757		1,163
	48	86	107	113	258	533	417	417	450
	72	70	28	20	43	170	284	284	328
ST (percent of total added)[c]	24	3,256 (106)		3,198 (104)	1,457 (47)		589 (19)		582 (
	48	2,889 (94)	2,840 (92)	2,136 (69)	1,901 (62)	1,617 (52)	1,185 (38)	951 (31)	942 (
	72	2,889 (94)	3,037 (98)	2,840 (92)	2,827 (92)	2,444 (79)	1,500 (49)	1,151 (37)	1,235 (
Total recovered (percent of total ST added)[d]	24	3,256 (106)		3,198 (104)	2,133 (69)		1,352 (44)		1,785 (
	48	2,978 (97)	2,956 (96)	2,280 (74)	2,179 (70)	2,231 (72)	1,805 (59)	2,743 (89)	3,004 (
	72	2,980 (97)	3,072 (100)	2,865 (93)	2,935 (95)	2,621 (85)	2,582 (84)	2,442 (79)	2,592 (

[a] Tr, Less than 0.5 nmol/70 ml of medium.
[b] ND, None detected.
[c] 1 mg (3,086 nmol) of ST added at 0 time.
[d] Total nanomoles = nanomoles of AFB_1, AFM_1, OMS, and ST; % total nanomoles = (total nanomoles/3,086 nmol) × 100.

AFB_2 with very low levels of AFM_2; no other aflatoxins were detected. AFB_2 production was highest in control cultures transferred after 72 h in GM and then incubated for more than 48 h in RM. In cultures supplemented with ST, higher levels of AFB_2 and AFM_2 were detected for most periods of incubation. AFM_2 appeared earlier and in higher levels in cultures supplemented with ST than in controls.

Added ST disappeared from cultures containing all three ages of mycelia at a steady rate until 48 h of incubation, after which little additional decline took place (Table 3). Several other metabolites were also identified from ST-supplemented cultures. These included substantial levels of AFB_1 and OMS and low levels of AFM_1.

In general, the levels of AFB_1, AFM_1, and OMS increased with the length of incubation. The highest AFB_1 recovery was with 48- and 72-h-old mycelia incubated for an additional 48 or 96 h in RM; the highest OMS recovery was with 24-h-old mycelia incubated for 96 h in RM. Produce recovery, estimated as the sum of the molar amounts of ST, OMS, AFB_1, and AFM_1 present in the culture, varied with the age of the mycelia. ST was most efficiently metabolized by 24-h-old mycelia; only 19% of the ST added was recovered after 24 h of incubation, whereas 72-h-old mycelia showed 49% recovery of ST at 24 h.

Finally, addition of AFB_1 to 48-h-old mycelia followed by 24 h of incubation in RM resulted in the appearance of AFM_1 (20 mmol), whereas the addition of AFM_1 to 48-h-old mycelia followed by 24 h of incubation in RM resulted in the recovery of unchanged AFM_1.

DISCUSSION

Data in Tables 1 and 2 support previous findings (8, 17) that strain SRRC 141 accumulates high levels of AFB_2 rather than AFB_1. An additional observation not previously reported in the appearance of AFM_2. In animal cells, AFM_1 can arise from AFB_1 (12), and it seems reasonable to suggest a similar step in this fungus, e.g., AFB_2 being the precursor of AFM_2 in a reaction mediated by a monooxygenase. T rapid conversion of ST to AFB_1 and, after a short lag pha to AFM_1, indicates that enzymes responsible for AFB_1 a AFM_1 biosynthesis are also present in SRRC 141. Furt support for AFM_1 arising from AFB_1 is the observation t when AFB_1 is added to resting cultures of the fungus, i converted to AFM_1, whereas addition of AFM_1 does form AFB_1. This contrasts with earlier work (11), in wh radiolabeled AFB_1 was fed to a wild-type culture and no label was detected in AFM_1. Problems with the transpor the isotope into the mold, diffusion within the mold cell dilution of the label with biosynthesized AFB_1 may expl the lack of conversion of radiolabeled AFB_1 to radiolabe AFM_1 in that study.

AFB_2 formation is generally higher in cultures sup mented with ST compared with that in controls, especi after 48 and 72 h of incubation (Table 3). This observa can be explained if it is assumed that the conversion zymes are inducible or, more probably, if AFB_1 is conve to AFB_2, in which case a metabolic grid operates (Fig. 2

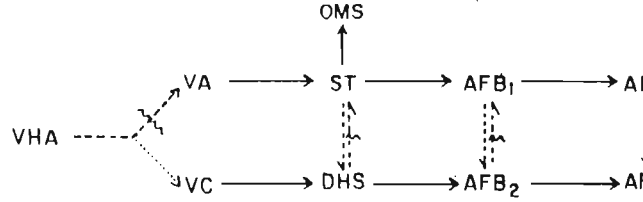

FIG. 2. Proposed metabolic grid for late stages of aflat biosynthesis. Arrows may represent more than one step. The s lines indicate proven reactions (this and previous work); the do lines indicate possible reactions. The wavy line indicates the po lated metabolic block in the mutant SRRC 141 with a com enzyme catalyzing the same transformation on a number of diffe substrates.

the enzyme normally responsible for the transformation of VHA to versicolorin A (VA) is missing in SRRC 141, it would explain the appearance of AFB_2 without AFB_1 in the mutant. Furthermore, this same enzyme might also catalyze the transformation of dihydrosterigmatocystin to ST, AFB_2 to AFB_1, and AFM_2 to AFM_1 (Fig. 2).

One odd result is that the amount of AFB_2 decreased in resting cell cultures utilizing 96-h-old mycelia after 48 h of incubation (Table 2). No concomitant production of AFM_2 was observed, so it seems that AFB_2 can also be degraded to some other unknown product.

OMS has been found in wild-type toxigenic *A. flavus* (4). Its appearance in this system has two possible explanations. ST added to whole cells may be incorporated in such a way that it comes into contact with a methyltransferase that is located in a part of the cell not normally associated with aflatoxin biosynthesis. Alternatively, it is possible that ST is normally never present in the cell at concentrations high enough for the methyltransferase to produce OMS in detectable quantity. The ST added artificially in this system is far in excess of that occurring naturally.

In the presence of dichlorvos, AFB_1-accumulating strains are blocked in aflatoxin production and accumulate VHA (5, 24). Similarly, when this high-AFB_2-accumulating strain is treated with dichlorvos, VHA accumulates. In an earlier study, dichlorvos caused VHA accumulation and inhibited aflatoxin production in a VA-accumulating mutant of *A. parasiticus* but did not inhibit versicolorin C production (3).

This high AFB_2-accumulating strain accumulates versicolorin C but not VA, indicating that a step between VHA and VA is probably blocked. This strain may have arisen by a mutation in an enzyme responsible for this conversion, perhaps VA synthase (22). It is not possible at this time to pinpoint the exact position at which the AFB_1 and AFB_2 biosyntheses diverge. A probable metabolic scheme is presented in Fig. 2. A similar scheme was proposed by Maggon et al. (13), who postulated that the AFB_1 arose via VA and ST and that the dihydrofurofuran aflatoxins arose as follows: versiconal $\rightarrow$versicolorin C$\rightarrow$ 5-hydroxydihydrosterigmatocystin $\rightarrow$ $AFB_2\rightarrow$ AFG_2.

Finally, summation of the molar amounts of ST, OMS, AFB_1, and AFM_1 present in the culture reveals an interesting trend in that the total recovered, in relationship to the amount of ST added, varied with the age of the mycelia and the time of incubation. This was not due to poor recovery or assay, since the total approached 100% at the beginning and increased at the end of the incubation period but dropped to as low as 44% in the middle (Table 3). Evidently, something is not accounted for in the mid-period of the experiments. It is tempting to suggest that the missing element is an intermediate between ST and AFB_1, since at this point, aflatoxin biosynthesis increases with respect to OMS production. It is hoped that the use of isotopically labeled ST will allow identification of this postulated intermediate.

ACKNOWLEDGMENTS

We thank G. Boudreaux and M. Legendre for performing the nuclear magnetic resonance and mass spectral analyses, respectively.

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Characterization of metabolites from a strain of *Aspergillus flavus* accumulating aflatoxin B_2

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A number of aflatoxins and anthraquinone pigments were isolated from a strain of *Aspergillus flavus*, several of which were fully characterized. The major metabolites isolated were aflatoxin B_2 and versicolorin C, which are normally only found as minor products from species of the genus *Aspergillus*. The identification of these products supports the proposal that aflatoxin B_2 can arise independently of aflatoxin B_1 and that, in this case, the branch in the pathway occurs at the versicolorins. Other metabolites charaterized were aflatoxin M_2, norsolorinic acid, and averufin.

S. Afr. J. Chem., 1985, 38, 107—109

'n Aantal aflatoksiene- en atrakinoonpigmente is vanuit 'n *Aspergillus flavus*-soort geïsoleer, waarvan sommige volledig gekarakteriseer is. Die hoofmetaboliete wat geïsoleer is, is aflatoksien B_2 en versikolorien C, wat normaalweg slegs as newe-metaboliete in spesies van die genus *Aspergillus* voorkom. Die identifikasie van hierdie produkte ondersteun die bewering dat aflatoksien B_2 onafhanklik van aflatoksien B_1 mag ontwikkel. Die ander metaboliete wat gekarakteriseer is, is aflatoksien M_2, norsoloriensuur, en averufien.

S.-Afr. Tydskr. Chem., 1985, 38, 107—109

Dedicated to the University of Natal
on the occasion of its 75th Anniversary

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Received 2 January 1985, revised 3 June 1985

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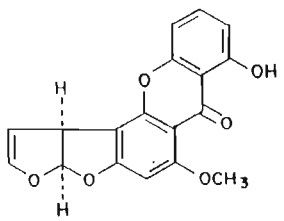
The aflatoxins are a group of mycotoxins produced b *Aspergillus flavus* and *A. parasiticus*. Of these, aflatoxin B is most commonly found and is also the most toxic an carcinogenic.[1] Its biosynthesis has been elucidated from th work of many groups using blocked mutants[2] and trace studies.[3,4] It is generally accepted that aflatoxin B_1 is forme from a C_{20} polyketide precursor via averufin → versicon acetate → versicolorin A → sterigmatocystin (**1**).



1 Sterigmatocystin

It has been proposed that aflatoxin B_1 is the precursor all the other commonly-occurring aflatoxins and indee there is evidence to support this supposition.[5] One su aflatoxin, aflatoxin B_2, is nearly always found in small quantities as a co-metabolite with aflatoxin B_1. Theoreticall aflatoxin B_2 could arise from aflatoxin B_1 by a simp hydrogenation of the isolated double bond in the dihydr bisfuran ring system.

On rare occasions, however, there have been reports the literature of aflatoxin B_2 occurring in fungal extracts the absence of aflatoxin B_1.[6]

These observations have been difficult to confirm in oth laboratories and consequently have often been dismissed cases of mistaken identity.

A strain of *A. flavus*, reputed to produce aflatoxin only, was investigated with a view to identifying all of t metabolites associated with the aflatoxins that it produce It was hoped that this study would conclusively prove tl aflatoxin B_2 could arise independently of B_1 and that lig would be shed on the biogenesis of the former metabol and its associated metabolites.

Results and Discussion

The major aflatoxin isolated from *A. flavus* (ATCC 241 was found to be aflatoxin B_2, and not the more us aflatoxin B_1, which was never detected in spite of extens culturing experiments. The basis for this identification

from ^{1}H n.m.r., where signals at δ6,39 (13-H_1), 4,10 (14-H_1), 2,20 (15-H_2), and 3,60 (16-H_2) revealed the bis-tetrahydrobisfurano system associated with the two series of aflatoxins and versicolorin C.[7,8] A parent ion of *m/e* 314 (M^+) in the mass spectrum with a characteristic M^+-29 peak, together with other physical data confirmed the identification.

The occurrence of aflatoxin B_2, in the absence of aflatoxin B_1, indicates that it can be biosynthesized independently, in contradiction to the traditionally held belief that aflatoxin B_1 is the precursor of the other aflatoxins.[5] This was confirmed by the addition of a known precursor of aflatoxin B_1, sterigmatocystin (**1**) to the fungus, which converted it into aflatoxin B_1 in a yield of more than 50%. Thus, the fungus is capable of forming aflatoxin B_1 provided that an appropriate precursor is present.

Of the other purple fluorescent compounds isolated, only aflatoxin M_2 could be identified as being related to the aflatoxins. This conclusion was made from its behaviour on chromatography, and the fact that its mass spectrum was identical to that of authentic material. The parent ion had *m/e* 330 (M^+), an M^+-17 peak indicating the presence of a hydroxyl group. The other physical data were consistent with this structural assignment.

Further study revealed that this metabolite appeared later in the growth cycle of the fungus, suggesting that it is a product from the metabolism of aflatoxin B_2. Metabolically, this suggestion makes sense, as it could be achieved by a one-step hydroxylation under the influence of a mono-oxygenase; indeed, this is how aflatoxin M_1 arises from aflatoxin B_1 in animal tissues.[9]

Thus we can conclude that aflatoxins B_1 and B_2 can arise independently of each other and then give rise to aflatoxins M_1 and M_2 respectively, by monohydroxylation.

All the other metabolites isolated from this strain of *A. flavus* were anthraquinone derivatives, which is in keeping with the known biosynthetic pathway of the aflatoxins. Versicolorin C, averufin, and norsolorinic acid were identified from their n.m.r. and mass spectral data, and comparison with authentic samples. Six minor pigments were also isolated but, of these, only versiconal hemiacetal acetate could be identified with any certainty from its mass spectral fragmentation pattern.

The identification of versicolorin C as the major anthraquinone pigment to be produced by *A. flavus* is unprecedented, although it has been isolated before from an aflatoxin-producing strain of *A. parasiticus*, as a minor product.[8] It was reasoned that the appearance of versicolorin C was not unconnected with that of aflatoxin B_2; probably some block in the aflatoxin biosynthetic pathway occurred, which gave rise to relatively large quantities of versicolorin C.

Experimental

M.p.s were determined on a Kofler hot-stage apparatus. Optical rotations were measured with a Schmidt & Hansch polarimeter at room temperature. ^{1}H N.m.r. spectra were recorded with a Varian CFT20 spectrometer using TMS as internal standard and deuteriodimethyl sulphoxide as the solvent, unless otherwise stated. Mass spectra were recorded on a Finnegan 4000 and a Varian MAT model CH7 spectrophotometers. Visible and u.v. spectra were recorded on an Hitachi model 220 spectrophotometer. Quantitation of metabolites was done on a Schoeffel SD 300 recording densitometer, that was calibrated with suitable standards. Preparative t.l.c. was performed on activated silica gel G plates (Merck art 7731) spread at a thickness of 0,3 mm. Two-dimensional qualitative t.l.c. was done using pre-coated aluminium-backed silica gel G plates (Merck 5553) cut into squares of 10 × 10 cm. Plates were developed with different solvent systems depending upon the metabolites to be separated. Anthraquinone pigments were detected by their orange colour and aflatoxin metabolites by their purple fluorescence under long wavelenth ultraviolet light.

Production of metabolites

A culture of *A. flavus* (ATCC 24109) originally isolated by Schroeder and Carlton,[10] was provided by Dr M.A. Klich (Southern Regional Research Centre, New Orleans), where it is held as SRRC 141. Cultures of this fungus were grown in a low-salts culture medium containing asparagine (1%), after that described by Reddy *et al.*,[11] in either 250 ml conical flasks (containing 70 ml medium) or in Fernbach flasks (containing 500 ml). Replacement medium was prepared after that described by Adye and Mateles.[12] Quantitative production of metabolites was done by culturing the fungus in Fernbach flasks on a bank shaker (160 r.p.m.) in a constant temperature room at 28°C for 10 days. Metabolites were solvent extracted from the dried mycelium, separated by preparative t.l.c. to homogeneity, and then recrystallized.

Addition of sterigmatocystin to fungal cultures

Cultures of *A. flavus* (ATCC 24109) were grown for two days in 250 ml conical flasks as previously described. The mycelium was filtered and washed with cold sterile distilled water. The damp mycelium (5 g) was resuspended in replacement medium (70 ml) in a 250 ml conical flask which also contained **1** (0,5 mg), added as a solution in acetone (Analar; 0,4 ml).

Aflatoxin B_2 (**3**)

The major purple-fluorescing metabolite was obtained as colourless crystals from acetone–chloroform, m.p. 302°C (decomp.) (lit.,[13] m.p. 303—306°C); $[\alpha]_D$ −440°. It co-chromatographed with authentic aflatoxin B_2, λ_{max} 220 (ε 21 000), 265 (12 500), and 363 (24 000) nm; *m/e* 314, 285, and 271; δ_H(CDCl$_3$) 2,20 (2H, m, 15-H_2), 3,06 (2H, t, *J* 1,2 Hz, 5-H_2), 3,32 (2H, t, *J* 1,2 Hz, 4-H_2), 3,60 (2H, m, 16-H_2), 3,90 (3H, s, OMe), 4,10 (1H, m, 14-H), 6,25 (1H, s, 9-H), and 6,39 (1H, d, *J* 1,2 Hz, 13-H).

Aflatoxin M_2

A purple-fluorescing metabolite was obtained in smaller quantity, which co-chromatograhed with aflatoxin M_2, λ_{max} 220 (ε 20 000) 265 (11 000), and 358 (21 000) nm; *m/e* 330, 313, 301, and 284.

Aflatoxin B_1

On addition of **1** to replacement culture of the fungus, a purple-fluorescing compound was observed on t.l.c., (0,6 mg was isolated from two experiments) just above aflatoxin B_2. The metabolite co-chromatographed with aflatoxin B_1, and gave a positive confirmatory test;[14] λ_{max} 221,264, and 363 nm; *m/e* 312, 284, 283, and 269. Lack of material precluded further physical measurements.

Versicolorin C

The major pigment was isolated as orange needles from acetone, m.p. 309°C (lit.,[13] m.p. 310°C); $[\alpha]_D$ 0°; λ_{max} 233

Chromatographic separation and determination of stable metal cyanide complexes in gold processing solutions

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A technique is described for the simultaneous separation and determination of the cyanide complexes of copper, nickel, cobalt, iron, and gold. The separation procedure utilizes the principles of ion-pair chromatography, *i.e.* the partitioning of ion pairs between a hydrophilic mobile phase and a hydrophobic column surface. The cyanide complexes are detected spectrophotometrically or, after the solution has passed through a suppressor, by conductivity measurement. The method is applied to gold solutions from the carbon-in-pulp process. The precision (relative standard deviation) was found to be better than 0,0201 for all complexes at concentrations of 5 mg l^{-1}, and better than 0,0138 at concentrations of 10 mg l^{-1}.

S. Afr. J. Chem., 1985, 38, 110—114

'n Tegniek vir die gelyktydige skeiding en bepaling van die sianiedkomplekse van koper, nikkel, kobalt, yster, en goud word beskryf. Die skeidingsprosedure maak gebruik van die beginsels van ioonpaarchromatografie, m.a.w. die verdeling van ioonpare tussen 'n hidrofiliese mobiele fase en 'n hidrofobiese kolomfase. Die sianiedkomplekse word spektrofotometries opgespoor, of deur die geleivermoë te meet nadat die oplossing deur 'n onderdrukker gestuur is. Die metode word toegepas op goudoplossings afkomstig van die koolstof-in-pulpproses. Daar is gevind dat die presisie (relatiewe standaardafwyking) beter as 0,0190 is vir alle komplekse met konsentrasies van 5 mg l^{-1}, en beter as 0,0138 met konsentrasies van 10 mg l^{-1}.

S.-Afr. Tydskr. Chem., 1985, 38 110—114

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Received 11 January 1985

Analytical procedures concerned with the determination of individual metal cyanide complexes have received little attention in the past. Usually two or three complexes are determined simultaneously as, for example, the hexacyanoferrates.[1] More recently, the Dionex Corporation issued a series of notes on the determination of some stable metal cyanide complexes in plating baths, by mobile-phase ion chromatography (MPIC).[2—4] In MPIC, which utilizes the principles of ion-pair chromatography, a suppressor device is used to reduce the conductivity of the mobile phase prior to conductometric detection. Carbon-based column packings with very hydrophobic surfaces are employed as the stationary phase. The hydrophilic mobile phase contains tetrabutylammonium hydroxide (2mM) as the pairing reagent, 0,2mM of sodium carbonate, and 40% acetonitrile. Under the conditions described, the separation of the complexes of cobalt(III), gold(I), and gold(III), and of gold(I), iron(III), iron(II), and gold(III), in that order, has been successfully demonstrated.[2—4]

Cuff[5] found recently that he had to increase the carbonate content of the eluent to 2mM to achieve successful separation of the gold(I) and gold(III) cyanides. He also found that it was not possible to reproduce the analysis of gold(I), iron(III), iron(II), and gold(III) complexes. Under all the conditions tested, the gold complexes were found to elute later than the iron complexes.

In the past, the determination of cyanides in process solutions from metallurgical plants for the recovery of gold was restricted to 'available' and 'total' cyanide. While the latter indicates the amount of strong metal cyanide complexes present, it gives no indication of their type and nature. Because of the interaction of strong metal cyanide complexes with the carbon or resins used in the recovery of gold and the occasional 'poisoning' of such materials, a knowledge of the kind of complex present can be of considerable importance. Similarly, characterization of the kind of cyanide complexes present in waters and effluents can result in more effective 'scrubbing' procedures and, thus, pollution of the environment can be prevented.

In the present study, therefore, it was decided that MPIC should be used for the separation and determination of cyanide complexes commonly encountered in gold processing solutions. Because of the discrepancies observed previously,[2—5] a more detailed investigation of the various factors affecting MPIC separation would be undertaken. An account of this study, which resulted in the separation of five commonly encountered metal cyanide complexes, is presented in this paper.

(ε 24 000), 255 (13 500), 266 (20 000), 291 (23 000), 324 (14 000), and 450 (8 500) nm; *m/e* 340, 325, 311, and 297; δ_H 2,15 (2H, m, 12-H_2), 3,80 (2H, m, 13-H_2), 4,10 (1H, m, 11-H), 6,47 (1H, d, *J* 1,2 Hz, 14H), 6,55 (1H, d, *J* 1,0 Hz, 7-H), 7,04 (1H, s, 4-H), and 7,08 (1H, d, *J* 1,0 Hz, 5-H).

Acknowledgements

The author thanks Mrs L.S. Lee for her assistance and stimulating discussions, Mr G.J. Boudreaux for n.m.r. spectroscopy, and Mr M. Legrane for mass spectrometry, all of the SRRC, New Orleans, and Dr J.W. Bennett of Tulane University, New Orleans for her encouragement. Thanks are also due to the SRRC, New Orleans for working facilities, the University of Natal, Council for Scientific and Industrial Research, Pretoria, and the U.S. Department of Agriculture for grants.

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MICROBIOLOGICAL REVIEWS, June 1988, p. 274–295
0146-0749/88/020274-22$02.00/0

Vol. 52, No. 2

Enzymes and Aflatoxin Biosynthesis

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INTRODUCTION

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

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

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

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

FUNGAL SECONDARY METABOLIC ENZYMES

Characteristics

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

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

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

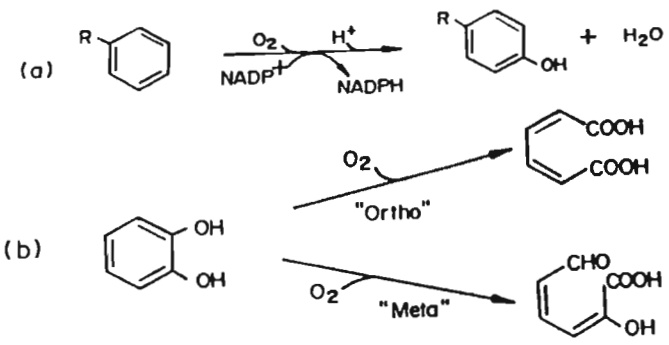

FIG. 1. Incorporation of molecular oxygen by oxygenases: (a) monooxygenases and (b) dioxygenases.

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

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

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

Isolation

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

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

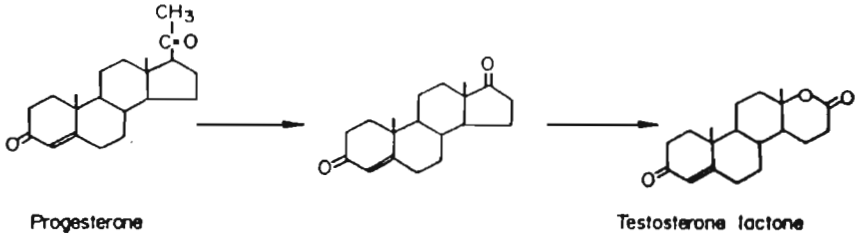

FIG. 2. Conversion of progesterone to testololactone by *P. lilicium* (153).

TABLE 1. Examples of oxygenases found in filamentous fungi

Reaction or enzyme	Substrate	Organism	Reference(s)
Cytochrome P-450	Aromatic ring	*Cunninghamella* spp.	44, 70, 71
Cytochrome P-450	Steroid	*Rhizopus nigricans*	31
Cytochrome P-450	Alkaloids	*Claviceps* sp.	5
Hydroxylation	Steroid	*Aspergillus ochraceus*	108
Hydroxylation	Steroid	*Aspergillus niger*	1, 102
Hydroxylation	Steroid	*Curvularia* sp.	145
Hydroxylation	Aromatic ring	*A. niger*	10, 29, 118, 17
Hydroxylation	Terpene	*A. niger*	76
Hydroxylation	Biphenyl	*A. parasiticus*	50
Hydroxylations	Various	Several	30, 173
Hydroxylation	Aromatic ring	*Penicillium patulinum*	139
Epoxidation	Cyclopenin	*Penicillium cyclopium*	197
BV	Steroid	*Cylindrocarpon* sp.	107
BV	Hydrocarbon	*Penicillium* sp.	4
BV	Hydrocarbon	*Fusarium lini*	181
Cleavage	Aromatic ring	*Penicillium patulum*	161
Cleavage	Various	Several	41
Cleavage	Quercetin	*A. flavus*	146
Lipoxygenase	Fatty acid	*Fusarium oxysporum*	159

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

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

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

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

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

Purification

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

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

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

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

AFLATOXINS

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

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

Biosynthesis and Primary Metabolism

All secondary metabolism stems from primary metabolism; therefore, the metabolic state of the latter will ultimately affect the former. Aflatoxin production is affected by catabolic activity (86, 87), reduced coenzymes level (23), energy charge (155), and metal ions (134). The role of these factors is difficult to define because of the complexity of the

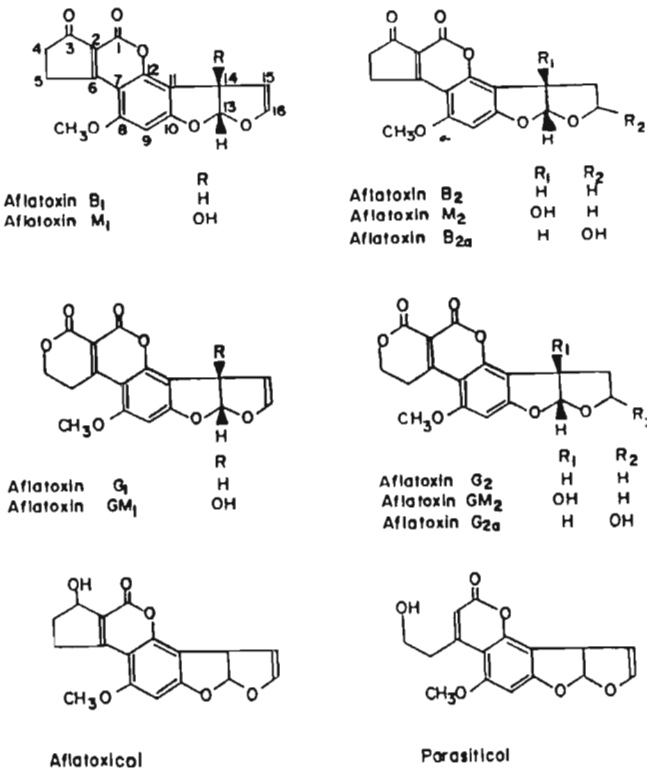

FIG. 3. Structures of the aflatoxins and closely related metabolites.

metabolic state of the organism. An added problem is that the metabolic activity of the fungus may be compartmentalized (12), and it is becoming clear that more powerful techniques such as recombinant deoxyribonucleic acid technology (15), immunohistology (91), and nuclear magnetic resonance spectroscopy of cells (160) will have to be applied.

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

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

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

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

stressed the importance of glycolytic activity together with oxygen availability.

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

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

Biosynthetic Pathway

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

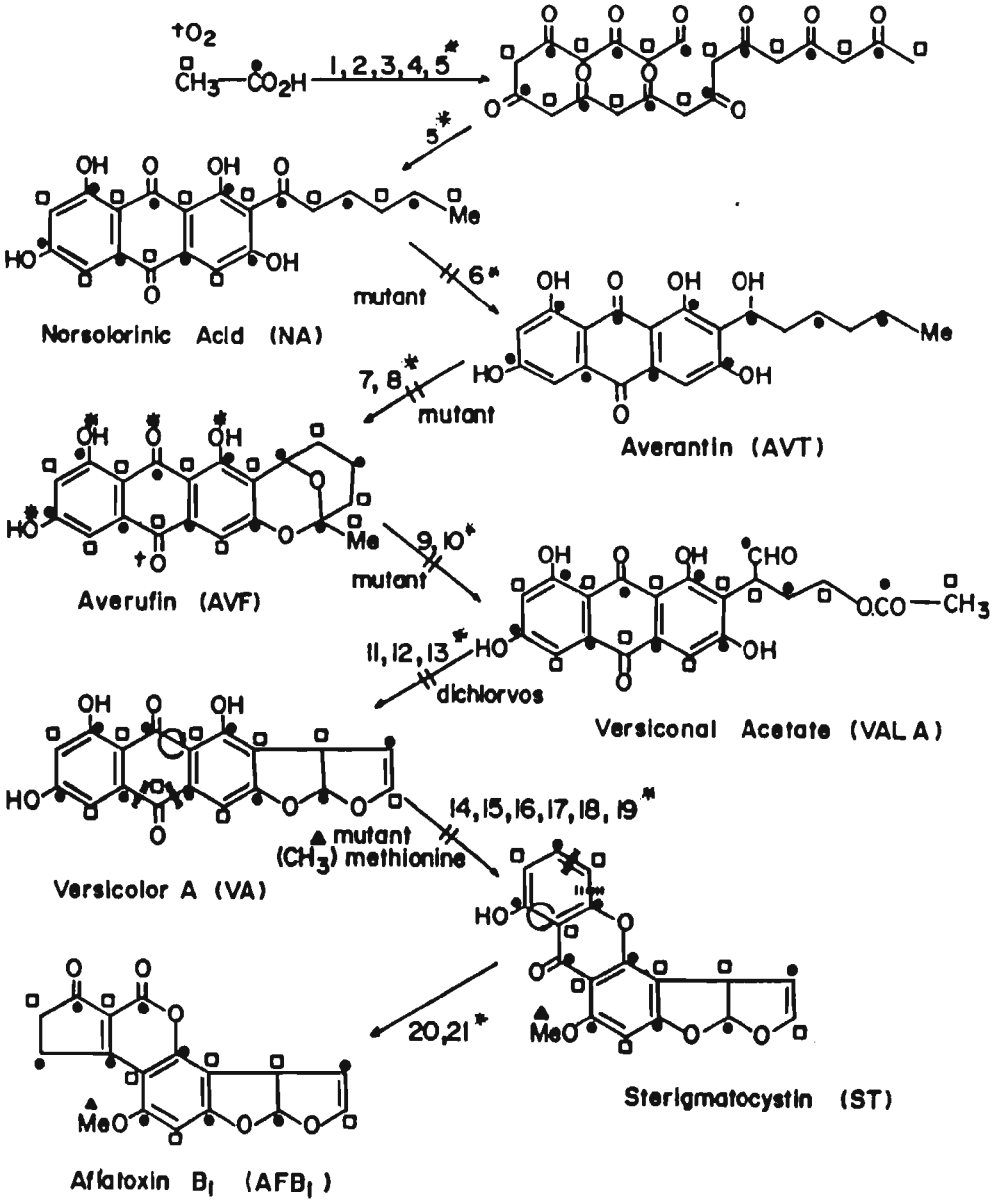

FIG. 4. Proposed biogenesis of AFB1 (189). *Minimum number of steps likely to be enzyme catalyzed. Reproduced with kind permission of the authors and Academic Press, Inc.

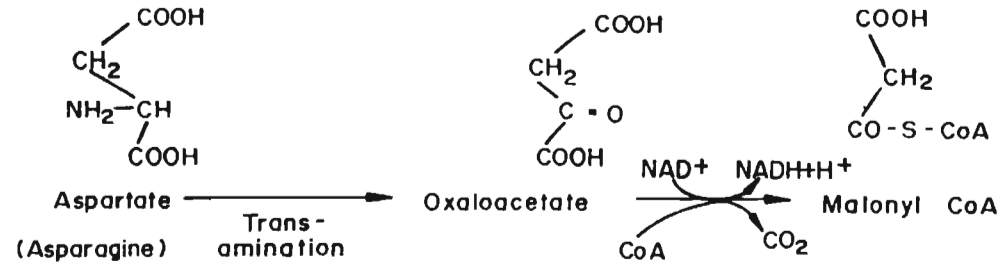

FIG. 5. Transamination of aspartate to oxaloacetate and then to malonyl CoA.

ENZYMES OF AFLATOXIN BIOGENESIS

Anthraquinone Biosynthesis

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

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

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

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

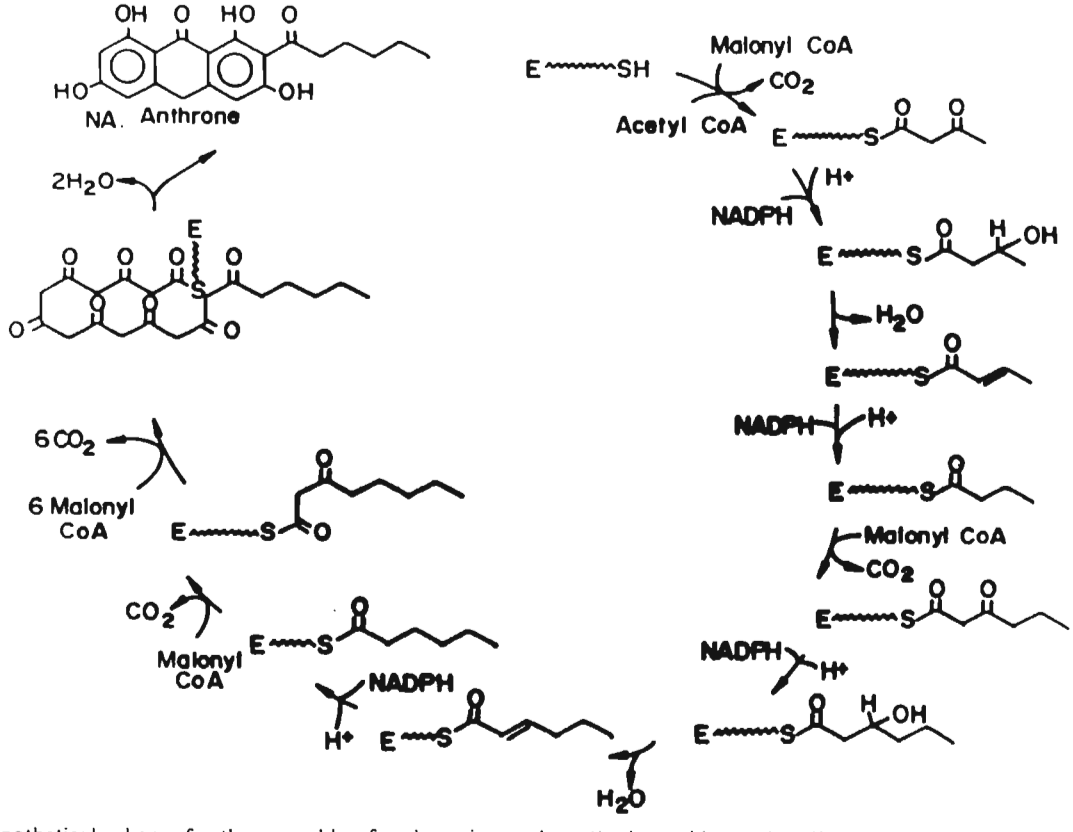

FIG. 6. Hypothetical scheme for the assembly of anthraquinones by a "polypeptide synthase" enzyme complex in *Aspergillus* species (16). Reproduced with kind permission of the authors and Academic Press, Inc.

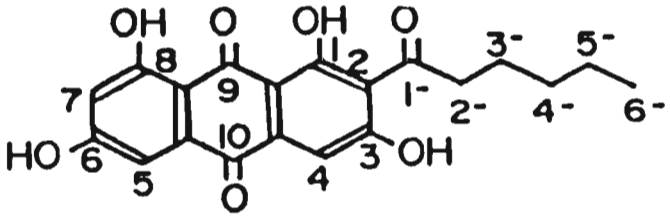

FIG. 7. Structure of NA.

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

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

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

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

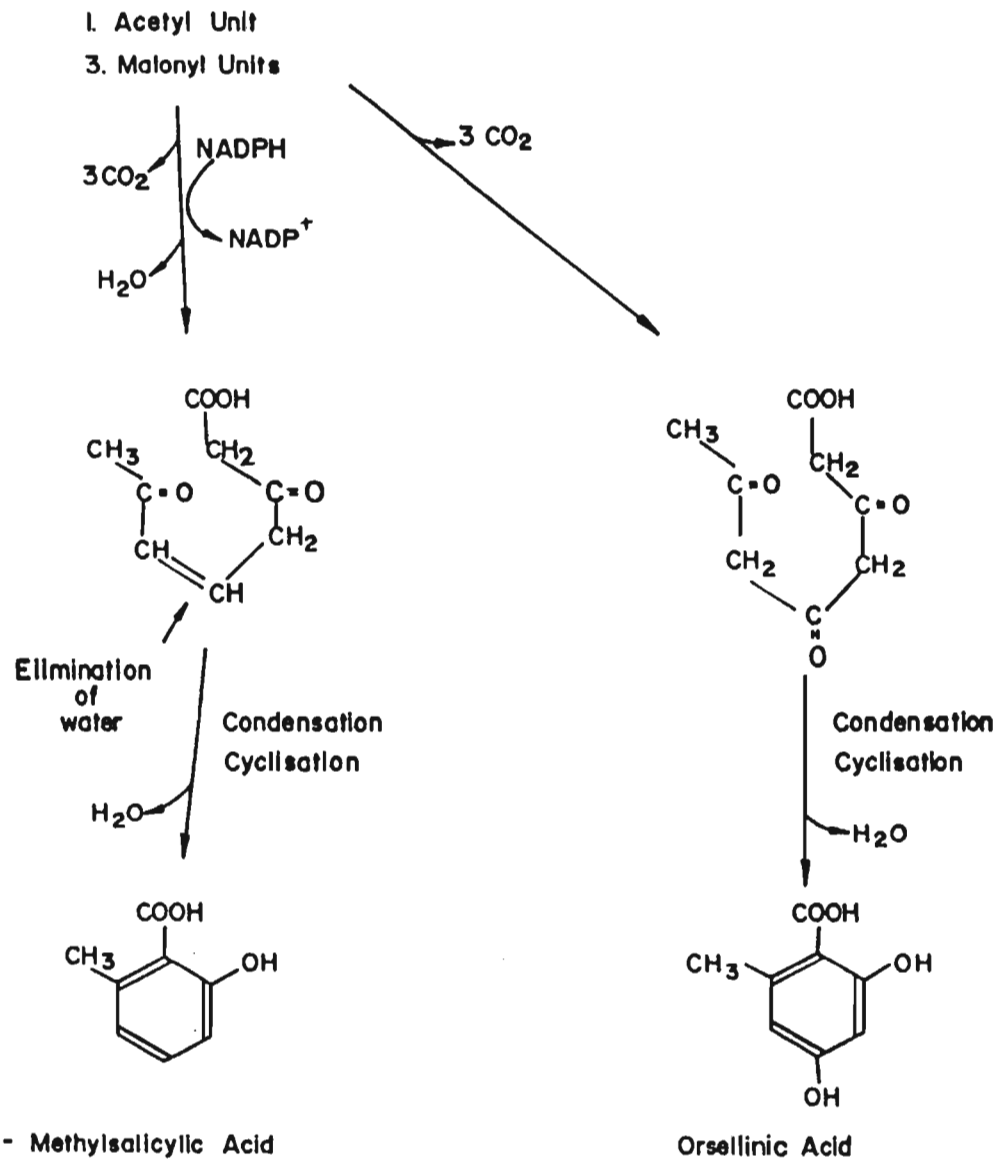

FIG. 8. Formation of a tetraketide and its conversion to 6-MSA or orsellinic acid.

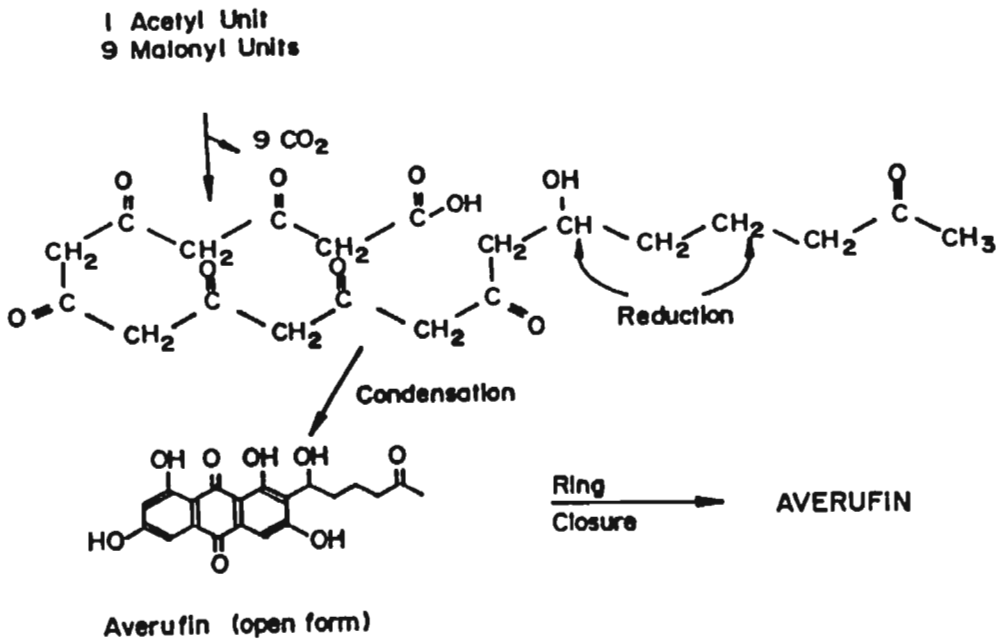

FIG. 9. AVF from the polyketide chain by reduction of 1′ and 3′ carbonyls.

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

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

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

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

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

Generation of the Bisdihydrofuran System

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

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

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

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

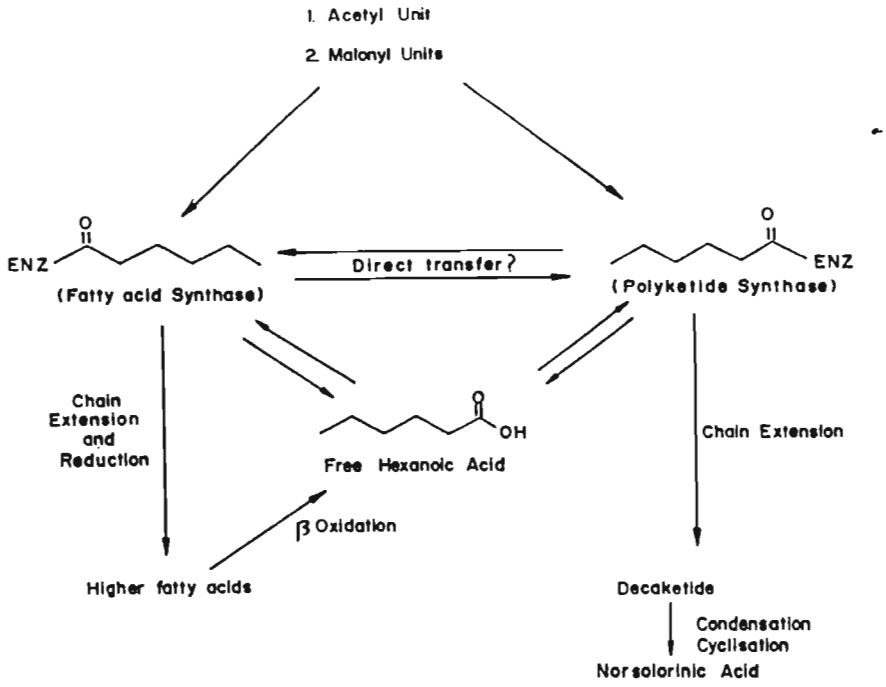

FIG. 10. Role of hexanoic acid in NA biosynthesis.

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

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

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

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

Acid hydrolysis of the ester group causes rapid ring closure to versicolorin C (VC) (175) (Fig. 15), a known metabolite of both *A. versicolor* (88) and *A. parasiticus* (Thus, a plausible suggestion is that the next step is cataly by an esterase, and in fact the addition of dichlorvos, inhibitor of acetyl choline esterase, to fungal cultures cau the accumulation of VAL A (204). Certain other organoph phorus compounds show a similar effect (64), and both th and dichlorvos do inhibit aryl esterases in *A. parasiticus* Other compounds also promote the accumulation of ments in aflatoxigenic strains of *Aspergillus*, e.g., benze (191), although this work was not repeatable by others (1

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

The question of the stereochemistry of the putative thraquinone precursors has been addressed by Towns (182) as this can influence reaction mechanisms. Stereos ificity is the hallmark of enzyme action, and it is surprising that when there is the choice between sev diastereoisomers one should be preferentially formed.

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

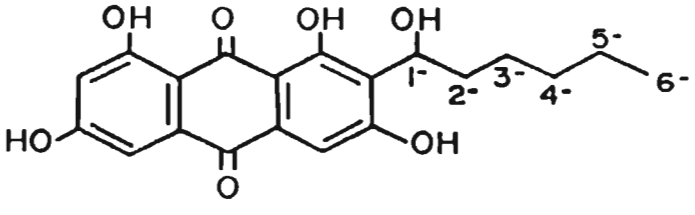

FIG. 11. Structure of AVT.

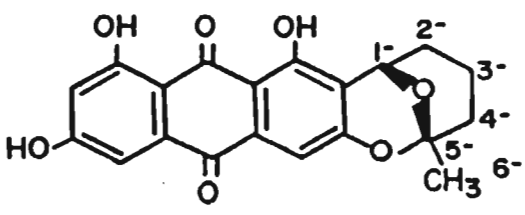

FIG. 12. Structure of AVF.

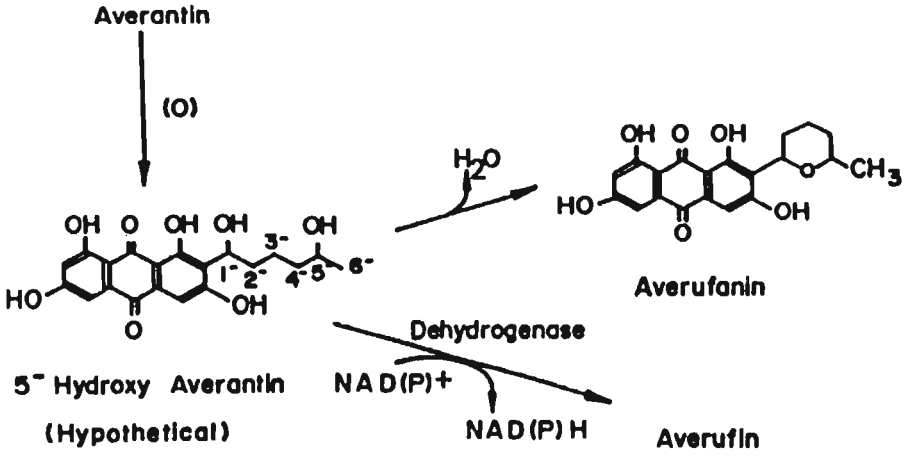

FIG. 13. AVT to AVF via oxidation of a monooxygenase to a ketone by means of a dehydrogenase.

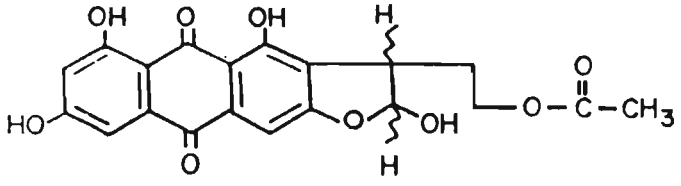

FIG. 14. Structure of VAL A.

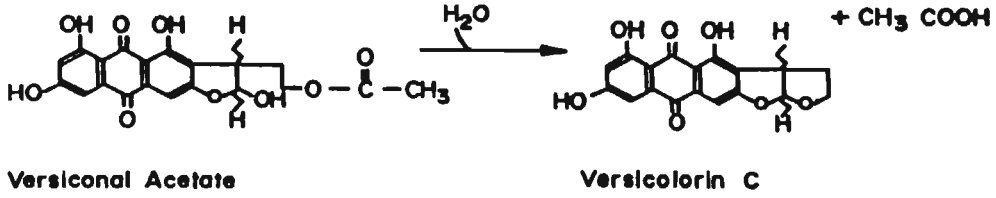

FIG. 15. Rapid ring closure to VC caused by acid hydrolysis of ester group.

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

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

The enzyme, versicoloronal synthase, is possibly me brane bound to facilitate substrate solubility and must ser at least two functions: (i) to generate an electron-defici center at position 2' of AVF, and (ii) to direct the reaction that an S conformation occurs at position 2' in the bisfura system. One model is given in Fig. 20. As hydroxylation inappropriate in generating the electron-deficient cen (184), an alternative is the removal of hydride, this bei accepted by an oxidized coenzyme such as $NADP^+$. T bonding electrons from the adjacent carbon (1') of AVF n attack this position, leaving this carbon (1') electron d cient and detached from the aromatic ring. The carbo now stabilized both electronically and spatially by a nucl

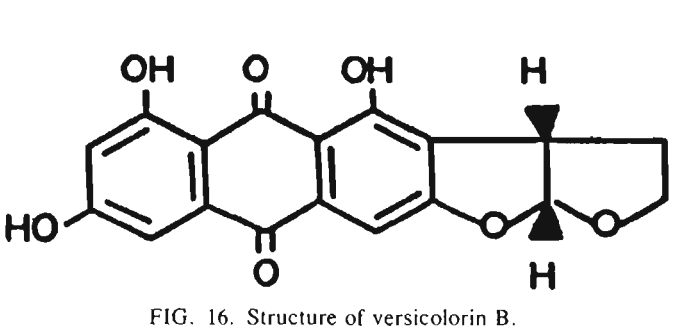

FIG. 16. Structure of versicolorin B.

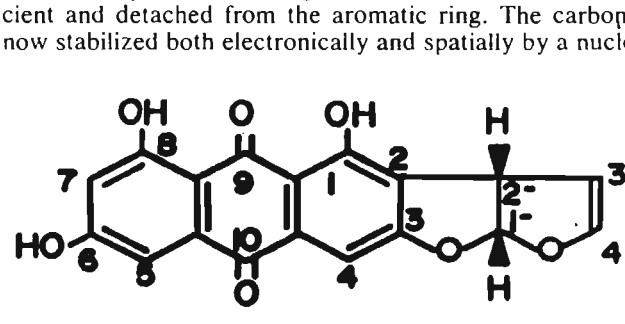

FIG. 17. Structure of VA.

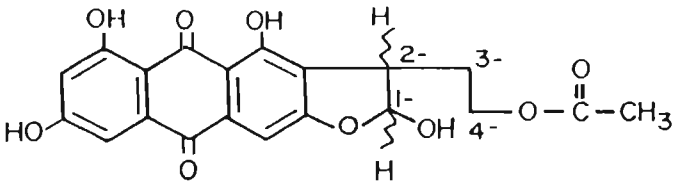

FIG. 18. Nomenclature of bisfuran system depicted as part of VAL A, the naturally occurring stereo arrangement at C-2' being for VA, etc.

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

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

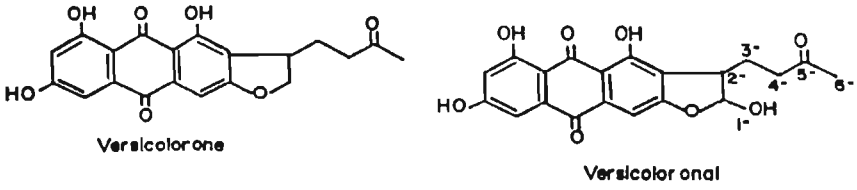

FIG. 19. Structures of versicolorone and versicoloronal.

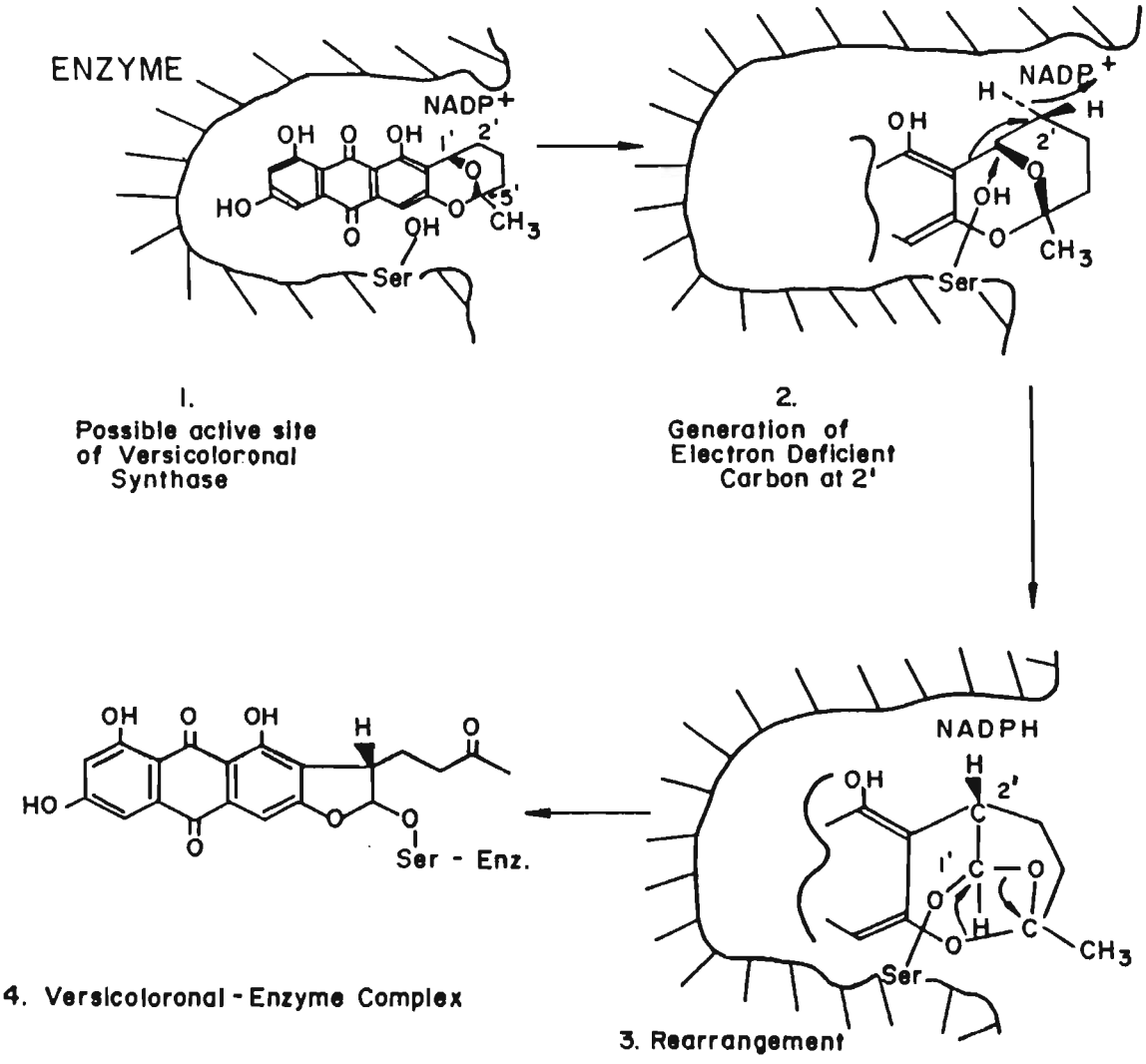

FIG. 20. Suggested mechanism for enzymatic conversion of AVF to versicoloronal.

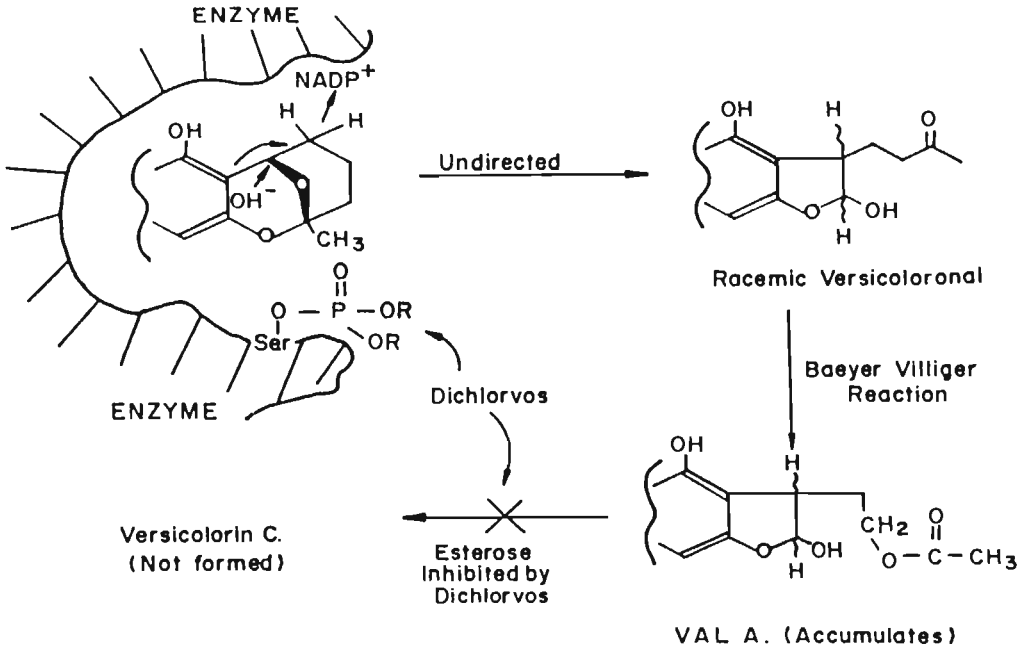

FIG. 21. Inhibition of versicoloronal synthase and VAL A esterase by dichlorvos.

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

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

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

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

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

Anthraquinone Modification

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

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

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

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

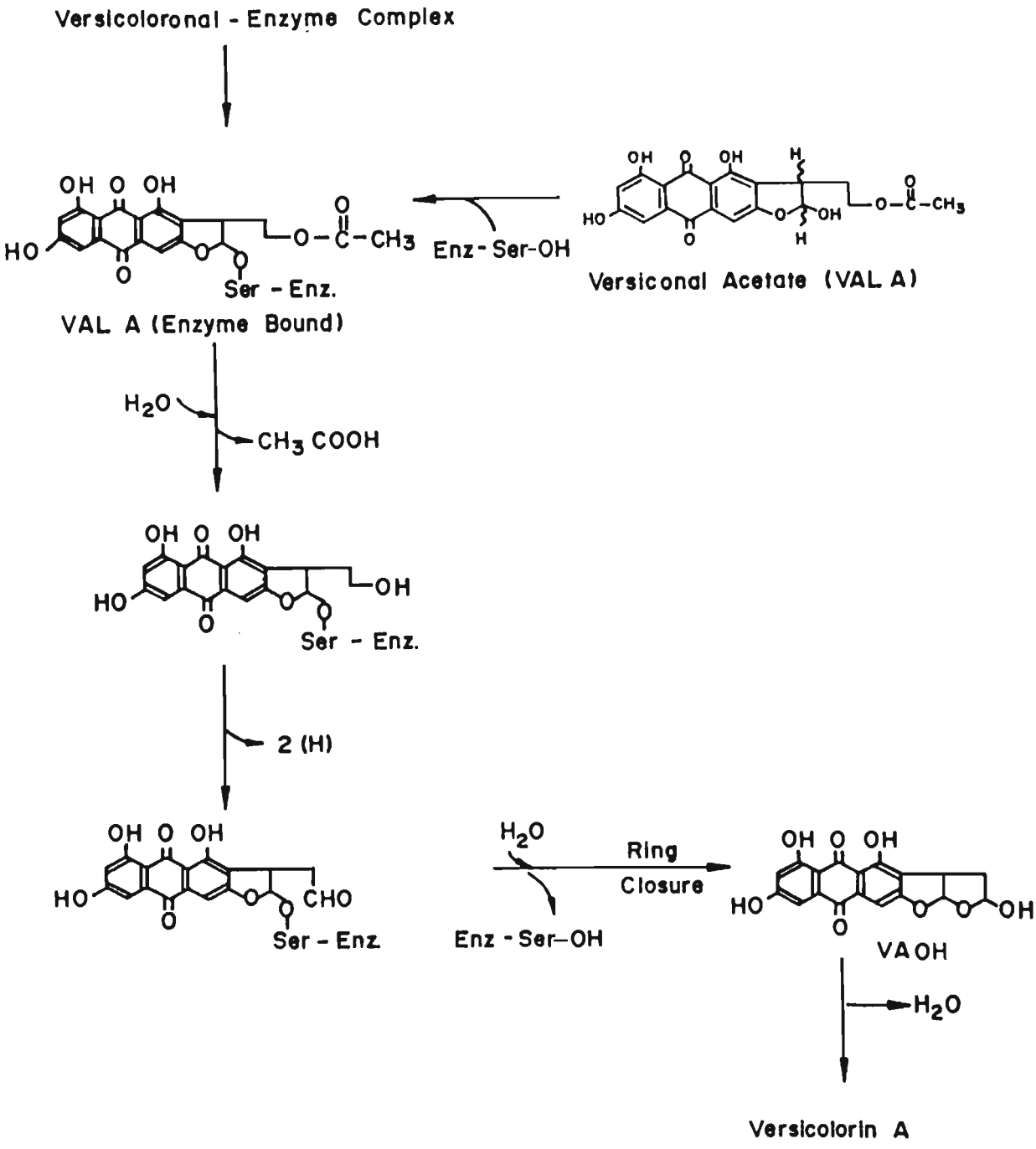

FIG. 22. System showing conversion of VAL A to VA.

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

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

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

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

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

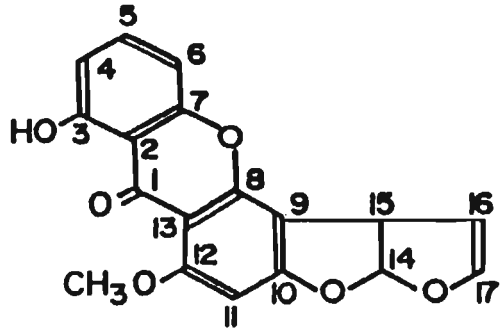

FIG. 23. Structure of ST.

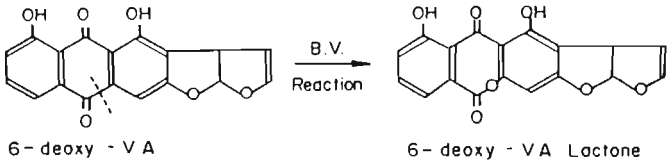

FIG. 24. Conversion of 6-deoxy-VA to 6-deoxy-VA lactone.

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

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

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

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

Other Aflatoxins

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

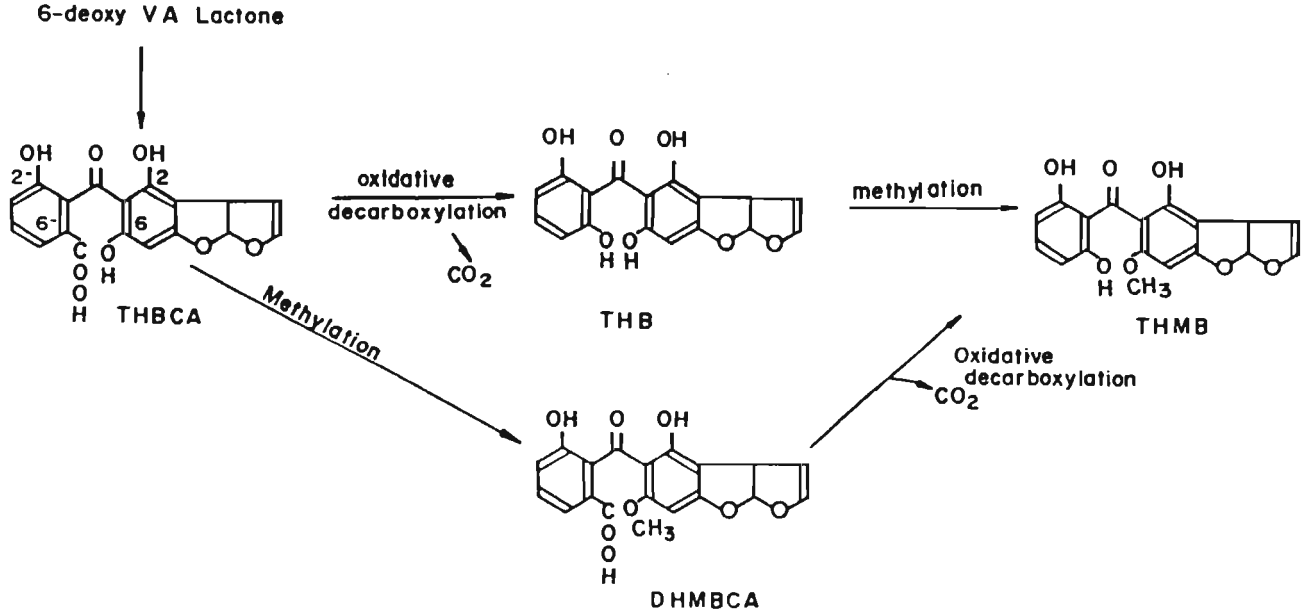

FIG. 25. Formation of 2,6,2',6'-tetrahydroxybenzophenone derivative (THB) and substituted 2,2',6'-trihydroxy,6-methoxy-benzophenone (THMB) or 2,2'-dihydroxy, 6-methoxy-6'-carboxylic acid (DHMBCA) from THBCA (substituted 2,6,2'-trihydroxybenzo-phenone-6'-carboxylic acid).

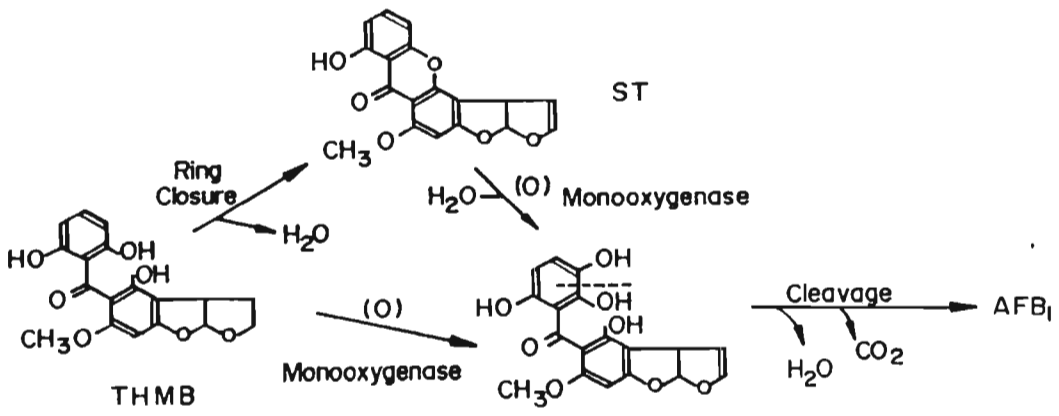

FIG. 26. ST from substituted 2,2',6'-trihydroxy,6-methoxy-benzophenone (THMB) via ring closure.

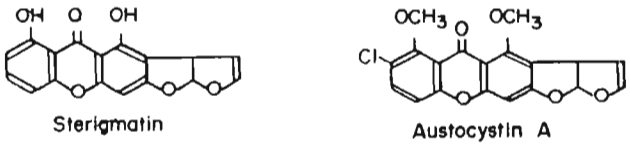

FIG. 27. Examples of linear xanthones.

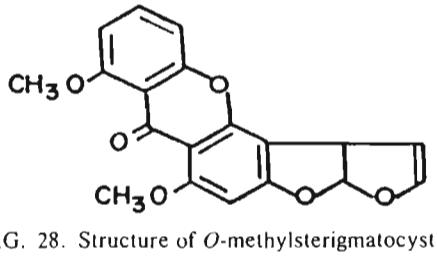

FIG. 28. Structure of *O*-methylsterigmatocystin.

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

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

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

This casts a new role for AFB2a and its xanthone, S hemiacetal, and anthraquinone, VA hemiacetal, analogs (4 as they could act as intermediates between the dihydro- ar tetrahydrobisfuran series. Possibly the metabolic grid d picted in Fig. 30 is more complicated and should include t pathway presented in Fig. 33.

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

GENERAL COMMENTS

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

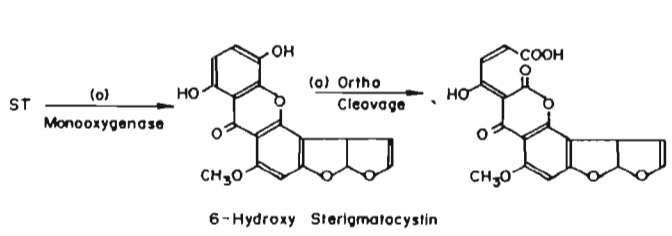

FIG. 29. ST to 6OHST to AFB1 via ortho cleavage.

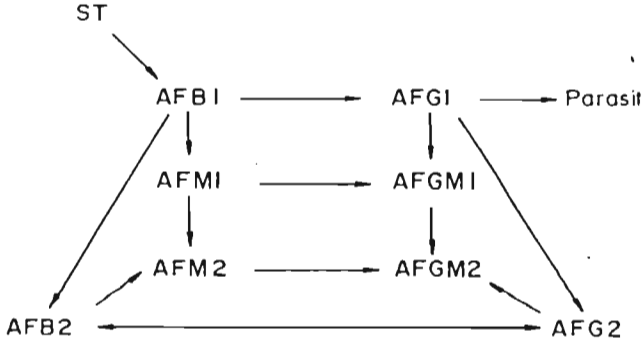

FIG. 30. Metabolic grid interconnecting major aflatoxins.

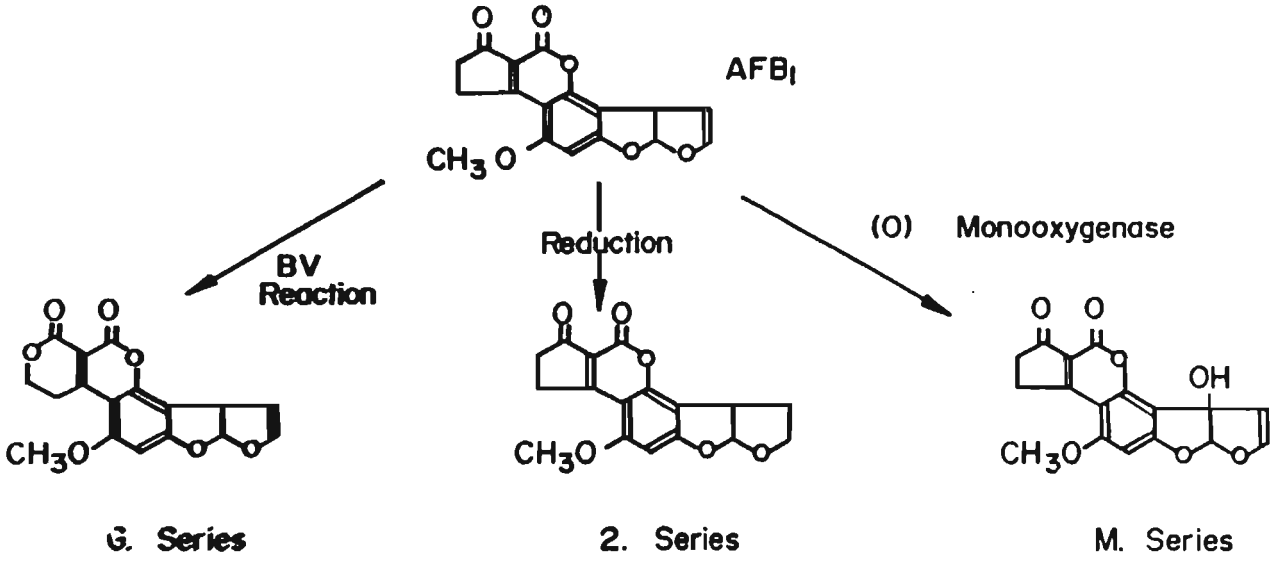

FIG. 31. Scheme showing that AFG1 could arise from AFB1 via a BV reaction and possible formation of AFB2 and AFG2.

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

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

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

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

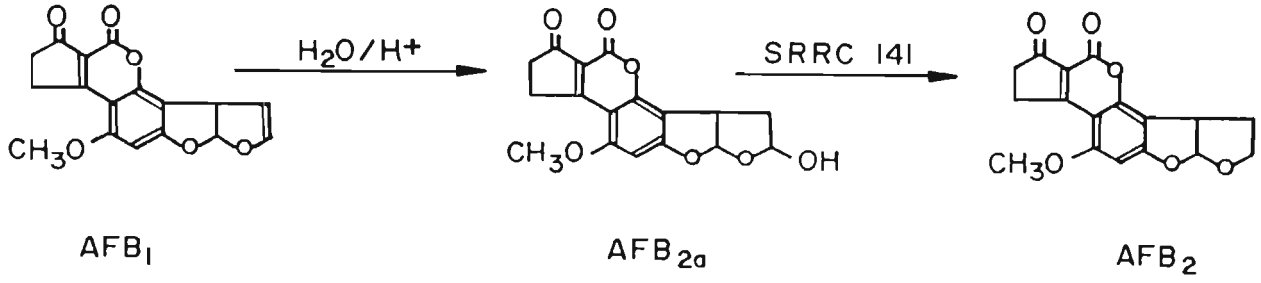

FIG. 32. AFB2a from AFB1 after addition of water.

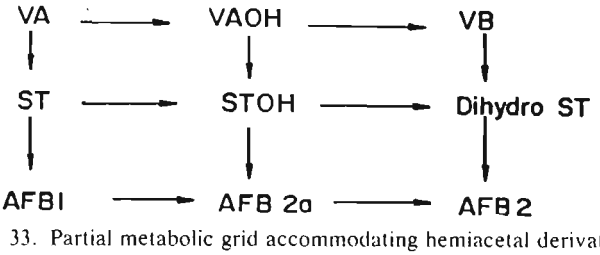

FIG. 33. Partial metabolic grid accommodating hemiacetal derivatives.

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

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

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

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

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

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

[e] Sequence of reactions may be different.

CONCLUDING REMARKS

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

ACKNOWLEDGMENTS

Current work on aflatoxin biosynthetic enzymes is supported by the C.S.I.R. and University of Natal, South Africa.

I sincerely thank my friends at the Southern Regional Research Laboratory, New Orleans, La.; Joan Bennett, Tulane University, New Orleans, La.; and Bill Tseng, Academica Sinica, Taiwan, for their support and inspiration to write this review.

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Vol. 166, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1990 Pages 38-42

THE PREPARATION OF AN ENZYME ASSOCIATED WITH AFLATOXIN BIOSYNTHESIS BY AFFINITY CHROMATOGRAPHY

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Received November 9, 1989

Summary: An affinity matrix for the purification of norsolorinic acid dehydrogenase, an enzyme involved in aflatoxin biosynthesis, was prepared by coupling norsolorinic acid to an agarose gel. This matrix was found to be ineffective in isolating active enzyme, and was therefore modified by methylation, using diazomethane. The methylated matrix produced a one-step purification of the enzyme from a crude homogenate, resulting in a 138-fold purification. The active isolate was found to contain one major and two minor bands upon non-denaturing electrophoresis, and all the norsolorinic acid dehydrogenase activity was associated with the major band. It was concluded that the matrix exhibited true affinity for the enzyme, and that affinity chromatography was a valuable approach to isolating other secondary metabolic enzymes involved in the biosynthesis of the aflatoxins.

Aflatoxins are biologically active, polyketide derived secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus*. Knowledge of this biosynthetic pathway arises mainly from studies using nuclear magnetic resonance with stable isotopes (1) and mutants of *A. parasiticus*, which have the pathway blocked at specific points (2). Regulation of the pathway involves complex controls, which are strongly influenced by specific cellular events in the growth cycle (3,4).

To date very few enzymes involved in the biosynthesis of the aflatoxins have been isolated and fully characterized. Recently, however, an homogeneous protein has been obtained from *A. parasiticus* with methyl transferase activity (5). The presence of this enzyme has been known for some time (6) when it was found to convert sterigmatocystin to O-methylsterigmatocystin. The enzymes responsible for the earlier steps in the pathway have received less attention. Consequently we investigated the conversion of norsolorinic acid (NA) to averantin (AVN) by NA dehydrogenase (7,8) (Fig. 1).

In order to isolate this secondary metabolic enzyme with the minimum number of separation steps and the maximum of purification and activity, the technique of affinity chromatography has been utilized. The results of this work are presented here.

0006-291X/90 $1.50

MATERIALS AND METHODS

Nicotinamide cofactors, ω–aminohexylagarose and toluene sulphonyl fluoride were purchased from Sigma Chemical Co., USA. N-methyl-N-nitroso-p-toluene-sulphonamide (Diazald) was obtained from Aldrich Fine Chemicals, USA.

A versicolorin A accumulating mutant of *A. parasiticus* (1-11-105 Wh1, kindly donated by Dr. J. W. Bennet) was used as the source of crude enzyme.

A cell free extract was prepared by inoculating 100ml of sterile, chemically defined medium (8) in 250ml Erlenmeyer flasks with a 1ml spore suspension ($1x10^6$ spores), and shake incubated (150 rpm) at 28°C for 84 hr.

Powdered lyophilised mycelia were prepared as previously described (6,9) and stored desiccated at 4°C until required.

The powdered mycelia (5g) were suspended in 100ml cold 20mM phosphate buffer (pH 7.0) containing 10% v/v glycerol, 2mM monothioglycerol, 1mM EDTA and 2mM magnesium chloride (buffer A), containing 0.5mM toluene sulphonyl fluoride as a protease inhibitor. The homogenate was gently titurated for 30 min and centrifuged at 30 000 × g. The resulting supernatant was used as the crude enzyme preparation.

Enzyme activity was assayed by adding 1ml of enzyme preparation to 2ml buffer A containing 10mM NADPH. The reaction was initiated by adding 20µg NA dissolved in 20µl dioxane. The mixture was then incubated for 1 hr at 28°C. The reaction was stopped by shaking with 10 ml ethyl acetate and the AVN so produced was assayed for as described previously (8).

Protein concentrations of the active enzyme preparations were determined spectophotometricaly at 230 and 260 nm (10).

Preparation of the affinity matrix (11). A sample of Î-aminohexylagarose (AHA) (20 ml gel) was suspended in 40 ml 0.2M sodium borate buffer (pH 9.3). Twenty ml of a solution of p-nitrobenzoyl azide (0.1M) in dimethylformamide (DMF) was added to the gel suspension, and the mixture was gently stirred at 50°C for 1 hr and then at 25°C for 12 hr. Trinitrobenzenesulphonic acid was used to check for the completion of the acylation reaction (12). The gel was washed with cold distiled water containing 50% DMF. The p-nitrobenzamidoalkylagarose was suspended in a solution of 0.2 M sodium dithionite in 0.5 M sodium bicarbonate (pH 8.5) and the mixture was shaken for 2 hr at 40°C. The gel was filtered, washed with distiled water and suspended in an equal volume of ice-cold 0.5M hydrochloric acid. Sodium nitrite (0.1M) was added to the gel suspension and gently stirred for 7 min in an ice-bath. The gel was filtered and washed thoroughly with cold distiled water. It was suspended in an equal volume of cold phosphate buffer (0.2M, pH 7.0). NA (10mg) was dissolved in 20 ml phosphate buffer (0.2M, pH 7.0) : DMF (2:1, v/v). The NA solution was added to the diazonium agarose suspension, which was maintained on ice. The formation of a coloured gel (brick-red) began immediately, and coupling was complete in 30 min. The NA-agarose matrix was warmed to room temperature, filtered, and washed with distiled water.

The affinity gel was methylated by reacting it with diazomethane (prepared as per manufacturer's instructions). The affinity gel was suspended in 50% aqueous acetone (20 ml). Diazomethane in ether (20 ml) was gently added to the NA-agarose slurry and the methylation reaction proceeded at 25°C for 6 hr. The reaction was complete when the ether layer became colourless. Excess ether was decanted, and the gel was washed with water. The gel was then filtered and suspended in buffer A.

The NA-agarose was packed into a glass column (1.1 × 20 cm) and equilibrated with buffer A. The crude enzyme preparation (60 ml) was applied directly to the column, and unbound proteins were eluted with buffer A at a flow rate of 20 ml/hr, until the absorbance at 280 nm of the eluate reached a constant value of about 0.05 units. The enzyme was eluted with a linear gradient of 0 - 0.5 M KCl (40 ml) in buffer A, 4 ml fractions being collected. Each fraction was then assayed for norsolorinic acid dehydrogenase activity.

The active enzyme fraction from the NA-agarose column was analysed for homogeneity by 7.5% non-denaturing polyacrylamide gel electrophoresis (PAGE).

RESULTS AND DISCUSSION

The preparation of the initial affinity matrix depended on the diazotised agarose derivative coupling to the hydroxy-anthraquinone moiety of NA, at either positions 4,5 or 7 (Fig. 1) as directed by the hyroxyl (phenyl) groups. The reaction proceeded smoothly and was irreversible, as evidenced by the brick-red colour of the gel that could not be dislodged from the gel by extensive washing with buffers at various pH values.

The initial purification of the enzyme by this ligand proved disappointing, however, in that little or no NA

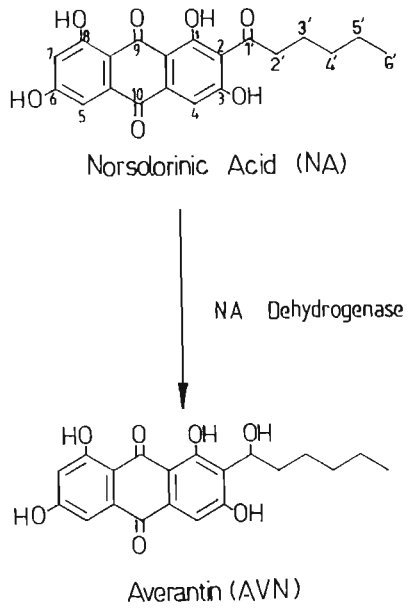
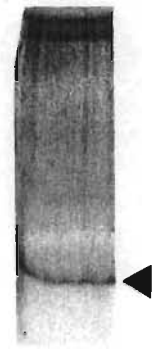


Figure 1. The conversion of norsolorinic acid to averantin by NA dehydrogenase.

dehydrogenase activity was recovered in the bound fraction. It was presumed that this was not due to a lack of specific binding between the enzyme and its immobilized substrate, since the points of attachment to the anthraquinone ring (either 5 or 7) would leave sufficient space for the enzyme to recognize the region of the molecule to which it normally binds (i.e. position 1') (Fig. 1).

A more likely reason for the loss of activity was the presence of phenol groups in the NA molecule, which seem to deactivate the enzyme when present in high concentrations. This effect has been noticed when assaying for enzyme activity using free substrate, and is particularly evident in purified enzyme preparations. It is presumed that this effect will be prevalent *in vitro* rather than *in vivo*, since in the latter case NA will tend to be associated with a membrane due to its hydrophobic character, and in addition, subsequent enzymes in the biosynthetic pathway will utilize NA, effectively preventing its accumulation. In order to test the potential adverse effects of phenolic groups, the affinity matrix was methylated by diazomethane, with conditions chosen to block only the more acidic groups at positions 5 and 7, leaving the less active hydroxyl group at position 1' available for specific enzyme recognition. That this treatment was effective was confirmed when the matrix changed colour from brick-red to orange.

When the crude enzyme preparation was applied to the methylated matrix, a highly active, purified enzyme solution was eluted in the bound fraction (Fig. 2). Non-denaturing PAGE of this fraction revealed one major



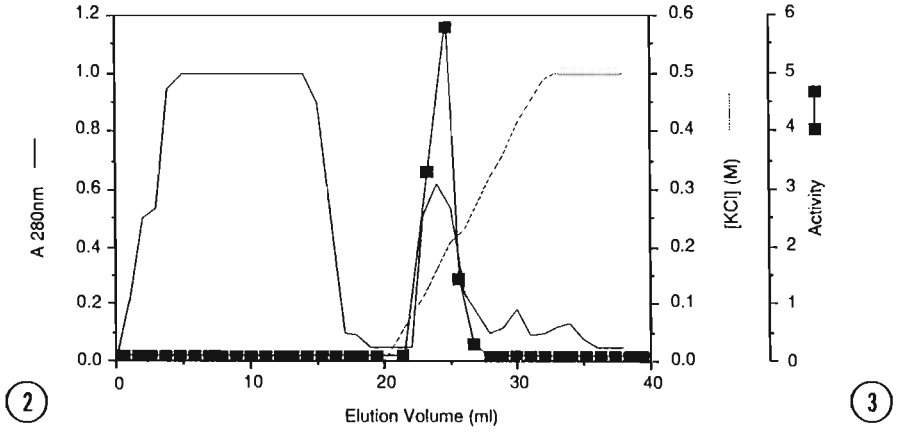


Figure 2. Elution profile for the affinity chromatographic separation of NA dehydrogenase on NA-Agarose. Crude enzyme (60 ml) was applied to 20 ml of the affinity matrix and washed with buffer A. Bound proteins were eluted by the addition of a linear gradient of KCl.
——— A 280 nm; ------- KCl; ■—■ NA dehydrogenase activity.

Figure 3. Electrophoretogram of NA dehydrogenase fraction after purification by affinity chromatography on NA-agarose.

and two minor protein constituents (Fig. 3). The major band, when excised and extracted from an unstained gel, contained all the NA dehydrogenase activity, whereas the two minor bands showed no activity towards NA.

Table 1. Purification of NA Dehydrogenase

Step	Vol. (ml)	Total Units [1]	Total Protein (mg)	Specific Activity (U/mg)	Purification Fold
Crude Extract	60.0	190	360.0	0.53	1
NA-Agarose	6.0	95	1.3	73.0	138

[1] A Unit is defined as that amount of enzyme which will catalyse the conversion of 1 nmole norsolorinic acid to averantin per minute at pH 7.0 and 25°C.

The matrix described herein appears to display specific affinity towards NA dehydrogenase, and yielded a 138-fold purification of the enzyme in a single step from the crude preparation (Table 1).

The rapid purification of NA dehydrogenase from large amounts of contaminating fungal proteins has allowed the full characterization of this enzyme, and suggests that affinity chromatography should find wide applicability in the isolation of other secondary metabolic enzymes in the aflatoxin pathway.

ACKNOWLEDGMENTS

This work was supported by grants from the University of Natal and the Council for Scientific and Industrial Research.

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Reprinted from Biochemical and Biophysical Research Communications 166, 38–42 (1990)

Mycopathologia **113**: 41–44, 1991.

The appearance of an enzyme activity catalysing the conversion of norsolorinic acid to averantin in *Aspergillus parasiticus* cultures

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Received 30 October 1989; accepted in revised form 11 June 1990

Key words: Aspergillus parasiticus, averantin, norsolorinic acid, secondary metabolic enzyme

Abstract

The activity of the enzyme responsible for the conversion of norsolorinic acid to averantin was studied in two strains of *Aspergillus parasiticus*. Cell-free extracts of the enzyme were purified from different aged mycelia and little activity was found prior to 24 hours after inoculation but this quickly reached a maximum at 48 hours and declined thereafter. Both strains of *A. parasiticus*, one in aflatoxin producing strain, the other a versicolorin A accumulating mutant, showed this trend. It was concluded that the enzyme responsible for this conversion was a secondary metabolic enzyme and was distinct from alcohol and mannitol dehydrogenases.

Introduction

Early elucidation of the mechanisms of aflatoxin biosynthesis centred on the establishment of intermediates in the pathway [1]. The identities of several intermediates were determined using radio-isotopes, nuclear magnetic resonance spectroscopy with stable isotopes and mutants of *Aspergillus parasiticus* [2, 3]. Regulation of the pathway involves complex controls, which are strongly influenced by specific cellular events in the growth cycle [4, 5].

Current research has concentrated on the terminal enzyme catalysed steps of aflatoxin B_1 (AFB_1) biosynthesis, i.e., the conversion of sterigmatocystin (ST) to *O*-methylsterigmatocystin (OMST) [6, 7] and both these substrates to AFB_1. The enzymes responsible for the earlier steps in the pathway have received less attention. Consequently we are currently studying the enzyme [8] responsible for the conversion of norsolorinic acid (NA) to averantin (AVN) (Fig. 1). Both these metabolites have been identified as intermediates in aflatoxin biosynthesis [1, 9].

In this study the activity of the enzyme, responsible for the conversion of NA to AVN, during the life cycle of *A. parasiticus* is investigated.

Materials and methods

The metabolites NA and AVN were isolated and purified [9, 10] from mutants of *A. parasiticus* (NOR-1) and (*ver-mu*-39) both kindly donated by Dr J.W. Bennett, University of Tulane. A versicolorin A accumulating mutant of *A. parasiticus* (1-11-105 Wh1) also supplied by Dr Bennett and an aflatoxin producing strain of *A. parasiticus*

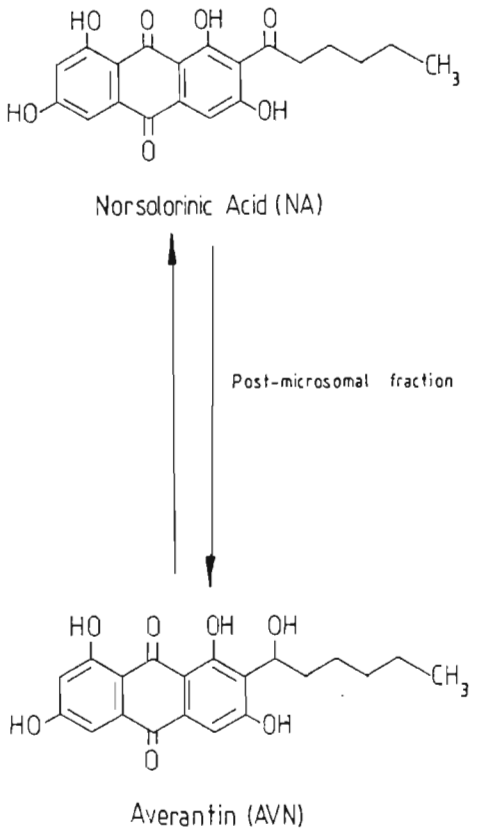

Fig. 1. Schematic representation of the conversion of norsolorinic acid to averantin.

(N1X) were maintained on potato dextrose agar (PDA) and used in the enzyme studies.

Cell-free preparation. One-hundred milliliters of sterile, chemically defined medium [11] in 250 ml Erlenmeyer flasks was inoculated with a spore suspension (10^6 spores per ml) of the stock culture and shake incubated (150 rpm) at 28 °C. At various time intervals (from 24 to 170 h) after inoculation, the mycelia from cultures were harvested from various flasks by vacuum filtration. The resultant mycelia were washed with cold sterile distilled water, lyophilised and weighed. Each lyophilised mycelium was fragmented by gently mulling in a dry chilled mortar. The powdered mycelium (2 g) was suspended in 50 ml cold 20 mM phosphate buffer (pH 7.0) containing 10% v/v glycerol, 2 mM monothioglycerol, 1 mM EDTA and 2 mM magnesium chloride (buffer A). Toluene sulphonyl fluoride (0.5 mM) (purchased from Sigma Chemical Co., St Louis, Mo.) was added. The homogenate was gently triturated for 30 min and centrifuged (30000 × *g*). The pellet was discarded and the supernatant was either assayed for enzyme activity or further centrifuged (105000 × *g*) to yield a post microsomal fraction (supernatant) and microsomal fraction (pellet). The latter was resuspended in a volume of buffer A equal to that of the original supernatant.

Enzyme assay. Enzyme activity was assayed by adding 1 ml of the preparation to 2 ml of buffer A containing either 10 mM NADPH or $NADP^+$. The reaction was initiated by adding the substrate, 30 μg NA dissolved in 30 μl dioxane. The mixture was then incubated for 1 h at 28 °C, the reaction being stopped by shaking with 10 ml ethyl acetate. The ethyl acetate was separated and the aqueous fraction further extracted with 10 ml ethyl acetate the organic solvent fractions being pooled, dried over anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen. The dried residue was redissolved in 100 μl ethyl acetate and a 20 μl portion was spotted onto the origin of a thin layer chromatography (tlc) plate (10 × 10 cm aluminium backed Kieselgel 60; Merck). The plate was developed in chloroform-ethyl acetate-acetone (85 : 15 : 10, v/v/v) which gave an R_F value for NA of 0.85 and AVN 0.64.

Quantitation of metabolites. This was done by high performance liquid chromatography (hplc) using a Varian model 5000 instrument equipped with a UV detector and integrator, on a reverse phase column (Spherisorb S50 DSI, Phase Separations, Clwyd, U K) and methanol-tetrahydrofuran (2 : 1, v/v) as the eluting solvent [12]. The retention times for NA and AVN were 10.1 and 8.2 min respectively with a flow rate of 2 ml/min.

Protein concentrations of the cell-free preparations were determined by measuring their absorbance at 230 and 260 nm [13].

Results and discussion

The enzyme activity catalysing the conversion of NA to AVN was found to be located in the post-microsomal fraction, i.e., in the cytosol. This is in keeping with the location of other simple dehydrogenases such as some alcohol dehydrogenases. From prolonged incubation of the reaction mixtures and lack of the appearance of other metabolites, it was concluded that other enzyme activities responsible for the conversion of AVN in the aflatoxin biosynthetic pathway, were inactive or absent. The promotion of the reaction in the desired direction (it is reversible) [8, 14, 16] (Fig. 1) was ensured by adding an excess of reduced coenzyme. In the presence of equal amounts of NADPH and $NADP^+$, the reaction in the direction of AVN was promoted. In the

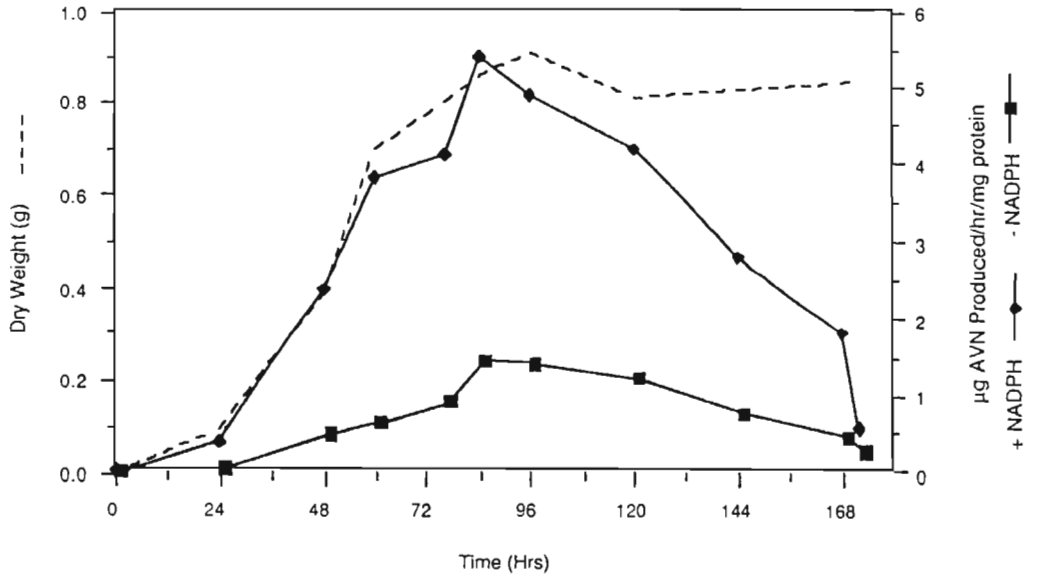

Fig. 2. Production of AVN by post-microsomal fraction of the mutant *Aspergillus parasiticus* (1-11-105 Wh1).

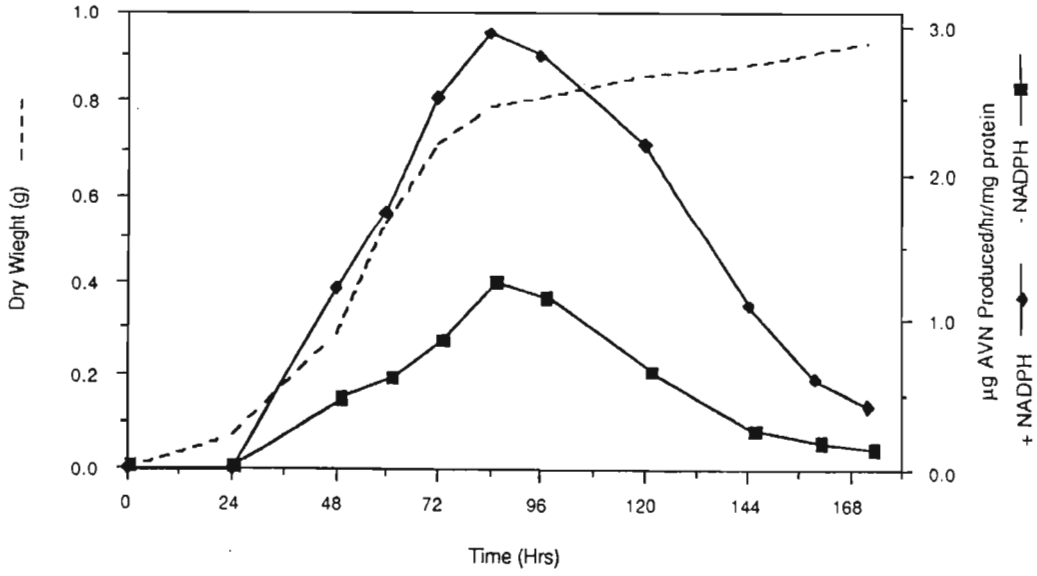

Fig. 3. Production of AVN by post-microsomal fraction of wild-type *Aspergillus parasiticus* (N1X).
---------- = Dry mycelial weight (g) —■— = − NADPH —◆— = + NADPH

absence of both cofactors AVN is produced by the post-microsomal fraction (Fig. 2, 3). For the purposes of this investigation, the enzyme activity responsible for the conversion of NA to AVN was monitored since NA is formed before AVN in the AFB_1 biosynthetic pathway [3, 9].

The enzyme activity was absent in 24 h old mycelium of both strains of *A. parasiticus*, when the organisms were in their trophophase. This then rapidly increased to a maximum conversion of NA by the praparation from 84 h old mycelium again for both fungal strains examined (Fig. 2 & 3). From the growth curve, this age of mycelium can be considered to be equivalent to the idiophase. Thence after the activity declined, indicating the final senescence of the organisms. These results indicate that the enzyme is of a secondary metabolic type and has no function in the growth of the organism. The variation of its activity with respect to age of mycelium is very similar to studies done on other secondary metabolic enzymes, e.g., sterigmatocystin methyl transferase [15] which confirms this view.

Addition of various other carbonyl and hydroxyl containing substrates in the presence of NADPH [16], i.e., acetone, acetophenone, mannitol, ethanol and propan-2-ol to the extract with the optimum activity (from 84 h old mycelium) had no detectable effect in enzyme activity showing that they were not competitive substrates. By deduction the enzyme has a degree of specifity and is not a general alcohol dehydrogenase.

Acknowledgements

This work was supported by grants from the University of Natal and the C.S.I.R.. Thanks is also due to R. Berry for technical assistance.

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Vol. 166, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1990 Pages 38-42

THE PREPARATION OF AN ENZYME ASSOCIATED WITH AFLATOXIN BIOSYNTHESIS BY AFFINITY CHROMATOGRAPHY

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Received November 9, 1989

Summary: An affinity matrix for the purification of norsolorinic acid dehydrogenase, an enzyme involved in aflatoxin biosynthesis, was prepared by coupling norsolorinic acid to an agarose gel. This matrix was found to be ineffective in isolating active enzyme, and was therefore modified by methylation, using diazomethane. The methylated matrix produced a one-step purification of the enzyme from a crude homogenate, resulting in a 138-fold purification. The active isolate was found to contain one major and two minor bands upon non-denaturing electrophoresis, and all the norsolorinic acid dehydrogenase activity was associated with the major band. It was concluded that the matrix exhibited true affinity for the enzyme, and that affinity chromatography was a valuable approach to isolating other secondary metabolic enzymes involved in the biosynthesis of the aflatoxins.

Aflatoxins are biologically active, polyketide derived secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus*. Knowledge of this biosynthetic pathway arises mainly from studies using nuclear magnetic resonance with stable isotopes (1) and mutants of *A. parasiticus*, which have the pathway blocked at specific points (2). Regulation of the pathway involves complex controls, which are strongly influenced by specific cellular events in the growth cycle (3,4).

To date very few enzymes involved in the biosynthesis of the aflatoxins have been isolated and fully characterized. Recently, however, an homogeneous protein has been obtained from *A. parasiticus* with methyl transferase activity (5). The presence of this enzyme has been known for some time (6) when it was found to convert sterigmatocystin to O-methylsterigmatocystin. The enzymes responsible for the earlier steps in the pathway have received less attention. Consequently we investigated the conversion of norsolorinic acid (NA) to averantin (AVN) by NA dehydrogenase (7,8) (Fig. 1).

In order to isolate this secondary metabolic enzyme with the minimum number of separation steps and the maximum of purification and activity, the technique of affinity chromatography has been utilized. The results of this work are presented here.

0006-291X/90 $1.50

MATERIALS AND METHODS

Nicotinamide cofactors, ω–aminohexylagarose and toluene sulphonyl fluoride were purchased from Sigma Chemical Co., USA. N-methyl-N-nitroso-p-toluene-sulphonamide (Diazald) was obtained from Aldrich Fine Chemicals, USA.

A versicolorin A accumulating mutant of *A. parasiticus* (1-11-105 Wh1, kindly donated by Dr. J. W. Bennet) was used as the source of crude enzyme.

A cell free extract was prepared by inoculating 100ml of sterile, chemically defined medium (8) in 250ml Erlenmeyer flasks with a 1ml spore suspension ($1x10^6$ spores), and shake incubated (150 rpm) at 28°C for 84 hr.

Powdered lyophilised mycelia were prepared as previously described (6,9) and stored desiccated at 4°C until required.

The powdered mycelia (5g) were suspended in 100ml cold 20mM phosphate buffer (pH 7.0) containing 10% v/v glycerol, 2mM monothioglycerol, 1mM EDTA and 2mM magnesium chloride (buffer A), containing 0.5mM toluene sulphonyl fluoride as a protease inhibitor. The homogenate was gently titurated for 30 min and centrifuged at 30 000 × g. The resulting supernatant was used as the crude enzyme preparation.

Enzyme activity was assayed by adding 1ml of enzyme preparation to 2ml buffer A containing 10mM NADPH. The reaction was initiated by adding 20μg NA dissolved in 20μl dioxane. The mixture was then incubated for 1 hr at 28°C. The reaction was stopped by shaking with 10 ml ethyl acetate and the AVN so produced was assayed for as described previously (8).

Protein concentrations of the active enzyme preparations were determined spectophotometricaly at 230 and 260 nm (10).

Preparation of the affinity matrix (11). A sample of Î-aminohexylagarose (AHA) (20 ml gel) was suspended in 40 ml 0.2M sodium borate buffer (pH 9.3). Twenty ml of a solution of p-nitrobenzoyl azide (0.1M) in dimethylformamide (DMF) was added to the gel suspension, and the mixture was gently stirred at 50°C for 1 hr and then at 25°C for 12 hr. Trinitrobenzenesulphonic acid was used to check for the completion of the acylation reaction (12). The gel was washed with cold distiled water containing 50% DMF. The p-nitrobenzamidoalkylagarose was suspended in a solution of 0.2 M sodium dithionite in 0.5 M sodium bicarbonate (pH 8.5) and the mixture was shaken for 2 hr at 40°C. The gel was filtered, washed with distiled water and suspended in an equal volume of ice-cold 0.5M hydrochloric acid. Sodium nitrite (0.1M) was added to the gel suspension and gently stirred for 7 min in an ice-bath. The gel was filtered and washed thoroughly with cold distiled water. It was suspended in an equal volume of cold phosphate buffer (0.2M, pH 7.0). NA (10mg) was dissolved in 20 ml phosphate buffer (0.2M, pH 7.0) : DMF (2:1, v/v). The NA solution was added to the diazonium agarose suspension, which was maintained on ice. The formation of a coloured gel (brick-red) began immediately, and coupling was complete in 30 min. The NA-agarose matrix was warmed to room temperature, filtered, and washed with distiled water.

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The initial purification of the enzyme by this ligand proved disappointing, however, in that little or no NA

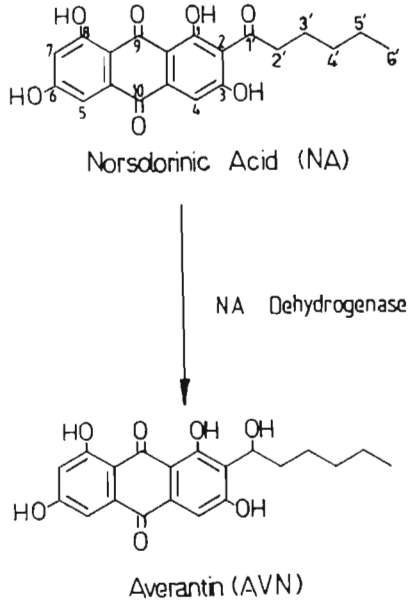

Figure 1. The conversion of norsolorinic acid to averantin by NA dehydrogenase.

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A more likely reason for the loss of activity was the presence of phenol groups in the NA molecule, which seem to deactivate the enzyme when present in high concentrations. This effect has been noticed when assaying for enzyme activity using free substrate, and is particularly evident in purified enzyme preparations. It is presumed that this effect will be prevalent *in vitro* rather than *in vivo*, since in the latter case NA will tend to be associated with a membrane due to its hydrophobic character, and in addition, subsequent enzymes in the biosynthetic pathway will utilize NA, effectively preventing its accumulation. In order to test the potential adverse effects of phenolic groups, the affinity matrix was methylated by diazomethane, with conditions chosen to block only the more acidic groups at positions 5 and 7, leaving the less active hydroxyl group at position 1' available for specific enzyme recognition. That this treatment was effective was confirmed when the matrix changed colour from brick-red to orange.

When the crude enzyme preparation was applied to the methylated matrix, a highly active, purified enzyme solution was eluted in the bound fraction (Fig. 2). Non-denaturing PAGE of this fraction revealed one major

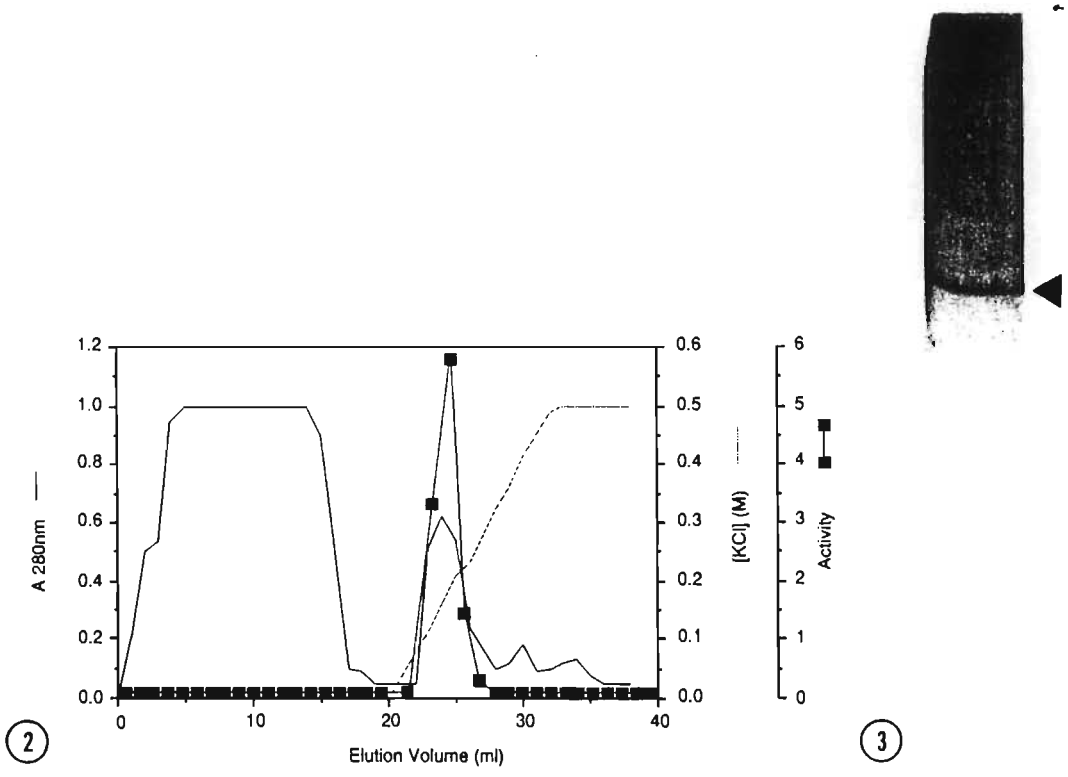

Figure 2. Elution profile for the affinity chromatographic separation of NA dehydrogenase on NA-Agarose. Crude enzyme (60 ml) was applied to 20 ml of the affinity matrix and washed with buffer A. Bound proteins were eluted by the addition of a linear gradient of KCl.
——— A 280 nm; ------- KCl; ■—■ NA dehydrogenase activity.

Figure 3. Electrophoretogram of NA dehydrogenase fraction after purification by affinity chromatography on NA-agarose.

and two minor protein constituents (Fig. 3). The major band, when excised and extracted from an unstained gel, contained all the NA dehydrogenase activity, whereas the two minor bands showed no activity towards NA.

Table 1. Purification of NA Dehydrogenase

Step	Vol. (ml)	Total Units [1]	Total Protein (mg)	Specific Activity (U/mg)	Purification Fold
Crude Extract	60.0	190	360.0	0.53	1
NA-Agarose	6.0	95	1.3	73.0	138

[1] A Unit is defined as that amount of enzyme which will catalyse the conversion of 1 nmole norsolorinic acid to averantin per minute at pH 7.0 and 25°C.

The matrix described herein appears to display specific affinity towards NA dehydrogenase, and yielded a 138-fold purification of the enzyme in a single step from the crude preparation (Table 1).

The rapid purification of NA dehydrogenase from large amounts of contaminating fungal proteins has allowed the full characterization of this enzyme, and suggests that affinity chromatography should find wide applicability in the isolation of other secondary metabolic enzymes in the aflatoxin pathway.

ACKNOWLEDGMENTS

This work was supported by grants from the University of Natal and the Council for Scientific and Industrial Research.

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Reprinted from Biochemical and Biophysical Research Communications 166, 38–42 (1990)

PREPARATIVE BIOCHEMISTRY, 21(2&3), 125-140 (1991)

THE AFFINITY PURIFICATION AND CHARACTERIZATION OF A DEHYDROGENASE FROM *Aspergillus parasiticus* INVOLVED IN AFLATOXIN B_1 BIOSYNTHESIS

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ABSTRACT

A two step scheme has been developed for the purification of a dehydrogenase from mycelia of 84 hours old *Aspergillus parasiticus* (1-11-105 Wh 1), which catalyzes the conversion of norsolorinic acid (NA) to averantin (AVN). The dehydrogenase was purified from cell-free extracts using reactive green 19-agarose and norsolorinic acid-agarose affinity chromatography. The latter affinity matrix was synthesised by attaching norsolorinic acid to ω-aminohexylagarose. The purified protein was shown to be homogenous on non-denaturing polyacrylamide gel electrophoresis. A final purification of 215-fold was achieved. Results of gel filtration chromatography indicated the approximate molecular mass of the native protein to be 140 000 daltons. The isoelectric point of the protein was about 5.5 as determined by chromatofocusing. The reaction catalyzed by the dehydrogenase was optimum at pH 8.5 and between 25° to 35°C. The Km of the enzyme for NA and NADPH was determined to be 3.45 μM and 103 μM respectively.

INTRODUCTION

The aflatoxins are toxic fungal secondary metabolites produced exclusively by strains of *Aspergillus flavus* (Link ex. Fries) and *A. parasiticus* Speare[1,2,3]. Limited progress has however, been made towards developing an understanding of the enzymes involved in aflatoxin B_1 (AFB_1) biosynthesis and their regulation and associated processes[4]. Mutants of *A. parasiticus* and *A. flavus* impaired in aflatoxin synthesis have been useful in studying the aflatoxin biosynthetic pathway, and the identities of several intermediates were determined using radio-isotopes and nuclear magnetic resonance spectroscopy with stable isotopes[1,3,5,6,7].

Secondary metabolic enzymes are subject to induction and regulation just as their primary counterparts[8]. An important difference, however, is that the reactions catalyzed by such enzymes are often controlled by enzyme concentration rather than substrate concentration, as found in primary metabolism[9].

Consequently, 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 on purification the small amount of enzyme present is sensitive to the slightest denaturation. Therefore, many secondary metabolic enzyme preparations fall far short of purification to a single protein, and often are hardly better than crude homogenates.

Recently, an homogenous protein has been obtained from *A. parasiticus* with methyl transferase activity that converted sterigmatocystin (ST) to O-methylsterigmatocystin (OMST)[10]. The purification of this enzyme involved a five step scheme resulting in a one hundred and sixty one fold purification.

The enzymes involved in the early stages of AFB_1 biosynthesis have received less attention. With this in mind, we investigated the conversion of norsolorinic acid (NA) to averantin (AVN) by NA dehydrogenase[4,11] (Figure 1). Preliminary studies showed that this enzyme was found to be located in the post-microsomal fraction, i.e., in the cytosol[11]. A partial purification of the NA dehydrogenase has been obtained using a single affinity chromatographic step[12].

In order to obtain a secondary metabolic enzyme with the minimum of separation steps and the maximum of purification, we have used affinity chromatography to purify the NA dehydrogenase. A two step affinity chromatographic procedure was used to purify the NA dehydrogenase and its physicochemical properties characterized.

MATERIALS AND METHODS

Chemicals. Nicotinamide cofactors, Reactive green 19-agarose (RGA), ω-aminohexylagarose and p-toluene sulphonyl fluoride were purchased from Sigma Chemical Co., USA. N-methyl-N-nitroso-p-toluene-sulphonamide (Diazald) was obtained from Aldrich Fine Chemicals, USA. All other chemicals were of the highest chemical purity.

Fungal strains and growth conditions. All anthraquinone accumulating *A. parasiticus* cultures were kindly donated by Dr J.W. Bennett, University of Tulane, New Orleans, USA.

A non-aflatoxigenic *A. parasiticus* (1-11-105 Wh1) mutant strain which accumulates versicolorin A (VA) was used as the source of enzyme. *Aspergillus parasiticus* (NOR-1) and *A. parasiticus* (AVN-1) were used for the production of NA and AVN.

Mycelia for cell free studies were prepared by inoculating 100 ml of sterile, chemically defined medium[13] in 250 ml Erlenmeyer flasks with a 1 ml spore suspension (*A. parasiticus* 1-11-105-Wh1, 1 x 10^6 spores), and shake incubated at 150 rpm and 28°C for 84 hr. The NA dehydrogenase activity is present at an optimum level under these conditions[11].

Norsolorinic acid and AVN were purified from mycelia of *A. parasiticus* NOR-1 and *A. parasiticus* AVN-1, respectively, by inoculating 400 ml yeast extract sucrose medium[14] in 1 l flasks with 2 ml spore suspensions (10^6 spores/ml) of either fungus. The flasks were incubated in static culture at 28°C for 15-20 days.

Purification of norsolorinic acid and averantin. The 15-20 day old pigmented mycelium was harvested by vacuum filtration, extracted with acetone until colourless and water was added to make a 30% (v/v) aqueous acetone solution. The acetone solution was extracted with hexane to remove lipid, and the pigments were then partitioned from acetone into chloroform (2 volumes). The chloroform extract was dried over anhydrous sodium sulphate and evaporated to dryness on a rotary evaporator (40°C). The dried residue was redissolved in 20 ml chloroform

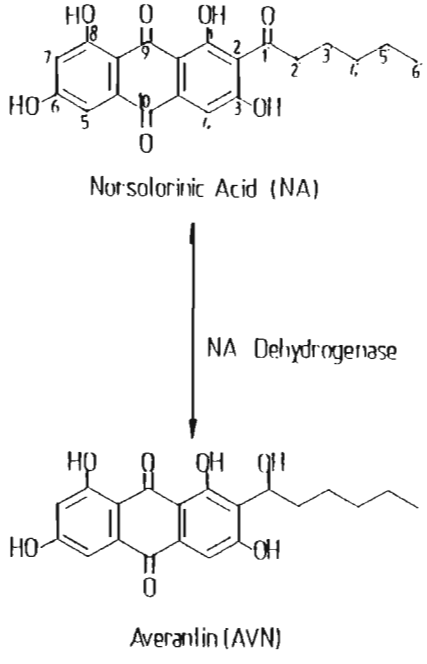

FIGURE 1

The conversion of norsolorinic acid to averantin by NA dehydrogenase.

and the metabolites were then purified by preparative thin layer chromatography (tlc)[15].

The purity of NA and AVN was checked using two dimensional analytical tlc. The metabolite was spotted on the origin of an aluminium backed precoated silica gel 60 plate (10 x 10 cm, Merck). The plate was then developed in a solvent system of chloroform: ethyl acetate: acetone: acetic acid (85: 15: 10: 1, v/v/v/v, CEAA in the first dimension R_f NA= 0.71; R_f AVN= 0.58) and then in petroleum ether: diethyl ether: acetone (7: 3: 2, v/v/v, PEA) solvent system in the second dimension (R_f NA= 0.41; R_f AVN= 0.23). The appearance of a single spot indicated a pure metabolite.

Preparation of cell free extracts. The 84 hr old mycelia were harvested from the growth medium by vacuum filtration and washed with copious amounts of cold sterile distilled water. The damp cake of mycelia was then lyophilised.

The lyophilised mycelia (20 g) were gently fragmented in a mortar with a pestle. The powdered mycelia were suspended in 400 ml cold 20 mM phosphate buffer (pH 7.0) containing 10% v/v glycerol, 2 mM monothioglycerol, 1 mM EDTA and 2 mM magnesium chloride (buffer A), containing 0.5 mM toluene sulphonyl fluoride as a protease inhibitor. The homogenate was gently triturated for 30 min (4°C) and centrifuged (30,000 x g, 20 min, 4°C). The supernatant was collected and stored on ice for the purification of the NA dehydrogenase. All subsequent purification steps were carried out at 4°C.

Enzyme assays. Norsolorinic acid dehydrogenase activity measurements were carried out at 25°C. The reaction mixtures routinely consisted of a final volume of 1 ml of buffer A containing 10 mM NADPH and the enzyme fraction. The assays were initiated by the addition of NA (30 µg dissolved in 30 µl dioxane). The concentrations of the substrates (NA and NADPH) were varied for Km determinations. The mixture was incubated for 1 hr at 28°C, the reaction being stopped by shaking with 10 ml ethyl acetate. The ethyl acetate was separated and the aqueous fraction further extracted with 10 ml ethyl acetate, the organic fractions being pooled, dried over anhydrous sodium sulphate and then evaporated to dryness under a stream of nitrogen. The dried residue was redissolved in 100 µl ethyl acetate and a 20 µl portion was spotted onto the origin of a tlc plate (10 x 10 cm). The plate was developed in CEAA which gave R_f values of 0.71 for NA and 0.58 for AVN respectively.

These metabolites were quantitated spectrophotometrically using extinction coefficients of ε_{235}= 24,500 or ε_{269}= 16,900 M^{-1} cm^{-1} for NA in ethanol and ε_{222}= 26,784 or ε_{262}= 15,810 for AVN in methanol[16, 17]. One unit of enzyme activity was defined as that amount of enzyme which will catalyse the conversion of 1 nmole NA to AVN per min at pH 7.0 and 25°C.

All experimental values from enzyme activity determinations represent the average of at least quadruplicate independent determinations, and linear regression analyses was used to calculate the linearity of plots during the determinations of Km values. The Km for AVN, $NADP^+$, NADH and NAD^+ was also determined (Table 2).

Protein determinations. Column effluents were continously monitored at 280 nm. Total protein concentration was determined by measuring the absorbance at 230 and 260 nm[18].

Synthesis of NA-agarose affinity matrix[12, 19]. Twenty ml of ω-aminohexylagarose was suspended in 40 ml 200 mM sodium borate buffer (pH 9.3). Twenty ml of a solution of p-nitrobenzoyl azide (100 mM) in dimethylformamide (DMF) was added to the gel suspension, and the mixture was gently stirred at 50°C for 1 hr and then at room temp. for 12 hr. Trinitrobenzenesulphonic acid was used to check for the completion of the acylation reaction[20]. The p-nitrobenzamidoalkylagarose gel suspension was washed with cold distilled water containing 50% DMF, suspended in a solution of 200 mM sodium dithionite in 500 mM sodium bicarbonate (pH 8.5) and shaken for 2 hr at 40°C. The gel was filtered, washed with distilled water and suspended in an equal volume of ice cold 500 mM hydrochloric acid. Sodium nitrite (100 mM) was added to the gel suspension and gently stirred for 7 min in an ice-bath. The gel was filtered and washed thoroughly with cold distilled water before being suspended in an equal volume of cold phosphate buffer (200 mM, pH 7.0). Norsolorinic acid (10 mg) was dissolved in 20 ml phosphate buffer (200 mM, pH 7.0) : DMF (2 :1, v/v) and added to the diazonium agarose suspension, which was maintained on ice. The formation of a coloured gel (brick-red) began immediately, and coupling was complete in 30 min. The NA-agarose matrix was warmed to room temp., filtered, and washed with distilled water.

The affinity gel was methylated by reacting it with diazomethane (prepared from Diazald as per manufacturer's instructions). The affinity gel was suspended in 50% aqueous acetone (20 ml). Diazomethane in ether (20 ml) was gently added to the NA-agarose slurry and the methylation reaction proceeded at 25°C for 6 hr. The reaction was complete when the ether layer became colourless. Excess ether was decanted, and the gel was washed with water, filtered and suspended in buffer A.

Gel electrophoresis. Non-denaturing polyacrylamide gel electrophoresis was performed on 7.5% uniform concentration gels, as described by Davis[21]. The gels were stained with Coomassie Blue R-250 and destained in 50% methanol/10% acetic acid (1 hr, 25°C) and then in 5% methanol/70% acetic acid (8 hr, 25°C).

RESULTS

Purification procedures.

Reactive green 19-agarose chromatography.

The cell free preparation (400 ml) was loaded onto an RGA column, equilibrated with buffer A, and then washed extensively with buffer A at a flow rate of 30 ml/hr. The enzyme was eluted with a linear gradient of 0-1.0 M KCl (160 ml) in buffer A (Figure 2). Fractions corresponding to the peak of activity, eluted at about 400 mM KCl, were pooled and dialysed exhaustively against 10 volumes of buffer A.

Norsolorinic acid-agarose affinity chromatography.

The active enzyme fractions from RGA were applied directly at a flow rate of 20 ml/hr on the NA-agarose column, equilibrated with buffer A. The enzyme was eluted with a linear salt gradient of 0-0.5 M KCl (40 ml) in buffer A (Figure 3). The methylated NA-agarose was found to have a high degree of binding specificity for the NA dehydrogenase, resulting in the removal of further contaminating protein in the unbound peak. The assay of fractions in the bound peak showed enzyme specific activity to be 215-fold higher than that in the crude cell free extract (Table 1).

Homogeneity. Non-denaturing PAGE analysis on the active NA-agarose fraction revealed an homogenous protein (Figure 4). The protein band, when excised and extracted in buffer A from an unstained gel, contained all the NA dehydrogenase activity (data not shown).

Enzyme characterization.

Sephacryl S-300 gel filtration chromatography (molecular weight determination).

The crude cell free preparation (40 ml) was loaded onto a Sephacryl S-300 column (2.5 x 95 cm), previously equilibrated with buffer A (2 column volumes) and eluted at a flow rate of 60 ml/hr (4°C). The void volume was estimated to be 100 ml (Figure 5).

Chromatofocusing (isoelectric point determination).

Fractions corresponding to the peak of activity (gel filtration chromatography), were pooled and concentrated to 5 ml by dialysing against a solid substrate (sucrose) at 4°C. The concentrated fraction was then dialysed against 25 mM imidazole buffer, pH 7.4, containing 10% (v/v) glycerol and 2 mM monothioglycerol. This fraction was applied to a PBE 94 chromatofocusing column (1.0 x 20 cm) which was previously equilibrated with the same imidazole buffer. Prior to loading the enzyme, a 5 ml portion of appropriately diluted Polybuffer 74 HCl, pH 4.0, containing 10% (v/v) glycerol and 2 mM monothioglycerol was applied to the column. A pH gradient was developed by applying 160 ml of the same Polybuffer 74 HCl, pH 4.0, at a flow rate of 40 ml/hr. The NA dehydrogenase eluted at a pH of approximately 5.5 (Figure 6). Regeneration of the column was carried out by washing with 10 mM sodium acetate, pH 4.0, with 1 M sodium chloride to release tightly bound proteins.

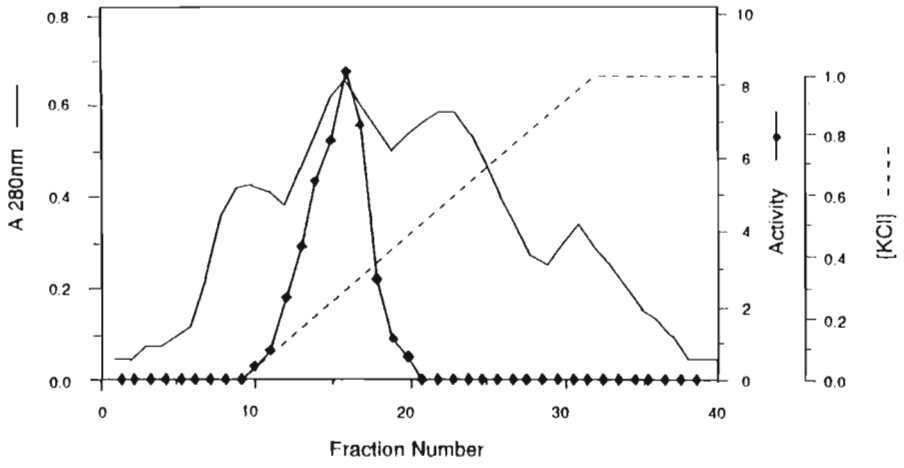

FIGURE 2

Reactive green 19-agarose affinity chromatography of crude cell-free preparation containing NA dehydrogenase activity.

Column; 2.0 x 25 cm : Buffer; buffer A (wash buffer) followed by 0 - 1 M KCl (160 ml) in buffer A (elution buffer) : Flow rate; 30 ml/h : Fractions; 5 ml. The unbound protein peak is not shown.

—— = A_{280}; —•— = Activity; - - - - = [KCl].

pH and Temperature optimum.
Enzyme assays with the purified protein were carried out in a solution buffered from pH 2.0 to 9.0 and measured at intervals of 0.5 pH units. A single pH optimum was recorded at pH 8.5, with a decline in activity below pH 6.5 and above pH 9.0 (Figure 7).
The effect of temperature was investigated by measuring NA dehydrogenase activity at 5°C intervals from 15°C to 60°C. The enzyme activity was found to be maximum at 35°C (Figure 8) under the conditions used but retained its activity for a longer period at 25°C.

Km for substrates.
The Km for both NA and NADPH was determined using Lineweaver-Burk plots. The Km for NA was calculated to be 3.45 µM (Figures 9a and 9b), while the Km for NADPH was found to be 103 µM (Figures 10a and 10b). The Km for AVN was estimated to be 3.72 µM, whilst that of $NADP^+$ was found to be 125 µM (Table 2).

DISCUSSION

The work reported was initiated with the object of studying the initial stages of aflatoxin biosynthesis, viz., the enzymatic conversion of NA to AVN. The presence

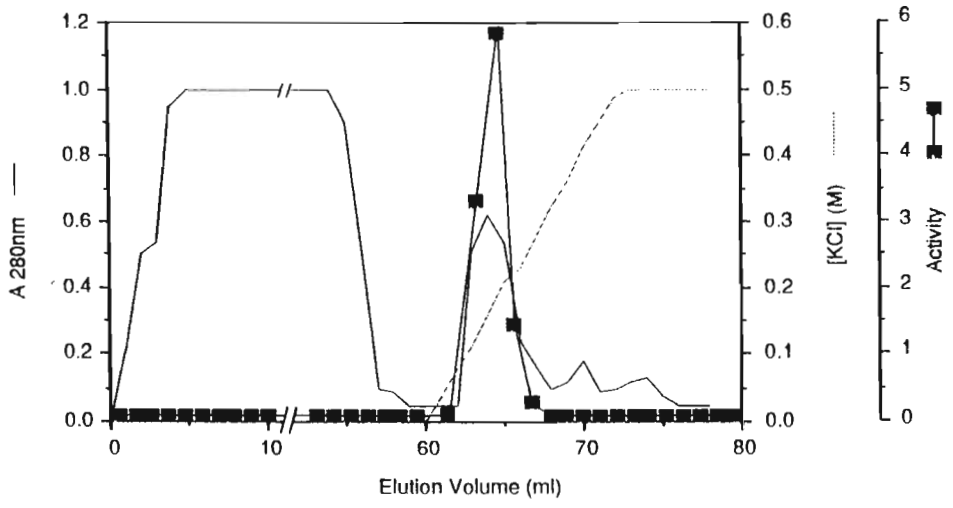

FIGURE 3

Norsolorinic acid-agarose affinity chromatography of pooled fractions from reactive green 19-agarose column containing the NA dehydrogenase activity.

Column; 1.1 x 20 cm : Buffer; Buffer A (wash buffer) followed by 0 - 5 M KCl (40 ml) in buffer A (elution buffer) : Flow rate; 20 ml/h : Fractions; 4 ml.

——— = A_{280}; ■———■ = Activity; ····· = [KCl].

TABLE I

Purification of Norsolorinic Acid Dehydrogenase

Step	Volume (ml)	Total Units[1]	Total Protein (mg)	Specific Activity (U/mg)	Purification (Fold)
Crude Extract	400	1330	2600.00	0.51	1
Reactive Green 19-Agarose	40	540	60.00	9.00	18
Norsolorinic Acid-Agarose	6	115	1.05	110.00	215

(1) A Unit is defined as that amount of enzyme which will catalyse the conversion of 1 nmol norsolorinic acid to averantin per minute at pH 7.0 and 25°C.

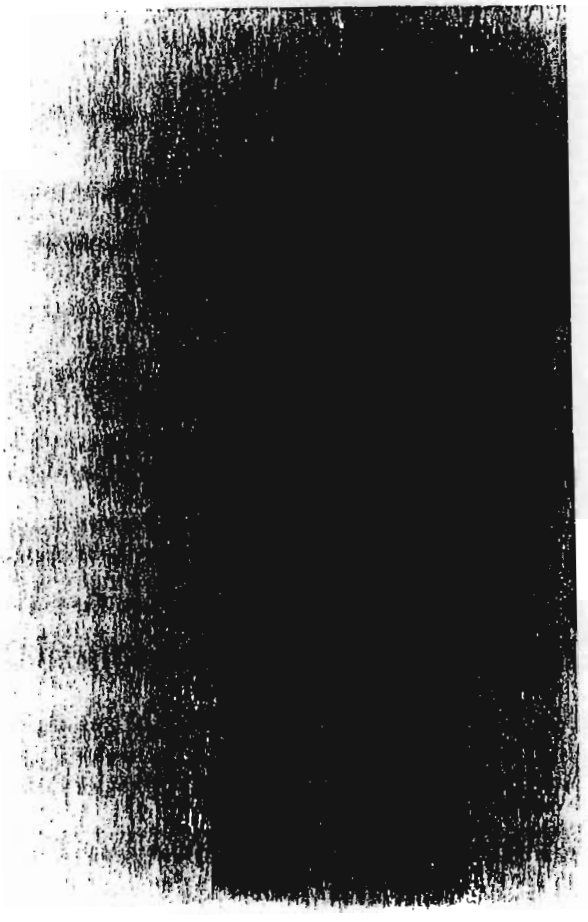

FIGURE 4

Non-denaturing polyacrylamide (7.5%) gel electrophoresis of 10 µg of purified NA dehydrogenase, migrating as a single band.

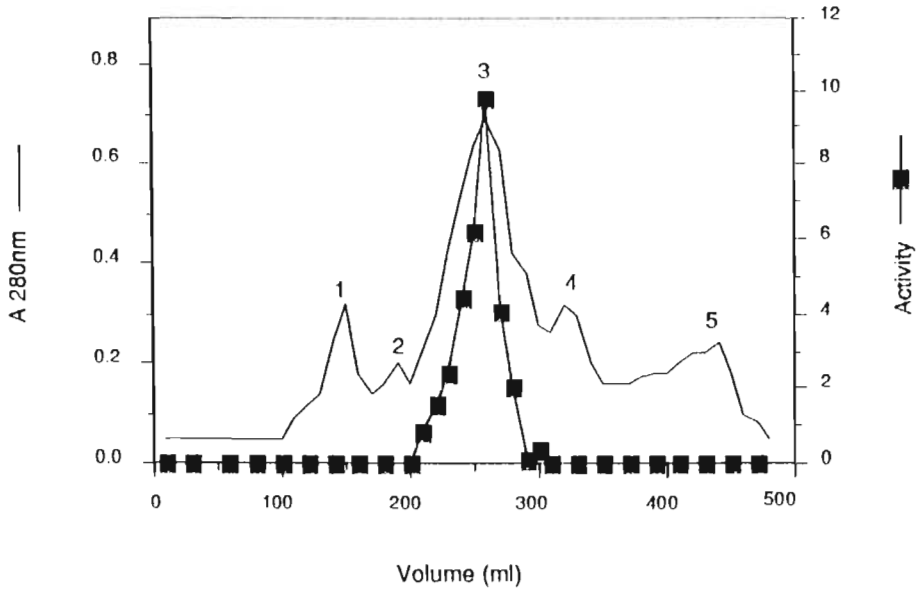

FIGURE 5

Sephacryl S-300 chromatography of NA dehydrogenase from a crude cell-free preparation of *A. parasiticus* (Wh 1). The void volume was determined by the elution of blue dextran. The molecular masses of standards, designated as numbers, are : 1 (catalase, 240 - kDa); 2 (yeast alcohol dehydrogenase, 150 kDa); 3 (lactate dehydrogenase, 140 kDa); 4 (bovine serum albumin, 68 kDa); 5 (myoglobin, 18.8 kDa).

Column; 2.5 x 95 cm : wash buffer; buffer A : Flow rate; 60 ml/h : Fractions; 10 ml.

——— = A_{280}; —■— = Activity.

of this NA dehydrogenase activity has been detected in cell free preparations from *A. parasiticus* mycelia[11,12]. In the current study the purification and properties of the NA dehydrogenase involved in aflatoxin biosynthesis (Figure 1) are reported.

The mutant (*A. parasiticus* Wh1), which accumulates VA, was used as the source of NA dehydrogenase, since its activity is higher than that of the wild type (*A. parasiticus* N1X)[11]. This mutant has also been used as the source of enzyme for activities catalyzing the conversion of ST to OMST and AFB_1[26]. In another study using this mutant, VA synthase activity was present in lower levels than the wild type[27]. This could be due to inhibition/repression by the product (VA), which is present at much higher levels in the mutant than in the wild type.

Starting from cell free preparations of *A. parasiticus* (1-11-105-Wh1) mycelium, two affinity chromatographic steps: RGA and NA-agarose led to the purification

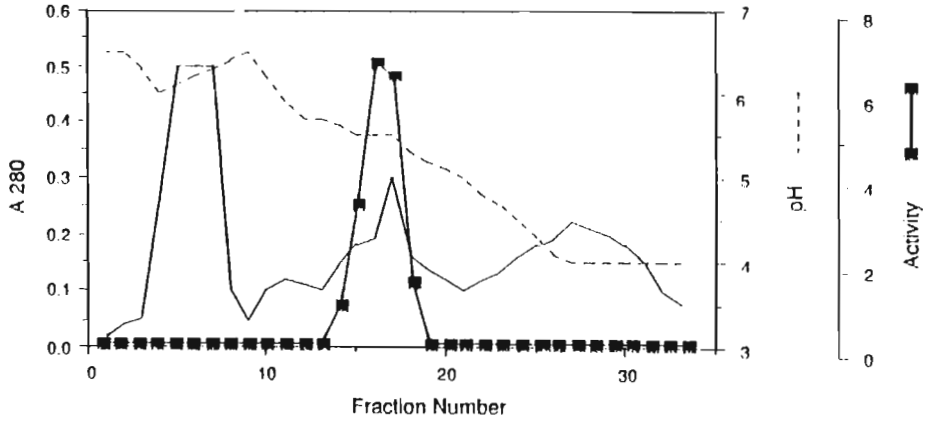

FIGURE 6

PBE 94 chromatofocusing chromatography of pooled fractions from the Sephacryl S-300 column containing NA dehydrogenase activity.

Column; 1.0 x 10 cm : buffer; imidazole buffer (pH 7.4, wash buffer) followed by Polybuffer 74 HCl (pH 4.0, elution buffer) : Flow rate; 45 ml/h : Fractions; 5 ml.

——— = A_{280}; ■——■ = Activity; - - - - = pH.

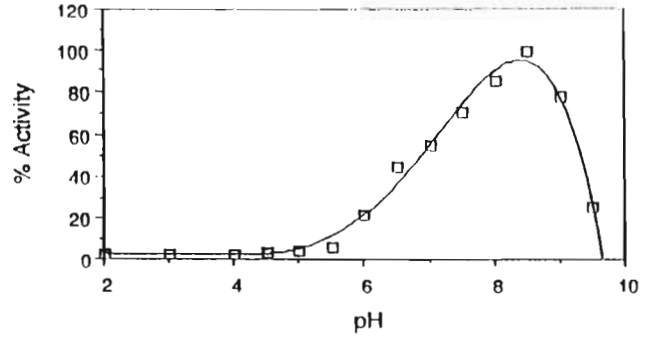

FIGURE 7

Effect of pH on NA dehydrogenase activity. The pH of the assay medium was adjusted using 50 mM glycine-HCl for pH 2.0 to 3.0, 50 mM sodium acetate for pH 3.5 to 5.5 and 50 mM potassium phosphate for pH 6.0 to 9.0. The enzyme concentration was fixed at 60 µg/ml. Enzyme assays were carried out for 15 min according to the procedure described in the Materials and Methods.

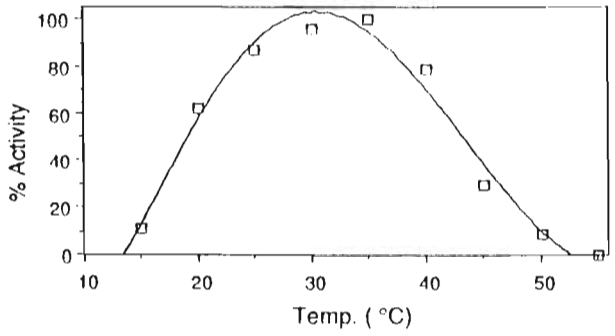

FIGURE 8

Effect of temperature on NA dehydrogenase activity. The enzyme assays were carried out for 15 min at pH 7.5 as described in Materials and Methods, at an enzyme concentration of 60 μg/ml.

of an homogenous protein. The purification provided nearly 1.05 mg of homogenous protein from a mycelial dry weight of approximately 20 g. The NA dehydrogenase appeared to be one of the minor proteins in *A. parasiticus* mycelium, since over a 215-fold purification was required to achieve homogeneity (Table 1). The amount of secondary metabolic enzyme appears low compared to the levels of some major enzymes of primary metabolism; however, secondary metabolic enzymes are formed only after rapid growth has ceased[4]. At this stage, most of the cell's synthesizing machinery has stopped functioning and nutrient supply has become a limiting factor. Secondary metabolic activities therefore are much less vigorous than primary metabolic activities occurring during rapid growth.

The removal of contaminating proteins by the first affinity matrix (RGA) resulted in an increase in enzyme activity. A possible explanation is that most or all the proteases are eluted in the unbound peak and therefore denaturation of the NA dehydrogenase is limited. The initial extraction buffer also contained toluene sulphonyl fluoride, which is a serine protease inhibitor. It is known that fungi have a high content of serine proteases[22] but it may be that other proteases are present which are not of this type. Another explanation for the increased enzyme activity could be due to the removal of a large amount of anthraquinone pigments in the pre-elution washing procedure. The phenolic nature of these pigments seems to cause denaturing of the enzyme. These pigments, although water insoluble, are associated with a membrane *in vivo* and they appear to be present as lipid complexes in the crude preparation.

The purification of the enzyme by the unmethylated NA-agarose affinity matrix proved disappointing in that no NA dehydrogenase activity was recovered in the bound fraction and the wash buffer. It was presumed that this was not due to a lack of specific binding between the enzyme and the immobilised NA, since the points of attachment to the anthraquinone ring (either 5 or 7) would leave

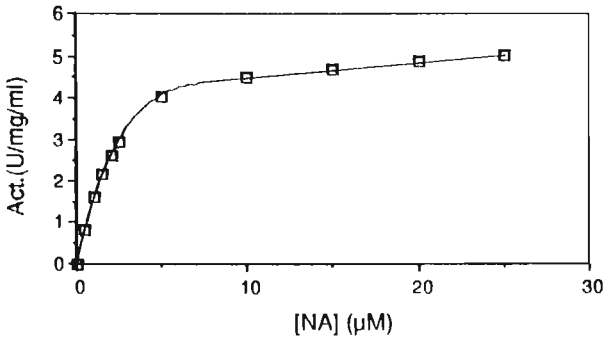

FIGURE 9a

Effect of norsolorinic acid concentration on NA dehydrogenase activity. The enzyme and NADPH concentrations were 60 μg/ml and 250 μM respectively.

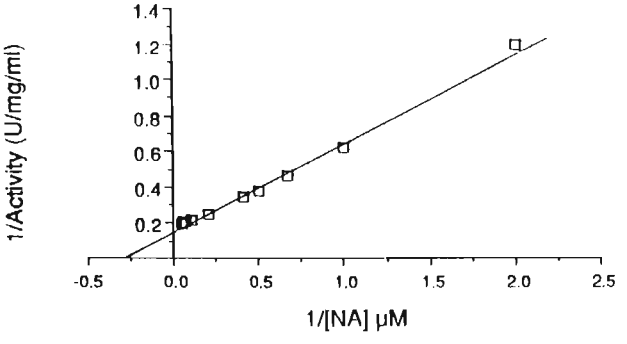

FIGURE 9b

Lineweaver-Burk plot of the data on effect of norsolorinic acid concentration on NA dehydrogenase activity.

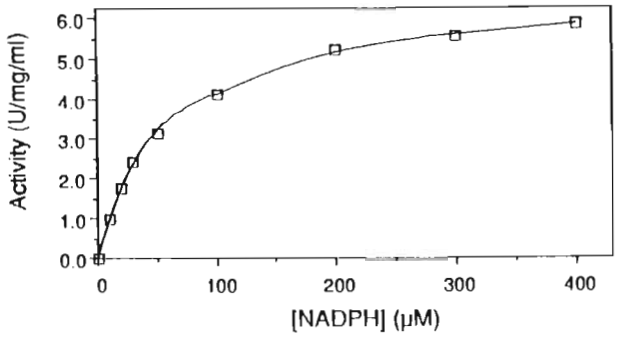

FIGURE 10a

Effect of NADPH concentration versus NA dehydrogenase activity. The enzyme and norsolorinic acid concentrations were 60 μg/ml and 50 μM respectively.

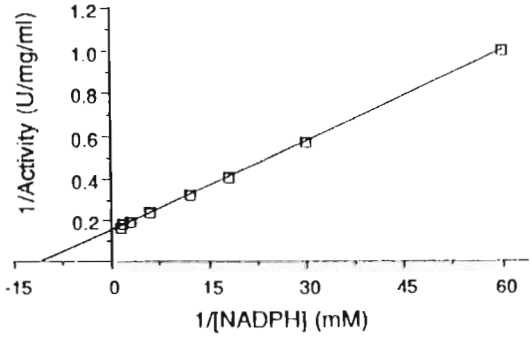

FIGURE 10b

Lineweaver-Burk plot of the data on effect of NADPH concentration versus NA dehydrogenase activity.

TABLE II

Km values for NA dehydrogenase, using NA and AVN with various nicotinamide cofactors

Substrate/Co-factor	Km (μM)	Substrate/Co-factor	Km (μM)
NA	3.45	AVN	3.72
NADPH	103.00	NADP'	125.00
NADH	115.00	NAD'	129.00

sufficient space for the enzyme to recognise the region of the molecule to which it normally binds (i.e. position 1', Figure 1)[12].

A feasible explanation for the loss of activity was the presence of phenol groups in NA, which seem to deactivate the enzyme. This effect has been noticed when assaying for the enzyme activity using free substrate, and is particularly evident in purified preparations. This effect will probably be prevalent *in vitro* rather than *in vivo*, since in the latter case NA will tend to be associated with a membrane due to its hydrophobic character. Thus the phenolic groups in the affinity matrix were methylated, by diazomethane in ether, a procedure that blocks only the more acidic groups at positions 5 and 7 leaving the less active carbonyl at position 1' (Figure 1) available for specific enzyme recognition[12]. Salt gradients proved to be a cheap and effective method of elution as evidenced by the highly purified NA dehydrogenase from NA-agarose, which migrated as a single band on non-denaturing PAGE (Figure 4).

The fractionation of the crude cell free preparation by gel filtration on Sephacryl S-300 showed the presence of a number of A_{280} absorption peaks of which peak 3 (Figure 5) contained the NA dehydrogenase activity. NA dehydrogenase eluted from the column at a position which was after yeast alcohol dehydrogenase but before lactate dehydrogenase, which was consistent with a molecular weight of 140 kD. This molecular weight is similar to that of other secondary metabolic dehydrogenases such as mannitol dehydrogenase[23].

The A_{280} absorption profile of the eluate from the chromatofocusing column displayed four peaks (Figure 6). The NA dehydrogenase activity migrated with the third, major protein peak at approximately pH 5.5. Thus the isoelectric point of this enzyme, as determined by chromatofocusing , is about pH 5.5.

A single pH optimum was recorded at pH 8.5, with a significant decline in activity below pH 6.5 and above pH 9.0 (Figure 7) which is within the range reported for the pH optima (pH 7.5 to 9.5) for other fungal dehydrogenases[23, 24].

The NA dehydrogenase activity was found to be maximum at 35°C (Figure 8) under the conditions used but retained its activity for a longer period at 25°C. The enzyme was rapidly denatured at temperatures higher than 40°C and at temperatures below 20°C, the enzyme showed low activity with only small amounts of AVN being produced. The temperature optima for the enzyme, between 25°C and 40°C, is consistent with that required (25° to 40°C) for maximal aflatoxin production *in vivo*[25].

The Km for NA (3.45 μM) is low which indicates that NA dehydrogenase has a high affinity for this substrate. The Km values for this enzyme should be viewed with caution due to an inadequate environment in terms of solubility of the hydrophobic substrate and product. The substrates were dissolved in dioxane, and it may be that the solvent and insolubility of substrate affected the reaction rate. It was noted, however that at all concentrations of substrates used the solution appeared homogenous indicating complete solubility of the substrate. The Km for AVN (3.72 μM) is slightly higher than that for NA indicating that the enzyme marginally favours the reaction from NA to AVN, at equivalent enzyme concentrations. This observation is further supported by the fact that the enzyme has a greater affinity for NADPH and NADH than NAD^+ and $NADP^+$ although both sets of Km values are of similar orders (Table 2).

The kinetic determinations for NA dehydrogenase posed a problem due to the hydrophobic nature of NA and AVN. As a result of the water insolubility of these metabolites, they were dissolved in a solvent, such as dioxane but not acetone as these metabolites were not fully soluble in this solvent. From the data obtained, Lineweaver-Burk plots seemed the most suitable for the determination of Km values. This method has also been used for Km determinations for other secondary metabolic enzymes such as mannitol dehydrogenase[23], VA synthase[27] and ST methyl transferase[10, 26] involved in AFB_1 formation. In the latter two enzyme systems, versiconal acetate , ST and OMST were used as substrates which are insoluble in water. These substrates were dissolved either in acetone or dimethylformamide. In one study[10] the velocity of the reaction for the methyl transferase was linear up to 3 hr with 12 μg enzyme and 0.5 mM S-adenosyl-methionine. This observation may be due to substrate insolubility; the amount added being in excess of its solubility coefficient ensuring saturation of the assay solution during the course of the reaction. In the case of the kinetic studies here, substrate insolubility of the levels added, did not seem to be a problem possibly due to complexes being formed between the mutual solvent (dioxane) and substrate.

In conclusion, the results show that affinity chromatography can be applied to the separation of secondary metabolic enzymes from crude homogenates with advantage, and in the case of aflatoxin biosynthesis the substrates can be immobilised through their anthraquinone moieties.

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Synthesis of Sterigmatocystin Derivatives and Their Biotransformation to Aflatoxins by a Blocked Mutant of *Aspergillus parasiticus*

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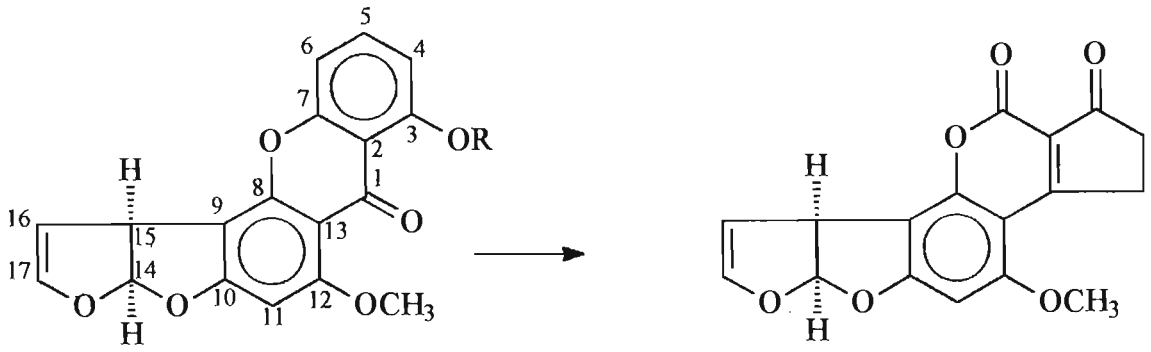
Abstract: Seven analogs of *O*-methylsterigmatocystin were synthesised as structural derivatives of the aflatoxin biochemical pathway. Their conversion to aflatoxin B_1 by whole cell feeding studies using a mutant of *Aspergillus parasiticus* is described.

Key words: *Aspergillus parasiticus,* sterigmatocystin, derivatives, specificity.

Introduction

Aflatoxins are a group of secondary metabolites produced by several species of moulds of the genus *Aspergillus* [1]. Aflatoxin B_1 (AFB_1)(**3**) is the most abundant of the group and is highly carcinogenic [2]. It is considered to be derived from the xanthone derivative, sterigmatocystin (ST)(**1**).



1. R = H

3.

2a. $R = CH_3$
2b. $R = CH_2CH_3$
2c. $R = CH_2CH_2CH_2$
2d. $R = CH_2(CH_2)_2CH_3$
2e. $R = CH_2(CH_2)_3CH_3$
2f. $R = CH_2CH{=}CH_2$
2g. $R = COCH_3$
2h. $R = COC_6H_5$

The incubation of possible intermediates with mutants of *Aspergillus parasiticus*, blocked early in the pathway that generated **3** illustrated their role as biogenetic precursors [3]. The currently accepted scheme for the aflatoxin pathway (Figure 1.) is based on data obtained from feeding studies using isotopically labeled precursors [4].

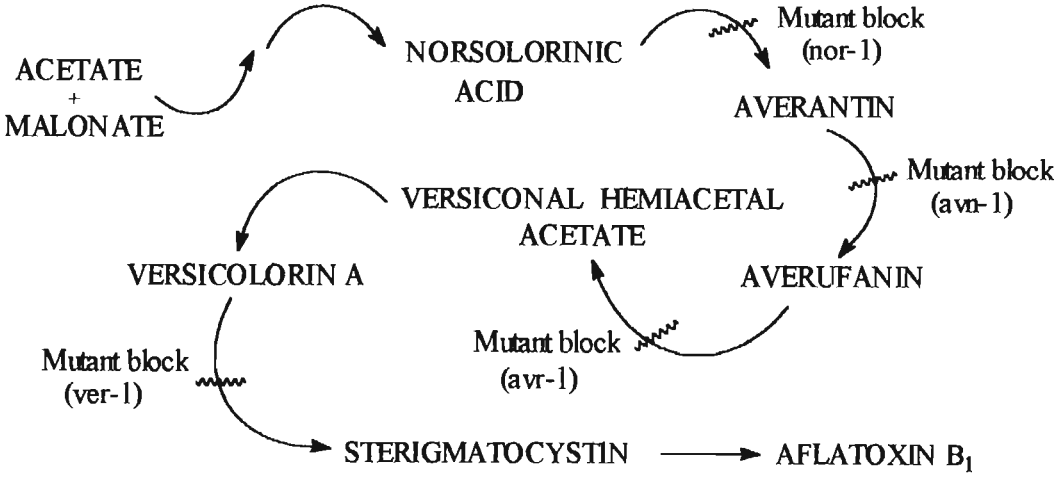


Figure 1. Scheme for aflatoxin biosynthesis and sites for blocked mutants [4].

The biosynthesis of **3** has been the subject of conflicting speculation and numerous reviews [5-8]. It has been proposed by some researchers [9-12] that the biosynthetic pathway involves the conversion of **1** to **3** via *O*-methylsterigmatocystin (OMST)(**2a**). As part of a program to investigate this step in AFB_1 biosynthesis, a series of derivatives of the free hydroxyl group at C-3 of **1** were prepared with a view to studying their conversion to **3** by active fungal systems.

We now report the synthesis [13] of alkyl and aryl sterigmatocystin derivatives (**2b-2h**) by employing aliphatic alkyl halides and acid chlorides. Their conversion to AFB_1 by whole cell feeding studies using a mutant of *A. parasiticus*, which lacks the enzymatic system to produce **1**, were studied in an attempt to elucidate the role of OMST in aflatoxin B_1 biosynthesis.

Materials and Methods

General

Melting points are uncorrected and obtained using an Electrothermal IA9000 digital melting point apparatus. High resolution masses and mass spectra were recorded on a Kratsos 9/50 mass spectrometer operating at an ionization potential of 70 eV. ^1H and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a Varian Gemini 300 spectrophotometer (operating at 300 MHz for ^1H and 75 MHz for ^{13}C).

High pressure liquid chromatography was carried out using a Perkin Elmer Liquid Chromatograph with a 20 µl injection loop equipped with a Perkin Elmer pump and linked to a fluorescence detector. A stainless steel (150mm x 4.60 mm) column packed with 5 micron particle C_{18} bonded Silica was used. The fluorescence excitation and emission wavelengths were set at 365 and 420 nm respectively. Ambient temperature and an isocratic operation with an aqueous mobile phase of acetonitrile: isopropanol: acetic

acid (10% / 5% / 5%) was used with a flow rate of 1.0 ml / min. All solvents were HPLC grade and filtered through a 0.45-μm filter (Millipore SA) prior to use.

Silica gel columns were prepared using Silica gel 60 (Merck Art:9385), and the slurry was packed employing the eluting solvents hexane: dichloromethane: ethyl acetate in the ratio 4:1:1. Silica gel (0.2mm) containing fluorescent indicator (F_{254}) on aluminium backed plates (Merck Art:5554) was used for analytical thin layer chromatography (t.l.c.) using the solvent system chloroform : ethyl acetate: isopropanol in the ratio 9:1:1.

Sterigmatocystin was isolated [10] from *A. versicolor* M1101, and was purified by preparative thin layer chromatography. Sterigmatocystin was recrystallized from acetone. *O*-methylsterigmatocystin was purchased from Sigma Chemical Suppliers (SA).
A range of alkylated and acylated sterigmatocystin derivatives were prepared.

General Procedure for Alkylation of Sterigmatocystin [13]

To a solution of **1** (10 mg; 30.9 μmoles) in dry acetone (5ml), was added anhydrous potassium carbonate (10.7 mg; 77.25 μmoles) and the alkyl halide (100 μl). The mixture was heated under reflux for 8 hours. The solids were removed by filtration, the filtrate was dried over anhydrous sodium sulphate, filtered and evaporated under a stream of nitrogen and gentle heat. The oil was neutralized with a solution of ammonia (2 ml of 12.5 %). The addition of deionised water yielded a precipitate which was filtered, washed and dried. The crude product was purified by gravity column chromatography using silica gel 60 (Merck Art: 9385) and the eluting solvents, hexane: dichloromethane: ethyl acetate (4: 1: 1). It was recrystallized from methanol.

General Procedure for Esterification of Sterigmatocystin [13]

To a solution of **1** (10 mg; 30.9 μmoles) in anhydrous pyridine (5 ml), was slowly added the acid chloride (100 μl). After the solution was left overnight at room temperature, more acid chloride (30 μl) was added and the mixture was left to stand for a further 45 minutes. The solution was heated under reflux for 15 minutes, poured onto ice (3 g) and left overnight. The product was extracted with chloroform (3 x 5ml), washed with 2M

hydrochloric acid (3 x 5ml) and then with deionised water (3 x 5ml). Thereafter, evaporation of the chloroform yielded a brown oil, which, on the addition of ethanol, gave a crude product. This was purified by gravity column chromatography using silica gel 60 (Merck Art: 9385) and the eluting solvents, hexane: dichloromethane: ethyl acetate (4: 1: 1). The compound was recrystallized from ethanol.

Sterigmatocystin (**1**). Yellow needles. Yield 110 mg. M.p. 244-246°C [lit [14] : 246°C]. R_f 0.93. [M^+] at *m/z* 324. δ_C ($CDCl_3$) 181.34 (C-1),108.87 (C-2), 162.31 (C-3), 111.24 (C-4), 135.67 (C-5), 105.89 (C-6), 154.96 (C-7), 154.03 (C-8), 106.52 (C-9), 164.57 (C-10), 90.51 (C-11), 163.29 (C-12), 105.96 (C-13), 113.24 (C-14), 48.06 (C-15), 102.50 (C-16), 145.37 (C-17), 56.79 (OCH_3). δ_H ($CDCl_3$) 6.81 (d, *J* 7.3 Hz,H-4), 7.48 (t, *J* 8.3, H-5), 6.73 (d, *J* 8.2, H-6), 6.42 (s, H-11), 6.81 (ddd, *J* 7.3, H-14), 4.79 (dd, H-15), 5.43 (dd, *J* 2.6, H-16), 6.48 (dd, *J* 2.4, H-17) and 3.97 (s, OCH_3)

O-Ethylsterigmatocystin (**2b**).Yellow needles. Yield 8.78 mg. M.p. 253-255 °C. R_f 0.87. [M^+ at *m/z* 352.0973] $C_{20}H_{16}O_6$ requires 352.0946. δ_H ($CDCl_3$) 6.91 (d, *J* 7.8, H-4), 7.48 (t, *J* 8.5, H-5), 6.74 (d, *J* 7.2, H-6), 6.38 (s, H-11), 6.78 (d, *J* 7.1, H-14), 4.79 (dd, *J* 2.8, 2.1, H-15), 5.43 (dd, *J* 2.5, *J* 2.6, H-16), 6.47 (t, *J* 2.7, H-17), 3.92 (s, OCH_3), 4.16 (q, *J* 7.0, H-1′) and 1.53 (t, *J* 7.1, H-2′)

O-Propylsterigmatocystin (**2c**). Pale yellow needles. Yield 10.63 mg. M.p. 213-215 °C. R_f 0.79. [M^+ at *m/z* 366.1100] $C_{21}H_{18}O_6$ requires 366.1107. δ_H ($CDCl_3$) 6.90 (d, 7.8, H-4), 7.45 (t, *J* 8.3, H-5), 6.73 (d, *J* 8.3, H-6), 6.38 (s, H-11), 6.78 (d, *J* 7.1, H-14), 4.77 (dd, *J* 2.8, 2.1, H-15), 5.43 (dd, *J* 2.7, 2.5, H-16), 6.47 (t, *J* 2.1, H-17), 3.92 (s, OCH_3), 4.04 (t, *J* 6.6, H-1′), 1.95 (st, *J* 6.8, H-2′) and 1.10 (t, *J* 7.3, H-3′)

O-Butylsterigmatocystin (**2d**). Pale yellow needles. Yield 9.63 mg. M.p. 180-182 °C. R_f 0.73. [M^+ at *m/z* 380.1253] $C_{22}H_{20}O_6$ requires 380.1260). δ_H ($CDCl_3$) 6.90 (d, *J* 7.90,

H-4), 7.45 (t, *J* 8.30, H-5), 6.73 (d, *J* 8.20, H-6), 6.37 (s, H-11), 6.7 (d, *J* 7.1, H-14), 4.77 (dd, *J* 2.8, 2.1, H-15), 5.42 (dd, *J* 2.6, 2.5, H-16), 6.47 (t, *J* 4.2, H-17), 3.92 (s, OCH_3), 4.08 (t, *J* 6.6, H-1′), 1.89 (m, H-2′), 1.57 (m, H-3′) and 0.97 (t, *J* 7.3, H-4′)

O-Pentylsterigmatocystin (**2e**). Pale yellow needles. Yield 9.37 mg. M.p. 152-154 °C. R_f 0.69. [M^+ at *m/z* 394.1427] $C_{23}H_{22}O_6$ requires 3394.1416. δ_H ($CDCl_3$) 6.90 (d, *J* 8.2, H-4), 7.45 (t, *J* 8.4, H-5), 6.74 (d, *J* 8.2, H-6), 6.37 (s, H-11), 6.77 (d, *J* 7.1, H-14), 4.78 (dd, *J* 2.8, 2.1, H-15), 5.43 (dd, *J* 2.6, 2.5, H-16), 6.47 (t, *J* 2.1, H-17), 3.92 (s, OCH_3), 4.07 (t, *J* 6.8, H-1′), 1.92 (m, H-2′), 1.49 (m, H-3′), 1.39 (m, H-4′) and 0.92 (t, *J* 7.3, H-5′).

O-Propenylsterigmatocystin (**2f**). Brown needles. Yield 8.99 mg. M.p. 204-206 °C. R_f 0.68 [M^+ at *m/z* 364.0939]$C_{21}H_{16}O_6$ requires 364.0947.δ_H ($CDCl_3$) 6.93 (d, *J* 8.2, H-4), 7.45 (t, *J* 8.5, H-5), 6.74 (d, *J* 8.2, H-6), 6.38 (s, H-11), 6.78 (d, *J* 7.2, H-14), 4.78 (dd, *J* 2.8, 2.1, H-15), 5.43 (dd, *J* 2.6, 2.5 H-16), 6.47 (t, *J* 2.6, H-17), 3.92 (s, OCH_3), 4.70 (dd, *J* 4.9,5.2, H-1′) 6.07 (m, H-2′), 5.31 (dd, *J* 9,2, 1.4, H-3′b) and 5.60 (dd, *J* 1.6, *J* 15.6, H-3′a).

O-Acetylsterigmatocystin (**2g**). Colourless needles. Yield 9.50 mg. M.p. 139-140 °C. R_f 0.63. [M^+ at *m/z* 366.0736] $C_{20}H_{14}O_7$ requires 366.0739. δ_H ($CDCl_3$) 7.30 (d, *J* 1.1, H-4), 7.59 (t, *J* 8.3, H-5), 6.92 (d, *J* 1.2, H-6), 6.40 (s, H-11), 6.91 (d, *J* 1.1, H-14), 4.79 (dd, *J* 2.8, 2.1, H-15), 5.42 (dd, *J* 2.7, 2.6, H-16), 6.49 (t, *J* 7.1, H-17), 3.93 (s, OCH_3) and 2.46 (s, CH_3).

O-Benzoylsterigmatocystin (**2h**).Colourless needles. Yield 9.45 mg. M.p. 257 259°C. [Lit[13] m.p. 258-260.] R_f 0.55. [M^+ at *m/z* 428.0891] $C_{25}H_{16}O_7$ requires 428.0895. δ_H ($CDCl_3$) 7.33 (d, *J* 7.1, H-4), 7.50 (t, *J* 7.8, H-5), 7.04 (d, *J* 6.6, H-6), 6.35 (s, H-11), 6.80 (d, *J* 7.1, H-14), 4.81 (dd, *J* 2.8, 2.5 H-15), 5.44 (dd, *J* 2.5, 2.6, H-16), 6.49 (t, *J* 2.3, H-17), 3.83 (s, OCH_3), 8.26 (d,7.1, Ha), 7.63 (m, Hb) and 7.60 (m, Hc).

Organism, Media and Culture Conditions

An aflatoxin blocked mutant of *A. parasiticus* (1-11-105 Wh 1) was maintained on potato dextrose agar. A spore suspension of approximately 10^6 spores, in 1 ml of 0.01 % sodium dodecyl sulphate, was inoculated in 10 x 100 ml of sterile solution of Reddy's medium [15] in 250 ml conical flasks. These were then incubated at 25° C in a rotary shaker at 180 r.p.m. for 96 hours. After incubation, the resultant mycelial pellets were pooled, collected on sterile cheese cloth, and rinsed twice with 50 ml of the resting medium. Mycelial pellets (3 g, wet weight) were added to resting medium (50 ml), supplemented with acetone (0.5 ml) containing 1.54 μmoles of the substrate and was incubated in a rotary shaker at 25°C and at 180 r.p.m .

Extraction of Aflatoxins and Sample Preparation for Thin Layer and High Pressure Liquid Chromatography

A wide bore disposable pipette was used to aliquot 5 ml of the incubated sample, at regular time intervals, and filtered. The filtrate was extracted with chloroform, dried over anhydrous sodium sulphate and evaporated to dryness *in vacuo*. The solid residue was dissolved in a minimum volume of chloroform, quantitatively transferred to vials and evaporated to dryness under a gentle stream of nitrogen and heat. The pellets were washed with 10 ml of an aqueous solution of 30% acetone, extracted with chloroform and treated as in the above case.

The dried residue was redissolved in 100μl chloroform and a 20μl portion was spotted onto the origin of a t.l.c. plate (10x10 cm aluminium backed Kieselgel 60). The plate was developed in CEI (chloroform: ethyl acetate : isopropanol in the ratio 90:5:5, by volume) and TEA (toluene: ethyl acetate: acetic acid in the ratio 50: 30: 4, by volume), air dried and the metabolites scanned for fluorescence under ultra-violet light. The t.l.c. plates were then sprayed with aluminium chloride in ethanol (20%, w/v) to test for the characteristic change in intensity and colour of fluorescence [16,17] of the metabolites

under ultra violet light. For HPLC analysis, the metabolites were dissolved in 500 µl of a derivatizing reagent viz., trifluoroacetic acid, the solution allowed to stand for 5 minutes and diluted with the mobile phase [18].

Results and discussion

The synthesis of the derivatives **2b-2h** from **1** required mild reaction conditions since earlier reports [13,19] showed that undesirable reactions occurred at the fused bisfuran ring when hot ethanolic potassium hydroxide was added or when **1** was refluxed vigorously with acetic anhydride in pyridine. Therefore the synthesis [13] of the ether derivatives were carried out using the modified Williamson synthesis with potassium carbonate as the base. Esterification was carried out using acid chlorides and the reactions performed at room temperature.

Although the separation of aflatoxins by HPLC is relatively simple and well defined, optimization of the separation and detection conditions of **3** was of some importance in order to minimize requirements for sample clean-up. The pre-column derivatization method using trifluoroacetic acid was the method of choice in the absence of post-column instrumentation since the hydration of AFB_1 and AFG_1 to AFB_{2a} and AFG_{2a} respectively, increased their fluorescence response in the reversed phase HPLC system [18]. As **3** was the aflatoxin of interest, efforts were focused on its quantification. It was found that detection at 10-1000 ppb gave a linear response, which was an adequate working range for the purpose of this study. Using this method, a sample clean-up for HPLC analysis was not necessary as there was no interference from other substances. It was found that a combination [20] of methanol, acetonitrile and water as the mobile phase, was not suitable for the isocratic chromatography with a C_{18} column, because of poor resolution and short retention time of the four major aflatoxins. The best separation and resolution was obtained by using an aqueous solvent system comprising of acetonitrile, isopropanol and acetic acid (10 %: 5 %: 5 %).

It was found that an incubation period of 48 hours in the whole cell feeding experiments, **1** and its derivatives **2a-2h** were converted to **3**. The identity of **3** was established by two dimensional thin layer chromatography with authentic standards. The derivatives **2a-2h** demonstrated a characteristic pale-blue fluorescence under ultra-violet illumination. Fluorescence of these compounds turned yellow-green when sprayed with aluminium chloride. Compound **1** exhibited a brick- red fluorescence under ultra-violet illumination which changed to yellow when sprayed with aluminium chloride. Compound **3** displayed a characteristic blue fluorescence and no colour change occurred in the presence of aluminium chloride.

The results indicated that the blocked mutant of *A. parasiticus* was able to convert **2a-2h** to **3**. The transformation of **1** and **2a** to **3** has been repeatedly reported [10,11,21] and the enzymatic activities involved in these conversions have been well established [22,23]. Although the position of **2a** as an intermediate in AFB_1 biosynthetic pathway is generally agreed, it is not clear either from the point of metabolic simplicity or enzyme specificity, why methylation of **1** is an obligatory process in the pathway. It is apparent from these findings that the enzyme(s) responsible for the conversion of **1** did not display a high degree of substrate specificity, since it was unable to recognize the difference between the various groups, either as ether or ester functional groups.

In order to investigate the question of relative specificity [9] of the enzyme(s) involved, a time course study of the various substrates was done. With this regard, both the culture fluid and mycelial fractions were monitored by HPLC for the production of **3** at regular time intervals. The time course for the biotransformation of selected derivatives into **3** in the culture fluid and mycelial fractions are presented in Figure 2 and Figure 3 respectively.

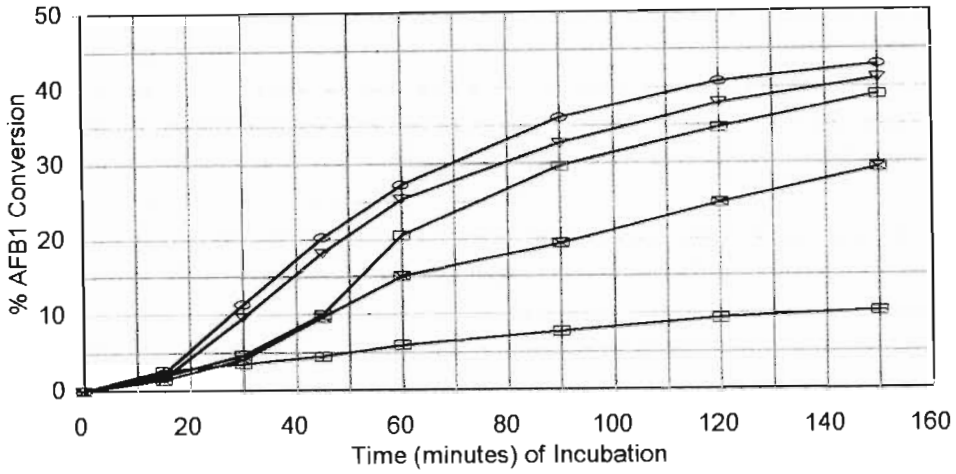


Figure 2. Time Course for the Biotransformation of ST Derivatives to AFB_1 in the Filtrate Fraction. KEY: **2c** (o); **2b** (∇); **2a** (□); **1** (□); **2h** (□)

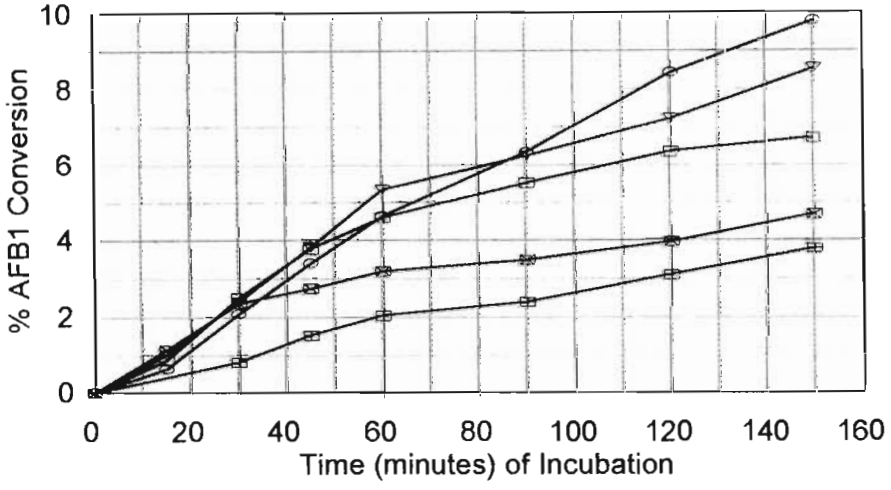


Figure 3. Time Course for the Biotransformation of ST Derivatives to AFB_1 in the Pellet Fraction. KEY: **2c** (o); **2b** (∇); **2a** (□); **1** (□); **2h** (□)

The results from both the culture fluid and mycelial fractions indicate a general decrease in the rate of conversion in the order **2c** > **2b** > **2a** > **1** > **2h**. Studies [24,25] have shown that electron donating alkyl groups present on a substrate can affect the

catalysis of enzymes generally by increasing the rate of conversion to products. It may be possible that a similar event is occurring in this case, although the trend is reversed in that the hydroxyl group of **1** is more electron donating than the alkyl groups of **2a-2h**. Although the propyl group of **2c**, by inductive effects, is more electron donating than the derivatives of **2**, it is unlikely that such minor contributions will account for the differences in the percentage conversion to **3**. It seems much more likely that there is a permeability effect with the uptake of the compounds through the fungal membrane, even though the addition of acetone to the fungal system used in previous studies [16] gave satisfactory results. Since **2c** is the least polar of the derivatives, it might be able to penetrate the cell membrane more easily and thus reach the active site for conversion to **3**. Hence the effect being measured is one of membrane permeability rather than enzyme specificity. The fact that all the derivatives were converted, however, reveals that the enzyme(s) involved in the conversion are non specific as far as the side chain is concerned.

This study does not resolve the question as to whether the alkylation of **1** is obligatory in the biogenesis of **3**.

Acknowledgments

We thank the Foundation for Research and Development for financial support (Grant number 2035387). We also thank J.W.Bennett of the University of Tulane for a gift of a culture of the mutant *Aspergillus parasiticus* (1-11-105 Wh1).

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The role of *O*-methylsterigmatocystin in aflatoxin B_1 biosynthesis

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Abstract

A cell free preparation was derived from a blocked mutant of *Aspergillus parasiticus* that was capable of converting sterigmatocystin (ST) to aflatoxin B_1 (AFB_1). Optimum conditions were determined for the conversion of ST to AFB_1, which revealed that the addition of the coenzymes, NADPH and SAM, increased the rate of conversion. Various derivatives, including ST and coenzymes NADPH and SAM were added to this preparation in an attempt to elucidate the final stages of AFB_1 biosynthesis. Time course experiments showed that ST and *O*-methylsterigmatocystin (OMST) were converted to AFB_1 at the same rate, whereas *O*-propylsterigmatocystin (OPST) was converted at a slower rate. It was concluded that ST had to be converted to an *O*-alkyl derivative prior to metabolism to AFB_1. Under natural conditions this is probably OMST but the results also indicate that other alkyl groups might substitute, suggesting that the enzyme responsible for the conversion exhibits relative specificity.

INTRODUCTION

The biosynthetic pathway to the carcinogenic mycotoxin, aflatoxin B_1 (AFB_1) is now generally agreed upon, particularly since more recent studies on its underlying molecular biology, have lent support to it [1]. A scheme is given in Fig. 1 [2], which indicates the formation of the anthraquinone derivative via the polyketide pathway, thence through other various modified anthraquinone metabolites to yield the characteristic bis-dihydrofurano ring system and finally via a xanthone derivative, sterigmatocystin (ST) to the substituted coumarin, AFB_1.

Whilst the overall pathway seems feasible and is supported by a large amount of experimental evidence, some of the precise mechanistic details, although subject to conjecture and deduction from metabolic studies, are not fully understood or described. This is a result of the lag between enzymological studies and those involving natural product chemistry, whole cell investigation and, as mentioned, the more recent molecular biological work. One such area of uncertainty is that involving the conversion of ST to AFB_1 in the latter parts of the pathway. There is strong evidence to show [3] that the methyl derivative of ST, i.e., *O*-methylsterigmatocystin (OMST) is an obligatory intermediate between ST and AFB_1. Whilst it might be argued that there is no reason why this should be so, on applying the principle of Occam's Razor, it seems an unnecessary step, as the methyl group does not appear in the final product, i.e., AFB_1. It is tempting to suggest, therefore, that OMST is either a side shunt metabolite or part of a metabolic grid. Thus a range of derivatives of ST and ST itself may lead to AFB_1, due to the enzyme(s) responsible for the conversion, exhibiting the secondary metabolic activity known as relative specificity.

Previous work, including that from our own laboratories has attempted to resolve this point [4]. A series of homologous O-alkyl derivatives of ST were synthesized and fed to whole cells of a blocked mutant of *Aspergillus parasiticus* (Wh1-11-105) capable of converting ST and OMST to AFB_1. The propyl derivative (OPST) was converted more rapidly than

the others, including the natural substrates ST and OMST. This did not resolve the question of which putative intermediates were involved in AFB_1 biosynthesis, as all were converted. The fact that OPST was converted the quickest of the substrates could be explained on the basis of polarity. It being the least polar, meant that it would penetrate the cellular membrane more rapidly. Thus part of the reaction rate was due to the rate of diffusion through the membrane.

In order to resolve this confounding factor, a cell free preparation was prepared from a blocked mutant of *A. parasiticus*, which is capable of converting ST to AFB_1. The various potential substrates were added to this in order to measure their conversion rate to AFB_1. The results are presented in this study.

Material and Methods

Materials

All chemicals were of Analar grade. Coenzymes (NADPH and SAM) were obtained from Boehringer Mannheim, South Africa.

Preparation of Substrates

Sterigmatocystin was prepared from a producing culture of *Aspergillus versicolor* (M1101) and the various homologues of ST, i.e., propyl (OPST) and methyl (OMST) were prepared by the modified Williamson synthesis, as previously described [4].

Production of Mycelium

An AFB_1 blocked mutant of *A. parasiticus* (Wh1-11-105) was maintained on potato dextrose agar. A spore suspension was prepared in sterile sodium dodecyl sulphate solution (0.01 %) of approximately 10^6 spores per ml and the solution was left standing for 15 minutes. Conventional aseptic techniques were used to inoculate the spore suspension (1 ml) into Erlenmeyer flasks (5 x 250 ml) containing sterile Reddy's medium (100 ml) [5]. The flasks were incubated at 28 °C in shake culture at 150 rpm. After 96 hours of incubation the mycelia pellets were harvested by filtering through double layer cheesecloth. The mycelia was washed with ice cold 20 mM phosphate buffer (pH 7.2) dried by vacuum filtration, freeze-dried and stored in an airtight container at -70°C.

Preparation of cell-free extract

A sample of freeze-dried mycelium (0.5 g) was gently ground to a fine powder in a dry chilled mortar. The powdered mycelium was suspended in ice cold 20 mM phosphate buffer (pH 7.2)(10 ml) and gently stirred for 15 minutes. The homogenate was centrifuged at 20 000 x g for 20 minutes at 4 °C and filtered through glass wool. The supernatant was used as the cell free extract (CFE). The protein content of the CFE was determined by the Bradford assay [6].

Conversion of substrates

Enzyme activity was determined by adding the CFE (500 µl) (final protein concentration = 1 mg/ml) to the phosphate buffered solution (pH 7.5) (400 µl) in a 10 ml test tube. To this mixture coenzymes (NADPH and/or SAM)(50 µl) were added to give a final concentration of 1.5 mM. The mixture was then incubated on a slow shaker (100 rpm) at 27 °C for 5 minutes. The reaction was started by adding the substrate (31 nmol) dissolved in acetone (50 µl). The reaction was stopped after 1 hour by adding chloroform (3 ml) and gently shaking. The chloroform layer was removed and passed through a small bed of

anhydrous sodium sulphate. The reaction mixture was extracted twice more with chloroform (3 ml) which was also passed through the sodium sulphate. The pooled extracts were evaporated to dryness and stored until further analysis. All experiments were conducted in triplicate and the results are expressed as a mean, unless otherwise indicated.

The optimum conversion conditions of the CFE were determined by incubating ST, as the substrate and varying the pH between 6.4 to 7.6 at 27 °C; the temperature between 22 – 32 °C at a pH of 7.2; and protein concentration between 0.5 – 3.0 mg/ml at a pH of 7.5 and temperature of 27 °C all for 1 hour. Time course reactions for the different substrates were done at a temperature of 28 °C, a pH of 7.2 and a protein concentration of 1 mg/ml

Methods of analysis: Thin Layer Chromatography

The dried residues were dissolved in chloroform (100 μl) and a portion (20 μl) was spotted onto the origin of a thin layer chromatography (tlc) plate (10x10 cm aluminium backed Kieselgel 60 Merck). The plate was developed in chloroform: ethyl acetate: propan-2-ol (CEI) (90: 5: 5, v/v) air dried and examined under long wave UV light for fluorescent spots. It was then sprayed with 20 % aluminium chloride in ethanol and heated to 120 °C and re-examined under UV light.

High Performance Liquid Chromatography

The quantitation of the various substrates and AFB_1 was done by high performance liquid chromatography (hplc) using a Spectra Physics UV 6000 LP system with a 20 μl injection loop, linked to a diode array detector. A C_{18} Lichrosphere (250 x 4.60 mm; 5 microns) reverse phase column was used with a gradient elution programme consisting of acetonitrile and water; time 0-10 min. 35 % acetonitrile; time 20 min. 20 % acetonitrile. The dried residues were dissolved in acetonitrile (10 ml) and an aliquot (50 μl) was injected using the loop.

The quantity of AFB_1 produced was calculated from the integrated peak area of the chromatograph and a back-fit straight-line equation from the calibration graph of authentic AFB_1. From this the percentage substrate conversion to AFB_1 was calculated.

Results and discussion

The results of a recent investigation [4] indicated that in whole fungal cell experiments with the conversion of O-alkyl derivatives of ST to AFB_1, these derivatives were subject to, at different extents, a permeability effect. Thus the actual rate of conversion of ST and its derivatives to AFB_1 could not be compared because of a time delay to penetrate the cellular membrane and reach the active enzyme site. Thus to obviate this problem, a CFE was prepared that was active in the conversion of ST and its derivatives to AFB_1. Initial investigation with this preparation were done using the conditions reported by Singh and Hsieh [7] i.e., at pH 7.5 and 27 °C using 50 times more cofactors (NADPH and SAM) than substrate. This established that the CFE was capable of converting ST to AFB_1, as shown by tlc and quantitated by hplc. A control with no substrate added, indicated that the preparation was not capable of producing AFB_1 without added ST intermediates. On incubating ST with the enzyme and cofactors over the pH range chosen, the optimum was found to be pH 7.2 (Fig.2). Similarly the optimum temperature was found to be 28 °C (Fig.3), which is in agreement to that found by Singh and Hsieh [7]. The effect of protein concentration is shown in Figure 4 (r^2 0.98) and as expected gives a linear increase in reaction rate with increasing protein content. Singh and Hsieh [7] reported a curvilinear response, for a similar investigation, and suggested that this was possibly due to a reduced oxygen supply.

It was also important to investigate the effect of cofactors, because it is known that NADPH is required for the conversion of ST to AFB_1 [8] and, if OMST is an obligatory intermediate, SAM is also required. Endogenous cofactors would be present in the CFE but not necessarily in the correct form due to lack of regeneration [8]. Figure 5 and Table 1 shows that the CFE is capable of converting ST to AFB_1 without added cofactor but their addition does increase the rate of conversion. Enhancement is greater by the addition of NADPH than SAM but greatest when both are added. The requirement for NADPH is not unexpected, as the type of monooxygenase proposed in the conversion would require it, and the favourable effects of SAM suggest that OMST is an intermediate in the pathway, as it is the source of the methyl group. In subsequent experiments both these cofactors were added in excess to ensure optimum conversion rates.

Time course experiments for the three substrates, i.e., ST, OMST and OPST using the determined optimum conditions were then carried out (Table 2). The rates of conversion for ST and OMST were statistically indistinguishable, although the rate for OMST was marginally higher (Fig.6). The conversion of OPST was slower, confirming that its superior conversion rate in previous experiments [4], using whole cells, was due to cell permeability effects. Lower conversion rates as compared to other experiments reported here, is due to variation in the activity of the CFEs. A plot of the results show that the reaction rate over the first hour is essentially linear, which agrees with other work [9].

The results of this study are encouraging in that a suitable CFE can be routinely prepared, which is useful for studying the final stages of AFB_1 biosynthesis. The addition of exogenous cofactor indicates that OMST is an intermediate in the pathway, although the presence of sub-optimal concentration of cofactor, does not allow a clear-cut answer. The conversion of the propyl analogue, although at a slower rate, also clouds the issue. Two explanations are possible: either the enzyme(s) responsible for the conversion exhibit relative specificity, whereby a series of ST homologues can be converted to AFB_1 but at different rates; or all the homologues are converted to a common intermediate, e.g., ST, prior to conversion. The former hypothesis is favoured from these results, which is in keeping with the characteristics of certain secondary metabolic enzymes, i.e., relative specificity [10]. Further experimentation is being done in this laboratory to resolve the question.

Acknowledgments

The authors gratefully acknowledge Professor J W Bennett, Tulane University, for the culture of *A. parasiticus* and the Foundation for Research and Development (FRD), South Africa, for a grant (Grant No, 2035387) to support this work.

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Table 1. The Effect of Co-Factors on the Production of AFB_1 from ST (10 μg) in Cell-Free Extracts for a Reaction Time 1 Hour at 28 °C and pH 7.2.

Co-Factors Added (1.5 mM)	Integrated Peak Area	AFB_1 Produced (ng/10 ml)	AFB_1 Produced (μg/assay)	AFB_1 Produced (μg/assay)[a]	% Conversion to AFB_1
NONE	23030 26112	102 115	1.02 1.15	1.08 ± 0.09	11.21
SAM	29733 30627	130 134	1.30 1.34	1.32 ± 0.03	13.71
NADPH	31379 36874	137 160	1.37 1.60	1.49 ± 0.16	15.47
SAM + NADPH	56003 65303	241 280	2.41 2.80	2.61 ± 0.28	27.10

[a]Values for AFB_1 represents the mean and standard deviation of experiments conducted in duplicate.

Table 2. The Enzymatic Conversion of Selected Substrates (30.86 nmol) to AFB_1 at 28 °C and pH 7.2, by Cell-Free Extracts in the Presence of 1.5 mM SAM and 1.5 mM NADPH.

Substrate Added	Time (minute)	Integrated Peak Area	AFB_1 Produced (ng/10 ml)	AFB_1 Produced (µg/assay)	AFB_1 Produced (µg/assay)[a]	% Conversion to AFB_1
ST	15	10782 7163 14116	51 35 65	0.51 0.35 0.65	0.5 ± 0.15	5.19
ST	30	23765 13507 23003	105 62 101	1.05 0.62 1.01	0.89 ± 0.23	9.24
ST	45	42365 35036 38357	183 152 166	1.83 1.52 1.66	1.67 ± 0.15	17.34
ST	60	58358 55887 64869	251 240 278	2.51 2.40 2.78	2.56 ± 0.19	26.58
OMST	15	6953 11850 16502	34 55 75	0.34 0.55 0.75	0.55 ± 0.21	5.71
OMST	30	25234 25244 33644	111 111 147	1.11 1.11 1.47	1.23 ± 0.21	12.77
OMST	45	42259 45223 50920	183 195 219	1.83 1.95 2.19	1.99 ± 0.18	20.67
OMST	60	60514 61790 63870	260 265 274	2.60 2.65 2.74	2.66 ± 0.07	27.62
OPST	15	6425 12357 8676	32 57 42	0.32 0.57 0.42	0.44 ± 0.12	4.57
OPST	30	14504 16566 10432	66 75 49	0.66 0.75 0.49	0.63 ± 0.13	6.54
OPST	45	26600 22777 24296	117 101 107	1.17 1.01 1.07	1.08 ± 0.08	11.21
OPST	60	27435 30347 52007	121 133 224	1.21 1.33 2.24	1.59 ± 0.56	16.51

[a]Values for AFB_1 represents the mean and standard deviation of experiments conducted in triplicate.

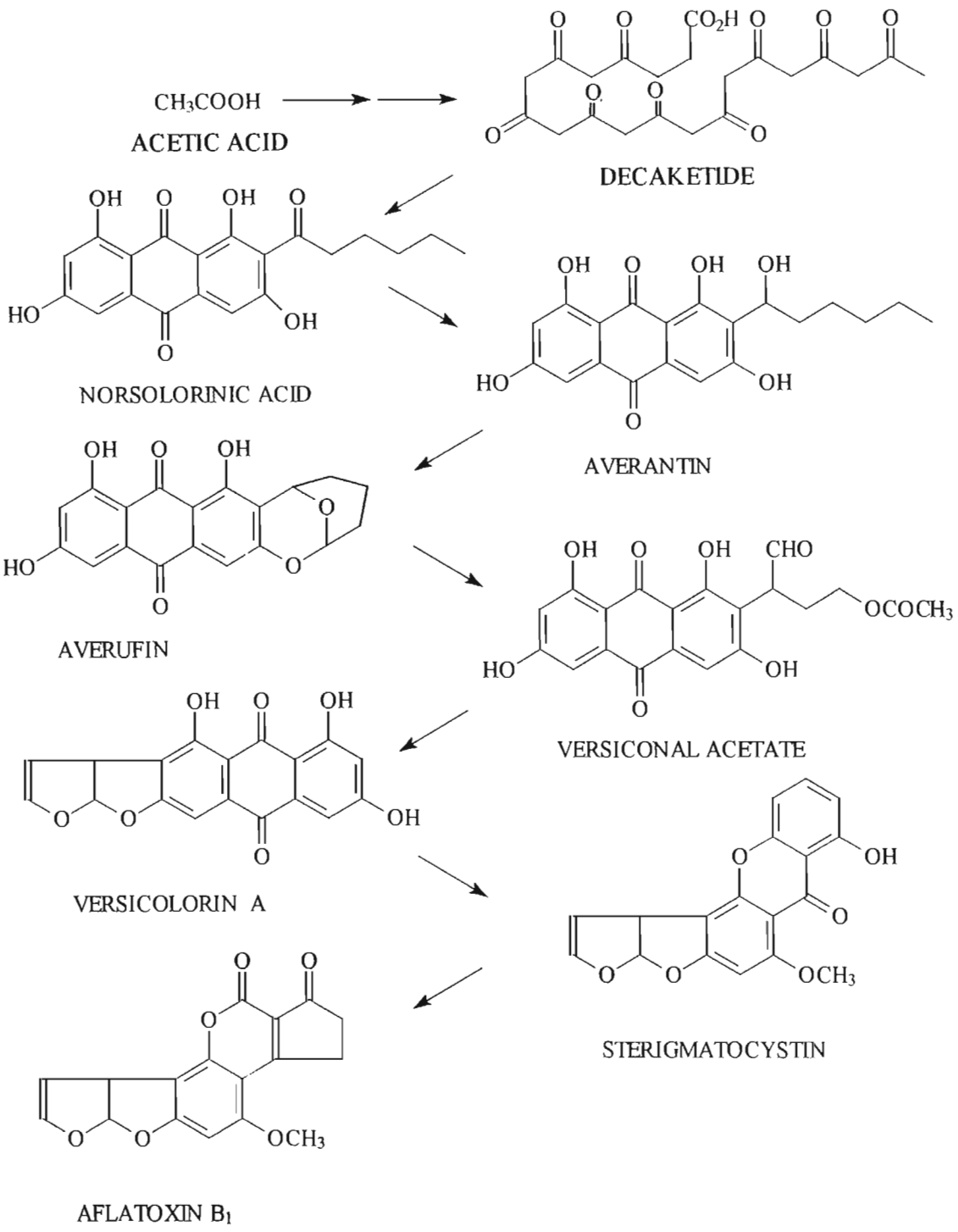

Figure 1. Proposed Pathway for the Biogenesis of Aflatoxin B_1.

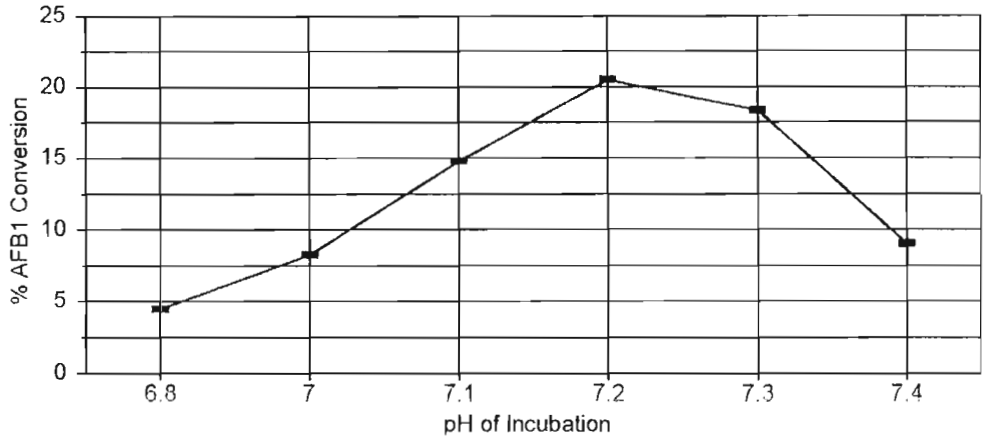

Figure 2. The Effect of pH of Incubation for the Enzymatic Conversion of ST to AFB_1 at 27 °C for a Reaction Time of 1 Hour.

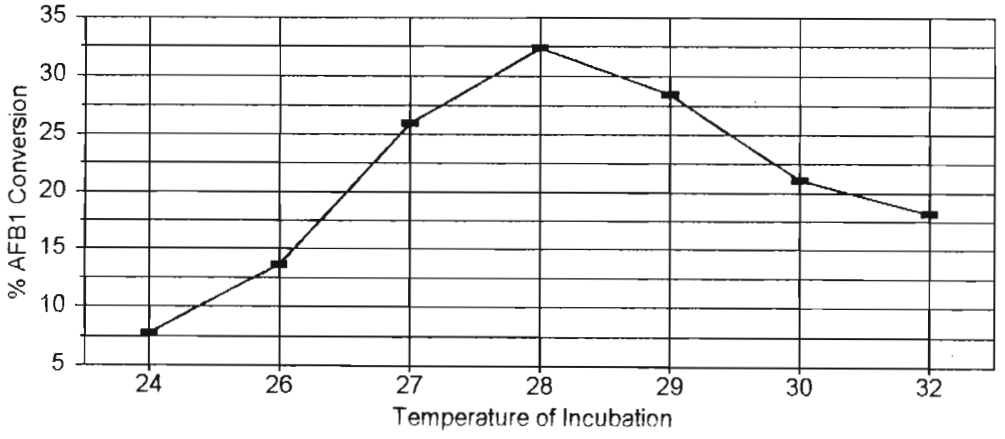

Figure 3. The Effect of Temperature of Incubation for the Enzymatic Conversion of ST to AFB_1 at p 7.2 for a Reaction Time of 1 Hour.

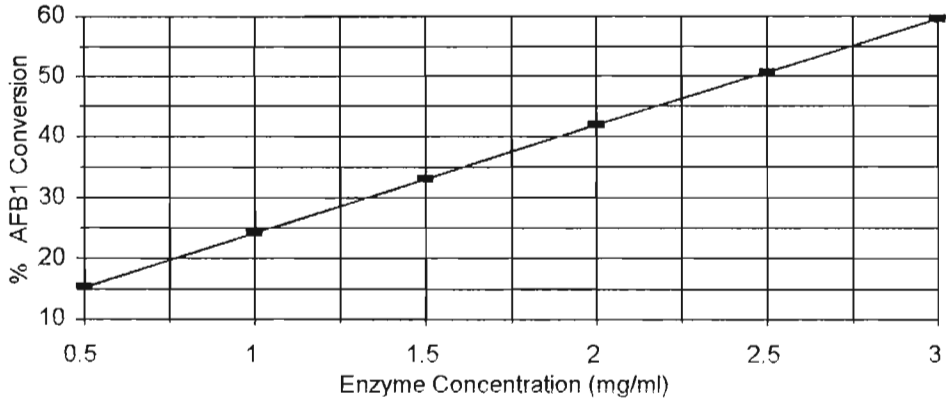

Figure 4. The Effect of Cell Free Extract Protein Concentration on the Conversion of ST to AFB_1 at pH 7.5, Temperature 27 °C for a Reaction Time of 1 Hour.

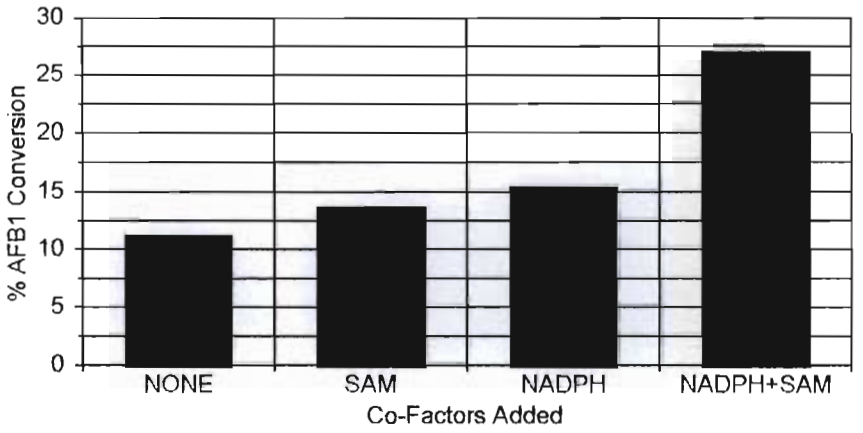

Figure 5. The Effect of Co-Factors (1.5 mM) on the Conversion of ST to AFB_1 in Cell Free Extracts for a Reaction Time of 1 Hour at 28 °C and pH 7.2.

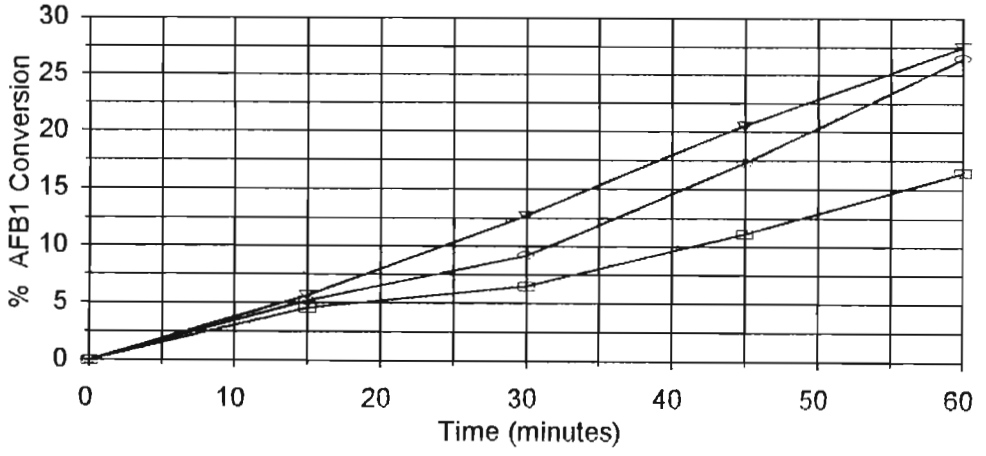

Figure 6. The Enzymatic Conversion of ST, OMST and OPST to AFB_1, in the Presence of 1.5 mM NADPH and 1.5 mM SAM, at 28 °C and pH 7.2.

The conversion of sterigmatocystin and its derivatives to aflatoxin B_1 by a partially purified enzyme system

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Abstract

From previous studies it is not clear as to whether O-methylsterigmatocystin (OMST) was an obligatory intermediate in the conversion of sterigmatocystin (ST) to aflatoxin B_1 (AFB1) by a cell free extract (CFE) from a blocked mutant of *Aspergillus parasiticus* or whether it was one of several possible O-alkyl ST derivatives that could act in this capacity. In order to resolve this difficulty various O-alkyl derivatives of ST and ST itself was added to a to a CFE that had been separated from low molecular weight metabolites and cofactors. By adding various combinations of the coenzymes NADPH and SAM, it was shown that ST had to be converted to an O-alkyl derivative before its conversion to AFB1. Under natural conditions it is proposed that O-MST is that alkyl derivative but the observations indicated that O-propylsterigmatocystin (OPST) is more rapidly converted to AFB1 than OMST. It was concluded that the enzyme(s) responsible for the conversion exhibited relative specificity and that OPST was a more preferred substrate than OMST or that propyl was a better leaving group than methyl under the experimental conditions used.

Introduction

Earlier studies on the role of sterigmatocystin (ST) and O-methylsterigmatocystin (OMS) in the biosynthesis of aflatoxin B_1 (AFB1) have not clearly resolved whether OMS is an obligatory intermediate or not [1] (Fig.1). Various O-alkyl derivatives of ST were synthesised and added to cells of a blocked mutant of *Aspergillus parasiticus* (Wh1-11-105) that was capable of converting ST and OMS to AFB1. This showed that several homologues were converted to AFB1 with the O-propyl (OPST) derivative being converted more rapidly than either ST or OMS in a time course study. It was concluded that this result could be explained on the basis of polarity of the substrate, as OPS was the least polar of those tested, which allowed it to penetrate the fungal cellular membrane more rapidly.

In order to resolve this difficulty, similar experiments were conducted with a cell free preparation derived from the blocked mutant of *A.parasiticus*, [2]. This showed that ST and the various homologues investigated, could be converted to AFB1 and consequently there was still not a clear cut answer to the question as to the role of OMS. The question resolved itself into two possibilities. Either OMS is an obligatory intermediate in the biosynthesis or

the enzyme system exhibits relative specificity and can convert various O-alkyl derivatives of ST, at various rates to AFB1. This may or may not include ST itself.

To decide between these two possibilities, a cell free extract from *A.parasiticus* was prepared, which was partially purified so that the enzyme system was separated from all small molecular weight compounds, including endogenous coenzymes and substrates. The results and conclusions from this study are reported in this presentation.

Materials and methods

Sephadex G-25 (particle size 100-300microns) was purchased from Pharmacia Fine Chemicals (SA). Dialysis tubing (10mm; molecular weight retention >10000) and other chemicals were purchased from Sigma Chemical Suppliers. O-Alkyl derivatives of ST were prepared as previously described [1].

Preparation of Partially Purified Enzyme

Production of mycelium. An AFB1 blocked mutant of A. parasiticus (Whl-11-105) donated by Dr J W Bennett, Tulane University was maintained on potato dextrose agar. A spore suspension of the fungus was used to inoculate 100ml of sterile Reddy's medium [3] in 250 ml conical flasks. The flasks were incubated at 28°C in shake culture at 150 rpm. After 96 hours of incubation the mycelia pellets were harvested by filtering through double layer cheesecloth. The mycelia was washed with ice cold 20 mM phosphate buffer (pH 7.2) dried by vacuum filtration, freeze-dried and stored in an airtight container at –78°C.

Preparation of cell-free extract. A sample of freeze-dried mycelium (0.5 g) was gently ground to a fine powder in a dry chilled mortar. The powdered mycelium was suspended in 10 ml ice cold 20 mM phosphate buffer and gently stirred for 15 minutes. The homogenate was centrifuged at 20000 xg for 20 minutes at 4°C and filtered through glass wool. This was used as the cell free extract (CFE). The protein content of the CFE was determined by the Bradford assay [4].

Fractionation by Molecular exclusion chromatography. A chromatography column (280 x 33mm) was packed with Sephadex G-25 gel in phosphate buffer (pH 7.2) and equilibrated with buffer at a flow rate of 30 ml per hour at 4°C. The CFE (2 mg/ml) was applied directly to the column and then eluted with buffer at a flow rate of 25 ml per hour. Fractions (1.5 ml) were collected and monitored at 280 nm to ascertain protein elution. Fractions containing protein were pooled, placed in a dialysis sac that had been prepared by soaking for 24 hours in deionised water, and dialysed against solid sucrose at 4°C for 1.5 hours. The concentrated protein solution (CPS) containing the enzyme activity, about 1ml, was used in conversion experiments.

Assay Methods

Enzyme catalysed reactions. Enzyme activity was determined by adding 500 μl of the CPS (final protein concentration = 250 μg/ml) to 400 μl of phosphate buffer (pH 7.2) in a 10 ml test tube. To this mixture, coenzymes (NADPH and/or SAM) (50 μl) were added to give a final concentration of 1.5 mM. The mixture was then incubated on a slow shaker (100 rpm) at 27°C for 5 minutes. The reaction was started by adding the substrate (31 nmol) dissolved in acetone (50 μl). The reaction was stopped after 1 hour by adding dichloromethane (3ml) and gently shaking. The dichloromethane layer was removed and passed through a small amount of anhydrous sodium sulphate. The reaction mixture was extracted twice more with dichloromethane (3ml) which was also passed through the sodium sulphate. The pooled extracts were evaporated to dryness under nitrogen for further analysis. All experiments were in triplicate and the results expressed as a mean, unless otherwise indicated. The substrates tested included: ST; OMST; OPST; and O-benzoylsterigmatocystin (OBST).

Thin Layer Chromatography. The dried residues were dissolved in chloroform (100 μl) and a 20 μl portion were spotted onto the origin of a thin layer chromatography (tlc) plate (10x10 cm aluminium backed Kieselgel 60 Merck). The plate was developed in chloroform/ethyl acetate/propan-2-ol (CEI) air dried and examined under long wave UV light for fluorescent spots. It was then sprayed with 20% aluminium chloride in ethanol and heated to 120°C and re-examined under UV light.

High Performance Liquid Chromatography. The quantitation of the various substrates and AFB1 was done by high performance liquid chromatography (hplc) using a Spectra Physics UV 6000 LP system with a 20μl injection loop, linked to a diode array detector. A C_{18} Lichrosphere (250x4.60 mm; 5 microns) reverse phase column was used with a gradient elution programme consisting of acetonitrile and water; time 0-10 min. 35% acetonitrile; time 20 min. 80% acetonitrile. The residue was dissolved in acetonitrile and made to 10 ml and 20 μl was injected using the loop.

The quantity of AFB1 produced was calculated from the integrated peak area of the chromatograph and a back-fit straight-line equation from the calibration graph of authentic AFB1. From this the percentage substrate conversion to AFB1 was calculated.

Results and discussion

A plot of the absorption at 280 nm of the effluent from the chromatography column is given in Figure 2. From an inspection of this, it seemed likely that fractions 9-17 would contain the total protein (including enzyme activity) particularly as they exhibited turbidity associated with the salting out of large proteins. On incubating these pooled fractions with ST and cofactors no conversion to AFB1 was observed. This was improved by repeating the incubation with pooled fractions 9 – 30 but this only gave a 3% conversion to AFB1 after 5hours incubation. On concentrating the enzyme by dialysis

against sucrose, a conversion of 13.6% for ST to AFB1 in the presence of NADPH and SAM was obtained. On incubating ST under the same conditions with either SAM or NADPH or no coenzyme at all no conversion was observed (Table 1).

This result was critical in that it demonstrated that gel filtration had removed cofactors from the mycelial extract and that ST is not converted to AFB1 in the absence of NADPH or SAM. It can, therefore, be concluded that in the natural pathway to AFB1 biosynthesis OMST is an obligatory intermediate. As OPST had been converted to AFB1 in crude cell free extracts in previous experiments [2] various O-alkyl derivatives were tested for conversion by the CFE in the presence of NADPH but in absence of SAM. The results are presented in Table 2. All three substrates (OMST, OPST and O-benzoylsterigmatocystin (OBST) were metabolised to AFB1 at different rates, hence the enzyme was displaying relative specificity, a well know phenomena in secondary metabolism [5]. As might have been anticipated, the aryl group as represented by OBST, are not favoured substrates, as the natural one OMST has a small alkyl group. The interesting observation was that O-PST is more rapidly converted than OMST itself, in these experiments, which is in contrast to previous results with a crude CFE [2]. Speculations on the reason(s) for these observations are as follows: the converting enzyme(s) has a hydrophobic patch around the active site that recognises longer alkyl chains, e.g. propyl, better than shorter ones, i.e., methyl; or propyl is a better leaving group than methyl in the enzyme catalysed reaction, where the alkyl group is lost. Further work using other homologues is required to resolve this question.

Finally there is not obvious answer to the question posed earlier [1] as to why ST has to be alkylated prior to conversion to AFB1. Usually free phenols are preferred substrates for oxygenases involved in aromatic cleavages [e.g., 6] O-alkyl ethers being more recalcitrant towards metabolic conversions. It may be that methylation under natural conditions is part of a detoxification mechanism and that the oxygenases involved in the conversion are also responsible for the metabolism of other substituted aromatic compounds that the fungus comes into contact with. This latter suggestion can only be resolved once we have a full understanding of fungal oxygenase systems involved in the breakdown of aromatic compounds.

Acknowledgements

The authors gratefully acknowledge Professor J W Bennett, Tulane University, for the culture of *A.parasiticus* and the FRD, South Africa, for a grant to support this work.

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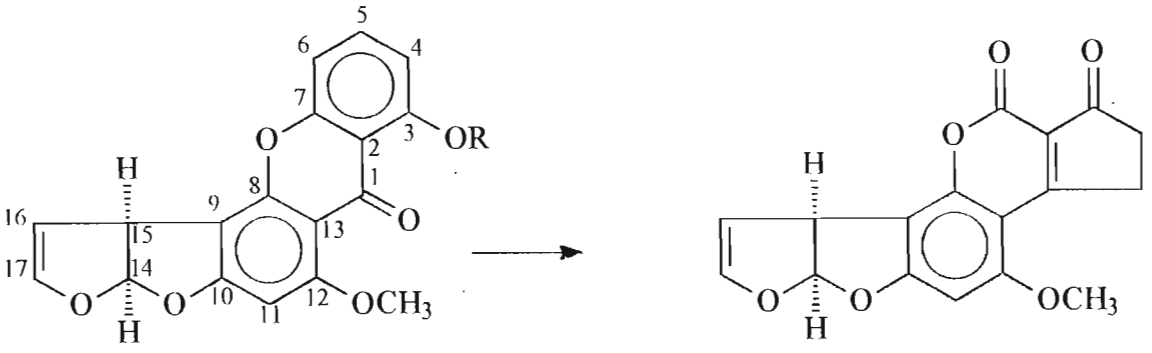

1. R = H

3.

2a. $R = CH_3$
2b. $R = CH_2CH_3$
2c. $R = CH_2CH_2CH_2$
2d. $R = CH_2(CH_2)_2CH_3$
2e. $R = CH_2(CH_2)_3CH_3$
2f. $R = CH_2CH{=}CH_2$
2g. $R = COCH_3$
2h. $R = COC_6H_5$

Figure 1. Scheme for the Biosynthesis of AFB_1 from Sterigmatocystin Derivatives.

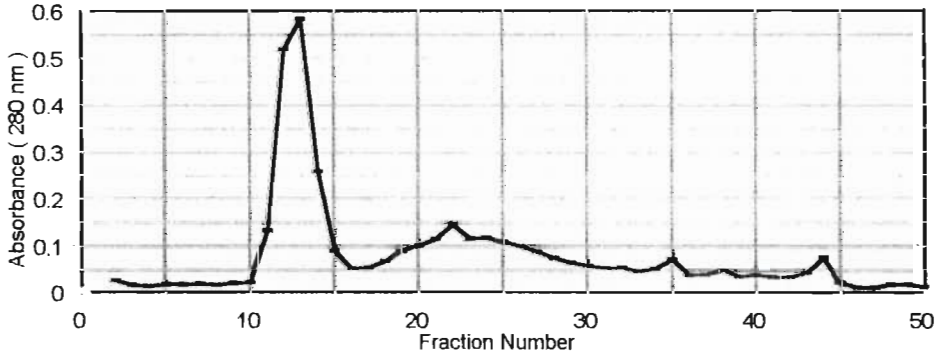

Figure 2. Protein Elution profile after Separation of the Cell Free Extract by Gel Chromatography with Sephadex G-25.

Table 1 : Enzymatic Reaction of Combined Fraction 9-30 at 28 °C and pH 7.2 with ST (10 μg) as Substrate in the Presence of 1.5 mM SAM and 1.5 mM NADPH.

Integrated Peak Area	AFB_1 Produced (ng/ml)	AFB_1 Produced (μg/assay)	AFB_1 Produced (μg/assay)[a]	% Conversion to AFB_1
71269[b]	304	0.30	0.31 ± 0.01	3.22
72569[b]	310	0.31		
322431[c]	1361	1.36	1.31 ± 0.08	13.61
295883[c]	1249	1.25		

[a] Values for AFB_1 represents the mean and standard deviation of experiment conducted in duplicate. [b]Values obtained for undialysed pooled fractions 9-30 for a reaction time of 5 hours. [c]Values obtained for dialysed pooled fractions 9-30 for a reaction time of 3 hours.

Table 2. [a] Enzymatic Reaction of Combined Fraction 9-30 , After Dialysis, at 28 °C and pH 7.2 for a Reaction Time of 3 Hours with ST (10 μg) as Substrate.

Substrate (30.86 nmol)	Co-factors added (1.5 mM)	% Conversion to AFB_1
ST	None	ND
ST	SAM	ND
ST	NADPH	ND
ST (BOILED ENZYME)	SAM + NADPH	ND

[a] Enzymatic reaction conducted in duplicate.
ND = not detected

Table 3. [a] Enzymatic Reaction of Combined Fraction 9-30 , After Dialysis, at 28 °C and pH 7.2 for a Reaction Time of 3 Hours with Different Substrates.

Substrate	Integrated Peak Area	AFB_1 Produced (ng/ml)	AFB_1 Produced (μg/assay)	AFB_1 Produced (μg/assay)[a]	% Conversion to AFB_1
OMST	468573	1975	1.98	1.97 ± 0.02	20.46
OMST	462997	1952	1.95		
OPST	897993	3780	3.78	3.77 ± 0.02	39.15
OPST	891679	3754	3.75		
OBST	72629	311	0.31	0.32 ± 0.01	3.32
OBST	73703	315	0.32		

[a] Values for AFB_1 represents the mean and standard deviation of experiment conducted in duplicate

CHAPTER 3:

THE ELIMINATION AND METABOLISM OF MYCOTOXINS

3.1 PAPERS AND STUDIES

Production and degradation of patulin by *Paecilomyces* sp. a common contaminant of silage
Paper 10 by Anderson, Dutton and Harding

The inhibition of aflatoxin biosynthesis by organo-phosphorus compounds
Paper 11 by Dutton and Anderson

The interaction between additives, yeasts and patulin production in silage
Paper 14 by Dutton, Westlake and Anderson

Comparison of two defined media for inhibitor and incorporation studies of aflatoxin biosynthesis
Book Chapter 1 by Bennett, Kofsky, Bulbin and Dutton

T-2 Toxin metabolism by rumen bacteria and its effect on their growth
Paper 23 by Westlake, Mackie and Dutton

Effect of several mycotoxins on the specific growth rate of *Butyricvibrio fibrinosolvens* and toxin degradation *in vitro*
Paper 24 by Westlake, Mackie and Dutton

Destruction of aflatoxin during the production of hydrolysed vegetable protein
Paper 26 by Williams and Dutton

In vitro metabolism of mycotoxins by bacterial, protozoal and ovine ruminal fluid preparations
Paper 27 by Westlake, Mackie and Dutton

Metabolism of aflatoxin B_1 by *Petroselinum crispum* (parsley
Paper 30 by Howes, Dutton and Chuturgoon

The effects of aflatoxin B_1 on germinating maize (*Zea mays*) embryos
Paper 37 by McLean, Berjak, Watt and Dutton

The influence of aflatoxin B_1 on the *in vitro* germination and growth of excised, mature *Zea mays* embryos
Paper 39 by McLean, Berjak, Watt and Dutton

Effects of aflatoxin B_1 on *in vitro* cultures of *Nicotinia tabacum* I. Callus growth and differentiation
Paper 40 by McLean, Watt, Berjak and Dutton

Effects of aflatoxin B_1 on *in vitro* cultures of *Nicotinia tabacum* II. Root and shoot development in tobacco plantlets
Paper 41 by McLean, Berjak, Watt, Dutton and Snyman

Cellular interactions and metabolism of aflatoxins
Paper 42 by McLean and Dutton

Aflatoxin B_1 - its effects on an *in vitro* system
Paper 44 by McLean, Watt, Berjak and Dutton

The effect of a commercial herbicide on aflatoxin lipid biosynthesis in *Aspergillus parasiticus*
Paper 50 by Chuturgoon, Punchoo, Bux, and Dutton

3.2: INTRODUCTION

Once the dangers of the aflatoxins and other mycotoxins were appreciated, vigorous efforts were made to prevent their formation in agricultural commodities or eliminate them once present. Prevention is always better than cure and aflatoxin contamination is an excellent proof of the maxim. Aflatoxins are not only acute toxins, causing death at low concentrations but are also carcinogenic, teratogenic, and mutagenic. They are resistant to destruction by heat and normal culinary preparative procedures, thus the prevention of their entry into the food chain is the first line of defence. In the case of aflatoxin this can be achieved by good farming practise, as the producing fungus is a saprophyte. Thus healthy crops in the field not exposed to abnormal stress, e.g., drought are relatively free from aflatoxin contamination. Even groundnut, which is a crop associated with aflatoxin contamination, is not susceptible at water activities outside a narrow window (Dorner *et al.* 1989). An additional strategy is the development of crop strains resistant to the production of aflatoxins (Anderson *et al.* 1996). This is particularly important in the protection of maize, as this cereal is persistently infected with *Fusarium* spp., in particular *F. moniliforme* and *F. subglutinans*, which are classed as "field fungi". These fungi are responsible for producing a whole range of toxins, including the trichothecenes, moniliformin, zearalenone and the fumonisins. The situation is so bad in KwaZulu Natal, that at least 50% of all samples analysed (Paper 43) are positive for some level of fumonisin B_1 (FB1) which is associated with various animal disease and oesophageal cancer in humans (Rheeder *et al.* 1992).

As agricultural commodities brought in from the field are naturally contaminated with fungal spores, the next line of defence is to prevent their germination and growth. This is traditionally achieved by drying the crop. It is assumed that any cereal stored at below 14% water content will be safe. This is a reasonable rule of thumb assumption, provided that the material is not stored for long periods, i.e., longer than a season, as some fungi grow at below this level and that all other parameters are consistent. For example, temperature is very important. Fluctuations in this factor, particularly in poorly ventilated storage areas causes humidity changes, which can result in water condensation. Metal structures such as bins, silos and ships holds are particularly vulnerable. Such water condensate can cause hot spots where fungi will

of fungal species to proliferate. As the conditions vary, various fungi become "stressed" and produce secondary metabolites, viz, mycotoxins. Thus materials should be stored, dry, in a water proof, cool, ventilated store, where other storage pests cannot enter, i.e., insects and rodents, which would also set up infection sites. These precautions are very important, where subsistence farming takes place. It is imperative that in rural areas simple measure are taken to ensure that fungal infection is minimised. Chemical pre-treatment of cereals using derivatives of propionic and related acids is possible but this can be expensive and if not applied correctly cause infecting fungi to produce secondary metabolites due to "chemical" stress (Al-Hilli & Smith 1979).

Once agricultural materials are infected, there is little than can be done to remove produced toxins by chemical means. As mentioned most are resistant to heat and treatment with chemical reagents is problematical. Ammoniation has been treated on a commercial scale to destroy aflatoxins in oilseeds (Lee *et al.* 1984). This is effective but increases the cost of the product and renders the material unfit for human consumption. Many other chemicals, ranging from ozone (Dwarakanath *et al.* 1968) to chlorine (Samarajeewa *et al.* 1991), in the destruction of AFB1, have been tried with varying success but all suffer from the same disadvantages as ammoniation.

For rural people the use of family members in sorting the crop and physically removing visibly infected material is an option. This material can be fed to animals to avoid waste and thus the animal acts as a screen. Carry over of the toxin is possible but this is usually at a much reduced level >200 parts dilution. The practise of using infected maize specifically for beer production is not, however, a safe method, particularly where FB1 is a major contaminant. This is likely to be concentrated in the final product.

Not unconnected with the chemical destruction of AFB1, is its metabolism in various biological systems and the prevention of its formation by various metabolic inhibitors. It was shown early on that fungal cultures could metabolise AFB1 to other products (Cole *et al.* 1972). Plants (Schoental & White 1965) are affected by AFB1 and animal tissues metabolise AFB1(Swensen *et al.* 1974) (this being important in the aetiology of mycotoxicoses in animals and man). In the case of humans this is covered in Medical Aspects in Chapter 5.

The prevention of AFB1 production by various agents, added to fungal cultures has been the subject of much research. This has two main objectives, one being the prevention of AFB1 formation in commodities, the other was to identified compounds that would specifically inhibit steps in the biosynthesis of AFB1. The former approach has not been commercially effective. It seems likely that certain spice extracts, e.g., garlic, clove oil and turmeric (Sinha *et al.* 1993), can prevent AFB1 formation and in some cases inhibit fungal growth. In the latter case the first specific inhibitor of AFB1 biosynthesis was dichlorvos (Rao & Harein 1993), an organophosphorous insecticide. This inhibited AFB1 production and caused the accumulation of an orange pigment named versiconal hemiacetal acetate (VHA), which was subsequently found to be an intermediate in the pathway (Schroder *et al.* 1974). Other organic chemicals were later found to block AFB1 production (Wheeler *et al.* 1989) but none are as specific in their action as dichlorvos.

COMMENTARY

The modification of mycotoxins by micro-organisms was reported in four publications: Papers 10, 14, 24, and 27. The first of these papers (Paper 10) reports on the production and degradation of patulin by *Paecilomyces* spp. This has some importance in wholesome silage production. Normally silage is produced by fermenting plant material, e.g., grass, under anaerobic conditions. This precludes infection by filamentous fungi, which are aerobes, but not by certain yeasts and bacteria that can operate under anaerobic conditions. The problem in most farm silage production is that the clamp where the silage is made cannot be kept strictly anaerobic. Further, when the clamp is opened and exposed to air, fungi rapidly develop from dormant spores. Many fungi are not deterred by the lower pH found in silage. The fungus *Paecilomyces* is commonly found in poor silage made in temperate climates and many strains produce high levels of the mycotoxin, patulin. The poisoning of cattle by infected silage is very variable and this is not altogether explained by the presence of different species and non-toxigenic and toxigenic strains. The finding that *Paecilomyces* spp can degrade patulin as well as form it, could in part explain this variation in observations. Paradoxically infected silage consumed by cattle after a longer period of growth may be exposed to lower concentrations of patulin. It would depend upon conditions as to whether patulin was being produced or degraded and the relative rates of these two apposed processes.

Another investigation, Paper 14, showed that the whole ecology of the silage clamp and the effect of additives, played a role in mycotoxin appearance and disappearance. Clearly the situation in silage fermentation is quite complex. However, it was shown that high yeast levels and addition of a commercial preparation Sylade favoured low patulin levels. One useful technique used in this study was the γ-irradiation of plant material to achieve microbial sterility thus avoiding gross chemical modification and breakdown of the material caused by other methods.

Studies were done the effects of rumen digestion on mycotoxin stability. This is important where ruminants are fed rations contaminated with mycotoxins. Firstly it complicates the metabolic conversions of the mycotoxins by the animals own tissues and body fluids. Secondly if the rumen is capable of degrading mycotoxins this might be a way in which contaminated agricultural commodities might be used with out compromising the animal or the consumer. Levels of contamination might of course be all important, as high concentrations might affect the rumen flora and cause problems for the animal. Paper 24 showed that a principle bacteria found in rumen, *Butyrivibrio fibrisolvens*, was not affected by AFB1 but did not degrade it in a simple culture fluid but it was capable of degrading various trichothecenes, mainly by deacetylation. More complex experiments described in Papers 23 and 27, showed that quite high levels of the highly toxic trichothecene T-2 toxin did not affect the rumen microorganisms tested and again other trichothecenes were degraded to varying degrees. Most interestingly lower levels of T-2 toxin actually stimulated *B. fibrisolvens* growth and this was taken to mean that the organism was actually using the mycotoxin as a carbon source. It was concluded in Paper 27 that the action of the rumen conferred a degree of resistance to mycotoxin poisoning in ruminant animals.

Following this study I co-supervised Janet Lee's masters (MT20) where various sheep tissue homogenates were incubated with trichothecenes, including T-2 toxin. As

might have been expected, liver and kidney homgenates were able to modify these toxins, as was brain. However, those derived from heart were not, which might explain the higher detrimental effects of these toxins on the cardio-vascular system of animals.

The metabolism of mycotoxins by plant tissue is also of some importance. As has been described many mycotoxins enter the human food chain via contamination of crops and it is a dangerous assumption to assume that these toxins only affect animals and not other eukaryotic organisms. Over the last 40 years it has been shown that mycotoxins can adversely affect plants (e.g., Marasas *et al.* 1971), can be modified by plants (Ruhland *et al.* 1996) and can be accumulated (Prince 1989) by them. In order to ascertain what plants did to AFB1, young parsley plants were placed in a solution of the toxin and allowed to imbibe the solution (Paper 30). Parsley was chosen as it has been shown to have strong secondary metabolic activity. On analysing the plants after 2 days incubation, a new fluorescent compound was observed on TLC of the extracts. This proved to be aflatoxicol A, a reduced derivative of AFB1 (Detroy & Hesseltine 1970). It was apparent that the plants contained a dehydrogenase that could convert the cyclopentenone ketone group to a secondary alcohol. On incubating AFB1 with cell free homogenate of parsley, a completely different result was obtained, where two more polar derivatives were observed. Clearly the plants contained other enzymes systems capable of metabolising AFB1, which were not available to the toxin in whole plants.

A series of experiments were done on the effects of AFB1, principally by Michelle McLean for whom I acted as a co-supervision in collaboration with Professor Pat Berjak and Dr Paula Watt, on maize (Papers 37, 39, and 44) and tobacco (Papers 40, and 41). The results clearly showed that both callus and plantlets of both species were adversely affected by the toxin. Immunocytochemistry (ICC) and electron microscopy (EM), showed abnormalities of sub-cellular structures and the presence of the toxin in the vacuole, cytoplasm and nucleus. Growth of differentiated tissue, including root and leaf development was inhibited often in a dose dependent fashion. The fact that the nucleus was labelled is of some significance, as it indicates that plant DNA is alkylated by AFB1, which is probably a major contributor to cell necrosis and inhibition of cell division, mirrored in animal tissues. This in turn suggests that plants have cytochrome P_{450} systems capable of "activating" FB1 via its epoxide, as in certain animal cells, e.g., liver (see Chapter 5).

After the discovery of the effects of dichlorvos on cultures of *A. parasiticus* other workers and myself and Murray Anderson added a whole range of potential inhibitors to fungal cultures in resting medium. Up to date no chemical inhibitions studies have improved upon the effects of dichlorvos, although some emulate it, including, naled (Schroder *et al.* 1974). We found that naled (an OP compound similar in structure to dichlorvos) chlormephos, ciodrin, phosdrin and trichlorphon had the same action on the fungus in that AFB1 production was lowered with the appearance of orange pigments, the principle one being VHA (Paper 11, Book Chapter 1).

After reading an article in TIBS (Harwood 1988) on the effects of propionate herbicides, I wondered if fungal systems would respond in the same manner, i.e., they block acetyl Coenzyme A carboxylase in monocotyledonous plants. This is an enzyme responsible for generating malonyl CoA an important precursor in fatty acid

and polyketide biosynthesis. This idea was tested by myself and Chuturgoon (paper 50) by adding DiclofopMethyl to cultures of *A. parasiticus*. Aflatoxin biosynthesis was completely blocked and the growth of the fungus strongly inhibited. This result was exciting in that it presented a possible control of monocotyledonous weeds and AFB1 biosynthesis in dicotyledonous crops such as groundnut. Unfortunately the manufacturers of DiclofopMethyl were not interested in this possibility and the only field trials done were inconclusive due to an unfortunate mix up in applying the herbicide.

A study was done by a Masters student, Keith Williams, in collaboration with Beecham Ltd, on the feasibility of using AFB1-contaminated groundnut protein for the production of total vegetable protein hydrolysate (TVPH) (Paper 26). This is produced by the hydrolysis of vegetable proteins, such as groundnut, by strong hydrochloric acid in a pressurised vessel at elevated temperatures. The hydrolysate is cooled and neutralised with sodium carbonate to yield a mixture of amino acids and salt which is used as a flavouring in products such as packet soups. It seemed to me that this process should be capable of totally destroying the aflatoxins. In order to demonstrate this a scaled down pressure vessel was fabricated and used to make TVPH in the laboratory from groundnut protein both naturally contaminated with AFB1 and spiked with radiolabelled AFB1. The results were very encouraging. The toxin was completely destroyed as evidence by chromatography of extracts of the product and tracing of the added label, which was removed in a charcoal filtration step use in the process. In bioassay tests, fertile egg and Ames test, the product was shown to be free from toxicity and mutagenicity.

J. Sci. Food Agric. 1979, 30, 229–232

Production and Degradation of Patulin by *Paecilomyces* Species, a Common Contaminant of Silage

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(*Manuscript received 27 July 1978*)

The production and degradation of patulin by a common silage contaminant, *Paecilomyces* sp., is described. Utilising protoplasts of the parent organism, 80% degradation of radiolabelled patulin occurs over the 3-h period studied and it is proposed that this degradation effect may account for anomalies in the investigation of suspected mycotoxin poisoning.

1. Introduction

Patulin is a fungal metabolite which has been found to be produced by a number of *Aspergillus* and *Penicillium* species and also by *Byssochlamys* sp.,[1] the perfect stage of *Paecilomyces* sp. Investigations of contaminated feedstuffs for patulin, a recognised mycotoxin, have at times proved contradictory. Analysis of contaminated commercial apple juice has revealed levels of up to 45 parts 10^{-6}.[2,3] Conversely, a corn–soya-bean meal contaminated with *Penicillium patulum* which had been implicated in the deaths of certain farm animals, produced patulin in culture experiments but proved negative for patulin in the meal.[4] Toxicity studies, including evidence that patulin is a carcinogen, have been discussed in an excellent review by Scott.[5]

Removal or detoxification of patulin from feed has received little attention although it has been shown to be unstable in alkaline conditions.[6] There is, however, an increasing interest in the degradation of mycotoxins and several systems have been shown to be capable of degrading certain mycotoxins. Most work in this field involves the degradation of aflatoxins, e.g. by ammoniation,[7] metabolism by microorganisms[8] including the aflatoxin producing cultures themselves[9] and protoplasts.[10]

The results reported here describe the production of patulin by a species of *Paecilomyces* isolated from silage, grown in various media, and its subsequent degradation by whole cultures and protoplasts of the producing organism.

2. Materials and methods

2.1. Culturing of *Paecilomyces* species

Paecilomyces sp. (266), kindly supplied by Mr A. Hacking, now of ADAS Aberystwyth, Wales, was maintained on potato dextrose agar. A spore suspension was prepared in 0.1% w/v sodium lauryl sulphate from a 14-day-old culture. Flasks (250 ml conical) containing the desired medium (50 ml) were inoculated with 1 ml of the spore suspension (10^7 spores) and incubated at 25°C for various time intervals on a rotary incubator (100 rev min^{-1}).

Reddy's medium[11] was a chemically defined low salt smedium. Yeast extract sucrose (YES) contained yeast extract (2% w/v) and sucrose (10% w/v). Potato dextrose broth (PDB) contained glucose (2% w/v), dipotassium hydrogen phosphate (0.1% w/v) and boiled potato extract (50% v/v). All preparations were sterilised at 15 psi for 15 min.

0022-5142/79/0300-0229 $02.00

2.2. Patulin estimation

Samples containing patulin were extracted with ethyl acetate (100 ml ethyl acetate per 50 ml sample). The solvent fraction was dried over anhydrous sodium sulphate and concentrated by rotary evaporation, the residue was then dissolved in acetone and spotted on to a thin layer chromatoplate (t.l.c.); silica gel G, 250 μm thick. The t.l.c. plate was developed in chloroform:acetic acid (9:1 v/v) and patulin was identified as an absorbent spot by observing the t.l.c. plate under short wave u.v. light, ($R_F = 0.32$). The silica containing the absorbent spot was then scraped off and the patulin was eluted by washing the silica with acetone which was then evaporated to dryness in a rotary evaporator. The patulin extract was redissolved in a known volume of ethanol and estimated spectrophotometrically at 275 nm ($\Sigma = 14\,540$).[5]

^{14}C-patulin was prepared from ^{14}C-glucose using the method of Bu'lock and Ryan.[12] The activity of the purified patulin was estimated in a Packard Tricarb Model 3300 liquid scintillation counter. Once the background count had been subtracted, actual counts were determined using channels ratio method to allow for quenching.

2.3. Protoplast preparation

Protoplasts were prepared using the technique outlined by Peberdy and Issacs.[13] Once a pellet of 'clean' protoplasts had been obtained (by centrifugation of a protoplast suspension, 500 *g* for 10 min) they were resuspended in PDB (10 ml) and incubated at 30°C in a Warburg constant volume flask. A wick of filter paper impregnated with 0.1% w/v KOH was placed in the centre well, this was renewed at the beginning of every incubation period. Patulin was dissolved in *NN*-dimethyl formamide and added to the protoplast suspension; incubation samples (1 ml) were removed at zero time and at various time-intervals thereafter. Samples were extracted with ethyl acetate as previously described and once the total extract had been run on the t.l.c. plate patulin was removed and estimated. In the ^{14}C-patulin experiment, silica, above the patulin spot up to the solvent front was removed and extracted with acetone, this was termed the top fraction. Silica below the patulin spot down to the origin was removed, extracted with acetone and termed the bottom fraction.

3. Results and discussion

The media investigated for their ability to stimulate patulin production by *Paecilomyces* sp. were selected on the basis of their established ability for producing mycotoxins. YES medium has been used in screening for the production of fungal metabolites whereas PDB has already been shown to be suitable for producing patulin in cultures of *Penicillium urticae*[14] and Reddy's is a chemically defined medium which stimulates aflatoxin production in *Aspergillus* sp.[11]

As may be expected, considerable difference in patulin production can be observed for these three media; PDB showed the greatest production over 4 days of incubation (590 mg per litre per g mycelium) see Table 1. However the major difference between the three media can be seen in the degradation of patulin. This was greatest in the PDB medium between day 4 and day 9, while the level in the YES medium follows a similar pattern but to a lesser extent. The culture in Reddy's medium does not show this production/degradation effect but only a continual production over the 9-day period.

It seems reasonable to assume that the loss of patulin in PDB and YES media is not due to chemical degradation as it is stable at the acid pH which exists in these cultures. This view is confirmed by the slower but steady increase in patulin concentration in the culture grown on Reddy's medium.

Because it has been shown that aflatoxins are degraded by protoplasts derived from their producing cultures of *Aspergillus flavus*,[10] protoplasts derived from *Paecilomyces* sp. were investigated for their ability to carry out a similar process with patulin. Tables 2 and 3 show the degradation of patulin by protoplasts derived from 8-day-old cultures of *Paecilomyces* sp. In Table 2 it can be seen that patulin disappears from the PDB grown culture, while a similar control experiment without protoplasts showed no change in patulin concentration over the 3-h incubation period; thus the degradation effect must be due to the presence of protoplasts. Addition of ^{14}C-patulin to proto-

Table 1. Production of patulin by *Paecilomyces* sp. in three different media

Medium used and pH[a]	Age of mycelium and amount of patulin produced (mg per litre per g mycelium)		
	2 Day	4 Day	9 Day
Reddy's	>0.05	108.4	172.7
pH	4.5	3.5	2.8
PDB	85.0	590.0	102.9
pH	5.4	4.4	6.8
YES	0.05	184.7	50.8
pH	6.5	5.4	4.2

[a] pH at time of harvest.

Table 2. Degradation of patulin by protoplasts derived from 8-day-old mycelium of *Paecilomyces* sp. and suspended in PDB

Incubation time (h)	Total patulin in medium (μg)	
	With protoplasts	Medium only
0	150	150
1	143	150
2	102	149
3	42	149

Table 3. Degradation of ^{14}C patulin[a] added to protoplasts derived from 8-day-old mycelium of *Paecilomyces* sp. and suspended in PDB

Incubation time (h)	Percentage loss of ^{14}C patulin	Percentage gain[b] of ^{14}C in top fraction	Percentage gain[b] of ^{14}C bottom fraction	Percentage gain[b] of $^{14}C\ CO_2$
1	12	0.05	8	1.3
2	26	1	10	1.9
3	80	2	15	2.3

[a] Patulin added = 0.003 μCi.
[b] Expressed as a percentage of ^{14}C patulin added.

plasts shows an 80% loss of label over 3 h of incubation with 15% appearing in the t.l.c. bottom fraction. It seems likely that the labelled degradation products remained in the aqueous fraction, however, this was not investigated further. The activity trapped in the KOH-paper indicates that the degradation of patulin involves decarboxylation(s).

The observed rapid production and degradation of patulin probably accounts for anomalies in the investigation of suspected cases of mycotoxin poisoning involving patulin. Hence it is possible that samples of feedstuff recently contaminated with patulin, when fed to animals, would cause mycotoxicoses. From our results it seems likely that later examination of the feedstuff could prove negative for patulin due to its subsequent degradation, although it would still be possible to isolate the causal organism. This situation seems to be a potential hazard in silage clamps, particularly when they have been opened, allowing the common silage contaminants such as *Paecilomyces* sp. to proliferate rapidly at the newly exposed surface, and hence to produce patulin. Poor husbandry

techniques would result in patulin poisoning in animals fed on such materials, and there would be great difficulty in detecting the source of the toxin for the reason suggested above.

In order to investigate this hazard further we intend to study the production and degradation of patulin in silage under controlled conditions.

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Reprinted from
Journal of Food Protection Vol. 43, No. 5, Pages 381-384 (May, 1980)

Inhibition of Aflatoxin Biosynthesis by Organophosphorus Compounds

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(Received for publication July 27, 1979)

ABSTRACT

The effect of a range of organophosphorus and various other compounds on production of aflatoxin by *Aspergillus flavus* was investigated. Five organophosphorus compounds - Chlormephos, Clodrin, Naled, Phosdrin and Trichlorphon- at concentrations of 20 and 100 μg/ml of culture fluid were found to have activity similar to Dichlorvos, in that they lowered the level of aflatoxin produced and caused formation of several anthraquinone pigments. Two of these pigments have not previously been described, one was named Versicol and a suggested structure is presented, whilst the other compound was shown to be its acetate derivative. A rationale is suggested for the required elements of structure, which are necessary for an organophosphorus compound to have Dichlorvos-type activity. Two unrelated compounds, ammonium nitrate and Tridecanone were also found to elicit Dichlorvos-type activity. It is likely that tridecanone or its breakdown products competitively inhibit enzymes involved in aflatoxin biosynthesis. It is possible that this inhibition effect explains the lowering of aflatoxin production in lipid-rich commodities infected by *A. flavus*.

Results from several studies have shown that the biosynthesis of aflatoxin B_1 (I) by *Aspergillus* species may be inhibited by a number of compounds, e.g. Dichlorvos (*10*), dimethyl sulfoxide (*4*) and sulfite (*8*). Dichlorvos (VIB) and the related compound Naled (VIC), at a concentration of 20 μg/ml of culture fluid reduced aflatoxin production with a concomitant appearance of versiconal hemiacetal acetate (II), suggesting that a specific step in the biosynthetic pathway had been blocked (*12*). This effect thus provided a powerful tool for further metabolic studies and also suggested a possible method for inhibiting aflatoxin biosynthesis in agricultural commodities infected with aflatoxin-producing strains of fungi.

As both Dichlorvos and Naled are organophosphorus insecticides capable of inhibiting acetyl choline esterase activity in insects, it was initially suspected that they inhibited esterase activity in aflatoxin-producing organisms and thus caused accumulation of the ester versiconal hemiacetal acetate (*9*). This hypothesis was supported by the observation that Dichlorvos does inhibit general esterase activity in cell-free extracts derived from *Aspergillus flavus*, although in similar cell-free extracts it was also noted that oxygenase activity was also inhibited (*1*). This latter observation lent credence to the view that oxygenase inhibition is principally responsible for the specific metabolic block in aflatoxin biosynthesis.

The investigation reported here explores the ability o various types of organophosphorus compounds t specifically inhibit aflatoxin biosynthesis. It was envi saged that the structural features required to be presen in organophosphorus compounds for their specific inhibitor action upon aflatoxin biosynthesis would b identified. Predictions with regard to the specific inhibi tory power of various organophosphorus compound could then be deduced and this in turn may lead to more effective control of aflatoxin formation i contaminated stores.

MATERIALS AND METHODS

We are indebted to the companies who supplied the followin pesticides: Chlormephos (Murphy Chemical Co.), Cyanthoate, Phen thoate (Montedison) Clodrin, Gardona, Phosdrin (Shell Chemical Co.) Dichlorvos, Trichlorphon (Bayer UK Ltd.), Dicrotophos, Monocroto phos, Phosphamidon (Ciba-Geigy), Durbusan, Nellite, Plondrel Ronnel (Dow Chemical Co.) and Naled (Chevron Chemical Co.). Othe chemicals were purchased as indicated: ammonium nitrate Anala (B.D.H.), 2- and 3- tridecanone (Koch Light), tributyl phosphate (Koc Light), trimethyl phosphate (Sigma). (See Fig. 1).

Cultures and inhibition studies

An aflatoxin-producing strain of *A. flavus* (*NI*) was maintained o potato dextrose agar and used throughout this study. The chemicall defined medium of Reddy et al. (*11*) was used as the standard liqui culture medium. A 1-ml volume of spore suspension (approximatel 1×10^6 spores) was used to inoculate 70 ml of medium in a 250-m conical flask, which was then incubated on a rotary shake (150 revs/minute, 25 C). To avoid sporicidal action, the require potential inhibitor was added, (dissolved in a maximum of 0.4 ml acetone), after 48 h of incubation, and at a range of concentrations. (F the purpose of this report only those concentrations at 20 μg/ml an 100 μg/ml are included). Usually there were at least four separate tes done for each inhibitor concentration. Control flasks were set up in th same manner but without addition of the potential inhibitor. Followin a further 4-day incubation period the mycelium was filtered off an washed with acetone (10 ml), chloroform (20 ml) and ethyl aceta (10 ml) in sequence. These washings were then shaken with the cultu filtrate and the organic layer was separated and dried over a minimu amount of anhydrous sodium sulfate. The culture filtrate was furth extracted with ethyl acetate (20 ml) and this extract was used to wa the sodium sulfate which had dried the main extraction. Both extrac were combined and evaporated to dryness on a rotary film evaporato The residue was dissolved in methanol and made up to 10 ml in volumetric flask.

Analysis of extracts

The ultra-violet and visible absorption spectrum of the methan solution of the extract was recorded on a Pye-Unicam 18 spectrophotometer, (dilutions being made with methanol whe necessary). A 50-μl sample of the methanol solution was spotted onto silica gel-G thin-laer plate (0.25 mm thick) and this was develop

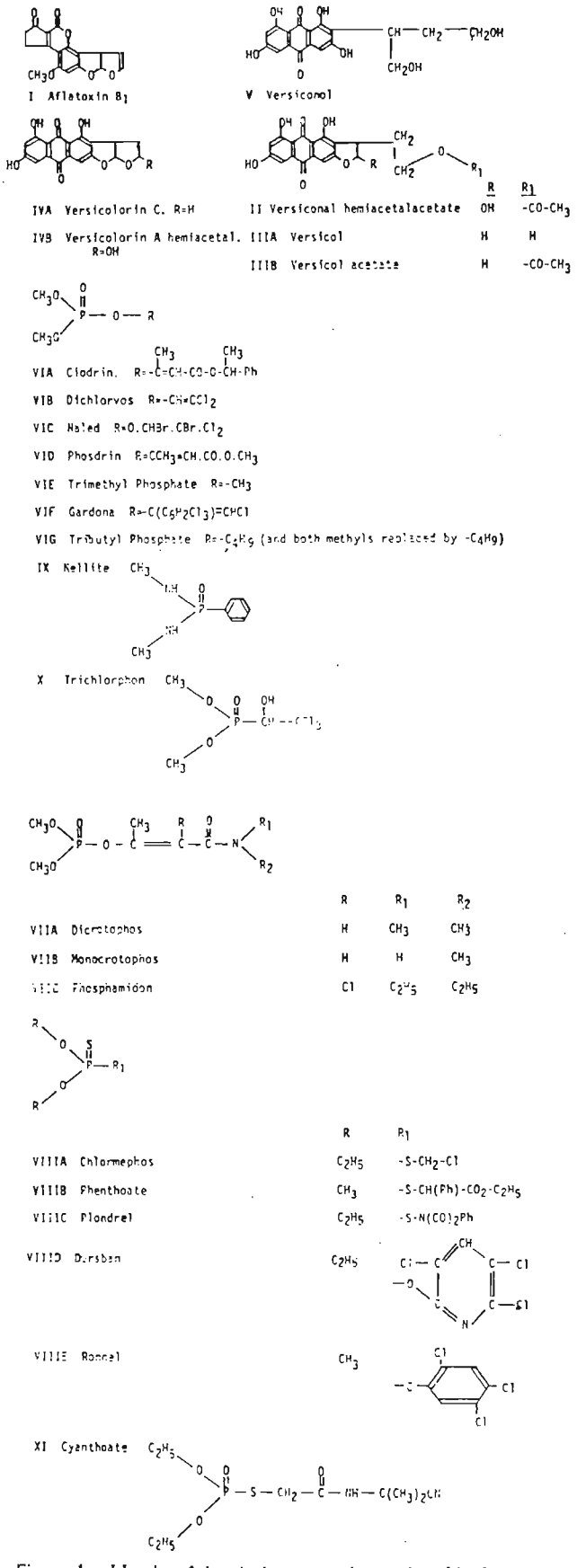

Figure 1. *Identity of chemical compounds mentioned in the text.*

using toluene-ethyl acetate-acetone-acetic acid 50:35:15:2 v/v (TEAA) as the solvent system. Standard quantities of versiconal hemiacetal acetate, versicolorin C and aflatoxin B_1 were also chromatographed, and thus by comparison it was possible to ascertain the approximate concentration levels of these metabolites in the original extracts. More accurate assessments of concentrations were made from the absorbance maxima in absorption spectra, aflatoxin being calculated in terms of aflatoxin B_1 (λ_{max} = 363nm, ε = 21,000) and anthraquinones in terms of versiconal hemiacetal acetate (λ_{max} = 450nm, ε = 7,400). In cases where larger amounts of anthraquinones were present, it was necessary to quantitatively isolate the aflatoxins by thin-layer chromatography and determine their concentrations separately since anthraquinones absorb to a moderate degree at 363 nm. Compounds were prepared for mass spectrometry by preparative thin-layer chromatography using silica gel G layers (0.5 mm) and various solvent systems depending upon the polarity of the compound to be isolated. Mass spectrometry was carried out on an A.E.I. MS9 mass spectrometer. Metabolites were identified by comparing their behavior on chromatography and mass spectrometry with that of authentic compounds.

RESULTS AND DISCUSSION

The results presented in Table 1 are those obtained for organophosphorus compounds which exhibited similar inhibition of aflatoxin biosynthesis in *A. flavus* to that of Dichlorvos while those in Table 2 are the results for compounds which showed no action or had a general inhibitory effect on *A. flavus* metabolism. The addition of Dichlorvos to shake cultures of *A. flavus* resulted in formation of a group of seven related anthraquinone pigments. Three of these compounds were produced in relatively large quantities, i.e. versiconal hemiacetal acetate (II), versicolorin C (IVA) and an unknown pigment (R_f 0.3 in the solvent system described; TEAA). The structure of this unknown compound, from mass spectral data (Table 3), is consistent with that of IIIA and the name versicol is suggested for the compound. The four other anthraquinones were all produced in smaller quantities; these included: averufin (*14*), averufanin (*3*), versiconol (V) and a fourth unknown compound (R_f 0.4). On hydrolysis this unknown compound yielded versicol. Mass spectral and chromatographic properties were consistent with this compound being versicol acetate (IIIB). Versicol acetate may arise from versiconal hemiacetal acetate by direct reduction and thence to versicol by hydrolysis.

Examination of the structure of the pesticides capable

TABLE 1. *Compounds shown to have similar inhibitory activity to Dichlorvos.*

Compound added	Concentration added (μg /ml)	Aflatoxin concent. (μ mol/flask)	Anthraquinone concentration (μ mol/flask)	Mycelial dry weight (g)
Control	—	8.4	0.1	2.0
Dichlorvos control	20	2.5	1.8	2.0
Chlormephos	20	5.2	4.1	2.1
	100	2.5	6.2	1.9
Ciodrin	20	4.4	6.4	1.6
	100	3.0	9.4	1.9
Naled	20	2.2	2.7	1.8
	100	2.1	0.7	1.6
Phosdrin	20	3.1	3.6	2.0
	100	2.0	3.2	1.8
Trichlorphon	20	4.4	2.9	1.9
	100	5.0	8.0	1.8
Ammonium nitrate	150 mg/ml	2.5	5.3	0.9
2-Tridecanone	1.5 mg/ml	7.0	2.8	2.0
	7.5 mg/ml	2.5	5.0	2.5
3-Tridecanone	1.5 mg/ml	7.5	3.2	2.9
	7.5 mg/ml	3.1	6.4	3.0

TABLE 2. *Compounds shown not to have similar inhibitory activity to Dichlorvos.*

Compound added	Concentration added (μ /ml)	Aflatoxin concent. (μ mol/flask)	Anthraquinone concentration (μ mol/flask)	Mycelial dry weight (g/flask)
Control	—	9.4	.1	2.1
Dichlorvos control	20	3.5	4.7	2.4
Cyanthoate	20	6.3	0.1	2.1
	100	3.0	0.1	2.8
Dicrotophos	20	9.3	0.2	3.0
	100	9.2	0.3	2.9
Durbusan	20	10.2	0.1	1.6
	100	10.1	0.1	1.4
Gardona	20	9.1	0.0	2.0
	100	9.1	0.0	2.0
Monocrotophos	20	1.4	0.4	1.2
	100	1.3	0.4	1.1
Nellite	20	10.5	0.1	1.4
	100	3.8	0.1	1.3
Phosphamidon	20	6.6	0.1	2.0
	100	3.8	0.1	1.8
Plondrel	20	6.8	0.1	1.9
	100	6.0	0.1	1.5
Ronnel	20	5.5	0.1	1.4
	100	3.3	0.1	0.9
Trimethyl phosphate	20	7.8	0.1	2.4
	100	11.9	0.1	2.9
Tributyl phosphate	20	10.0	3.2	2.1
	100	10.2	5.5	1.8

of eliciting pigment formation similar to Dichlorvos revealed that in general they possess two small alkyl groups, usually methyl, esterified to a phosphorus atom. Dichlorvos (VIB), Ciodrin (VIA), Phosdrin (VID) and Naled (VIC) have a third more complex group esterified to the phosphorus atom while Trichlorphon (X) has a carbon attached directly to this atom (i.e. it is a phosphonate compound). Finally Chlormephos (VIIIA) differed in that it has two oxygens replaced by sulfurs (i.e. it is a phosphorothiolate). A common feature in these compounds, however, is that they all possess an electron-deficient carbon atom one removed from the phosphorus atom. It is this character which presumably makes the phosphate ester bonds somewhat labile and therefore much more reactive towards target enzymes. Thus if an organophosphorus compound is to have Dichlorvos-like activity it must have at least this latter feature in its structure. Naled (VIC) is similar in structure to Dichlorvos (VIB); however, its lower specific activity is probably due to the presence of a bulky bromine atom on the carbon adjacent to the ester oxygen. In other examples, bulky groups also appear to abolish Dichlorvos-type activity, e.g. a phenyl group as in Phenthoate (VIIIB), Gardona (VIF), Ronnel (VIIIE) and Nellite (IX) or as chloropyridine in Durbusan (VIIID). The presence of nitrogen also appeares to affect activity, this is reflected in the lack of activity of Phosphamidon (VIIC), Monocrotophos (VIIB) and Dicrotophos (VIIA) in spite of the fact that they are very similar in structure to the active compound Phosdrin (VID).

From the evidence it appears that a phosphate ester group is involved in inhibition and therefore it seems likely that a group such as a serinyl hydroxyl is present in one part of the active site of the enzyme which is specifically inhibited. It seems therefore that the inhibition process is at least in part similar to that found in the inhibition of acetylcholine esterase by a phosphorylation process (*7*).

Simple alkyl phosphates, e.g. trimethyl phosphate (VIE), lack the ability to inhibit aflatoxin biosynthesis and this may be explained by the absence of the electron-deficient carbon atom present in the active compounds. However, there was one exception to the lack of activity in simple phosphate esters and this was tributyl phosphate (VIG). This compound did elicite pigment formation in *A. flavus* but the pigments formed did not correspond chromatographically to those produced by addition of Dichlorvos. Four compounds were isolated: versicolorin A, versicolorin C, versicolorin A hemiacetal (IVB) (*6*) [a compound which has been identified in cultures of *Aspergillus parasiticus* (1-11-105 Whl) in our laboratories], and a fourth compound (R_f 0.76) which has not yet been identified.

When ammonium nitrate was added to the culture fluid (10-15% w/v), pigments similar to those produced by Dichlorvos treatment were extracted. This effect may be due to the simultaneous action of both ions since neither ammonium nor nitrate added individually produced the same effect. The addition of 2- or 3-tridecanone as solid (1 g) also resulted in Dichlorvos type

TABLE 3. *Mass spectral data for various metabolites.*

Peak heights quoted as percentage									
Versicolorin A		Versicolorin C		Versicolorin A hemiacetal		Versicol		Versicol acetate	
m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
338	70	340	65	356	55	342	56	384	60
325	48	325	60	339	28	240	12	356	16
310	77	311	82	338	88	330	4	349	22
309	72	297	100	323	20	324	24	342	22
281	100			313	36	311	76	341	22
				311	85	309	26	340	20
				310	88	298	88	325	100
				309	100	297	100	324	100
						386	16	323	100
						385	12	311	60
								310	60
								309	100
								298	60
								297	100

action. At the temperature of the incubator (25 C) they melted into an oily layer which became visibly orange in color as the incubation period progressed. Eventually the tridecanone is metabolized, leaving behind an orange residue. It is possible that these compounds act by solvating the anthraquinones and hence allowing them to pass through the cell membrane which is normally impermeable, thus denying them as intermediates in aflatoxin biosynthesis. In addition it is also possible that tridecanones compete for oxygenases present in the hydrophobic regions of the fungal cell (cf. *5*) and hence by a saturation effect limit the rate of anthraquinone metabolism. Such mechanisms are in accordance with other investigations which suggest that aflatoxin formation may be inhibited by naturally occuring long-chain compounds such as fatty acids (*2*).

It must be stressed that some of these conclusions are tentative and that compounds found to lack inhibitory activity may do so for a number of reasons, e.g. lack of penetration of the cell membranes, fungal detoxification (*13*) etc. Thus it is evident that further investigations are required before the specific inhibitory processes in aflatoxin biosynthesis are to be fully understood and it seems likely that this may only be achieved by utilizing cell-free systems.

ACKNOWLEDGMENTS

The authors are grateful to Professor Y. Hatsuda for a gift of versiconol and to Dr. J. Bennett for a subculture of *A. parasiticus* (1-11-105 Whl).

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Reprinted from Volume 26 of DEVELOPMENTS IN INDUSTRIAL MICROBIOLOGY
A Publication of the Society for Industrial Microbiology, 1985

CHAPTER 34

Comparison of Two Defined Media for Inhibitor and Incorporation Studies of Aflatoxin Biosynthesis

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Aflatoxins are typical secondary metabolites of *Aspergillus flavus* and *A. parasiticus*. As secondary metabolites, the production of aflatoxins is sensitive to changes in environmental parameters such as substrate, and the kinetics of their production is correlated with the cessation of growth during a special stage called idiophase. We have compared two major approaches to studying aflatoxin biosynthesis at the beginning of idiophase (48 h) using two defined media. In one set of experiments, selected organophosphates were added to the defined media at this time; in the second set of experiments, a blocked aflatoxin mutant was presented with a known late intermediate of aflatoxin biosynthesis and transferred to resting media. Despite the different compositions of the media, comparable results were obtained. Of the 15 organophosphates tested, four exhibited dichlorvos-like activity in both media. Inhibition of aflatoxin production and concomitant accumulation of an orange pigment was observed for ciodrin, phosdrin, hexamethyl phosphorous triamide, and triphenyl phosphate. In the resting cell experiments, a blocked aflatoxin-negative mutant was grown in the two different media and then transferred to a resting cell medium in the presence of sterigmatocystin, a known intermediate in aflatoxin biosynthesis. After additional incubation, both B and G aflatoxins were detected from mycelia pregrown in both media. Moreover, chromatographic profiles of mycelial extracts were also similar. We conclude that the differences in aflatoxin production observed on these two media are more quantitative than qualitative.

INTRODUCTION

Aflatoxins are secondary metabolites produced by certain strains of the common molds *Aspergillus flavus* and *A. parasiticus*. These mycotoxins are acutely toxic and carcinogenic to many animal species, and aflatoxin contamination of foods and feeds is a major problem in agriculture. The aflatoxin literature has been reviewed (Goldblatt 1969; Heathcote and Hibbert 1978; Steyn 1980; Bennett and Christensen 1983).

Various media have been devised for the laboratory production of aflatoxins. Highest yields were obtained with natural substrates, such as peanuts, wheat, or rice, or with complex liquid media containing supplements, such as yeast extract or corn steep liquor (Diener and Davis 1969; Venkitasubramanian 1977). Early work showed that defined media traditionally used for culturing fungi, such as Czapecks-Dox medium and Raulin's medium, were poor substrates for aflatoxin

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production (Diener and Davis 1969). Subsequently, two defined media were devised especially for supporting high levels of aflatoxin production. The first of these (AM) was formulated by Adye and Mateles (1964) and has been widely used in studies of aflatoxin biosynthesis (Hsieh and Mateles 1970; Singh and Hsieh 1977; Townsend et al. 1982).

The second (RLSM) was formulated by Reddy et al. (1971) and produced higher yields of total aflatoxin than did the AM medium. Each of these defined media contained a carbon source, NH_4SO_4, $MgSO_4 \cdot 7H_2O$, and various trace elements. However, the proportion of constituents varied between the two media, and RLSM contained 10 g of L-asparagine per liter.

Different laboratories around the world have adopted one or the other of these defined media for studies of aflatoxin biosynthesis, raising the question: Are equivalent results obtained? In this study we have compared the two media for use in two major approaches to the study of aflatoxin biosynthesis: (1) a screening study of organophosphates as possible inhibitors of aflatoxin biosynthesis, and (2) a precursor-feeding study using an aflatoxin-negative mutant and whole cell resting cultures.

Materials and Methods

Fungal strains. The aflatoxigenic wild type strain was *A. parasiticus* (NRRL 5862, SU-1). The aflatoxin-negative strain was a versicolorin-A accumulating mutant of *A. parasiticus* designated *ver-1* (ATCC 36537), which was originally isolated by ultraviolet light irradiation (Lee et al. 1975).

Media and culture conditions. Stock cultures were maintained on potato dextrose agar plus 0.5% yeast extract. The two defined liquid growth media, AM and RLSM, were formulated by Adye and Mateles (1964) and Reddy et al. (1971), respectively. The resting medium (AM-RM) was formulated by Adye and Mateles (1964). AM was composed of the following: sucrose, 50.0 g; $(NH_4)_2SO_4$, 3.0 g; KH_2PO_4, 10.0 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; $Na_2B_4O_7 \cdot 10H_2O$, 0.7 mg; $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$, 0.5 mg; $Fe_2(SO_4)_3 \cdot 6H_2O$, 10.0 mg; $CuSO_4 \cdot 5H_2O$, 0.11; $ZnSO_4 \cdot 7H_2O$, 17.6 mg; and 1,000 ml deionized H_2O.

RLSM was composed of the following: sucrose, 85.0 g; L-asparagine, 10.0 g; (NH_4SO_4), 3.5 g; KH_2PO_4, 0.75 g; $MgSO_4 \cdot 7H_2O$, 0.35 g; $CaCl_2 \cdot 2H_2O$, 0.75 g; $ZnSO_4 \cdot 7H_2O$, 10.0 mg; $MnCl_2 \cdot 4H_2O$, 5.0 mg; $(NH_4)_2\ Mo_7O_{24} \cdot 9H_2O$, 2.0 mg; $Na_2B_4O_7$, 2 mg; $FeSO_4 \cdot 7H_2O$, 2 mg; and 1,000 ml deionized water.

AM-RM was composed of the following: glucose, 15.0 g; KH_2PO_4, 5.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; and the same trace minerals as AM.

All liquid cultures were grown at 27–28 C in a gyratory shaker in the dark.

Organophosphates. The following 16 organophosphates were tested: dichlorvos (dimethyl-2,2-dichlorovinyl phosphate), ciodrin [dimethyl-1-methyl-2(1-phenylcarbetho) vinyl phosphate] phosdrin [methyl-3-(dimethoxyphosphinyloxy) crotonate], dimethyl methylphosphonate, triethyl phosphate, triethyl-2-phosphonopropionate, hexamethyl phosphorous triamide, diethyl-2-bromoethyl

phosphate, triethyl phosphonocrotonate, trimethyl phosphonoacetate, ethyl-diethoxy-phosphinyl formate, triphenyl phosphate, triethyl phosphonoacetate, tris-(2-chloroethyl) phosphate, methyl-dichlorophosphate and diethyl-chlorothiophosphate. For each experiment, a freshly prepared 1% stock solution of a given organophosphate was made in acetone. Cultures of wild type *A. parasiticus* SU-1 were grown in 20 ml of AM or RLSM for 48 h, and then the appropriate amount of organophosphate stock solution was added with a micropipet into the culture in order to deliver 20 ppm. Control flasks contained 35 μl acetone alone. Treated cultures were incubated an additional 72 h before extraction.

Presentation of sterigmatocystin to whole cells in resting cell cultures. One hundred ml of AM or RLSM were inoculated with a dense spore suspension of the *ver-1* mutant and incubated in the dark for 48 h on a rotary shaker. The resultant mycelial pellets were collected in cheesecloth and thoroughly washed with AM-RM. Then 1 g (wet wt) of the pellets was added to 9.8 ml of AM-RM in a 50-ml Ehrlenmeyer flask. To this was added 0.65 μmol of sterigmatocystin (Sigma) dissolved in 0.2 ml acetone. Control flasks contained 0.2 ml acetone without the sterigmatocystin. The resting cell cultures were incubated an additional 48 h on the rotary shaker and then extracted.

Mycelial extractions. After the appropriate times of incubation, wet mycelial pellets were filtered through cheesecloth and then soaked in acetone (25–40 ml) for 2 h. The acetone extract was filtered through a Buchner funnel into a side arm flask, diluted to 70% with deionized water, and poured into a separatory funnel. The aqueous acetone mixture was extracted twice with 30 ml of chloroform and evaporated to dryness under a hood. The resultant dried mycelial extract was resuspended in 1 ml of chloroform or acetone prior to chromatographic analysis.

Thin-layer chromatography and identification of metabolites. Mycelial extracts were separated by thin-layer chromatography (tlc) on 250-mm silica gel G plates (Analtech). Extracts and standards were delivered using a glass syringe calibrated in microliters. The silica gel plates were developed in an unlined covered tank containing 100 ml of the developing solvent. The developing solvent for aflatoxins was chloroform:acetone (9:1, v/v); the developing solvent for pigments was toluene:ethyl acetate:acetone:acetic acid (60:20:15:2 v/v/v/v). Developed plates were observed under long wave ultraviolet light. The authentic standards of aflatoxins (B_1, B_2, G_1, and G_2) and of versiconal hemiacetal acetate (VHA) were provided by Mrs. L. S. Lee, Southern Regional Research Center, New Orleans, LA. Aflatoxins were quantified visually according to the method of Pons et al. (1969).

A two-dimensional tlc analysis was performed on mycelial extracts that produced an orange pigment. Aluminum-backed silica gel 60 plates (Merck 5388) cut into 10-cm squares were spotted with 20 μl of extract in the lower left-hand corner. The plates were first developed in chloroform:acetone (9:1, v/v), dried with a hot air blower, and then developed in toluene:ethyl acetate:acetone:acetic acid (60:25:15:2, v/v/v/v). Developed plates were observed under long wave ultraviolet light. Control plates with an authentic standard of VHA were developed in the same tanks as experimental plates.

Results and Discussion

The results of tlc analysis of mycelial extracts from cultures of wild type *A. parasiticus* grown on AM or RLSM media, with or without 16 organophosphates are presented in Table 1. Controls produced high levels of aflatoxin and no orange pigment. Reduction in aflatoxin production was observed for eight compounds: dichlorvos, ciodrin, diethyl chlorothiophosphate, tris-(2-chlorethyl) phosphate, phosdrin, hexamethyl phosphorous triamide, triethyl 4-phosphocrotonate, and triphenyl phosphate. For five of these compounds (dichlorvos, ciodrin, phosdrin, hexamethyl phosphorous triamide, and triphenyl phosphate) aflatoxin inhibition was correlated with production of an orange pigment. The orange pigment had the same R_f as an authentic sample of VHA in both one-dimensional and two-dimensional tlc.

The first organophosphate to be associated with aflatoxin inhibition was dichlorvos based on experiments using wheat, rice, peanuts, and corn (Rao and Harein 1972). Hsieh (1973) later reported inhibition of aflatoxin production in defined AM medium; subsequently, dichlorvos treatment of aflatoxigenic strains was associated with the accumulation of an orange pigment (Schroeder et al. 1974; Yao and Hsieh 1974) and the characterization of this orange pigment as VHA (Cox et al. 1977; Fitzell et al. 1977). Both C^{14} and C^{13} isotopic-labeling studies indicated that VHA was an intermediate in aflatoxin biosynthesis (Bennett and Christensen 1983). In our screen of organophosphate compounds, four exhibited inhibition of aflatoxin biosynthesis correlated with the accumulation of an orange pigment with the same chromatographic mobility as an authentic standard of VHA. Results were similar in both AM and RLSM. Dutton and Anderson (1980) have previously reported dichlorvos-like activity in RLSM for chlormephos, ciodrin, naled, phosdrin, and trichlorphon.

The insecticidal activity of dichlorvos and other organophosphates is due to their inhibition of cholinesterase activity. This and previous studies (Hsieh 1973; Dutton and Anderson 1980) show that only some organophosphates inhibit aflatoxin production. One possible explanation for the differential activity is that the mechanism of aflatoxin inhibition is not similar to that of the cholinesterase inhibition. In fact, Dutton and Anderson (1980) postulated that dichlorvos and other organophosphates affecting aflatoxin production did so by an inhibition of fungal oxygenase activity. Other explanations for the inactivity of the certain organophosphates is that there is differential solubility or sensitivity to fungal detoxification.

The results of the experiments with resting cell cultures of *ver-1* with and without sterigmatocystin are presented in Table 2. The versicolorin A-accumulating mutant is normally blocked in aflatoxin production. When this mutant was grown for 2 d in AM or RLSM and then transferred to AM-RM in the presence of 0.65 μmol sterigmatocystin, aflatoxins were recovered after 48 h of incubation. Both B and G aflatoxins were detected, with similar concentrations produced by both AM-grown and RLSM-grown cultures. The chromatographic profiles of mycelial extracts were also similar except that AM-grown cultures produced a blue-green compound with an R_f similar to that of aflatoxin B_{2a}. This compound was usually absent in chromatograms of RLSM-grown cultures.

The kinetics of aflatoxin biosynthesis are similar to those of many other fungal

TABLE 1. *Presence of aflatoxins and an orange pigment after thin-layer chromatography of wild type* Aspergillus parasiticus *grown on two defined media, with and without 16 selected organophosphates*

Organophosphate[a]	AM Medium		Reddy Medium	
	Aflatoxins[b]	Orange pigment[c]	Aflatoxins[b]	Orange pigment[c]
Control	++++	−	++++	−
Dichlorvos	++	+	++	+
Ciodrin (33)	++	+	++	+
Dimethyl methylophosphonate (35)	++++	−	++++	−
Triethyl phosphate (37)	++++	−	++++	−
Methyl dichlorophosphate (27)	++++	−	++++	−
Diethyl chlorothiophosphate (34)	+++	−	++	−
Tris(2-chloroethyl)phosphate (29)	++	−	+++	−
Phosdrin (32)	++	+	+++	+
Triethyl phosphonoacetate (35)	++++	−	++++	−
Trimethyl phosphonoacetate (35)	++++	−	++++	−
Hexamethyl phosphorous triamide (45)	+++	+	+++	+
Triethyl 4-phosphonocrotonate (35)	+++	−	+++	−
Triethyl 2-phosphonopropionate (36)	++++	−	++++	−
Ethyl diethoxyphosphinyl formate (36)	++++	−	++++	−
Diethyl 2-bromoethyl phosphonate (35)	++++	−	++++	−
Triphenyl phosphate (35)	+++	+	+++	+

[a] Numbers in parentheses indicate μl of 1% stock solution of compound added to defined media.
[b] Fluorescence under long wave ultraviolet light, with (++++) equivalent to controls.
[c] Orange pigments with same R_f as an authentic standard of versiconal hemiacetal acetate. (+) = presence; (−) = none detected.

TABLE 2. *Recovery of aflatoxins after presentation of 0.65 μmol sterigmatocystin (ST) to a blocked aflatoxin-negative mutant*

Growth Medium[a]	With or Without ST (0.65 μmol)	Aflatoxin (nmol)	
		B_1	G_1
AM	ST	391	Trace[b]
AM	control	ND[c]	ND
RLSM	ST	387	Trace
RLSM	control	ND	ND

[a] See methods for description of media.
[b] Trace = less than 10 nmol.
[c] ND = none detected.

secondary metabolites, i.e., the onset of biosynthesis is usually correlated with the cessation of growth during a special stage called idiophase. The mycelia of our blocked mutant were grown in AM or RLSM and transferred to resting medium at the beginning of idiophase. No aflatoxins were detected in controls, but cultures of both AM-grown and RLSM-grown mycelia produced similar levels of aflatoxin in the presence of sterigmatocystin. Sterigmatocystin is a known precursor of aflatoxin biosynthesis (Hsieh et al. 1973).

Aflatoxins are acetate-malonate derived secondary metabolites. In controlled liquid fermentations, the events of secondary metabolism follow a predictable pattern that can be manipulated by changing the composition of the medium, the temperature of incubation, and other experimental parameters (Foster 1947; Drew and Demain 1977). Since secondary metabolism is so sensitive to changes in the substrate used for growth, there has been difficulty in comparing the results from different laboratories on the events initiating aflatoxin biosynthesis. For example, many of the C^{14}-precursor incorporation studies have been conducted by growing cells in AM medium and transferring to a resting cell culture (Bennett and Christensen 1983); studies on carbohydrate metabolism have involved growth in a peptone medium (Abdollahi and Buchanan 1981); and experiments on the interface between primary and secondary metabolism have employed RLSM (Maggon et al. 1977; Clevstrom et al. 1983).

In summary, we have compared mycelia pregrown in two different defined media for 48 h. In one set of experiments, selected organophosphates were added at the beginning of idiophase; in the second set of experiments, a blocked aflatoxin mutant was presented with a known late intermediate of aflatoxin biosynthesis and transferred to resting media at this time. Despite the different compositions of the media, qualitatively comparable results were obtained in both sets of experiments, although quantitative differences in levels of aflatoxins produced were detected. In the future, workers employing either AM or RLSM may be more confident in making inter-laboratory comparisons of their data on aflatoxin biosynthesis.

Acknowledgments

We thank John Floyd, Louise Lee, and Eivind Lillehoj for their advice and en-

couragement. This research was supported by a Cooperative Agreement from the U.S. Department of Agriculture (#532569).

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The interaction between additives, yeasts and patulin production in grass silage

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Abstract

Both laboratory-prepared and sterile farm silage was found to support growth of *Paecilomyces sp.* and patulin production. The formation of patulin was affected by the levels of yeast present in the silage, and it was found that there was an inverse relationship between yeast population levels and patulin concentration.

The commercial silage additive, 'Sylade' had a greater lethal effect on yeast and fungi than 'Add F', the latter allowing the formation of patulin by *Paecilomyces sp.* in the silage.

In an attempt to produce better silage in a consistent manner, a number of silage additives, e.g. 'Sylade' and 'Add F', have been introduced. These quickly lower the pH of the silage which results in the promotion of lactic acid-producing bacilli and preserve the silage from clostridial activity. The effects of these additives on the fungal flora and in particular those capable of forming mycotoxins has received little attention apart from that of Di Menna and co-workers (2) and Escoula (3).

One common fungal contaminant of silage is *Paecilomyces sp.*, the imperfect stage of *Byssochlamys*, which is capable of forming the mycotoxin patulin (7) and has been associated with illness and death in dairy cattle (9). The production of patulin on a natural substrate is complicated by the fact that it is subject to degradation by both producing-species of *Paecilomyces* (1) and by yeast (4). Furthermore it has been shown in fermenting apple juice that patulin above certain concentrations is toxic to the strains of yeast involved in its degradation (6). Consequently it can be inferred that there must be a relationship between the concentration of patulin and the level of a yeast population in any commodity where the two occur.

In this report the effects of patulin and yeast upon each other in silage and the influence on this interaction by the commercially available additives, 'Sylade' and 'Add F' are described.

Materials and methods

Culturing

Paecilomyces sp. was maintained as previously described (1). Where required spore suspensions were prepared in the range 1×10^6-10^7 cm^{-3} in distilled water from 14-day-old cultures.

Microbial analysis on the silage was conducted by a serial dilution method using Ohio Agricultural Experimental Station agar (OAES) (5) for the isolation of yeasts and fungi at 25° and 30° and nutrient agar (Oxoid) with the addition of actidione (0.5%) (NAA) for the enumeration of total bacteria.

Mixed yeast cultures were isolated from farm silage on OAES and used to prepare a suspension of cells in distilled water, (about 1×10^6 cells cm^{-3}).

Silage preparation

For the preparation of laboratory-made silage perennial rye grass (*Lolium perenne*), dry matter content 24%, was obtained from The Grasslands

Research Institute, Hurley, Berks. This was packed into Pyrex glass tubes (38 × 200 mm) as four 10-g portions which allowed subsequent sampling of the silage to be done at four levels. The tubes were sealed with rubber bungs which had been fitted with fermentation traps and then incubated for various time periods at 25 °C.

Farm silage was obtained from a Nottinghamshire farm and where necessary sterilized by γ-radiation, (6 MCi total). This was then packed into tubes in a similar manner to the ryegrass in order to simulate silage clamp conditions.

Where required the spore suspensions of *Paecilomyces sp.* or yeasts were sprayed into the silage at a rate of 10^3 spores g^{-1} silage.

The addition of a 'Sylade' and 'Add F' was done by spraying a known amount of silage at a rate equivalent to 2.5l Kg^{-1}. Patulin solution was sprayed onto the silage at a rate of 100 cm^3 Kg^{-1}, the actual concentration of solution depending upon the amount of patulin required in the sample of silage.

Extractions and detection of patulin

Patulin was extracted from silage using an adaptation of a multi-mycotoxin method (8). The silage (40 g) was extracted with ethyl acetate (100 cm^3) and filtered. The extract was then reduced to a volume of 10 cm^3 using a rotary vacuum evaporator. The concentrated extract was dialysed overnight against 30% (v/v) aqueous acetone (100 cm^3) and then the dialysate was extracted with chloroform (2 × 50 cm^3 portions). The combined extracts were then dried over anhydrous sodium sulphate and evaporated to a standard volume (2 cm^3) by rotary evaporation.

Patulin was determined by h.p.l.c. using a Waters Associated Model 600 system and Whatman Partisil PXS 10/25 ODS II reverse phase column for separation. The solvent system used was methanol: water:acetic acid 100-99-1(v/v) with a flow rate of 1 cm^{-3} min^{-1}. Detection was done with a Waters Associated Model 440 UV spectrophotometer which had a dual wavelength facility of 254 and 280 nm.

Results and discussion

The formation of patulin in silage

Attempts to produce patulin in farm silage inoculated with a high patulin-producing strain of *Paecilomyces sp* was unsuccessful in spite of the fact that the fungus could be isolated at levels greater than 10^3 propagules g^{-1} silage at any point in the 56-day incubation period. It was concluded that patulin was being degraded by microflora in the silage, as it was unlikely that an increase in *Paecilomyces sp.* propagules could occur without patulin production.

Identical experiments with sterilized farm silage confirmed this opinion as patulin was formed in all the various silage levels monitored (Table 1). These results also showed that there is a decrease in the production of silage towards the bottom of the silage and also of the fungal propagules isolated both at 25 °C and 37 °C. Thus the degree of anaerobiosis in the silage can be correlated with both fungal growth and patulin production. This agrees with an observation previously made (10) that patulin is only detected in small pockets within the silage in the field where semi-aerobic conditions prevail.

The effect of patulin on silage microflora

In order to confirm the degrading action of yeast, patulin was added to laboratory-prepared silage which had been deliberately inoculated with a high level of yeast. The results show that patulin was

Table 1. Patulin content and growth of *Paecilomyces sp.* as estimated by viable propagules at various levels in sterilized-farm silage.[a]

Tube quarter	Patulin content mg Kg^{-1} [b]		*Paecilomyces* propagules g^{-1}	
	Day 7	Day 14	Day 7	Day 14
Upper	4.25	4.32	6.2×10^6	6.6×10^6
Upper-middle	0.98	1.09	4.6×10^5	2.2×10^6
Lower-middle	0.72	1.04	3.7×10^4	1.1×10^5
Bottom	0.17	0.88	3.4×10^3	9.0×10^5

[a] Mean of two experiments.
[b] Calculated on the basis of an 82% recovery.
[c] Isolated after five days incubation on OAES. Similar results were obtained for the incubation at 37 °C.

Table 2. The breakdown and effect of added patulin on the microorganisms in laboratory-prepared silage inoculated with yeast.[a]

Time days mg Kg^{-1}	Microbial count g^{-1}			Patulin content[d]	Patulin breakdown (%)
	Bacteria[b]	Yeast[c]	Fungi[c]		
0	2.45×10^6	4.5×10^5	2.1×10^5	10.00	0
2	1.2×10^6	10	4.3×10^5	10.00	0
4	1.4×10^6	0	8.8×10^3	7.80	22
6	1.75×10^6	0	2.6×10^4	7.56	24

[a] Mean of two experiments

[b] Isolated after three days incubation on NAA at 25 °C.

[c] Isolated after five days incubation on OAES at 25 °C. Similar results were found for the incubation of 37 °C after three days.

[d] Calculated on a recovery of 82%. Patulin initially added at a rate of 10 mg Kg^{-1} silage.

degraded at a rate of 23% of that added, (10 mg Kg^{-1}), over a six-day incubation period (Table 2). This cannot be accounted for by the action of *Paecilomyces sp.* as the incubation period was too short (1) and therefore it must be due to the presence of the yeast. It was also observed that there was a decrease in the yeast population which can be explained by the toxicity of the patulin. This result agrees with observations made on fermenting apple juice (4).

On repeating the experiment with various concentrations of patulin in farm silage (Table 3), it was found that there is a threshold level of patulin between 10 and 20 mg patulin Kg^{-1} silage, above which the yeast did not proliferate but below which they were able to grow and degrade patulin. The number of propagules of *Paecilomyces sp.* present were also affected in that they increased with decrease in patulin concentration. This was in contrast to total bacterial population which remained constant at all levels of patulin tested.

These results show that high levels of patulin can be produced in silage when contaminated with *Paecilomyces sp.* It is also evident that the amounts of patulin formed will depend upon the presence of yeasts. If these proliferate rapidly before the onset of patulin production they will control the amount of patulin formed by degrading it. However if patulin is produced in quantity prior to yeast proliferation then their levels will be depressed. From a practical point of view it would seem that an initial rapid production of high levels of yeast in silage is to be encouraged when contamination with *Paecilomyces sp.* and other patulin-forming fungi are likely to be a problem.

The effect of silage additives

When laboratory-made silage is treated with 'Add F' and 'Sylade' differences in the microbial populations were observed (Table 4). The addition of 'Sylade' completely inhibited the growth of yeast

Table 3. The effect of various added concentrations of patulin on the microbial flora in farm-made silage.[a]

Patulin added mg Kg^{-1}	Microbial counts g^{-1} silage			Patulin recovered mg Kg^{-1} [d]	Patulin breakdown (%)
	Bacteria[b]	Yeast[c]	Fungi[c]		
20.0	4.5×10^6	0	2.2×10^3	16.50	17.5
10.0	3.9×10^6	4.6×10^2	9.6×10^4	7.75	22.5
1.0	4.5×10^6	4.0×10^4	6.5×10^5	0.25	75.0
0.1	4.1×10^6	4.2×10^4	1.9×10^5	0	100
0	5.5×10^6	7.2×10^4	2.7×10^5	0	-

[a] Mean of two experiments.

[b] Isolated after three days incubation on NAA at 25 °C.

[c] Isolated after five incubation on OAES at 25 °C. Similar results were obtained for the incubation at 37 °C after three days.

[d] Calculated on a recovery of 82%.

Table 4. Yeast and fungal populations and patulin production in laboratory-prepared silage treated with various additives and inoculated with *Paecilomyces.*

Incubation period days	Sylade treated			Add F treated		
	Yeasts[a]	Fungi[a]	Patulin mg Kg^{-1}	Yeasts[b]	Fungi[b]	Patulin g Kg^{-1} [c]
2	0	10	0	1.1×10^2	1.2×10^3	0
4	0	10	0	2.1×10^3	3.0×10^3	0
6	0	10	0	10	5.0×10^3	0.4
8	0	1.1×10^3	0	10	7.2×10^3	0.5
10	0	1.6×10^1	0	2.2×10^1	3.4×10^4	0.4
12	0	10	0	10	1.0×10^3	0.4
14	0	10	0	10	2.7×10^3	0
28	0	3.2×10^2	0	10	3.1×10^4	0
56	0	10	0	10	1.2×10^3	0

[a] Isolated after five days on OAES. Natural inoculm from the rye grass plus added *Paecilomyces.* Similar results were obtained for the incubation at 37 °C over three days.

[b] Isolated after five days incubation on OAES. Natural inoculum from the rye grass plus added *Paecilomyces.* Higher counts were obtained for the incubation at 37 °C over three days.

[c] Calculated on a recovery of 82%.

and greatly suppressed the growth of the fungi where the highest count obtained was 1.1×10^3 propagules g^{-1}. In the case of 'Add F' the levels of yeasts and fungi were very similar to that of the control (Table 5) apart from the yeast cultured at 37 °C which were more prolific in the treated silage. Treatment with 'Sylade' prevented the formation of patulin, which is explained by the inhibition of fungal growth, whereas 'Add F' permitted the production of patulin but at a reduced concentration as compared with the control (Table 5).

The practical implication of these results is that the commercial silage additive 'Sylade' effectively controls the formation of and the growth of fungi in silage. This could be used as a criterium for using 'Sylade' in preference to 'Add F' in the production of silage, although it should be borne in mind that this conclusion is based on results where *Paecilomyces sp.* artificially predominates which is unlikely to occur in efficiently made silage.

Table 5. Yeast and fungal populations and patulin production in laboratory-prepared silage inoculated with *Paecilomyces.*[a]

Incubation Period days	Yeasts[b]	Fungi[b]	Patulin content mg Kg^{-1} [c]
2	0	2.6×10^3	0
4	1.2×10^3	4.0×10^3	0
6	0	1.0×10^3	0
8	10	1.3×10^4	1.2
10	1.2×10^2	2.5×10^3	1.5
12	10	2.6×10^4	1.1
14	0	2.5×10^3	0.5
28	1.7×10^2	5.7×10^4	0
56	1.3×10^2	6.3×10^3	0

[a] Mean of two experiments.

[b] Isolated after five days incubation on OAES at 25 °C. Natural inoculum plus *Paecilomyces.* Similar results were obtained for the incubation at 37 °C.

[c] Calculated on a recovery of 82%.

Acknowledgements

We wish to thank Mr A. Hacking (M.A.F.F./A.D.A.S., Aberystwyth); Dr M. Woolford (The Grasslands Research Institute, Hurley, Berks) and Mr A. Buckle (M.A.F.F./A.D.A.S., Shardlow) for their advice on various aspects of the work performed and also to Mr A. Hacking for patulin-producing strains of *Paecilomyces.* We also wish to thank BP, Chemicals Division, Hull, for the supply of 'Add F' and 'Sylade'.

This work has been supported by a grant from the S.R.C., U.K.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1987, p. 587–592 Vol. 53, No. 3
0099-2240/87/030587-06$02.00/0

T-2 Toxin Metabolism by Ruminal Bacteria and Its Effect on Their Growth

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Received 15 August 1986/Accepted 3 December 1986

The effect of T-2 toxin on the growth rates of different bacteria was used as a measure of its toxicity. Toxin levels of 10 μg/ml did not decrease the growth rate of *Selenomonas ruminantium* and *Anaerovibrio lipolytica*, whereas the growth rate of *Butyrivibrio fibrisolvens* was uninhibited at toxin levels as high as 1 mg/ml. There was, however, a noticeable increase in the growth rate of *B. fibrisolvens* CE46 and CE51 and *S. ruminantium* in the presence of low concentrations (10 μg/ml) of T-2 toxin, which may indicate the assimilation of the toxin as an energy source by these bacteria. Three tributyrin-hydrolyzing bacterial isolates did not grow at all in the presence of T-2 toxin (10 μg/ml). The growth rate of a fourth tributyrin-hydrolyzing bacterial isolate was unaffected. *B. fibrisolvens* CE51 degraded T-2 toxin to HT-2 toxin (22%), T-2 triol (3%), and neosolaniol (10%), whereas *A. lipolytica* and *S. ruminantium* degraded the toxin to HT-2 toxin (22 and 18%, respectively) and T-2 triol (7 and 10%, respectively) only. These results have been explained in terms of the presence of two different toxin-hydrolyzing enzyme systems. Studies with *B. fibrisolvens* showed the presence of a T-2 toxin-degrading enzyme fraction in a bacterial membrane preparation. This fraction had an approximate molecular weight of 65,000 and showed esterase activity (395.6 μmol of *p*-nitrophenol formed per min per mg of protein with *p*-nitrophenylacetate as the substrate).

The trichothecenes are a chemically related group of biologically active metabolites of which T-2 toxin is an important member (16). The importance of these mycotoxins cannot be overemphasized, especially in many parts of the world where a large proportion of the diet of an animal is composed of products that are naturally infected with fusaria and related fungal species capable of trichothecene production.

T-2 toxin reportedly causes a toxicosis usually associated with hemorrhaging and was first reported by Hsu et al. (9), who demonstrated that toxin levels of 2 mg/kg of mouldy corn were associated with death in 20% of a dairy herd. However, ruminants are generally less susceptible to trichothecene toxicoses than are monogastric animals (13). In an attempt to elucidate the role of ruminal fermentation in toxin degradation, experiments were conducted on sheep fistulated in the lower part of the abomasum, through which a culture of *Fusarium sporotrichioides* was introduced. The treatment caused acute toxicosis within 30 min, whereas an animal fed the same diet per os for a period of 6 days remained clinically healthy (13). It was concluded that the relative resistance of ruminants to toxic compounds of *Fusarium* spp. results from the complex ruminal digestion process. Other studies have also shown that T-2 toxin and a number of other mycotoxins considered to be toxic to domestic ruminants were metabolized to less toxic products by microorganisms in whole rumen fluid in vitro (11). Ochratoxin A was degraded to ochratoxin α and phenylalanine in bovine ruminal fluid (10). Deoxynivalenol was degraded to a de-epoxidation product (12), whereas HT-2 toxin was identified as a breakdown product of T-2 toxin after incubation in ovine ruminal fluid (11).

Since the detoxification of T-2 toxin involved deacetylation, experiments were conducted with pure cultures of ruminal bacteria known to have esterase activity. The effect of T-2 toxin on growth rate was monitored, in addition to the formation of T-2 toxin breakdown products. A crude enzyme preparation with esterase activity was isolated from *Butyrivibrio fibrisolvens* and shown to hydrolyze T-2 toxin. (This research was conducted by K. Westlake, in partial fulfillment of the requirements for the Ph.D. degree from the University of Natal, Pietermaritzburg, Republic of South Africa, 1985).

MATERIALS AND METHODS

Organisms and sources. Bacterial cultures were maintained on agar slopes (4) above liquid nitrogen. *B. fibrisolvens* CE46, CE51, CE52, and CE56 have been previously characterized (15). The other bacteria utilized were *Anaerovibrio lipolytica* 5S (7, 8) and *Selenomonas ruminantium* ATCC 19205. The tributyrin-hydrolyzing bacteria KTB9, KTB10, KTB13, and KTB16 were isolated from ovine whole rumen fluid on medium 10 of Caldwell and Bryant (5) modified to contain tributyrin (1%, vol/vol) as the sole energy source. The isolations were conducted in an anaerobic cabinet (model 1024; Forma Scientific, Marietta, Ohio) with a 30% CO_2/65% N_2/5% H_2 gas phase.

Growth rate and toxicity studies. All growth rate studies were conducted in specially marked, narrow-neck, 1-oz. (ca. 29-ml) McCartney bottles equipped with tight-fitting rubber bungs (Sarmcol 254; Howick, Natal, South Africa). These bottles were first gassed with an anaerobic gas mixture (30% CO_2–65% N_2–5% H_2), sealed, and sterilized. Medium 10 containing glucose (1%, wt/vol) was dispensed into the bottles in 10-ml amounts with a repeating syringe.

Stock cultures of bacteria maintained on agar slopes were allowed to thaw. Three serial transfers in liquid culture were done, and the resulting inoculum was used in growth rate studies.

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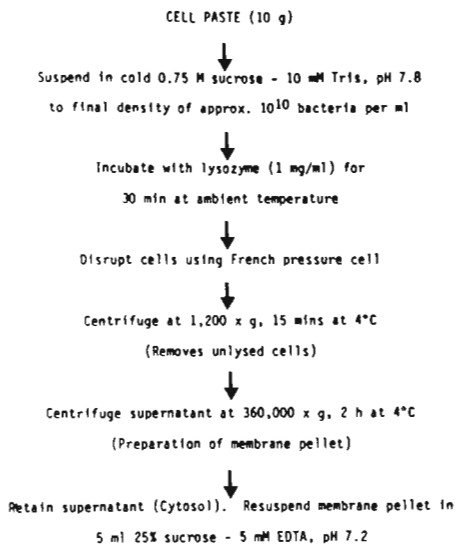

FIG. 1. Flow chart for preparation of membrane and cytosol fractions from *B. fibrisolvens* CE51.

T-2 toxin, prepared as a concentrated solution in dimethylformamide (DMF), was injected into the McCartney bottles which contained medium to achieve the required concentration. Medium injected with DMF only served as a control. All test and control media were inoculated in triplicate. After equilibration at 39°C, each bottle was inoculated with 0.2 ml of bacterial inoculum, and the optical density at 578 nm was immediately determined (Eppendorf photometer 1260 with a modified cell holder for 1-oz. McCartney bottles). Subsequent optical density readings were taken at 15- to 30-min intervals until the stationary phase was reached. At this point each bottle was opened, and the supernatant was extracted three times with an equal volume of ethyl acetate. After drying with anhydrous $(NH_4)_2SO_4$, evaporation of the solvent at 60°C under a stream of N_2, and reconstitution in 200 μl of ethyl acetate, the extract was analyzed by gas-liquid chromatography (K. Westlake, M. F. Dutton, R. K. Berry, J. D. Lee, and P. R. Barrowman, submitted for publication).

Optical density data were entered onto a minicomputer (Olivetti P6060), and a linear regression program was used to calculate growth rates and correlation coefficients.

Preparation of bacterial suspensions for use in enzyme studies. Twenty liters of sterile anaerobic medium 10 plus $NaHCO_3$ (9.1%, wt/vol) was prepared. The complete medium was then gassed with an anaerobic gas mixture at a pressure of approximately 3.4 kPa for a minimum of 12 h.

Bacterial inoculum (200 ml) was prepared and added to the vessel containing 20 liters of medium via the transfer line by using an anaerobic gas mixture to create a positive pressure. The medium was incubated at 39°C. Samples were taken at regular time intervals until an optical density of 0.8 to 1.0 had been attained (12 to 24 h). The medium-dispensing line was then connected to the inlet line of a zonal rotor of a centrifuge (model RC-2; Ivan Sorvall, Inc., Norwalk, Conn.), and the medium was centrifuged (27,500 × *g*; 4°C; flow rate, ca. 1.5 liters/h). After centrifugation, the rotor tubes were transferred to an anaerobic cabinet, and the pelleted bacteria were washed into a 400-ml screw-cap bottle with an anaerobic diluent (3). The bottle was tightly sealed, and the bacteria were stored at −10°C for further use.

Cell fractionation and enzyme studies. Although *B. fibrisolvens* is classified as a gram-negative bacterium, the cell wall ultrastructure is more typical of gram-positive bacteria (2, 6). Membrane and cytosol fractions of this bacterium were prepared as detailed in Fig. 1. Resulting cytosol and membrane fractions were treated with ammonium sulfate at 4°C for 60 min before centrifugation (12,000 × *g*, 15 min, 4°C). Fractions of 0 to 30, 30 to 40, 40 to 50, and 50 to 60% ammonium sulfate concentration were collected and assayed for esterase activity.

Gel filtration of active fractions was performed on Sephadex G-150 (40/120 mesh). The dry gel was allowed to swell for 24 h in 0.05 M phosphate buffer, pH 7.0, before being used to pack the column (70 by 2.5 cm). Samples were eluted with 0.05 M phosphate buffer (pH 7.0), and the effluent was collected in 6-ml fractions (Golden Retriever model 328; ISCO, Lincoln, Nebr.) The protein concentration was mea-

TABLE 1. Effect of T-2 toxin on the specific growth rate (μ) and lag time (*t*) of different strains of *B. fibrisolvens*, *S. ruminantium*, and *A. lipolytica* grown on 1% (wt/vol) glucose[a]

Bacterial species and strain	μ[b]		*r*[c]		*t* (h)	
	Without T-2 toxin	With T-2 toxin[d]	Without T-2 toxin	With T-2 toxin	Without T-2 toxin	With T-2 toxin
B. fibrisolvens						
CE46	0.69	0.90	0.994	0.981	5.5	6.3
CE51	0.64	0.76	0.997	0.995	4.4	6.2
CE52	0.60	0.61	0.989	0.922	0.6	1.6
CE56	0.54	0.51	0.992	0.992	0.3	0.8
S. ruminantium	0.80	0.90	0.997	0.998	1.4	1.3
A. lipolytica	0.52	0.52	0.994	0.991	3.3	4.2
Tributyrin hydrolyzers						
KTB9	0.52	No growth	0.993		0.2	
KTB10	0.56	No growth	0.995		0.8	
KTB13	0.55	No growth	0.994		0.2	
KTB16	0.46	0.56	0.995	0.991	0.3	0.0

[a] Values represent the mean of experiments conducted in triplicate.
[b] At time $t - 1$.
[c] *r*, Correlation coefficient.
[d] At 10 μg/ml.

TABLE 2. Effect of different concentrations of T-2 toxin on the growth rate (μ) and lag time (t) of *B. fibrisolvens* CE51 grown on 1% (wt/vol) glucose[a]

T-2 concn (μg/ml)	μ[b]	r	t (h)
0	0.55	0.997	1.2
0.1	0.52	0.999	0.7
0.5	0.55	0.998	0.5
1.0	0.55	0.998	0.4
2.0	0.48	0.998	0.4
10.0	0.51	0.998	0.5
50.0	0.51	0.999	0.9
100.0	0.53	0.999	0.4
500.0	0.51	0.998	0.6
1,000.0	0.49	0.997	0.4

[a] Values represent the mean of experiments conducted in triplicate.
[b] At time $t - 1$.

sured at 280 nm with an ISCO model UA-2 analyzer. Protein pack samples were pooled for enzyme analysis.

Toxin degradation by fractions obtained after gel filtration on Sephadex G-150. Pooled protein fractions were incubated at 39°C for 10 min, and then T-2 toxin prepared in DMF was added at 100 μg/ml. Samples (10 ml each) were withdrawn at various time intervals and extracted three times with an equal volume of ethyl acetate. The pooled extracts were then dried at 60°C under a stream of N_2 and analyzed by gas-liquid chromatography.

Protein determination. Protein was measured by the Bio-Rad assay procedure (Bio-Rad protein assay kit II; Bio-Rad Laboratories, D-8000, Munich, Federal Republic of Germany). A calibration curve prepared by using bovine serum albumin (1.2 mg/ml) was linear between 0 and 120 μg of protein.

Measurement of esterase activity. Esterase activity was assayed by monitoring the release of *p*-nitrophenol from *p*-nitrophenylacetate at 405 nm on a Beckman 25 scanning spectrophotometer at 39°C. The reaction mixture consisted of 2.0 ml of 0.1 M phosphate buffer (pH 6.0) containing 1 mM dithiothreitol, 0.5 ml of enzyme and cell extract, and 0.01 ml of *p*-nitrophenylacetate (0.251 M) in DMF.

Formation of *p*-nitrophenol was measured against a blank, which was 2.5 ml of 0.1 M phosphate buffer (pH 6.0) containing 1 mM dithiothreitol and 0.01 ml of *p*-nitrophenylacetate (0.251 M) in DMF.

RESULTS

Effect of T-2 toxin on growth rate and lag time of pure cultures of ruminal bacteria. Growth rate experiments in batch culture containing 1% (wt/vol) glucose as the carbon source were used to assess the toxicity of T-2 toxin to different ruminal anaerobic bacteria. The results indicate that T-2 toxin, added at a concentration of 10 μg/ml, had no measurable inhibitory effect on the growth rate of *B. fibrisolvens* CE46, CE51, CE52, and CE56; *S. ruminantium*; and *A. lipolytica* (Table 1). Notably, the growth rate of strains CE46 and CE51 and *S. ruminantium* appeared to be stimulated by the presence of T-2 toxin. The correlation coefficients indicated good agreement between experiments performed in triplicate (Table 1). There was a tendency for lag times to increase in the presence of T-2 toxin, but subsequent experiments did not confirm this trend. Four unidentified tributyrin-hydrolyzing bacterial isolates were isolated from the rumen and tested for susceptibility to T-2 toxins. Three of these isolates were completely inhibited by the presence of T-2 toxin (10 μg/ml), whereas the fourth isolate (KTB16) was not affected (Table 1). The tributyrin-hydrolyzing isolate KTB16 was morphologically similar to the other *B. fibrisolvens* strains.

B. fibrisolvens CE51 was selected for further study to determine the effect of T-2 toxin concentration on growth rate and lag time. Even at toxin concentrations as high as 1 mg/ml, there was no significant effect on growth rate or lag time (Table 2). Although the stimulation of growth rates in the presence of T-2 toxin, as reported in Table 1, was not confirmed in this experiment, this can probably be ascribed to differences in inocula and media.

Degradation of T-2 toxin and the formation of toxic breakdown products by pure cultures of ruminal bacteria. All strains of *B. fibrisolvens*, grown with glucose as the energy

TABLE 3. Degradation of T-2 toxin (10 μg/ml) and formation of less toxic breakdown products by different strains of *B. fibrisolvens*, *S. ruminantium*, and *A. lipolytica* grown on 1% (wt/vol) glucose or cellobiose[a]

Energy source and bacterium	Toxin concn (% of added T-2)				Recovery (%)
	T-2	HT-2	T-2 triol	Neosolaniol	
Glucose					
B. fibrisolvens					
CE46	69	16	3	9	97
CE51	57	22	3	10	92
CE52	48	33	12	0	93
CE56	28	38	9	14	89
S. ruminantium	62	18	10	0	90
A. lipolytica	59	22	7	0	88
Cellobiose					
B. fibrisolvens					
CE46	61	12	14	0	87
CE51	60	29	5	7	91
CE52	75	13	5	0	93
CE56	67	8	6	9	90

[a] Values represent the mean of experiments conducted in triplicate.

TABLE 4. Effect of different concentrations of T-2 toxin on toxin degradation and the formation of breakdown products by *B. fibrisolvens* CE51 grown on 1% (wt/vol) glucose[a]

T-2 concn (μg/ml)	Toxin concn (% of added T-2)				Recovery (%)
	T-2	HT-2	T-2 triol	Neosolaniol	
100	61	20	9	0	90
500	44	20	12	11	87
1,000	42	27	12	10	91

[a] Values represent the mean of experiments conducted in triplicate.

source, were able to degrade T-2 toxin to HT-2 toxin, T-2 triol, and neosolaniol, except for strain CE52, which degraded T-2 toxin to HT-2 toxin and T-2 triol only (Table 3). *S. ruminantium* and *A. lipolytica* degraded T-2 toxin to HT-2 toxin and T-2 triol when grown on glucose. *B. fibrisolvens* CE56 was the most efficient strain at deacetylating T-2 toxin to breakdown products, with only 28% of the added T-2 toxin left at the end of incubation (Table 3). When *B. fibrisolvens* was grown on cellobiose, the percentage of degradation of T-2 toxin was generally lower, especially for strain CE56. Furthermore, strains CE46 and CE52 both degraded T-2 toxin to HT-2 toxin and T-2 triol only. Notably, rates were also lower with cellobiose as the energy source.

The effects of different concentrations of T-2 toxin on its breakdown by *B. fibrisolvens* CE51 are presented in Table 4. T-2 toxin added at concentrations of 100, 500, and 1,000 μg/ml was degraded to a similar extent as is reported in Table 3, even at the highest concentration. Breakdown products formed from T-2 toxin were HT-2 toxin, T-2 triol, and neosolaniol.

Isolation of esterase enzyme from *B. fibrisolvens* capable of deacetylating T-2 toxin. Esterase activity was present in both cytosol and membrane fractions prepared from *B. fibrisolvens* CE51. However, there was a significant increase in esterase activity (169.6 μmol of *p*-nitrophenol formed per min per mg of protein) after treatment with 0.25% (wt/vol) Triton X-100 (Table 5). This membrane fraction was further purified by precipitation with ammonium sulfate. The highest esterase activity (395.6 μmol of *p*-nitrophenol formed per min per mg of protein) was present in the 50 to 60% fraction (Table 5). Gel filtration of this fraction on Sephadex G-150 yielded several peaks. Each of the eluted protein peaks was assayed for esterase activity, and a single peak (protein peak 1) showing the greatest amount of esterase activity was isolated. This peak was shown to have an approximate molecular weight of 65,000. After incubation of this peak with T-2 toxin (10 μg/ml), HT-2 and T-2 triol were identified as breakdown products in the incubation mixture (Fig. 2). After a 1-h incubation, 42% of the added T-2 toxin had been degraded, giving a T-2 toxin degradation rate of 4.2 μg/h per μg of protein by the purified esterase preparation.

TABLE 5. Esterase activity in cytosol and membrane fractions of *B. fibrisolvens* CE51

Fraction	Esterase activity[a]
Cytosol	96.7
Membrane fraction	95.9
Membrane fraction + Triton X-100 (0.25%, wt/vol)	169.6
$(NH_4)_2SO_4$ precipitation of membrane + Triton X-100 fraction	
0–30%	129.4
30–40%	12.2
40–50%	3.2
50–60%	395.6
Supernatant	5.1

[a] In micromoles of *p*-nitrophenol formed per minute per milligram of protein.

DISCUSSION

Previous work has shown that the trichothecenes generally have little effect on bacteria. In their studies, Bamburg and Strong (1) showed that T-2 toxin exhibited no growth inhibition of *Escherichia coli*, *Staphylococcus aureus*, or *Bacillus subtilis* at concentrations below 1 mg/ml. Other workers (18) showed that the trichothecene trichodermin binds to ribosomes, particularly to the 60S subunits of eucaryotic cells. The binding of trichodermin was inhibited by the presence of other trichothecenes. Therefore, the specific binding capacity of trichothecenes to eucaryotic ribosomes may provide an explanation for the lack of T-2 toxicity, as observed in this study (Tables 1, 3, and 5).

In the present study, correlation coefficients obtained in growth rate experiments indicate that linear growth on bacteria occurred in nearly all cases, with good agreement between experimental replications. Except for three strains of tributyrin-hydrolyzing bacteria, T-2 toxin (10 μg/ml) had no toxic effect on the growth rate of the bacteria tested (Table 1), and *B. fibrisolvens* CE51 was not inhibited (Table 2).

The increase in the growth rate of *B. fibrisolvens* CE46 and CE51 and *S. ruminantium* may indicate that T-2 toxin is being utilized by these bacteria as an additional energy source. Previous studies (17) have shown that T-2 toxin, diacetoxyscirpenol, neosolaniol, nivalenol, and fusarenone-

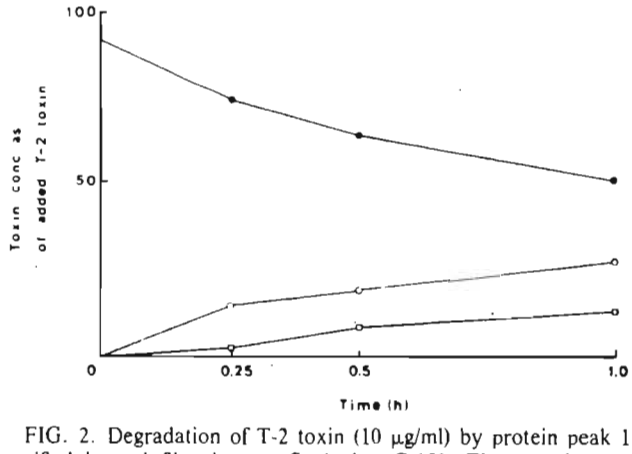

FIG. 2. Degradation of T-2 toxin (10 μg/ml) by protein peak 1 purified by gel filtration on Sephadex G-150. The protein was obtained from the precipitate of the 50 to 60% $(NH_4)_2SO_4$ saturated fraction of the cell membrane preparation of *B. fibrisolvens* CE51. Symbols: □, T-2 triol; ○, HT-2; ●, T-2.

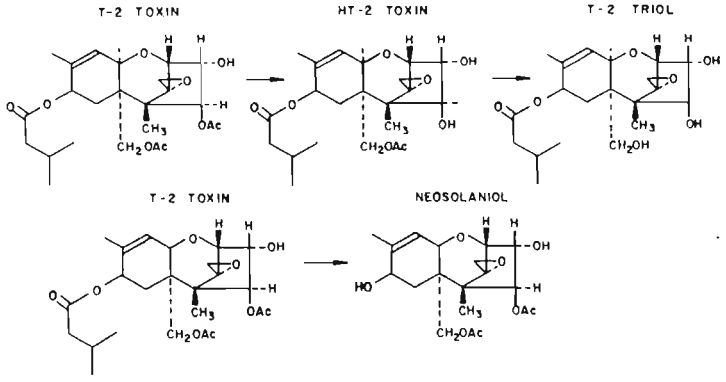

FIG. 3. Pathways of T-2 toxin degradation in *B. fibrisolvens* CE51.

X were assimilated by the soil bacterium *Curtobacterium* sp. strain 114-2. T-2 toxin was first deacetylated to HT-2 toxin, with further deacetylation to produce T-2 triol. Thus, although sufficient carbon source was present as glucose (1%, wt/vol), it is possible that a mechanism similar to that operating in *Curtobacterium* sp. strain 114-2 was responsible for the degradation of T-2 toxin, as shown in Tables 3 and 4.

Because KTB9, KTB10, and KTB13 were isolated on a medium containing tributyrin as the sole energy source, these bacteria must possess esterase enzyme activity. Therefore, the fact that the growth of these bacteria was inhibited by T-2 toxin, whereas others were not inhibited, indicates that either some degree of enzyme specificity is required for breakdown of T-2 toxin or the mechanism and site of action of T-2 toxin are different in these bacteria.

The degradation of T-2 toxin by *B. fibrisolvens* suggests the presence of at least two different enzymes differing in their specificity for the side-chain groups on the toxin molecule. Enzymic hydrolysis at C-3 and subsequently at C-4 would lead to the production of HT-2 toxin and T-2 triol, whereas a single hydrolytic cleavage at C-8 would lead to the production of neosolaniol (Fig. 3). The only previous report that we found of neosolaniol production from T-2 toxin by microorganisms was that of actinomycete fungi (19).

S. ruminantium and *A. lipolytica* hydrolyzed T-2 toxin to HT-2 toxin and T-2 triol only, which may indicate a difference in enzyme complement between these bacteria and *B. fibrisolvens*. No de-epoxidation transformation products were measured in these studies because of the lack of suitable standards.

The degradation of T-2 toxin by a series of deacetylation reactions suggests the involvement of a nonspecific esterase-like enzyme or a number of specific esteraselike enzymes, because, in all experiments, the percent recovery data indicate that there was little or no T-2 toxin degradation by nonenzymatic hydrolysis. For each of the bacteria *B. fibrisolvens*, *A. lipolytica*, and *S. ruminantium*, the esterase activity in whole homogenates was found to be 473.8, 239.6, and 135.7 μmol of *p*-nitrophenol formed per min per mg of protein, respectively. Further enzyme studies were conducted with *B. fibrisolvens* CE51, because this bacterium was shown to be capable of T-2 toxin degradation. The growth rate of this bacterium noticeably increased in the presence of T-2 toxin, and it was shown to have the highest esterase activity. In these investigations, only those fractions showing the highest esterase activity were selected for further purification work. The esterase activities found in the various fractions (Table 5) show that the highest activity was found in the membrane fraction after treatment with Triton X-100. The significant increase in esterase activity in the presence of Triton X-100 suggests that the enzyme is closely membrane associated.

Upon incubation with T-2 toxin, the protein peak isolated after column chromatography hydrolyzed T-2 toxin to HT-2 toxin (26%) and to T-2 triol (13%) after a 1-h incubation (Fig. 2). No degradation was shown in the control experiment. Thus, it is likely that the protein peak isolated is responsible for at least some of the T-2 toxin-degrading ability of *B. fibrisolvens* CE51. Because neosolaniol was not produced, further evidence is presented for the existence of a second T-2 toxin-degrading enzyme and, therefore, a certain degree of enzyme-substrate specificity.

The presence of an esterase in *B. fibrisolvens* capable of hydrolyzing aromatic esters has been previously reported (14). Thus, it is possible that such an enzyme, which normally facilitates the utilization of energy sources within the rumen, also degrades T-2 toxin.

The presence of a T-2 toxin-degrading protein fraction from *B. fibrisolvens* reinforces previous observations of Kiessling et al. (11) that whole rumen fluid can deacetylate T-2 toxin and may therefore provide a detoxification mechanism for the host animal. Although T-2 triol and neosolaniol were not detected in the studies of Kiessling et al. (11), the possible dilution of T-2 toxin-hydrolyzing activity by other microorganisms incapable of such reactions may have resulted in levels of these two toxins below the detection limit.

ACKNOWLEDGMENTS

Financial support for K. Westlake, from the Council of Scientific and Industrial Research and University of Natal, South Africa, is gratefully acknowledged.

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Journal of Food Protection, Vol. 51, No. 11, Pages 887-891 (November 1988)

Destruction of Aflatoxin During the Production of Hydrolysed Vegetable Protein

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(Received for publication February 5, 1988)

ABSTRACT

An investigation into the breakdown of aflatoxins during the hydrolysis of artificially contaminated vegetable protein was conducted using a laboratory scale reactor. The conditions were selected to emulate a commercial process used to produce a protein hydrolysate used in processed food and soup ingredients. Chromatographic analysis showed that aflatoxins at relatively high concentration are totally destroyed and removed from the product. Residues from extracts were free of mutagenic properties, as monitored by the Ames test.

The toxicity and carcinogenicity of the aflatoxins (Fig. 1), particularly aflatoxin B_1, are well documented *(1)*. Because of these properties, contaminated materials are virtually unusable in human food and therefore represent a large economic loss to the world's food supply *(2)*. Hence much effort has gone into developing methods to detoxify commodities contaminated with aflatoxin. Such methods of detoxification include: ammoniation *(3)*, the prime method of commercial detoxification; treatment with peroxide *(4)*; or alkali *(5)*; and solvent extraction *(6)*. Apart from problems with residual toxicity, the main difficulty with all these methods is extra cost of the treatment and change in quality and character of the treated material.

One commercial process that seems to hold some promise of destroying aflatoxin in plant protein, especially that from peanut, is the hydrolysis of such proteins to form a product consisting mainly of amino acids, for use in the food industry, as packet soup ingredients and meat extenders. The method involves treating protein with hydrochloric acid at an elevated temperature and pressure for up to 10 h; the product is then neutralized, treated with charcoal, filtered, and spray dried. This process seems particularly useful in the utilization of peanut meal, because this material is often contaminated with aflatoxin *(7)*, which makes it unfit for commercial use.

The study described in this paper covers the behavior of aflatoxins under the conditions used by Beecham (S.A.) Ltd., for the preparation of vegetable protein powder both in hydrolysis tubes and in a small scale reactor.

MATERIALS AND METHODS

Reaction of pure aflatoxins

Labeled (^{14}C) aflatoxin B_1: 279 µg (15.3 µCi/mmol); aflatoxin B_2: 67 µg (24.4 µCi/mmol); aflatoxin G_1: 109 µg (70.5 µCi/mmol); and aflatoxin G_2: 7 µg (42 µCi/mmol) [prepared after the method of Hsieh & Mateles *(8)*] were individually dissolved in chloroform (1 ml) and placed in separate pyrex hydrolysis tubes (5 mm x 160 mm). The solvent was evaporated under a stream of nitrogen, after which, hydrochloric acid (3 M, 3 ml) was added and then frozen in liquid nitrogen. The tubes were evacuated and sealed by means of a flame. They were placed in an oven at 120° for 9 h, cooled and then carefully opened for further analysis. The contents of each tube were washed into separate beakers and stirred with a magnetic stirrer, while sodium carbonate was added to achieve a final pH of 5.6. Degradation products were then extracted as described in the following relevant section.

Hydrolysis of protein plus aflatoxins

Experiments with commercial peanut protein were carried out exactly as in the experiment described in the previous section, except that a chloroform solution containing 69.6 µg aflatoxin B_1 (27.4 µCi/mmol) and 45.6 µg aflatoxin B_2 (9.5 µCi/mmol) was added to the defatted peanut meal (1.725 g) in the hydrolysis tube. The mixture was made as homogeneous as possible by mixing with a thin glass rod. The rod was washed with about 5 ml of chloroform, which was added back to the mixture. The chloroform was then removed with a stream of dry nitrogen. The tubes and their contents were then treated as in the previous section, except that after hydrolysis and cooling, they were frozen in liquid nitrogen. Upon opening, the tubes were placed in a sealed beaker with strips of filter paper soaked in sodium hydroxide solution to trap any released carbon dioxide. After thawing, the contents of each tube were adjusted to pH 5.6, with sodium carbonate, and a solution of hot sodium chloride solution (0.552 g in 1.7 ml water) containing activated charcoal (0.042 g) was added and stirred for 30 min. The mixture was filtered through a Whatman No. 1 filter paper on a Buchner apparatus and the residue was washed with 10 ml of warm distilled water. The residues and washings were then stored at 4°C for further analysis.

Pilot scale experiment

A scaled down model of the industrial reactor used to hydrolyse

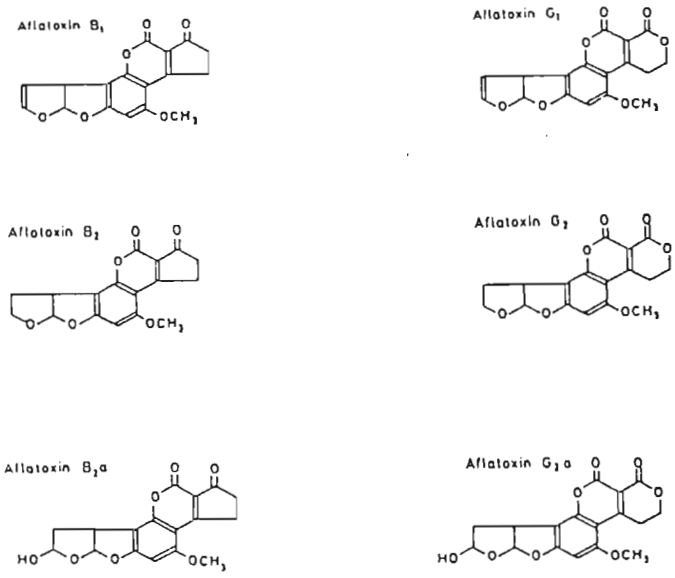

Figure 1. *Structure of the four major aflatoxins and aflatoxins B_{2a} and G_{2a}.*

vegetable protein was fabricated by Beechams (S.A.) Ltd. to a scale of one to a thousand. In essence this reactor is a stainless steel pot lined with an acid resistant coating with an acid resistant sealable lid. The reactor was equipped with an anchor stirrer, inlet and outlet ports and enclosed with a steam jacket.

The peanut meal (1250 g), which was naturally contaminated with unlabeled aflatoxins (B_1: 321 µg/Kg; B_2: 41.5 µg/Kg; G_1: 161 µg/Kg; G_2: 15 µg/Kg) was mixed with radio-labeled aflatoxin dissolved in 200 ml chloroform in a 2500-ml beaker. The chloroform was then removed by placing the beaker in the air flow of a fume hood overnight. The dry meal was then transferred to the reactor and 3 M hydrochloric acid (2174 ml) was added. The experiment was done with labeled aflatoxin B_1 (81 µg, 647 µCi/mmol), B_2 (74.6 µg, 372 µCi/mmol), G_1 (194 µg, 239 µCi/mmol) G_2 (89 µg, 387.8 µCi/mmol), and once without any addition of label as a control experiment.

The reactor was set up and the reaction initiated by passing steam (160 kPa; 129°C) into the reactor bowl jacket; the mixture was stirred at a rate of 54rpm. Hydrolysis was terminated after 9 h by switching off the steam supply and circulating cold water through the steam jacket for 90 min. The cooled vessel was purged with argon gas, the exhaust gas being passed through barium hydroxide solution to trap carbon dioxide.

The reactor was opened and the contents together with water washings were emptied into a 25-L bucket. The contents of the bucket were then neutralized to pH 5.6 over a period of 4 h by stirring with commercial sodium carbonate. After the neutralization step a hot solution of sodium chloride (400 g in 1200 ml water) and activated charcoal (30 g) were added and the mixture was stirred for a further 30 min. The slurry was filtered through needle cloth on a large Buchner apparatus; the residue was washed again with hot water (500 ml) and the final residue and total filtrate was stored at 4°C for further analysis.

Extraction of degradation products

The products from all the reactions were treated in essentially the same manner as follows: the filtrates were extracted in a separatory funnel with three equal volumes of chloroform. Each extract was passed through a small bed of anhydrous sodium sulphate to remove moisture. The sodium sulphate was washed with 5 ml of chloroform. The dried extracts were pooled, evaporated to near dryness on a rotary evaporator using low heat (60°), and the concentrated was transferred quantitatively to a small vial. The extract was evaporated to dryness using a stream of dry nitrogen and stored at 4°C. The aqueous phase was extracted three times with equal volumes of ethyl acetate which were pooled, dried and evaporated as for chloroform extract. The extracted aqueous phase was freeze dried and the lyophilisate was extracted with three volumes of warm methanol equal to the original aqueous phase volume. The pooled methanol extracts were evaporated to dryness.

The washed, filtered, charcoal-containing residues from the hydrolysis tube experiments were extracted successively with chloroform (3 x volume of filtrate) ethyl acetate, methanol, and aqueous ethanol (80%). The extracts were evaporated to dryness or in the case of the aqueous ethanol to a volume of 5 ml. All fractions were stored at 0°C for further examination.

The residues from the small reactor were extracted in a similar fashion excepting that a soxhlet apparatus was used. The residue was extracted for 24 h with each solvent in turn, the extracts then being evaporated as for the aqueous extracts.

Analysis of amino acids

A portion of the dried hydrolysed protein product was dissolved in water and analysed for amino acid content using a Beckman 119 Amino Acid Analyser according to the manufacturers instructions.

Analysis of aflatoxin degradation products

The extracts were dissolved in a minimum of a suitable solvent and then chromatographed in two dimensions on 10 x 10cm aluminium backed thin layer chromatography (TLC) plates (cut from 20 x 20cm Merck 5553). The amount spotted onto the plate varied, but for the small scale experiments the amount was typically equivalent to a tenth of the total extract and for the pilot experiment one hundredth. Normally the plates were developed in the first dimension with chloroform-ethyl acetate-propan-2-ol (90:5:5v/v/v) and in the second with diethyl ether-methanol-water (94:4.5:1.5 v/v/v), but for the methanol and aqueous extracts, they were developed in propan-2-ol-water-concentrated ammonia (7:2:1 v/v/v) and butanol-water-acetic acid (12:5:3 v/v/v). The plates were viewed under ultra violet (UV) light and fluorescent and absorbent spots were marked. Various spray reagents were used to detect the products, i.e. sulphuric acid in ethanol (20%), Pauly's reagent *(9)*, and 2,4 dinitrophenyl hydrazine (DNPH) *(9)*.

High performance liquid chromatography was used as a means of assaying the known aflatoxins. The system was a Waters system equipped with 501 pumps, 420 fluorescence detector and 710B Wisp autosampler. Elution was done isocratically with acetonitrile-acetic acid-water (14.18:0.82:85 v/v/v) on a Novo pak C_{18} column. Aflatoxin B_1 and G_1 were derivatised with trifluoroacetic acid prior to chromatography *(10)*.

Radioautography and scintillation counting

Radioautography was carried out using 20 x 20cm glass backed thin layer chromatograms developed in the solvent systems described in the previous section. X-ray plates (Cronex MRF-31) were taped onto each plate and were left in the dark for 60 d and examined for dark spots after development.

A small portion of each ^{14}C containing extracts was evaporating to dryness with a stream of nitrogen in a scintillation vial. Scintillation cocktail concentrate (Lumax Ltd.) diluted to 4% in toluene was added (10 ml) to each vial. After dark adaptation,

ey were counted in a Beckman LS 3801 liquid scintillation unter set up for the Beckmann "H number" method *(11)*. olor quenched samples were bleached according to the method Neame *(12)*. Barium carbonate, recovered by filtration from e carbon dioxide entrapment experiments, was counted in a ial containing scintillation fluid after dark adaptation.

ioassays

The extracts were subjected to the Ames mutagenicity test *13)*, using *Salmonella typhimurium* (Tester strain TA 100) in ie presence of Aroclor 1254-rat liver S-9, as a means of detect-ig aflatoxin-degradation products with residual mutagenic activity *14)*. Dimethyl sulphoxide (DMSO) or water was used to dis-olve the control and various test extracts.

RESULTS AND DISCUSSION

Acid hydrolysis of aflatoxins in the absence of protein

The major portion of the radioactivity recovered from :he labeled aflatoxins treated with acid in the absence of proteins was found to be in the chloroform extract, ranging from 28% of the quantity added as aflatoxin G_1 to 55% for aflatoxin G_2, indicating that the major degradation products were relatively non-polar. In the case of aflatoxin B_1, a proportion of the radioactivity was found in the methanol (8.7%) and aqueous ethanol extracts (10.1%), showing that some polar products had been generated. In terms of total radioactivity, less counts were recovered for aflatoxin G_1 (65%) than the other aflatoxins (B_1: 92%, B_2: 90%, & G_2: 91%). This effect may be explained on the basis of extra reactivity of aflatoxin G_1 due to the molecule possessing both the double bond in the terminal furan ring and the lactone ring, which allows greater polymerisation of the products of degradation.

On analysis of the chloroform extracts from aflatoxins hydrolysed in the presence of protein, it was found that aflatoxins B_{2a} and G_{2a} (about 0.1% in terms of aflatoxins B_1 and G_1 added) were present in experiments containing aflatoxin B_1 and G_1 respectively, as shown by the TLC and HPLE. Of the aflatoxins examined, aflatoxins B_2 and G_2 were the most resistant to breakdown; between 10-11% of that added being recovered as opposed to 0.1% for aflatoxins B_1 and G_1. Thus it can be concluded that aflatoxins B_2 and G_2 are less susceptible to destruction by the acid treatment than B_1 and G_1, probably because the double bond in the latter molecules can add water in the presence of acid to form aflatoxins B_{2a} and G_{2a} (Fig. 1), allowing further destruction of the bisdihydrofurano ring system.

Various fluorescent spots other than known aflatoxins and their derivatives, were observed on two dimensional chromatograms of the chloroform extracts but not all of these gave a positive spot on the radioautographs, indicating low concentrations. Due to the low concentrations and complexity of the TLC pattern (at least fifteen fluorescent spots were generated from aflatoxin B1) these were not investigated further. It can be assumed that the coumarin ring is still intact because the compounds were fluorescent, although several gave positive results with Pauly's and DNPH reagent indicating the presence of phenol and reactive carbonyl groups.

It can be concluded that aflatoxins subjected to the hydrolytic procedure in the sealed tubes, without any other material present, break down into products with the coumarin ring intact, implying that the bis-furano ring moiety is modified. It is likely that open ring forms of the coumarin system produce in the reaction, are reformed again on neutralization.

Acid hydrolysis of proteins in the presence of aflatoxins

Amino acid analysis of the products from both the hydrolysis tubes and reactor experiments showed the presence of amino acids in similar proportions as for the commercial product. This result indicates that the laboratory scale experiments emulated the conditions present in the industrial reactors used to prepare the protein hydrolysate.

The presence of vegetable proteins and the addition of charcoal to the final reaction mixture after the acid hydrolysis, completely changed the pattern of products formed by the reaction as observed on TLC. Both the hydrolysis tube and reactor experiments (Table 1) gave similar results, although the former gave poorer total recovery possibly due to an effect of scale.

An important result was that in all cases none of the original aflatoxin or the water adduct products, aflatoxin B_{2a} and G_{2a} were detected in extracts from the acid hydrolysis of proteins with aflatoxins. Furthermore, the major part of the radioactivity added as aflatoxin, was found to be in the residue which is a mixture of unhydrolysed insoluble material and charcoal. The removal of aflatoxin as an insoluble material was most encouraging, as it indicates that even if aflatoxin or toxic degradation products are present, they are removed by the charcoal treatment. The main bulk of the counts in all the residues that could be solubilised were those in the methanol fraction indicating that products with a greater polarity than the original aflatoxins, had been generated. It is probable that the amino acids liberated during the hydrolysis had reacted with exposed reactive groups (e.g. carbonyl) on the aflatoxin molecule to form water soluble products.

Several fluorescent radioactive spots were observed on chromatograms of methanol extracts from the residue: all had much lower RF values than the original aflatoxin. About 50% of the radioactivity was associated with the origin of the chromatogram and this could not be resolved further in the more polar TLC solvent systems. Thus identification of this material was virtually impossible. Furthermore, the metabolites at the origin of the chromatograms gave negative results with the spray reagents used. This latter result supports the notion that active groups such as carbonyls and phenols had reacted with the amino acids or other components of the meal, although fluorescence was observed under UV light, indicating that the coumarin ring system was still intact.

The amount of radioactive CO_2 trapped as barium carbonate, was always less than 1% of that added, so although

TABLE 1. *The distribution of radioactivity found in various fractions of hydrolysed protein and insoluble residue produced in the reactor.*

Fraction	Activity (dps) retrieved from added labeled aflatoxin as indicated (%)*			
	B_1	B_2	G_1	G_2
Hydrolysed Protein				
Precipitated Salt*	82 (1.3)	72 (2.2)	148 (2.8)	73 (1.9)
Trapped $^{14}CO_2$	37 (0.6)	16 (0.5)	16 (0.3)	14 (0.4)
Chloroform	137 (2.2)	23 (0.7)	29 (0.5)	25 (0.6)
Ethyl acetate	105 (1.7)	109 (3.3)	24 (0.5)	15 (0.4)
Insoluble Residue				
Chloroform	711 (11.4)	275 (8.4)	399 (7.6)	285 (7.4)
Ethyl acetate	836 (13.5)	113 (3.5)	141 (2.7)	109 (2.8)
Methanol	2096 (33.7)	1577 (48.2)	1976 (37.8)	1849 (47.8)
Aqueous ethanol	220 (3.5)	237 (7.2)	186 (3.6)	202 (5.2)
Remaining residue	661 (10.6)	344 (10.5)	1592 (30.4)	788 (20.4)
Total	4885 (78.6)	2765 (84.5)	4510 (86.3)	3358 (86.9)

*Activity recovered as a percentage of that added (dps), ie. B_1 = 6216, B_2 = 3271, G_1 = 5228, G_2 = 3866.
*Precipitated on extraction with ethyl acetate.

TABLE 2. *Salmonella/Microsome mutagenicity assay of fractions isolated from the protein hydrolysed in the small reactor.*

Aflatoxin*	Fraction	Revertants/plate** Solvent/control*	test
B_1	standard	126 (DMSO)	981
G_2	standard	126 (DMSO)	1133
Hydrolysed Protein			
B_1	Chloroform	140 (water)	123
B_1	Ethyl acetate	102 (water)	162
B_1	Total product	126 (DMSO)	161
G_1	Chloroform	140 (water)	87
G_1	Ethyl acetate	102 (water)	106
G_1	Total product	126 (DMSO)	150
Residue			
B_1	Chloroform	140 (water)	103
B_1	Ethyl acetate	102 (water)	86
B_1	Methanol	107 (water)	183
G_1	Chloroform	140 (water)	103
G_1	Ethyl acetate	102 (water)	102
G_1	Methanol	107 (water)	166

*Amount added per plate equivalent to 0.05 μg B_1 and 0.04 μg G_1 in original spiked protein, as calculated from radioactivity; added in 0.1ml solvent. Standard B_1 added = 0.05 μg; G_1 = 0.2 μg.
**Tester strain used, TA 100; results are means of triplicates.
*Carrier solvent or fraction from hydrolysed protein without added aflatoxin.

decarboxylation does take place, it does not seem to be the main route of degradation.

Mutagenicity tests

All the fractions tested from the pilot scale experiment showed low reversion rates in the Ames test, as compared to aflatoxin B_1 and G_1 controls (Table 2). The results support the hypothesis that the bisfurano system is destroyed by the process as it is the double bond in this moiety of in aflatoxins B_1 and G_1 that is considered to be responsible for their carcinogenic and mutagenic properties *(15)*.

In conclusion, it seems that the production of food extenders by acid hydrolysis of proteins, could become important in the commercial use of peanut and other vegetable proteins, contaminated with aflatoxins. As a consequence, contaminated proteins that cannot be used at the present, could be utilized in the production of hydrolysed vegetable protein for use as a food flavour or free amino acids. This would represent a considerable saving in protein resources.

ACKNOWLEDGMENTS

We thank the University of Natal and C.S.I.R. South Africa for a bursary for K. Williams; Beecham (S.A.) Ltd. for supporting the work and N.R.I.N.D., M.R.C., Tygerberg, Cape, for carrying out the Ames tests.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1987, p. 613–614
0099-2240/87/030613-02$02.00/0

Vol. 53, No. 3

Effects of Several Mycotoxins on Specific Growth Rate of *Butyrivibrio fibrisolvens* and Toxin Degradation In Vitro

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Received 15 August 1986/Accepted 3 December 1986

Four strains of *Butyrivibrio fibrisolvens* did not degrade aflatoxin B_1. Acetyl T-2 toxin, T-2 toxin, HT-2 toxin, deoxynivalenol, diacetoxyscirpenol, verrucarin A, zearalenone, and ochratoxin A did not affect the specific growth rate of *B. fibrisolvens* CE51 significantly, but all were degraded to greater or lesser extents. Breakdown products were produced as a result of deacetylation reactions.

Previous studies have shown that ruminants are more resistant to mycotoxin poisoning than monogastric animals (6), while preparations of whole rumen ingesta have been shown to be capable of degrading T-2 toxin (4), deoxynivalenol (5), zearalenone (4), and ochratoxin (3, 4). These data support the theory that the rumen is responsible for conferring a degree of mycotoxin resistance in ruminants. Pure cultures of certain rumen bacteria were capable of degrading T-2 toxin to HT-2 toxin, T-2 triol, and , in some cases, neosolaniol (11). In addition (11), a protein preparation isolated from the membrane fraction of *Butyrivibrio fibrisolvens* CE51 was able to degrade T-2 toxin to HT-2 toxin and T-2 triol.

In the present study, the toxicity of a number of different mycotoxins for *B. fibrisolvens* CE51 was investigated, and toxin degradation was monitored.

The methods used in monitoring the effect of mycotoxins on bacterial growth rate have been described elsewhere (11). Aflatoxin analysis was performed by the method of Berry et al. (1) after extraction in chloroform. Trichothecene analysis was performed by the method of Westlake et al. (K. Westlake, M. Dutton, R. Berry, J. Lee, and P. Barrowman, submitted for publication) after extraction in ethyl acetate. All toxins were purchased from Sigma Chemical Co., except for deoxynivalenol, which was a generous gift from P. G. Thiel (Medical Research Council, Tygerberg, South Africa). The bacteria used have been described previously (10). Because aflatoxin B_1 has previously been shown to be toxic for a number of different bacteria (8), initial toxicity studies on four different strains of *B. fibrisolvens* were conducted with this mycotoxin. The results (Table 1) showed that aflatoxin B_1 (10 μg/ml) was not toxic for any of the strains tested, as judged by bacterial growth rate in the presence and absence of toxin. Furthermore, there was no significant degradation of aflatoxin B_1. Data concerning the susceptibility of rumen bacteria to aflatoxin B_1 are contradictory. In in vitro studies with whole rumen fluid, Mathur et al. (8) and Kiessling et al. (4) could not detect breakdown products of aflatoxin B_1, while Engel and Hagemeister (2) showed that 40% of the added aflatoxin B_1 was degraded. However, because it has been shown that diet (7) and time of sampling (9) affect rumen microbial composition and numbers, a direct comparison of these results cannot be made because of differences in diets, time of sampling of rumen fluid, toxin concentration, or incomplete data concerning any of these parameters. The results obtained in the present study (Table 1) showed that these strains of *B. fibrisolvens*, one of the predominant rumen bacteria, could not detoxify aflatoxin B_1. Assuming that microbial activity is solely responsible for toxin degradation within the rumen, this would indicate that diet may play an important role in conferring aflatoxin B_1 resistance in ruminants and may explain differences in previous in vitro studies (2, 8).

TABLE 1. Effect of aflatoxin B_1 (10 μg/ml) on specific growth rate and the extent of aflatoxin breakdown by four different strains of *B. fibrisolvens* with 1% (wt/vol) glucose as the sole energy source[a]

Strain	With aflatoxin B_1		Without aflatoxin B_1		% Toxin remaining after incubation to stationary phase
	μ (h^{-1})	r	μ (h^{-1})	r	
CE46	0.67	0.997	0.64	0.998	95
CE51	0.54	0.992	0.53	0.996	91
CE52	0.53	0.997	0.53	0.988	98
CE56	0.48	0.996	0.49	0.985	95

[a] Values represent the means of experiments conducted in triplicate.

Further studies were conducted with only *B. fibrisolvens* CE51. None of the mycotoxins tested effected any notice-

TABLE 2. Effect of different mycotoxins (10 μg/ml) on the specific growth rate of *B. fibrisolvens* CE51 with 1% (wt/vol) glucose as the sole energy source[a]

Toxin	μ (h^{-1})	r
None (control)	0.53	0.998
Acetyl T-2 toxin	0.50	0.997
T-2 toxin	0.52	0.995
HT-2 toxin	0.49	0.998
Deoxynivalenol	0.51	0.998
Diacetoxyscripenol	0.51	0.997
Verrucarin A	0.55	0.998
Zearalenone	0.56	0.998
Ochratoxin A	0.52	0.997

[a] Values represent the means of experiments conducted in triplicate.

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TABLE 3. Degradation of acetyl T-2 toxin, T-2 toxin, and HT-2 toxin by *B. fibrisolvens* CE51 grown with 1% (wt/vol) glucose as the sole energy source

Toxin added (10 μg/ml)	Breakdown product (% added toxin)			
	T-2 toxin	HT-2 toxin	T-2 triol	Neosolaniol
Acetyl T-2 toxin	29	10	0	0
T-2 toxin		23	8	4
HT-2 toxin			7	0

able change in the growth rate of this bacterium (Table 2), but all were degraded to greater or lesser extents. The breakdown products of zearalenone, verrucarin A, diacetoxyscirpenol, and deoxynivalenol were not identified because of a lack of suitable standards; however, acetyl T-2 toxin was degraded to T-2 toxin and HT-2 toxin; T-2 toxin was degraded to HT-2 toxin, T-2 triol, and neosolaniol; and HT-2 toxin was degraded to T-2 triol (Table 3). In another study (11), the presence of two enzymes with different substrate specificities was postulated to explain the production of HT-2 toxin, T-2 triol, and neosolaniol from T-2 toxin by *B. fibrisolvens* CE51. We propose that we were unable to detect neosolaniol as a degradation product of acetyl T-2 toxin and T-2 triol because the concentrations present were below the detection limits of the method.

It is possible that the enzyme responsible for the deacetylation of T-2 toxin is a fairly nonspecific esterase that may also deacetylate diacetyoxyscirpenol to monoacetoxyscirpenol (4). In previous in vitro studies with zearalenone, this toxin was degraded to zearalenol (3), while in similar studies, deoxynivalenol was degraded to a de-epoxidation product (5). In many cases, the breakdown products are less toxic than the parent compound either by virtue of increased polarity and therefore decreased cell permeability or by removal of a toxic functional group.

Because *B. fibrisolvens* can deacetylate T-2 toxin and some of its derivatives, this particular bacterium may play an important role in increasing the resistance of ruminants to trichothecene toxicity. It is not possible to assess the degree of importance until studies have been conducted with other major rumen bacteria, but these results indicate that diet may play an important role in determining toxin resistance inruminants by regulating the relative numbers of bacteria capable of toxin degradation.

Bursary support for K. Westlake from the Council for Scientific and Industrial Research and the University of Natal is gratefully acknowledged.

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Animal Feed Science and Technology, 25 (1989) 169–178
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

In Vitro Metabolism of Mycotoxins by Bacterial, Protozoal and Ovine Ruminal Fluid Preparations

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(Received 19 April, 1988; accepted for publication 14 November 1988)

ABSTRACT

Westlake, K., Mackie, R.I. and Dutton, M.F., 1989. In vitro metabolism of mycotoxins by bacterial, protozoal and ovine ruminal fluid preparations. *Anim. Feed Sci. Technol.*, 25: 169–178.

Mycotoxin degradation studies with ovine rumen fluid in vitro have shown that degradation of both aflatoxin B_1 and G_1 was < 10% when added at levels of 1.0 and 10.0 $\mu g\ ml^{-1}$. However, at these concentrations microbial activity was partially inhibited as determined by the percentage inhibition of digestion of alfalfa hay. T-2 toxin, HT-2 toxin, deoxynivalenol and diacetoxyscirpenol were all degraded by ovine rumen fluid in vitro when added at a level of 10 $\mu g\ ml^{-1}$. There was no significant degradation of neosolaniol. T-2 toxin (10 $\mu g\ ml^{-1}$) was degraded to HT-2 toxin (6.0 $\mu g\ ml^{-1}$) and T-2 triol to (1.5 $\mu g\ ml^{-1}$) after 12 h incubation.

Bacterial and protozoal fractions prepared from ovine rumen fluid were capable of T-2 toxin degradation and, although the protozoal fraction was more active in this respect, it was also the most sensitive to the toxic effects. A cell-free preparation from whole ovine rumen fluid was unable to degrade T-2 toxin.

These results support the theory that the rumen plays an important role in conferring a certain degree of toxin resistance in ruminant animals.

INTRODUCTION

Although toxicoses associated with trichothecene toxins have been reported in ruminant animals (Hsu et al., 1972), there is evidence to suggest that ruminants are more resistant to this group of toxins than monogastric animals and that the rumen is the major organ involved in the detoxification process (Kurmanov, 1977). This has been further substantiated by the work of Hult et al. (1976) who described the in vitro degradation of ochratoxin A by rumen in-

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0377-8401/89/$03.50

tional set of triplicate bottles containing DMF but without added toxin served as controls.

After thorough mixing, 20-ml samples were withdrawn at various time intervals for toxin analyses. The sample bottle was quickly regassed between samples. In the aflatoxin studies, the aqueous layer containing undegraded lucerne hay was retained and filtered through a previously dried and weighed 9 cm Whatman filter paper (grade 01). This was dried to constant weight at 70°C and the decrease in sample weight with incubation time was used as a qualitative index of microbial activity.

Experiments using bacterial and protozoal preparations

Approximately 2 l of whole rumen ingesta was sampled from 4 sheep fed milled lucerne hay. The ingesta was withdrawn 2 h after the morning feed through ruminal cannulae using gentle suction. The ingesta was strained through 2 layers of cheese cloth and allowed to stand for approximately 1 h at 39°C in an anaerobic cabinet (65% N_2–30% CO_2–5% H_2 gas phase; Forma Model 1024) during which time protozoa settled on the bottom of the flask. After discarding partially-digested forage, the protozoa were sedimented (200×g 15 min, 4°C). The centrifuge tubes were returned to the anaerobic cabinet, the supernatant decanted and the combined protozoal pellets resuspended in 25 ml anaerobic diluent (protozoal inoculum). The decanted supernatant constituted the bacterial inoculum to which was added dioctyl sodium sulphosuccinate (1 mg ml^{-1}).

Modified medium 10 with 1% (w/v) glucose as sole carbon and energy source (Westlake et al., 1987a) was prepared in 200-ml amounts in 4 oz McCartney flat bottles. The antibiotics streptomycin (2 mg ml^{-1}) and penicillin (2 mg ml^{-1}) were added to 4 of the bottles. Protozoal inoculum (5 ml) was added to the remaining 4 bottles. After pre-incubation for 15 min at 39°C, T-2 toxin (100 μg ml^{-1} in DMF) was added to 2 each of the media inoculated with bacteria and protozoa and an equal volume of DMF added to the remaining 4 bottles. After thorough mixing, samples were taken after 0 and 6 h of incubation. Protein analyses were performed using the method of Lowry et al. (1951). The numbers of holotrich and entodiniomorph protozoa were determined after dilution with formal saline (10% (v/v) CH_2O + 0.85% (w/v) NaCl) and enumeration in a Nageotte counting chamber (0.50 mm, Walter Schrenck Erben, D-6238, Hofheim, F.R.G.) under a phase-contrast microscope (Nikon Optiphot, 100× magnification).

Toxin extraction, analysis and recovery

Rumen fluid and rumen fluid preparations containing toxin were extracted with 3 equal volumes of solvent (chloroform for aflatoxin and ethyl acetate for

gesta. Similarly, Kallela and Vasenius (1982) showed degradation of the *Fusarium* toxin, zearalenone.

More recently, Kiessling et al. (1984) described the degradation of ochratoxin A, zearalenone, T-2 toxin and diacetoxyscirpenol by rumen ingesta in vitro but were unable to show any degradation of aflatoxin B_1 and deoxynivalenol. However, using in vitro studies with bovine rumen fluid, King et al. (1984) showed that deoxynivalenol was almost completely degraded to a single de-epoxidation product within 24 h, while Ivie (1976) also demonstrated an epoxide to olefin transformation by rumen ingesta. Furthermore, Engel and Hagemeister (1978) showed up to 40% degradation of aflatoxin B_1 in in vitro studies with rumen ingesta. Thus, results concerning the toxin-degrading ability of rumen ingesta are conflicting. The importance of these toxins cannot be over emphasised, especially in animal feedstuffs, where a large portion of animals' diet in many parts of the world comprises products that are naturally infected with fungal species capable of trichothecene production.

In the present study the effect of ovine rumen ingesta on the degradation of a number of different mycotoxins including aflatoxin B_1 and G_1, T-2 toxin, HT-2 toxin and deoxynivalenol has been studied together with microbial susceptibility to the toxic effect of T-2 toxin.

MATERIALS AND METHODS

General

This work was conducted at two laboratories. Initial studies with rumen fluid, were conducted at the Department of Biochemistry, University of Natal, Pietermaritzburg, South Africa, while later studies with bacterial and protozoal fractions from rumen fluid were conducted at the Department of Rumen Biochemistry, Animal and Dairy Science Research Institute, South Africa.

Experiments using rumen fluid

Mature intact donor sheep were maintained on a lucerne hay diet. Whole rumen fluid samples were withdrawn 1 h after the morning feed into a sealed flask at 39°C using a stomach tube. After dilution of whole rumen fluid with prewarmed anaerobic McDougall's artificial saliva buffer (20 ml rumen fluid:80 ml buffer), toxins (listed in Table 1) made up as concentrated solutions in dimethylformamide (DMF) were added to a final concentration of 10.0 μg ml^{-1} (unless otherwise stated). For aflatoxin experiments, 0.5 g alfalfa hay (oven dried and milled to pass 1 mm screen) was added to each 100 ml screw-cap polythene bottle, prepared in triplicate. After flushing with CO_2 the bottle was sealed and incubated for 12 h in a horizontal position at 39°C. An addi-

tional set of triplicate bottles containing DMF but without added toxin served as controls.

After thorough mixing, 20-ml samples were withdrawn at various time intervals for toxin analyses. The sample bottle was quickly regassed between samples. In the aflatoxin studies, the aqueous layer containing undegraded lucerne hay was retained and filtered through a previously dried and weighed 9 cm Whatman filter paper (grade 01). This was dried to constant weight at 70°C and the decrease in sample weight with incubation time was used as a qualitative index of microbial activity.

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Modified medium 10 with 1% (w/v) glucose as sole carbon and energy source (Westlake et al., 1987a) was prepared in 200-ml amounts in 4 oz McCartney flat bottles. The antibiotics streptomycin (2 mg ml^{-1}) and penicillin (2 mg ml^{-1}) were added to 4 of the bottles. Protozoal inoculum (5 ml) was added to the remaining 4 bottles. After pre-incubation for 15 min at 39°C, T-2 toxin (100 μg ml^{-1} in DMF) was added to 2 each of the media inoculated with bacteria and protozoa and an equal volume of DMF added to the remaining 4 bottles. After thorough mixing, samples were taken after 0 and 6 h of incubation. Protein analyses were performed using the method of Lowry et al. (1951). The numbers of holotrich and entodiniomorph protozoa were determined after dilution with formal saline (10% (v/v) CH_2O + 0.85% (w/v) NaCl) and enumeration in a Nageotte counting chamber (0.50 mm, Walter Schrenck Erben, D-6238, Hofheim, F.R.G.) under a phase-contrast microscope (Nikon Optiphot, 100× magnification).

Toxin extraction, analysis and recovery

Rumen fluid and rumen fluid preparations containing toxin were extracted with 3 equal volumes of solvent (chloroform for aflatoxin and ethyl acetate for

all other toxins). The organic layer was collected in 100-ml round-bottomed flasks after passing through a bed of anhydrous sodium sulphate. The solvent was evaporated by rotary vacuum evaporation and reconstituted in a small volume of extraction solvent before transferring to a half dram vial for further evaporation of the solvent under a stream of nitrogen. After drying, the sample was reconstituted in 0.5 ml solvent. Aflatoxins were analysed according to the method of Berry et al. (1984) on a Varian model 5000 HPLC equipped with a Beckman Ultrasphere-ODS column. Trichothecene analyses were performed according to the method of Westlake et al. (submitted for publication).

In all toxin degradation investigations, the percentage recovery of toxin was determined after analysing the amount of toxin recovered at time 0. Subsequent toxin concentrations were adjusted to 100% recovery according to the determined percentage recovery at time 0.

Source of toxins

All toxins used in this study were obtained from Sigma Chemical Co., with the exception of deoxynivalenol, which was a generous gift from Dr. P.G. Thiel (MRC, Tygerburg, South Africa).

RESULTS

Degradation of toxins by ovine rumen fluid

Initial studies showed that there was little degradation of aflatoxin B_1 by rumen digesta when added at concentrations of 1.0 and 10.0 μg ml^{-1} (Table 1). These toxin concentrations were higher than the toxin concentrations encountered in vivo and therefore responses were more easily measured.

The percentage inhibition of digestion of milled and dried lucerne hay (Table 1) was used as a measure of toxic activity and showed that, as measured after 3 h incubation, the inhibitory effect was least at the lowest aflatoxin B_1 concentration (50%). The percentage inhibition of digestion when aflatoxin B_1 was added at 10.0 μg ml^{-1} was 67%.

In a similar study with aflatoxin G_1 (Table 1), toxin degradation values were also very low (30 μg l^{-1} h^{-1}) while the percentage inhibition of digestion of lucerne hay was lower than those values obtained upon incubation with aflatoxin B_1 (20% and 40% aflatoxin G_1 was added at 1.0 and 10.0 μg ml^{-1}, respectively).

These experiments were extended to include the trichothecene toxins T-2 toxin, HT-2 toxin, deoxynivalenol, diacetoxyscirpenol and neosolaniol. However, the effect on digestion of lucerne hay was not determined.

Initial rates of toxin degradation were measured over the period from 0 to 3 h of the 12 h incubation period. The results showed (Table 1) that, of the

TABLE 1

In vitro degradation of several mycotoxins by ovine ruminal fluid

Toxin added and concentration ($\mu g\ ml^{-1}$)	Inhibition (%) Lucerne Hay	Degradation rate[a] ($\mu g\ l^{-1}\ h^{-1}$)	Recovery (%)
Aflatoxin B_1			85
1.0	50	30.0 ± 0.05 (± 0.08)[b]	
10.0	67	166.7 ± 14.0 (± 23.6)	
Aflatoxin G_1			85
1.0	20	30.0 ± 0.05 (± 0.08)	
10.0	40	233.0 ± 6.6 (± 11.14)	
T-2 toxin (10.0)	ND	2220 ± 111 (± 187.3)	78
HT-2 toxin (10.0)	ND	1833 ± 41 (± 69.2)	76
Deoxynivalenol (10.0)	ND	2230 ± 366 (± 617.7)	55
Diacetoxyscirpenol (10.0)	ND	2066 ± 54 (± 91.1)	80
Neosolaniol (10.0)	ND	133 ± 24 (± 40.5)	68

Figures represent mean values of experiments conducted in triplicate.
[a]Calculated from the initial 3 h of a 12 h incubation period.
[b]95% confidence limits.
ND = not determined.

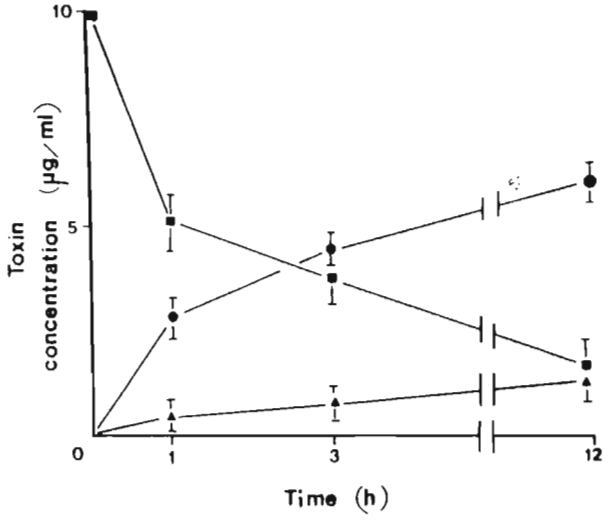

Fig. 1. Production of trichothecene metabolites after incubation of T-2 toxin (10 $\mu g\ ml^{-1}$) with rumen contents for 12 h at 39°C. Figures represent average value of experiments conducted in triplicate. (■, T-2 toxin; ●, HT-2 toxin; ▲, T-2 triol)

trichothecene toxins, all but neosolaniol showed degradation rates of approximately 2 mg $l^{-1}\ h^{-1}$. Degradation of neosolaniol was slow (133 $\mu g\ l^{-1}\ h^{-1}$) ~ 17-fold lower than the rates for T-2 toxin and deoxynivalenol.

The data in Fig. 1 show the disappearance with time of T-2 toxin from rumen digesta after incubation at 39°C and the appearance of HT-2 toxin and T-2 triol.

The final concentrations of the 3 toxins were 1.8, 6.03 and 1.5 μg ml^{-1} for T-2 toxin, HT-2 toxin and T-2 triol, respectively. HT-2 toxin was therefore the major metabolite. The change in toxin concentration was much greater in the first 3 h than in the subsequent 9 h. The mean average recovery of T-2 toxin as T-2 toxin, HT-2 toxin and T-2 triol was 93%.

Mycotoxin degradation by bacterial and protozoal fractions from ovine rumen fluid

In order to determine the active fraction responsible for toxin degradation, bacterial, protozoal and cell-free preparation were obtained from whole rumen digesta. The results showed that both the bacterial and protozoal fractions were capable of toxin degradation while there was no degradation by the cell-free preparation (Fig. 2). Since equal volumes of rumen fluid were used in the preparation of each replicate of protozoal and bacterial fractions, the protozoal fraction was more effective than the bacterial fraction at degrading T-2 toxin although after 12 h incubation there was only a 11% difference in T-2 toxin concentration between the two.

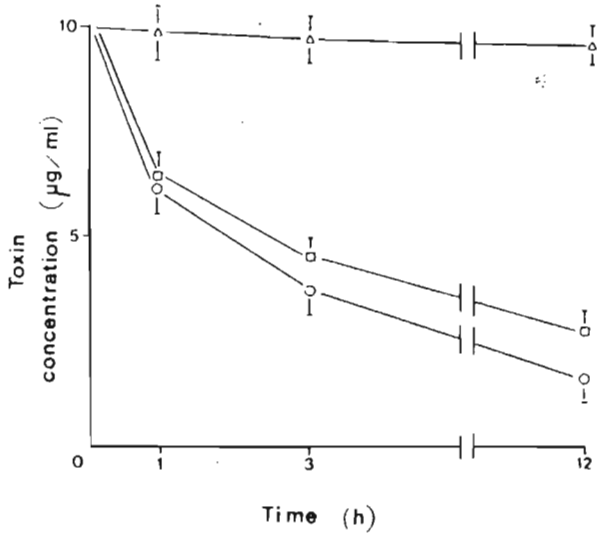

Fig. 2. Degradation of T-2 toxin by protozoal (○), bacterial (□) and cell-free (△) preparations from whole rumen fluid. Figures represent average values of experiments conducted in duplicate.

Susceptibility of bacterial and protozoal preparations from ovine rumen fluid to T-2 toxin

During this study, duplicate samples of the bacterial and protozoal preparations were taken at 0 and 6 h to determine total protein concentration and protozoal counts. Protein concentration measurements (Table 2) showed that, for the bacteria, total protein in the presence of T-2 toxin was higher when compared to protein concentration values in the absence of T-2 toxin. However, in the protozoal preparation, the difference in protein concentration at 0 and 6 h was markedly less in the presence of T-2 toxin.

These results were in agreement with a decrease in protozoal numbers in the absence of T-2 toxin compared to protozoal numbers incubated in the presence of T-2 toxin (Table 3). There was very little difference in the ratio of holotrich

TABLE 2

Susceptibility of bacterial and protozoal preparations from ovine ruminal fluid to T-2 toxin (10 μg ml^{-1}), as determined by total protein measurement[a]

Time (h)	Protein concentration (μg ml^{-1})			
	Bacteria		Protozoa	
	−toxin	+toxin	−toxin	+toxin
0	1013 ± 290	978 ± 178	1155 ± 35	805 ± 63
6	1607 ± 226	1652 ± 136	1828 ± 366	917 ± 71
Difference	594	674	673	112

[a]Figures represent mean values of experiments conducted in duplicate.

TABLE 3

Toxicity of T-2 toxin (10 μg ml^{-1}) to a protozoal preparation from ovine ruminal fluid, as determined by direct counting[a]

Time (h)	Protozoal count (numbers ml^{-1} × 10^{-3})		Ratio (Holotrichs: Entodinimorphs)	
	−toxin	+toxin	−toxin	+toxin
0	36.0 ± 2.0	39.7 ± 0.1	0.44 ± 0.07	0.41 ± 0.05
6	21.1 ± 0.7	20.5 ± 0.3	0.43 ± 0.08	0.36 ± 0.08
Difference	14.9	19.2	0.01	0.05

[a]Figures represent mean values of experiments conducted in duplicate.

to entodiniomorph protozoa when incubated with and without toxin (Table 3).

DISCUSSION

Results obtained in previous studies concerning degradation of aflatoxin B_1 by rumen ingesta were conflicting. In vitro studies using whole rumen fluid, showed no detectable breakdown products of aflatoxin B_1 (Mathur et al., 1976; Kiessling et al., 1984) while Engel and Hagemeister (1978) showed that 40% of the added aflatoxin was degraded. In the present study (Table 1) both aflatoxin B_1 and G_1 were degraded despite observed microbial toxicity as measured by the inhibition of digestion of lucerne hay. Aflatoxin B_1 was shown (Table 1) to be more toxic than aflatoxin G_1 and this is in agreement with previously determined microbial toxicity values (Ciegler et al., 1967). The observed rate of T-2 toxin degradation (Table 1) shows quite close agreement with that of Kiessling et al. (1984) who in a similar study, calculated a degradation rate of 1730 ± 384 $\mu g\ k^{-1}\ h^{-1}$ for T-2 toxin. In their study, they also measured the degradation rates of diacetoxyscirpenol and deoxynivalenol. For diacetoxyscirpenol the measured rate was less than half (845 ± 45 $\mu g\ l^{-1}\ h^{-1}$) that determined in the present investigation (2066 ± 54 $\mu g\ l^{-1}\ h^{-1}$) while deoxynivalenol was not degraded at all. In other work by King et al. (1984), deoxynivalenol (10 $\mu g\ g^{-1}$) was almost completely transformed into a single de-epoxidation product after 24 h-incubation in vitro with bovine rumen fluid.

It has been shown (Westlake et al., 1987a,b) that pure cultures of rumen bacteria differ in their ability to degrade T-2 toxin. Further, both diet and time of sampling affect rumen microbial composition and numbers (Mackie et al., 1978; Michalowski and Muszynski, 1978; Leedle et al., 1982, 1986). Because of these factors and the fact that there will also be variation in rumen microbial composition between animals, it is impossible to make a direct comparison of data obtained by different authors. However, under the conditions prevailing in the present study, deoxynivalenol was degraded at a rate similar to that of T-2 toxin, although breakdown products were not identified.

Incubation of T-2 with ovine rumen fluid in vitro showed that the breakdown products were the deacetylated derivatives HT-2 toxin and T-2 triol (Fig. 1). Work by Westlake et al. (1987a) has shown that pure cultures of *Butyrivibrio fibrisolvens*, a rumen bacterium, can degrade T-2 toxin to these 2 products plus neosolaniol, while the rumen bacteria *Selenomonas ruminantium* and *Anaerovibrio lipolytica* can degrade T-2 toxin to HT-2 toxin and T-2 triol alone. These bacteria were specifically chosen because of their known esterase activity and other functional groups of rumen bacteria may not possess this same activity. However, these 3 bacteria are normally present in the rumen of animals at quite high levels and may, therefore, play a significant role in the toxin degradation observed in the present study. Recent studies using bovine rumen

microorganisms showed that T-2 toxin, diacetoxyscirpenol and deoxynivalenol were transformed to a variety of de-epoxy and deacylated products (Swanson et al., 1987). The biotransformation to deacylated products was more rapid than to de-epoxy products.

In vitro studies on T-2 toxin degradation by bacterial, protozoal and cell-free preparations from ovine rumen fluid showed that all toxin-degrading ability was associated with the microorganisms and that the protozoal fraction was slightly more active than the bacterial fraction (Fig. 2). However, protein concentration and viable count data concerning the effect of T-2 toxin on these 2 fractions, showed that the protozoal fraction was much more susceptible to toxin effects than the bacterial fraction and in fact that the bacterial fraction grew better in the presence of toxin (Tables 2 and 3). For the protozoal fraction, these data appeared to be contradictory, for despite apparent protozoal susceptibility to the toxic effects of T-2 toxin this fraction had the highest toxin-degrading ability (Fig. 2). However, the rate of T-2 toxin degradation by the protozoal fraction was much greater during the initial 3 h of incubation followed by a decrease in protozoal numbers after this period as a result of inhibition by T-2 toxin.

For the bacterial preparation, T-2 toxin did not appear to exert any toxic effects, as judged by protein concentration values (Table 4), yet as with the protozoal preparation toxin-degrading ability was greatest over the first 3 h of incubation. This is most likely due to substrate depletion and accumulation of end products.

The apparent differences in T-2 toxicity to the bacterial and protozoal preparations are not surprising since previous studies have shown that the trichothecene toxins are much more toxic to eukaryotic than prokaryotic organisms (Bamburg and Strong, 1971).

Since the rumen bacteria appear to have increased resistance to the toxic effects of the trichothecenes, they may play a more significant role than the protozoa in conferring increased resistance to these toxins in ruminant animals, despite lower toxin-degrading ability. The effect of diet on rumen microbial composition and numbers has been discussed previously and as a result, the diet of ruminant animals may be an important determinant in the relative toxin resistance of these animals.

ACKNOWLEDGEMENTS

Bursary support for K. Westlake from the C.S.I.R. and the University of Natal, Pietermaritzburg, South Africa, is gratefully acknowledged.

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Mycopathologia **113**: 25–29, 1991.

Metabolism of aflatoxin B_1 by *Petroselinum crispum* (parsley)

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Received 30 October 1989; accepted in revised form 30 April 1990

Key words: Aflatoxicol, aflatoxin B_1, parsley, *Petroselinum crispum*

Abstract

On administration of aflatoxin B_1 to whole parsley (*Petroselinum crispum*) plants, a derivative was formed, which was shown to be aflatoxicol by its chromatographic properties and mass spectrometry. Optimum conditions for the production of the derivative was on the second day after administration of the toxin to the plants, which were 90 days old after germination. Cell-free preparations of parsley were found not to produce aflatoxicol A from added aflatoxin B_1; instead they formed two new derivatives, which from chromatographic properties, were shown to be more polar than either aflatoxin B_1 or aflatoxicol A.

Introduction

Aflatoxins are potent hepatocarcinogens formed by *Aspergillus parasiticus* and *Aspergillus flavus*. The major aflatoxin, aflatoxin B_1 is converted by mammalian systems, including detoxification systems such as the cytochrome P450 complex, to various derivatives [1]. The principal products (Fig. 1) that have been identified, are the hydroxylated derivatives: aflatoxin M_1, aflatoxin P_1, aflatoxin B_{2a} and a reduced derivative aflatoxicol [2].

Microorganisms are also capable of modifying aflatoxin B_1, e.g., to aflatoxicol by *Absidia repens* [3] and to tetrahydroxyaflatoxin B_1 by *Rhizopus arrhizus* [4]. Attempts to demonstrate any of these conversions by plants have not been successful and this includes crops plants such as peas and wheat [5] and maize [6]. This lack of activity may be of economic significance, as it has been shown that certain plants can take up aflatoxins from the soil [6] and thereby pass them on to the human consumer in an unchanged form.

In this study parsley was chosen to investigate metabolic modifications of aflatoxin B_1, as it has a well studied secondary metabolism [7] which includes partially characterised monooxygenases that could be involved in the hydroxylation of xenobiotic substances such as mycotoxins.

Materials and methods

All solvents were of analar grade; aflatoxin B_1 and aflatoxicol A was purchased from Makor Chemicals Ltd., Israel.

Plant material

Plants used in the experiments were grown as follows: parsley (*Petroselinum crispum*) seeds

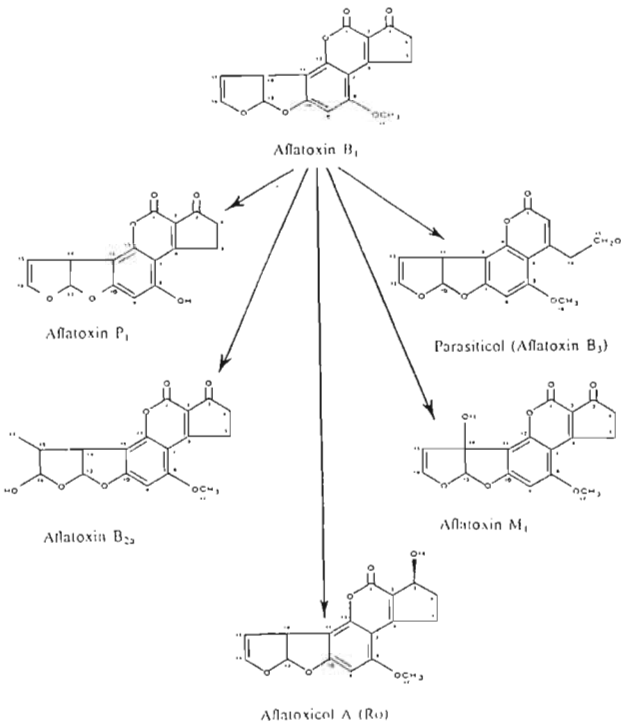


Fig. 1. Aflatoxin B_1 and some of its metabolic products.

were purchased from a local seed merchant and sown in commercial bark medium, which was kept moist throughout the growing period. The germinated seeds were grown in a phytotron at ambient tempertaure and normal lighting conditions. The plants were harvested after 90 days of incubation after germination, as this was found to give consistent results.

In vivo studies

Parsley plant roots were gently washed free of potting medium with sterile distilled water. Three scintillation vials were prepared; two containing 20 ml of aflatoxin B_1 solution (2 μg/ml in aqueous acetone 0.5% v/v); and one containing 20 ml aqueous acetone (0.5% v/v) without aflatoxin B_1 to act as a control. Ten parsley plants were selected so as to be as uniform as possible in size and shape. Five were added to one of the solutions containing the aflatoxin and five to the control salution. The remaining vial was used as a check for chemical degradation occurring during the experiment. All three vials were exposed to normal daylight conditions for 2 days. These experiments were repeated five times to ensure reproducibility. Similar experiments were carried where aflatoxicol A was substituted for aflatoxin B_1.

After two days the plants were removed, washed with a small amount of aqueous acetone (0.5% v/v) gently ground in a mortar and lyophilized. The remaining solutions and combined washings were also lyophilized. The dried residues were extracted sequentially with three 5 ml portions of chloroform, acetone and ethanol, the individual solvent extracts being pooled. The solvents were then evaporated under nitrogen using a Reacti-therm (Pierce Chemical Co) and redissolved in 0.5 ml of the original solvent. Twenty μl were then spotted onto the origin of a 10 x 10 cm two dimensional aluminium backed silica gel chromatoplate (Merck Darmstadt). The plate was developed in chloroform : ethyl acetate : propan-2-ol (90/5/5 v/v) in the first dimension and toluene : ethyl acetate : formic acid (6/3/1 v/v/v) in the second. After drying the fluorescent metablites were observed under long wave ultraviolet light.

Quantitation was done using a Water's 501 high performance liquid chromatograph equipped with a Water's 490 fluorimeter. Acetonitrile : methanol : water (3/3/4 v/v/v) was used as the elution phase on a C_{18} matrix-packed column (Radial-Pak).

In several experiments leaves, stems, and roots of plants treated with aflatoxin B_1, were separately examined for conversion products. The experiments were also repeated using stems and leaves that had been separated from the root system by carefully cutting with a sharp razor blade. These two portions were separately incubated in the imbibing solutions and treated as above.

Indentification of metabolites

The extracts from several experiments where aflatoxin B_1 had been added to plants, were pooled

and subjected to preparative thin layer chromatography using the aforementioned solvent systems. Conversion products from the added aflotoxin B_1 were isolated in this way and were repeatedly separated until a single spot was obtained upon two-dimensional qualitative tlc.

Ultraviolet spectroscopy was done using an Hitachi 220 spectrophotometer; the compounds being dissolved in spectral grade methanol. Mass spectrometry was carried out on a Hewlett Packard 5988A, electron impact 70eV, direct insertion probe mass spectrometer.

Cell-free preparations

All preparations of cell-free extractions were done in equipment kept at 4 °C unless otherwise indicated.

Parsley plants, 20–30 g wet weight, were lyophilised and then gently ground in a mortar in 40 ml 0.05 M potassium phosphate buffer (pH 7.6) containing 14.0 mM 2-mercaptoethanol and 2.0 mM EDTA with a small amount of acid-washed sand and Polyklar AT (BDH Chemicals U.K.) added at a rate of 1 g per 1 g wet weight tissue [8]. The resultant slurry was centrifuged at 10000 × *g* for 20 min. and the supernatant was retained as the crude enzyme preparation. Fresh tissue was also ground with acid washed sand and also by prior freezing in liquid nitrogen. The crude enzyme extracts then being prepared as for the lyophilised sample.

Three samples (13 ml) of each preparation were dispensed into vials. One acted as a control; NADH (final concentration 0.5 mM) was added to one; and NADPH (final concentration 0.5 mM) to the third. Aflatoxin B_1 was then added to all the vials to give a final concentration of 2 μg/ml. All the reaction mixtures were then incubated overnight at 20 °C and then extracted with three equal volumes of chloroform. The chloroform extracts from each experiment were separately pooled, dried by passing through a small bed of anhydrous sodium sulphate, evaporated, and examined using tlc, described above.

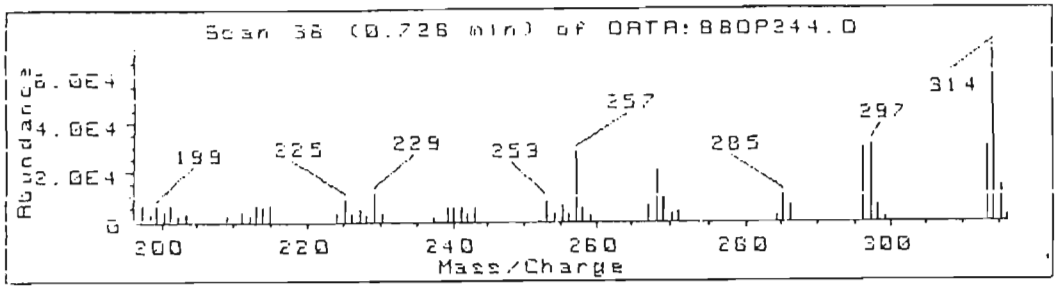
Examination of parsley root exudates

Parsley plants were incubated, as above, for 2 days in 2 vials each containing distilled water (20 ml). After this period the plants were removed and aflatoxin B_1 (5 μg/ml) was added to one vial. Both vials were incubated at 20 °C overnight and then analysed for aflatoxin derivatives as described. To eliminate microbial action, the experiment was repeated after the roots of the plants had been treated with sodium hypochlorite (3%) for 5 min., followed by washing with sterile distilled water. In addition chloramphenicol and streptomycin (50 nM) were added to the imbibing solution.

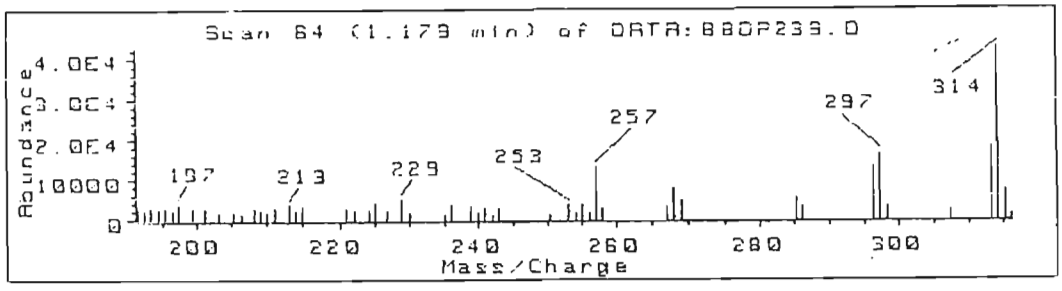
Results and discussion

Plants fed aflatoxin B_1 consistently yielded a new purple fluorescent compound, which was found primarily in the chloroform extract. This substance had RF values of 0.53 and 0.58 in the first and second dimension, respectively, in two dimensional tlc. This substance was not observed in extracts of the control experiments. The chromatographic properties of the metabolite was identical to that of aflatoxicol A and on spiking one of the extracts with pure aflatoxicol A, yielded a single fluorecent spot on tlc, indicating that the substance was aflatoxicol A. Ultraviolet and mass spectrometry of the compound isolated by preparative tlc gave spectra that were identical to those obtained from authentic aflatoxicol (Fig. 2). The compound was aflatoxicol A; the yield based on aflatoxin B_1 added, under the conditions used, was approximately 40%. Experiments where aflatoxicol A was fed to the plants, yielded aflatoxin B_1 indicating that the reaction was reversible.

Because aflatoxicol was found in all plant tissues examined, i.e., roots, stem, and leaf, the stems plus leaves were separated from the roots and both these systems were capable of converting aflatoxin B_1 to aflatoxicol A. Apparently the enzyme(s) responsible for the conversion ramifies through the whole plant and is likely to be a dehydrogenase, considering the reaction



(i) Standard aflatoxicol



(ii) Derivative

Fig. 2. Comparison of the mass spectra of pur aflatoxicol with that of the aflatoxin.

involved and the results of other studies [9]. Interestingly the imbibing solutions were also capable of forming aflatoxicol A, although at a lower conversion rate (15%). The treatment of parsley roots with sodium hypochlorite or antibiotics did not affect this reaction so the effects of microorganisms were ruled out. This was further supported by lack of any turbidity or growth in the solutions and the lack of colonies on plating out these inbibing solutions onto nutrient agar. The conclusion drawn from these results is that the enzyme(s) responsible for the formation of aflatoxicol A must pass into the imbibing solution from the root system. Further studies will be needed to decide whether this is an artifact of the experiment or a natural phenomena.

All three cell-free preparations gave similar results in that two new fluorescent compounds were found in the chloroform extract with RF values of 0.31 and 0.18 respectively in the first dimension and 0.22 and 0.15 respectively in the second. These results were unexpected in that no aflatoxicol was observed and the two substances were more polar than the original aflatoxin B_1, as evidenced by their behaviour on tlc. These compounds were isolated from the lypholised preparation, as this gave the best conversion (5%) of both compounds; at least double that afforded by the other methods. These compounds were not aflatoxin B_{2a}, M_1 and P, as they did not co-chromatograph with any of these standard compounds on tlc. Unfortunately insufficient material was available to characterise them further.

Clearly the intact parsley plant can convert aflatoxin B_1 to aflatoxicol A, probably mediated by a dehydrogenase. Whether this is NAD or NADP linked is impossible to say from the present results. Disruption of the plant to form a cell-free preparation, evidently releases enzymes that do not normally come into contact with aflatoxin B_1 in the intact plant. Presumably these enzyme(s) are in structures that aflatoxin B_1 cannot penetrate.

A difficulty that needs explanation is that no aflatoxicol is formed in the crude enzyme system, which ought to occur as dehydrogenases are usually associated with the cytosol of cells. Furthermore, incubation of aflatoxin B_1 with a combination of centrifuged cell debris and supernatant did not result in the formation of aflatoxicol A. One explanation is that in the crude enzyme preparation a relatively specific enzyme(s) is present that rapidly converts both aflatoxin B_1 and aflatoxicol A to more polar analogous products as evidenced by the appearance of two compounds on tlc. If the conversion of aflatoxin B_1 to aflatoxicol is slow (rate limiting) then this would explain the absence of the latter product.

This system needs more detailed investigation to ascertain the differences between intact plants and disrupted material. The results do show that parsley unlike other plant systems has the capability of modifying aflatoxins and this may have commercial possibilities.

Acknowledgements

Thanks are due to Mr R.K. Berry and Mr M. Watson for their technical assistance, to the Department of Horticulture, University of Natal for phytotron space and to the University of Natal and C.S.I.R. Pretoria for financial assistance.

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Mycopathologia **119**: 181–190, 1992.

The effects of aflatoxin B_1 on immature germinating maize (*Zea mays*) embryos

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Received 8 August 1991; accepted in revised form 26 February 1992

Keywords: aflatoxin B_1, electron microscopy, *in vitro*, immature maize embryo, *Zea mays*

Abstract

Immature maize (*Zea mays* L.) embryos were treated with aflatoxin B_1 concentrations, ranging from 0.1 $\mu g\ ml^{-1}$ to 25 $\mu g\ ml^{-1}$. Below 5 $\mu g\ ml^{-1}$ aflatoxin B_1, root and shoot elongation was not significantly inhibited. Ultrastructurally, root tip cells showed little deterioration, except a possible diffused clearing in mitochondria and plastids. As the toxin concentration was increased above 5 $\mu g\ ml^{-1}$, shoot, and particularly root elongation, was progressively inhibited. Associated with this, there was an apparent decrease in the ribosome population. Furthermore, membranes, particularly the vacuolar membrane, became abnormal and vacuolar distension occurred. At 20 and 25 $\mu g\ ml^{-1}$, these effects were exacerbated, and mitochondria and plastid structure was disrupted. At these concentrations, there was evidence of a disruption in lipid metabolism. The results are discussed in the context of known aflatoxin effects on cellular control mechanisms and ultrastructure in animal systems.

Introduction

Aspergillus flavus and *Aspergillus parasiticus* are moulds commonly associated with developing crops in the field and with seeds in storage [1]. These fungi produce several mycotoxins, including aflatoxin B_1 which is a potent hepatotoxin and hepatocarcinogen [2]. In the past, research has centred primarily on the effects of this toxin on animals, and animal cells in culture. More recently, however, there has been increasing interest in the potential deteriorative effects the toxin may have on seed viability and seedling vigour, and on a number of edible plant products, many of them staple foods [3, 4]. To this end, research work has mainly investigated toxin effects on biochemical (amino acid; enzyme activities) and physiological (germination; root and shoot elongation) parameters.

A. flavus and *A. parasiticus* are ubiquitous in nature, frequently occurring in agricultural soils and contaminating growing plants [5]. Since both the fungi and their metabolites gain access to the plant under field conditions [5], the potential effects on seeds at varying stages of development are of interest. It is well known that susceptibility to disease is dependent on factors such as plant age, tissue differentiation, and nutritional status, as well as environmental factors including temperature and relative humidity [6]. In order to

avoid such variables and ensure that the effects of various levels of aflatoxin B_1 alone on the embryos were being monitored, the present investigation reports on the possible ultrastructural effects of this toxin on immature *Zea mays* embryos cultured aseptically *in vitro*.

Materials and methods

Zea mays seeds (var. PNR 6363) were supplied by the Pioneer Seed Company, Greytown, Natal, South Africa. Pure crystalline aflatoxin B_1 was purchased from the Council for Scientific and Industrial Research, Pretoria, South Africa. A stock solution, prepared from crystalline aflatoxin, was dissolved in DMSO and diluted with sterile distilled water. The DMSO concentration in any treatment never exceeded 0.1%.

Maize embryo excision and incubation with aflatoxin B_1. Immature ('milk stage', 2 months prior to harvest) maize seeds were surface-sterilized by washing in 1% sodium dodecyl sulphate for 5 minutes, followed by a 10 minute immersion in 1% sodium hypochlorite. They were then rinsed three times in sterile distilled water. The embryos were excised aseptically, rinsed briefly with 1% sodium hypochlorite and washed thoroughly in sterile distilled water. Embryos were plated individually onto a maize embryo medium (pH 5.8) [7] to which aflatoxin B_1 was added (after autoclaving) in two dose ranges [0.1, 0.5, 1.0 or 2.0 $\mu g\,ml^{-1}$ (low dose) and 5, 10, 20 or 25 $\mu g\,ml^{-1}$ (high dose)] and incubated at 25 ± 3 °C, with a 16 hour photoperiod, 200 $\mu E\,m^{-2}\,sec^{-1}$ light intensity. Thin layer chromatography confirmed that aflatoxin B_1 was stable under the culture conditions.

In order to assess the possible effects of DMSO, embryos were incubated for 9 days on a maize embryo medium containing either 0.005% or 0.4% DMSO, and root tips were processed for electron microscopy as described below. Ultrastructurally, apart from a fractional increase in the degree of vacuolation at the higher concentration (four times the concentration at 25 $\mu g\,ml^{-1}$ AFB_1), the DMSO had no deleterious effects on cellular organelles, nor were root measurements of this material statistically different from the control material ($p \leq 0.05$, LSD). It was thus decided that for all treatments, material cultured on the unadulterated medium would serve as the control.

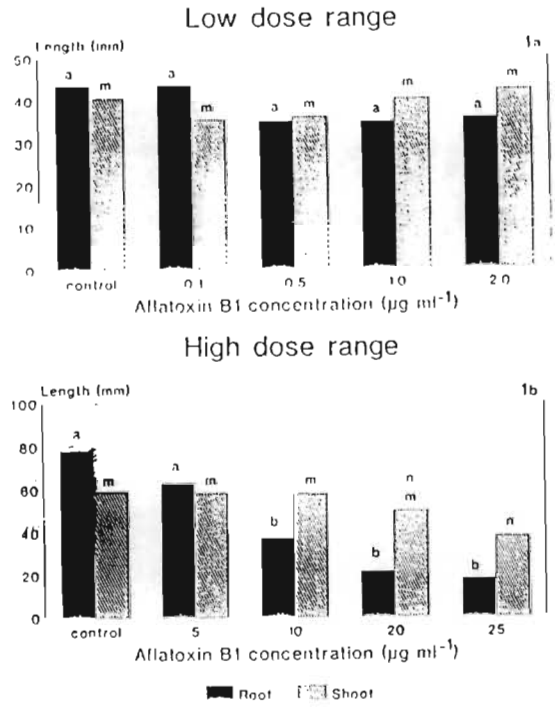
Germination assessment and root and shoot elongation of embryos. Radicle and shoot extension were recorded every two days after plating. Final measurements were made following 9 days of incubation, after which root tips were excised and processed for electron microscopy.

Electron microscopy of cultured material. The tips of the primary roots of cultured embryos were immersed in Karnovsky's fixative (pH 6.0), post-fixed at 4 °C in 1% aqueous osmium tetroxide, block-stained with uranyl acetate during ethanol dehydration and embedded in epoxy resin. Ultrathin sections were post-stained with uranyl acetate and lead citrate. Sections were viewed using either a Jeol 100C or a Zeiss EM10B transmission electron microscope.

Results

Effects of aflatoxin B_1 on root and shoot length

Figures 1a & 1b show the data for root and shoot elongation for the two toxin dose ranges presently utilised (0.1–2.0 $\mu g\ ml^{-1}$ and 5–25 $\mu g\,ml^{-1}$ aflatoxin B_1). In the higher dose range, there was a concentration-related inhibition of root and shoot elongation, this being more noticeable for root growth. At concentrations of 10, 20 and 25 $\mu g\,ml^{-1}$ aflatoxin B_1, mean root length was significantly different from both the control and the 5 $\mu g\,ml^{-1}$ toxin level ($p \leq 0.05$, LSD). A dose level of 10 $\mu g\,ml^{-1}$ toxin, was correlated



Figs 1a & b. Root and shoot length for immature *Zea mays* embryos treated with two dose ranges (0.1–2.0 μg ml^{-1}; 5–25 μg ml^{-1}) of aflatoxin B_1. Different letters for root (a or b) and shoot (m or n) measurements are significantly different ($p \leq 0.05$, LSD).

with a 53% inhibition of root elongation, and this value increased to 77% at 25 μg ml^{-1} aflatoxin B_1. The greatest inhibition (43%) between consecutive dose levels of toxin on root tissue occurred with a doubling in toxin concentration from 10 μg ml^{-1} to 20 μg ml^{-1}. The most marked inhibition of shoot elongation was observed at 25 μg ml^{-1}, with the increase in toxin concentration from 20 to 25 μg ml^{-1} accounting for the greatest degree of inhibition (24%) between two concentrations. In contrast, in the lower dose range, no concentration-dependent trend was observed and no statistically significant differences were observed amongst these treatments ($p \leq 0.05$, LSD).

Germination for all treatments was in excess of 90%. In contrast, in a previous study in which embryos were treated with up to 100 μg ml^{-1} aflatoxin B_1, embryo viability was reduced to 10% (unpublished data).

Ultrastructural effects

Since it was obvious from growth assessments that the roots were more severely affected by the aflatoxin B_1 treatment than the shoots, this study focussed on the cytological changes in root tips. The substructure of cells presented a mosaic of greater or lesser deterioration, and the alterations reported here are general rather than absolute trends. The cells of control roots (Figs. 2 and 3) were characterised by starch-containing plastids, a well-developed endoplasmic reticulum (not shown), and compact mitochondria, the dense matrices reflecting a high level of metabolic activity of these organelles. A striking feature of control tissue was the dense nature of the cytoplasm, resulting partly from the large number of ribosomes (Fig. 3).

Dose levels from 0.1 to 2.0 μg ml^{-1} aflatoxin B_1 appeared to have little effect on the ultrastructural features of treated roots. A similar disposition of ribosomes, rough endoplasmic reticulum, Golgi bodies, mitochondria and plastids as in the control tissue, was observed (Fig. 4). There were, however, occasional areas of cytoplasmic dissolution (Fig. 5). Although not observed frequently, there were instances of abnormal aggregations of lipid droplets in some cells (Fig. 6). An ultrastructural feature observed in some mitochondria, particularly in cells treated with 1.0 and 2.0 μg ml^{-1} aflatoxin B_1, was a diffuse central region (Fig. 7). There was evidence that plastid morphology had been affected. Some of these organelles presented as bodies with little internal detail (Fig. 7).

A dose level of 5 μg ml^{-1} of aflatoxin B_1 produced marked ultrastructural changes in the root tip cells. The most noticeable of these was the increase in the degree of vacuolation. These cell components were observed to have swelled markedly and there was evidence of their fusion (Fig. 8). Localised tonoplast dissolution was also apparent. Mitochondria, plastids and endoplasmic

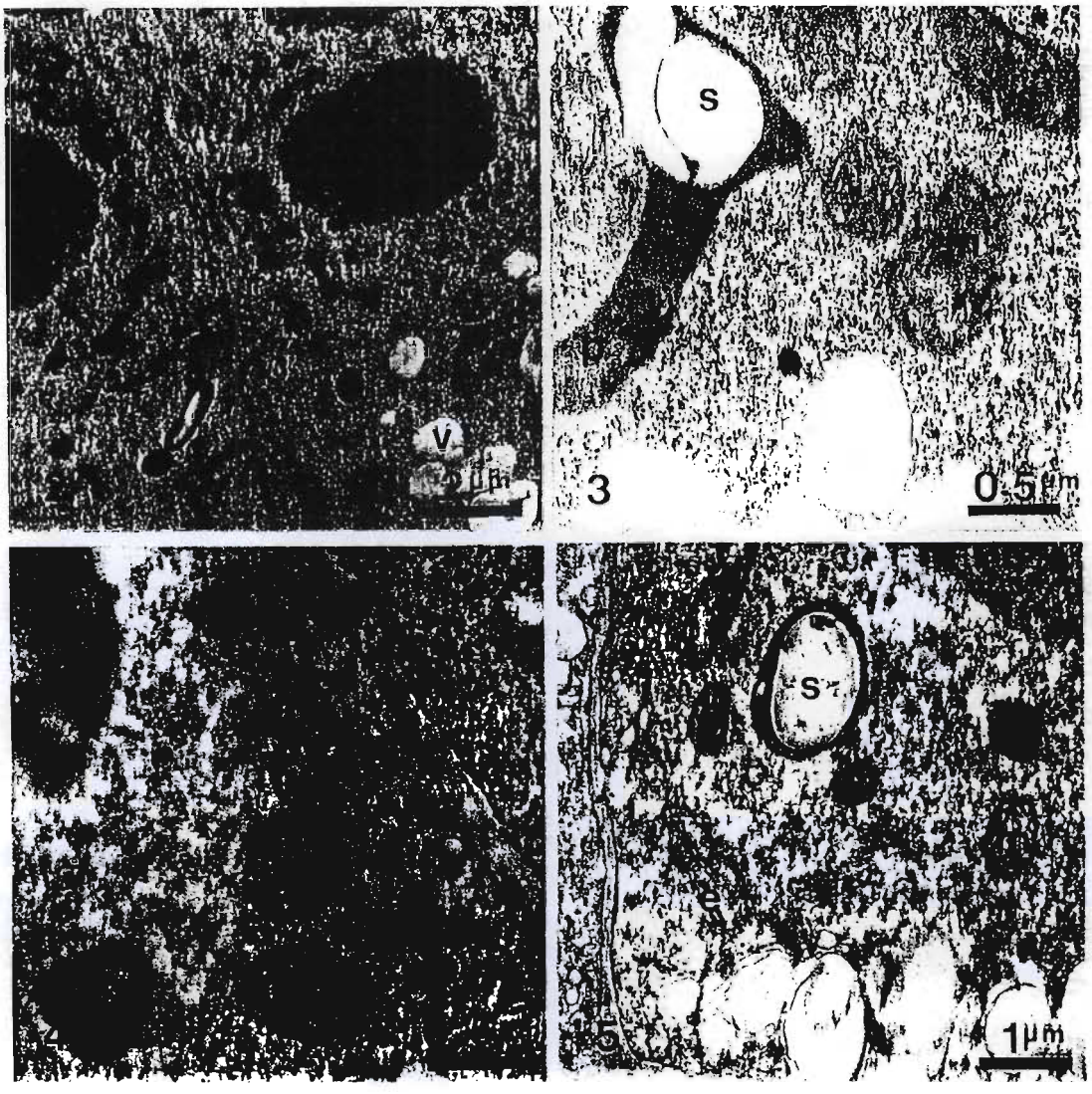


Plate 1. Figs 2 & 3. Cells of control root tips exhibit an abundance of cytoplasmic ribosomes; mitochondria (m) with well-developed cristae; starch-containing (s) plastids (p) and small vacuoles (v). Fig. 2. ×7 500; Fig. 3. ×24 000; *Figs 4 & 5.* Cells treated with 0.1 µg ml^{-1} aflatoxin B_1 (AFB_1). Fig. 4 illustrates the compact mitochondria (m) and the large number of cytoplasmic ribosomes. Fig. 5 suggests that slight cytomatrical dissolution has occurred. Mitochondria (m), short strands of rough endoplasmic reticulum (e), vacuoles (v) and starch-containing (s) plastids (p) are visible. Fig. 4. ×24 000; Fig. 5. ×12 400.

reticulum generally showed no ultrastructurally visible evidence of abnormality (Fig. 9). There was, however, a marked loss of density of the cytomatrix which could be attributed to a decrease in the ribosome population (Figs. 8 and 9).

An increase in toxin concentration above 5 µg ml^{-1} resulted in an exacerbation of subcellular deterioration observed to occur more sporadically at lower dose levels, viz. tonoplast disruption, vacuolar distention and cytoplasmic dilution (Fig. 10). Additionally, there was severe derangement of the ultrastructure of the organelles. The originally dense matrix of the plas-

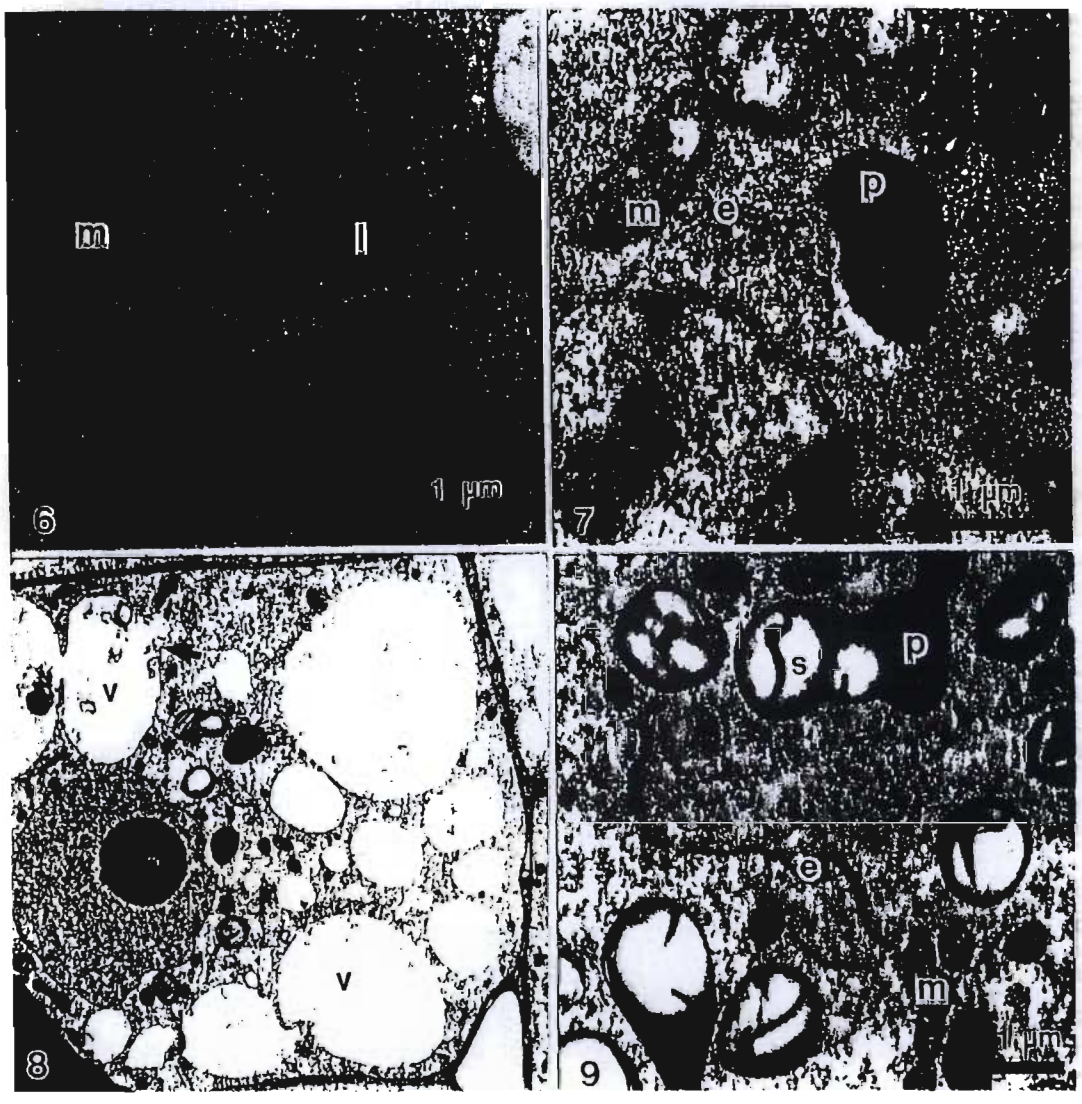


Plate II. Fig. 6. An unusual aggregation of lipid droplets (l) in cells treated with 1.0 μg ml^{-1} AFB_1. Occasional mitochondria (m) appear with a noticeable diffuse central region. ×19 500; *Fig. 7.* In cells treated with 2.0 μg ml^{-1} AFB_1, many mitochondria (m) showed a diffuse central zone. Short strands of rough endoplasmic reticulum (e) and a plastid (p) lacking internal detail are visible. ×22 400; *Figs 8 & 9.* An increase in the size and number of vacuoles (v), with localised tonoplast (arrowheads) dissolution in cells treated with 5 μg ml^{-1} AFB_1. Fig. 9 illustrates the presence of rough endoplasmic reticulum (e) and plastids (p) with large starch grains (s). There is an apparent decrease in the density of ribosomes, while mitochondria (m) are intact, although compacted organelles. Fig. 8. ×7 850; Fig. 9. ×10 000.

tids had become relatively diffuse, and often starch deposits appeared depleted. The ER cisternae had apparently broken up. Most mitochondria too, appeared intact but with swollen cristae (Fig. 11).

As the toxin concentration was increased to 20 and 25 μg ml^{-1}, extensive lysis of the tonoplast and plastid membranes was observed (Fig. 12). Accompanying this, was considerable cytoplasmic dissolution (Figs 12 & 13). Mitochondrial integrity, too, appeared disrupted (Fig. 13). Some cells appeared completely collapsed, with large lipid coalescences (Fig. 14).

While the cytoplasm and its organelles de-

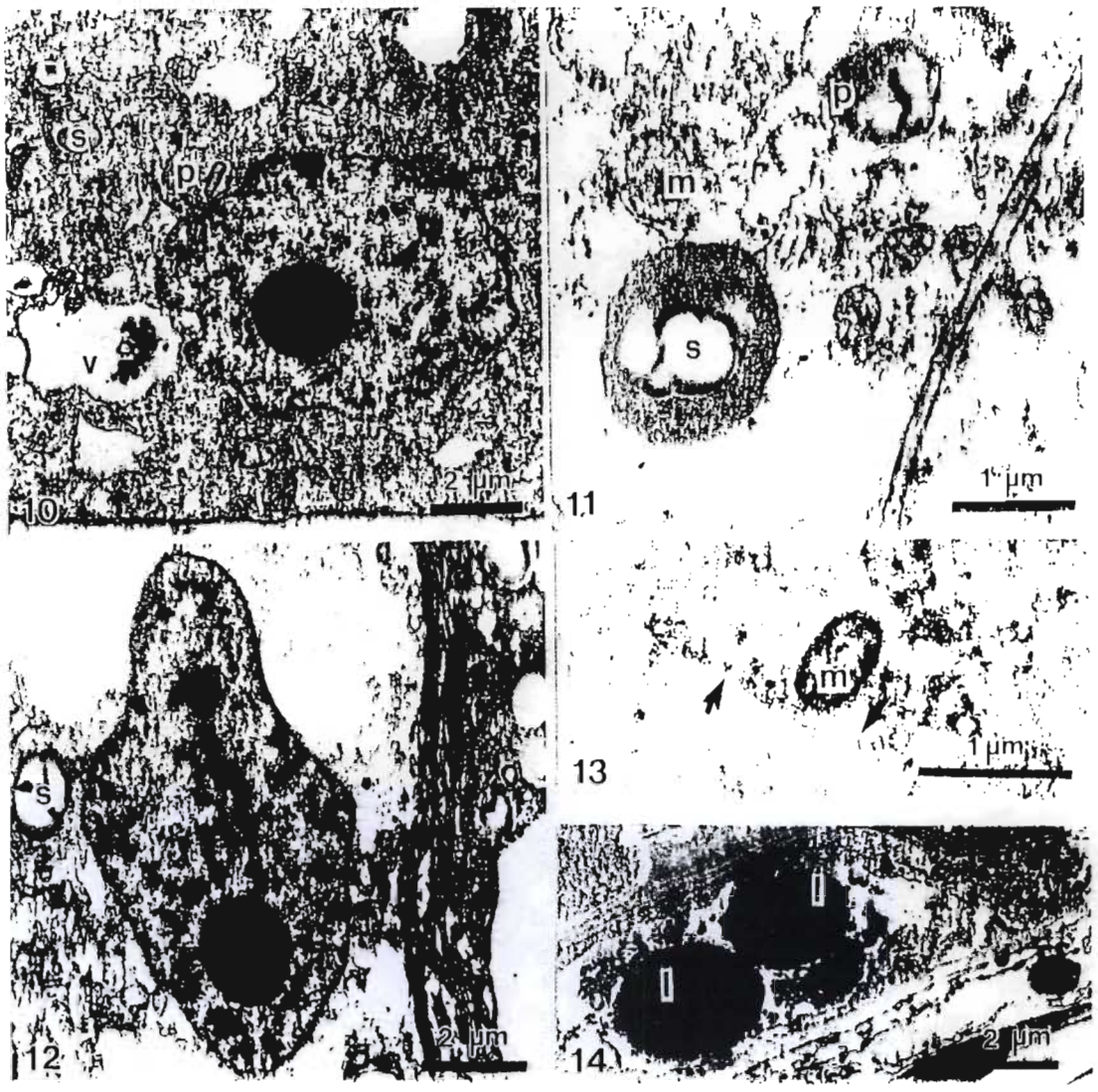


Plate III. Figs 10 & 11. In this tissue, treated with 10 µg ml^{-1} AFB_1, some cells exhibit a large number of cytoplasmic ribosomes (Fig. 10), while in others, these particles appear largely depleted (Fig. 11). A common feature, however, is the deterioration of the plastids (p), with apparent starch (s) depletion. The mitochondria (m) are apparently intact, but cristae appear to be somewhat swollen. Fig. 10 ×6 300; Fig. 11 ×16 500; *Figs 12 & 13.* In this material exposed to 20 µg ml^{-1} AFB_1, plastid structure has been disrupted such that starch grains (s) appear free in a disorganised cytoplasm. The nucleus remains intact albeit abnormal in appearance (Fig. 12). A mitochondrion (m) lacking detail, membrane fragments (arrowheads) and cytoplasmic dissolution suggest severe cell deterioration (Fig. 13) Fig. 12 ×7 000; Fig. 13 ×19 800; *Fig. 14.* Large accumulations of lipid (l) in a deteriorated cell in a root tip treated with 25 µg ml^{-1} AFB_1. ×5 000.

teriorated, the nucleus and nucleolar components appeared morphologically intact. The chromatin, however, was quite abnormal, and a distinct loss of nuclear matrix homogeneity had occurred (Fig. 12).

Discussion

Above 5 µg ml^{-1}, aflatoxin B_1 resulted in concentration-dependent deteriorative effects on excised maize embryos. Inhibition of root elongation was

more marked than was shoot elongation. Such observations may have significant effects on the development of the plant in the field, as impaired root development could result in a decrease in nutrient uptake, and hence decreased vigour and ultimately, yields.

Other workers [8, 9, 10] investigating aflatoxin B_1-induced inhibition of germination and root and shoot elongation suggested that there are varying degrees of susceptibility amongst different plant species. In lettuce and several members of the Cruciferae, 100 $\mu g\,ml^{-1}$ aflatoxin was reported as having little or no inhibitory effect on seed germination [8, 9], while the same toxin concentration had an inhibitory effect on the germination of *Lepidium sativum*, another crucifer [10]. Lower concentrations (1, 2 and 10 $\mu g\,ml^{-1}$) did not impair seed germination in *L. sativum*, but as the toxin concentration increased to 100 $\mu g\,ml^{-1}$, germination decreased to zero (65% at 25 $\mu g\,ml^{-1}$; 10% at 50 $\mu g\,ml^{-1}$)[10]. Similarly, two varieties of soybean treated with 2.5–10 $\mu g\,ml^{-1}$ aflatoxin B_1 failed to show any significant changes in germination, growth and radiolabel uptake [11]. In the present study also, germination appeared to be unaffected by concentrations up to 25 $\mu g\,ml^{-1}$ aflatoxin B_1, but higher concentrations resulted in a delay in germination or a loss of axis viability (unpublished data).

In the toxin concentration ranges used in the present investigation (0.1–2.0 $\mu g\,ml^{-1}$ and 5–25 $\mu g\,ml^{-1}$), aflatoxin B_1 appears to have little or no qualitative effect on axis viability. In all instances, germination, assessed as the emergence of the radicle, exceeded 90%. In the high dose range, there was, however, a severe inhibition of radicle elongation following this initial protrusion, suggesting an interruption in the synthesis of new material for continued growth, which might be interpreted as a diminuition of vigour. In a review article, Dashek and Llewellyn [12] are of the opinion that 'rather high concentrations of aflatoxin are required to alter the germination percentage of most seeds'. The results of the present investigation appear to support this observation.

Toxicity of aflatoxin B_1, therefore might be suggested to be manifested once the supply of preformed metabolites has been exhausted and it is possible that the synthesis of new molecules for shoot and root extension cannot be initiated. In the present case, it could be suggested that as the concentration of aflatoxin B_1 increases, more of the DNA may be inactivated by binding, thereby inhibiting RNA synthesis and hence protein synthesis. Tripathi and Misra [13], working on maize seeds, found that aflatoxin B_1 was capable of binding with DNA, as measured by viscometry, difference spectrophotometry and equilibrium dialysis. In animal systems, aflatoxin B_1 binds specifically to (the guanine residues of) DNA [14], implying that there may be similar mechanisms of inhibition operating in both plant and animal systems.

Inhibition of root and shoot growth by aflatoxin B_1 was observed at much lower toxin concentrations than was germination. Results from the present investigation suggest that toxin concentrations above 5 $\mu g\,ml^{-1}$ cause both physiological abnormality and deteriorative ultrastructural effects in *Zea mays* roots. Other researchers have reported such inhibition at low toxin concentrations. For example, *Pimpinella anisum* seeds treated with 2.5 $\mu g\,ml^{-1}$ aflatoxin B_1, exhibited a 71% suppression of root elongation [15]. In *Lepidium sativum*, however, at 100 $\mu g\,ml^{-1}$ aflatoxin B_1, root and hypocotyl elongation were inhibited by 91% and 93%, respectively [8], although inhibition was less marked in other cruciferous species [9].

At low toxin concentrations (0.1–2 $\mu g\,ml^{-1}$), there was no related disruption of root and shoot development. This was confirmed ultrastructurally, although there was evidence of initial interference with mitochondrial, and to a lesser extent, plastid structure. At higher toxin concentrations, inhibition of root and shoot elongation was accompanied ultrastructurally by derangement of cytoplasmic constituents – disso-

lution of membranes, particularly the tonoplast, loss of ribosomes, organellar disruption and disappearance of the endoplasmic reticulum. The increased inhibition of root and shoot extension as aflatoxin B_1 concentration increased is suggested to be correlated with the increasing disruption of the organelles.

There is a paucity of literature on the ultrastructural effects of *Aspergillus* toxins on plant tissue. A common observation in the few reports that exist, however, appears to be the presence of abnormal lipid bodies in the cytoplasm of cells. These have been reported in wheat embryos infected with *A. glaucus* [16]; *Lepidium sativum* seedlings exposed to culture extracts of *A. sulphureus* [17]; and *Lepidium sativum* roots exposed to aflatoxin B_1 [8]. In animal tissue, lipid body accumulation has also been reported, for example, in monkey kidney epithelial cells treated with sterigmatocystin and aflatoxin B_1 [18] and rat liver tissue treated with T-2 toxin [19].

From the ultrastructural studies presented, lipid metabolism appears to be affected. These effects are, however, more noticeable at higher toxin concentrations (above 25 $\mu g\ ml^{-1}$), and abnormality becomes more exaggerated when the dose level is increased to 50 and 100 $\mu g\ ml^{-1}$, where lipid droplets appeared to coalesce to form large lipid bodies (unpublished data). The underlying cause of this abnormality has not been elucidated, and is not explained in the literature. However, as the present study eliminates all fungal influence, other than toxin effects, it seems that these are a major factor in lipid coalescence. This phenomenon has been suggested to be diagnostic of fungal infected maize seeds [20], but might, more specifically, be diagnostic of toxin-elaborating seed-borne fungi.

Other major ultrastructural alterations induced by aflatoxin B_1 in this study included an increase in the number of vacuoles, many of which appeared to have been autophagic, and general cytoplasmic plasmolysis and necrosis. These features were observed in material subjected to aflatoxin B_1 concentration in excess of 5 $\mu g\ ml^{-1}$. This symptom, observed with *A. sulphureus* toxins, has been suggested to correspond to a stimulated self-phagocytosis (autophagy) [17]. Those authors consider this a non-specific response to the toxin.

The similarity of deteriorative changes that appear to be induced by different toxins on the various tissues in both plant and animals is interesting. Mollenhauer et al. [19] have remarked on the similarity in cellular response of liver cells to T-2 toxin and to aflatoxin, although there was recovery following T-2 intoxication. This could suggest certain common underlying features between the reported effects of aflatoxin B_1 exposure and these observed here in root tips of 9 day old *Zea mays* seedlings.

Much attention has been directed towards the ultrastructural alterations in nuclear and nucleolar morphology in plant and animal tissue following toxin exposure. Crisan [9] reported irregularly-shaped nuclei of treated *Lepidium sativum* roots which exhibited a less granular nucleoplasm, an increase in interchromatin granules and ring-shaped nucleoli with macrosegregation and nucleolar capping. These changes are similar to the characteristic changes reported in aflatoxin B_1- and sterigmatocystin-treated animal tissue [18], and those induced by inhibitors of DNA-dependent RNA synthesis, e.g. actinomycin D [21]. Nuclear alterations, except chromatin clumping, were not an obvious feature in the cells of *Zea mays* roots currently being investigated.

However, if the primary effect of aflatoxin B_1 is on the DNA, then nuclear morphological abnormality might merely be a gross secondary manifestation.

Growth of seeds and seedlings involves the synthesis of new proteins, including enzymes, and other components for cell division and extension. A consistent feature of this ultrastructural investigation at the higher dose level was the marked decrease in the density of cytoplasmic ribosomes. Disruption of ribosome structure or function would necessarily interfere with protein synthesis. Current preliminary investigations suggest that at aflatoxin B_1 concentrations of both 5 and

25 μg ml^{-1}, there is a marked inhibition of [^{3}H]-leucine incorporation into TCA-precipitable protein in excised maize embryos (unpublished data). This is in agreement with the work of other authors who found similar decreases in radiolabelled amino acid uptake in aflatoxin B_1-treated plant tissue [22, 23]. There is evidence in the animal literature that aflatoxin B_1 interferes with protein synthesis, by binding to the DNA, thereby inhibiting mRNA synthesis [18].

There is biochemical evidence that several inhibitors of protein synthesis, including aflatoxin B_1, interfere with ribosome structure. Floyd et al. [24] compared the sedimentation coefficient of RNA species affected by actinomycin D and aflatoxin B_1. Actinomycin D inhibited the synthesis of 45S RNA, while aflatoxin B_1 inhibition was more general, affecting the 28, 35 and 45S RNA. In the present investigation, toxin concentrations above 5 μg ml^{-1} caused a severe depletion of cytoplasmic ribosomes, which is in agreement with the biochemical evidence of other workers.

For animal tissue, there is some consensus regarding the mode of action of aflatoxin, in terms of metabolic activation by hepatic microsomes. The matter is not clear-cut in the plant literature. In order for aflatoxin to exert its toxic effects on plant cells, it must be taken up from the culture medium, and the inhibition of germination and root and shoot elongation suggest that the toxin does enter the plant cells. There are reports of aflatoxin B_1 being isolated from soybean roots and shoots [22] and organelles [23], but accounts of the fate of aflatoxin in various plant species, following its absorption, are somewhat inconsistent. Reiss [25] extracted only aflatoxin B_1 from wheat and pea kernels incubated in 10 and 100 μg ml^{-1} toxin. In parsley seedlings, aflatoxicol and an unidentified metabolite have been isolated following incubation with aflatoxin B_1 [26]. Preliminary investigations suggest that aflatoxin B_1 is taken up into the embryonic axes of germinating maize seeds. However, there is presently insufficient data in the literature to elucidate the ultimate fate of aflatoxin. The possibility exists that plants may exhibit a spectrum of 'detoxifying mechanisms', and so could be categorised as 'resistent' or 'suspectible' species, as with animals. Work is currently being undertaken to elucidate the mode of action and fate of aflatoxin in cultured plant tissues.

Acknowledgements

The authors would like to thank Mrs T Naicker and Mrs S Bux for their technical assistance, and the University of Natal for financial assistance.

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Journal of Natural Toxins, Vol. 2, No. 1, 1993
Printed in U.S.A.

THE INFLUENCE OF AFLATOXIN B_1 ON THE *IN VITRO* GERMINATION AND GROWTH OF EXCISED, MATURE *ZEA MAYS* EMBRYOS

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(Received August 20, 1992; Accepted October 21, 1992)

ABSTRACT

Aflatoxin B_1, a mycotoxin produced by *Aspergillus flavus* and *A. parasiticus*, inhibited root elongation of germinating excised *Zea mays* embryos from 5.0 μg ml^{-1}. Shoot elongation was inhibited from 2.0 μg ml^{-1} AFB_1 AFB_1. At 10, 20, and 25 μg ml^{-1}, inhibition of root elongation was more marked than was the inhibition of shoot elongation. Inhibition of root and shoot growth was apparent from the first week of toxin exposure. Removal of the toxin source resulted in a reversal of the inhibitory trend on shoot elongation within 1 w of regrowth. Following 4 weeks in potting soil without a toxin source, any toxin-induced differences in percentage wet and dry mass were overcome. AFB_1 was isolated from the roots and shoots of seedlings following 9 days of exposure to toxin. After 4 weeks potted, AFB_1 was recovered from the roots, but not the shoots of seedlings. These findings suggest that AFB_1 is taken up by maize seedlings and at the concentrations used, has reversible phytotoxic effects.

INTRODUCTION

The fungi responsible for producing aflatoxin B_1 (AFB_1), *Aspergillus flavus* and *A. parsiticus*, are ubiquitous and frequently found associated with agricultural soils, developing crops and stored seeds (Christensen and Sauer, 1982), often causing loss of seed quality and viability. Since AFB_1 is the most potent hepatocarcinogen known (Krogh, 1987), the fate of this mycotoxin in the developing plant or the stored seed is of considerable economic, veterinary, and medical importance.

Maize, apart from being an important component of animal feed, is a staple food in large parts of India, Central and South America, and Africa, areas with climates conducive to fungal proliferation. In the countries concerned, loss of seed quality and viability, or livestock death has severe economic implications for these all-too-often impoverished communities. While research has been directed towards the biochemistry of this toxin and its effects when consumed by animals, far less has been documented regarding resultant plant and seed pathology. These considerations prompted the present investigation of the

response of maize embryos to controlled treatment with purified AFB_1. Other current studies, aimed at ascertaining the effects of AFB_1-producing strains of *Aspergillus* spp., should clarify the responses to the continued presence and anabolic activity of the fungi, *per se*.

MATERIALS AND METHODS

Aflatoxin B_1

Pure (>99%) crystalline AFB_1 was obtained from the Council for Scientific and Industrial Research, Pretoria, South Africa. A stock solution of 1000 μg ml^{-1} AFB_1 was prepared by dissolving 10 mg of toxin in 400 μl DMSO. Sterile distilled water was added to give a final volume of 10 ml.

Zea mays caryopses (seeds)

Mature maize seeds (PNR 6363) were obtained from the Pannar Seed Company (Greytown, Natal, South Africa), and were stored at 4 ± 2°C until required.

Prior to excision of the embryos, caryopses were rinsed in 2% Hibitane$_R$ (50 mg ml^{-1} chlorohexidine gluconate; ICI Pharmaceuticals, South Africa) for 15 min, washed thoroughly with sterile distilled water and allowed to imbibe at room temperature for 18-24 h. Embryos were excised, washed for 15 min with a 2% Hibitane solution, soaked for 10 min in a 0.1 mg ml^{-1} penicillin/streptomycin mixture, followed by a 5-min immersion in 1% sodium hypochlorite. Embryos were rinsed three times in sterile distilled water and then plated aseptically onto a maize embryo germination medium (pH 5.8) (Reinert and Yeoman, 1982) into which AFB_1 at two dose ranges [0.1, 0.5, 1.0, 2.0 μg ml^{-1} (low) and 5.0, 10.0, 20.0, 25.0 μg ml^{-1} (high)] had been incorporated following autoclaving of the medium. Embryos were allowed to germinate and establish for 9 d at 25 ± 3°C, 16 h photoperiod at 200 μE m^{-2} sec-1. Three replicates of 20 embryos were assessed for each treatment. In each dose range, control material comprised embryos grown on the following media: no DMSO, and DMSO in the lowest and the highest AFB_1 concentration for each dose range [i.e., DMSO concentration in 0.1 and 2.0 μg ml^{-1} AFB_1 (low dose range) and 5.0 and 25 μg ml^{-1} AFB_1 (high dose range).

Germination Assessments and Growth Measurements

Germination was scored as the emergence of the radicle from the coleorhiza, and expressed as a percentage.

Following 9 d of incubation, root and shoot lengths were measured and the means compared statistically (one-way analysis of variance, LSD, $p \leq 0.05$).

Fresh Mass and Percentage Dry Mass of Seedlings

Fresh mass and percentage dry mass values of seedlings from each replicate were determined. For dry mass determination, individual seedlings were dried in an oven at 60°C until constant mass was attained.

Regrowth of Seedlings

To determine whether any observed inhibitory effects induced by AFB_1 were permanent, the regrowth of seedlings was assessed in two ways after their removal from the toxin source. In the first instance, seedlings were planted in sterile potting soil and placed in a greenhouse exposed to natural diurnal conditions. Seedlings were

provided with tap water and allowed to establish. After four weeks, the fresh mass and percentage dry mass were determined. In the second instance, following measurement of root and shoot lengths, seedlings were transferred to larger sterile glass jars containing a soft maize embryo medium (0.3% agar). The roots were introduced into the agar and seedlings were allowed to establish for one week (25 ± 3°C; 16 h photoperiod; 200 μ m^{-2} sec^{-1}). After 1 week, root and shoot lengths and seedling weights were recorded and compared with initial values (i.e., after 9 d exposure to toxin).

Aflatoxin B_1 Extraction and Detection by TLC

Plant Material

In an attempt to determine the fate of AFB_1, the toxin was extracted from two (high dose range) and three (low dose range) 9 d old seedlings cultured *in vitro* and two 4 w old potted plants (high dose range). For *in vitro* material, roots, shoots, and the transition zone tissue (remains of embryonic axis once roots and shoots had emerged) were separated and the toxin extracted from each component. In potted plants, no clearly defined transition zone remained; therefore, assessment was based on roots and shoots separately. The toxin was extracted three times from homogenized material with a chloroform:methanol (4:1) mixture. The extracts were pooled, dried over anhydrous sodium sulphate, and evaporated until the volume was reduced to 0.5 ml. Twenty μl were spotted onto the origin of 10 x 10 cm aluminum-backed, silica gel chromatoplates (Merck, Darmstadt). Plates were developed in chloroform:ethyl acetate:isopropyl alcohol (90:5:5 v/v). After drying, the plates were viewed under long wave ultraviolet light. Approximately 2 ng (Reiss, 1973) to 3 ng (Pons and Goldblatt, 1969) of AFB_1 can be detected on alumina coated plates.

Medium

The stability of AFB_1 under the culture conditions was checked. Tubes containing media into which AFB_1 had been incorporated were incubated for 9 d. The toxin was extracted and chromatographed as described above and the R_f value compared with that of the AFB_1 stock.

Statistics

All results were compared statistically (one-way analysis of variance, LSD, $p \leq 0.05$). Different letters on figures for any one parameter (e.g., root length) represent significantly different values. As a result of the different controls, material in the low and high dose ranges are considered independently.

RESULTS

Germination and Growth Measurements

The percentage germination for all treatments in both the low and the high toxin dose ranges was 100%.

In the low dose range, AFB_1 had no significant effect on root length (Fig. 1a), while shoot length was unaffected until a concentration of 2 μg ml^{-1} was attained. In the high dose range, however, there was a severe inhibition of root growth at 5 μg ml^{-1}, the lowest toxin concentration in the range (Fig. 1b). Root elongation continued to be significantly and increasingly inhibited with increasing toxin concentration. Mean shoot length also followed a significant, but less marked, trend of reduced growth. For all treatments in the high dose range, shoot lengths were significantly different from the

control seedlings. Furthermore, mean shoot length at 20 and 25 μg ml^{-1} was significantly different from the lower concentrations (Fig. 1b).

Figures 2a and 2b show mean shoot length of seedlings originally germinated on a medium containing the indicated AFB_1 concentration, before and following incubation for 1 week on an unadulterated maize embryo germination medium. Initially, in the low dose range, 2 μg ml^{-1} AFB_1-treated shoots were significantly shorter than those of control material. However, following a week without AFB_1, this inhibitory effect had been overcome (Fig. 2a).

In the high dose range, the initial stepwise inhibition of shoot lengths that was recorded following 9 day incubation with AFB_1 was overcome once exogenous toxin was no longer suppied (Fig. 2b). Following 1 week on a toxin-free medium, no statistically significant differences were found between material initially grown at any of the AFB_1 concentrations and controls. Roots for all treatments (high and low dose ranges), showed little or no regrowth over the week of incubation following toxin removal (data not shown). If shoot growth, following 1 week without a toxin source, is measured as a percentage increase over the initial starting value (i.e., value after 9 day incubation with AFB_1), then the difference between high and low dose treatments is exacerbated (Fig. 3). In the low dose range, no significant differences were observed (Fig. 3). In the high dose range, however, the higher the initial toxin concentration tc which the embryos were exposed, the greater the recovery, as measured by the increase in shoot length (Fig. 3).

While germination (assessed as the emergence of the radicle) was 100% for all treatments, the rate of root and shoot elongation was markedly slower with increasing toxin concentrations above 2 μg ml^{-1} (Figs. 4a, 4b, 5a, and 5b). Figures 4a and 5a demonstrate the similar trend of minimal effects for both root and shoot elongation at all concentrations in the low toxin dose range. On the other hand, the concentrations in the high dose range retarded the growth of both roots (Fig. 4b) and shoots (Fig. 5b). For root tissue, while initial growth showed only slight retardation, any inhibition manifested between 2 and 6 days was exacerbated between 6 and 9 days (Fig. 4b); at all the higher concentrations, little growth was observed during this latter 3 day period. The inhibition of elongation of shoots followed a less obvious trend, with some degree of recovery apparent between 6 and 9 days (Fig. 5b).

Fresh Mass and Percentage Dry Mass of Seedlings

Following 9 days of incubation in the presence of AFB_1, in the low dose range, the fresh mass of only the 2 μg ml^{-1}-treated material was significantly lower than that of the control seedlings (Fig. 6a). No differences were observed between toxin treatments. Following 4 weeks in potting soil without AFB_1, no differences between any treatments were recorded.

In the high dose range, the fresh mass of seedlings from all treatments was significantly different from control material (Fig. 6b). The lowest (5 μg ml^{-1}) and the highest (25 μg ml^{-1}) concentrations also differed significantly. However, once potted without a toxin source, seedlings recovered and after the 4 week period, all groups exhibited similar fresh mass values.

Figure 7a demonstrates that no differences in the dry mass of seedlings occurred for the low concentrations, either at 9 days or after 4 weeks. In the high

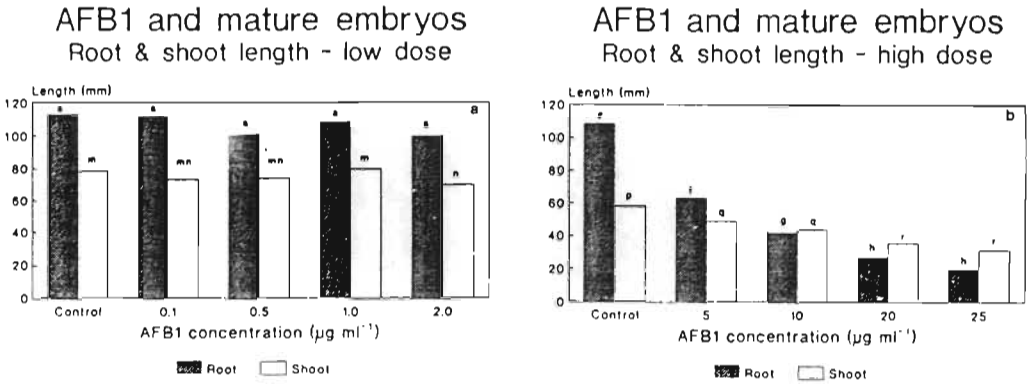

Figure 1. Mean root and shoot length of mature maize seeds treated with aflatoxin B_1 for 9 days.
Fig. 1a. Low dose range (0.1 - 2.0 μg ml^{-1} AFB_1).
Fig. 1b. High dose range (5.0 - 25.0 μg ml^{-1} AFB_1).
a,e,m,p, etc. Differing letters for a measured parameter indicate a significant difference (LSD, $p \leq 0.05$).

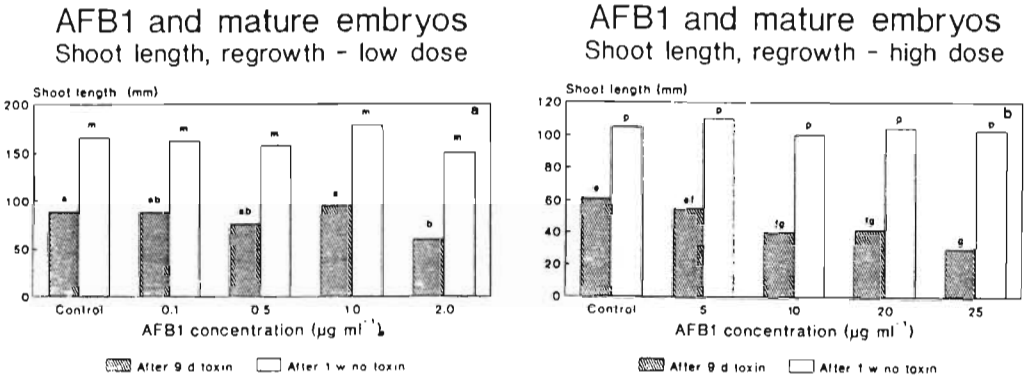

Figure 2. Mean shoot length for aflatoxin B_1-treated seedlings following 1 week regrowth on a toxin-free medium. Seedlings had previously been exposed to aflatoxin B_1 for 9 days prior to the regrowth period.
Fig. 2a. Low dose range (0.1 - 2.0 μg ml^{-1}).
Fig. 2b. High dose range (5.0 - 25.0 μg ml^{-1}).
a,e,m,p, etc. Differing letters for a measured parameter indicate a significant difference (LSD, $p \leq 0.05$).

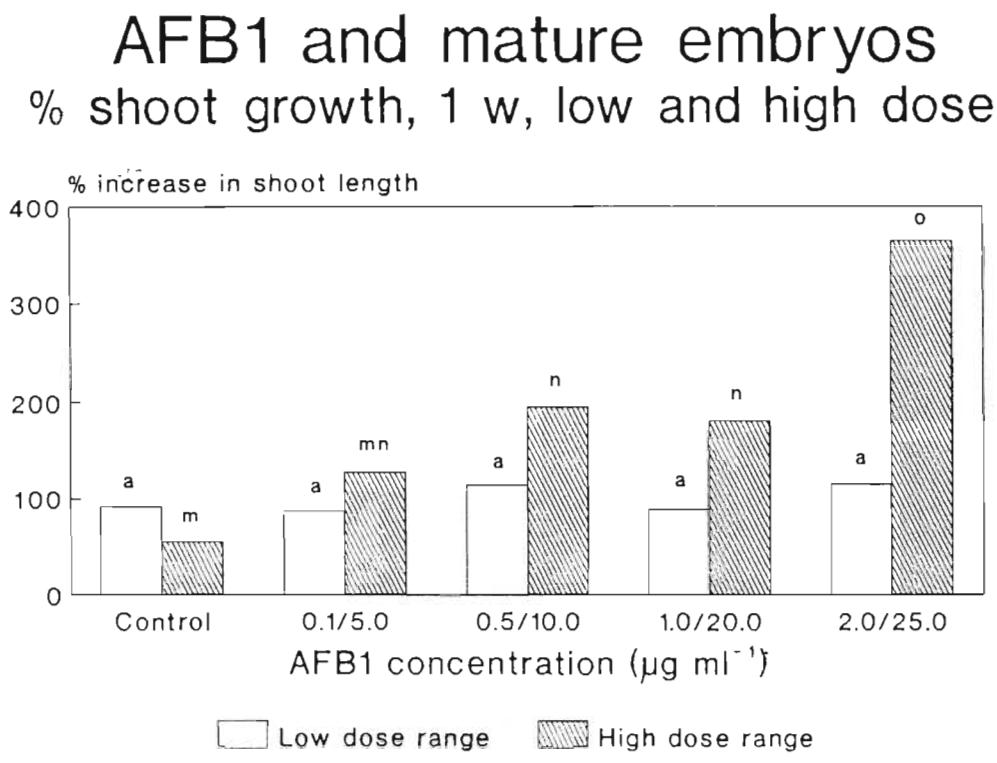

Figure 3. Percentage increase in shoot length following 1 week regrowth on a toxin-free medium (low and high dose ranges). Seedlings had previously been exposed to aflatoxin B_1 for 9 days.
[a,m, etc.] Differing letters for a measured parameter indicate a significant difference (LSD, $p \leq 0.05$).

dose range, dry mass of *in vitro*-grown seedlings increased as the toxin concentration was raised (Fig. 7b). However, once potted under normal conditions, without any toxin, these dry mass values were similar for all treatments.

Aflatoxin B_1 Extraction

Aflatoxin B_1 was isolated from the roots and the transition zone tissue of 9 day old seedlings treated with 5-25 μg ml^{-1} AFB_1. Additionally, AFB_1 was extracted from the shoot tissue of 20 and 25 μg ml^{-1}-treated material. In the low dose range, however, toxin could be detected only in the transition zone tissue of 1 and 2 μg ml^{-1}-treated material, although AFB_1 persisted in the agar after 9 days of incubation. Following 4 weeks of growth without AFB_1, this toxin was still isolated from the roots of 5-25 μg ml^{-1}, but not from any shoot tissue. No other metabolites of AFB_1 could be visualized under UV illumination.

DISCUSSION

Researchers have reported on the phytotoxicity of the mycotoxins known to be potentially hazardous to man and other animals. Toxins investigated include

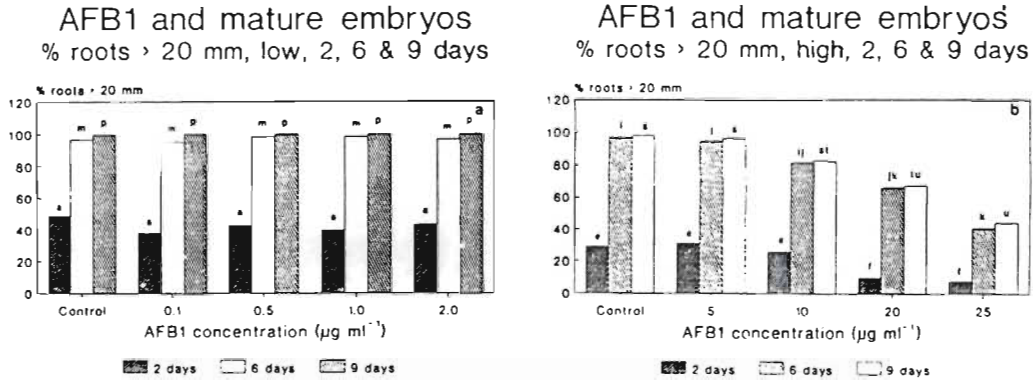

Figure 4. Percentage roots attained a length in excess of 20 mm at 2, 6, and 9 days incubation in the presence of aflatoxin B_1.
Fig. 4a. Low dose range (0.1 - 2.0 μg ml^{-1}).
Fig. 4b. High dose range (5.0 - 25.0 μ ml^{-1}).
a,e,m,p. etc. Differing letters for a measured parameter indicate a significant difference (LSD, $p \leq 0.05$).

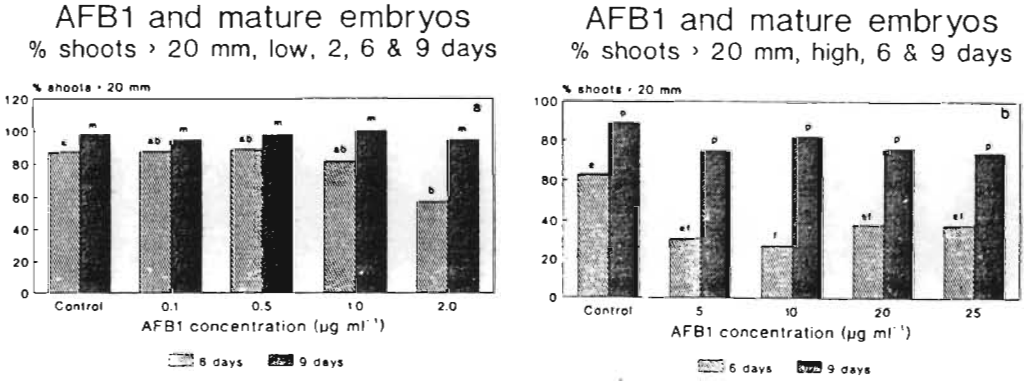

Figure 5. Percentage shoots attaining a length in excess of 20 mm following 6 and 9 days indubation in the presence of aflatoxin B_1.
Fig. 5a. Low dose range (0.1 - 2.0 μg ml^{-1}).
Fig. 5b. High dose range (5.0 - 25.0 μg ml^{-1}).
a,e,m,p. etc. Differing letters for a measured parameter indicate a significant difference (LSD, $p \leq 0.05$).

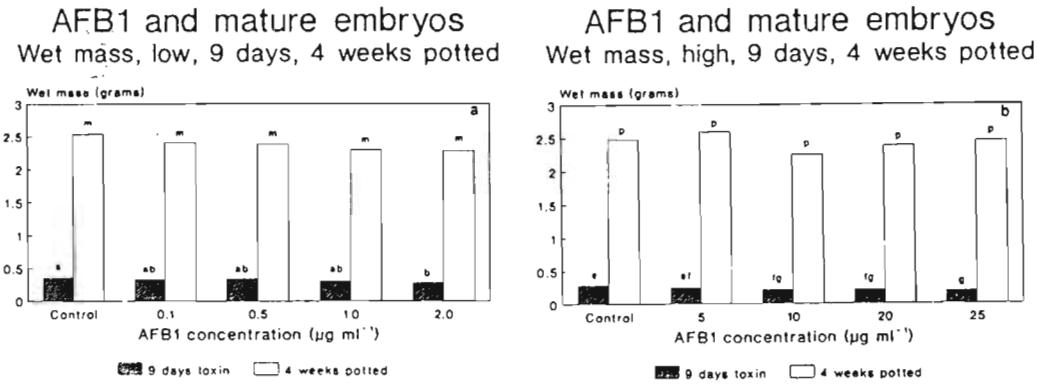

Figure 6. Wet mass of seedlings following 9 days incubation aflatoxin B_1 and following 4 weeks potted without a toxin source.
Fig. 6a. Low dose range (0.1 - 2.0 μg ml^{-1}).
Fig. 6b. High dose range (5.0 - 25.0 μg ml^{-1}).
a,e,m,p., etc. Differing letters for a measured parameter indicate a significant difference (LSD, $p \leq 0.05$).

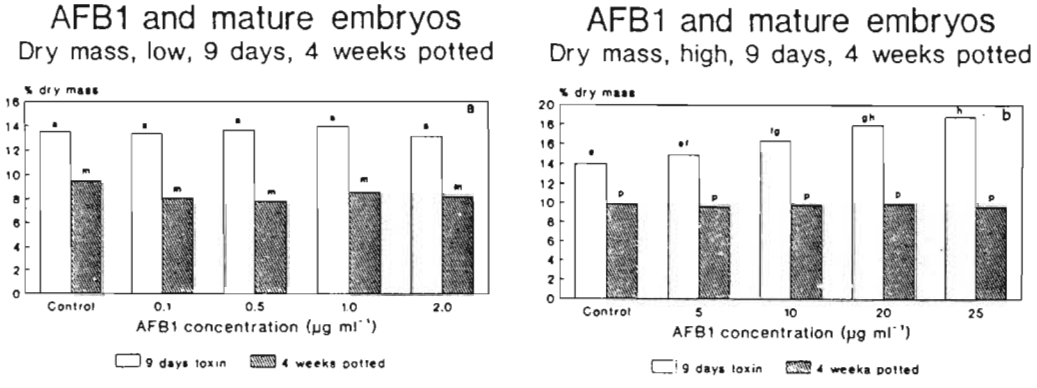

Figure 7. Percentage dry mass of *Zea mays* seedlings treated with aflatoxin B_1 for 9 days and following 4 weeks without a toxin source.
Fig. 7a. Low dose range (0.1 - 2.0 μg ml^{-1}).
Fig. 7b. High dose range (5.0 - 25.0 μg ml^{-1}).
a,e,m,p. etc. Differing letters for a measured parameter indicate a significant difference (LSD, $p \leq 0.05$).

patulin (Ellis and McCalla, 1973), citrinin (Damodaran et al., 1975), penicillic acid (Sassa et al., 1971), and fumonisin B_1 (van Asch, 1990). However, the phytotoxic effects of AFB_1 have received considerably more attention than any of the other mycotoxins (Schoental and White, 1965; Asahi et al., 1969, Truelove et al., 1970; Crisan, 1973a,b; Brodnik et al., 1978; Young et al., 1978; Misra and Tripathi, 1980; Llewellyn et al., 1982; Dashek and Llewellyn, 1983; Chatterjee, 1988; McLean et al., 1989, 1990a,b). Most of those authors have reported on the toxin-associated effects on germination, root and shoot growth, and protein and RNA synthesis. Only a few investigtors have attempted to examine the ultimate fate of AFB_1 once it has been assimilated into the plant tissue (Mertz et al., 1980; Reiss, 1984; Walker et al., 1985; Howes et al., 1991), although there is agreement that AFB_1 is absorbed by the plant.

In the present investigation, AFB_1 could not only be isolated from the roots, but also the transition zone tissue and the shoots of germinating embryos exposed to 20 and 25 μg ml^{-1} AFB_1 for 9 days. This suggests that the toxin is taken up by the roots and translocated to aerial plant parts. Following a period of growth (4 weeks) in the absence of AFB_1, the toxin could not be detected in the shoots by TLC, although it was still isolated from the roots. It would appear, then, that the shoot tissue of *Zea mays* seedlings is capable of "metabolizing" AFB_1. Whether this involves a metabolic degradation or inactivation is not known. Since shoot growth had normalized after 9 days, even at the highest concentration, and no metabolites of AFB_1 were detected on the TLC plates, inactivation of the toxin seems more probable. After 9 days of incubation *in vitro*, at 5 and 10 μg ml^{-1} AFB_1, the toxin could be isolated only from root and transition zone tissue. In the low dose range (0.1 - 2.0 μg ml^{-1}), however, toxin could be detected only in the transition zone tissues and not from the shoots of 2.0 μg ml^{-1}-treated seedlings. More sensitive techniques such as HPLC are required to confirm and perhaps extend these preliminary findings, as low concentrations of AFB_1 and its metabolites may be beyond the detection of TLC. If, however, the present indications are confirmed, then the nature of the mechanism of toxin metabolism can be narrowed to a process (and possibly an organelle type) that occurs in shoot tissue only.

Mertz et al. (1980) found that 2 days after transfer to an AFB_1-free solution, treated maize seedlings exhibited a 75% and a 50% reduction in toxin concentration in root and in stem-leaf tissue, respectively. Following transfer to soil for 13 days, these already lowered levels were further reduced by 80% and 86%, respectively.

Despite the attenuation of AFB_1 levels described by Mertz et al. (1980), and the results reported presently, the evidence suggests that the toxin is translocated. In terms of the evidence of Mycock et al. (1992), that the fungus (*A. flavus*) itself will persist and move through the growing plant, a continuing source of AFB_1 could well be available throughout the life cycle. Since AFB_1 is taken up and translocated, there are important consequences regarding the next seed generation. The results of Mertz et al. (1980), who injected ^{14}C-labelled AFB_1 into an incision in the stem subtending the developing ear 14 days after pollination, support this observation. Thirty days later, while endosperm tissue had only traces of toxin, embryos and pericarps contained detectable levels of AFB_1.

These findings have important

repercussions as regards agricultural practices. Not only could developing plants be exposed to AFB_1 contamination systemically or by airborne *Aspergillus flavus* or *A. parasiticus* inoculum, but AFB_1 can be absorbed from the soil and translocated through the plant. If contaminated plant parts are returned to the soil, a farming practice which is quite common, then it is possible that AFB_1 may be absorbed by the root system and translocated to the foliage, and perhaps even the developing caryopses. In 1977, in the southeastern United States, the corn crop was severely contaminated with aflatoxin and many fields were destroyed by ploughing the stover and grains back into the soil (McMillan et al., 1978). In view of the present observations and those of Mertz et al. (1980), this action may retrospectively be viewed as unsound. Not only would such practices impose a potential health hazard as the soil levels of AFB_1 would be elevated, but growth and development, and hence productivity of the subsequent crop may be jeopardized.

In the present study, AFB_1 could still be isolated from roots a month after removal of the toxin source, suggesting that some of the toxin may be "bound" in the root tissue. The precise nature of such an interaction is unknown. In animal systems, AFB_1 forms adducts with the DNA, preferentially at the guanine residues (Benasutti et al., 1988). Extracted DNA from maize seedlings was found to bind AFB_1, as measured by equilibrium dialysis, difference spectrophotometry and viscosity determination (Tripathi and Misra, 1981). This suggests that a similar mechanism may operate in both plant and animal tissues. Other workers have found that the mitochondrial DNA of rat liver has a three to four-fold greater affinity for AFB_1 than does nuclear DNA (Niranjan et al., 1982). Similarly, Asahi et al. (1969) found that exposure of root slices of sweet potato to AFB_1 resulted in a preferential inhibition of mitochondrial DNA replication, without apparently interfering with mitochondrial enzyme activities. Those authors expressed the opinion that in sweet potato root slices, AFB_1 inhibited replication of both mitochondrial and nuclear DNA, without having any effect on cytoplasmic or mitochondrial protein synthesis. With regard to cytomatrical ribosomal function, the opinion of Asahi et al. (1969) is in direct contradiction to that of Black and Altschul (1965) who were of the opinion that their data supported aflatoxin as an inhibitor of protein synthesis.

Additional binding sites, such as that of the plasma membrane and/or the nuclear envelope should not be excluded. For example, in sugar cane cells, helminthosporoside, a host-specific toxin produced by *Helminthosporium sacchari*, was found to bind to membrane protein (Strobel and Hess, 1974), although the results of this investigation have been disputed (see Scheffer and Livingston, 1984).

The ultimate fate of AFB_1 in the plant is controversial, as there are conflicting reports regarding the occurrence, or lack of degradation products of AFB_1. A number of researchers have not isolated any breakdown products of AFB_1 in the plant systems investigted. Mertz et al. (1980), finding a decrease in toxin levels, assumed a metabolic degradation, although no fluorescing compounds except AFB_1 were detected. Similarly, Reiss (1984) failed to detect any other mycotoxins in AFB_1-treated peas and wheat kernels. Howes et al. (1991), however, found that in root, stem, and leaf tissue of AFB_1-treated parsley seedlings, aflatoxicol A was extracted. On feeding plants with aflatoxicol A, AFB_1 was detected, indicating that the reaction is reversible. Those

authors suggest that the enzyme responsible, probably a dehydrogenase, is found throughout the plant. The disparity in the findings of Howes et al. (1991) and those of Mertz et al. (1980) and the present investigation suggests that more sensitive methods are required for detection of metabolic products, and/or that plant species may differ in their ability to metabolize AFB_1.

Using cultured, excised soybean roots, treated with exogenously applied AFB_1, Walker et al. (1985) found that the bulk fo the toxin was recovered from the 80,000 g fraction (microsome fraction). In animal systems, activity of microsomal enzymes results in the metabolism of AFB_1 to AFB_{2a}, AFP_1, AFM_1, AFQ_1, and AFB_1 epoxide (Heathcote and Hibbert, 1978; Hsieh, 1987). AFB_1 epoxide appears to be the active carcinogenic form of AFB_1 (Busby and Wogan, 1984) [see Cole and Cox (1981) for chemical structures]. Assessment of whether plant microsomes, especially in the root tissue, are capable of metabolizing AFB_1 to its carcinogenic form requires serious consideration because of the potential health risk it poses to both man and his stock animals, especially where root crops are involved. Further studies using the more sensitive HPLC techniques will be carried out to ascertain whether this (or any other) metabolite does accumulate in plant tissues, especially when a continuing source of AFB_1 is supplied.

The morphometric results of the present investigation indicate that a continuing supply of AFB_1 will inhibit the growth of plant tissues. While germination (assessed as the emergence of the radicle) was unaffected for all treatments, mean root and shoot lengths and the rate of elongation of these plant parts were inhibited by toxin concentrations above 1 $\mu g\ ml^{-1}$. The findings of other workers suggest that there may be differing susceptibilities of various plant species to the influence of AFB_1. In 11 cruciferous species, following exposure to 100 $\mu g\ ml^{-1}$ AFB_1, inhibition of root elongation ranged from 22% to 91%, while for shoot elongation, this inhibition ranged from 29% to 93% (Crisan, 1973b). The same concentration, however, severely inhibited the germination of *Lepidium sativum*, another crucifer (Schoental and White, 1965). Those authors reported that germination was reduced from 65% of 25 $\mu g\ ml^{-1}$ to zero at 100 $\mu g\ ml^{-1}$. Dashek and Llewellyn (1983) expressed the opinion that inhibition of germination of most seeds might require relatively high concentrations of AFB_1.

In the present study, while initial radicle protrusion was unaffected, elongation of shoots and particularly roots to lengths in excess of 20 mm was severely inhibited above concentrations of 2 $\mu g\ ml^{-1}$. An AFB_1-induced inhibition of root elongation has been reported for *Pimpinella anisum* (71% suppression of root elongation at 2.5 $\mu g\ ml^{-1}$ and 82% inhibition at 5 $\mu g\ ml^{-1}$) (Llewellyn et al., 1982) and for *Lepidium sativum* (91% and 93% inhibition of root and hypocotyl extension, respectively, at 100 $\mu g\ ml^{-1}$ (Crisan, 1973a). It would appear that while initial germination and elongation processes are relatively unaffected by the toxin, a stage is reached at which suppression of growth occurs. Perhaps this stage reflects a primary effect, and an inability for new products required for continued growth to be synthesized, or, as a secondary effect, the inhibition of uptake of nutrients from the medium as a result of the accumulation or presence of AFB_1 in root tissue. Preliminary results, using [^{3}H] sucrose, suggest that uptake by germinating embryos may be impaired by 25 $\mu g\ ml^{-1}$ AFB_1 during the initial 72 h or germination. Additionally, formation of some toxic metabolite(s) cannot be ruled out at this stage.

In the present investigation, if seedlings removed from AFB_1-containing medium were allowed to grow for a week without exogenous toxin, all original suppression of shoot elongation that was observed, even at the highest toxin concentration (25 $\mu g\ ml^{-1}$), was overcome. This suggests that the presence of the toxin has a reversible inhibitory effect on the shoots, but does not indicate whether this is a primary or a secondary effect. Withdrawal of the toxin may allow nutrients to be absorbed by the root system, or overcome any temporary blockage of anabolic processes that might have occurred in the presence of AFB_1. In this regard, Schoental and White (1965) found that concentrations of AFB_1 as low as 2.5 $\mu g\ ml^{-1}$ could induce albinism (chlorophyll deficiency) in *Lepidium sativum* seedlings. Crisan (1973a), on the other hand, found no chlorosis in seedlings of lettuce treated with 100 $\mu g\ ml^{-1}$ AFB_1. This again suggests differences in species susceptibility to AFB_1.

There are several reports on the inhibition of DNA, RNA and/or protein synthesis resulting from exposure of plant tissues to AFB_1 (Asahi et al., 1969; Truelove et al., 1970; Young et al., 1978). Tripathi and Misra (1981) reported that AFB_1 treatment of germinating maize seeds resulted in the suppression of RNA, protein and DNA synthesis, in that order. Furthermore, at concentrations below which these differences were recorded, AFB_1 was found to inhibit chromatin-bound DNA-dependent RNA polymerase activity (Tripathi and Misra, 1981). From these observations, there does appear to be some consistency with the findings for animal systems. In this regard, using purified RNA polymerase from rat liver, Yu (1977) has demonstrated that RNA polymerase II is preferentially inhibited, probably directly, and that RNA polymerase I inhibition results from impairment of template function. Earlier, Kunimoto et al. (1974) found that in HeLa or LS1787 cells treated with AFB_1, there was impaired nucleoside and nucleotide uptake.

Misra and Tripathi (1980) and Chatterjee (1988) have reported that in maize, AFB_1 inhibits α-amylase activity, thereby preventing the hydrolysis of starch to sucrose. Similarly, Black and Altschul (1965) found that a mixture of aflatoxin B and G suppressed gibberellic acid-induced α-amylase release in barley seeds. Production of α-amylase in imbibing or germinating seeds requires *de novo* synthesis of mRNA (Chandra and Varner, 1965). If AFB_1 binds to DNA and impairs template function, the inhibition of α-amylase synthesis would be inevitable. In this regard, Chatterjee (1988) found that exogenously applied sucrose or α-amylase could overcome such inhibition. In the normal situation in germinating peanuts, Singh et al. (1974) found that free reducing sugars increased up to 96 h of germination and then remained constant. With increasing AFB_1 concentration, however, the levels of free-reducing sugars decreased. It was suggested as early as 1965 that the aflatoxins might be involved at the level of DNA. Lilley (1965) noted significant increase in the number of abnormal anaphases and an inhibition of mitosis following treatment of *Vicia faba* roots with a mixture of aflatoxins.

In the present investigation, fresh mass measurements (in the 5-25 $\mu g\ ml^{-1}$ concentration range) indicated a temporary inhibitory effect correlated with the presence of AFB_1. However, once the toxin was removed, the seedlings recovered, and after 4 weeks, no significant differences in fresh mass were observed. These results are in agreement with the growth trend exhibited by the shoots following toxin

removal. However, they still do not elucidate whether the inhibition results from the physical presence of the toxin (intracellular binding and inhibition) or as a result of impaired uptake of nutrients. The significant increase in dry mass with increasing AFB_1 concentration (in the high dose range) exhibited over the first 9 days of growth is surprising. However, it might well attest to the abnormal accumulation of some tissue/cell component. Correlative electron microscopy, which is currently underway, may resolve the present unexpected anomaly.

Aflatoxin B_1, up to 25 μg ml^{-1}, has no lethal effects on excised mature maize embryos. In the presence of the toxin, both root and shoot elongation are inhibited by doses of 2 μg ml^{-1} and more. Concomitantly, fresh mass values showed a similar declining trend. Removal of the toxin allowed the stunting to be overcome, implying a temporary inhibition. However, higher AFB_1 concentrations can result in severe decreases in germination in some seeds (Schoental and White, 1965). In such instances, irreversible deleterious alterations are likely to be induced, such that no recovery or repair is possible as long as the toxin persists. There are, however, divergent observations relating viability to toxin concentration.

Aflatoxin B_1 may thus be metabolized differently (mechanism; time) by different seed species, and even by different cultivars, leading to the proposal that the mechanism involved in loss of viability resulting from exposure to AFB_1 may depend on the seed type (Harman and Pfleger, 1974).

From these results and those of other workers, there is increasing evidence that a number of mycotoxins may also be regarded as phytotoxins. As such, their status in plant pathology needs to be investigated, particularly considering the ubiquitous nature of many of these mycotoxin-producing fungi.

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Effects of aflatoxin B_1 on in vitro cultures of *Nicotiana tabacum* var. Samsun

1: Callus growth and differentiation

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Received 3 May 1993; accepted in revised form 12 October 1993

Abstract. Calli of *Nicotiana tabacum* (tobacco) were treated with two dose ranges of aflatoxin B_1 (0.1–2.0 μg ml^{-1} – low dose; 5–25 μg ml^{-1} aflatoxin B_1). The ability of calli to recover following 3 weeks of toxin exposure was also investigated. The I_{50} (50% inhibition) value for fresh mass accumulation was approximately 2 μg ml^{-1} AFB_1. Fresh mass accumulation was significantly lower than the control value from 0.5 μg ml^{-1} AFB_1. Following 3 weeks growth without a toxin source, the growth of calli up to and including 10 μg ml^{-1} AFB_1, was significantly greater than control calli, indicating reversibility of the toxic effects. With increasing toxin concentration, chlorophyll content of callus was inhibited from 0.5 μg ml^{-1}. Transfer to a toxin-free medium resulted in a degree of recovery (up to 0.5 μg ml^{-1}). In the dose range 5–25 μg ml^{-1}, the levels of chlorophyll were drastically reduced, with no recovery following AFB_1 removal. Electron microscopy revealed a disruption of chloroplast structure as an early deteriorative event in AFB_1 exposure of callus cells. Protein levels were less sensitive, with inhibition manifested only in the high dose range. Shoot development occurred at all concentrations, but was significantly inhibited from 5 μg ml^{-1} AFB_1. Recovery following toxin removal was minimal at these higher AFB_1 concentrations. The number of necrotic calli increased progressively from 5 μg ml^{-1} as toxin levels increased.

Key words: Aflatoxin B_1, Callus, Differentiation, Electron microscopy, Organogenesis, Tobacco

Introduction

The aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus*. Aflatoxin B_1 (AFB_1) has been found to be carcinogenic, mutagenic, teratogenic and toxic to animals, including man [1]. The phytotoxic effects of AFB_1 have been the subject of numerous investigations [2–16] but, with few exceptions [6; 12–16], those studies have focused on toxin-associated effects on germination and root and shoot growth. Furthermore, the molecular and physiological bases of the detrimental effects of the toxin at both cellular and whole plant level are poorly understood, although for animal tissue there is some consensus that the mode of action of aflatoxin involves metabolic activation by microsomes [see 17].

Plant tissue culture procedures have progressed to a level that most species can be regenerated in vitro. Large numbers of cultures can be produced in the laboratory with morphogenetic development being strictly controlled by manipulations of the nutrient media and other environmental parameters. Consequently, the effect of various types of stresses at successive stages of organ differentiation may be investigated. This is important as expression of traits at the organ and whole

plant levels is not necessarily the same as at the level of undifferentiated cells [18]). The production and culture of undifferentiated cell masses (callus) also permit the mechanism of action of a compound, introduced into the culture medium, to be studied independently of the process of translocation. Much of the fundamental research on the control of plant cell differentiation and plantlet establishment has been performed on *Nicotiana tabacum*, mainly as a result of the ease with which tobacco can be manipulated in culture.

Hence, in vitro cultures, such as the organogenic tobacco callus used in this study, provide appropriate model experimental systems for investigations on the effects of AFB_1 (and other natural toxins) at the anatomical, morphological, biochemical and genetic levels, as well as in the elucidation of possible relationships between susceptibility to the toxin, cellular differentiation and tissue organisation. Such information could prove valuable for the possible future development of in vitro selection of AFB_1-tolerant cell lines.

Materials and methods

Plant material and growth conditions. *Nicotiana tabacum* var. Samsun plants were grown from seeds in vermiculite and watered every second day with Long Ashton nutrient solution [19]. The plants were maintained in a greenhouse, under natural diurnal conditions, and were discarded before flower initiation.

Callus initiation and plantlet regeneration. Young leaves from the plant apex were surfaced-sterilised in 1% Hibiscrub (50 mg ml^{-1} chlorohexidine, ICI Pharmaceuticals) for 10 min, followed by immersion in 1% sodium hypochlorite for 15 min. After thorough rinsing in sterile, deionised water, the leaves were cut into small pieces (approximately 2 × 2 mm) and plated onto a callus induction medium containing MS nutrients [20] (2% sucrose, 2.3 μM kinetin, 11 μM IAA, and 1% agar, pH 5.8). The leaf discs were incubated in the dark at 25 ± 3 °C for 2 weeks. At the end of this period, the calli produced on the edges of the leaf explants were excised and subcultured onto fresh medium. These callus cultures were maintained at a 16 h photoperiod, 200 μmoles m^{-2} sec^{-1} photon flux density and 25 ± 3 °C, for 10 to 14 days, after which they were transferred to an aflatoxin-containing medium.

Aflatoxin B_1 treatments. A range of aflatoxin B_1 (CSIR, SA) concentrations (0.1–25 μg ml^{-1}) was prepared from a stock solution and added to autoclaved medium (MS, 2% sucrose, 1% agar, pH 5.8). The control calli were grown on the following media: no DMSO, and DMSO concentrations equivalent to those found in the lowest and highest AFB_1 concentration for each dose range [i.e. DMSO concentration in 0.1 (0.004% DMSO) and 2 (0.08% DMSO) μg ml^{-1} (low dose range) and 5 (0.2% DMSO) and 25 (1% DMSO) μg ml^{-1} (high dose range) AFB_1]. Individual experimental calli were weighed, transferred to aflatoxin-containing medium and incubated for 3 weeks, under a 16 h photoperiod, at 200 μmoles m^{-2} sec^{-1} photon flux density and 25 ± 3 °C. After the 3-week exposure to the toxin, the calli were placed on an AFB_1-free medium for a further 3 weeks.

Growth measurements. Callus fresh mass was assessed on a weekly basis, and protein and chlorophyll content determined at the end of the period of exposure to the toxin, and after subsequent growth on toxin-free medium. The incidence of shoot production and the development of necrotic calli were recorded. Protein was extracted as recommended by Wetter [21] and assayed by the Folin-Lowry method [22]. Chlorophyll determinations were performed according to Bruinsma [23].

Electron microscopy of cultured material. Small pieces of callus were immersed in Karnovsky's fixative (pH 6.0) at room temperature overnight, washed in 0.2 M sodium cacodylate buffer (pH 6.0) and post-fixed in 1% aqueous OsO_4 for 2 h

at 4 °C in the dark. Tissue was then dehydrated in a graded acetone series. During dehydration in 75% acetone, the material was block-stained for 1 h (4 °C, dark) with saturated uranyl acetate. The material was then immersed in a 50:50 acetone:epoxy (Spurr) resin mixture [24] overnight, followed by infiltration with pure epoxy resin (8–12 h) and polymerised in fresh resin (8–12 h at 70 °C). Sections were stained for 10 min with 2% uranyl acetate, post-stained with lead citrate [25] for 30 min, and viewed with either a Zeiss EM10B or a JOEL 100C transmission electron microscope at an accelerating voltage range of 60–100 kV.

Statistics. Results were assessed using one-way analysis of variance (ANOVA) (LSD, $p \leq 0.05$). Sets of data were compared with the appropriate DMSO controls. In the figures, values sharing the same alphabetical character for any measured parameter are not significantly different.

Results

Callus growth, chlorophyll and protein content

The growth responses of tobacco calli, incubated in the presence and absence of AFB_1, can be seen in Fig. 1. The two different controls correspond to the levels of DMSO present in the low (0.1 and 2 $\mu g\ ml^{-1}$ = control 1) and high (5 and 25 $\mu g\ ml^{-1}$ = control 2) dose ranges of AFB_1 tested. Uptake of AFB_1 into the callus cells was confirmed using TLC (results not shown).

It is evident from the data that, after three weeks in culture, suppression of growth was directly related to the concentration of aflatoxin supplied in the medium (Figs 1 & 2A). The I_{50} value (50% inhibition) for fresh mass increase was found to occur at approximately 2 $\mu g\ ml^{-1}$ AFB_1 (Fig. 2A). Thereafter, incremental toxin levels resulted in increasingly greater numbers of calli that turned brown, a feature usually representative of senescence and death (Fig. 2B). At the highest concentration of AFB_1 tested (25 $\mu g\ ml^{-1}$), fresh mass accumulation was inhibited by approximately 94%, and 90% of the calli appeared dead (brown and gelatinous). It was also evident that fresh mass accumulation was inhibited slightly by the levels of DMSO in control 2 (Fig. 1).

After 3 weeks of aflatoxin treatment, calli were transferred to an AFB_1-free medium for a further 3 weeks (Fig. 1). After this second incubation period, the calli serving as control 2 did not fully recover from the negative effects of the DMSO to which they had been subjected previously. However, growth repression by AFB_1 supply up to 10 $\mu g\ ml^{-1}$ was reversed after a 3 week incubation on toxin-free medium (Figs 1 & 2A). In contrast, calli which were apparently largely necrotic at the end of the aflatoxin treatment (10–25 $\mu g\ ml^{-1}$) did not recover (Fig. 2B).

Callus chlorophyll content was sensitive to the effects of AFB_1 as inhibition occurred even at the lowest dose of AFB_1 (0.1 $\mu g\ ml^{-1}$), but was only significantly different from the controls at 1 $\mu g\ ml^{-1}$ (Fig. 3A). The extent of the reduction in chlorophyll levels ranged from approximately 20% to 80% at AFB_1 concentrations of 0.1–0.5 $\mu g\ ml^{-1}$ and 20–25 $\mu g\ ml^{-1}$, respectively. Results obtained after removal of the stress (Fig. 3A) indicate that severe, irreversible damage, occurred following exposure to aflatoxin concentrations in excess of 2 $\mu g\ ml^{-1}$ only. Calli treated with 0.1–0.5 $\mu g\ ml^{-1}$ AFB_1 recovered well, with chlorophyll levels exceeding those of control values.

Protein levels did not differ significantly from the control up to the 10 $\mu g\ ml^{-1}$ AFB_1 treatment (Fig. 3B). However, above this concentration, callus protein content was drastically reduced. In such cases, subsequent transfer to AFB_1-free medium for 3 weeks did not reverse the effects of the toxin and total protein content of calli remained low.

Ultrastructural characteristics of callus cells

Callus cultures undergoing organogenesis exhibit some degree of heterogeneity as they comprise a

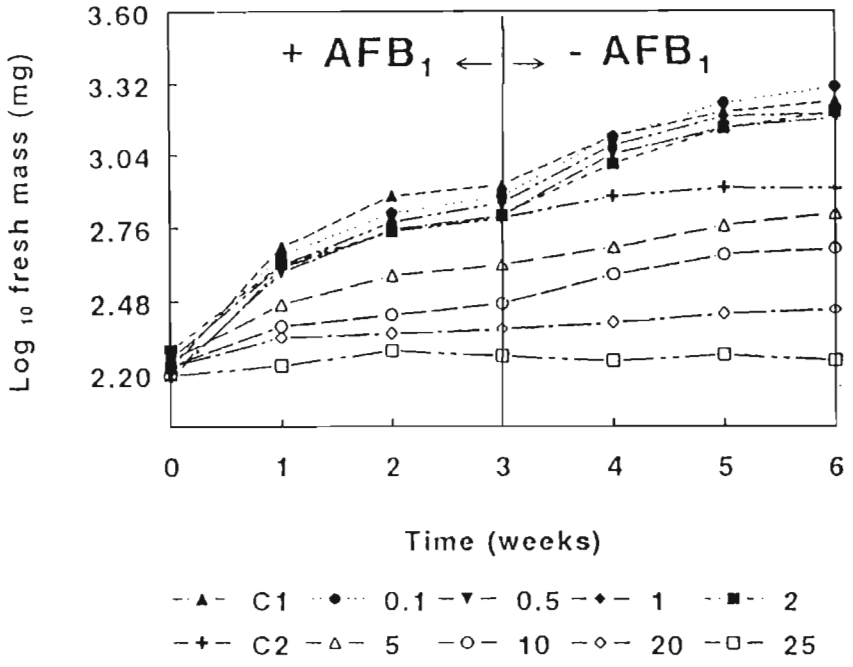

Fig. 1. Growth curves of calli cultured on a medium containing a range of AFB_1 concentrations (0.1–25 μg ml^{-1}) for 3 weeks and then subcultured onto a toxin-free medium for a further 3 weeks. Controls C1 and C2 correspond to the control calli for the low dose range (0.1–2.0 μg ml^{-1}) AFB_1 and the high dose range (5–25 μg ml^{-1}) AFB_1, respectively.

mass of cells at different developmental stages [26], viz. quiescent cells, those preparing for division and cells at various stages of differentiation. To minimise variability, callus material used for transmission electron microscopy was selected from areas, which appeared healthy and were devoid of necrosis. Cells of control calli (including DMSO controls) were found to be large, with a vacuole that occupied a major proportion of the cell volume (Figs 4 & 5). The cytoplasm almost invariably formed a relatively small peripheral rim, and appeared diffuse with ribosomes dispersed amongst the membrane-bound organelles. In some cells, the nucleus was spherical and central (Figs 4 & 5), while in others it tended to be flattened and peripheral (Fig. 5). In nuclei of control material, the chromatin had a regular appearance (Figs 4 & 5). Mitochondria generally appeared circular in profile (Figs 4 and 5), suggesting spherical and/or oval shapes, with well developed tubular cristae in an uniformly dense matrix. Both small, round proplastids and well developed chloroplasts were evident (Figs 4 & 5). Fully differentiated chloroplasts were generally characterised by the presence of large starch grains, which may be attributed to free availability of carbon and a high ratio of carbon to nitrogen in the growth medium.

Cells from calli grown in the presence of 0.1 and 0.5 μg ml^{-1} AFB_1 generally appeared ultrastructurally similar to those of the control. However, some cells exhibited apparently degenerating plastids and nuclei with an abnormal arrangement of the chromatin (Figs 6–8). These features became increasingly evident in cells treated with 1 and 2 μg ml^{-1} AFB_1 (Figs 9–13), although some ultrastructurally normal chloroplasts and mitochondria were still present (Figs 9 & 11). At these toxin concentrations, mitochondrial morphology varied from apparently normal (Fig. 11), with well developed cristae and a regular matrix, to deranged, with poorly developed cristae (Fig. 10) or only a few abnormally swollen cristae (Figs 12 & 13). Some plastids too, exhib-

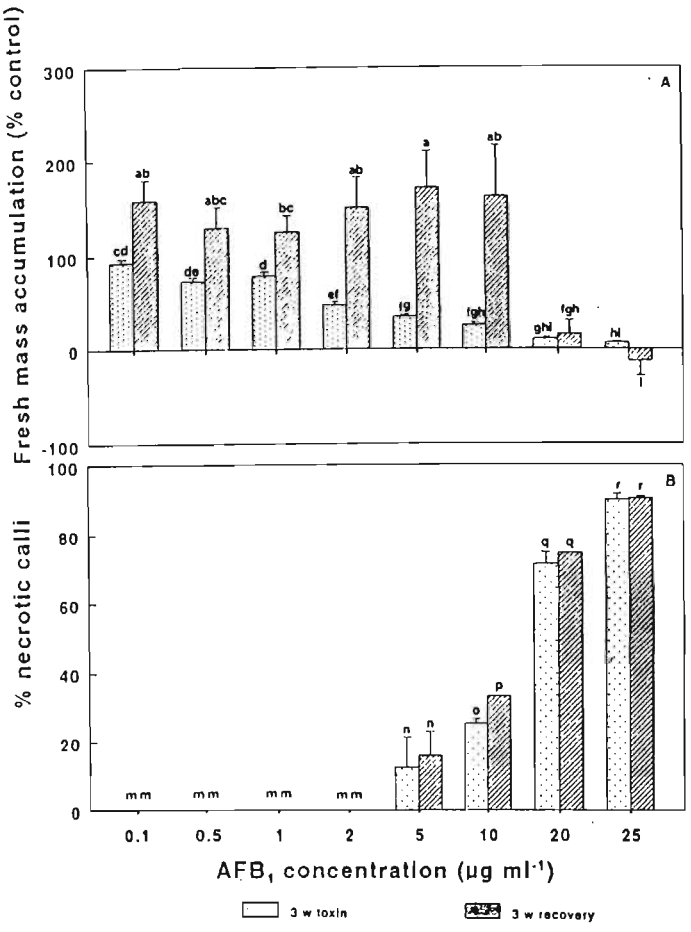

Fig. 2. The effect of AFB_1 on: (A) fresh mass accumulation, expressed as a percentage of the control value. Alphabetical letters have been assigned according to a multiple range test. Values for treated calli were significantly different from the control, except 0.1 (3 w toxin); 0.5 & 1 (3 w recovery); 5 and 10 µg ml^{-1} (3 w recovery); (B) the percentage of necrotic calli, following 3 week toxin exposure and then following a 3 week recovery period (no toxin). All controls have been assigned the letter 'm', according to a multiple range test, implying that only from 5 µg ml^{-1} AFB_1 were values significantly different from the controls.

ited deterioration (Fig. 13). Despite such organellar abnormalities, many polysomes were observed (Figs 9, 10 & 12).

In general, the deteriorative subcellular effects observed at the lower toxin levels became progressively exacerbated in callus cells subjected to the higher AFB_1 concentrations (5–25 µg ml^{-1}) (Figs 14–17). At these toxin levels, most of the chloroplasts were disrupted (Figs 14 & 15) and nuclear morphology was highly abnormal (Fig. 16). A characteristic feature of cells treated with 10 to 25 µg ml^{-1} AFB_1 was the presence of large numbers of lipid droplets (Figs 14 & 17), which were frequently apparently coalescing (Fig. 17).

Shoot regeneration via indirect organogenesis

After six weeks in culture (3 weeks exposure to toxin, followed by 3 weeks without a toxin source), control calli of *N. tabacum* exhibited a high degree of cellular and organ (shoot) differentiation (Fig. 18A). The extent of shoot formation and establishment appeared to be unaffected by either DMSO (Fig. 18A) or AFB_1 supplied

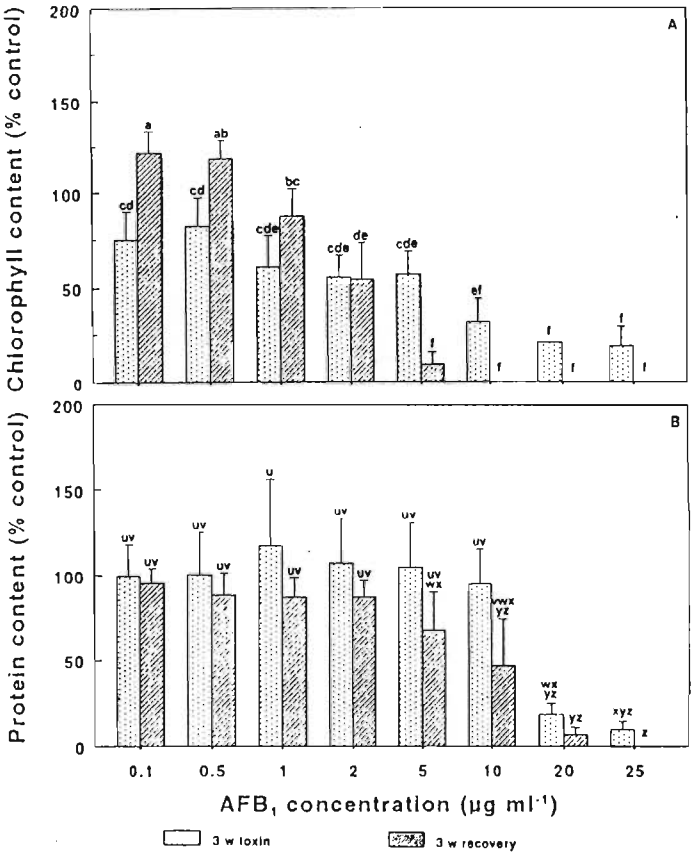

Fig. 3. The effect of AFB_1 on: (A) callus chlorophyll content, expressed as a percentage of the control. In the low dose range, following 3 w of toxin exposure, 1 and 2 µg ml $^{-1}$ were statistically different from the control. Following 3 w after toxin removal, only 2 µg ml^{-1} AFB_1-treated calli had significantly different chlorophyll levels. In the high dose range, all values (3 w toxin; 3 w recovery) were significantly lower than the control value; (B) protein content, expressed as a percentage of the appropriate control. Only 20 & 25 µg ml^{-1}-treated calli had protein levels significantly lower than the control. Following 3 w without a toxin source, significant differences were recorded from 10 µg ml^{-1} AFB_1.

in the 0.1–2 µg ml^{-1} range (not illustrated). In contrast, at the higher range of AFB_1 tested, shoot development (number of shoots per callus) was repressed from 5 µg ml^{-1}, even following a growth period of 3 weeks in the absence of toxin, as seen in Figure 18B. Similarly, the number of calli undergoing organogenesis was affected significantly by the AFB_1 treatments, only at concentrations greater than 5 µg ml^{-1} (data not presented). In the presence of 25 µg ml^{-1} AFB_1, only 14% of the calli produced shoots. The status at this concentration remained statistically unchanged upon transfer to medium devoid of toxin.

Discussion

It has been documented for a number of plant species that AFB_1 inhibits seed germination, as well as root and shoot elongation. Susceptibility to this toxin appears to vary, according to the plant species, plant organ, and the metabolic or growth process investigated [3–16; 39; 40; 42–44]. Seeds of a number of species, including several lettuce cultivars and some members of the Cruciferae, appear to tolerate AFB_1 concentrations of 100 µg ml^{-1} [5; 6], whereas in immature embryos of *Zea mays*, germinated in vitro, shoot and root elongation was severely repressed by 5 µg ml^{-1}

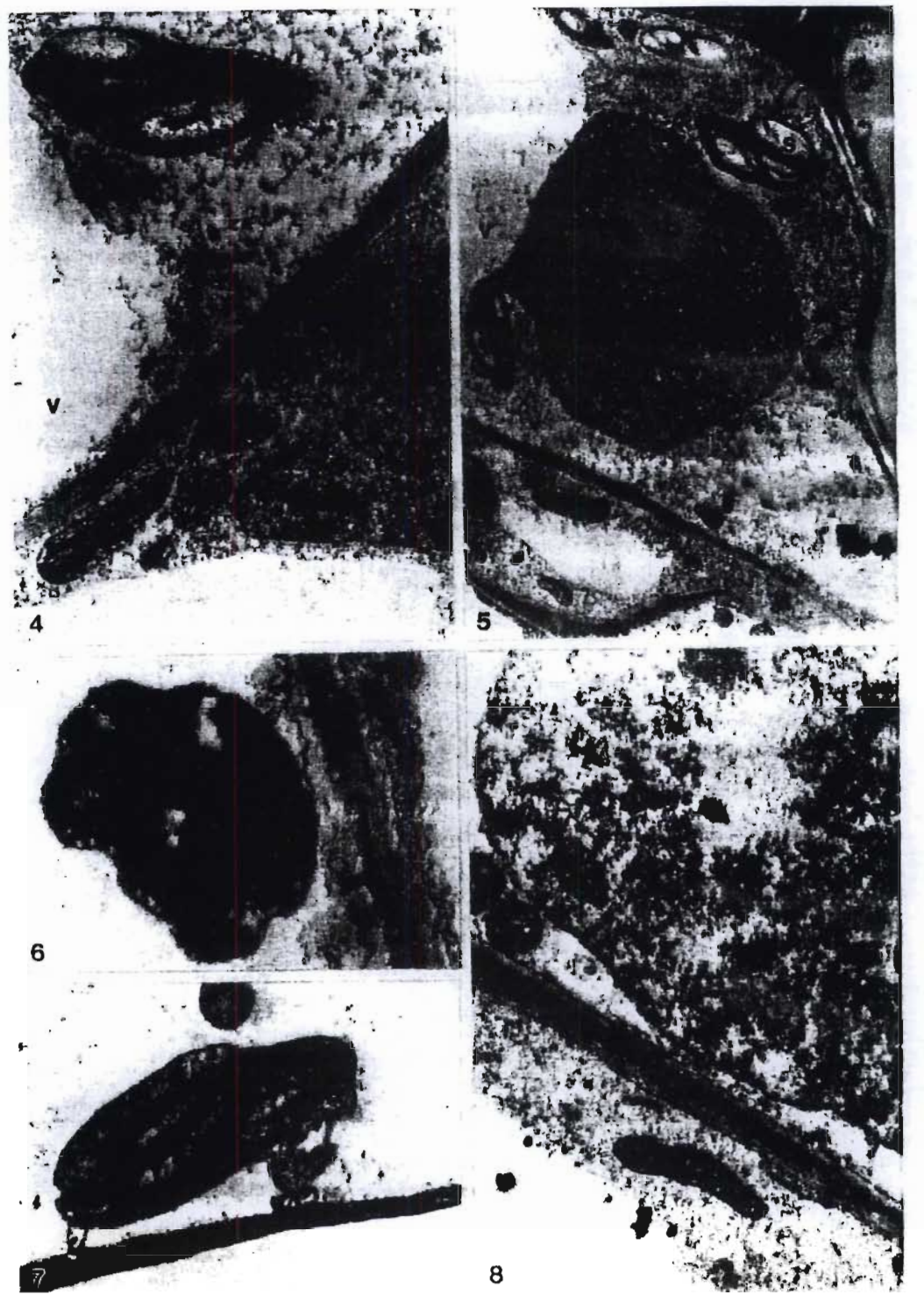

Plate I. *Figs 4 & 5.* Cells from control calli usually contained a large vacuole (v), mitochondria (m) with well-developed cristae, and chloroplasts (p) showing well developed grana and often containing starch (s) grains. Fig. 4. ×21 500; Fig. 5. ×6 500;
Figs 6 & 7. Chloroplasts exhibiting severe structural disruption in cells treated with 0.1 (Fig. 6) and 0.5 (Fig. 7) μg ml^{-1} AFB_1. Fig. 6. ×20000; Fig. 7. ×13000;
Fig. 8. Apparent deterioration of nuclear integrity in a cell treated with 0.5 μg ml^{-1} AFB_1. m = mitochondrion; Fig. 8 ×13000.

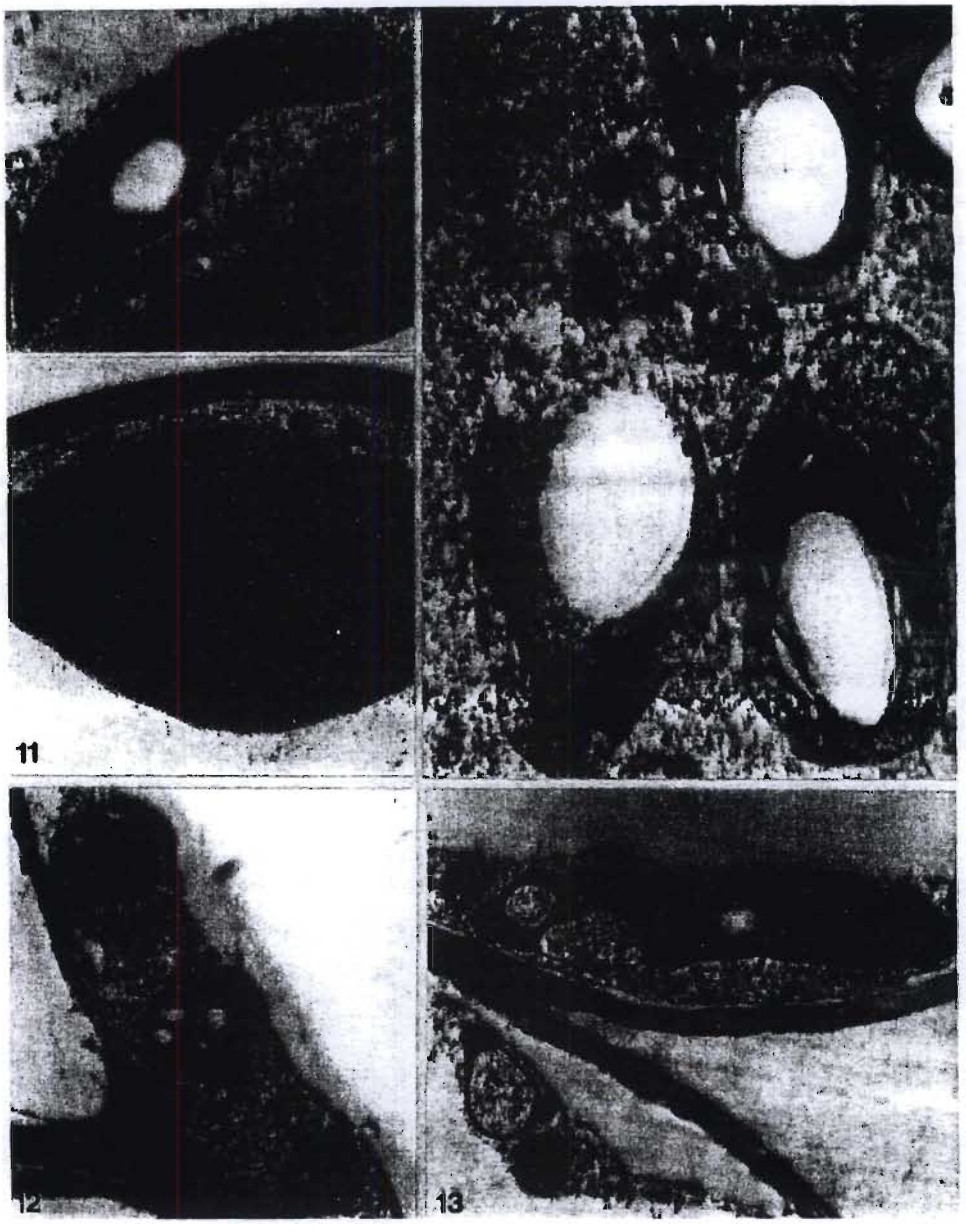

Plate II. *Figs 9–13.* Organelles from cells exposed to 1.0 μg ml^{-1} AFB_1 for 3 weeks. The chloroplasts in Fig. 10 exhibits swelling (*) and disruption of the organellar membrane (arrows). The cristae in the mitochondrion (m) in Fig. 10 are poorly developed. Note the persistence of cytomatrical polysomes (Figs 9 & 10). s = starch grains; Fig. 9; ×16800; Fig. 10. ×12500; Fig. 11. ×5000; *Figs 12 & 13.* Mitochondria from cells treated with 2.0 μg ml^{-1} AFB_1 exhibit a swelling of the scarse cristae. Polysomes remain prevalent (Fig. 12). Chloroplasts (Fig. 13) showing the presence of many plastoglobuli which might be an indication of thylakoid membrane breakdown. Fig. 12. ×16500; Fig. 13. ×13000.

AFB_1 or more but appeared unaffected by doses in the 0.5–1.0 μg ml^{-1} range [16].

Our study indicates that callus cells of *N. tabacum* are extremely susceptible to AFB_1, in that a 3 week exposure to 0.5–1.0 μg ml^{-1} was sufficient to bring about an approximate 25% decrease in fresh mass accumulation, relative to the controls. This sensitivity to aflatoxin may be ascribed to the fact that, in contrast to seeds and embryos, calli do not comprise fully differentiated tissues

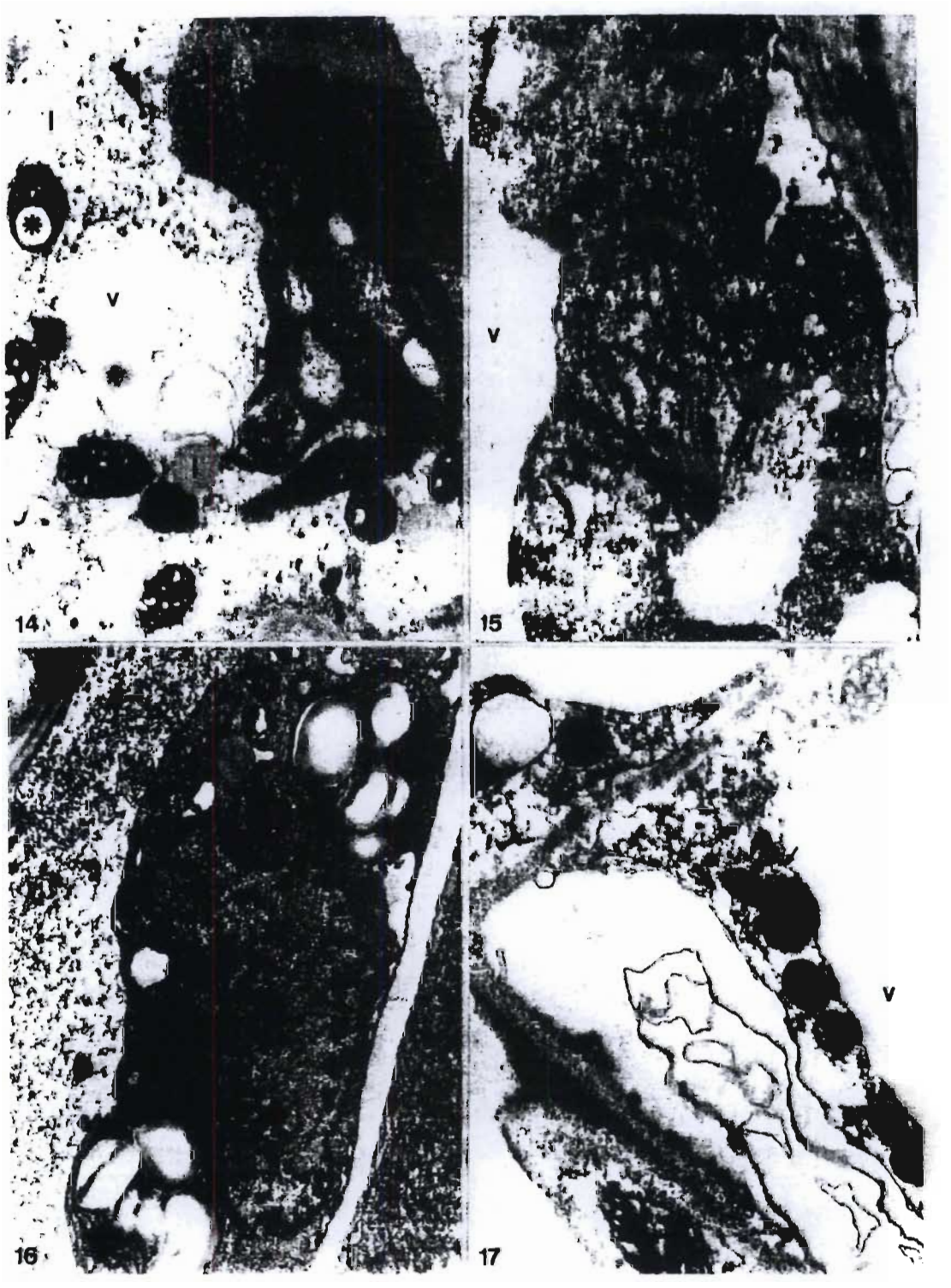

Plate III. *Figs 14–16.* In cells treated with 10 μg ml^{-1} AFB_1, chloroplast (p) morphology is severely disrupted. While some mitochondrial profiles (m) appear regular, others exhibit severe internal vesiculation (*), presumably as a result of abnormal swelling of the cristae. The nucleus in Fig. 16 exhibits abnormal condensation of the chromatin. I = lipid; Fig. 14. ×16500; Fig. 15. ×12400: Fig. 16. ×10000; *Fig. 17.* A deteriorated cell from callus treated with 25 μg ml^{-1} AFB_1. Despite this, some mitochondria (m) have a regular appearance. Lipid (I) droplets are numerous, with apparent fusion (arrows). Fig. 17. ×19000.

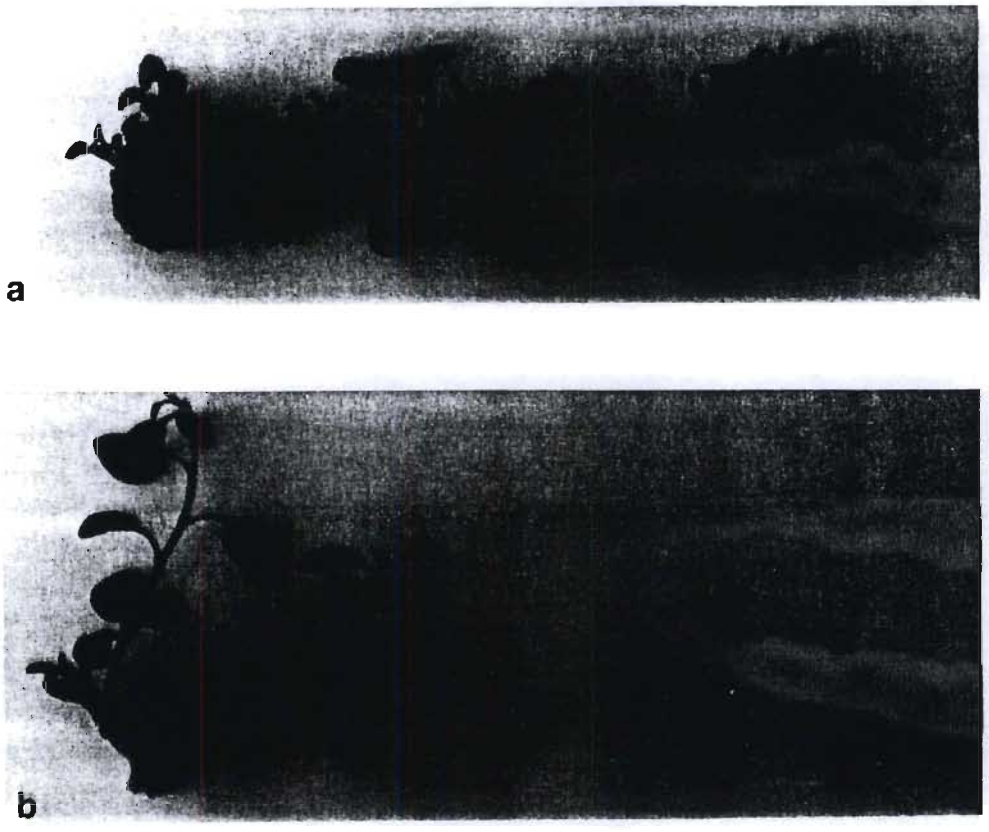

Plate IV. *Fig. 18.* (a) Control calli following 6 weeks of incubation (3 weeks toxin exposure; 3 weeks recovery with no toxin). From left to right: no DMSO; DMSO concentration found in 5 (0.2% DMSO) and 25 (1% DMSO) μg ml^{-1} AFB_1; (b) Calli treated with 5–25 μg ml^{-1} AFB_1 following a 3 week toxin exposure and a 3 week recovery period. From left to right: 5, 10, 20 and 25 μg ml^{-1} AFB_1.

and the mechanism(s) required for dealing with the toxin may either not be present or not be sufficiently developed. Unorganised callus consists of parenchyma cells that are highly vacuolated and have small amounts of cytoplasm and, in some cases, well developed chloroplasts [27–29]. According to those reports, in tobacco, organogenesis is initiated when cell files (areas of preferential active cell division) are formed adjacent to xylem and tracheid elements within the callus mass. Cell files then give rise to meristemoids, which are meristem-like aggregates of small, non-polar, non-vacuolated cells that form shoot and root primordia. Hence, in tobacco callus, fresh mass increase during organogenesis may be attributable to cell division and cell growth within the callus mass, as well as the presence and growth of the regenerating shoot primordia.

The aflatoxin concentration which caused 50% inhibition of fresh mass increase was 2 μg ml^{-1} (Fig. 1). At this AFB_1 concentration, while chlorophyll levels were inhibited by approximately 45% (Fig. 2A), polysomes were still prevalent (Figs 9, 10 & 12) and neither protein content (Fig. 2B) nor shoot formation were affected significantly. These results, together with findings by other workers [29–31] that relatively high protein levels and rates of protein synthesis are associated with organogenesis, seem to indicate that a 3 week exposure to 2 μg ml^{-1} AFB_1 has no significant effect on organ differentiation. It is suggested, therefore, that under these con-

ditions, the observed inhibition in fresh mass accumulation was due to a repression of cell growth within the callus mass. This is supported by the findings that, upon transfer of the calli to an aflatoxin-free medium, the fresh mass increase of 0.1–10 μg ml^{-1}-treated calli exceeded that of the control calli (Figs 1 & 2A). The % calli developing shoots increased for all treatments once the toxin had been removed, but not to the same degree of recovery observed for fresh mass values (Fig. 2A). It could be argued that, as the toxin was supplied in the growth medium, one would expect a decreasing toxin concentration gradient to exist between the abaxial (closest to the medium) and adaxial callus faces, resulting in a reduced effect on shoot primordia. However, this should not have occurred as the meristemoids are more frequent in the lower or central parts of the callus due to quantitative interactions between growth factors active over physiological gradients [32, 33].

A number of authors have suggested that AFB_1 may interfere with mitosis: Lilley [34] reported a significant increase in the number of abnormal anaphases and an inhibition of mitosis in the roots of *Vicia faba* seedlings after treatment with a crude aflatoxin mixture. Similarly, Jacquet et al. [35] reported that mixed aflatoxins induced chromosomal abnormalities in meristematic cells of *Allium cepa* and *A. ascalonicum*, while Reiss [36] observed chromosome bridges, C-mitoses and a reduction of the mitotic index in *A. cepa* treated with AFB_1. More recently, Packa [37] reported that deoxynivalenol, deoxyacetylscirpenol and F-2 toxin generally decreased the mitotic index in several cereals and field beans, and resulted in increased numbers of metaphases, C-metaphases, abnormal chromosomes and chromosome bridges. That author interpreted such events as evidence for a disruption in the functioning of the mitotic spindle.

There is evidence that AFB_1 (or mixed aflatoxins) interferes with the normal-functioning of chloroplasts. Slowatizky et al. [42]) observed that etiolated maize leaves, treated with AFB_1, did not green upon exposure to fluorescent light. Other authors have reported the development of chlorotic areas in plants treated with AFB_1 (e.g. *Lemna minor* and *Nasturium officianale* [43, 39], *Cucumis sativus* L. [44]). In the present study, disruptive effects on cell ultrastructure were recorded initially in calli treated with 1 and 2 μg ml^{-1} AFB_1 and became gradually more severe with increasing toxin levels. The initial and most apparent subcellular abnormality was the deterioration in plastid morphology, an observation which supports the early progressive decline in chlorophyll content of the callus recorded with increasing AFB_1 concentration (Fig. 2A). Alterations in mitochondrial structure (swelling of cristae, diffuse matrix) were also detected, but apparently only after plastids had been affected, viz. in callus cells treated with 2 μg ml^{-1} AFB_1.

The number of articles reporting on the ultrastructural alterations induced by AFB_1 in animal, and particularly plant, cells is limited. In the plant literature, the electron microscopical evaluation of toxin-induced alterations in cell ultrastructure has been restricted largely to the host-specific toxins, especially that of *Helminthosporium maydis* (HmT toxin) [see 38 for review]. A few ultrastructural investigations have been conducted on mycotoxins such as AFB_1 [6, 12–16; 39], metabolites produced by *A. sulphureus* [40], and fumonisin B_1 [41]. These observed effects of AFB_1 on cellular organelles of tobacco callus appear similar to those described by several workers, and reviewed by Hanchey [38] for a number of host-selective toxins. In reviewing the effects of such toxins, that author reports that HmT toxin causes swollen mitochondria with few cristae and a decreased matrix density, and cercosporin causes a thickening of the mitochondrial boundary membrane and swollen cristae. Irregularly-shaped plastids with few granal lamellae have been observed with tentoxin treatments, and plastid envelope rupture is a common feature in victorin-treated leaves [38].

A noticeable feature of callus cells cultured on high levels of aflatoxin (10–25 μg ml^{-1}) was the occurrence of large, abnormal lipid bodies in the cytoplasm. Their appearance in cells as a re-

sponse to the presence of various *Aspergilli* or their toxins has been reported for a number of species, for example *A. glaucus*-infected wheat embryos [45], aflatoxin-treated *Lepidium sativum* roots [6], *Zea mays* embryos [16], and *L. sativum* roots treated with *A. sulphureus* metabolites [40]. Although the underlying cause of this abnormality has not been elucidated, this phenomenon is regarded as an indication of fungal-induced disrupted lipid metabolism [46]. It is interesting to note that in the present case, large lipid bodies were generally detected in cells treated with those levels of AFB_1 which caused 80–100% callus deterioration.

In conclusion, the data for both the ultrastructural and growth parameters indicate that, in differentiating callus cultures, a 3 week treatment with AFB_1 concentrations up to 2 $\mu g\ ml^{-1}$ causes ultrastructural cellular changes which are manifested as a 50% decrease in fresh mass accumulation, an effect which is reversed upon removal of the stress. At this level of toxin exposure, shoot development is unaffected. In contrast, cells treated with AFB_1 concentrations higher than 20 $\mu g\ ml^{-1}$ exhibit morphological, biochemical and ultrastructural characteristics which seem to reflect a critical event/(s) affecting the survival of the differentiating callus. This is the point at which the ability of the cells to differentiate and produce shoots is irreversibly impaired. This initial investigation has been extended to include differentiated plantlets of *Nicotiana tabacum*, and is presented in a subsequent report.

Acknowledgements

The authors wish to thank Mrs T. Naicker, Electron Microscopy Unit, Faculty of Medicine, University of Natal, for expert technical assistance, and the Foundation for Research Development and University of Natal Research Fund for financial assistance.

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Pergamon

0163-7258(94)00054-9

Pharmac. Ther. Vol. 65, pp. 163–192, 1995

0163-7258/95 $29.00

Associate Editor: K. D. BHOOLA

CELLULAR INTERACTIONS AND METABOLISM OF AFLATOXIN: AN UPDATE

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Abstract—The aflatoxins are a group of closely related mycotoxins that are widely distributed in nature. The most important of the group is aflatoxin B_1 (AFB_1), which has a range of biological activities, including acute toxicity, teratogenicity, mutagenicity and carcinogenicity. In order for AFB_1 to exert its effects, it must be converted to its reactive epoxide by the action of the mixed function mono-oxygenase enzyme systems (cytochrome P450-dependent) in the tissues (in particular, the liver) of the affected animal. This epoxide is highly reactive and can form derivatives with several cellular macromolecules, including DNA, RNA and protein. Cytochrome P450 enzymes may additionally catalyse the hydroxylation (to AFQ_1 and AFM_1) and demethylation (to AFP_1) of the parent AFB_1 molecule, resulting in products less toxic than AFB_1. Conjugation of AFB_1 to glutathione (mediated by glutathione *S*-transferase) and its subsequent excretion is regarded as an important detoxification pathway in animals. Resistance to AFB_1 toxicity has been interpreted in terms of levels and activities of these detoxifying pathways. This article reviews the multiple reactions and effects attributed to aflatoxin, with particular reference to the interaction of aflatoxin with nucleic acids and proteins, and the contribution this mycotoxin has in disease development and in the promotion of hepatocellular carcinoma (HCC). The anti-mutagenic properties of several dietary factors are also considered in this article. Undoubtedly, the most important aspect of aflatoxin action is its putative role in the development of human cancer, in particular, HCC. Recently, there has been a renewed interest in this aspect and experimental evidence is rapidly accumulating at the molecular level, indicating aflatoxin as an important consideration in the aetiology of human HCC.

Keywords—Aflatoxin, anti-mutagenic, cytochrome P450, glutathione *S*-transferase, metabolism, mycotoxin.

CONTENTS

Abbreviations—AF, aflatoxin; DAS, diallyl sulphide; DES, diethylstilbestrol; ER, endoplasmic reticulum; GST, glutathione *S*-transferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; NLS, nuclear location sequence; PLC, primary liver cancer.

1. INTRODUCTION

1.1. Mycotoxins

Mycotoxins are secondary metabolites produced by fungi in foods and feeds, which, on ingestion, can result in the illness or death of animals, including humans (Hayes, 1981). The diseases caused by these mycotoxins are called mycotoxicoses and generally are classified according to the symptoms resulting from ingestion (Smith and Moss, 1985). The classical mycotoxicosis is ergotism, where, during the 9th and 10th centuries, there were numerous records of gangrenous ergotic outbreaks, with limbs rotting and falling off. The Order of St Anthony was established in the 11th century to provide hospitals for those suffering from this condition, which was known as St Anthony's fire. It was not until about 1850 or so that it became known that the disease was associated with the consumption of cereals, especially rye, contaminated with the plant pathogenic, ergot-producing (then not known) fungus, *Claviceps purpurea* (Smith and Moss, 1985; Berry, 1988).

Through the years, there have been numerous outbreaks of different mycotoxicoses in humans and other animals that have tended to be endemic to certain areas. Examples include yellow rice disease in Japan and sheep facial eczema in New Zealand. In the 1940s and 1950s, certain fungal metabolites were discovered to have useful antibiotic properties, with the unique property of being selectively toxic towards pathogenic bacteria, while remaining relatively harmless to animals (including humans) during treatment. It was also about this time that it was recognised that some diseases could be attributed to certain fungal toxins involuntarily ingested with food. In the Ukraine, the use of over-wintered wheat for bread-making resulted in an epidemic of a fatal disease known as alimentary toxic aleukia. Although Soviet scientists investigated the occurrence, little was known about illnesses associated with fungal toxins, let alone an awareness of the many mycotoxins. For example, Forgacs and Carll (1962) described mycotoxicoses as "neglected diseases", while at about the same time, Garner (1961) wrote that "in only a few instances has it been shown that extracts of fungi are harmful".

It was, however, a singular event that occurred in 1960 in Britain that established the significance of mycotoxicoses at the international level. This was the death of thousands of turkey poults, ducklings and chicks, and so became known as 'Turkey-X' disease (Butler, 1974). Analysis of the feed established the presence of a fungus, *Aspergillus flavus*, and thin layer chromatography identified several compounds that fluoresced under ultraviolet illumination. These compounds were named *aflatoxins*. More recent evidence suggests that the symptoms described in the field cases during the 1960s are not consistent with those known to be produced by the aflatoxins alone. With current knowledge and expertise, the findings would indicate that at least one other mycotoxin known to be produced by *Aspergillus flavus* (amongst other fungi), cyclopiazonic acid, may have been involved (Cole, 1986).

Since then, many more toxic fungal secondary metabolites have been identified, and as technology advances, the list becomes longer. While Huff *et al.* (1988) have reported that at least 200 substances of fungal origin are sufficiently toxic to warrant mycotoxin status, others report much higher figures (Cole and Cox, 1981; Watson, 1985). Watson (1985), on reviewing the literature, considers that 432 fungal compounds can be regarded as toxins, although only about one-quarter of these are toxic to mammals. Currently, hitherto unknown toxic compounds are being isolated and identified continually. Particular reference to the fumonisins (Bezuidenhout *et al.*, 1988) may be made in this regard. The present article utilises the extensive reviews of Kiessling (1986) and Hsieh (1987) as a basis for updating and summarising the more recently published data on aflatoxin B_1 (AFB_1) metabolism.

1.2. Aflatoxins

Although 17 compounds, all designated as aflatoxins, have been isolated, the term usually refers to four metabolites of this group of bis-furocoumarin metabolites produced by *Aspergillus flavus* and/or *Aspergillus parasiticus*. These are named AFB_1, B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2), all of which occur naturally (Fig. 1). The four compounds are distinguished on the colour of their fluorescence under long-wave ultraviolet illumination (B = blue; G = green), with the subscripts relating to their relative chromatographic mobility. AFB_1 is usually found in the highest concentrations, followed by AFG_1, AFB_2 and AFG_2. *Aspergillus flavus* produces only AFB_1 and AFB_2, while *Aspergillus parasiticus* produces these and additional compounds. The order of acute and chronic toxicity is $AFB_1 > AFG_1 > AFB_2 > AFG_2$, reflecting the role played by epoxidation of the 8,9-double bond (Wogan, 1966) and also the greater potency associated with the cyclopentenone ring of the B series, when compared with the six-membered lactone ring of the G series. Aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2) are hydroxylated forms of AFB_1 and AFB_2. Aflatoxin B_{2a}

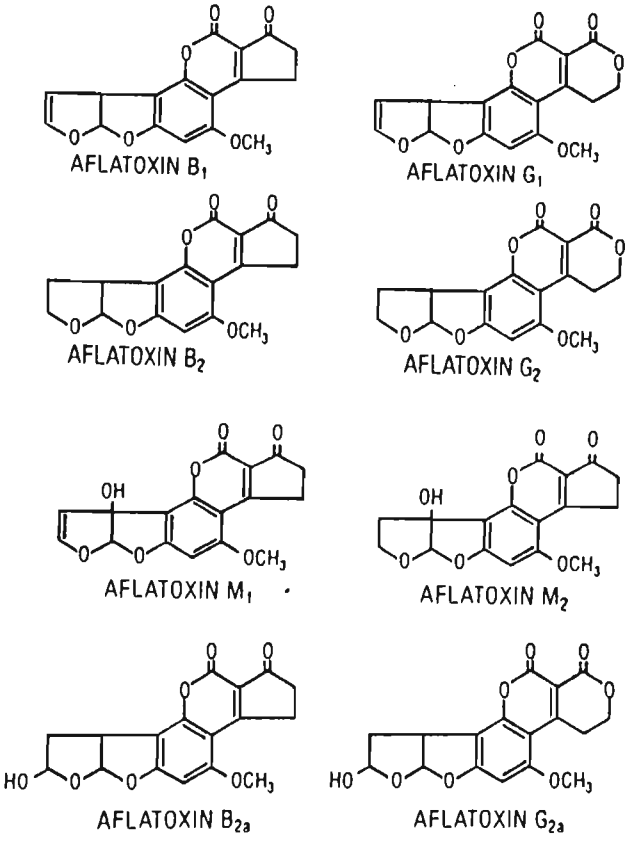

Fig. 1. Structures of aflatoxins B_1, B_2, G_1, G_2, M_1, B_{2a} and G_{2a}.

(AFB_{2a}) and aflatoxin G_{2a} (AFG_{2a}) are 8,9-hydrated products of AFB_1 and AFG_1 (Dutton and Heathcote, 1968). These compounds are relatively non-toxic when compared with AFB_1 and AFG_1.

The aflatoxins are freely soluble in moderately polar solvents (e.g. chloroform and methanol), especially in dimethylsulphoxide, and also have some water solubility. These compounds are very stable at high temperatures, with little or no destruction occurring under ordinary cooking conditions or during pasteurisation. The presence of the lactone ring in their structure makes the aflatoxin molecule susceptible to alkaline hydrolysis. Acid treatments (e.g. propionic acid) are also used frequently for their detoxification.

1.3. Aflatoxicosis

Toxicologically, aflatoxin may be regarded as a quadruple threat — as a potent toxin, a mutagen, a teratogen and a carcinogen (Ueno and Ueno, 1978). The lethal toxicity of AFB_1, however, varies in different animals: from extremely susceptible (sheep, dog, rat) to resistant species (monkey, chicken, mouse). There are no toxicity values for humans, but there is ample epidemiological evidence from case studies in Africa, South East Asia and India to implicate aflatoxins in the incidence of liver cancer and infant mortality (Hsieh, 1986). AFB_1 has also been reported to form adducts with DNA and so may play a role in the development of extrahepatic cancers. In this regard, Dvorackova *et al.* (1981) previously have implicated AFB_1 in the development of lung cancer. In comparing the ability of AFB_1 to bind to bladder and tracheobronchial tissues derived from several animals, Stoner *et al.* (1982) found that extrahepatic binding of AFB_1 to DNA was higher in AFB_1-resistant species than in susceptible species.

Although the epidemiological evidence relating AFB_1 to primary liver cancer (PLC) appears convincing, as yet, it is circumstantial. Stoloff (1989) is of the opinion that a correlation between high levels of aflatoxin in the diet and liver cancer does not prove a causal relationship. In countries where the incidence of liver cancer is high, the hepatitis B virus (HBV) is also common. Since this virus is known to be oncogenic, it is likely that liver carcinomas may arise from contributions of both agents (Hsieh, 1986). A further factor to consider in each case is the general malnutrition that prevails in one form or another in these areas. Lack of certain nutritional factors, e.g. protein or vitamin A, may predispose an individual to the toxic or even carcinogenic effects of AFB_1 (Smith and Moss, 1985; Newberne, 1987; Decoudu *et al.*, 1992). Recently, Prabhu *et al.* (1989) have reported that in rats, a copper deficiency enhanced the conversion of AFB_1 to its reactive metabolite, resulting in greater AFB_1–DNA adduct formation and increasing the risk of liver cancer.

2. CELLULAR METABOLISM

2.1. Activation

Activation of AFB_1 is important in any mycotoxicological consideration of the effects of AFB_1 on organisms. AFB_1 in itself is not carcinogenic, but is metabolised by the body to produce an ultimate carcinogenic metabolite (Swenson *et al.*, 1974), AFB_1–8,9-epoxide, formed by oxidation of the 8,9-vinyl ether bond (Fig. 2). Patterson (1973) traced the biotransformation of AFB_1 in susceptible cells by a pathway later modified by Ueno and Ueno (1978). Following transport across the plasma membrane, the AFB_1 molecule is activated by microsomal (smooth/tubular endoplasmic reticulum (ER)-associated) mixed-function mono-oxygenases (requiring cytochrome P450, NADPH and molecularoxygen)toformthehighlyreactiveAFB_1–8,9-epoxide(Swenson*et al.*,1974).Additionally,the nuclear envelope of rat hepatocytes is also reported to contain all the enzymes necessary for the metabolic activation of AFB_1 (Kasper and Gonzalez, 1982). The AFB_1 epoxide may bind to nuclear DNA, resulting in nuclear damage, or may bind to sex-linked sites on the ER. This binding to the latter may result in ribosomal detachment and polysome degradation. AFB_1 may also be *reversibly* converted by an NADPH-reductase to aflatoxicol. The aflatoxicol thus may act both as a sink and a reservoir for AFB_1 (Patterson, 1973; Hsieh *et al.*, 1977; Wong and Hsieh, 1978). The microsomal mono-oxygenase system is also responsible for transforming the AFB_1 into polar molecules such as AFM_1, aflatoxin P_1 (AFP_1) and aflatoxin Q_1 (AFQ_1). AFM_1, AFP_1 and AFQ_1 can be eliminated by

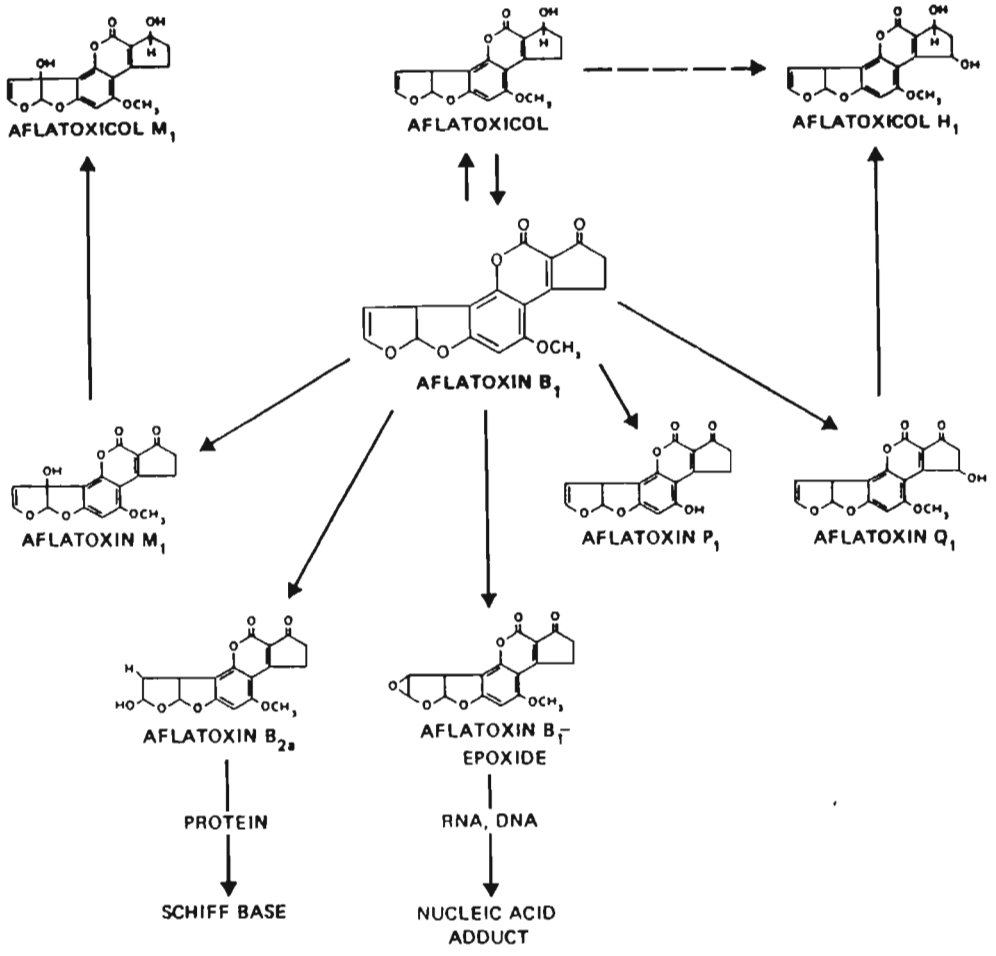

Fig. 2. Metabolism of aflatoxin B_1 (World Health Organization, 1979).

the hepatocytes, but the epoxide binds to nucleic acids and proteins and is thought to be the carcinogenic form of AFB_1 (Swenson *et al.*, 1974). The AFB_1-epoxide may become hydrated to its dihydrodiol (8,9-dihydro-8,9-dihydroxy AFB_1), followed by rearrangement to a putative dialdehyde phenolate intermediate, which is capable of condensing with primary amino acid groups of proteins and other cellular constituents, forming Schiff bases (Neal and Colley, 1979). AFB_{2a}, thought to be a hydrolytic product of the AFB_1 or its conjugates, in the phenolate form, binds to proteins, forming Schiff bases (Fig. 2). This AFB_{2a} may then cause the acute toxic effects of AFB_1 (Hsieh *et al.*, 1977; Hsieh, 1987). The decreased toxicity of AFB_{2a} when administered orally can be explained on the basis of non-absorption in the gut (Thompson *et al.*, 1992).

Amstad *et al.* (1984) have postulated an alternative mode of action for AFB_1 to this direct mechanism of binding to critical intracellular macromolecules. AFB_1 may exert its genotoxic effects by an indirect mechanism: through being membrane-active, via the intermediacy of active oxygen, lipid hydroperoxidases and small aldehydes (Amstad *et al.*, 1984). In that study, sister chromatid exchanges were induced in human lymphocytes at very low levels of covalent AFB_1–DNA adducts, which could not be explained entirely in terms of a direct genotoxic action.

2.2. Detoxification

Detoxification reactions of mycotoxins invariably involve conjugation of the toxin to glucuronic acid, sulphate or glutathione (Hsieh, 1987). The major detoxification reaction of AFB_1 is conjugation of the reactive epoxide to glutathione (mediated by glutathione *S*-transferase, GST) (Degen and Neuman, 1978, 1981). The AFB_1–glutathione conjugate is excreted primarily through the bile

(Hsieh, 1987). The conjugate, however, is reported to have the potential to be hydrolysed by the intestinal microflora, to release the AFB_1 for reabsorption and enterohepatic circulation (Hsieh and Wong, 1982). AFB_1–8,9-epoxide might also be detoxified by the UDP-glucuronyl-transferase, sulphotransferase and possibly the epoxide hydrolase systems (Hayes *et al.*, 1991a).

Most of the other aflatoxins (AFP_1, AFH_1, AFG_1, AFM_1) form glucuronide or sulphate conjugates and can be excreted in the urine (Wong and Hsieh, 1980). AFB_1 (or its epoxide) may be hydroxylated to form AFQ_1 and AFM_1 (Roebuck and Wogan, 1977; Raney *et al.*, 1992b) or demethylated to form AFP_1 (Roebuck and Wogan, 1977). The relative resistance or susceptibility of different animal species may depend then, not only on differences between activation of AFB_1, but also on differing abilities for its conversion to conjugation products that can be excreted (Hsieh *et al.*, 1977; Roebuck and Wogan, 1977). In this regard, Roebuck and Wogan (1977) have reported that resistant species (e.g. monkey, mouse and human) were able to excrete AFQ_1 and AFP_1, while the more susceptible species (e.g. duck and rat) produced aflatoxicol and no AFP_1.

2.3. Interaction with Biomolecules

2.3.1. *Nucleic Acids*

Nucleophilic hetero-atoms (e.g. nitrogen and oxygen) in the organic bases of nucleic acids are susceptible to electrophilic attack by metabolites of mycotoxins, forming covalent adducts. Any alteration in nucleic acid (both DNA and RNA) structure effected by these adducts will impair DNA and RNA template activity, resulting in inhibition of DNA, RNA and ultimately protein synthesis. The possible resultant point mutations may lead to the manufacture of non-functional molecules (Hsieh, 1987). Adduct formation *in vivo* may result, therefore, in transformation of cells, or even cell death, depending on the severity of impairment of template activity (Hsieh, 1987). Ewaskiewicz *et al.* (1991), however, have reported that low doses of AFB_1 may result in transient non-covalent AFB_1–DNA binding, which forms prior to AFB_1 activation and DNA adduct formation.

Both AFB_1–epoxide and, to a lesser extent, its hydration product, the dihydrodiol form of AFB_1, react with nucleic acids. The epoxide specifically makes an electrophilic attack on the N^7 position of guanine of DNA and RNA (Croy and Wogan, 1981a,b; Croy *et al.*, 1978; Essigmann *et al.*, 1980; Benasutti *et al.*, 1988), while the dihydrodiol forms a Schiff base with amino groups of the bases (Hsieh, 1987). The dihydrodiol is highly reactive and binds to proteins at the site of its formation (Neal and Colley, 1979). While AFB_1–N^7-guanine (*trans*-2,3-dihydro-2-(N^7-guanyl)-3-hydroxy AFB_1) is the major adduct formed, other metabolites of AFB_1 have the ability to form adducts with DNA, in particular, AFM_1–N^7-guanine and AFP_1–N^7-guanine (Essigmann *et al.*, 1982). The structure of the epoxide formed may be an important consideration in the affinity of the molecule for DNA. For example, the cyclopentenone ring fused to the lactone ring of the coumarin allows intercalation with DNA, while the less planar delta-lactone ring of aflatoxins G_1 and G_2 reduces DNA binding affinity by approximately one order of magnitude (Raney *et al.*, 1990).

The N^7-guanyl adduct is unstable and may either undergo spontaneous, non-enzymatic depurination or be stabilised by the opening of the imidazole ring to yield pyrimidyl adducts [2,3-dihydro-2-(N^5-formyl-2,3,6-triamino-4-oxopyrimidine-N^5-yl)-3-hydroxy AFB_1 (AFB_1 FAPY) and 8,9-dihydro-8-(2-amino-6-formamide-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxy AFB_1 (AFB_1 III)] within 24 hr of AFB_1 exposure (Hayes *et al.*, 1991a). The pyrimidyl adducts are not lost spontaneously, but appear to be removed catalytically by DNA repair enzymes. The presence of the N^7-guanyl adduct in the urine of exposed individuals arises as a result of this spontaneous depurination. Depurination at guanine residues could lead to a GC → TA conversion during replication, while the formamidopyrimidine derivatives are repair-resistant and thus, relatively persistent, resulting in mutations if present at the time of DNA replication (Croy and Wogan, 1981b).

2.3.2. *Proteins*

Besides being important structural and functional cellular components, proteins also act as cellular receptors, having nucleophilic nitrogen, oxygen and sulphur hetero-atoms in their functional groups

(Hsieh, 1987). The structure and activities of proteins may be altered by non-specific-irreversible-covalent (conformational change resulting in denaturation or blocking of binding sites) and specific-reversible-non-covalent (competitive binding) binding with mycotoxins. Proteins that bind mycotoxins reversibly may act as reservoirs of the toxin, prolonging toxin exposure, or they may serve as carriers in the transport of reactive metabolites (Ch'ih and Devlin, 1984; Hsieh, 1987).

There is evidence that some AFB_1 molecules become cytoplasmically bound to molecules destined for the nucleus (Ch'ih and Devlin, 1984; Ch'ih *et al.*, 1993). The former researchers have proposed the presence of a cytoplasmic binding protein(s). On entering the cell, AFB_1 is translocated (non-covalently bound) to microsomes (Ewaskiewicz *et al.*, 1991) for activation, facilitated by this cytoplasmic binding protein. The majority of the epoxide is detoxified and is removed rapidly from the cell as water-soluble, polar metabolites (Ch'ih and Devlin, 1984). A portion of the activated AFB_1 is translocated to various subcellular sites where covalent binding occurs, first to cellular macromolecules (e.g. rER) and then later in the nucleus, and finally in mitochondria (Ch'ih and Devlin, 1984). More recently, several cellular proteins (e.g. pyruvate kinase > albumin > carbonic anhydrase > pancreatic RNase > histones) were found to bind AFB_1, while the presence of a nuclear location sequence (NLS) (as is found in histones) markedly increased nuclear translocation and activation of the AFB_1 in the nucleus (Ch'ih *et al.*, 1993). Such findings exemplify the opportunistic nature of AFB_1.

Mycotoxin binding to functional proteins may inhibit protein activity, particularly in the case of enzymes. If biosynthesis of the protein is unaffected, then effects on the protein can be reversed, as soon as the non-functional proteins are replaced by *de novo* synthesis. Proteins involved in biosynthetic pathways, neurotransmission, hormone functions, membrane transport and immune mechanisms are critical factors when considering the biochemical and physiological effects of mycotoxins. In addition, binding to molecules distal from the active site or to inert proteins may represent a detoxification and sequestering mechanism and as such, may act as a toxin sink (Hsieh, 1987).

2.4. Inhibition of ATP Generation

At acute mycotoxin exposure levels, inhibition of cellular energy production is a major metabolic effect (Hsieh, 1987). In this regard, AFB_1, AFG_1 and AFM_1 inhibit oxygen uptake in tissue homogenates (Smith and Moss, 1985). The aflatoxins act on the electron transport system, with AFB_1, AFG_1 and AFM_1 inhibiting the electron transport chain between cytochromes b and c or c_1 (Site II) in rat liver mitochondria (Doherty and Campbell, 1972, 1973). AFB_1 is also known to act at the cytochrome oxidase level (Kiessling, 1986; Betina, 1989). It would appear, however, that the biochemical effects of AFB_1 on liver mitochondria do not require metabolic activation (Hsieh, 1987), although Niranjan and Avadhani (1980) have reported the presence of a cytochrome P450 type of mono-oxygenase system in rat liver mitochondria that is capable of generating electrophilic reactive metabolites that could covalently modify mitochondrial DNA, RNA and proteins.

Uncoupling of oxidative phosphorylation results in depletion of cellular ATP. As a result, sodium and potassium gradients within the cell are affected and mitochondria swell (Hsieh, 1987). AFM_1 has been found to uncouple *in vitro* oxidative phosphorylation and inhibit electron transport (Pai *et al.*, 1975). AFB_1 is a similar uncoupler, but is more effective as an electron transport inhibitor, also inhibiting ATPase activity (Hsieh, 1987). AFB_1 may also inhibit rat liver oligomycin-sensitive Mg^{2+} ATPase (i.e. ATP synthase) of the inner mitochondrial membrane (Hayes, 1978).

2.5. Immunocytochemical Localisation of Aflatoxin B_1

Recent immunological advances have made it possible to obtain from commercial sources a wide range of antibodies directed against many naturally occurring compounds. By means of a primary antibody and a secondary antibody to which was attached a 5-nm colloidal gold probe (all available commercially; Sigma Immunochemicals, St Louis, MO), an indirect immunocytochemical technique was designed to detect AFB_1 within the cells of plant tissues. This indirect immunocytochemical technique involved the use of a primary antibody (anti-AFB_1), directed against an antigen (AFB_1).

The immunological reaction was visualised electron microscopically by means of a secondary antibody (raised against the primary antibody) to which was attached a 5-nm gold probe. Ultrathin sections of tissues were then exposed to the above immunological reagents. The use of appropriate controls (first level [method] and second level [adsorption]) confirmed the validity of the positive results obtained. Following a continuous supply of AFB_1 in the medium, AFB_1 could be immuno-located within the nucleus (specifically associated with the nucleoplasm rather than within the nucleolus), the vacuoles and the cytoplasm of the stem cells of regenerating tobacco plantlets (*Nicotiana tabacum*) and root tips of excised, germinating embryos of maize (*Zea mays*) cultured *in vitro* (Fig. 3). Occasional gold particles were associated with the cell walls and with organelles (mitochondria and plastids). The results of this immunocytochemical investigation confirm the published evidence for animals, that AFB_1 acts directly on the nucleic acids, particularly the DNA (Meneghini and Schumacher, 1977). It is probable that several of the measured decreases (e.g. RNA and protein syntheses) following AFB_1 exposure will then be secondary manifestations resulting from AFB_1-DNA binding.

3. EFFECT ON MACROMOLECULAR BIOSYNTHESIS

3.1. DNA

Inhibition of macromolecular biosynthesis is a major metabolic effect of mycotoxins and may lead to failure to replace essential molecules, particularly functional proteins, possibly resulting in cell death (Hsieh, 1987). Mast cell stimulation, as a result of tissue damage, could cause inflammation, leakage of body fluids and subsequent haemorrhage (Hsieh, 1987), although AFB_1 itself does not appear to stimulate histamine release from these cells (Bent *et al.*, 1993). If the animal survives, cell regeneration may promote the expression of existing DNA lesions and, hence, the possible development of the tumorous condition (Hsieh, 1987).

One of the first measurable effects of AFB_1 on cells and tissues is inhibition of DNA synthesis. In the liver, this inhibition occurs at toxin concentrations, which apparently are not inhibitory to RNA or protein synthesis (Meneghini and Schumacher, 1977), suggesting interference of DNA synthesis to be a primary biochemical effect. It would appear that AFB_1 blocked the initiation step in DNA replication rather than the elongation process. Inhibition may result from covalent binding of AFB_1 to DNA and proteins, leading to modification of DNA template activity and/or inactivation of certain enzymes in DNA synthesis (Hsieh, 1987). Covalent binding of AFB_1 to membrane proteins may also reduce uptake of thymidine and other precursor nucleotides necessary for DNA synthesis (Kunimoto *et al.*, 1974).

3.2. RNA

Synthesis of rat liver RNA is inhibited rapidly by AFB_1 (Sporn *et al.*, 1966; Lafarge and Frayssinet, 1970; Yu, 1977, 1981), especially nucleolar RNA synthesis, related to formation of rRNA (18S and 28S) and rRNA precursors (32S and 45S) (Roy, 1968; Yu, 1977). This inhibition is due primarily to reduction of DNA template activity and inhibition of RNA polymerase II, an enzyme largely responsible for mRNA synthesis (Gelboin *et al.*, 1966; Pong and Wogan, 1970; Saunders *et al.*, 1972; Yu, 1977), and from impairment of nucleotide transport (Kunimoto *et al.*, 1974; Akinrimisi *et al.*, 1974). On the other hand, RNA polymerase I activity was largely unaffected by AFB_1 administration (Yu, 1977). Yu (1983) found that after activation *in vitro* and *in vivo*, AFB_1 binds preferentially to the physiologically active regions of the nucleolar chromatin of rat liver cells, possibly explaining the measured decreases in RNA synthesis. In a previous report, Yu (1981) had suggested that AFB_1 may interfere with RNA chain elongation. Additionally, chromosomal proteins may play a role in the binding of AFB_1 to DNA, since removal of these proteins resulted in a substantial loss of this specific binding (Yu, 1983). Contrary to this, however, Ch'ih *et al.* (1993) have found that several extranuclear proteins (e.g. albumin, pyruvate kinase) could bind AFB_1 more effectively than could histone proteins.

AFB_1 disrupts post-transcriptional processing of nuclear RNA for the manufacture of rRNA from nucleolar RNA precursors (Harley *et al.*, 1969), interfering with cleavage of the 45S RNA (into 18S and 28S rRNA) in rat liver (Hsieh, 1987). Transfer RNA processing is interrupted similarly, resulting

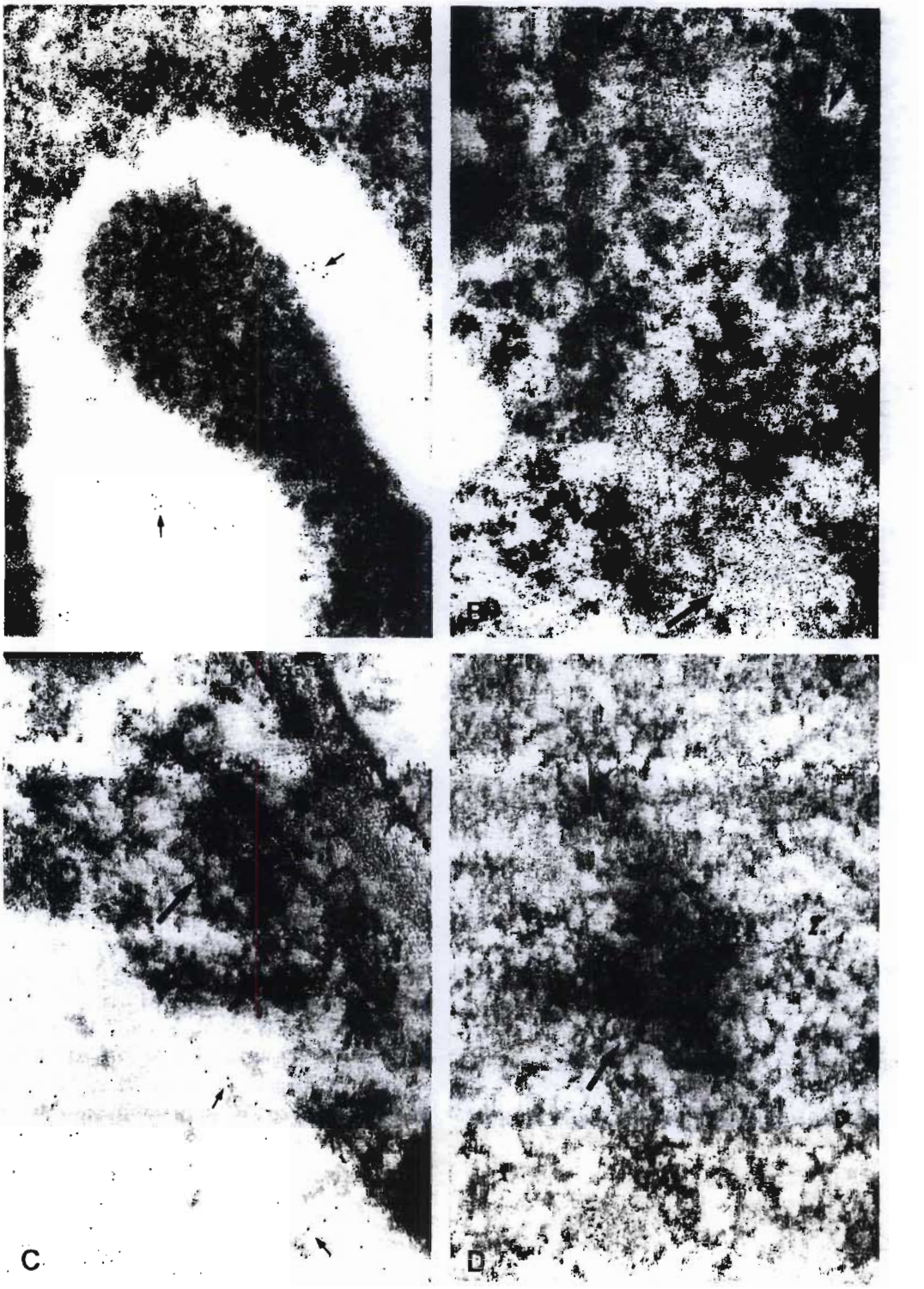

Fig. 3. Immunocytochemical localisation of aflatoxin B_1 in plant cells (magnification = 75,000 ×). **A** and **B**: Gold particles within the vacuole (**A**) and nucleus (**B**) of root tip cells of excised, germinating embryos of *Zea mays*. **C** and **D**: Localisation of aflatoxin B_1 within the cytoplasm and vacuole (**C**) and nucleus (**D**) of stem cells of regenerating plantlets of *Nicotiana tabacum*.

in elevated cytoplasmic levels of the 5S precursor of tRNA (Hsieh, 1987). In rats treated with AFB_1, Irvin and Wogan (1984) found that rDNA regions of liver DNA were preferentially accessible to AFB_1 modification, which may be explained in terms of the diffuse conformation within the transcribing gene.

Alterations in nuclear and nucleolar morphology are some of the most prominent effects of aflatoxin (and several other mycotoxins) in treated animal cells (Terao and Ueno, 1978). Ultrastructural morphological changes to the nucleolus that frequently have been reported include a gradual redistribution of nucleolar components (macrosegregation), resulting in segregation of granular and fibrillar components, fragmentation and the development of ring-shaped nucleoli. These observations may be visible manifestations of the measured alterations in nucleolar RNA synthesis (Roy, 1968; Yu, 1977) or the apparent accessibility of rDNA regions by AFB_1 (Irvin and Wogan, 1984).

3.3. Protein

Inhibition of protein synthesis by mycotoxins such as AFB_1, may arise directly from inactivation of biosynthetic enzymes, or indirectly by alteration of DNA template activity, or inhibition of RNA synthesis and maturation, translation, and/or interference with amino acid transport (Hsieh, 1987). Ultrastructurally, degranulation (detachment of ribosomes) from ER frequently has been reported in AFB_1-treated cells (Terao and Ueno, 1978). Such observations may arise as a result of disruptive changes, including direct damage to the ER membranes, interference with the ribosome binding sites on the membrane, interference with the ribosomal cycle, inhibition of the release of newly synthesised proteins and a suppression of mRNA synthesis (Terao and Ueno, 1978). As a result of this dissociation of ribosomes, ER-mediated protein synthesis is likely to be disrupted. In this regard, Viviers and Schabort (1985) found AFB_1-induced alterations in the phosphoprotein patterns in soluble and insoluble rat liver fractions, possibly by changes in amount and properties of specific proteins, the substrate proteins themselves and specific phosphatases. It is possible that *de novo* protein synthesis may also be affected.

3.4. Carbohydrate and Lipid Metabolism

Several animal species, when administered AFB_1, exhibit reduced hepatic glycogen levels and elevated serum glucose levels (Kiessling, 1986). These may arise from either an inhibition of glycogenic enzymes (e.g. glycogen synthase), an inhibition of glyconeogenesis, a decrease in glucose transport into hepatocytes or an increase in the activity of enzymes metabolising glycogen precursors (e.g. glucose 6-phosphate dehydrogenase) (Kiessling, 1986; Hsieh, 1987).

AFB_1 is known to cause lipid accumulation in the liver (Hamilton, 1975). This is generally believed to arise as a result of impaired lipid transport rather than increased lipid biosynthesis. Chou and Marth (1975) have reported an increase in hepatic lipid levels in mink injected with AFB_1, although there was no observable difference in [^{14}C]acetate uptake. Based on their findings, Chou and Marth (1975) have suggested that such hepatic lipid increases result from reduced oxidation of fats or increased lipid synthesis. In this regard, damage to mitochondria [which is frequently observed in AFB_1-treated cells (Terao and Ueno, 1978)] may result in decreased oxidation by these organelles, with a concomitant accumulation of lipids in the liver. The possibility that AFB_1 (or its metabolites) may alter the mobility of lipids is not overlooked by these workers.

This alteration in lipid transport or synthesis occurs at dietary toxin concentrations that do not affect growth rate or RNA synthesis (Hsieh, 1987). In chickens, AFB_1 not only affected lipid synthesis and transport, but appeared to influence lipid absorption and degradation (Tung *et al.*, 1972). Thus, impaired triacylglyceride transport (in chickens, at least) is a primary lesion and is not a secondary effect resulting from impaired nucleic acid metabolism (Tung *et al.*, 1972).

4. SPECIFIC EFFECTS

4.1. Immune Response

Several review articles on impairment of the immune response by mycotoxins in several experimental animal species have been published (Pier, 1973, 1986, 1992; Richard *et al.*, 1978; Pier *et al.*, 1980, 1986; Pier and McLoughlin, 1985; Pestka and Bondy, 1990, 1994; Sharma, 1993). Generally, an inhibition of protein synthesis could result in an alteration of serum protein concentrations, leading to suppression of non-specific, humoral substances. Subacute doses of AFB_1 in guinea pigs resulted in complement deficiency (Thurston *et al.*, 1972), delayed interferon production in turkeys (Pier, 1973; Pier and McLoughlin, 1985) and delayed lymphokine activity (Pier *et al.*, 1977). At higher doses, AFB_1 lowered levels of immunoglobulin G and immunoglobulin A in chicks (Giambrone *et al.*, 1978), leading to an impairment of acquired immunity. Recently, Pier (1992) has reported that mycotoxins may reduce the efficacy of acquired immunity during vaccination. Pier *et al.* (1986) found that *in vitro* exposure of B-lymphocytes to AFB_1 (and T-2 toxin) caused suppression of the lymphogenic response. Exposure of 18-day-old chick embryos to AFB_1 was found to induce dose-related increases in sister chromatid exchanges in T-lymphocytes (2-fold increase) and B-lymphocytes (6- to 8-fold increase). AFB_1 also reduced the mitotic index of B-cells and reduced the progression of B-lymphocytes, and to a lesser extent, T-lymphocytes, through successive rounds of replication (Potchinsky and Bloom, 1993). In human lymphocytes, low doses of AFB_1 were found to cause mitotic aberrations in a dose-dependent manner (Amstad *et al.*, 1984).

AFB_1 affects the cell-mediated immune response, causing a reduction in the response of T-lymphocytes to phytohaemagglutinin, thymic involution and failure to develop immunity following vaccination in turkeys (Pier *et al.*, 1972) and in chickens (Giambrone *et al.*, 1978). Experimentally, AFB_1 has been found to reduce antibody production, inhibit the phagocytic ability of macrophages, reduce complement, decrease T-cell number and function and cause thymic aplasia (Richard *et al.*, 1978; Pier, 1986; Reddy *et al.*, 1987).

Haemopoiesis also appears to be affected by AFB_1. Cukrová *et al.* (1991) found that a dose of AFB_1 as low as 0.5 $\mu g\ mL^{-1}$ exerted a strong suppression of myelopoiesis in bone marrow cultures. Recently, exposure of rats to aflatoxin resulted in an initial suppression of granulocyte and monocyte colony-forming units in the bone marrow (Cukrová *et al.*, 1992a,b), possibly as a result of an inhibition of mRNA transcription.

Impairment of the efficiency of the mononuclear phagocytic system has been observed. AFB_1 was found to suppress the activity of Kupffer cells in the liver (Mohapatra and Roberts, 1985), while others have reported an inability of bovine macrophages to produce interleukin 1, when presented with *Listeria monocytogenes* and other bacteria, following pretreatment of the animals with 10 $\mu g\ mL^{-1}$ AFB_1 (Kurtz and Czuprynski, 1992). *In vitro* exposure of chicken peritoneal macrophages to AFB_1 resulted in a dose-dependent increase in cellular damage and a decrease in macrophage adherence ability (Neldon-Ortiz and Qureshi, 1992). If mixed-function oxidases were added to this culture system, in addition to these observations, reduced phagocytic ability of macrophages was detected at much lower AFB_1 concentrations. It is likely that on addition of mixed-function oxidases, AFB_1 was more readily metabolised to its reactive metabolite, resulting in exacerbated cellular damage.

Secondary mycotoxin-related diseases may result from impairment of the immune system. Animals showed increased susceptibility to candidiasis, coccidiosis, salmonellosis and general immunologic deficiency. Thus, mycotoxins could predispose livestock to infectious diseases, resulting in decreased productivity (Pestka and Bondy, 1990), and possibly mortality as a result of complications.

4.2. Hormonal Effects

Steroid hormones regulate cellular functions by specifically and non-covalently binding to cytoplasmic receptor proteins and membranes in target cells. Following activation, the hormone–receptor complexes are transported to the nucleus and there induce selective gene transcription (mRNA) by binding to chromatin acceptor sites (Guyton, 1987). AFB_1 is known to bind covalently to DNA (specifically at the guanine residues), thereby possibly decreasing nuclear acceptor sites for hormone receptor complexes, with a consequent reduction in the activity of the

hormone. AFB_1 is known to reduce, in a dose-dependent manner, the nuclear acceptor sites for the glucocorticoid–cytosol receptor complex in rat liver (Wogan and Friedman, 1968). The formation of the hormone–receptor complex appeared unaffected (Hsieh, 1987). An interesting discussion involving a possible AFB_1–steroid hormone–ER–lysosomal enzyme pathway in the development of tumorous cells was presented by Money-Kyrle (1977).

AFM_1 is known to compete with oestradiol for the uterine cytosol receptor site at concentrations at which AFB_1, AFB_2 and AFG_1 were ineffective (Kyrein, 1974). AFB_1 inhibits the binding of polysomes to ER, thereby inhibiting protein synthesis. Incubation with corticosterone (but not hydrocorticosterone) reduced the effect of AFB_1, presumably by competing for the polysome-binding sites on the ER membrane (Williams and Rabin, 1969). *In vitro*, sex-linked binding sites of smooth or degranulated microsomes from rat livers were completely inhibited by AFB_1 (i.e. selective binding of testosterone by female liver microsomes and of oestradiol by male liver microsomes) (Blyth *et al.*, 1971; Sunshine *et al.*, 1971; Kiessling, 1986; Hsieh, 1987).

4.3. Mutagenic and Teratogenic Effects

AFB_1 (or more correctly, its epoxide) is the most potent mutagen of the aflatoxins, and there is a strong correlation between the ability of aflatoxins to be mutagenic and carcinogenic (Smith and Moss, 1985). AFB_1 causes chromosomal aberrations (chromosomal fragments, with occasional bridges, chromatid bridges and chromatid breakages) and DNA breakage in plant and animal cells (World Health Organization, 1979; Smith and Moss, 1985). It also produces gene mutations in bacterial test systems (Ames' test), where activation by rat or human microsomal preparations is essential (Wong and Hsieh, 1976).

Several mycotoxins, including AFB_1 are teratogenic (Hayes, 1981; Smith and Moss, 1985). Mycotoxins, which are potent inhibitors of protein synthesis, might be expected to cause impairment of development of primordia and differentiation in the foetus.

4.4. Carcinogenic Effects

4.4.1. *Initiators And Promoters*

Transformation of cells to the tumorous state is a two-step process: initiation and promotion (Hsieh, 1987). In the initiation step, the biochemical lesions produced in RNA, and particularly DNA, become 'fixed' features following cell division (Hsieh, 1987). Rapidly dividing cells are more at risk from mutation than are quiescent cells, since during DNA replication, adducts are converted to mutations and the time required for DNA repair may be insufficient (Hayes *et al.*, 1991a). Altered cells are potentially cancerous, but must undergo promotion. Under favourable conditions, promotion will occur, and transformed cells may become malignant, proliferating independently of normal cellular regulatory mechanisms (Hsieh, 1987).

Carcinogenic chemicals may be classified as initiators, promoters or both. The latter category (initiator and promoter), which includes AFB_1, AFG_1, AFM_1, sterigmatocystin, versicolorin, luteoskyrin and rugulosin, are referred to as complete carcinogens. Ochratoxin A, zearalenone and the trichothecenes are generally regarded as promoters (Hsieh, 1987). The importance of co-contamination of food by more than one mycotoxin-producing fungus, therefore, cannot be overemphasised, e.g. fumonisins and AFB_1 (Ueno *et al.*, 1993).

AFB_1 has been reported to bind to DNA in a selective, non-random manner in rats: i.e. it binds specifically to hepatic mitochondrial DNA (Niranjan *et al.*, 1982), nuclear ribosomal RNA gene sequences of liver DNA (Irvin and Wogan, 1984) and transcriptionally active regions of liver nucleolar chromatin (Yu, 1983). This binding is related to the accessibility of these areas of DNA to the toxin. Such areas generally lack histones (Yu, 1983), while the rDNA regions maintain a diffuse conformation due to high transcriptional activity (Irwin and Wogan, 1984). Recently, Ch'ih *et al.* (1993) investigated the *in vitro* binding ability of AFB_1 to various proteins. Binding ability of AFB_1 to histones was comparatively low. Additionally, nuclear translocation and activation of AFB_1 and AFB_1–protein conjugates were assessed using rat liver nuclei. Proteins containing a NLS, e.g. histones and albumin–NLS, facilitated AFB_1 translocation into the nucleus, where activation and adduct formation occurred (Ch'ih *et al.*, 1993).

AFB_1 is reported to be capable of covalently binding to mitochondrial DNA with a 3- to 4-fold greater affinity than for nuclear DNA (Niranjan *et al.*, 1982). Lesions in mitochondrial DNA are persistent, perhaps reflecting a lack of *appropriate* excision repair mechanisms in this organelle. As a result, mitochondrial transcription and translation may be persistently inhibited by these lesions, contributing to neoplastic transformation of the cell (Hsieh, 1987).

Methylation of DNA may be inhibited by covalent binding of AFB_1 to DNA, thereby altering gene expression and cellular differentiation. Then, oncogenes may be activated, precipitating oncogenic transformation of mammalian cells by producing heritable transcriptional mutations in these genes (Wilson and Jones, 1983).

4.4.2. *The* Ras *Oncogenes and Hepatocellular Carcinoma*

Ras proto-oncogene activation by several carcinogens in tumour development has been well documented (Reynolds *et al.*, 1987; Balmain and Brown, 1988). More recently, AFB_1 has been demonstrated to activate the Ki-*ras* gene in rat liver. In this regard, in the final stages of AFB_1-induced rat liver hepatocellular carcinoma (HCC), two activating mutations in the codon 12 region of Ki-*ras* genes (GGT → GAT (McMahon *et al.*, 1987) and GGT → TGT (Sinha *et al.*, 1988)) have been identified. Soman and Wogan (1993) have confirmed the Ki-*ras* codon 12 GGT → GAT mutation in rat liver, suggesting the involvement of this genetic mutation in the development of AFB_1-induced HCC in rats. There is, however, no evidence that *ras* gene mutations occur in human HCC (Bailey and Williams, 1993).

4.4.3. *The* p*53 Gene and Hepatocellular Carcinoma*

Recently, evidence has been accumulating regarding the development of human HCC, with respect to aflatoxin, the *p*53 tumour-suppressor gene, and more specifically, codon 249 of this gene. Hsu *et al.* (1991) have found in Chinese patients a striking mutational specificity in the third base position of codon 249 of the *p*53 gene, resulting primarily in a G → T substitution. In Southern African and Asian patients, this transversion was detected at codon 249 in about 50% of the analysed HCC tumours (Hsu *et al.*, 1991; Bressac *et al.*, 1991). In non-human primates, however, no mutations at codon 249 were detected in AFB_1-induced tumours (Fujimoto *et al.*, 1992). The data for rats suggest that AFB_1 alone is not sufficient to account for the specificity of the *p*53 mutations in HCC. While Lilleberg *et al.* (1992) are of the opinion that alteration of the *p*53 suppressor gene is involved in HCC induction in rats, the results of Hulla *et al.* (1993) regarding the specificity of the *p*53 mutation, however, suggest that AFB_1 is not responsible for these lesions.

Hsieh *et al.** attempted to assess the correlation between mutations at codon 249 and the level of AFB_1–DNA adducts in the liver tissue of HCC patients from a high AFB_1 risk area (Taiwan) and a low AFB_1 risk area (Japan). The AGG → AGT transversion was found in 21% of Taiwanese patients and none of the Japanese patients. AFB_1–DNA adducts, however, were found in tumorous and non-tumorous tissues from both groups of patients. Furthermore, AFB_1–DNA adducts were found in 50% of patients lacking the *p*53 mutation. It is the opinion of Hsieh *et al.* that adducts reflect recent AFB_1 exposure and so may not be a reliable index of earlier AFB_1 exposure that may have precipitated the induction of HCC.

In Japan, HCC is the third leading cause of cancer-related deaths (Nose *et al.*, 1993). Nose *et al.* (1993) have detected *p*53 gene alterations in only 30% of HCC patients, and invariably only in advanced cases, suggesting that *p*53 gene alteration may be a late event in tumorigenesis of HCC in Japan. Additionally, in Japan, hepatocarcinogenesis is often associated with a persistent HBV (or hepatitis C virus) infection, rather than with aflatoxin exposure (Nose *et al.*, 1993).

Chen *et al.* (1992) have reported that in Taiwanese patients, 70% of the HCC smears assessed were positive for AFB_1–DNA adducts. The findings relating the involvement of both HBV and AFB_1 in HCC development are controversial (Santella *et al.*, 1993). The results of these recent investigations serve to reinforce the idea of a multifactorial aetiology for the development of HCC. In attempting

*Hsieh, D. P. H., Atkinson, D. N. and Zhao, M.-S. (1992) Aflatoxin-DNA adducts and *p*53 gene alterations in human liver tumors. In: *Proceedings of the VIII International Symposium on Mycotoxins and Phycotoxins*, November 1992, Mexico City, Mexico, p. 36.

to determine the agent(s) involved in regional development of liver cancer, many factors need to be considered: physiological and ethnic differences; additional microbial agents (other than aflatoxigenic fungi) and local contaminants of foods and feeds. Wogan (1992)* has suggested that a possible synergistic response may exist between chemical and viral agents in the environment and has considered the possibility of other mycotoxins acting as mutagenic and carcinogenic agents. Of these mycotoxins, the fumonisins and sterigmatocystin are the most likely candidates. While these mycotoxins have been found to be relatively potent liver carcinogens in experimental animals, little is known about human exposure (Wogan, 1992). It is the opinion of Fujimoto *et al.* (1992) that the development of the mutation in codon 249 of the *p*53 gene in human HCC is likely to involve environmental carcinogens other than AFB_1, or that the HBV hepatitis is a prerequisite for AFB_1-induced G → T transversion in the codon. It is also probable, as is suggested by Puisieux *et al.* (1991), that the *p*53 mutational hotspots identified in different tumours are selected targets for specific environmental carcinogens.

In a study on Gambian children, Wild *et al.* (1992) found that the majority of individuals (75–100%) had AFB_1-albumin adducts. Children who were positive for HBV surface antigens had higher adduct levels than children with markers of past infection or who had never been infected with the virus. There were highly significant differences between three major ethnic groups, necessitating consideration of other physiological factors, such as polymorphism in cytochrome P450 and GST. In this regard, Hollstein *et al.* (1993) found an AGG → AGT transversion at codon 249 and an ATC → AAC transversion at codon 254 in 15 Taiwanese HCC patients. All but one patient were negative for AFB_1–liver adducts and AFB_1–serum albumin adducts. On genotyping patients for GST, it was found that 12 of the 15 patients possessed the null genotype.

Ozturk *et al.* (1991) have provided some of the best evidence relating AFB_1 ingestion with HCC development. They noted a specific mutation in the *p*53 tumour-suppressor gene in hepatoma tissue from patients at high risk of AFB_1 exposure. In their study, in four countries where AFB_1 intake was high, 22% of tumour samples had the characteristic mutation at codon 249 of the *p*53 gene, in comparison with less than 1% in tumours from patients from countries where the risk of AFB_1 intake was low. HBV infection was commensurately high in countries where AFB_1 contamination of food was prevalent. Ozturk *et al.* (1991) then compared similar incidents of HCC and HBV in a high AFB_1 intake area (Mozambique) with a low AFB_1 intake area (Transkei). In Mozambique, 53% of patients exhibited this mutation, while only 8% of the patients had this mutation in the Transkei. Considering that both groups had similar HBV exposure levels (approximately 11%), it was suggested that HBV was not responsible for the difference in incidence of codon 249 mutations. Those findings substantiate earlier reports of van Rensburg *et al.* (1985) that the estimated daily intake of AFB in Mozambique was approximately four times that of Transkeians. The ratio of HCC incidence in these two areas was similar, suggesting an aetiological role for AFB_1 as a procarcinogen in the development of liver cancer. However, Kolars (1992) still is of the opinion that HBV (or other agents of chronic liver disease endemic to particular areas) may be a prerequisite for AFB_1-mediated HCC. In this regard, Hsing *et al.* (1991) found that in 65 counties in China, HCC mortality rates were significantly linked to the prevalence of HBV surface antigen positivity. Incidence was higher where there was elevated levels of blood cholesterol, greater liquor consumption and a diet high in rapeseed oil and mouldy corn. No significant correlation was found between mortality and the levels of AFB_1 in urine. It is the opinion of Hsing *et al.* (1991) that HBV infection contributes to the substantial variation in liver cancer mortality in China, but they recognise the importance of dietary and environmental factors.

Patel *et al.* (1992) assessed patients from the United Kingdom, other countries of low AFB_1 intake and countries of high intake for *p*53 gene mutation at codon 249. The incidence of the mutation was low in all samples, and it is their opinion that other environmental factors need to be considered regarding the aetiology of human HCC. More recent work, however, has demonstrated the presence

*Wogan, G. N. (1992) Experimental and epidemiological evidence associating aflatoxin exposure, liver cancer risk and the involvement of oncogenes and tumor suppressor genes in liver carcinogenesis in humans. In: *Proceedings of the VIII International IUPAC Symposium on Mycotoxins and Phycotoxins*, November 1992, Mexico City, Mexico, p. 60.

of AFB_1–DNA adducts in a range of tissues taken from autopsy specimens in the United Kingdom (Harrison *et al.*, 1993).

Indirect evidence for AFB_1 involvement in HCC comes from Chongming Island, a high risk region for HCC near Shanghai. Since the 1960s, there has been a marked decrease in the use of maize, a commodity invariably high in aflatoxins. Concomitantly, a subsequent regression in the local incidence of liver cancer has been recorded (Ross *et al.*, 1992). In another study, Yu (1992) measured AFB_1 intake and AFM_1 excretion in 81 households in 10 villages in the Chinese province of Guangxi and found a positive correlation between PLC mortality and AFB_1 intake from maize and peanut oil, but interestingly not from rice. Groopman *et al.* (1992b), in the Guangxi Autonomous Region, when analysing total AFB_1-N^7-guanine excretion in urine plotted against total AFB_1 exposure, found a correlation of 0.8, suggesting that measuring excreted AFB_1 is a good indication of the level of AFB_1 consumption and adduct formation.

Yap *et al.* (1993), on reviewing the incidence of HCC, HBV and AFB_1 intake, concluded that both HBV and AFB_1 are risk factors and, in fact, may have a cumulative effect on HCC development. Yap *et al.* (1993) comment further that while HBV increases the likelihood of HCC, it is not essential for the development of HCC. Similarly, Zhang and co-workers (Zhang *et al.*, 1991), investigating the presence of AFB_1–DNA adducts and of HBV surface antigens in Taiwanese HCC patients, concluded that both AFB_1 and HBV may be involved in HCC development in Taiwan. In another study, Wu-Williams *et al.* (1992) have utilised the data generated for HBV, AFB_1 and HCC incidence in southern Guangxi, China, to generate models evaluating the relative importance of AFB_1 and HBV in the development of HCC. While purely additive models fitted the data poorly, multiplicative relative risk and interactive excess risk models provided satisfactory descriptions of that data and the data for the United States, a low risk area.

Recently, Wild and co-workers (Wild *et al.*, 1993), in assessing the numerous investigations involving HCC, HBV and aflatoxin, have concluded that despite the plausibility of an interaction between these two aetiological agents in HCC development, strong evidence supporting an interactive mechanism has not been elucidated.

In an extensive review of the research involving AFB_1–DNA adduct studies, Choy (1993) has commented that from both ingestion and injection studies, the dose–response of DNA adduct formation (mainly in rats) is linear, with no apparent threshold value. Based on these assessments, Choy (1993) has warned that extrapolation of this data to humans should be viewed critically, since human AFB_1–DNA adduct data are incomplete, although Groopman *et al.* (1993) concluded from their study that the presence of AFB_1–guanine adducts in urine is a good non-invasive marker for exposure to AFB_1 and the risk of genetic damage. Additional investigations undoubtedly will improve the risk assessment for humans with respect to AFB_1, and perhaps elucidate the individual contribution of AFB_1 and HBV to the development of HCC.

4.4.4. *Cytochrome P450, Glutathione S-transferase and Hepatocellular Carcinoma*

In the recent literature, efforts at understanding AFB_1 toxicity and the enigma of HCC development appear to concentrate on the GST enzymes (resulting in AFB_1 detoxification) and, to a lesser extent, on the cytochrome P450 bio-activation (and in some instances, detoxification) isoenzymes. Many of these studies are still in their early stages, but the general opinion is that a protective effect is afforded by GSTs in different tissues, as measured by decreases in AFB_1–DNA adduct formation with increased GST activity (Hayes *et al.*, 1991a; Coulombe, 1993).

Metabolism of AFB_1 involves oxidative reactions by members of the cytochrome P450 supergene family of isoenzymes. Different cytochrome P450 isoenzymes can result in AFB_1 metabolites of varying carcinogenic potential; for example, in humans, the formation of DNA–AFB_1 adducts depends on activation by cytochromes P450IA2, P450IIA3, P450IIIA4 and P450IIB [decreasing order] (Aoyama *et al.*, 1990) and cytochrome P450IA enzymes metabolise the detoxification of AFB_1 to AFM_1 (Koser *et al.*, 1988). Other cytochrome P450 isoenzymes are responsible for the conversion of AFB_1 to other less toxic metabolites: AFQ_1 (in humans, by P450IIIA (Forrester *et al.*, 1990)) and AFP_1 (Hayes *et al.*, 1991a).

At least 10-fold differences in cytochrome P450IIIA and P450IA expression have been observed between individuals (Watkins, 1990). In humans, it would appear then that the cytochrome P450IIIA

family is responsible for both AFB_1 epoxidation and the formation of AFQ_1 (Forrester *et al.*, 1990). The ability of a cytochrome P450 to catalyse both the activation and the detoxification of AFB_1 has been reported elsewhere (Guengerich *et al.*, 1992). AFM_1 production by human hepatic microsomes from different individuals correlates with the level of P450IA2 (Forrester *et al.*, 1990), while in rats, the $P_3$450 cytochrome appears to be involved (Koser *et al.*, 1988).

The ability of tissue to bio-activate AFB_1 is an important consideration in understanding the ability of AFB_1 to induce toxic, mutagenic or carcinogenic transformation in cells. In this regard, Imaoka and co-workers (Imaoka *et al.*, 1992) have investigated the genotoxic and mutagenic activation of AFB_1 on *Salmonella typhimurium* by rat hepatic, renal and pulmonary microsomal fractions and purified cytochrome P450 enzymes. Hepatic microsomes displayed the greatest mutagenic activation, while renal microsomes had the lowest activity. Additionally, cytochrome P450IIC2 (a major hepatic cytochrome P450 in male rats) had the highest activating ability, while renal forms of P450 (e.g. cytochromes P450IVA2 and P450 K-4) exhibited the lowest activities. It would appear that the greater ability of hepatic microsomes (as compared with pulmonary and renal equivalents) to bio-activate AFB_1 is dependent on the different classes of cytochrome P450s present in that tissue.

Various hepatic cell populations (hepatocytes, Kupffer and endothelial cells) have been found to differ in their AFB_1-bio-activating ability (Schlemper *et al.*, 1991). Ten-fold higher AFB_1 concentrations were required by non-parenchymal (Kupffer and endothelial) cells to obtain a similar number of *Salmonella typhimurium* TA98 revertants (as compared with parenchymal cells). In freshly isolated cells, AFB_1 was found to bind (although differentially) to DNA in both parenchymal and non-parenchymal cells in a dose-dependent manner (Schlemper *et al.*, 1991).

Metabolic activation of AFB_1 was studied using human cell lines that expressed individual cytochrome P450s (Crespi *et al.*, 1991). Cells expressing cytochrome P450IA2 were the most sensitive (at 10 ng mL^{-1}) to the toxic and mutagenic effects of AFB_1. Cells expressing cytochrome P450IIIA4 were 5- to 10-fold less sensitive than those expressing P450IA2. The least sensitive cells expressed cytochrome P450IIIA6, while cells resistant to 1 μg mL^{-1} AFB_1 expressed no cytochromes (Crespi *et al.*, 1991).

The ability of an organism (or a tissue) to form the AFB_1-epoxide might explain the sensitivity of trout and quail to AFB_1. A cytochrome P450 isolated from the livers of β-naphthoflavone-treated rainbow trout had a 15-fold greater ability to form AFB_1–8,9-epoxide than did either the phenobarbital-induced or β-naphthoflavone-inducible rat liver cytochrome P450s (Williams and Buhler, 1983). Similarly, Neal *et al.* (1986) found a cytochrome P450 with a particularly high epoxidation ability in quail liver microsomes.

In vitro epoxidation of AFB_1 was determined using liver microsomes from rats of different ages (as measured by adduct formation with calf thymus DNA). Newborn rats were capable of minimal AFB_1–DNA binding when compared with adults. Levels of the AFB_1–glutathione conjugate were similarly low in neonatal rats (Behroozikha *et al.*, 1992). These findings suggest that the immature liver is less efficient than the mature organ at activating and detoxifying foreign chemicals.

Kitamura *et al.* (1992) were able to transfect MCF-7 breast cancer cells with a plasmid containing cytochrome P450IIIA7 complementary DNA, obtaining three cell lines. These transgenic cell lines showed 8- to 10-fold higher sensitivity to AFB_1 than did the parental MCF-7 cells. These results would suggest that expression of this class of cytochrome P450 promoted the formation of reactive AFB_1 metabolites.

Expression of cytochrome P450 enzymes can be influenced by exogenous agents (Nebert *et al.*, 1991), e.g. cytochrome P450IIIA enzymes are inducible by glucocorticoids and rifampin (Watkins, 1990), and cytochrome P450IA enzymes can be induced by polycyclic hydrocarbons in cigarette smoke and by dietary 'green plant' flavones (Nebert *et al.*, 1991). In this regard, environmental agents might influence the susceptibility to AFB_1-mediated hepatocarcinogenesis by altering the expression of individual cytochrome P450 enzymes that either activate or detoxify AFB_1 (Kolars, 1992). In this regard, the observations of Lin *et al.* (1991) suggest that smoking might have a protective effect on individuals at risk of developing HCC. In that study, in a Fujian province considered to be a high AFB_1 intake area, the risk of hepatoma was significantly increased in non-smokers. One interpretation by Lin *et al.* (1991) is that smoking could impart protection, as cytochrome P450IA enzymes may be induced, thereby possibly promoting metabolism of AFB_1 to AFM_1 (essentially a detoxification reaction), rather than activation to more reactive metabolites by other cytochrome

P450 enzymes. Contrary to this, however, cytochrome P450IA2 has been shown elsewhere to be the most important cytochrome P450 isoenzyme promoting AFB_1 binding to DNA in humans (Aoyama *et al.*, 1990). Interestingly, Raney *et al.* (1992b) have postulated (based on experimental evidence) cytochrome P450IIIA4 to be the dominant enzyme in human liver microsomes involved in both the oxidation of AFB_1 to its epoxide (activation) and hydroxylation of AFB_1 to AFQ_1 (detoxification).

While it would appear that cytochrome P450 enzymes may be important considerations in explaining the relative susceptibilities of different animal species to AFB_1 (and many other noxious substances), the evidence implicating the protective effects of GST enzymes against AFB_1–DNA adduct formation in tissues is equally compelling. Although much of the work is either *in vitro* or utilises rats as experimental animals, the importance of AFB_1–glutathione conjugation as a significant detoxification mechanism cannot be ignored. The GSTs comprise a supergene family of enzymes that have been subdivided into 5 classes: α, μ, π, τ and microsomal (Hayes *et al.*, 1991a). However, little is known about the specific GSTs responsible for detoxifying AFB_1. In rats, the α-GSTs appear to have the greatest ability to metabolise 8,9-epoxides (Coles *et al.*, 1985).

It is well documented that animal species have differing susceptibilities to the mutagenic or carcinogenic effects of AFB_1 (Wong and Hsieh, 1976, 1980; Hsieh *et al.*, 1977; Roebuck and Wogan, 1977). In comparing AFB_1 toxicity in mice and rats, it is generally accepted that mice fall into the 'resistant' category, while rats are highly 'susceptible'. It was predicted that this difference was likely to depend (among other factors) on the differing abilities to detoxify AFB_1 (Hsieh *et al.*, 1977; Wong and Hsieh, 1980). When the proteins of complementary DNAs of rat GST Yc_1 and of mouse GST Yc were expressed from a prokaryotic expression vector in *Escherichia coli*, mouse isoenzyme activity towards AFB_1–8,9-epoxide had a 50-fold higher conjugating activity than did the equivalent isoenzyme of the rat (Beutler *et al.*, 1992). Beutler and colleagues are of the opinion that the α class GST Yc isoenzymes in mouse liver protect these animals from the hepatotoxic effects of AFB_1, perhaps explaining the differing (and marked) susceptibilities of these two animal species to AFB_1.

In a series of mouse whole body autoradiographic studies, a group of Swedish researchers have interesting results regarding the extrahepatic tissue localisation of AFB_1 (Larsson *et al.*, 1992; Larsson and Tjälve, 1992). Pretreatment of adult mice with a glutathione-depleting agent resulted in accumulation of tissue-bound label (AFB_1) in the nasal olfactory and respiratory mucosae, as well as the mucosae of the nasopharynx, trachea and oesophagus (which was not observed in non-pretreated mice) (Larsson and Tjälve, 1992). The authors of the latter study are of the opinion that glutathione is normally responsible for scavenging AFB_1 in these tissues, thereby preventing AFB_1–DNA adduct formation. In additional studies, AFB_1–DNA adduct formation was also located in several extrahepatic sites in rainbow trout (uveal melanin, vitreous humor, kidneys, olfactory rosettes and pyloric caecae) (Larsson *et al.*, 1992). Furthermore, in autoradiographic studies in 1- and 5-day-old mice, a marked localisation of [^{3}H]-AFB_1 was found in the nasal olfactory mucosa. *In vitro* incubation of nasal olfactory mucosa with AFB_1 demonstrated marked binding in this tissue. If, however, glutathione was added to the incubation medium, this binding was reduced. Autoradiography of [^{3}H]-AFB_1 in pregnant mice showed labelling of the foetal olfactory mucosa (at day 18 but not at day 14) (Larsson and Tjälve, 1992). It would appear then that *in vivo* accumulation of AFB_1 in extrahepatic tissues of infant mice may be related to low GST activity in the tissues of these animals, or alternatively, to the development of AFB_1 bio-activating enzymes (cytochrome P450s).

More recently, in whole body autoradiography of [^{3}H]-AFB_1 in marmoset monkeys, AFB_1 was localised in several extrahepatic sites, including the nasal olfactory (quantitatively the greatest binding) and respiratory mucosae, the mucosae of the nasopharyngeal duct, pharynx, larynx, trachea and oesophagus and the melanin of the eyes and hair follicles (Larsson and Tjälve, 1993). In addition, in *in vitro* micro-autoradiography, AFB_1 could be detected in the epithelial lining of several areas of the respiratory and alimentary tracts and the liver. If a cytochrome P450 inhibitor was added to the incubation medium, this binding was no longer apparent. Interestingly, in this study, the grey matter of the brain exhibited a greater binding capacity than did the white matter. The possible interaction between the binding of AFB_1 to melanin, photo-activation of AFB_1 upon UV exposure, and the development of skin tumours in albino mice has been discussed, the possible relevance of which may previously have been overlooked (Larsson and Tjälve, 1993).

Tjälve *et al.* (1992) have found that microsomal preparations of bovine olfactory mucosa have a greater affinity than liver microsomes to induce covalent binding of AFB_1 to calf thymus DNA and microsomal proteins. Addition of glutathione to these preparations decreased AFB_1–DNA binding. When cytosolic fractions of mouse liver (where AFB_1 resistance may be related to high hepatic GST activity) were added to the olfactory mucosal incubation medium, the decrease in AFB_1–DNA binding was more pronounced. The nasal olfactory mucosal tumours, which are found in relatively high frequencies in cattle in developing countries (many animals exhibiting signs of severe aflatoxicosis), might be explained in terms of the high AFB_1 bio-activating ability of bovine olfactory mucosa (i.e. P450 involvement) and perhaps lower levels of GST activity (Tjälve *et al.*, 1992).

Several other researchers have shown GST enzymes to be important protective agents against AFB_1–DNA adduct formation, e.g. Mandel *et al.* (1992) using low protein diets in 3-week-old rat weanlings and Liu *et al.* (1991) in comparing the ability of human liver fractions and lymphocytes to deal with aflatoxin and other foreign chemicals. Liu *et al.* (1991) found a highly significant correlation ($r = 0.88$) between AFB_1–DNA adduct concentrations and GST μ class activity. Tsuji *et al.* (1992), in comparing species and sex differences in AFB_1-induced GST placental forms, concluded that glutathione and GST play an important role in modulating hepatic AFB_1–DNA adducts.

Interestingly, human liver cytosolic fractions conjugated epoxide isomers to glutathione to a lesser extent than did similar cytosolic preparations from rats or mice (Raney *et al.*, 1992a). Moss and Neal (1985) previously had reported that human hepatic GSTs do not play an important role in protecting against AFB_1. The information, however, is too scant for any conclusions to be drawn regarding the physiological importance of GSTs in detoxification in humans.

When neonatal rats were exposed (first, third and fifth day) to diethylstilbestrol (DES) [previously used as an anabolic compound with oestrogenic properties], and then at 5 months of age treated with a single dose of AFB_1, DES-pretreated animals showed a 35% decrease in AFB_1–DNA adduct formation and a 2-fold increase in the levels of α-GST. Results suggest that neonatal DES treatment resulted in long-term protective increases in basal α-GST levels, causing lower levels of DNA adduction following adult exposure to AFB_1 (Zanger *et al.*, 1992). More specifically, Gopalan *et al.* (1992) found that in *in vitro* rat studies, the different classes of α-GSTs induced were dependent on the foreign chemical used. For example, the highest catalytic activity with microsome-mediated AFB_1–epoxide conjugation was observed with GST 3-3, while for synthetic AFB_1–epoxide conjugation, GST 4-4 appeared to be important. Thus, in rats, α-GST 3-3 may play an important role in inactivation of AFB_1-epoxide generated *in vivo* (Gopalan *et al.*, 1992).

4.5. Aflatoxin B_1 Transport and Repair of Aflatoxin–DNA Adducts

Transport of foreign chemicals out of cells involves two possible families of efflux pumps: the P-glycoprotein pump (specific for hydrophobic compounds) and the glutathione *S*-conjugate carrier (specific for drug–glutathione conjugates), both of which may play a role in eliminating AFB_1 from the cell (Hayes *et al.*, 1991a). The involvement of these two pumps in AFB_1 toxicity has generally not been researched. In one of the few articles pertaining to this line of work, Burt and Thorgeirsson (1988) have shown that AFB_1 induces the mRNA coding for the P-glycoprotein in mouse liver, thus implicating this pump in transport of the toxin.

Little information is available concerning removal of covalently bound AFB_1 from mammalian cells. The major adduct formed is the chemically unstable AFB_1–N^7-guanyl adduct, which is lost spontaneously (when mutations are likely to arise) from DNA to yield apurinic sites. The other two adducts (AFB_1 FAPY and AFB_1 III) are not lost spontaneously and may be catalytically removed by DNA repair enzymes (Hayes *et al.*, 1991a). In transformed xeroderma pigmentosum cells, Waters *et al.* (1992) found that up to 40% of AFB_1-induced genetic lesions were repaired after 6 hr, indicating that DNA repair may be important following AFB_1 exposure. Ball *et al.* (1990) have shown significant interspecies differences in repair capacity (AFB_1–DNA adduct removal) in cultured tracheal epithelium. This variation may be a factor accounting for the difference in the susceptibility of species to cancer of the respiratory tract. Leadon *et al.* (1981) have reported that the AFB_1–N^7-guanine adduct is removed spontaneously and enzymatically in fibroblasts, probably by nucleotide excision

repair mechanisms. The AFB_1–N^7-guanine adduct, however, may be converted to non-repairable, persistent AFB_1–formamidopyrimidine lesions.

Following a single dosing with AFB_1, maximum liver DNA adduct levels were measured after 2 hr. By 24 hr, 88% of the AFB_1–DNA adducts had been removed (Wogan *et al.*, 1980). Newberne and Wogan (1968) have postulated that in Fischer rats, the rapid removal of DNA adducts may be related to the requirement for multiple exposure to AFB_1 for the induction of tumours.

Thus, susceptibility and resistance to the toxic and carcinogenic effects of AFB_1 may depend on several factors: expression of bio-activating cytochrome P450s, detoxifying cytochrome P450s, GST activity, effective removal of AFB_1 detoxification products from the cell, and the ability for excision of AFB_1 adducts from DNA, and finally, repair of damage to nucleic acid (Hayes *et al.*, 1991a; Coulombe, 1993). While there may be volumes of relevant literature, there is still a need for research into particular aspects (toxicological, physiological and biochemical) of AFB_1 toxicity.

4.6. Anti-mutagenic Substances

If the GST detoxification mechanism is important in affording protection (resistance) against AFB_1–DNA adduct formation, then this resistance might be inducible and acquired (Hayes *et al.*, 1991a). Any factor that stimulates GST activity would have the potential to enhance intrinsic resistance mechanisms (Hayes *et al.*, 1991b). In this regard, several natural food extracts and, interestingly, anti-schistosomal drugs (e.g. oltipraz, a dithiolethione) have been found to enhance GST activity, and hence, AFB_1–glutathione conjugation. In male rats fed a diet supplemented with 0.03% 1,2-dithiole-3-thione and 250 μg AFB_1 kg^{-1} day^{-1} (0–4 days; 7–11 days), there was a 76% diminution in hepatic adducts and a 62 and 66% decrease in urinary AFB_1–N^7-guanine and serum AFB_1–albumin adducts, respectively (Groopman *et al.*, 1992a). Similarly, Kensler *et al.* (1992) have reported that male rats fed 1,2-dithiole-3-thione (0.001–0.003%) all showed signs of elevated hepatic GST activities. Four- to 6-fold increases in the specific activities of aflatoxin–glutathione conjugation were recorded in cytosolic preparations of livers of animals fed 1,2-dithiole-3-thione. Several extrahepatic organs also showed elevated GST activity (Kensler *et al.*, 1992). Roebuck *et al.* (1991) claim that oltipraz (0.075%) offers complete protection against AFB_1-induced hepatocellular neoplasms, reducing mortality and hepatic AFB_1–DNA adducts in rats 3 months after dosing. This anti-schistosomal drug thus appears to be a general inducer of detoxification enzymes and might warrant use as a protective agent.

Bolton *et al.* (1993), based on the results of rat trials, have suggested that oltipraz may exert considerable activity against the cytotoxic and auto-promoting action of repeated exposure to AFB_1. It is suggested that oltipraz be utilised in intervention trials in human populations frequently consuming AFB_1-contaminated foods, and more particularly, in areas with high incidences of liver cancer (Bolton *et al.*, 1993).

There is a wealth of information concerning the anti-mutagenic properties offered by several plant products (Wattenberg, 1985; Brockman *et al.*, 1992; Ueno, 1993). Pretreatment of male rats with geniposide (as a penta-acetyl derivative), an extract of *Gardenia fructus*, resulted in consistent elevation of the activities of GST and γ-glutamylcysteine synthase (Wang *et al.*, 1992). Wang's group is of the opinion that defence mechanisms of rat hepatic tissue may incorporate the enhanced GST activity and induction of γ-glutamylcysteine synthase for glutathione biosynthesis. Geniposide was found to inhibit the damage to DNA caused by AFB_1 in C3H10T1/2 cells (Tseng *et al.*, 1992). Additionally, the inhibitory effect of AFB_1 on DNA synthesis was also overcome. In another study, geniposide was capable of inhibiting the growth and development of C-6 glioma cells in rats (Wang *et al.*, 1993).

The suppressive effects of another plant product, crocetin (a carotenoid), on hepatocellular lesions induced by AFB_1 were investigated in male rats fed AFB_1 and crocetin for 10 days. Thirty-five weeks later, the group that had received AFB_1 and crocetin exhibited a 40% reduction in liver lesions as compared with the AFB_1-alone group (Wang *et al.*, 1991). The crocetin-only group exhibited no lesions. The protective effects of crocetin may result from elevated GST activity and decreased formation of hepatic AFB_1–DNA adducts (Wang *et al.*, 1991).

GST-inducing activity has been documented for active compounds in lemongrass oil (from *Cymbopogon citratus*) and galanga root oil (from *Alpinia galanga*) (Zheng *et al.*, 1993). In female A/J

mice, D-limonene (from lemongrass oil) resulted in a 2.4- to 3-fold increase in GST activity in liver and small and large intestinal mucosae, while geraniol (in lemongrass oil) induced a 2.5-fold increase in intestinal mucosae only. The *trans*-cinnamate (from galanga root oil) induced increased GST activity in the liver and intestines (Zheng *et al.*, 1993). Limonene is a component of several essential oils and is often used for flavourings and colourings and so, these oils may be chemo-protective agents that can be used as dietary supplements.

Recently, grapefruit juice has been demonstrated in *in vitro* experiments with human liver microsomes to have anti-carcinogenic properties against AFB_1 (Guengerich and Kim, 1990). Naringenin, an aglycone of the flavonoid naringin, most abundant in grapefruit and related citrus, was found to inhibit the activation of AFB_1.

A number of plant phenolic compounds have been tested for their ability to modulate microsome-mediated activation of AFB_1 and influence covalent adduct formation of activated metabolites with DNA. Bhattacharya and Firozi (1988) report that polyhydroxyl flavonols, e.g. robinetin, quercetin, fisetin and morin, were active in this regard. In *Salmonella typhimurium*, kaempferol and rutin were very active in inhibiting AFB_1 mutagenicity by up to 50% (Francis *et al.*, 1989). Catechin, a phenolic flavonoid, effected a dose-dependent reduction (up to 40%) in AFB_1–DNA binding (Prasanna *et al.*, 1987). Observations from this last study indicate that mixed-function oxidases (cytochrome P450-dependent), essential for the metabolic activation of these carcinogens, exhibit differing sensitivities to the various inhibitors of their activity.

Other food additives, such as tumeric, garlic and asafoetida, have been shown to contain anti-oxidants, which scavenge free radicals and inhibit chemically-induced carcinogenesis (Unnikrishnan and Kuttan, 1990). Soni *et al.* (1993) found that AFB_1-induced fatty acid changes, necrosis and biliary hyperplasia were inhibited by daily doses of tumeric, curcumin and ellagic acids (all containing anti-oxidants). Garlic and asafoetida inhibited necrosis and degeneration of the tissue, but biliary hyperplasia persisted (Soni *et al.*, 1993).

Garlic extracts have long been recognised to possess anti-mutagenic properties. Tadi *et al.* (1991) tested the effects of two garlic extracts (ajoene and diallyl sulphide (DAS)) on the metabolism and binding of AFB_1 to DNA. These two organo-sulphur extracts inhibited AFB_1 binding to calf thymus DNA and reduced adduct formation. GST levels appeared unaffected by ajoene and DAS, suggesting that AFB_1 metabolism and DNA binding is influenced by inhibition of Phase 1 (activation) enzymes (cytochrome P450s). Additionally, DAS selectively induced cytochrome P450IIB1/2 [reported elsewhere to activate AFB_1 (Aoyama *et al.*, 1990)] in rat liver, primarily due to transcriptional activation, while the levels of cytochrome P450IIE1 were unaffected (Pan *et al.*, 1993). Furthermore, while levels of cytochrome P450IIB1/2 mRNA were elevated in liver, stomach and duodenal tissue, levels in lung and nasal mucosae remained unchanged. This serves to reinforce the idea of tissue selective activation/inactivation by foreign chemicals.

Extracts of four Chinese medicinal plants traditionally used in the treatment of lung, liver and rectal tumours (*Oldenlandia diffusa* (Rubiaceae); *Scutellaria barbata* (Labiatae); *Astragalus membranaceus* (Leguminosae); *Ligustrum lucidum* (Oleaceae)), produced concentration-dependent inhibition of histidine-independent revertant (His^+) *Salmonella typhimurium* colonies induced by AFB_1 (Wong *et al.*, 1992). Additionally, extracts of three of these plants inhibited AFB_1–DNA binding and reduced the formation of organo-soluble metabolites of AFB_1. In some instances, when used in combination, the effects were additive. Similarly, Chulasiri *et al.* (1992) have found two flavonoids (hispidulin and hortensin) from *Millingtonia hortensis* (Bignoniaceae) to have anti-mutagenic properties against AFB_1 in *Salmonella typhimurium* without being cytotoxic to the bacterial cells. Three water- and ethanol-soluble extracts of a Chinese green tea exhibited inhibitory effects on the development of precancerous enzyme-altered hepatocellular foci in rats (Qin, 1991). To date, only a single extract of green tea [(−)-epigallocatechin] has been identified (Ueno, 1993). Ruan and co-workers (Ruan, 1992; Ruan *et al.*, 1992) reported that eight natural foods (sesame, chestnut, dad-lily, laver, red Chinese date, bamboo shoot, kelp and green tea), regularly sold in markets in China, exhibited anti-mutagenic properties, with the more stable anti-mutagenicity being shown by dad-lily and green tea. Interestingly, the mutagenicity of *Salmonella typhimurium* induced by *Aspergillus versicolor* was inhibited by dad-lily, bamboo shoots and green tea, while, red Chinese date inhibited the mutagenicity induced by *Aspergillus ochraceus*. In addition to *Aspergillus flavus*, these two *Aspergillus* species are frequently found associated with maize in that area (Ruan *et al.*, 1992).

Brockman *et al.* (1992) have provided an extensive summary regarding the antimutagenic properties of the retinoids (vitamin A and its derivatives) and the carotenoid, β-carotene. With respect to the anti-mutagenicity against AFB_1, most dietary antimutagens were active against AFB_1. In the light of these findings, Brockman *et al.* (1992) have questioned the current view that AFB_1 is a potent mutagen and carcinogen *in vivo*, particularly in animals and humans on a well-balanced diet.

Recently, chlorophyllin, a non-toxic derivative of chlorophyll, has been shown to inhibit AFB_1–DNA adduct formation in a dose–responsive manner in *in vivo* animal models, suggesting it as a promising candidate for chemo-prevention (Dashwood *et al.*, 1991).

Wattenberg (1985) has divided the chemo-preventative agents (largely dietary), into three categories, depending on the level at which they are effective at preventing tumour development (Fig. 4). Thus, chemo-protectants are classified as compounds that *prevent* formation of carcinogens, as *blocking* agents, preventing the carcinogen from reacting with cellular molecules or as *suppressing* agents that inhibit the neoplastic change in cells following binding of the carcinogen to cellular components. The phenols appear to be effective at all three levels (Wattenberg, 1985).

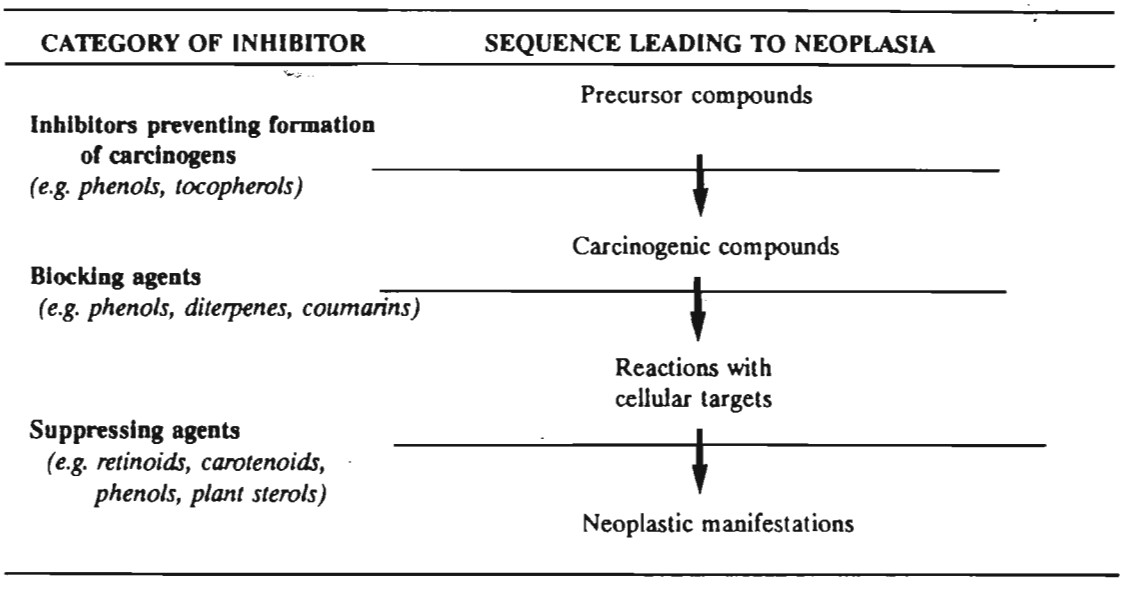

Fig. 4. Categories of chemo-protective agents, based on the stage at which they exert their protective effects. Adapted from Wattenberg (1985).

5. DIET AND CANCER

Dietary compounds may inactivate transcription of cytochrome genes, the products of which may be responsible for epoxide formation of several xenobiotic chemicals. Free radicals have long been thought to be involved in tumour development (Harman, 1962; Mavelli and Rotilio, 1984; Troll and Wiesner, 1985). Considering that a number of potential mutagens and carcinogens are diet-related, it would be impossible to eliminate them completely. Since the generation of free radicals appears to be more or less a universal factor contributing towards tumour development, any compound that can scavenge the oxygen and hydroperoxide radicals, thereby preventing their peroxidation of critical cellular components, would be beneficial.

Many other dietary factors (e.g. selenium, vitamin E, sulphur-containing amino acids, copper and zinc) are known to affect the anti-oxidative ability of an organism (Huang and Fwu, 1993). Protein deficiency has been presumed to impair the anti-oxidative defense system (Chow, 1988). Recently, Huang and Fwu (1993) have demonstrated that the degree of protein deficiency in rats affects the extent of depression of the activities of the anti-oxidative enzymes (e.g. glutathione peroxidase, superoxide dismutase) and hence, the degree of tissue lipid peroxidation. An AFB_1-containing diet supplemented with methionine was found to attenuate the AFB_1-induced increase in hepatic glutathione in domestic fowl (Beers *et al.*, 1992).

Dietary lipids have been demonstrated to modulate the metabolism and toxicity of xenobiotics by regulating the activities of Phase 1 (activation) and Phase 2 (detoxification) metabolism (Yang *et al.*, 1993), with cytochrome P450 enzymes being differentially activated (Yoo *et al.*, 1992).

Bailey and Williams (1993) have provided a review on food-related carcinogens and anti-carcinogens, discussing the concept of free radical scavengers (particularly in the diet) as chemo-protectants against tumour development. Ueno (1993) has also reviewed the concept of diet/toxin interactions and has emphasised the protective role afforded by free radical scavengers.

6. STRUCTURALLY RELATED MYCOTOXINS

Sterigmatocystin is structurally similar to AFB_1 with respect to dimensions and configuration of the bisdihydrofuran moiety, resulting in the possibility of metabolic activation at the same site, the $C_8 = C_9$ double bond. Sterigmatocystin, therefore, may operate metabolically in the same fashion as AFB_1. Sterigmatocystin–AFB_1 adducts have been reported (Reddy *et al.*, 1985; Gopalakrishnan *et al.*, 1992), and sterigmatocystin was found to be cytotoxic and mutagenic to cultured Chinese hamster cells (Noda *et al.*, 1981).

7. OTHER MYCOTOXINS

Jelinek *et al.* (1989) has drawn attention to the significant concentrations of a large number of mycotoxins produced by *Fusarium*, *Penicillium* and *Aspergillus* species found naturally contaminating foods and feeds. Only a few of the mycotoxins (such as AFB_1, ochratoxin A, citrinin and to a lesser extent, T-2 toxin, and the ergot alkaloids) have been investigated in sufficient depth such that a risk assessment can be attempted. Newberne (1993), however, has reported than several mycotoxins (including the fumonisins, a number of trichothecenes and ochratoxin) are now under review by the WHO–IARC and several national public health agencies. It is reported, however, that AFB_1 is currently the only mycotoxin that is regulated by the FDA (Coulombe, 1993).

Ruan (1991) considers the possibility of co-mutagenic effects of more than one fungal metabolite in the incidence of liver cancer in Fusui county, China. The geographical variation in incidence of PLC indicates involvement of specific factors, whether these be genetic or environmental epidemiological factors. The evidence involving AFB_1 and HBV is equally provocative and both factors must be considered in any risk assessment.

The fumonisins, which are now being reported as frequent contaminants of crops (Sydenham *et al.*, 1993; Ueno *et al.*, 1993), have received specific attention as causal agents in certain pathological conditions in humans and animals. In this regard, fumonisin B_1 has been epidemiologically associated with human oesophageal cancer in some regions of the Transkei, South Africa (Sydenham *et al.*, 1990), but as with many diseases of suspected mycotoxin aetiology, the evidence is only circumstantial. The pathologies associated with fumonisin B_1 are interesting, since, unlike AFB_1, which is known to be hepatotoxic to many animals, fumonisin mycotoxicoses are manifested in different organs, depending on the animal involved. Fumonisin B_1 results in equine leukoencephalomalacia (Kellerman *et al.*, 1990; Wilson *et al.*, 1992), liver cancer in rats (Gelderblom *et al.*, 1991) and pulmonary oedema in swine (Colvin and Harrison, 1992). Methods of isolation and purification of these mycotoxins have been developed only recently and so, the full extent of the potency of these mycotoxins has not yet been characterised completely. Mention should also be made of the possibility (or likelihood) of the co-contamination of foods and feeds by more than one mycotoxin, making diagnosis of the disease condition difficult. The area of multiple mycotoxin exposure, particularly relating to humans, warrants intensive investigation (Bach and McLean, 1993).

8. CONCLUSIONS

Since the discovery of the aflatoxins in the early 1960s, the study of mycotoxins has passed from a fringe area of vague inter-relationship with nutritionally derived disease to a large-scale

multidisciplinary research effort investigating many fungal metabolites. The research endeavours are ongoing and not least with respect to the studies on AFB_1, the most commonly occurring of the aflatoxins.

One of the most significant discoveries with respect to AFB_1 was its carcinogenic properties when Butler (1964) showed that of all the naturally occurring carcinogens, the rat exhibited the greatest susceptibility to AFB_1. Not surprisingly, the impact of this discovery and the accumulating evidence has led to the implication of AFB_1 in the development of human HCC. It may be that, in the final analysis, aflatoxin together with other environmental agents, such as the HBV and other mycotoxins, will provide a model that explains the origins and mechanisms of cancers that are dependent on an interaction between environmental and genetic factors.

Acknowledgements—The authors acknowledge the services of the Electron Microscope Unit, Faculty of Medicine, University of Natal. Research was made possible through funding from the Faculty of Medicine, University of Natal, South Africa.

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FOOD ADDITIVES AND CONTAMINANTS, 1995, VOL. 12, NO. 3, 435–443

Aflatoxin B_1—its effects on an *in vitro* plant system

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The phytotoxic effects of aflatoxin B_1 (AFB_1) on *in vitro* cultures of differentiating calli and regenerating plantlets of *Nicotiana tabacum* were assessed. Callus appeared more sensitive to the effects of AFB_1, with fresh mass accumulation and callus chlorophyll levels affected at low (approximately 0·5 μg/ml) aflatoxin concentrations. Transmission electron microscopy revealed early deteriorative alterations in chloroplast morphology. Inhibitory effects of the toxin (up to and including 10 μg/ml) on callus fresh mass accumulation were reversed following a 3 week toxin-free recovery period. In tobacco plantlets, root and leaf development, and root and leaf mass were significantly inhibited in a dose-dependent fashion with increasing AFB_1 concentration above 0·5 μg/ml. Inhibitory effects on plantlet root development were more pronounced that on leaf development.

Keywords: aflatoxin B_1 (AFB_1), regenerating plantlets, tissue culture, tobacco callus, transmission electron microscopy

Introduction

It is well documented that aflatoxin B_1 (AFB_1), produced by *Aspergillus flavus* and *Aspergillus parasiticus*, is a potent hepatocarcinogen in many animal species (Hsieh *et al.* 1977), and has been implicated as an aetiological agent in the development of hepatocellular carcinoma in several African and Asian communities (Wogan 1992). Pre-harvest contamination of crops, especially peanuts and maize, by aflatoxin is frequently reported, and is of agricultural, economic and medical concern (Nichols 1983, CAST 1989). Traditionally, the aspergilli have been regarded as saprophytes, being mainly responsible for the deterioration of stored seeds (Christensen and Sauer 1982). Evidence has been accumulating regarding the opportunistic behaviour of these fungi, and probably more correctly, should be regarded as facultative necrotrophs (Lillehoj 1987), invading developing seed in the field and elaborating several mycotoxins. Furthermore, Mycock *et al.* (1992) have shown that *A. flavus* may be systemically transmitted, thereby being present in the plant body from sowing to harvest.

The involvement of *Aspergillus* sp. as plant pathogens has not been seriously entertained, but there is accumulating evidence that the aflatoxins (and the mycotoxins produced by *Fusarium* and *Penicillium* sp.) are phytotoxic compounds. The phytotoxic effects of the aflatoxins have been investigated, with respect to seed germination, and the inhibition of root and hypocotyl elongation (Reiss 1978, Dashek and Llewellyn 1983). However, elucidation of the precise mode of action of AFB_1 in the plant cell has been neglected. The present investigation utilizes an *in vitro* plant tissue culture system (*Nicotiana tabacum*) to assess the phytotoxic effects of AFB_1 on several biochemical and morphological processes.

0265-203X/95 $10·00

Materials and methods

Tobacco callus

Callus was initiated from young apical leaves of tobacco (*Nicotiana tabacum* var. Samsun) plants. The apical leaves were removed, surface-sterilized for 10 min in 1% (v/v) Hibiscrub (50 mg/ml chlorohexidine, ICI Pharmaceuticals, South Africa), followed by a 15 min wash in 1% (w/v) sodium hypochlorite. Leaves were then cut aseptically into small discs (approximately 2 mm × 2 mm) and plated onto a callus induction medium (pH 5·8), containing MS nutrients (Murashige and Skoog 1962), 2% (w/v) sucrose, 1% (w/v) agar, 2·3 μM kinetin and 11 μM indole acetic acid, and incubated in the dark at 25 ± 3°C for 2 weeks. At the end of this period, the initiated callus tissue was subcultured onto fresh medium, and placed under a photoperiod regime (16 h light at 200 μM/m^2/s photon flux density), and 25 ± 3°C. Following 10–14 days, calli had established sufficiently to be transferred to medium containing AFB_1 (0·1–2 μg/ml and 5–25 μg/ml), incorporated into the medium following autoclaving. As dimethyl sulphoxide (DMSO) was used to dissolve the crystalline AFB_1 for the stock solution, the controls for each dose contained the appropriate DMSO concentrations.

Individual calli were weighed and transferred to sterile culture tubes containing the AFB_1-containing medium. Tubes were sealed with Parafilm and calli were incubated for 3 weeks at 25 ± 3°C (16 h photoperiod; 200 μM/m^2/s photon flux density).

Tobacco plantlets

Calli were induced and allowed to differentiate (approximately 4 weeks, see above), after which they were transferred to a plantlet regeneration medium (MS nutrients, 2% sucrose, 1% agar, pH 5·8, hormones omitted). After 2–3 weeks incubation, tobacco plantlets of approximately 8–10 cm had established. Explants (approximately 2 cm) containing the apical bud and the leaf immediately subtending it were sampled and then inserted into the AFB_1-containing medium (0·5, 2, 10 and 25 μg/ml). Control plantlets were grown on the appropriate DMSO-containing medium. Explants were incubated for 3 weeks (16 h photoperiod at 200 μM/m^2/s photon flux density, and 25 ± 3°C).

Growth measurements

Tobacco callus. Following exposure of calli of AFB_1, parameters including fresh mass accumulation, callus chlorophyll and protein levels, percentage calli developing shoots and/or becoming necrotic, were assessed. Transmission electron microscopy (TEM) of callus material was also performed. After AFB_1 exposure, calli were transferred to a fresh AFB_1-free medium, and allowed to recover for 3 weeks. The same parameters were assessed, but no microscopical analysis was done.

Tobacco plantlets. Leaf and root mass, leaf number, leaf chorophyll and protein content and the percentage plantlets developing roots were determined. Data not presented in this report are discussed elsewhere (McLean *et al* 1994a, b).

Results were compared statistically (one way analysis of variance, LSD, $p \leq 0{\cdot}05$); different letters on figures represent significantly different values.

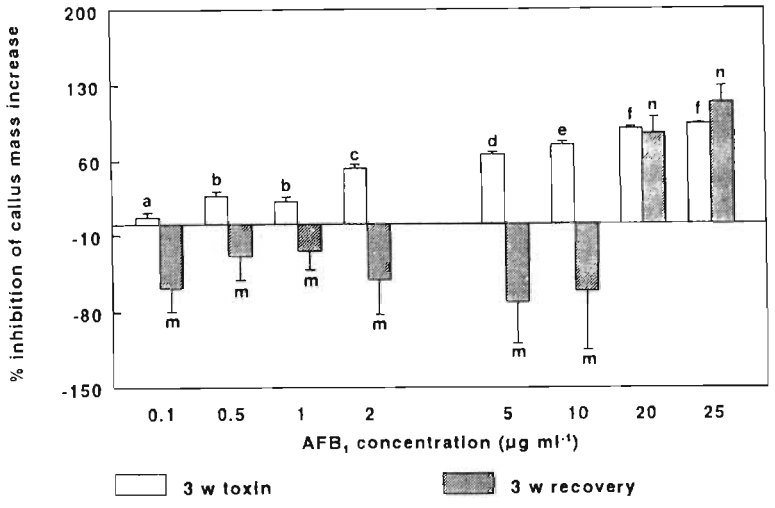

Figure 1. Percentage inhibition of callus fresh mass accumulation following a 3 week exposure period to AFB_1, and a 3 week toxin-free recovery period. A negative value corresponds to a fresh mass accumulation in excess of control values.

Results

Tobacco callus

The major trends observed in tobacco calli with increasing AFB_1 are presented in figures 1 and 2, and tables 1 and 2. Callus fresh mass accumulation (figure 1) and chlorophyll levels (figure 2) were significantly affected at AFB_1 concentrations as low as 0·5 and 1 µg/ml, respectively. The electron micrographs accompanying figure 2 confirm the early and progressive deterioration in chloroplast morphology with increasing AFB_1 concentration. Shoot development appeared relatively unaffected until 5 µg/ml AFB_1, while necrosis was apparent only in the high dose range of toxin (table 1). Browning of areas of calli in immediate contact with the medium was observed at all toxin concentrations, suggesting a direct toxic effect.

Tobacco plantlets

The number of leaves developing, and leaf mass per plantlet were significantly inhibited from 10 and 2 µg/ml AFB_1, respectively (figure 3). Similar data for root development are presented in figure 4. Leaf/root mass and leaf number showed an initial increase at 0·5 µg/ml AFB_1 before dose-dependent inhibitory effects were observed. The percentage rooted plantlets differed significantly from controls from an AFB_1 concentration of 0·5 µg/ml and, for root mass, the value was 10 µg/ml, after which a dose-dependent decrease with increasing toxin concentration was observed. A comparison of the I_{50} (50% inhibition) values for several parameters for tobacco calli and plantlets is presented in table 2.

Discussion

From the data presented in table 2, it is apparent that differentiating calli of *Nicotiana tabacum* are more sensitive to the phytotoxic effects of AFB_1 than are the more differentiated regenerating plantlets. Of the parameters measured, callus chlorophyll and fresh mass accumulation were negatively affected at low AFB_1

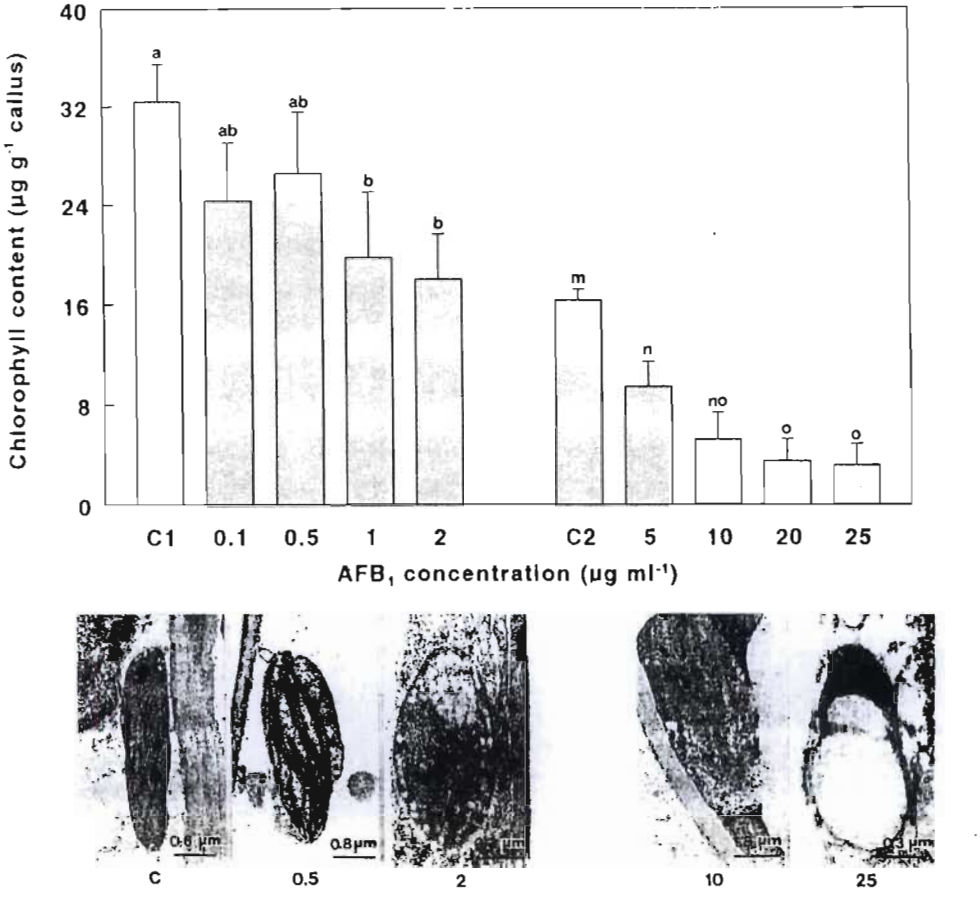

Figure 2. Callus chlorophyll content (0·1–25 µg/ml AFB_1) and chloroplast morphology (Control, 0·5, 2, 10 and 25 µg/ml AFB_1) following 3 weeks exposure to AFB_1. Values from the low and high dose ranges were not compared statistically.

concentrations. Such low doses have been reported to cause little or no effect on germination of various seeds (Jones *et al.* 1980, Llewellyn *et al.* 1982) and excised embryos (McLean *et al.* 1993a), or on root hypocotyl extension in a number of seedlings (Llewellyn *et al.* 1984, McLean *et al.* 1993a).

Higher AFB_1 concentrations were required to effect inhibitory responses in tobacco plantlets, when compared with callus, suggesting that differential tissue may be able to deal more effectively with the toxin. Uptake of AFB_1 from the culture medium has been confirmed by thin layer chromatography, and by immunocytochemical localization, using a gold probe (McLean *et al.* 1992). In this latter investigation, AFB_1 was immunolocated predominately in the nucleus, cytoplasm and the vacuole of stem cells of tobacco plantlets.

Of the parameters assessed in tobacco plantlets, root development was more seriously affected than leaf development. This is not surprising, considering that root initiation occurred in or in close proximity to the AFB_1-containing medium. Decreases in leaf mass and leaf number per plantlet followed a similar dose-dependent trend with increasing toxin concentration. Translocation of the toxin through the plantlet was indicated by the isolation of AFB_1 from root, stem and leaf tissue of AFB_1-exposed

Table 1. Percentage calli developing shoots and percentage calli becoming necrotic following 3 weeks of AFB_1 exposure.

AFB_1 concentration	% Calli with shoots	% Necrotic calli
Control 1	93 ± 3^{ab}	0^{m}
0·1 μg/ml	82 ± 2^{abcde}	0^{m}
0·5 μg/ml	89 ± 3^{abcd}	0^{m}
1 μg/ml	71 ± 21^{cde}	0^{m}
2 μg/ml	80 ± 0^{bcde}	0^{m}
Control 2	88 ± 4^{abc}	0^{m}
5 μg/ml	69 ± 11^{def}	13 ± 9^{n}
10 μg/ml	63 ± 17^{ef}	25 ± 1^{o}
20 μg/ml	49 ± 9^{f}	72 ± 4^{p}
25 μg/ml	14 ± 2^{g}	$90 \pm .2^{q}$

Table 2. Concentration of AFB_1 resulting in 50% inhibition (I_{50}) of the parameters measured for tobacco callus and tobacco plantlet *in vitro* systems.

	AFB_1 concentration ($\mu g\ ml^{-1}$)	
Parameter	Callus	Plantlet
Fresh mass	1–2*	Root ± 5 Leaf 10*–25
Chlorophyll content	5*–10	> 25
Protein content	10–20	> 25
% calli with shoots	20*–25	—
% rooted plantlets	—	10–25*
Leaf number	—	> 25

* I_{50} value closer to this value.

plantlets (data not presented). Observed inhibitory effects on leaf development may therefore arise from a direct toxic effect (i.e. the presence of the toxin in the tissue, possibly acting on a specific site(s)).

In animal cells, AFB_1 is known to be activated by mixed function microsomal enzymes to form the 8,9-AFB_1 epoxide (Swenson *et al.* 1974), which then binds specifically to the N^7 residues of the guanine bases of nuclear DNA (Benasutti *et al.* 1988), nucleolar DNA (Yu 1983) and also to mitochondrial DNA (Niranjan *et al.* 1982). As a result of impaired DNA template activity, transcriptional and translational processes are affected. DNA-dependent RNA polymerase activity is also inhibited (Gelboin *et al.* 1966). AFB_1 is capable of binding to DNA extracted from germinating *Zea mays* seedlings (Tripathi and Misra 1981). Furthermore, in AFB_1-treated maize seeds, those authors found decreases in DNA, RNA and protein synthesis (in that order), as well as an inhibition of DNA-dependent polymerase activity. In tobacco plantlets (and also in *Zea mays* root tips), AFB_1 has been immunolocated in the nucleus of stem cells (McLean *et al.* 1992). Those data suggest that the primary site of action of AFB_1 in the plant cell may mirror that in animal cells.

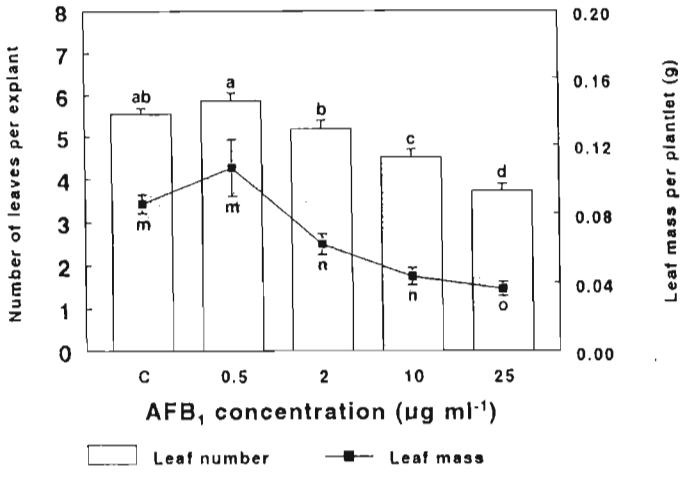

Figure 3. Number of leaves developing and leaf mass per plantlet following a 3 week exposure period of AFB_1.

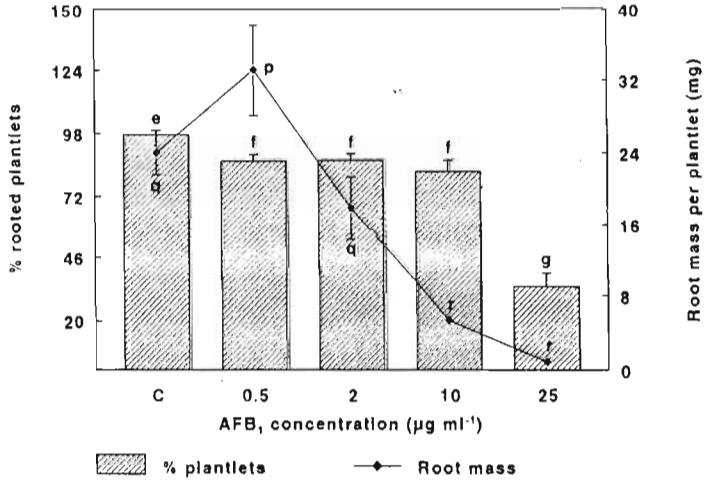

Figure 4. The effect of a 3 week exposure period of AFB_1 on the percentage plantlets developing roots, and on root mass per plantlet.

Since root development in tobacco plantlets was severely retarded above 10 μg/ml AFB_1, where roots generally developed above the medium (attesting to a direct toxic effect), the stunting of plantlets observed may be precipitated by impaired nutrient uptake and a consequent nutrient deficiency. Scanning electron microscopy of roots from these plantlets has revealed an inhibition of root hair development, especially at 25 μg/ml AFB_1, where areas of roots were completely devoid of root hairs (data not presented). These observed effects on the roots may have resulted in stunted plantlets, since any factor that inhibits root development is likely to cause a reduction in plant growth (Knoche and DuVick 1987).

When tobacco calli were transfered to an AFB_1-free medium for 3 weeks, following 3 weeks exposure to toxin, fresh mass accumulation of all calli (up to and including 10 μg/ml AFB_1) exceeded that of the respective controls. This apparent reversal of inhibition would indicate that normal metabolic events could proceed, once

Table 3. Summary of ultrastructural alterations observed in tobacco callus

Feature	Concentration	Ultrastructural observations
Chloroplasts	From 0·1 μg/ml	Starch degradation; large plastoglobuli; disruption to disappearance of grana; loss of membrane definition: membrane rupture; internal diffuse areas
	1 μg/ml	Most organelles degenerate with loss of membrane integrity; deterioration of thylakoids; starch depletion
	2 μg/ml	All of the above; loss of osmiophilic properties of grana
	5–25 μg/ml	Few intact organelles remain; most are severely deteriorated with membrane damage, diluted matrix, disrupted grana; many plastioglobuli
Mitochondria	0·1 μg/ml	Initial swelling of cristae in a few organelles
	From 1 μg/ml	Central diffuse clearing; loss of integrity of outer membrane; internal swelling of cristae
	2 μg/ml	Extensive swelling of cristae in some organelles
	5–25 μg/ml	All of the above, although some apparently normal mitochondria persist
Nucleus	0·1 μg/ml	Occasional nucleus with diffuse nucleoplasm
	from 0·5 μg/ml	Abnormal clumping of condensed chromatin; granular and fibrillar elements in nucleoplasm
	5–25 μg/ml	Largely abnormal nuclei with diffuse or abnormal clumping of chromatin
Lipid bodies	10–25 μg/ml	Increase in number; apparent fusion

the toxin had been removed, as has been reported for tobacco calli treated with T-2 toxin (Helgeson *et al.* 1973). Such an observation substantiates the probability of a direct toxic effect on AFB_1 exposure. Since growth could exceed that of controls by up to 60%, the possibility exists that cells may be able to metabolize (degrade) or sequester the toxin such that it is no longer inhibitory of mitosis or other metabolic processes. The immunolocalization of AFB_1 in the vacuole of tobacco plantlet stem cells suggests that the vacuole may act as a sink for AFB_1 (or its metabolites). The plant vacuole, apart from several other functions, has been reported previously to sequester noxious substances (Wink 1993).

Reversal of toxin-induced inhibition of fresh mass accumulation did not occur above 10 μg/ml AFB_1. It is probable that at these higher AFB_1 concentrations, the damage incurred during the exposure period was lethal to most of the cells in the callus mass. Repair, on removal of the offending compound, could therefore not be initiated. Electron microscopy following AFB_1 exposure revealed, however, that a few cells of 20 and 25 μg/ml-treated calli contained an apparently normal complement of organelles (see table 3 for summary of ultrastructural observations). These cells could perhaps represent 'resistant/tolerant' cells, and it may thus be possible to screen for the presence of resistance/tolerance of AFB_1 in the tobacco callus bioassay.

From the data presented, there is no doubt that AFB_1 is phytotoxic to differentiating tobacco calli, and to a lesser extent, to regenerating tobacco plantlets. AFB_1 probably

exerts its effects directly, by being absorbed and translocated, and/or indirectly, by imposing a nutrient stress on the *in vitro* plant system. The tobacco culture system can be used as a simple, sensitive bioassay system for the screening of potential environmental toxic compounds before trials are taken to the field.

Acknowledgements

The services of the Electron Microscopy Unit, Faculty of Medicine, University of Natal, Durban, are duly acknowledged. Research was made possible through funding from the University of Natal.

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Effects of aflatoxin B_1 on in vitro cultures of *Nicotiana tabacum* var. Samsun

2: *Root and shoot development in tobacco plantlets*

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Received 3 May 1993; accepted in revised form 12 October 1993

Abstract. The effects of aflatoxin B_1, (0.5–25 μg ml^{-1}) on in vitro root and shoot development in young tobacco explants were investigated. Despite an initial apparent stimulatory effect on most measured parameters at 0.5 μg ml^{-1} AFB_1, the number of leaves, root and leaf mass per plantlet were progressively inhibited with increasing AFB_1 concentration. The number of explants developing roots was reduced to 34% at the highest (25 μg ml^{-1}) AFB_1 concentration, following 3 weeks exposure to the toxin. Leaf chlorophyll content at this toxin concentration was significantly lower than that measured for control plantlets. Thin layer chromatography confirmed the absorption of AFB_1 by the plantlets. Using immunocytochemical techniques, AFB_1 was immunolocated predominantly in the vacuoles, the nucleus and the cytoplasm (possibly intravesicularly). The results are discussed in terms of this immunolocation within the cell.

Key words: Aflatoxin B_1, Immunocytochemistry, Regeneration, Tissue culture, Tobacco plantlets

Introduction

Aflatoxin B_1 (AFB_1), a potent hepatotoxin produced as a secondary metabolite by *Aspergillus flavus* and *A. parasiticus*, has been recognised to have phytotoxic effects [see 1], although there has been a hesitancy to label it a phytotoxin. There is a general lack of knowledge involving mycotoxins in the development of plant disease syndromes. For example, although members of the genus *Fusarium* have been recognised as important plant pathogens, and are known to be producers of a broad spectrum of mycotoxins, little is known about the rôle of these mycotoxins in plant disease. Research into molecular aspects of some of the disease syndromes in which *Fusarium* species have been associated, may implicate these toxins in disease development. Similarly, the toxins of the Aspergilli and the Penicillia need to be evaluated in this regard.

Tissue culture techniques facilitate the study of effects of herbicides, pesticides and even fungal and bacterial toxins on plant tissues at different stages of growth, differentiation and development. Such in vitro systems also allow researchers to investigate the specific molecular and biochemical effects of a toxin without the biotic and abiotic variables which may operate under field or greenhouse conditions. Although the situation in the whole plant would be far more complex, extrapolation of conclusions drawn from in vitro work would be a logical progression.

The earlier study in this series [1] investigated the effects of AFB_1 on several growth parameters, including shoot development, of the relatively undifferentiated tobacco (*Nicotiana ta-*

bacum) callus. It appeared that tobacco callus cells were sensitive to relatively low doses (I_{50} value for fresh mass increase was 2 μg ml^{-1} AFB_1), but that once shoot primordia had developed (i.e. differentiation had commenced), shoot growth continued relatively unaffected by the toxin. For this reason, the present study was initiated to investigate root and shoot development (plantlet establishment) in differentiated tobacco explants, in an attempt to ascertain whether differentiated plants are better able to cope with the potential phytotoxic effects of AFB_1. The ultimate fate of AFB_1 at the cellular level was also investigated, by means of immunocytochemical localisation of AFB_1.

The comparison of the effects of AFB_1 on shoot and root development in differentiated (tobacco explants) and undifferentiated material (tobacco callus), should provide greater insight into the phytotoxic effects of AFB_1 on plant tissue than would an investigation on established plants alone. The use of in vitro culture systems is promoted as a simple and useful tool, enabling the researcher to investigate specific aspects of the the action of one particular factor, presently AFB_1, that might otherwise be obscured.

Materials and methods

Plantlet initiation from tobacco callus. Tobacco calli were initiated as described previously [1]. Differentiating calli with visible shoot primordia were subcultured onto a medium [2] (MS; 1% agar; 2% sucrose, pH 5.8) from which nutrients plant growth regulators had been omitted and incubated at 200 μmoles m^{-2} sec^{-1} photon flux density, with a 16 h photoperiod and an incubation temperature of 25 ± 3 °C. Within 2–3 weeks of incubation, plantlets with well developed root and shoot systems had established.

Root development and shoot regeneration. The apical parts of these tobacco plantlets were excised aseptically. All parts of the shoot (leaves and petiole), except the apical bud and the leaf immediately subtending it, were removed (see insert of Fig. 1A). Explants of a similar size were selected. These were then placed onto a growth medium (MS nutrients plus 2% sucrose; 1% agar, pH 5.8; without plant growth regulators) into which different concentrations of AFB_1 had been incorporated following autoclaving.

Aflatoxin B_1 treatment. Aflatoxin B_1 was aseptically incorporated into the regeneration medium from a stock solution [1] such that concentrations of 0.5, 2, 10 and 25 g ml^{-1} of toxin were achieved. Experimental controls comprised plants grown in the medium containing the following: no DMSO, and DMSO at the concentrations found in 0.5 (0.02% DMSO) and 25 (1% DMSO) μg ml^{-1} AFB_1.

Growth measurements. Explants were incubated for 3 weeks (200 μmoles m^{-2} sec^{-1} photon flux density, 16 h photoperiod, 25 ± 3 °C). At weekly intervals, leaf number and root development were recorded. At the termination of the incubation period, root and leaf tissue (including the petiole) were excised and mass of roots and leaves per plantlet determined. Chlorophyll content of leaves (petiole removed) was assessed, according to the method of Bruinsma [3].

Extraction of aflatoxin B_1 from plant tissue. Root, stem and leaf tissue were homogenised separately in 1 ml of distilled water using an Ultraturax. Aflatoxin was extracted three times by the addition of 3 ml, 2 ml and finally 1 ml of a chloroform:methanol (4:1) mixture. The solvent phases were pooled, and poured over anhydrous sodium sulphate. The sodium sulphate was washed with an additional 2 ml of solvent mixture. The chloroform:methanol volume was reduced to 0.5 ml by evaporation. Ten μl of extract were spotted onto Kieselgel thin layer chromatographic plates (Merck, Darmstadt) and allowed to run in a chloroform:ethyl acetate:isopropyl alcohol (6:3:1) mixture. Plates were viewed under long wave UV light and compared with the AFB_1 standards run simultaneously.

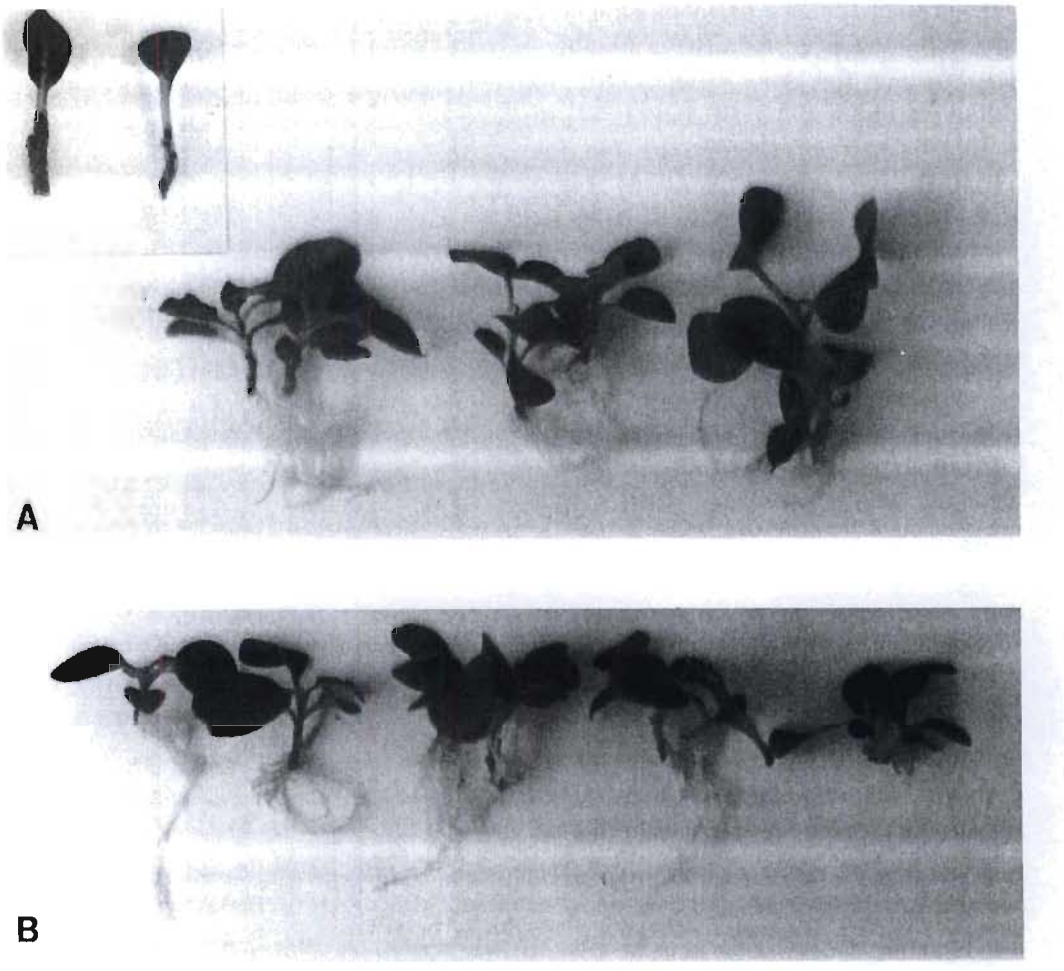

Fig. 1. (A) Control plantlets [from left to right: no DMSO; DMSO as found at 0.5 (0.02% DMSO), and 25 (1% DMSO) μg ml^{-1} AFB_1]. *Inset*: Explant with apical bud and single leaf used for plantlet establishment; (B) Plantlets treated (from left to right) with 0.5, 2, 10 and 25 μg ml^{-1} AFB_1 for 3 weeks. Root development at 25 μg ml^{-1} AFB_1 was severely impaired.

Immunocytochemical localisation of aflatoxin. Explants of tobacco plantlets were incubated for 11 days in the presence of 20 μg ml^{-1} AFB_1, as described above. Cross-sections of stem tissue were microwave-fixed and embedded in a low viscosity resin (VCD/HXSA) [4]. Microwave fixation involved a 5 min pre-incubation in 2.5% glutaraldehyde containing 0.5% caffeine (in 0.5 M phosphate buffer, pH 6.5), a 6 sec exposure to microwaves, followed by a 15 min post-fixation in fresh fixative [5]. An acetone series (15 min immersion with 2 changes of each of 30, 50, 75, 90 and 100%) was used for dehydration of specimens. Stem tissue was then infiltrated with resin by immersing the specimens in the following acetone:resin mixtures: 70:30 for 1 h; 50:50 for 2 h and 30:70 for 1 h (2 changes each). Specimens were then placed in fresh pure resin overnight. The resin was renewed and allowed to polymerise at 70 °C for 8–12 h.

Ultrathin sections were cut for immunolocalisation of AFB_1, which involved the following procedure: etching of resin by a 3 min treatment with 5% H_2O_2, incubation of sections mounted on nickel grids in a serial dilution (1:100 to 1:1000) of polyclonal rabbit anti-serum to AFB_1 (Sigma

Immunochemicals), followed by incubation with a 5 nm IgG immunogold probe (Sigma Immunochemicals). Washing was done using 0.5 M Tris buffer containing either 0.2% BSA (pH 7.2) or 1% BSA (pH 8.0), when appropriate. For the method control, the primary antibody (antiserum to AFB_1) was replaced with phosphate-buffered saline. To check the specificity of the antibody for the antigen, a serial dilution of AFB_1 (1–100 μg ml^{-1}) was incubated with the antiserum for aflatoxin for 24 h prior to use.

Sections were stained for ten minutes with 2% uranyl acetate, washed twice in sterile distilled water and post-stained with lead citrate [6] for 30 min. Sections were then viewed with a Joel 100C transmission electron microscope at an accelerating voltage range of 60–100 kV. Electron micrographs were taken at a magnification of 33000 or 50000.

Statistics. Results were assessed using one way analysis of variance (ANOVA, LSD; $p \leq 0.05$). Differing alphabetical characters on Figures reflect significant differences for any measured parameter.

Results

Plant regeneration and growth

In the present experimental system, two processes were investigated: the development of shoots from the apical bud, and root development from the already differentiated explant, resulting in the establishment of tobacco plantlets. At all AFB_1 concentrations, leaf production and root development occurred. However, with increasing toxin concentration, both of these parameters were significantly inhibited (Figs 1B, 2A & 3A). While the number of leaves per explant was significantly inhibited from 10 μg ml^{-1} AFB_1 (Fig. 2A), a significant inhibition of accumulation in leaf mass per plantlet was observed from 2 μg ml^{-1} of toxin (Fig. 2B). Although leaf area was not determined, these measurements concur with the observed visible decrease in leaf size at the higher toxin concentrations (Fig. 4).

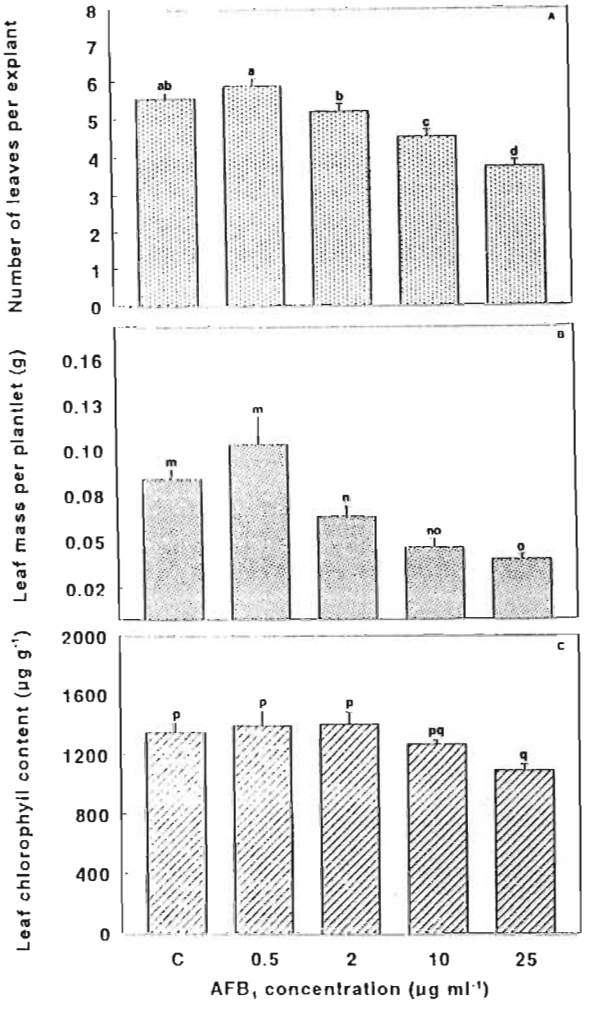

Fig. 2. The effects of AFB_1 on regenerating tobacco plantlets treated with 0.5–25 μg ml^{-1} for 3 weeks. (A) Number of leaves developing per plantlet; (B) Leaf mass (g) per explant; (C) Chlorophyll content of excised leaves (μg chlorophyll per gram of fresh tissue). Differing alphabetical characters reflect significant differences for any measured parameter.

During the incubation period, it became apparent that one or more of the regenerated leaves of the plantlets incubated in the presence of 25 μg ml^{-1} AFB_1, and to a lesser extent, at 10 μg ml^{-1}, had developed chlorotic areas, mainly manifested as a blanching in the midvein area and as interveinal chlorosis. Extracted chlorophyll levels substantiated these observations (Fig. 2C). At the

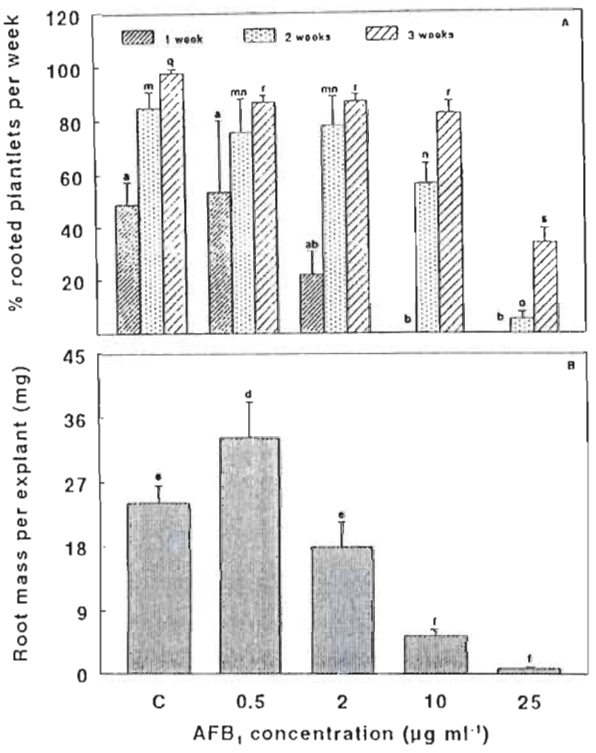

Fig. 3. The effects of 0.5–25 μg ml^{-1} AFB_1 on (A) the percentage of plantlets developing roots, assessed on a weekly basis for 3 weeks, and (B) mass of roots produced per plantlet following 3 weeks of toxin exposure.

highest AFB_1 concentration, chlorophyll content of leaves of treated plantlets was 82% of that of control plants (i.e. 18% inhibition).

The inhibitory trend of the toxin with increasing concentration was more pronounced when root development was assessed (Fig. 3). At 10 and 25 μg ml^{-1} AFB_1, plantlets failed to develop roots during the first week of toxin exposure, compared with a value of approximately 50% in control plantlets and plantlets treated with lower doses of toxin. Following 3 weeks of toxin exposure, root development (compared with control plants) was significantly inhibited, even at the lowest toxin concentration (0.5 μg ml^{-1} AFB_1) tested. Root development was particularly severely inhibited at 25 μg ml^{-1}. Only 34% of plantlets developed roots. A significant increase in mass was, however, observed at 0.5 μg ml^{-1} AFB_1 which might be interpreted as a stimulatory effect.

Plantlets treated with 10 and 25 μg ml^{-1} AFB_1 were considerably shorter than those incubated with lower toxin concentrations. Leaf arrangement had a rosette appearance, as internodal distance was reduced at these higher toxin concentrations. (Fig. 1B).

Immunocytochemical localisation of aflatoxin

Uptake of aflatoxin B_1 by the establishing tobacco plantlets was confirmed using thin-layer chromatography. AFB_1 was extracted from stem, root and leaf tissue of 10 and 25 g ml^{-1} AFB_1-treated material. Immunolocation of the AFB_1 molecules in the stem tissue of the plantlet was then attempted, as shoot extracts gave the largest fluorescent spot under long wave UV illumination.

Visualisation of 5 nm gold particles in tissue used for immunolocation of specific antigens (in this case AFB_1) can be considered a positive reac-

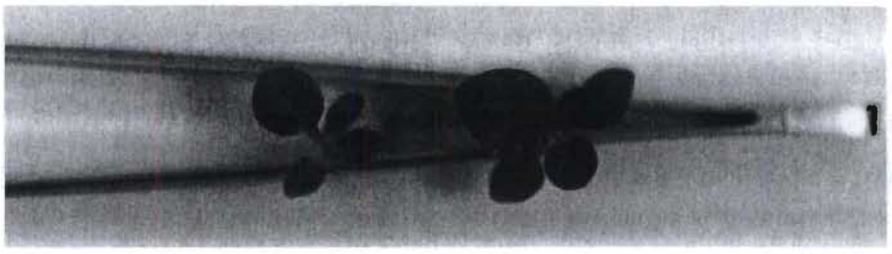

Fig. 4. Chlorotic spots visible on regenerated plantlet exposed to 10 μg ml^{-1} AFB_1 (right), and reduced leaf size in plantlets treated with 25 μg ml^{-1} AFB_1 (left).

tion provided the method control yields a negative response, i.e. a lack of gold particles associated with the sections. Furthermore, a positive or first level control (adsorption control), where the antigen and primary antibody are incubated together prior to treatment of section (in order to bind the primary antibody), should yield reduction of gold particles associated with sections, i.e. a reduction in the immunolocalisation of the antigen (AFB_1). The method controls were completely devoid of 5 nm gold particles (Figs 5 & 6), while a few isolated electron dense particles were observed in the cytoplasm of the positive control sections (Figs 7 & 8), indicating almost complete adsorption of the antigen (AFB_1) by the antibody at the lowest toxin concentration (1 $\mu g\ ml^{-1}$ AFB_1). These observations indicate the specifity of the gold probe for AFB_1.

In this initial investigation of 20 $\mu g\ ml^{-1}$ AFB_1-treated stem tissue from tobacco plantlets, gold particles could be visualised primarily in the vacuoles and the nuclei of the cells (Figs 9–12). Gold particles were also observed in the cytoplasm of the cells. On closer examination, it would appear that the gold particles are located within small vesicles (Figs 9 & 10). In the nucleus, clusters of gold particles were frequently observed to be associated with the visible chromatin.

Discussion

AFB_1 at relatively low concentrations was able to affect several growth parameters of regenerating tobacco plantlets. This is similar to the previous observations for tobacco callus [1]. For these young, differentiated establishing plantlets, root development was more severely affected than was the ability to develop leaves. This could result from direct toxic effects of AFB_1 to those plant parts in immediate contact with the toxin-containing medium. At the higher toxin concentrations, stem tissue in contact with the medium exhibited a browning, necrotic response. Roots in these plantlets emerged above the level of the medium. Such an observation suggests a direct toxic effect on contact with the AFB_1 in the medium.

The trend of decreasing leaf mass and the reduction in the number of leaves regenerated per plantlet with increasing toxin concentration attests to the inhibitory effects of toxin exposure at sites distal from the toxin source. Such inhibitions may arise from an impairment of nutrient uptake from the medium as a result of physiological or biochemical events occurring at the root level. Alternatively (considering that only 34% of plantlets at the highest AFB_1 concentration developed roots), or additionally, the AFB_1 translocated to aerial plant parts may directly inhibit certain metabolic processes, such as chlorophyll and protein synthesis.

TLC confirmation and immunocytochemical localisation of AFB_1 in the stem tissue of tobacco plantlets provide unequivocal evidence for the translocation of the toxin from the roots to aerial plant parts. Other workers have reported an uptake and translocation of AFB_1 to leaf-stem tissue, warning of the potential health hazard to consumers of consumption of commodities grown in AFB_1-contaminated soil [7].

Compared with the response of callus tissue [1], the differentiated cells of the tobacco plantlets appear less susceptible to the phytotoxic effects of AFB_1. In callus, the I_{50} value for fresh mass accumulation was 2 $\mu g\ ml^{-1}$, and many were unable to survive at 25 $\mu g\ ml^{-1}$, as measured by the number of necrotic calli. In tobacco plantlets, however, although at the highest AFB_1 toxin concentration, a mere 34% of explants developed roots (in comparison with nearly 100% in controls), the I_{50} values for root and leaf mass per explant were 10 $\mu g\ ml^{-1}$, and approximately 5 $\mu g\ ml^{-1}$, respectively. Such findings would indicate that differentiated cells of tobacco plantlets are less susceptible to the toxic effects of AFB_1 than the relatively undifferentiated cells of tobacco callus.

The measured decrease in chlorophyll content of the regenerated leaves, as well as the presence of chlorotic areas on leaves in treated plantlets, suggests that AFB_1 affects chlorophyll synthesis.

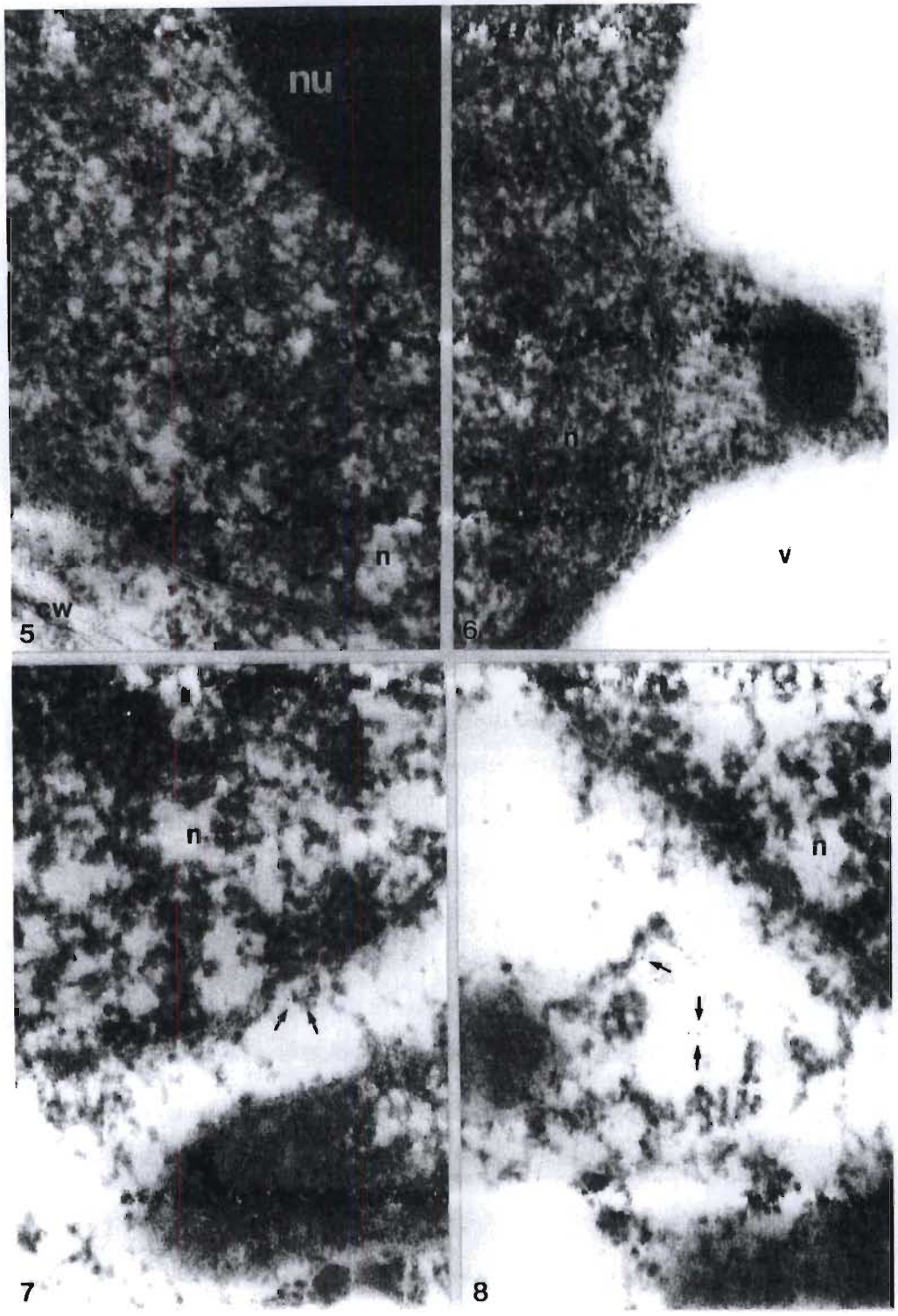

Plate I. *Figs 5 & 6.* Method controls (primary antibody replaced with phosphate buffered saline) of stem tissue from tobacco plantlets treated with 20 μg ml^{-1} AFB_1 for 11 days. Micrographs appear devoid of gold particles. Figs 5 & 6 ×65000. *Figs 7 & 8.* Adsorption controls (primary antibody and 1 μg ml^{-1} AFB_1 incubated together 24 h prior to use). Most areas appear devoid of gold particles. A few isolated particles were observed in the cytoplasm of the cells (arrows). Figs 7 & 8 ×65000; cw = cell wall; n = nucleus; nu = nucleolus; v = vacuole.

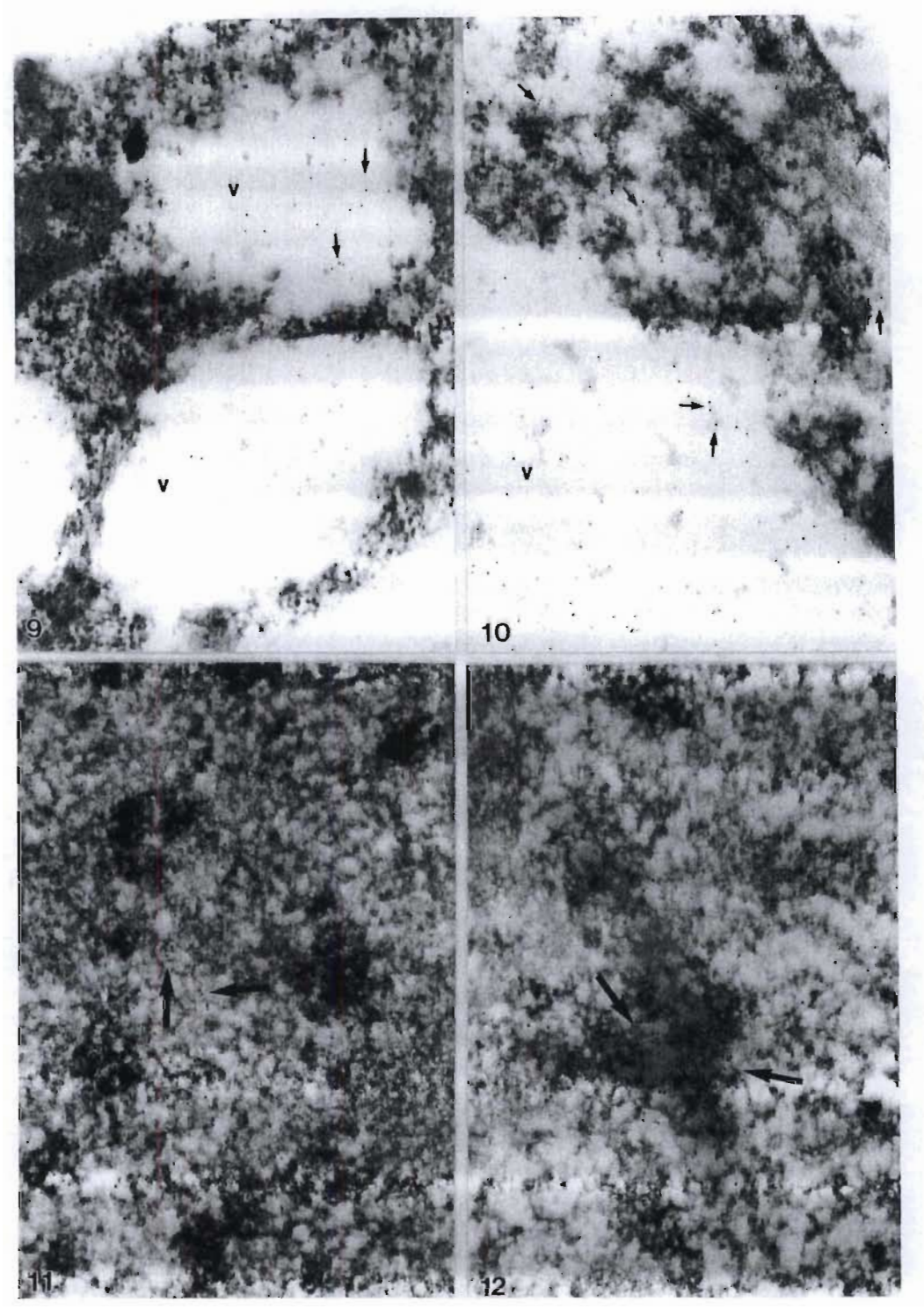

Plate II. *Figs 9–12.* Stem tissue of tobacco plantlets treated with 20 μg ml^{-1} AFB_1 for 11 days. Gold particles (arrows) are present in the vacuoles (v), cytoplasm (Figs 9 & 10) and in the nucleus (Figs 11 & 12). In Fig. 12, a cluster of gold particles is associated with an irregular clump of chromatin. Fig. 9. ×43000; Figs 10–12. ×65000.

There are several reports indicating that the aflatoxins, as well as other mycotoxins, interfere with the normal functioning of chloroplasts. Disruption of chloroplast structure, manifested as an inhibition of grana development was observed in etiolated leaves of *Zea mays* exposed to light after AFB_1 (and AFM_1,) had been applied directly to the leaf surface [8]. Such spots failed to green. The effect was, however, partially reversed when either sucrose of δ-aminolevulinic acid was added. Since sucrose is known to increase protochlorophyll formation [9]) and that δ-aminolevulinic acid is an early precursor in chlorophyll synthesis [10], those authors are of the opinion that AFB_1-induced greening and inhibition of grana formation may result from disruption of normal chlorophyll synthesis.

Other workers have reported either a disruption of chloroplast structure [11] or the appearance of chlorosis [12–15] in a number of plant species exposed to aflatoxins. In a prior study on tobacco callus treated with AFB_1 [1], disruption of chloroplasts was one of the initial ultrastructural observations following toxin exposure. Other mycotoxins are also responsible for the development of chlorosis, e.g. moniliformin causing interveinal chlorosis in corn and tobacco plants [16].

An aspect of AFB_1 interaction/metabolism in plant cells that warrants investigation is the interference with normal plant growth regulator control. The phenomenon of 'rosetting' (shortening of internodal distances) observed in regenerating tobacco plantlets at higher toxin doses is similar to that reported for moniliformin-treated tobacco plants [16]. In that study, the effect was reversible, provided the toxin source was removed. A few researchers have attempted to investigate the possible interactions between AFB_1 and gibberellins [17–18] and auxins [19–21]. T-2 toxin and its interaction with plant growth regulators has also been investigated [22, 23]. While the results of those studies are interesting, their data are too fragmentary to be conclusive and additional investigation is necessary.

The immunocytochemical localisation of AFB_1 in the stem tissue of young tobacco plantlets provides some of the most conclusive evidence of the fate of this mycotoxin in plant cells. The appearance of the label in the nucleus and, to a lesser extent, in the nucleolus is in agreement with the findings for AFB_1-treated animal cells. Aflatoxin B_1 binds to macromolecules in the animal cell – principally to the DNA, RNA and proteins [24]. Most interest has centred around the binding of the toxin to DNA, since AFB_1 has long been implicated as a causal agent for liver cancer in many African communities [25]. It is, however, not AFB_1 itself that binds to DNA. Microsomal activation of AFB_1 results in the formation of an epoxide that binds preferentially to the N7 residue of guanine, thereby blocking m-RNA synthesis [24].

As to whether AFB_1 (or its epoxide) binds to the DNA of the plant cell is still the subject of conjecture. Tripathi and Misra [26] extracted the DNA from maize seeds and incubated it in the presence of AFB_1. As assessed by viscometry, difference spectroscopy and equilibrium dialysis, AFB_1 was found to bind to the DNA in vitro. This may provide the initial evidence that the ultimate destination of this mycotoxin in plant and animal cells is similar. The immunocytochemical localisation of AFB_1 in the nucleus of tobacco stem cells in the present investigation is further evidence for the nucleus being a target for the toxin. It does, however, not imply any specific binding of the toxin to the nucleic acids. In this regard, the present study is being expanded to the isolation of DNA from AFB_1-exposed plant tissue in an attempt to assess the existence or absence of DNA-guanine adducts.

The localisation of gold particles in the vacuoles of tobacco stem cells is interesting. Plant vacuoles contain a number of enzymes, especially hydrolases. Visualisation of the gold probe in this organelle suggests that vacuoles may be used by the plant tissue as a 'dump' for noxious compounds. In the relatively harsh acidic environment of the vacuole, toxin may become bound, metabolised, detoxified or inactivated by one or several mechanisms. Howes et al. [27] postulated

that the ability of parsley plants to metabolise AFB_1 to aflatoxicol A, a less toxic compound, was brought about by a dehydrogenase enzyme found throughout the plant. In the present investigation, on TLC plates, no other metabolites of AFB_1 were visualised that were not present in the medium following 3 weeks incubation. However, these observations do not preclude the possibility of their existence, since they may occur in concentrations too low for detection on TLC plates. A factor that must be considered in this investigation, is that while the immunogold probe has a high specificity for AFB_1, it will also cross-react with AFG_1, AFB_2 and AFG_2, but not AFB_{2a}, AFG_{2a} or AFM_1 (Sigma Immunochemicals). High pressure liquid chromatography of plant extracts is presently underway to detect possible trace levels of any AFB_1 metabolites.

Gold particles observed in the cytoplasmic region appeared to occur within vesicles. If (as is probable) such vesicles are ER-derived, one can deduce the microsomal connection, as microsomes contain fragments of rounded-off ER obtained as a result of cell fractionation. Such vesicles (in vivo) might directly be internalised by vacuoles or be so after a Golgi-associated step. Either way, one can speculate about an ER [microsome] – (Golgi) – vacuole pathway, accounting for the accumulation of AFB_1 intravacuolarly.

This and the companion study (tobacco callus [1]) provide valuable information regarding the interaction between the plant cell and AFB_1. The findings of these two investigations suggest that differentiated tissues possess more effective mechanisms for dealing with AFB_1 than do the less differentiated tissue of the callus. Furthermore, the results of this study support the report of Mertz et al. [7] that AFB_1 can be absorbed and translocated to aerial plant parts. These important findings may have considerable economic implications in the agricultural community with regard to ploughing techniques, seed set, and levels of AFB_1 present in the soil or in the plant infected with *Aspergillus flavus* or *A. parasiticus*.

Although several questions (i.e. ultimate fate of toxin; possible binding to DNA; detoxification etc.) remain largely unanswered, some of the aspects of toxin metabolism have been addressed. Tissue culture, where micro-environmental conditions can be accurately manipulated by the researcher, provides a useful tool for investigating some of the unanswered questions regarding the rôle of toxins (and perhaps mycotoxins) as disease determinants. The two in vitro systems investigated (callus and plantlets) exhibit different sensitivities to AFB_1. In selecting an in vitro plant system for testing for genotype tolerance, the researcher must decide at what level this tolerance operates. This will depend on the developmental stage at which a plant is likely to be exposed to toxin, i.e. developing seed (likely to be more sensitive), or plants that have already established (likely to be less sensitive).

Acknowledgements

The authors would like to thank Mrs T Naicker (Electron Microscope Unit, Faculty of Medicine, University of Natal) and Mr J Wesley-Smith (Electron Microscope Unit, Dept. of Biology, University of Natal) for their expertise in different aspects of the techniques employed. This research was made possible by funds obtained from the University of Natal Faculty of Medicine Research Fund and the Foundation for Research and Development.

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Mycotoxins and Phycotoxins - Developments in Chemistry, Toxicology and Food Safety, ed by M. Miraglia, H. van Egmond, C. Brera, J. Gilbert
Printed in the U.S.A., 1998

32

The Effect of a Commercial Herbicide on Lipid and Aflatoxin Biosynthesis in *Aspergillus Parasiticus*

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ABSTRACT

Arylphenoxypropionates like diclofop methyl (DCM) exert their selective herbicidal action by inhibition of acetyl coenzyme A carboxylase (ACC) in monocotyledonous plants. This enzyme, which catalyses the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA, is of key importance in both the biosynthesis of fatty acids and polyketides. This reaction is the first committed step in fatty acid biosynthesis in plants and other organisms and forms the basic building blocks of aflatoxin formation through polyketide synthesis during idiophase of fungal growth. Diclofop methyl affected *Aspergillus parasiticus* growth by as much as 70% over a seven day period indicating that an essential process in the fungus has been adversely affected. On measuring lipid production, it was found that the total amount of lipid produced per unit mass of mycelium was less than that of the control. This is consistent with the known action of DCM on plants, i.e. inhibition of ACC. More significantly, the production of aflatoxin B_1 was inhibited, supporting the supposition that DCM inhibits all acetate-derived metabolites that depend on malonyl-CoA for building units.

1. INTRODUCTION

The dangers presented by the aflatoxins in world food supplies are a well documented fact (Gundu Rao and Harein, 1972). Consequently much effort has

KEY WORDS: toxigenic fungi, arylphenoxypropionate herbicide, acetyl coenzyme A carboxylase, inhibition, polyketides

been expended on either preventing their occurrence or removing them once present (Piva *et al.*, 1995). Detoxification of contaminated foodstuffs has not yet been shown to be commercially viable and, as the aflatoxins are comparatively stable to heat and other food processing (Samarajeewa *et al.*, 1990) it seems that the best approach is to prevent the formation of aflatoxin.

Various methods of toxin elimination have been attempted, including the breeding of crop strains resistant to infection by toxigenic strains of *Aspergillus parasiticus* and *A. flavus* (Scott and King, 1988), improved storage of crops (Goldblatt, 1971) and removal of contaminated seeds (Cole, 1989). The effects of various pesticides on the production of aflatoxin by *Aspergillus* spp. have been studied at various times (Dutton and Anderson, 1980), after it was shown that dichlorvos inhibited aflatoxin B_1 (AFB_1) biosynthesis (Gundu Rao and Harein, 1972). These investigations seem, however, to be based on an empirical approach and it is unlikely that a practical field treatment could be devised that would, at one and the same time, limit aflatoxin production and control a crop pest.

A group of novel herbicides, known as propionates, more precisely arylphenoxypropionates, e.g., diclofop methyl (DCM) (Fig 1), were developed in the 1980s which selectively eliminated monocotyledonous plants without adversely affecting dicotyledonous ones (Hoppe, 1985). It was found that these compounds acted by specifically inhibiting acetyl CoA carboxylase (ACC), a key enzyme in fatty acid biosynthesis. Malonyl CoA, the product from the reaction catalysed by ACC, is the source of acetate units used to form fatty acid. It seemed a reasonable assumption that as polyketide synthase requires a source of malonyl CoA polyketide biosynthesis would also be inhibited (Dutton, 1988). As aflatoxins are modified polyketides, inhibition of their production by application of propionate herbicides to dicotyledonous crops, such as groundnut, to control monocotyledonous weeds would appear to be a possible option. Initial trials (unpublished data) indicated that all the commercial propionate herbicides used to treat *A. parasiticus* in liquid cultures not only inhibited aflatoxin production but also fungal growth itself.

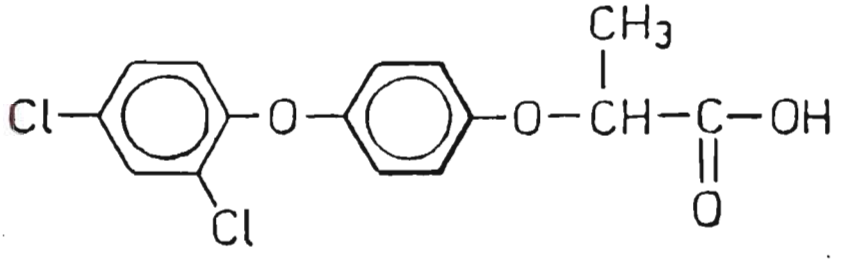

Figure 1. Chemical structure of diclofop methyl.

This investigation examined the effects of DCM on fungal growth, lipid and aflatoxin production by *A. parasiticus*.

4. MATERIALS AND METHODS

All chemicals used in this study, unless otherwise indicated, were purchased from Merck (South Africa). A sample of DCM was kindly donated by Hoechst Chemicals.

Aspergillus parasiticus (CMI 91019b) was maintained on potato dextrose agar (PDA). A spore suspension was prepared in sterile sodium dodecyl sulphate solution (0.01%) of approximately 10^6 spores per ml (Chuturgoon and Dutton, 1991).

Sterile liquid medium (Reddy *et al.*, 1971), 100 ml in 250 ml plugged erlenmeyer flasks was inoculated with 1 ml of spore suspension and then placed in a shake incubator (28°C, 180 rpm) and harvested at suitable time intervals for up to 7 days (Chuturgoon and Dutton, 1991). DCM dissolved in methanol was added to the inoculated flasks at time zero to achieve a final concentration of 50 µg/ml growth medium. Control flasks were prepared by adding the same volume of methanol. All experiments were done in triplicate. On harvesting, the mycelial pellets were filtered off on a buchner apparatus, lyophilised and weighed. The culture fluid was retained for aflatoxin determination.

Lipids were extracted by fragmenting the lyophilised mycelium (1 g) to a fine powder and suspending in a mixture (15 ml) of chloroform: methanol:distilled water (5:10:4) (Shih and Marth, 1974). The resultant homogenate was filtered and chloroform (5 ml) was added to the filtrate. The organic phase was passed through anhydrous sodium sulphate, and dried in a vacuum desiccator over anhydrous $CaCl_2$ for 2 days. Weight of the dried residue was taken as a measure of lipid production.

Aflatoxins were extracted from the culture fluid as described previously (Reddy *et al.*, 1971). Total aflatoxin concentration was determined by high performance liquid chromatograpy using pre-column derivatisation (Berry *et al.*, 1984).

3. RESULTS AND DISCUSSION

In liquid culture DCM severely inhibited mycelial growth, as shown in Figure 2, to a third of that found in the control. This indicates that DCM intereferes with a fundamental metabolic process important in the growth of the fungus. Support for this is the lower level of total lipid in the treated mycelium, which was half of that in controls on a lipid to mycelium ratio, after 4 days incubation (Fig. 3).

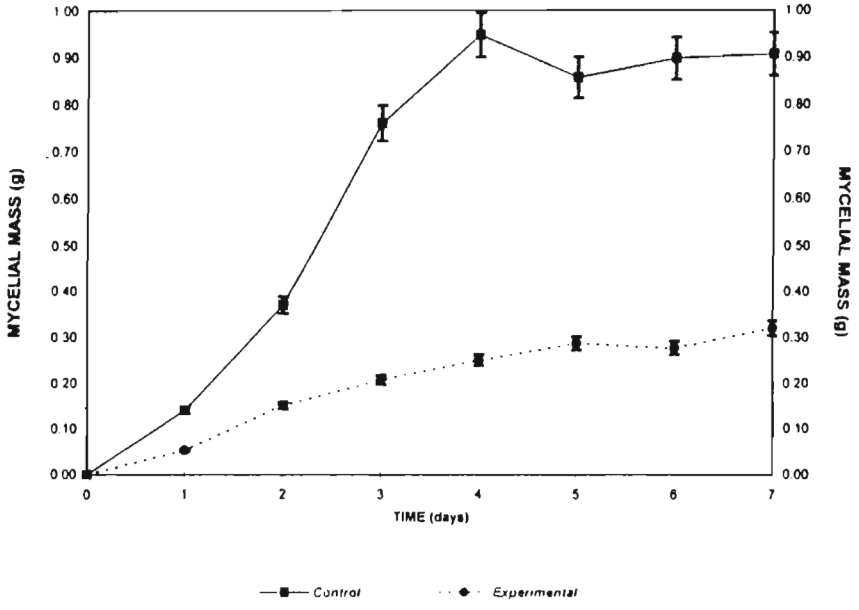

Figure 2. The effect of DCM (50 µg/ml) on *A. parasiticus* mycelial growth over a seven day period for control and experimental cultures.

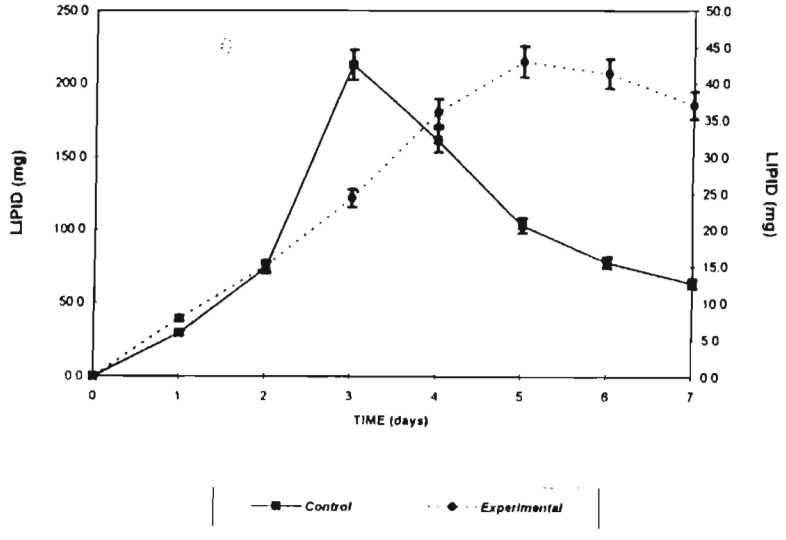

Figure 3. The effect of DCM (50 µg/ml) on *A. parasiticus* total lipid production over a seven day period for control and experimental cultures.

A more dramatic effect was noted on AFB_1 production which was inhibited by approximately two orders of magnitude (Fig. 4). These results strongly support the notion that DCM inhibits a metabolic step common to both general lipid and AFB_1 biosynthesis and that is likely to be the production of malonyl CoA by the inhibition of ACC. Several interesting points arise from this conclusion. Firstly it would suggest that propionate herbicides could be useful agri-chemicals to use in treating dicotyledonous crops, such as groundnut, for the control of monocotyledonous weeds, toxigenic strains of fungi and aflatoxin production. This might also be extended to monocotyledonous crops such as maize genetically engineered to resist the effects of these herbicides.

Another important point is that if a specific step is being blocked in AFB_1 biosynthesis then these compounds would be useful in studying the early part of the biosynthetic pathway. Putative aflatoxin precursors could be added to DCM inhibited cultures in order to test their conversion to final product. Furthermore it is not clear whether polyketide synthase shares the same ACC enzyme as fatty acid synthase. If so then malonyl CoA becomes a diverging point; if not, do the two ACC enzymes have equal sensitivity to the herbicide? The difference in degrees of inhibition of fatty acid and AFB_1 observed in these experiments could be explained on that basis, although it is more likely to be a difference in the amounts of the two synthases present, as polyketide synthase concentration is rate limiting.

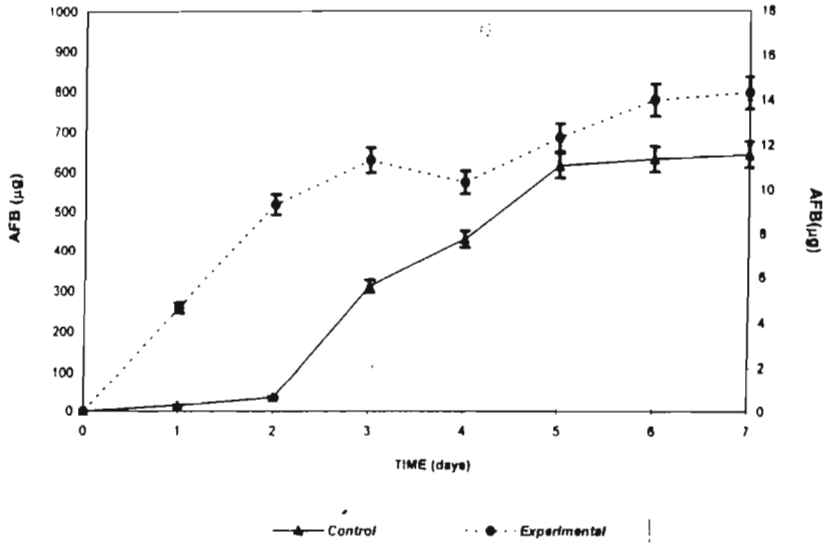

Figure 4. The effect of DCM (50 µg/ml) on *A. parasiticus* total aflatoxin production over a seven day period for control and experimental cultures.

These results show that propionate herbicides such as DCM have the ability to interfere with fungal growth through their ability to inhibit fatty acid biosynthesis. More importantly the biosynthesis of aflatoxins is strongly inhibited, which offers hope of developing a method for controlling monocotyledonous weeds, fungi and aflatoxin in crops such as groundnut in a cost effective manner.

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CHAPTER 4:

OCCURRENCE OF MYCOTOXINS

4.1 PAPERS AND STUDIES

The separation of aflatoxin biosynthetic intermediates by high performance liquid chromatography
Paper 15 by Berry, Dutton and Jeenah

Occurrence of mycotoxins in cereals and animal feedstuffs in Natal, South Africa
Paper 19 by Dutton and Westlake

Mycotoxins found in supermarket foods
Paper 21 by Dutton and Westlake

The incidence of mycotoxins in the South African poultry industry
Paper 22 by Dutton and Westlake

Incidence of mycotoxins and fungi feedstuffs in Natal, South Africa 1994
Paper 43 by Dutton and Kinsey

Analysis of agricultural commodities in Natal from 1984-1993 for mycotoxins
Paper 46 by Dutton and Kinsey

4.2: INTRODUCTION

After the outbreak of Turkey X disease and its elucidation as a mycotoxicosis, rapid strides were made in identifying other mycotoxins (the current count is over 300) derived from three main genera of fungi, namely *Aspergillus*, *Fusarium* and *Penicillium* spp. This automatically led to an examination of agricultural commodities for their presence, as well as an understanding of their modes of action and involvement in episodes of disease. Initially TLC was used to identify mycotoxins in extracts from fungal cultures foods and feeds but as time progressed more advanced instrumentation was employed, in particular HPLC. In addition, GC was employed where trichothecenes were involved, as these do not have any chromaphore for ease of detection. Later GC/MS was used (Chaytor & Saxby 1982) and double focussing mass spectrometers (Placencia *et al.* 1990), capillary electrophoresis (Maragos 1995) and most recently of all LC/MS (Aikyama *et al.* 1998).

Many surveys of cereals such as wheat (Shotwell *et al.* 1976) and maize (Shotwell *et al.* 1971) for general mycotoxins, and specific ones, such as DON, fumonisin and aflatoxin have been carried out. It is interesting to note that certain mycotoxins not only predominate in certain climatic zones but also in certain crops and at certain times during their life span, i.e., growth, harvesting and storage. This can assist in

deciding which toxins to look for in a certain situation. However, it is very difficult to be sure that any toxin, from those known, is not present in any commodity that can act as growth substrate for fungi. For the laboratory involved in mycotoxin analysis two clues are available. The first is to screen the commodity for fungi and then to look for toxins that are known to be produced by the fungi found. This is not fool proof as a toxin may be present due to the prior dominance of another specie or a fungus may be present that is non-toxigenic. The other pointer is disease symptoms in animals that have eaten the material, as most toxins have known characteristic symptoms after ingestion , e.g., ergot cause abortion and nervous syrnptoms (Robbers 1979), ochratoxin, kidney damage (Cooper 1979) and zearalenone precocious sex organ development, particularly in pigs (Aucock *et al.* 1980).

4.3 COMMENTARY

After my arrival in Natal in 1980, I found that there was nothing being done to find out what mycotoxins were present in local agricultural commodities. Organisations such as the Natal Regional Veterinary Service (RVS) were being brought problems possibly of mycotoxin in origin but with no means of investigating them. With Ken Westlake, I set up a modest screening programme based on that used by the Ministry of Agriculture, Fisheries and Food (MAFF) which depended on a dialysis clean up and TLC (Patterson and Roberts 1979). In addition we also screened for fungi using the MAAF method based on the use of Ohio Station agar (Kaufman *et al.* 1963). This was also adopted by the RVS under the expert hands of Penny Anderson.

These methods were used with great success, as it is possible to screen for at least 20 of the more common toxins and adapt to several more where necessary. Almost immediately sample began to arrive and a database of mycotoxin incidence began to be built up. This has continued to date resulting in over 7000 samples being analysed with an increasing throughput.

Although this sounds most praiseworthy, several factors have to be borne in mind. Firstly the samples sent are often not statistically taken and, therefore, not representative of a bulk commodity. The method is not no very sensitive for some of the toxins, as it has by its very nature to be a compromise between the detection of various metabolites with a great variation in their chemistry. The quantitation is not very accurate as it has to be done by inspection of spots on TLC plates against known standards. In spite of this, however, valuable information can be passed back to the farmer, miller etc. and the results are generally quite adequate for their purpose. Where greater accuracy is needed the sample may then be submitted to an instrumental method specifically developed for that toxin. This is routinely done where aflatoxin and zearalenone are quantitated from the neutral fraction using HPLC. The method used for AFB1 is that developed by us (Paper 15).

The more recent discovery of the fumonisins posed a problem for the dialysis method, as their analysis is not possible using this method, because of their polarity. To overcome this a sample of the material had to be separately analysed using the method of Sydenham *et al.* (1992). During his current Masters project, Paul Chelule (MT17) has developed a method for fumonisin clean up from the same extract prepared for the dialysis method. The acetonitrile/aqueous potassium chloride

solution is passed through a SAX cartridge. The fumonisins are eluted and analysed using the Sydenham method.

Another innovation introduced in the screening programme was that of cytotoxicity testing (Robb *et al.* 1983). This is done using human cell lines, usually hep-2 which is a liver cancer cell culture. The results are quantitated using the MTT test (Smith *et al.* 1992) which in essence measure viability through NADH generation (Liu *et al.* 1997). This was developed by D Pillay in her Masters (MT21) and has proved highly efficient as a monitor of samples, which are toxic but which do not contain the 20 mycotoxins detected by the dialysis method. Naturally the method picks up toxins other than mycotoxins, which can be useful to clients.

The first two years of results were published in 1985 (Paper 19) and showed that AFB1 was the most commonly found mycotoxin (>27% of all samples) and *Aspergillus spp.* including *A. flavus* group was the most commonly found fungus. In addition supermarket foods were also analysed and we had the unfortunate experience of being threatened with litigation for analysing peanut butter and reporting the fact that it had high levels of aflatoxin. At that time quality control of these commodities was not as good, as it should have been, reflecting the poor understanding and assessment of mycotoxins in commodities at that time. In association with local chicken producers, a survey of chicken feed was carried out in order to find answer for unexplained losses in productivity and a cause for chicken ascites (waterbelly; Paper 22).

More recent publications on our results (Papers 43 and 45) still indicate AFB1 as being the most commonly found mycotoxin (>14% of all samples) but this is being rapidly over taken by FB1, because of its common occurrence in South African maize. Results quoted in Paper 43 showed that over 94% of the maize and feed samples analysed for FB1 in 1994 were positive for FB1

Currently work is being done in the Ngcolose and Mphise regions of the Tugela Valley with the rural population. Part of this study is to analyse "plate ready" food for mycotoxins and to try to correlate the results with the appearance of biomarkers (Chapter 5) particularly fumonisin, in serum, urine and faeces (Papers 55, & 57). In agreement with the studies done on agricultural commodities, FB1 figures high on the list of contaminants but other *Fusarium* mycotoxins are also routinely found, including, ZEA, trichothecenes and also aflatoxins. Efforts are being made to advise the local population through "imbezos" to minimise their exposure these mycotoxins by improved storage methods and food selection.

Journal of Chromatography, 283 (1984) 421–424
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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Note

Separation of aflatoxin biosynthetic intermediates by high-performance liquid chromatography

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(Received August 29th, 1983)

During the investigation of the biosynthesis of aflatoxin B_1 (AF) by cell-free extracts of *Aspergillus flavus*, various putative precursors and their products had to be separated and quantified. The precursors include the anthraquinones norsolorinic acid (NA), averufin (AVF), versiconal hemiacetal acetate (VHA), versicolorin A (VA), and the xanthone derivative sterigmatocystin (ST). The structures of these compounds are shown in Fig. 1.

Previous studies[1–6] had used thin-layer chromatography for the separation of these metabolites, but even using two-dimensional systems it is not possible to separate all of them; in particular the AVF–VA pair is difficult to resolve. For this reason a rapid and effective separation was sought which could also afford a quantification of the amounts present. These criteria were ideally met by high-performance liquid chromatography (HPLC), and a method of separation was developed which is described here.

EXPERIMENTAL

Materials and reagents

Standards were obtained by purifying metabolites from various strains and mutants of *Aspergillus* species[7,8] and aflatoxin B_1 was obtained from Sigma (St. Louis, MO, U.S.A.).

All organic solvents were of analytical reagent grade, and HPLC-grade water was obtained by passing water previously purified by a Millipore R/Q system (Millipore, Bedford, MA, U.S.A.) through a Millipore Norganic cartrige.

Apparatus

A Varian Model 5000 liquid chromatograph Aerograph Operations, Walnut Creek, CA, U.S.A.) fitted with a column oven and a Varian Vari-Chrom UV Visible detector (operated at 325 nm and 0.1 a.u.f.s.), was used. A Hewlett-Packard HP3390A plotting integrator (Palo Alto, CA, U.S.A.) was used to calculate peak areas and component concentrations. The column was a Spherisorb S5ODS1 5-μm reversed-phase column (25 cm × 4.5 mm I.D. stainless steel) (Phase Separations, Clwyd, U.K.), and was preceded by a guard column dry-packed with 40-μm Vydac RP (4 cm × 4 mm I.D.) (Varian). Samples (0.5–10.0 μl) were injected onto the column by means of a six-port loop injector.

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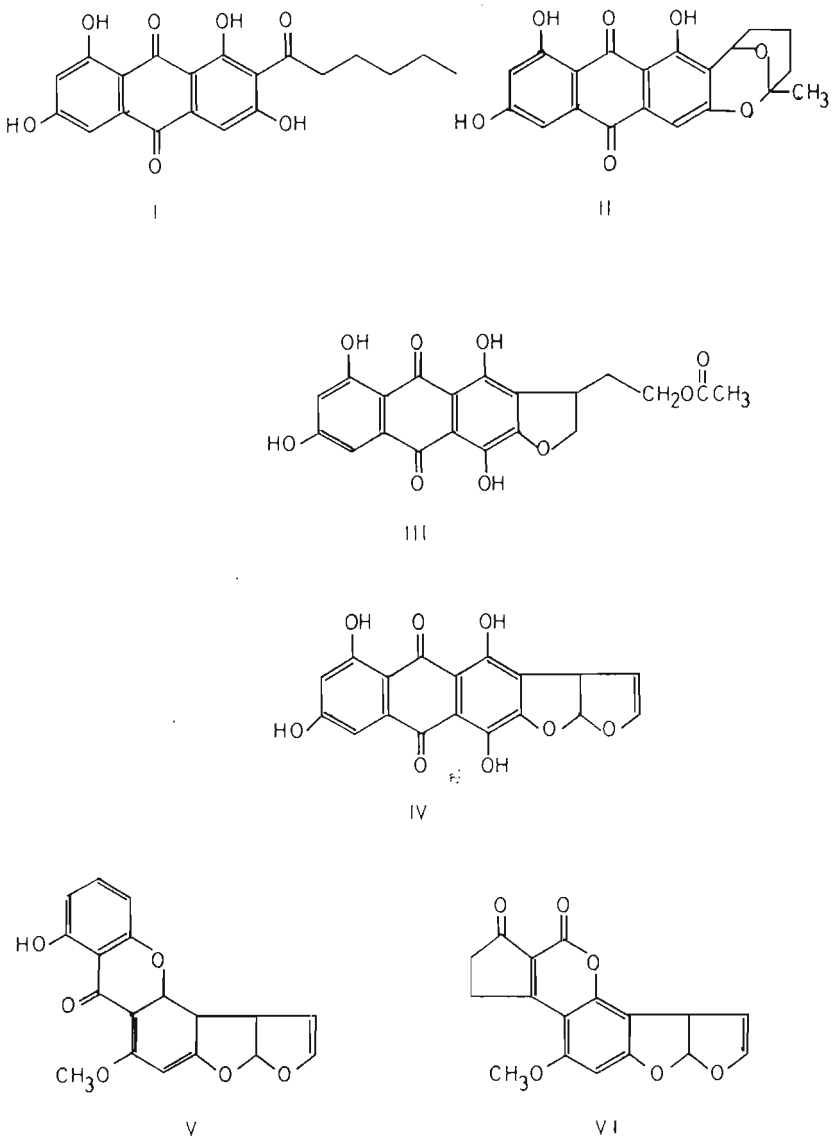

Fig. 1. Structures of aflatoxin biosynthetic intermediates. I = Norsolorinic acid; II = averufin; III = versiconal hemiacetal acetate; IV = versicolorin A; V = sterigmatocystin; VI = aflatoxin B_1.

RESULTS AND DISCUSSION

Of several solvent systems investigated, a ternary solvent system consisting of methanol tetrahydrofuran (2:1, Solvent A) and water (Solvent B) at a flow-rate of 2.0 ml/min and a temperature of 50°C with a linear gradient from 45% B to 25% B over 6 min was found to effect the separation.

A typical chromatogram is shown in Fig. 2. Retention times (min) were: aflatoxin B_1, 1.92 ± 0.04; VHA, 2.67 ± 0.24; ST, 3.89 ± 0.09; VA, 5.93 ± 0.22; AVF,

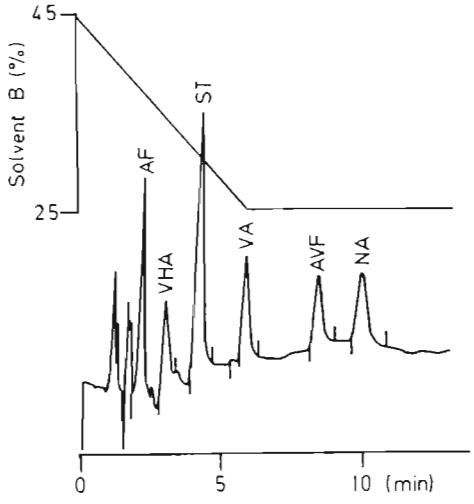

Fig. 2. Chromatogram of aflatoxin biosynthetic intermediates: aflatoxin B_1 (AF), versiconal hemiacetal acetate (VHA), sterigmatocystin (ST), versicolorin A (VA), averufin (AVF), norsolorinic acid (NA). Solvent A = methanol-tetrahydrofuran (2:1); solvent B = water. Gradient, 45% B to 25% B over 6 min; flow-rate, 2 cm^3 min^{-1}; temperature, 50°C.

8.52 ± 0.21; NA, 10.05 ± 0.31. These times are the means of 12 runs over a period of 30 days; their reproducibility was excellent, and quantification was reproducible to 2% at the 5–20-μg level.

Although this solvent system gave a good separation of all of the components, it was occasionally desirable to separate only some of them. In particular, we were interested in separating AVF from VA and VHA, and ST from aflatoxin B_1. In the former case, an isocratic solvent consisting of acetonitrile tetrahydrofuran water (25:20:55) gave a good separation, with the components eluting in the following times (min): VHA, 2.12; VA, 2.98; AVF, 5.07. It should be noted that aflatoxin B_1 coelutes with VA under these conditions.

An isocratic solvent system of acetonitrile–water (1:1) gave a convenient separation of ST (retention time 5.87 min) and aflatoxin B_1 (2.50 min). Under these conditions VA eluted in 3.87 min.

If it is necessary to separate the major aflatoxins, this can be achieved by using an isocratic system consisting of acetonitrile–methanol (3:2, v/v) (45%) and water (55%). The following retention times (min) were obtained: aflatoxin G_2, 3.00; aflatoxin G_1, 3.22; aflatoxin B_2, 3.61; aflatoxin B_1, 4.00.

In order to demonstrate the effectiveness of the system reported here, chromatographs of crude extracts from *A. parasiticus* strain W49 (Donkersloot), which accumulates AVF, and strain 1-11-105 Wh1 (Bennett), which accumulates VA are shown in Figs. 3 and 4, respectively.

Separation of the major aflatoxins was achieved by using an isocratic system consisting of acetonitrile–methanol (3:2) (45%) and water (55%). The following retention times (min) were obtained: aflatoxin G_2, 3.00; aflatoxin G_1, 3.27; aflatoxin B_2, 3.61; and aflatoxin B_1, 4.00.

The metabolites O-methylsterigmatocystin (OMS), dihydrosterigmatocystin

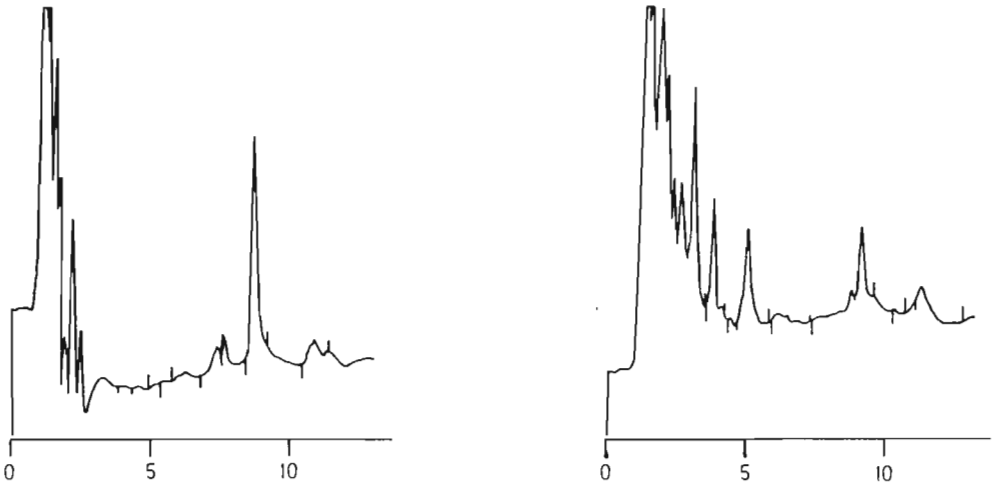

Fig. 3. Chromatogram of a crude extract from *A. parasiticus* strain W49. Chromatographic conditions as for Fig. 2.

Fig. 4. Chromatogram of a crude extract from *A. parasiticus* strain 1-11-105 Wh 1. Chromatographic conditions as for Fig. 2.

(H_2-ST), averantin and versicolorin C were tested on the methanol-tetrahydrofuran system, and it was found that a good separation of OMS, H_2-ST and ST was obtained, with retention times (min) of 2.57, 3.63 and 4.03, respectively. Averantin could be resolved from VA and AVF, with retention times (min) of 7.95, 5.95 and 8.26 respectively. Versicolorin C, however, could not be resolved from VA using this solvent system.

These solvent systems have therefore proved useful as a means of investigating aflatoxin biosynthetic intermediates quickly and accurately.

ACKNOWLEDGEMENTS

We are grateful to Dr. Joan W. Bennett of the Department of Botany, Tulane University, New Orleans, LA, U.S.A., and to Dr. J. A. Donkersloot of the National Institute of Dental Research, Bethesda, MD, U.S.A. for fungal mutants. We are also grateful to the Council for Scientific and Industrial Research, Pretoria and the University of Natal Development Fund, Pietermaritzburg, South Africa for financial assistance.

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DUTTON & WESTLAKE: J. ASSOC. OFF. ANAL. CHEM. (VOL. 68, NO. 5, 1985)

CHEMICAL CONTAMINANTS MONITORING

Occurrence of Mycotoxins in Cereals and Animal Feedstuffs in Natal, South Africa

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During the period 1982–1983, just under 800 samples of agricultural commodities, comprising cereals, compound feeds, hay, and silage, were examined for molds and mycotoxins. Aflatoxin B_1 showed the highest incidence rate; it occurred in over 27% of all samples analyzed, the highest levels being found in peanut meal at 1500 ppb. Other mycotoxins detected were patulin and a number of trichothecene toxins at incidence rates in all commodities of 5.6 and 3.1%, respectively. The commodities at highest risk were oil seeds, excluding soya bean; the latter was found to be fairly free from contamination with mycotoxins. The most prevalent fungi were *Aspergillus flavus* and *parasiticus*, which were found in over 22% of all samples, whereas *Penicillium* spp. showed the lowest incidence of genera, specifically identified in 8.3% of all samples examined. This latter finding explains in part the low incidence of *Penicillium* mycotoxins.

Since mycotoxins were brought to the attention of agriculturalists in the early 1960s by the outbreak of Turkey X disease in England (1), scientists in South Africa have made significant contributions to their study (e.g., 2–4). Most of these studies, however, have been of an academic nature or have concerned specific outbreaks of mycotoxicoses (5, 6). Very little has been done to estimate the general occurrence of mycotoxins and their producing fungi in agricultural commodities. Government agencies and food firms do carry out routine screening, usually for the aflatoxins, but because these results remain unpublished, there is little information available for the agriculturalist attempting to assess the mycotoxin problem.

During 1980, an analytical system was set up at the University of Natal, South Africa, to examine various commodities for the presence of mycotoxins and fungal contaminants. This was done to assess the prevalence of such contaminants in South African agricultural commodities, and so raise the level of knowledge concerning this problem to that in other countries.

The work reported here is for the period from the beginning of 1982 to the end of 1983 and covers materials produced in South Africa.

Experimental

Collection of Samples

Samples were sent to our laboratory from many sources, but the main suppliers were the Veterinary Research Laboratories at Allerton, Pietermaritzburg, several millers and animal feed suppliers, farmers, and farm cooperatives. All materials were either currently in use or marketed in Natal, and at least 50% of them were produced in the province, although it was not possible to be more specific than this because of insufficient data. To our knowledge, all samples were of South African origin, but certain ones were unspecified and may well have been imported; this applies to less than 5% of all samples examined. The samples examined were unrepresentative in that either they were suspected of being infected with fungi, or they were taken by nonstatistical methods. The latter situation arose because the actual sampling was performed by persons other than the authors, e.g., farmers or milling operatives. Normally, 1 kg samples were presented for analysis, and these were thoroughly mixed and milled in a C&M Junior laboratory mill, 2 mm mesh size, before analysis.

Over 700 samples of commodities, such as maize and other cereal grains and flours, oil seeds and their products, compound feeds, lucerne, hay, fish meal, and compound feeds, were obtained and analyzed.

Extraction and Cleanup

The initial screening method (7) was that used by the Ministry of Agriculture, Fisheries and Food in the United Kingdom (8). The method involves extracting a milled 25 g sample of material with aqueous acetonitrile followed by defatting and a cleanup step using dialysis. Two fractions are obtained; a neutral one containing the aflatoxins, trichothecenes, patulin, sterigmatocystin, zearalenone (F-2 toxin), and neutral *Alternaria* toxins; and an acidic one containing ochratoxin, citrinin, tenuazonic acid, cyclopiazonic acid, kojic acid, and penicillic acid. When examining samples of forage that were suspected of being infected with fungi, we used the screen to look for dicoumarol in the neutral fraction, because it has been reported that this modified grass metabolite can be generated by the action of fungi such as *Aspergillus fumigatus* (9).

Thin Layer Chromatography

These mycotoxins are separated by thin layer chromatography (TLC) on 2-dimensional silica gel G aluminum-backed chromatoplates, 10 × 10 cm (cut from 20 × 20 cm plates, Merck Cat. No. 5553), using various solvent combinations, as recommended by Patterson and Roberts (10). Detection was carried out initially by viewing under longwave UV light and then by using various spray reagents: 3-methyl-2-benzothiazoline hydrazone hydrochloride solution (MBTH) for patulin (11), Pauly's reagent (12) for kojic acid and penicillic acid (with prior treatment for the latter with ammonia vapor (13), alcoholic aluminum chloride (14) for sterigmatocystin, and Ehrlich's reagent (15) for cyclopiazonic acid. The trichothecenes were detected by sulfuric acid in combination with anisaldehyde (16) or chromotropic acid (CTA) (17) or by nicotinamide-acetyl pyridine reagent (NAP) (18). The anisaldehyde reagent was also used to locate tenuazonic acid.

TLC Confirmation

Confirmation of positive results was done by comparison with known standards run on the same chromatoplate and was further verified by spiking the extract with the relevant toxins. Co-chromatography in the 2 solvent systems used and correct color characteristics under UV light with spray reagents were considered sufficient confirmatory evidence.

Determination

The level of mycotoxin contamination was estimated by visual comparison with a series of standards, and for aflatoxins, accurate quantitation was done by liquid chromatography, using a Varian Model 5000 liquid chromatograph

Received February 19, 1985. Accepted April 16, 1985.

Table 1. LC determination of incidence of aflatoxin B_1 in South African agricultural commodities during 1982–1983

Commodity[a]	Samples: No. examined	Samples: No. positive	% Positive	Aflatoxin B_1 concn, ppb (μg/kg)
Maize	155	39	25.2	<5–1500
Other cereals[b]	71	10	14.0	<5–300
Oil seeds[c]	73	31	42.5	<5–2000
Soya bean	34	5	14.7	<5–20
Animal feed	42	12	28.6	<5–40
Poultry feed	144	50	34.7	<5–100
Other feeds[d]	194	68	35.1	<5–400
Fish meal	41	1	2.4	20
Other meals[e]	30	0	0	—
Forage[f]	10	0	0	—
Total, mean	794	216	27.2	<5–2000

[a]Each commodity heading includes harvested original materials plus products such as flour and defatted meals.
[b]Includes oats, wheat, and barley.
[c]Includes peanut, sunflower, and cotton seed; soya bean considered separately.
[d]Includes unspecified materials destined for animal consumption.
[e]Includes blood, carcass, and bone meals.
[f]Includes hay, lucerne, and silage.

Table 2. LC determination of incidence of *Aspergillus flavus*[a] propagules in South African maize during 1982–1983

No. propagules/g	Aflatoxin B_1 concn, ppb, no. samples: ND[b]	5	5–9	10–19	20–99	100
ND	78	12	—	3	4	1
<10	19	5	—	1	1	1
$10–10^3$	12	1	1	1	2	—
10^4	3	—	—	1	—	1
10^5	2	—	2	—	—	1
10^6	1	—	—	—	—	—
$>10^7$	1	—	—	—	—	—

[a]Includes both *Aspergillus flavus* and *parasiticus*.
[b]ND = not detected.

(Varian Instrument Group, Palo Alto, CA) fitted with a Beckman Ultrasphere-ODS column, 4.6 × 25 cm (Beckman Instruments, Inc., Berkeley, CA). The solvent system used was methanol–acetonitrile–water (1 + 1 + 3, v/v/v), and the absorbance of aflatoxin was measured at 365 nm using a Varian Vari-Chrom UV detector. (Where available, mycotoxin standards were obtained from Sigma Chemical Co., St. Louis, MO; others were kindly donated by P. Thiel, MRC, Tygerberg, South Africa, and Y. Ueno, Tokyo University.)

Mycological examination was done by weighing out a 1 g sample (wet weight), which was suspended by agitation in 10 mL sterile Ringer's solution, and then serially diluted to 10^{-6} with sterile Ringer's solution. Samples of 1 mL were then pipetted into sterile Petri dishes to which molten Ohio Agricultural Station Agar (19), at 42°C, was added, sufficient to adequately cover the bottom of the dish. The dishes were swirled to ensure thorough mixing and then allowed to stand at room temperature until the agar had set. The dishes were then incubated at 25°C for 5 days and scored as viable fungal propagules/g for the main mycotoxin-producing genera, i.e., *Aspergillus, Penicillum,* and *Fusarium* (*Aspergillus flavus,* including both *A. flavus* and *A. parasiticus,* was scored separately from the rest of the *Aspergillus* spp.). The rest of the detected fungi were grouped as "Others;" these were unidentified fungi or those which were not major toxin producers, such as *Mucor, Rhizopus,* and *Trichoderma* spp.

All the results were entered into a computer data base used to expedite data retrieval and coordination.

Results and Discussion

The results are summarized in Tables 1–4. The most commonly found mycotoxin was aflatoxin B_1; this was particularly true of maize, oil seeds, and feeds. It was often accompanied by aflatoxin B_2 (about 40% of the time), although in smaller quantity, and sometimes by aflatoxins G_1 and G_2 (about 20% of the time for aflatoxin G_1).

Because maize is a major crop in South Africa, the results for this commodity are given in a little more detail. Table 2 attempts to correlate aflatoxin B_1 incidence with that of *Aspergillus flavus* (this includes *A. parasiticus*), so that the detection of the fungi might indicate incidence and level of aflatoxin contamination. The results (Table 2) reveal that the majority of samples fell in the range < 5 ppb with a fungal count ≤ 10 propagules/g. It is clear that there is little correlation between aflatoxin incidence and *A. flavus* count; many samples with viable fungal propagules contained no toxin, whereas a majority of those containing toxin had no fungal contamination or had counts of < 10 propagules/g.

A similar situation was observed for the other toxins and fungal contaminants recorded in Tables 3 and 4; thus we can conclude that prior screening for fungal contamination may have intrinsic value but cannot replace proper chemical analysis to detect specific mycotoxins.

The range of aflatoxin B_1 concentrations in Table 2 is broken down into 5 categories to give a comparison of the levels found with those permitted by South African law. The law covers 2 areas, that of food for human consumption, which states that the total concentration of aflatoxins in such foods must not exceed a total of 10 ppb with a 5 ppb limit on aflatoxin B_1, and that of animal feedstuffs, which varies depending on the age and species of animal, although in general the upper limit is 20 ppb total (20). It is not the intention of the authors to discuss legal limits, which is a controversial question, but an appreciable number of the samples found to contain aflatoxin were at levels which we considered to be highly dangerous because of possible contamination of the human food chain, albeit our sampling regime was nonstatistical.

The results from Table 3 show that patulin is the next most common mycotoxin found after aflatoxin B_1, although patulin was not found in any of the maize samples. It was detected in other commodities (apart from fish meal) in about 10% of all samples tested.

Trichothecene toxins were also detected from time to time during the survey period in approximately 5% of all samples investigated, including maize. Of the known trichothecenes, diacetoxyscirpenol was the most commonly found, with T-2 toxin and deoxynivalenol (vomitoxin) occurring occasionally, although the bulk of the positive samples resulted from unassigned trichothecene, as determined by their reaction with NAP and CTA spray and after comparison with standards. In such samples (2 of which were found), fusarenone X and neosolaniol were tentatively identified by comparison with standards but could not be distinguished from each other with the chromatography systems used. Both the NAP reagent and chromatropic acid were invaluable for indicating the presence of other trichothecenes, which could not be identified because of a lack of suitable standards.

The incidence of zearalenone (F-2 toxin) was much less than that of the other *Fusarium* toxins, i.e., the trichothecene toxins, and was mainly confined to maize samples, particularly those which were visibly infected with mold. This may explain why F-2 toxin was not found in the feed samples, because moldy maize either would not have been used for feed or would have been diluted with sound material, which,

Table 3. Incidence of mycotoxins other than aflatoxin B_1 in South African agricultural commodities assessed by mycological examination

Commodity (no. samples)	Mycotoxin[a], no. samples					
	Kojic[b] acid	Patulin	Sterigma-tocystin	Tricho-thecene[c]	Other[d]	F-2 toxin
Maize (155)	3	ND	4	6	3	9
Other cereals (71)	ND	8	ND	5	ND	ND
Oil seeds (107)	ND	8	ND	1	3	1
Soya bean (34)	ND	ND	ND	ND	ND	ND
Animal feed (42)	ND	3	ND	3	1	1
Poultry feed (144)	ND	10	ND	1	ND	ND
Other feeds (194)	ND	13	ND	8	ND	ND
Fish meal (41)	ND	ND	ND	ND	ND	ND
Other meals (30)	ND	1	ND	1	ND	ND
Forage (10)	ND	2	ND	1	ND	ND
Total (794)	3	45	4	25	7	11

[a]ND = not detected; several samples had multimycotoxin contaminations: 1 sample, deoxynivalenol and F-2; 1 sample, aflatoxin B_1/trichothecene toxin; 6 samples, patulin/trichothecene toxin; 6 samples, more than 1 trichothecene toxin; and 8 samples, patulin/aflatoxin B_1.
[b]Always found in the presence of aflatoxin.
[c]Includes deoxynivalenol (3), T-2 toxin (6), diacetoxyscirpenol (7), and "others" (9).
[d]"Other" represents metabolites giving reactions with spray reagents indicative of trichothecenes, but which could not be confirmed by other methods; 2 were tentatively identified as neosolaniol and fusarenone X.

Table 4. Incidence of different species of fungal propagules in South African agricultural commodities during 1982–1983

Commodity[a]	Species[b], no. samples				
	Aspergillus	*Aspergillus flavus*[c]	*Fusarium*	*Penicillium*	Other
Maize (4)	49	57	44	12	42
Other cereals (6)	23	13	18	3	17
Oil seeds (7)	13	23	7	5	25
Soya bean (9)	12	9	3	6	8
Animal feed (0)	16	11	6	6	16
Poultry feed (6)	32	38	37	15	54
Other feeds (30)	63	24	43	13	46
Fish meal (14)	14	4	1	4	5
Other meals (6)	2	5	5	1	15
Fodder (1)	6	2	4	1	10
Total (74)	230	176	168	66	238

[a]Commodity composition same as that for Table 1. Numbers in parentheses are samples where no fungi were detected.
[b]Main mycotoxin producing species considered only; "Other" includes species that are not major toxin producers, e.g., *Rhizopus*, *Mucor*, and *Trichoderma* sp. Most positive samples contain more than one genera.
[c]Counted separately from the other *Aspergillus* spp. and includes *A. parasiticus*.

although not a desirable practice, is a common method used in the disposal of suspect material.

In the case of soya beans and their products, the incidence and levels of mycotoxins including aflatoxin B_1 were much lower than for maize and oil seeds, namely, 14.7% of samples investigated. Those showing contamination with aflatoxin came from a series of samples from 2 farms with visibly moldy soya bean meal; the presence of aflatoxins and their levels were confirmed by liquid chromatography. The low incidence and levels of aflatoxin are in keeping with the general observation that soya beans are resistant to contamination by aflatoxin even though they are capable of supporting fungal growth (21).

In contrast to surveys by other workers (8, 22), ochratoxin and citrinin were never detected in any samples. This is in strong contrast to the situation in the United Kingdom (8) where these toxins and sterigmatocystin were the main contaminants of barley and other cereals. Sterigmatocystin was also an uncommon contaminant and was only found in 2 cases of disease in swine that had been fed maize stored in small farm silos. The presence of this contaminant was deduced by its characteristic R_f values in the solvents used, color under UV light, and characteristic bright yellow-green fluorescence with aluminum chloride spray.

Other mycotoxins that do not seem to be a problem in South African feedstuffs are penicillic acid, cyclopiazonic acid, and tenuazonic acid. Dicoumarol was conveniently detected by our method in the neutral fraction. Spiked samples showed good recovery (85%) and could be detected on thin layer chromatography plates by viewing under UV light and then spraying with Pauly's reagent, which gave a strong orange-yellow positive reaction. No dicoumarol was ever detected in forage samples even though several of these were heavily infested with fungi and were associated with hemorrhagic symptoms in cattle.

Table 4 shows that most of the agricultural commodities presented for investigation contained viable fungal propagules of species that are known mycotoxin producers. Maize showed consistently high counts of all the fungi determined, including *Fusarium* sp. which presumably entered the commodity before harvest because it does not compete very well with storage fungi such as *Aspergillus* sp. (23). The occurrence of *Fusarium* sp. in feeds probably reflects their maize content; maize is used as a common energy source, particularly in poultry feed. Overall, the incidence of *Penicillium* sp. was low compared with that found in more temperate climates (8). This may partly explain the absence of mycotoxins such as ochratoxin, citrinin, and penicillic acid.

Conclusion

The results from this study clearly reveal that South Africa, in common with other countries, has a mycotoxin problem

in its agricultural industry. Considering the vast tonnage of materials produced and consumed (e.g., 8–9 million tons of maize annually), the actual incidence of mycotoxin contamination is low and does not approach crop losses caused by the action of insect pathogens and other factors. However, mycotoxins do represent a threat and can make their presence at times strongly felt, especially during the drought crisis suffered in South Africa in the last few years.

Acknowledgments

We thank A. Kinsey for sample preparation and extraction; R. K. Berry for assistance with computation; Y. Ueno, Tokyo University of Science, Tokyo, for samples of fusarenone X and neosolaniol; P. Thiel, M. R. C., Tygerberg, Cape Town, for tenuazonic and cyclopiazonic acids; Epol Ltd South Africa, C.S.I.R. South Africa, and University of Natal for grants; and the Regional Veterinary Laboratories, Pietermaritzburg, for their cooperation and samples.

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Reprinted from the *Journal of the Association of Official Analytical Chemists*.
Vol. 68 #5

MYCOTOXINS FOUND IN SUPERMARKET FOODS

M F Dutton and K Westlake

The results of a survey of selected foods for the presence of mycotoxins are presented. Aflatoxin was found in peanut butter and maize meal, patulin in apple juice and *Alternaria* toxins in tomato paste. The implications of these findings are discussed together with the general background to the mycotoxin problems.

SAMEVATTING

Die resultate van 'n ondersoek na die teenwoordigheid van mikotoksiene in 'n reeks geselekteerde voedsels, word voorgelê. Aflatoksien is in grondboontjiebotter en mieliemeel gevind, terwyl patulien in appelsap en *Alternaria*-toksiene in tamatiepasta gevind is. Die implikasies van hierdie bevindings word teen die agtergrond van die algemene mikotoksienprobleme bespreek.

When most food or feed is damp, it becomes a suitable substrate for the growth of moulds. We are all familiar with the higher fungi, such as the Basidiomycetes, e.g. mushrooms. However, many fungi are less prominent and form a mat-like growth called a mycelium, and also can produce variously coloured spores, e.g. blue-green growths on mouldy bread. When these fungi grow on feed and food, they not only cause taint but will also produce metabolites and if these are poisonous to animals and man they are called mycotoxins (Gr. mykes = mushroom + toxicon = poison for arrows). Although not all fungi produce mycotoxins, there are some species capable of producing more than one. On the other hand, some mycotoxins are produced by more than one species.

There is a widely held misconception that aflatoxin, in particular aflatoxin B_1, is synonomous with mycotoxin. This has come about because of the wide notoriety that this metabolite has had in the press recently. Its reputation is well earned as it is highly toxic, carcinogenic, teratogenic, mutagenic and embryotoxic; however, there are many other mycotoxins known. In fact, over 200 such compounds have been documented (Cole and Cox, 1981), although of these only about 20 have any economic significance. The structures of a selection of the more important ones are given in Fig. 1.

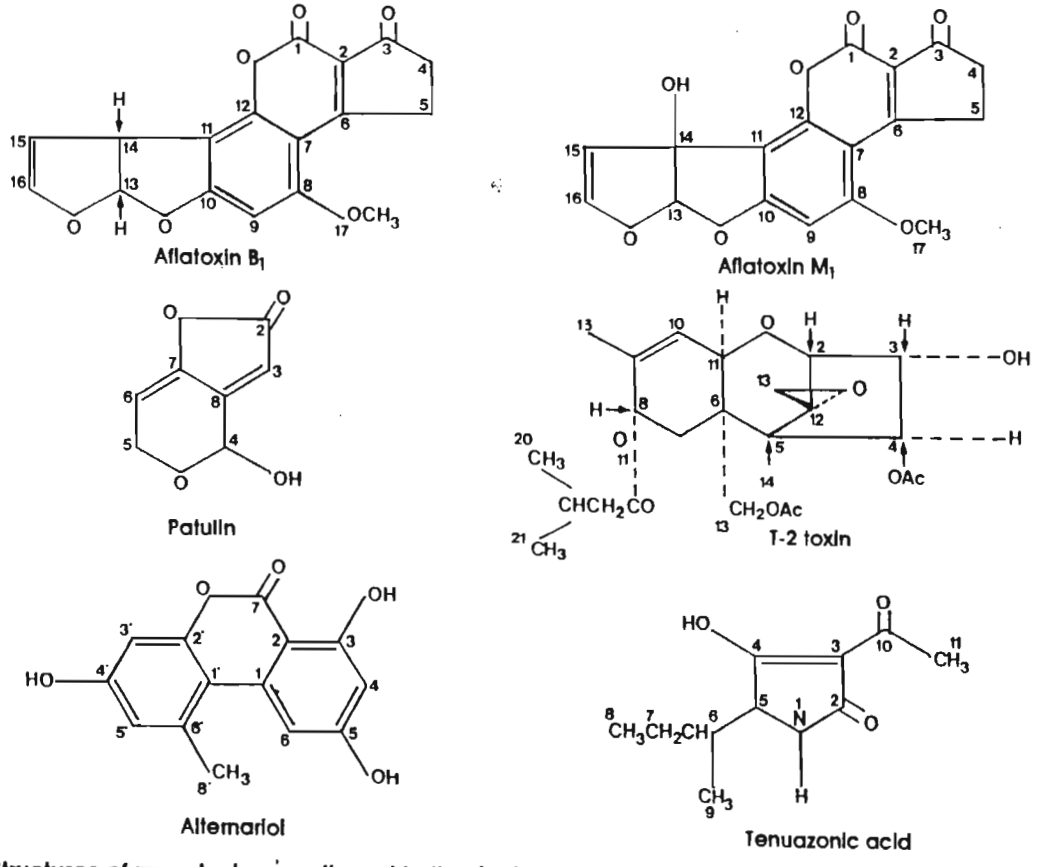

FIGURE 1: Structures of mycotoxins mentioned in the text

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Supported by a University of Natal Development Foundation Grant.

Acknowledgements
Thanks are due to Mrs A Kinsey and Mr A Chuturgoon for technical assistance.

Much of the work done on the occurrence and effects of mycotoxins concerns farm animals, as these are more likely to consume feeds that have been contaminated with fungi and hence mycotoxins. The main danger to the human consumer is the possibility of carry-over of toxins into meat (Polan *et al*, 1974), eggs (Jacobsen and Wiseman, 1974) and milk (Patterson and Anderson, 1982). Of these milk is the more likely to be contaminated as it has been shown that dairy cattle consuming aflatoxin B_1 excrete a slightly less toxic modified form known as aflatoxin M_1 (Nabney *et al*, 1967) (see Figure 1 for the structure).

With respect to the human consumer, surprisingly little has been done in surveying food from Western countries for mycotoxins, probably because of good quality control which minimises the possibility of mouldy foods reaching the table. In the third world the situation is different and there is strong evidence to suggest that much of the liver cancer in underdeveloped countries can be correlated directly to aflatoxin B_1 intake (e.g. Peers and Linsell, 1973; Van Rensburg *et al*, 1974). There are also other disease conditions attributed to mycotoxins (Table 1), but very often the evidence is of a statistical or circumstantial nature.

TABLE 1

Some human disease conditions linked to mycotoxins

Disease	Mycotoxin*	Producing organism
Ergotism	Ergot Alkaloids	*Claviceps purpurea*
Alimentary Toxic Aleukia (ATA)	Trichothecenes	*Fusarium spp*
Yellow Rice Poisoning	Islanditoxin	*Penicillium islandicum*
Balkan Nephropathy	Ochratoxin A	*Aspergillus ochraceus*
Liver Cancer	Aflatoxin B_1	*Aspergillus parasiticus*

* = *In some cases casual agent assumed from available evidence*

Several reports concerning the presence of mycotoxins in human foods and beverages have been made in the South African press in recent years, e.g. aflatoxin in sorghum beer and peanut butter. Quite recently there was a scare due to a report in parliament on the presence of aflatoxin B_1 in breakfast cereal. Fortunately most manufacturers in the food industry are aware of the mycotoxin problem and such contaminated materials are quickly withdrawn and the process, in this case breakfast cereal production, is more stringently monitored.

Since 1980 the Mycotoxin Unit at the University of Natal has been actively engaged in several projects involving mycotoxins and not least of these has been the survey of both animal feeds and human foods for the presence of mycotoxins. Thus in late 1982 we commenced a project to assay food in local supermarkets for mycotoxins in order to assess what problems, if any, were present. Investigations of this type tend to be done on a haphazard basis, i.e., various laboratories in different countries look for specific toxins which are of interest to them. Aflatoxin is a prime target because of its dangerous properties, ease of detection and ubiquitous nature. Investigation into this problem in South Africa depends mainly on the government food analysis services, and organisations such as the National Research Institute for Nutritional Diseases, M.R.C., Tygerberg and the National Food Research Institute, C.S.I.R., Pretoria. Many firms also do their own screening, particularly those in the oil seed business. It must be pointed out though that these firms are generally interested in aflatoxin alone.

Legislation specifically limits the amount of aflatoxin that may be present in a food commodity, the limit for aflatoxin B_1 being 5 ppb (5 μg/kg) in a total aflatoxin concentration not exceeding 10 ppb. Other mycotoxins are covered by a blanket statement referring to toxic substances of a fungal origin. As far as we are aware, the Mycotoxin Unit of the University of Natal is the only laboratory that screens materials on a regular basis for all the more common mycotoxins. This is not a happy situation as certain foodstuffs are susceptible to contamination with particular toxins and therefore require strict monitoring. A summary of these is given in Table 2.

TABLE 2

Commodities* associated with certain mycotoxins

COMMODITY	MYCOTOXIN
Apple and other fruit juice	Patulin
Barley	Citrinin, Ochratoxin, Sterigmatocystin
Cheese	Roquefortine, Aflatoxin M1
Groundnuts and other nuts	Aflatoxin, Tenuazonic Acid
Maize	Aflatoxin, Trichothecenes, Zearalenone
Meat and Meat products	Penicillic Acid, Aflatoxin, Aflatoxin M1
Milk and products	Aflatoxin M1
Oranges and other citrus	Tenuazonic Acid
Pasture	Sporidesmin, Tremorgens
Pepper and other spices	Sterigmatocystin
Tobacco	Aflatoxin
Tomatoes and tomato products	*Alternaria* Toxins
Wheat	Deoxynivaleñol

* *All food commodities have been found at times to contain aflatoxin*

The rest of this paper will be concerned with our method of mycotoxin analysis and the results of our survey for mycotoxins in foods destined for human consumption.

METHOD

The method of analysis used by the mycotoxin unit of the University of Natal is based on a multiscreening technique devised by the late Derek Patterson (Roberts and Patterson, 1975; Patterson and Roberts, 1979) of the Central Veterinary Laboratories, Weybridge, U.K. and it is used by the Ministry of Agriculture, Fisheries and Food, U.K. in the Agricultural Development and Advisory Service (A.D.A.S.).

An outline of the method is as follows: a weighed sample of food (25 g) is blended and extracted with aqueous acetonitrile. The sample is defatted by extraction with iso-octane and then retained for further treatment. The neutral chloroform fraction is then evaporated and taken up in a small volume of acetonitrile, which is then placed in a dialysis sac. The fraction is dialysed against aqueous acetone, the mycotoxins being found in the dialysate. They are then re-extracted into chloroform, evaporated to dryness and dissolved in a small volume (200 μl) of chloroform-acetone 1:1 v/v.

The retained extracted aqueous layer is acidified and extracted with chloroform to yield an acid mycotoxin fraction (Table 3). The chloroform is evaporated and the residue is dissolved in the same amount of solvent as the neutral fraction.

TABLE 3

Mycotoxins covered by routine screening

NEUTRAL FRACTION	ACID FRACTION
Aflatoxins B_1, B_2, G_1, G_2 and M_1	Citrinin
Alternaria Toxins: e.g., Alternariol	Kojic Acid
Patulin	Ochratoxin A
Sterigmatocystin	Penicillic Acid
Trichothecenes: e.g., T2 Toxin	Tenuazonic Acid
Zearalenone (F2)	

The extracts (20 μl) are then spotted on two-dimensional aluminium-backed silica gel G thin layer plates (10 x 10 cm) cut from 20 x 20 cm prepared plates (Merck Art 5 553). The chromatographs are then developed in pairs of solvents suited for their separation. In certain cases spray reagents are used to detect the presence of a particular mycotoxin.

In the case of liquid products a measured volume was freeze-dried and the solid residue was extracted as for solid products.

Samples of foods were bought at random from supermarkets in Pietermaritzburg and analysed in duplicate according to the above method.

RESULTS AND DISCUSSION

The results of the survey are given in Table 4.

Several commodities were found to contain mycotoxins, although the majority of the samples did not contain detectable levels.

It can be seen from Table 4 that the larger proportion of samples assayed were free from detectable quantities of mycotoxins and this indicates the good work done by government quality control services and the manufacturers themselves, although the fact that mycotoxins were found at all leaves no room for complacency.

The samples that proved positive were apple juice with patulin contamination, tomato paste which contained alternariol and maize flour and peanut butter which contained aflatoxin. To be fair it must be pointed out at this juncture that the samples which were shown to be positive were ones taken from a chronological series indicating that the occurrence is sporadic rather than permanent, as would be expected from this sort of problem.

Patulin is considered by many authorities to be a powerful mycotoxin (Scott, 1974), although at one stage it was considered for use as an antibiotic (Birkenshaw, *et al*, and Raistrick 1943. It has toxic properties; its LD_{50} for mice is 3 mg/kg but its instability, particularly in neutral to alkaline solutions, greatly reduce its toxic potential in foods. Fruit juices are somewhat susceptible to contamination with patulin, especially apple (Scott, *et al*, 1972) as apples are easily infected with *Penicillium expansum*, one of the several fungal species that produce patulin. Fermentation products such as cider are considered to be safe, as yeasts break down patulin during the fermentation process (Harwig, *et al*, 1973). Fortunately fruit juice manufacturers are aware of the problem and take precautions to prevent patulin formation in their products.

The presence of alternariol in certain samples of tomato paste is to be viewed in similar light to that of patulin in fruit juice. Tomatoes are susceptible to infection with *Alternaria* spp (Pero, *et al*, 1973) and hence can be expected to contain their toxins from time to time. The toxicity of these compounds is fairly low, e.g., the LD_{50} for alternariol in mice is greater than 200 mg/kg and they do not seem to have any other dangerous properties. A more potent toxin produced by *Alternaria* spp is tenauzonic acid (Fig 1); fortunately this toxin was not detected in our samples.

TABLE 4

The incidence of mycotoxins in food surveys from December 1982 to July 1984

	NUMBER OF SAMPLES	MYCOTOXINS FOUND*
Brown Bread Meal	3	Nil
Maize Meal	5	Aflatoxin B_1 (2)
Wheat Meal	4	Nil
Sorghum Malt	1	Nil
Rice	2	Nil
Pearl Barley	1	Nil
Home brew	2	Nil
Popcorn	1	Nil
Sugar Beans	1	Nil
Haricot Beans	1	Nil
Soya Beans	1	Nil
Kidney Beans	1	Nil
Sultanas	1	Nil
Raisins	1	Nil
Apple Juice	9	Patulin (2)
Guava Juice	1	Nil
Grape Juice	3	Patulin (1)
Apple Sauce	3	Nil
Tomato Paste	5	Alternariol (2)
Dried Fruit	1	Nil
Peach Juice	1	Nil
Peanut Butter	5	Aflatoxin B_1 (2)
Peanuts	2	Nil
Blue Cheese	1	Nil
Cottage Cheese	1	Nil
Other Cheeses	2	Nil
White Pepper	2	Nil
Muesli	2	Nil
Instant Coffee	1	Nil
Total	62	9

* *Nil = none detected. Number of positive samples in parenthesis*

In contrast, aflatoxin is a more serious problem. As already stated, it is a very dangerous substance, aflatoxin B_1 being the most hepatacarcinogenic compound to occur naturally.

Once aflatoxin B_1 is formed it is very resistant to degradation and prolonged cooking under normal conditions does not remove it to any extent. The main source of aflatoxin in food is peanuts, although nearly every other commodity one can think of has been shown to contain aflatoxin or to support its production (Stoloff, 1976). The main reason why peanuts are particularly susceptible is the very nature of the plant, in that the pods containing the nuts develop in the soil, which is rich in fungal inoculum. There is evidence to show (Pettit, *et al*, 1971) that the producing fungi can infect pods prior to harvest and under these conditions they can grow at as little as 8 % water content. Consequently peanuts when harvested may already contain aflatoxin irrespective of any further conditions and treatments. In general, however, peanuts that look sound, are free of mechanical and insect damage and have been stored properly, are not likely to be contaminated with aflatoxin. There are also sorting machines available to the food industry that can remove infected and potentially dangerous kernels.

Many peanut consignments are separated into oil and meal. As a generalisation, such oil is safe because most of the toxin remains in the meal and that which is carried over is destroyed by an alkaline wash of the oil. The meal, however, cannot be so easily decontaminated and as it is a valuable source of protein often finds its way into animal feeds. Manu-

facturers using peanut meal as a protein source have to be especially careful to ensure that the raw material is free from aflatoxin. The case of the contaminated breakfast cereal was in fact due to the use of such peanut meal.

Similar stringency has to be applied to the manufacture of peanut butter and in 1984 several samples were found to be contaminated, including those mentioned in this paper. Because of these cases, the manufacturers of these commodities are now taking extreme care, as much of the raw peanut stocks are "suspect" because of the recent drought conditions, which makes the plants more susceptible to fungal invasion and the fungi more likely to form the toxin (MacDonald *et al.* 1964). This situation may seem paradoxical in that fungi require moisture to grow, but if the fungi can initially grow in the pod, the subsequent low water stress can cause toxin production.

Maize is also prone to infection with aflatoxin-producing fungi and what was said with regard to drought conditions promoting aflatoxin formation in peanuts can also be applied to this commodity. In the U.S.A. much work has been done on this problem, (e.g., Lillehoj *et al.*, 1976) and we are hoping that similar studies on South African maize will be made in the near future.

From the preceding discussion it is clear that in the case of mycotoxins in food, eternal vigilance is necessary. The fact that young animals are much more susceptible to the effects of aflatoxin than old animals (Moreau, 1979) is an important consideration in monitoring both milk and peanut butter. It is not the intention of the Mycotoxin Unit at the University of Natal to usurp the rôle of the government analytical services, which do an excellent job, but rather to complement and to look out for danger areas and the less well-known mycotoxins, which can and do occur on occasions. In addition there is much research to be done on food commodities produced in South Africa in order to bring our knowledge up to the same standard as that in the U.S.A., for after all we are the major food producer in Africa.

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mastitis by 15% to 50%. This range seemed to be correlated to the use of teat dip and other factors.

In an experiment carried out by Griffin, *et al.* (1982) two methods of cup removal were used, with a bacterial culture injected into the claw piece during the last pulsations of milking. The first method was a 'gentle removal' with the claw and teat cup pulsation chamber vented, allowing the cups to fall off after milking. The second method, 'abrupt removal', involved pulling off one teat cup before milking and pulsation vacuum was shut off, thus allowing a sufficient drop in vacuum to remove the cups. During the experimental period, 25 of the 40 quarters subjected to abrupt cluster removal became infected, while only seven subjected to the gentle removal were infected. This indicated that the air admitted into the teat cup in the second method, caused teat impaction on the other quarters.

The evidence given in this article suggests that teat impaction as reported by Griffin, *et al.* (1982) may well have had a marked effect on the udder health of the Cedara herd.

Acknowledgements

The author wishes to thank Messrs P.G. Stewart, T.J. Dugmore, A. van Niekerk and Mrs A. Hockly for their assistance in the preparation of this report. Mrs M. Smith for the statistical analysis of the data and Dr R.W. Bryson for the somatic cell counts.

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The incidence of mycotoxins in litter, feed, and livers of chickens in Natal

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Received 17 April 1985

Mycotoxins, and more specifically aflatoxins, have been found in samples of feed, litter, and liver tissue obtained from broiler chicken houses that had a high incidence of ascites and were also associated with low growth rates. It is suggested that aflatoxin may act synergistically with other stress factors to promote the ascites condition.

Mikotoksiene en meer spesifiek aflatoksiene is gevind in monsters van voer, mis en lewerweefsel wat verkry is van braaikuikenhokke wat 'n hoë voorkoms van waterpens het. Dit is ook geassosieer met lae groeitempo's. Daar word voorgestel dat aflatoksien sinergisties mag optree saam met ander stresfaktore om die voorkoms van waterpens te bevorder.

Keywords: Aflatoxin, ascites, broiler chickens, synergism, South Africa

The presence of mycotoxins in poultry feed can affect the health of the birds seriously (Smith, 1982). Mycotoxins are highly poisonous secondary metabolites produced by several genera of fungi. They have been found in many animal feeds and have been shown to adversely affect animals ingesting them (Mirocha & Christensen, 1974). More than 200 mycotoxins are now known (Cole, & Cox, 1981) and each one will be more or less toxic depending on animal species. Aflatoxin B_1 has been shown, in laboratory trials, to be one of the most toxic and to cause a number of disease conditions in poultry, including impairment of kidney function (Tung, Wyatt, Thaxton & Hamilton, 1973), decreased mass gain, impaired immune response, hepatic necrosis, and haemorrhage (Pier, Richard & Cyszewski, 1980). Therefore, the occurrence of mycotoxins and toxigenic strains of fungi in agricultural products was investigated during 1982/83.

Samples of chicken litter, feed, and liver tissue were obtained from broiler houses in the Durban – Pietermaritzburg area of Natal, containing both healthy and diseased birds. These were taken on an *ad hoc* basis and were dependent on the location and frequency of disease symptoms. Random samples were taken where possible. The mycotoxins were analysed using a modification of the method of Patterson & Roberts (1979) and have been described previously (Dutton & Westlake, 1985).

A total of 142 poultry feed samples were analysed over a 2-year period and these revealed that samples from broiler houses where poor growth and ascites had been reported, had

Table 1 Incidence of mycotoxins in chicken feed, litter, and liver in cases of broiler disease

Sample type and disease condition	Aflatoxin B_1			Other mycotoxins[b]		
	Total no. of samples examined	Incidence (range)[a]	% positive	Incidence	% positive	Total positive (%)
Feed						
Control	25	3(T-20)	12	1	4	16
Poor growth[c]	75	18(T-80)	24	5	7	31
Ascites	30	9(T-100)	30	2	7	37
Other[d]	12	1(20)	8	ND	–	8
Litter						
Control	72	10(0 – 40)	14	ND	–	14
Poor growth[c]	63	11(T-1000)	17	3	5	22
Ascites	27	6(T-20)	22	ND	–	22
Other[d]	4	1(T)	25	ND	–	25
Liver						
Healthy	4	ND	–	ND	–	–
Poor growth[c]	9	ND	–	ND	–	–
Ascites	27	5(T-40)	19	ND	–	19
Other[d]	14	ND	–	ND	–	–

[a]Concentration (µg/kg) given in parenthesis where T = trace and ND = not detected.
[b]Comprises patulin and trichothecenes.
[c]Covers general malaise and nondescript diseases.
[d]Covers malabsorption and gizzard erosion.

obtained mainly from feed millers and hence represent the general occurrence of aflatoxin in feed samples. Furthermore, the highest level of aflatoxin B_1 found in feeds from houses with cases of ascites was 100 µg/kg in control samples. From Table 1 it can also be seen that mycotoxins other than aflatoxins were also found in feed samples and that the percentage occurrence was again associated with disease conditions.

The occurrence of mycotoxins in litter followed a similar trend to that found in feed samples (Table 1). Under normal circumstances one would not expect the presence of toxins in litter to affect the health of the chickens significantly. However, in one series of litter samples analysed, consisting only of groundnut hulls, aflatoxin B_1 was found to be present at levels in excess of 10 µg/g and was associated with a very poor growth rate of broiler chickens.

Examination of liver tissue showed the presence of aflatoxin B_1 in five out of 27 birds suffering from ascites (Table 1). This is the first time that aflatoxin has been found in such samples in this laboratory. It is possible that the timing of sampling is a critical factor, as aflatoxin B_1 is rapidly metabolized and excreted by animal tissues (Pier, Heddlestone, Cyszewski & Patterson, 1973; Harland & Cardeihac, 1975). This would result in depletion of toxin levels below detection limits within a short time after ingestion.

The incidence of fungi in the various materials examined is shown in Table 2. Those capable of mycotoxin production include members of the genera *Aspergillus, Penicillium,* and

Table 2 Incidence of toxin-producing fungi found in chicken feed and litter in 1982/83 for the Durban – Pietermaritzburg area of Natal

Sample type and disease condition	Total no. of samples	Number of samples with viable fungal propagules (% total)				
		Aspergillus spp.	*Aspergillus flavus*	*Fusarium* spp.	*Penicillium* spp.	Other[a]
Feed						
Control	25	10(40)	10(40)	5(20)	2(8)	8(32)
Poor growth[b]	75	3(4)	14(19)	15(20)	5(7)	19(25)
Ascites	30	1(3)	3(10)	9(30)	1(3)	3(10)
Other[c]	12	1(8)	3(25)	4(33)	2(17)	4(33)
Litter						
Control	72	29(40)	11(14)	8(11)	ND	71(99)
Poor growth[b]	63	7(11)	8(13)	3(5)	ND	9(14)
Ascites	27	ND	3(11)	1(4)	ND	ND
Other[c]	4	2(50)	2(50)	1(25)	ND	3(75)

[a]Other covers non-toxin producers and unidentified fungi; some samples contained more than one genera. Figure in parenthesis is percentage of total samples examined.
[b]Covers general malaise and nondescript disease.
[c]Covers malabsorption syndrome and gizzard erosion and two cases of aspergillosis.

Fusarium whilst fungi listed as other include species from the genera *Mucor, Trichoderma, Rhizopus* and in the case of litter, a large number of yeasts.

The results show widespread contamination of feed and litter with various fungal genera but with the highest incidence being found in control samples, perhaps owing to a lack of competition from other micro-organisms.

It was shown by Chang & Hamilton (1981) that aflatoxin increases the susceptibility of broilers to infectious bursal disease (IBD) and at the same time alters the symptoms of typical IBD. It was further shown that aflatoxin can exert a synergistic effect with other conditions such as excess heat, thereby increasing the severity of disease (Wyatt, Thaxton & Hamilton, 1975). It is therefore difficult to determine the possible influence of aflatoxin in a disease such as ascites but aflatoxin is likely to act synergistically with other stress factors to cause this condition.

In conclusion, this survey indicates that mycotoxins, especially aflatoxins, may depress production and could play a significant role in certain poultry diseases in South Africa.

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Mycopathologia **131**: 31–36, 1995.

Occurrence of mycotoxins in cereals and animal feedstuffs in Natal, South Africa 1994

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Received 14 February 1995; accepted in revised form 18 May 1995

Abstract

During the year of 1994, 417 samples of agricultural commodities, comprising: maize, compound animal feeds, oil seeds, soya bean, fish meal and forage were examined for fungi and over 20 mycotoxins using a multi-screen augmented with individual assays. Trichothecenes had the highest incidence of over 19% in all samples received, followed by aflatoxin at 6% and then zearalenone at 3%. Selected samples (73) were analyzed for fumonisin B_1 and of these, 69 (94%) were found to be positive. Because of this result and high incidence of *Fusarium* spp. (over 70%) in maize and maize containing feeds, which was higher than either *Aspergillus* spp. (19%) or *Penicillium* spp. (33%), attention is drawn to the actual and potential presence of fumonisin in the food chain.

Key words: Agricultural commodities, Analysis, Mycotoxin

Introduction

The introduction and refinements of modern instrumental chromatographic methods [1] tends to have made them supersede thin layer chromatography (TLC) as the main method of choice for analyzing mycotoxins. In developing countries, however, expensive modern instrumentation is often not available and where it is, it often cannot be adequately serviced and maintained. A possible future solution of this problem is the introduction of immuno-assay methods [2] which are specific and sensitive. At the moment these methods are limited to only certain mycotoxins, e.g., aflatoxin, ochratoxin and fumonisin and in some case are only used as sophisticated clean-ups, which then need the usual instrumental methods of quantitation.

It is likely that for most developing countries simple and relatively cheap methods of mycotoxin analysis, such as TLC, will be practical for the foreseeable future, particularly for mycotoxins that are peculiar to a geographical region. Indeed broad screening programmes using flexible methods of detection and also involving mycological surveys, are required to define the limits of the problem.

We have used a multi-mycotoxin screen [3] incorporating TLC as the first line of mycotoxin detection to analyze a range of agricultural commodities and in 1985 a paper was published on the results for 1981 to 1983 [4]. Since that time our analytical service has continued with improvements due to increasing experience and the application of additional methods.

To give an assessment of this screen and its potential application to regional use in African countries, the results for the year of 1994 is reported here. These results are particularly suitable as a yardstick, because the largest number of samples in any one year to date have been analyzed and this includes analysis for the more recently discovered fumonisin B_1. An additional point of interest is that the method was taught to delegates at a regional FAO mycotoxin workshop in Botswana [5], this was found to be well received and produced some interesting results.

Materials and methods

Collection of samples. Samples, mainly from farmers, feed companies and poultry companies were submitted for analysis, usually together with a completed

questionnaire (previously distributed to regular clients) giving details surrounding the circumstances of the sample, e.g., method of sampling, associated disease symptom(s) and condition of sample. Where possible, 1 kg samples were submitted but as little as 100 g in some cases. These were all ground and thoroughly mixed prior to analysis. The major materials analyzed were maize and its milled products, general feed, poultry feed and their components.

Extraction, cleanup, thin layer chromatography and confirmation. The methods applied were basically the same as those used in the previous report [4]. Briefly, for the multi-screen, the sample was extracted with aqueous acetonitrile, defatted with iso-octane, treated with sodium bicarbonate, which on extraction with chloroform, gave a neutral fraction and, after acidification with sulphuric acid, an acidic one. The neutral fraction was dialysed against aqueous acetone, re-extracted into chloroform and examined by TLC. The acid fraction was directly examined by TLC.

Several mycotoxins not amenable to analysis by the multi-screen were independently assayed and these included: aflatoxin in groundnut [6] ergot alkaloids [7] (extracted with ethyl acetate/ammonium hydroxide mixtures [8]), moniliformin [9], fumonisin B_1 [10], gliotoxin [11], and cyclopiazonic acid [12].

Aflatoxins were detected with ultraviolet light and other mycotoxins with various spray reagents as reported before [4] but zearalenone was located with diazotized dianisidine (in place of benzidine) [13]. Confirmation and semi-quantization was done with authentic standards obtained from Sigma Chemical Co, St Louis or Makor Chemicals, Jerusalem or CSIR, Pretoria or MRC, Tygerberg by spiking and visual comparison.

Further confirmation and accurate quantitation of aflatoxin [14] moniliformin [15] fumonisin B_1 [16] patulin [17] and zearalenone [18] was done using high performance liquid chromatography (Waters 501 or Spectra Physics P2000 both fitted with UV absorbance detector and fluorimeter) and for trichothecenes [19] by derivatization with trimethylsilylimidazole followed by gas chromatography and mass spectrometric detection, GC/MS (Hewlitt Packard 5890 series II fitted with an m/s 5971 detector).

Mycological examination. Fungal contamination and viable propagule count was done as previously reported [4] using Ohio Experimental Station Agar [20]. For identification purposes individual colonies were transferred to other suitable agar media and where necessary sent to either the National Fungal Collection, Pretoria or to the MRC, Tygerberg (Professor W.F.O. Marasas) for species identification.

Results and discussion

During the year 1994 a total of 417 samples of various agricultural commodities were analyzed for over twenty different mycotoxins and examined for the presence of contaminating fungi. This number of samples represents an increase in samples done in previous years which were between 150–200 [21]. The reason for this increase is not clear but it may be connected with an enhanced awareness of the mycotoxin problem, due to South Africa's formal re-entry into world markets.

The major number of samples handled were maize (198, 47.9%) (Table 1) reflecting the agricultural importance of this cereal in South Africa. Feeds were the next most numerous which also underlines the importance of maize, as this cereal was the usual energy source in them. Poultry feeds represented about a third of the total feeds, indicating the importance of poultry to Natal. The origin of these was local.

All the commodities listed in Table 1 were found to be amenable to the multi-screen mycotoxin analysis used, except intact oil seeds which were unsuitable due to high lipid content. These were analysed for aflatoxin using the CB method [6]. The incidence of aflatoxins is given in Table 2 which is restricted to those commodities showing positive results. The salient features are the higher than average values (up to 400 ppb) for the limited number of oil seeds examined and the absence of aflatoxin in maize. Notably, in most cases all four major aflatoxins were found with aflatoxin B_1 predominating.

Most of the other mycotoxins detected (Table 3) were presumably derived from *Fusarium* spp. and included several trichothecenes, i.e., deoxynivalenol, diacetoxyscirpenol, nivalenol and an unknown trichothecene. This latter metabolite gives positive reaction with the spray reagents used [22, 23] to detect trichothecenes and gives characteristic fragmentation patterns on gc/ms but does not correspond to available standards. Because nearly 20% of commodities tested contain this substance efforts are being made to identify it.

Other mycotoxins detected (Table 3) were cyclopiazonic acid, patulin and zearalenone. Patulin does not seem to be much of a problem in feeds and cereals although it was present in the one sample of silage.

Table 1. Commodities received in 1994 and positive for mycotoxins[a]

Commodity	No.	Aflatoxin	Trichothecene	Other[b]
Cereal-maize	198	0	16	36
Cereal-sorghum	7	0	0	0
Cereal-triticale	4		3	2
Cereal-wheat	5	0	11	0
Feed-general	84	11	20	27
Feed-pig	13	0	12	0
Fish meal	5	0	0	0
Feed-poultry	52	5	13	10
Litter-poultry	6	0	1	3
Oilseed-cotton	3	1	0	0
Oilseed-peanut	10	6	0	0
Oilseed-sunflower	1	0	0	0
Other[c]	14	3	3	0
Soyabean meal	14	3	3	0
Total	417	26	80	82
Percentage		6.2	19.2	19.7

[a]Fungal screening only = 116; aflatoxin screening only = 12; fumonisin B_1 analysis = 61.
[b]Toxins include: patulin, zearalenone, and cyclopiazonic acid.
[c]Other includes: cat food (1), hay (3), citrus pulp (2), meat product (1), chicken liver (2), spices (3), lucerne (1), mead (1).

Table 2. Summary of aflatoxin in commodities

Commodity	No.	Aflatoxin		Number of samples Total level (ppb)				
		No.	Type	<20	20–100	100–200	200–300	300–400
Feed-general	57	12	Afl 4	2	7	2	1	0
Feed-poultry	42	2	AFB_1	1	1	0	0	0
		3	Afl 4	1	2	0	0	0
Oilseeds[a]	13	7	Afl 4	0	1	0	5	1
Soyabean	14	3	Afl 4	3	0	0	0	0
Total	126	27		7	11	2	6	1
Percentage		20.6		5.6	8.7	1.6	4.8	0.8

[a]Includes 10 peanut and 3 cottonseed.

The incidence of zearalenone varies from year to year and prevalence seems to be linked to drought conditions during the maize growing season. In the past, cyclopiazonic acid has not been routinely tested for but recent concern with regards to its production by members of the *Aspergillus flavus* group [24] prompted specific screening where high levels of these fungi were detected. Two feeds so examined were found to contain between 200–400 ppb of this mycotoxin. It would seem prudent to make this a matter of routine where *A. flavus* is found. Two mycotoxins not detected were ochratoxin A and citrinin which is in strong contrast to the situation in temperate climates such as Europe where these toxins are routinely found.

Polar toxins such the fumonisins could not be analyzed by our screen and were investigated on request

Table 3. Mycotoxins other than aflatoxin and fumonisin found in commodities

Commodity	No.	Mycotoxin positive						
		DAS	DON	NIV	TRI	CA	F2	PAT
General feed	57	0	1	0	19	2	0	0
Pig feed	13	5	0	0	3	0	0	
Poultry feed	37	0	0	0	13	0	0	0
Litter	6	0	0	0	1	0	3	0
Maize	161	0	3	0	13	0	3	0
Triticale	2	0	1	0	1	0	1	0
Wheat	5	0	1	5	5	0	0	0
Soyabean	0	0	0	0	3	0	0	0
Other[a]	5	0	0	0	1	0	0	4
Total	286	5	6	5	59	2	9	4
Percentage		1.7	2.1	1.7	20.6	0.7	3.1	1.4

[a]Other = citrus pulp (2), curry powder (2), silage (1).
Note there are 14 samples with more than one mycotoxin present. DAS = diacetoxyscirpenol; DON = deoxynivalenol; NIV = nivalenol; TRI = unknown trichothecenes; CA = cyclopiazonic acid; F2 = zearalenone; PAT = patulin.

using a separate method [10]. This deviation from routine is one indicator of the flaw in the figures presented in Table 1. Of the 417 samples listed only 80 were analyzed for fumonisin B_1 (FB1) (Table 4); of these 66 were positive which is 85%. These results give cause for alarm although yellow maize, which is normally used for animal feed, showed a higher incidence of fumonisin contamination than white maize. Unfortunately clients dictate when fumonisin analysis is done, as it has to be charged separately. Because of this the statistics may be skewed in favour of finding the toxin, although the high incidence and levels of *Fusarium* spp. found in most commodities (Table 5) would indicate otherwise.

The results presented in Table 1 are unadjusted and 98 samples of the maize and 18 of the feed samples were sent for fungal screening only, which gives a final number of samples fully or partly analysed of 301. The former should be eliminated from the statistics, as they count as negatives in spite of the fact that many of these samples had high fungal counts (Table 5). In addition some samples (14) showed multiple mycotoxin contaminations. If these are eliminated also then 174 samples were contaminated with one or more mycotoxin giving a 57.8% overall contamination. This result is likely to be an under estimate, as many other mycotoxins are known that are not analyzed for and the detection limits for some toxins are rather high [3] resulting in lower levels not being detected. The sampling is biased in favour of toxin presence as most samples submitted were suspect.

Table 4. Incidence of fumonisin B_1 in maize and feed

Commodity	No.	Positive level (ppb)[a]			
			< 200	200–1000	< 1000
General feed	5	5	0	3	2
Poultry feed	32	27	0	21	6
Maize	35	32	4	23	5
Triticale	3	2	0	2	0
Miscellaneous	5	0	0	0	0
Total	80	66			
Percentage		82			

[a]Highest levels found general feed = 11000 ppb; poultry feed = 4000 ppb; maize = 2000 ppb.

The screening of samples for viable fungal propagules is a useful exercise in its own right as an indicator of mould contamination but it also complements the mycotoxin analysis and gives an indicator as to what mycotoxins could be present. The high incidence (69%) and high levels (203 samples had 1×10^6 or greater propagules/g) of *Fusarium* spp., principally *F. moniliforme*, in commodities examined in our laboratories is a matter for concern. The limited analysis for FB_1 in this study shows that this toxin occurs common-

Table 5. Fungi found in commodities

Commodity	No.	Number of positive samples[a]		
		Aspergillus spp.	*Fusarium* spp.	*Penicillium* spp.
Cereal-maize	198	18	186	78
Cereal-sorghum	7	2	5	4
Cereal-triticale	4	1	0	1
Cereal-wheat	5	1	1	1
Feed-general	84	26	49	18
Feed-pig	13	5	9	4
Feed-poultry	52	8	24	9
Litter-poultry	6	0	2	3
Oilseed-peanut	10	4	4	4
Oil seed-other	4	0	2	1
Other	20	9	4	8
Soyabean meal	14	4	4	7
Total	417	78	290	138
Percentage		18.7	69.5	33.1

[a] *Aspergillus* spp. includes *A. flavus* group; *Fusarium* spp. principally *F. moniliforme* and *F. subglutinans*.
All positives have > 10 propagules/g, majority in range of $1 \times 10^4 -> 10^6$.

ly in maize and this agrees with other investigations [25]. In our opinion it is imperative that routine analysis for the fumonisins in maize in Southern Africa is inaugurated as quickly as possible and should be as standard a procedure as checking oil seeds for aflatoxin.

The data presented here shows that it is possible to apply a multi-mycotoxin screen using TLC, as the principle method of detection and semi-quantitation, to a range of agricultural commodities. The method is capable of more precise results down to a lower detection limit if backed up by HPLC and GC/MS which can confirm TLC findings where used. This was the case with many samples where clients needed more definitive results. Judging from the people and organisations using our laboratory we apparently provide an independent service that is unique in Southern Africa. To give some idea of the usage 166 samples were sent in by feed companies, 103 by poultry companies, 66 by millers, 46 by farmers, 11 by agricultural co-operatives, 5 by insurance companies and 16 by universities and sister institutes.

In claiming that this system is suitable for use by regional laboratories to provide an analytical service several factors should be borne in mind. Considerable time and effort is required to obtain the necessary skill and experience to take full advantage of the method. The operator has to work quickly and accurately; to be able to score and interpret TLC plates after various treatments and under different conditions; to know when and how to perform confirmatory tests; to avoid spurious readings which give rise to false positives; to be able to do practical mycology; and to either identify fungi to at least genus level or know where to obtain speciation where necessary.

The operative must be able to use clues given by the presence of contaminating fungi and animal symptoms provided by clients to decide, which toxins are likely to be present, although the presence of any particular fungus does not guarantee the presence of its mycotoxins nor its absence that the toxin is not present. Probably the most important skill is to know the limits of the method. We are constantly improving our system but clearly a multi-screen cannot give the degree of sensitivity and accuracy that a method developed for a specific toxin can give. One innovation we have introduced which is an invaluable safety net is that of toxicity testing using human cell lines [26, 27]. This can be used to advise clients where the multi-screen extract is toxic even though routine mycotoxins have not been detected.

Acknowledgements

We thank Rainbow Chickens and Robertson's Spices for financial support; W.F.O. Marasas, P. Thiel and E. Sydenham, PROMEC, MRC Tygerberg for assistance with fungal identification and mycotoxin analysis; R. Berry, J. Geyser, and M. Wagner for technical support; The University of Natal for facilities; and clients who provided samples and information.

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S. Afr. J. Anim. Sci. 1996, 26(2)

Technical Note

A note on the occurrence of mycotoxins in cereals and animal feedstuffs in Kwazulu Natal, South Africa 1984–1993

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Received 15 August 1995; accepted 2 April 1996

During the period 1984–1993 just over 1600 samples of agricultural commodities, comprising maize, compound animal feeds, oil seeds, soyabean, fishmeal and forage were examined for fungi and over 20 mycotoxins using a multi-screen augmented with individual assay. Aflatoxin had the highest incidence in over 14% of all samples examined followed by trichothecenes at 10% and then zearalenone at 4%. Since 1989 selected maize samples with high levels of *Fusarium* spp. have been examined for fumonisin B_1 and of these (n = 20) in 1993, 90% were positive. Because of this result and high incidence of *Fusarium* spp. (32%) in maize and maize containing feeds, which was higher than either *Aspergillus* spp. (27%) or *Penicillium* spp. (12%), concern is expressed with regard to the potential presence of fumonisin in the food chain.

Gedurende die tydperk 1984–1993 is net meer as 1600 monsters van verskillende landbouprodukte, wat, onder andere, mielies, gemengde veevoedsel, oliesade, sojabone, vismeel en kuilvoer insluit, ondersoek vir die voorkoms van swamme en net meer as 20 mikotoksiene. Die mikotoksiene is opgespoor deur gebruik te maak van algemene opsporingstoetse wat daarna deur spesifieke individuele toetse uitgebrei is. Die aflatoksiene het die meeste voorgekom en is in 14% van al die monsters wat getoets is, gevind, gevolg deur die trichothecene (10%), en zearalenone (4%). Sedert 1989 is mieliemonsters met hoë vlakke *Fusarium* spp. getoets vir die voorkoms van fomonisien B_1 en in 1993 is 90% (n = 20) positief getoets. As gevolg van die uitslag, asook die hoë voorkoms van *Fusarium* spp. (32%) in mielies en mielie-bevattende voere, wat hoër as beide *Aspergillus* spp. (27%) of *Penicillin* spp. (12%) was, word kommer uitgespreek ten opsigte van die potensiële voorkoms van fumonisien in die voedselketting.

Keywords: mycotoxins, fungi, food

In 1985 results were published of a multi-mycotoxin screening used to analyse samples of agricultural commodities sent in by farmers and commercial companies in Natal from 1981 to 1983 (Dutton & Westlake, 1985). Since that time the analytical service has continued with improvements resulting from increasing experience and the application of additional methods.

The introduction of immunoassay (Coker, 1984) and refinement of chromatographic methods (Chu, 1984) for analysing mycotoxins tend to have made thin layer chromatography (tlc) obsolete. Multi-mycotoxin screens can also be criticised because they are not optimum for any particular toxin and at best represent a compromise between the range of toxins analysed in developing countries. However, a multi-screen using tlc as its first line of detection, can be put to good service, because it requires the minimum of equipment, can detect a range of toxins in a short period of time, and can be made semi-quantitative with the use of standards. Lack of detectability is not a problem with most mycotoxins, as levels responsible for disease in animals can be detected, which is what the practical farmer needs to know.

Ranged against this is the importance of having an experienced evaluator who can interpret results correctly and can read indicative signs such as disease symptom, fungi present and condition of the sample, with a degree of insight. The necessity of modern instrumentation for definitive quantitation and identification is also a limiting factor, particularly as developed countries have laws governing permitted mycotoxin levels in imported produce. In order to give an assessment of one multi-mycotoxin screen, the results from analysing agricultural commodities provided mainly by farmers, feed companies and other commercial concerns for the period 1984 to 1993 are reported here.

Collection of samples

Samples were submitted for analysis, mainly from farmers, feed companies and poultry companies, usually together with a completed questionnaire (previously distributed to regular clients) giving details surrounding the circumstances of the sample, e.g., method of sampling, associated disease symptom(s) and condition of sample. Where possible 1 kg samples were submitted but there was as little as 100 g in some cases. The samples were all individually milled and thoroughly mixed prior to analysis. The major materials analysed were maize and its milled products, general feed, poultry feed and their components.

Extraction, cleanup, thin layer chromatography and confirmation

The methods applied were basically the same as those used in the previous report (Dutton & Westlake, 1984). Briefly for the multi-screen, the sample was extracted with aqueous acetonitrile, defatted with iso-octane, treated with sodium bicarbonate which on extraction with chloroform gave respectively a neutral fraction and, after acidification with sulphuric acid, an acidic one. The neutral fraction was dialysed against aqueous acetone, re-extracted into chloroform and examined by tlc. The acid fraction was directly examined by tlc.

Several mycotoxins not amenable to the multi-screen analysis were independently assayed and these included: aflatoxin in groundnut (Stoloff & Scott, 1984) (other mycotoxins were not investigated in this commodity), ergot alkaloids (McLaughlin *et al.*, 1964) {extracted with ethyl acetate/ammonium hydroxide mixtures (Ware *et al.*, 1986)}; moniliformin (Scott & Lawrence, 1987); fumonisin B_1 (Sydenham & Thiel, 1992); gliotoxin (Richard *et al.*, 1989); and cyclopiazonic acid (Lansden, 1986).

Aflatoxins were detected with ultra violet light, and other mycotoxins with various spray reagents, as reported before (Dutton & Westlake, 1985) but zearalenone was located with diazotized dianisidine (in place of benzidine) (Malaiyandi *et al.*, 1983). Confirmation and semi-quantifition was done with authentic standards obtained from Sigma Chemical Co, St Louis or Makor Chemicals, Jerusalem or Council for Scien-

tific and Industrial Research, Pretoria or Medical Research Council, Tygerberg, by spiking and visual comparison.

Further confirmation and accurate quantifition of aflatoxin (Beebe, 1978) moniliformin (Thiel *et al.*, 1982) fumonisin B_1 Thiel *et al.*, 1991) patulin (Moller & Josefsson, 1981) and zearalenone (Prelusky *et al.*, 1989) were done using high performance liquid chromatography (Waters 501 or Spectra Physics P2000 both fitted with UV absorbance detector and fluorimeter) and for trichothecenes (Gilbert *et al.*, 1985) by derivatization with trimethylsilylimidazole followed by gas chromatography and mass spectrometric detection, gc/ms (Hewlitt Packard 5890 series II fitted with an m/s 5971 detector).

Mycological examination

Fungal contamination and viable propagule count was done as previously reported (Dutton & Westlake, 1985) using Ohio Experimental Station Agar (Kaufman *et al.*, 1963). For identification purposes individual colonies were transferred to other suitable agar media and where necessary sent to either the National Fungal Collection, Pretoria or to the Medical Research Council, Tygerberg (Professor W.F.O. Marasas) for species identification.

Although the total number of samples examined, 1602, was greater than in the previous report (794), it should be noted that the time period was 10 years as compared with two in the former. The lower annual average number is explained by the fact that included in the first report was the total turnover from a particular feed manufacturer wishing to ascertain the quality of their materials. In the current study the number of samples dealt with per year was fairly constant at between 100 and 200 per year up to 1993 when the number increased (Figure 1). This trend has continued for 1994 and by June had already reached the 305 mark (not presented in this report). The reason for this up-swing is difficult to explain but the increase in samples from the commercial sector (Figure 2) may be related to the re-entry of South Africa into world markets.

During the period covered by this study, we have recorded all our results in data base using commercially available software together with the occurrence of the mycotoxins, their

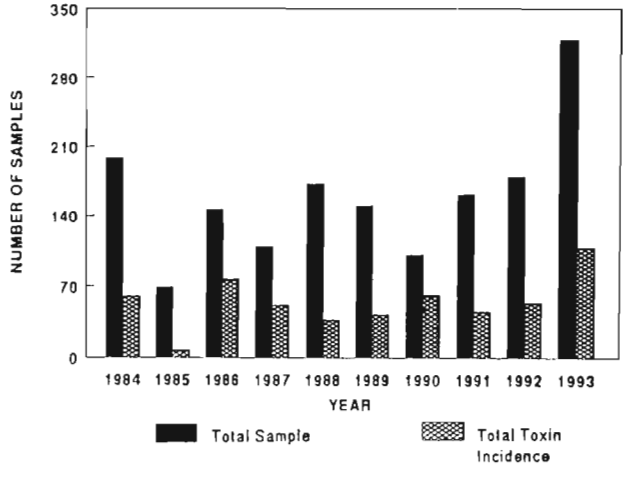

Figure 1 Number of samples per year and total mycotoxin incidence.

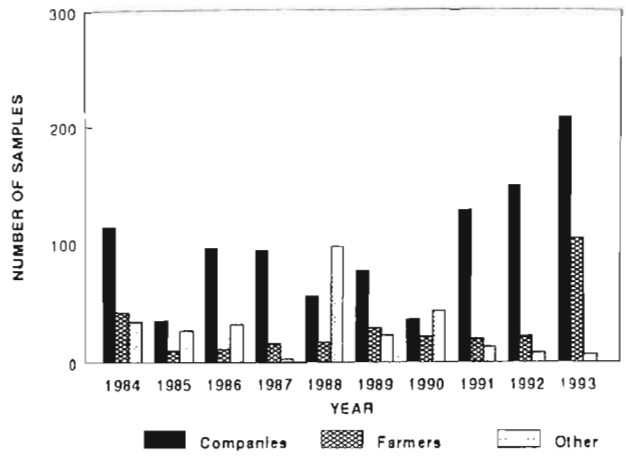

Figure 2 Sources of samples.

symptoms, and the fungi produced, which we obtained from the literature. This has proved very useful for generating statistics and directing which mycotoxins to look for based on information given by the client. The major commodities received were maize (408), general feed and components (346), and poultry feed (233). The latter figure was given separately to indicate the importance of mycotoxin analysis to the local poultry industry in Natal, where high production efficiency can be affected by the presence of sub-lethal levels of toxin in the feed. Minor but routine materials were groundnut (42), sunflower seed (22), cottonseed (38), soyabean (61); other cereals, mainly wheat (27); forage, mainly lucerne and hay (28); silage (8), poultry litter (37), fish meal (49), and various animal tissues and fluids (84). The latter were of both animal e.g., chicken liver and human, e.g., blood and urine origin. The balance was made up of food commodities (53) and miscellaneous items. Included in food commodities were 23 samples of apple juice which were examined specifically for patulin.

The source of the samples was of some interest. The main suppliers were poultry companies (346 samples), feed companies (299 samples), farmers (293 samples), and the University of Natal (121). The remainder in order of sample numbers came from food companies, co-operatives, insurance companies, millers, hospitals, rural homes, institutes, private individuals and supermarkets. The exact order, however, varied from year to year depending upon particular problems encountered.

Trends in mycotoxin incidence are similar to those observed in the previous report. Overall percentage of mycotoxin contamination was 35%. Allowing for double contaminations the figure is reduced to 30%, a similar figure (27%) to that found previously (Dutton & Westlake, 1985) (Table 1). Care should be exercised in interpreting these figures as they were biased for several reasons. Firstly there would have been a tendency towards finding mycotoxin contamination owing to the fact that the samples were drawn from suspect materials. Secondly where specific analyses had to be done, e.g., in the case of fumonisin, these were not carried out if the client did not request them (extra costs involved) or if there were no indicators such as symptoms or fungi to suggest such an analysis. This lead to an underestimation of contamination.

Table 1 Number of positive incidences of mycotoxins in the total number of samples analysed from 1984 to 1993

Mycotoxin	Incidence	Level PBB	Comment
Aflatoxin B_1/B_2	114	1–500	Both toxins
Aflatoxin (All)	115	1–500	All 4 toxins
Cyclopiazonic acid	1	ND	Insensitive
Fumonisin B_1[b]	43	TR–1000	By tlc
Gliotoxin[b,c]	1	ND	
Kojic acid	34	ND	
Oosporein[c,e]	4	ND	
Patulin	33	1–280	
Penicillic acid	6	ND	
Sterigmatocystin	4	ND	
Tenuazonic acid	2	ND	In litter
Trichothecenes			
Deoxynivalenol	22	ND	
Diacetoxyscirpenol	19	ND	
Nivalenol[c]	10	ND	
Trichothecene[c,d]	100	ND	Unknown
T-2 Toxin	3	ND	
Zearalenone	67	50–8000	
Total	594		

[a]Out of a total number of samples of 1602

[b] Analysis of these done separately and commenced in 1989

[c] Unconfirmed because of lack of standard

[d] Unknown metabolite which gives characteristic colour reactions of a trichothecene and is found in maize

[e] Oosporein associated with the presence of *Acremonium* spp. and appears as a yellow visible spot on tlc

[f] It has recently been determined that the multi-screen is insensitive to cyclopiazonic acid and a separate assay is now done when appropriate fungi are found

TR = trace < 200 ppb; ND = not quantitated, > 200 ppb

Another difficulty was that of sampling, as this was done by the person supplying the sample. Hence there was no guarantee that the results have any statistical validity, although efforts were made to ensure that representative random samples were submitted. In these cases a mixture of the samples was analysed. Lack of sensitivity would also overlook low level contamination by certain toxins, e.g. cyclopiazonic acid, the detection limits of the dialysis method for mycotoxins being given by Patterson (Patterson & Roberts, 1979). Finally it was not possible to screen for all known fungal toxic metabolites.

In order to address the latter problem we have now set up a cell toxicity test based on human cell lines and a tetrazolium salt assay coupled with microscopy (Robb *et al.*, 1990; Smith *et al.*, 1992). This has already proved its worth recently by showing cattle feed extracts to be highly cytotoxic in spite of the absence of detectable mycotoxins.

Aflatoxin B_1 was the most prevalent mycotoxin contaminating 14.3% of total samples, 7.2% of these containing all four aflatoxins, with trichothecenes next at 8.5% and then zearalenone (4.2%). A major contributor to the trichothecene figure is an unknown compound which gives positive response to the spray reagents used to detect these metabolites, i.e., 50% sulphuric acid in methanol, chromotropic acid reagent (Baxter *et al.*, 1983) and Kato's sulphuric acid in methanol chromotropic acid reagent (Takitani *et al.*, 1979). Initial gc/ms results support its identity as a trichothecene but no further identification was possible owing to a lack of a coincidental spectrum or standard. The incidence of the fumonisins should not be overlooked as these were not screened for until 1989. As these have to be analysed separately, not all samples are examined, as clients have the option to have this analysis at extra cost. Maize examined in another programme (Dutton *et al.*, 1993) was found to have a 50% incidence of fumonisin B_1 contamination and in this study it had an incidence of 90% (18/20) of samples examined in 1993. The result is biased, however, as the samples analysed were selected as being suspect. Considering the relative ease of detecting aflatoxin with respect to fumonisin it seems very likely that the latter is at least as ubiquitous in South African products as aflatoxin.

Other minor mycotoxin contaminants were patulin and kojic acid both at just over 2% of all commodities examined. The significance of these fungal metabolites is difficult to assess, as both have low toxicity, although patulin has been implicated as a carcinogen in the past and is now back in the news owing to its prevalence in apple concentrates. Minor incidences of other mycotoxins included cyclopiazonic acid, sterigmatocystin, gliotoxin, tenuazonic acid, and oosporein, although the latter two could not be confirmed owing to lack of standards. A detailed record of their incidence is included in Table 1.

By far the most predominant fungus found in the samples was *Fusarium* spp. (507 samples positive = 31.3%) which were mainly either *F. moniliforme* or to a lesser extent *F. subglutinans* as indicated where the fungus was identified down to species level. This reverses the order of prevalence between this genus and *Aspergillus* spp. as noted in the previous study (Dutton & Westlake, 1985), the latter incidence being 435 (26.9%) of which 284 belong to the *A. flavus* group. The third major genus was *Penicillium* spp. which had an incidence of 156 (9.6%). Other fungal genera included *Acremonium* (60; 3.7%), *Diplodia* (19; 1.2%), *Paecilomyces* (8; 0,5%), *Rhizopus* (24; 1.5%) and a group of unknown fungi with white non-sporing mycelium (68; 4.2%). There were 420 incidences where more than one fungal species occurred in a sample.

The high incidence of *Fusarium* spp. in samples indicated the common use of maize in South African feeds and other commodities, for it is likely that this is the source of *F. moniliforme* and *F. subglutinans* which are common contaminants of this cereal. This finding could have important repercussions as they are a source of fumonisin. The significance of this for human and animal health is not at the present clear but it seems likely that it may clarify previously unexplained animal disease conditions and production losses.

The distribution of mycotoxin incidences amongst commodities is given in Table 2. The results follow the pattern exhibited in the previous 1985 report (Dutton &

Table 2 Incidence of mycotoxins in South African agricultural commodities 1984–1993

Commodity	Mycotoxin (No. samples positive)[a]						
	Aflatoxin	Trichothecene	Kojic acid	Fumonisin B_1	Zearalenone	Patulin	Other[d]
Maize	35	56	17	10	21	3	6
Other cereal	7	4	0	0	2	1	0
Oil seed	37	2	0	0	3	1	0
Animal feed	57	69	9	14	31	2	7
Poultry feed	46	26	7	20	7	4	7
Fish meal	4	0	0	0	0	0	0
Animal tissue	0	0	0	0	0	0	
Forage[b]	6	1	0	0	0	0	2
Soyabean	9	0	0	0	0	1	0
Miscellaneous[c]	18	11	1	0	3	21	2
Total	229	169	34	44	67	33	20

[a] Total number of samples assayed = 1602. [b] Includes: hay, lucerne, and poultry litter. [c] Includes: bagasse, beer, brewer's grains, dry beans and food. [d] Includes: gliotoxin (1), cyclopiazonic acid (1), penicillic acid (5), oosporein (6), tenuazonic acid (2), sterigmatocystin (4).

Westlake, 1985).

After using the dialysis clean-up method for a multi-mycotoxin for over 13 years we have found that with modifications we can routinely assay for over 20 of the more commonly occurring mycotoxins. The method is, however, limited to the more non-polar toxins, and fumonisins and moniliformin, for example, have to be analysed using other clean-up methods.

For precise results the method is poor compared with specifically designed methods coupled to modern instrumental methods, as recoveries can be as low as 50% for certain toxins or even lower., e.g., cyclopiazonic acid. In experienced hands, however, and with a knowledge of the limitations of the methods, levels of toxins can be effectively gauged by visual comparison with known levels of standards run under similar conditions. Often this is all a local farmer or miller needs to know so that the correct remedial action can be taken. Where more precise figures are needed, samples can then be forwarded to centres which have more sophisticated methods at their disposal.

To screen for over 20 toxins, estimate the magnitude of contamination, and identify the major fungal contaminants costs R58 ($17) per sample for consumables using fairly basic laboratory equipment. We feel that this makes it highly suitable for use in Southern Africa where more fully equipped laboratories are not available for routine mycotoxin analysis.

Acknowledgements

We thank Rainbow Chickens and Robertsons Spices for financial support; W.F.O. Marasas, P. Thiel and E. Sydenham, PROMEC, MRC Tygerberg for assistance with fungal identification and mycotoxin analyses; R. Berry, J. Geyser and M. Wagner for technical support; The University of Natal for facilities; and clients who provided samples and information.

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CHAPTER 5:

HUMAN STUDIES

5:1 PAPERS AND STUDIES

An investigation into the passage of natural toxins across the digestive tract wall using the everted sac technique
Paper 36 by Thompson, Dutton, Bye and Dehrmann

Aflatoxins and kwashiorkor in Durban, South Africa
Paper 38 by Ramjee, Berjak, Adhikari and Dutton

Fumonisins, mycotoxins of increasing importance: their nature and their effects
Paper 45 by Dutton

Maize storage and health related problems for the indigenous rural community
Paper 48 by Gqaleni, Chuturgoon, and Dutton.

The detection and measurement of aflatoxin B_1 conjugates in humans in Natal, South Africa
Paper 49 by Dutton, Myeni, Early, Chuturgoon, and Bux

Analytical method for the determination of sphinganine and sphingosine in serum
Paper 51 by Castegnaro, Garren, Galendo, Gelderblom, Chelule, Dutton and Wild

The determination of fumonisin B_1 in faeces: a short term biomarker for assessment of exposure
Paper 55 by Chelule, Gqaleni, Chuturgoon and Dutton

Serum fumonisin B_1 levels in Black African women
Paper 56 by Reddy, Dutton, Chuturgoon, Moodley and Moodley

A study of the occurrence of fumonisin B_1 in a rural community in Kwazulu Natal, South Africa
Paper 57 by Chelule, Gqaleni, Chuturgoon and Dutton

5.2: INTRODUCTION

The question of the occurrence of human mycotoxicosis is a difficult one to answer for two main reasons. As with animal disease it is only by good fortune that the contaminated food, causing an acute mycotoxicosis, is available for analysis. Often symptoms appear after the food has been consumed or been disposed of. The metabolic effects of mycotoxins in animals, best monitor by the measurement of biomarkers, e.g., lysylAFB1 in serum, are poorly understood and quantitated. The second problem is that there is a lack of human volunteers to test the effects of toxins, assuming that ethical permission would be given. To extrapolate from animal models is the norm for testing the effects of molecules on humans (e.g., in pharmaceuticals).

This is not necessarily as sound as it seems in the case of mycotoxins, as there is often wide differences in species responses. These effects are well understood in attempts to develop risk factors and permissible limits for mycotoxin contamination of food. In such assessments the NUL is applied reduced by a fudge factor called the Safety Factor, which is a large number to ensure the best protection for the consumer (Kuiper Goodman 1991).

In order to assess human exposure, it is not possible to routinely measure mycotoxin loads in the diet routinely and therefore biomarkers of mycotoxin ingestion must be developed where possible. In first world countries, the problem is not be so acute, as mycotoxin intake can be minimised by legislation and monitoring of raw commodities. For other situations, such as those that prevail in rural Africa, this is not a possibility. Ideally a biomarker should be an easily measurable parameter in the individual, which responds to mycotoxin intake in a dose accumulative way. It should also have a fairly long half-life measured in weeks rather than days and, if possible obtainable by non-invasive means, e.g., from urine.

The first mycotoxin to be looked at from this point of view was AFB1, which forms adducts with macromolecules such as DNA and serum albumin. The formation of these adducts depends upon the "activation" of AFB1 in the liver to the epoxide AFB1O. The epoxide is reactive towards nucleophilic centres, such as guanine in DNA and lysine in proteins. Other amino acids with nucleophilic side chains also react (MT16). These adducts have some of the attributes looked for in an ideal biomarker. Base adducts from DNA of AFB1 can be measured in urine (Groopman *et al.* 1992). Similarly lysyl-AFB1 adducts can be isolated serum albumin, which has a half-life of around 20days. Although these are possible measurements, currently they need fairly sophisticated methods of analysis and are also beset by the problem of what the result really means in terms of toxin intake. I think that an immuno-dipstick type of analysis is required, which could then be correlated to dietary intake from food analysis. The alternative is to turn to an animal model with the difficulties mentioned above.

Other biomarkers are being developed for ochratoxin and fumonisin exposure. Of these only the latter interests us, because to date ochratoxin has not been found in South African foods with the exception of imported coffee. The FB1 marker depends upon its ability to interfere with sphingolipid metabolism. It inhibits the incorporation of sphinganine (SA) into ceramides, which not only has the effect of block cellular signalling (Yoo *et al.* 1996) and membrane component formation but the accumulation of sphinganine. Thus by measuring SA levels and comparing them with sphingosine (SO) it is possible to see an elevation, which in theory can be correlated to FB1 intake (Riley *et al.* 1993). As can be imagined there is quite a gap between theory and practise. Firstly the extraction of sphingoid bases from physiological fluids and tissues is highly variable, which dictates the use of internals standards to monitor efficiency of extraction. Secondly there is a definite difference in SA/SO ratios in males and females. It would seem likely that the menstrual cycle in females has an influence but is not yet clear why this difference is found.

In spite of difficulties encountered in identifying authentic cases of mycotoxins in humans, there are notorious exceptions, the classical one being ergotism. This is cause by the ingestion of ergot alkaloids produced *Claviceps purpurea* and related

fungi, in cereals, particularly rye (Robbers 1979). Other notable examples are yellow rice poisoning in Japan, alimentary toxic aleukia (ATA) in Russia and various aflatoxicoses. These are all cases, which have acute toxicoses cases resulting in death, which makes it easier to correlate the toxin with the lethal effect. There is a whole list of chronic conditions, however, which have been ascribed to mycotoxin ingestion but are difficult define precisely. These include liver carcinoma (Lindsell & Peers 1977) and kwashiorkor (Lamplugh & Hendrickse 1982) induced by aflatoxin; kidney nephthropy by ochratoxin (Krough & Elling 1976); and oesophageal cancer by fumonisin (Rheeder *et al.* 1992). These may be cause, in part, by long exposure to sub-lethal doses of the toxin, alongside other undesirable factors such as malnutrition, avitaminosis, smoking, and alcohol abuse. The identification of toxins in-patients with these diseases is not proof of cause. An example of this is kwashiorkor, where there is plenty of evidence to show abnormal AFB1 levels in such patients but this is now regarded as an effect rather than a cause (Hendrickse and Maxwell 1989).

5.3. COMMENTARY

The major part of my research work on human mycotoxicosis commenced in 1992 when I joined the Medical Faculty, University of Natal, Durban. This was understandable being an appropriate activity in the Faculty and because of access to physiological fluids and tissues from Patients, principally at King Edward VIII Hospital (KEH) through clinical colleagues.

One study, however, was done at Pietermaritzburg, before I left, which would have applications later on. This was the use of everted viable gut sacs for the study of active uptake of compounds and toxins (Paper 36). We were able to show, using rat tissue that AFB1 rapidly crossed the gut wall but not AFB2A. This is an important finding, as it would seem that, if AFB1 was converted to any degree by the acid conditions of the stomach to AFB2A, then this could not enter the host to cause further damage. Indeed any fermentations or treatments that lowered the pH of the food matrix would in effect, detoxify it.

Work on the role of AFB1 in disease in South Africa was commenced. This included an investigation into local cases of kwashiorkor(KR) (Paper 38). Several interesting points sprang form this study. Notably, it was extremely difficult to obtain control samples that were not positive for AFB1, a finding adequately supported by later studies, for AFB1 and other toxins. Furthermore control patients had higher levels of AFB1 in their urine than KR patients, the levels in serum being reversed. This latter finding could be interpreted, as meaning that KR patients had impaired liver/kidney function with respect to AFB1 excretion. Thus there is no evidence to support the notion that AFB1 is the primary cause of KR but increased AFB1 levels in the blood may be an effect. It is important to note that KR patients with higher levels of AFB1 in their blood were more likely to have complications with higher mortality later.

The cause of higher levels of hepatocellular carcinoma (HCC) in certain areas in Africa and other parts of the world has been the subject of many studies. Most of these supporting the role of AFB1 exposure as being the primary cause (e.g. Lindsell & Peers 1972). In spite of studies showing a direct correlation between AFB1 intake and cancer incidence there is, however, controversy surrounding this explanation,

because of the prevalence of hepatitis B (HB) in the same areas (Stoloff 1989). We did a detailed study (Paper 49) examining HCC patients for AFB1 conjugate in their serum (a biomarker), AFB1 in the liver and carcinoma tissue by immunocytochemical (ICC)/electron microscopy (EM) techniques. A substantial number of the patients examined were positive for AFB1 both in tissue (62%) and serum albumin. There also was a high number of patients, positive for HB virus, as shown by the presence of serological markers (95%). One of the patients of the two HB positives, used as controls, had AFB1 in their liver tissue, indicating the prevalence of AFB1 in their diet . As cellular pathology was more severe for patients having AFB1 and HB virus present it was conclude that both these factors had a role to play in the development of HCC.

One other interesting observation made in the HCC study was that AFB1 was found bound in various organelles. These included the nucleus, not unexpected considering the known binding of AFB1O to DNA; the endoplasmic reticulum (ER) and the mitochondria (MC). This posed the question of how did the mitochondria become labelled? It is known that mitochondria do have cytochrome $P_{450,}$ which can be capable of forming AFBO from AFB1 (Niranjan 1980). Hence it is quite feasible for AFB1 to enter the mitochondrion, become activated and bind to mitochondria DNA (mDNA) and/or mitochondrial protein. Alternatively the AFB1 becomes activated at the ER, which is rich in cytochrome P_{450} activity, binds to nascent "signal" proteins and is trafficked to the mitochondria. Actually the situation in terms of AFB1 activation, diffusion and trafficking of the toxin and its derivative and final location in the cell, is much more complicated. This is to be the focus of research in the future, where questions such as "what is the role of rough and smooth ER in these processes?" will be addressed.

Our approach to resolving this problem (MT22) was to incubate mitochondria with either AFB1 or AFB1O, isolate the protein by polyacrylamide gel electrophoresis (PAGE) and Western blot the protein with AFB1 antibody. If any protein band was labelled, it would indicate mitochondrial capability of AFB1 binding, providing the proteins were encoded by mDNA. Although it is true that trafficked protein could also be labelled, particularly from the matrix side of the inner mitochondrial membrane, they should not be as heavily labelled as proteins arising *de novo* within the matrix and directly exposed to AFB1O added initially as AFB1. More general labelling was expected from added AFB1O.

These studies are not just of academic importance, as mitochondria have been viewed as being involved with the formation of cancerous growths for a long time (Pederson 1978) and now are thought to have a direct connection with apoptosis. Certainly on reviewing the literature several inhibitory effects on mitochondria have been claimed for AFB1, including inhibition of ATP synthesis (and interference with electron transport (Obidoa 1986).

The study of the occurrence and effects of AFB1 in humans in Kwazulu Natal, although of some importance considering their ubiquitous occurrence, is only of secondary interest to my research group. A much more alarming scenario is beginning to develop surrounding the potential dangers of fumonisin. A comprehensive review of this mycotoxin, i.e., the most predominant of the group fumonisin B_1 (FB1) up to 1996 was published by myself (Paper 45). An indication of

the interest shown in this toxin is that the review is now inadequate and the number of papers that have appeared in the literature since, is almost double that given in the review.

Certain members of the Fusarium species, most notably, *F.moniliforme, F. subglutinans* and *F.proliferatum*, produce fumonisins. In humans it has been implicated in oesophageal cancer (see Chapter 4) and in animals, porcine lung oedema, equine leukoencephalomalcia (ELEM), and rat liver cancer. It is reputed to cause liver and kidney damage. Because of the general infection of all South African maize (and also of much of the rest of the world) with *F. moniliforme* and/or *F. subglutinans* (Paper 43) it is not surprising to find a high incidence of FB1 contamination in maize grain and all maize/corn products. The situation is greatly exacerbated in South Africa, because the majority of the population uses maize as a staple food.

As was reported in Chapter 4, a rural area in KwaZulu Natal was investigated for the presence of FB1 and other mycotoxins and for the presence of FB1 biomarker (So/Sa) ratios (Papers 51, 55 & 57). To date the results of this survey are worrying (Papers 51 & 55) and agree with other studies in the Transkei (Marasas *el al.* 1993). The biomarker study is making little sense, because there is no correlation whatsoever between the Black population in the Tugela Valley and controls from the French population. This of course may just reflect dietary and genetic differences but the alternative explanation that the Black population as a whole has skewed sphingoid base biosynthesis, because of continuous FB1 ingestion, is a real possibility.

This latter conclusion is gaining steady support from other studies. In order to develop a short-term biomarker we have been analysing faecal samples for the presence of FB1 and its hydrolytic products (Paper 55). The idea I had was that, as nearly all FB1 ends up in the faeces via the GIT or bile, then this was the place to look for the toxin. This would indicate what the FB1 intake there had been within the last 24-48 hours depending upon the personal habits of the individual. Of 24 children sampled for parasite estimation from the South Coast of Natal, 70% were positive for the presence of FB1. Similar results are now starting to come from the Tugela Valley faecal sampling programme (Paper 57). Both studies show that there is a statistical chance that rural people have six times more risk of being exposed to FB1, as compared with urban Black populations.

Because eclampsia and pre-eclampsia are relatively common in both Kwazulu Natal and the Transkei, I thought that, as a long shot, we should look to see if there was any evidence of FB1 in these patients. A study done by Mrs Reddy towards her Masters (MT15) showed that most of the patients examined, had FB1 in their blood, a remarkable finding and, furthermore, that there was a statistical difference between the levels found in eclamptic patients and controls and pre-eclamptic ones (Paper 56).

Examination of tissue is revealing similar results. The probing of oesophageal cancer (OC) tissue using ICC/EM showed that FB1 was present in the cells but not in the peri-cancer tissue (MT 23). Control experiments showed that the antibody used gave positive results for an OC cell line treated with FB1 but not the untreated control. This, however, does not rule out cross reactivity but as far as we know the antibody is

specific for FB1 and congeners. Interestingly only 8 of the 10 patient tissues examined gave the result. On examining patient records, it was found that the two negative results came from Indian patients brought up in Kwazulu Natal, whereas the other 8 were Black patients, most of whom had originated in the Transkei. It is the first real evidence that has been obtained to show that FB1 has anything at all to do with OC. Again this finding is remarkable and unexpected. It raises more questions than it answers. For example, why does OC tissue, in contrast to surrounding tissue, take up FB1 or is this the initiator? Does it enter from the oesophagous or from the blood system? How do cells and the body for that matter, take FB1 up, as it is highly polar and animal experiments show very low uptake from the GIT? What is the mechanism for the induction of OC, as well as all the other disease conditions caused in animals but not OC. These question are currently being addressed by members of my research team, in particular a Post Doctoral Fellow Dr O Tosomba.

If all this is not enough evidence to show that FB1 is present in the Black population of Kwazulu Natal, another study of placental tissue from pre-eclamptic and eclamptic patients is showing positive for FB1 by ICC/EM (MT19). In fact to date we have not been able to find negative control material and are hoping to get this from our collaborators who work on placenta in the United Kingdom. Whatever the effects of FB1 are in humans it is now very clear that our population in Kwazulu Natal is routinely exposed to this mycotoxin at relatively high levels. Considering the high number of patients with cancers, such as, penile, uterus, breast, liver and kidney; and chronic diseases, such as idiopathic congestive cardiopathy (Campbell 1990), kwashiorkor, and Mseleni joint disease; and conditions such as brain lesions, as well as those already discussed, it is quite possible that mycotoxins, in particular FB1, play a role. Several programmes are currently under way to examine these possibilities, e.g., zearalenone in breast and uterus cancer (PT10).

In collaboration with Dr N Gqaleni, dwellings in Kwazulu Natal are being assessed for living and air quality from the point of view of fungi. Fungi play an important role in the condition know as "Sick Building Syndrome" (SBS). Although most studies done in this area are directed at first world air condition buildings, it is becoming apparent that private homes are not immune from such problems (Smith *et al.*1992). Work on this problem in the informal housing sector began in 1997 with an Honours project by Miss Danaviah, which has now developed into a Masters project (MT25). The results showed that people living in a squatter camp, Malukai, south of Durban, were exposed to a wide range of fungi as their spores and viable propagules. Many of these isolates were toxinogenic, in that they were capable of producing mycotoxins. Currently the work is also being done in the poor housing in the Wentworth district of Durban. This urban area is of particular interest as it has the highest industrial pollution within the Durban conurbation.

Journal of Natural Toxins, Vol. 1, No. 2,
pp. 9-16 Printed in U.S.A.

AN INVESTIGATION INTO THE PASSAGE OF SELECTED NATURAL TOXINS ACROSS THE DIGESTIVE TRACT WALL USING THE EVERTED SAC TECHNIQUE

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(Received January 13, 1992; Accepted March 9, 1992)

ABSTRACT

Everted sacs derived from the rat alimentary tract demonstrated the passage of selected naturally occurring toxins from the mucosal to the serosal surface, indicating the potential of this system for studying the uptake of intact toxins from the digestive system of animals. Two dissimilar groups of toxins were used in this study, namely the plant toxins atractyloside and carboxyatractyloside and the mycotoxins aflatoxin B_1 and its water adduct aflatoxin B_{2a}.

Atractyloside and carboxyatractyloside were transported across all sections of the rat alimentary tract wall including intestine and colon, the optimum pH of the mucosal medium being between pH 5 and 7. From this result it is concluded that the kidney damage reported in rats fed extracts of *Callilepsis laureola*, a South African plant that produces these toxins, is caused by the direct effect of atractyloside. This also has implications in human intoxication caused by the ingestion of the tuber of this plant as an herbal medication.

The uptake of aflatoxin B_1 by everted sacs may not be due to active transport but is probably the result of passive diffusion. The impediment to aflatoxin B_{2a} may be explained, therefore, by its higher polarity. This result has significance in the poisoning of animals by aflatoxin B_1 as it is rapidly converted to aflatoxin B_{2a} in acid conditions which may be encountered in the stomach. This may be an important factor in the relative susceptibilities of animals to the effects of aflatoxin.

INTRODUCTION

The everted sac technique, pioneered by Wilson and Wiseman (1954) has been used extensively to study the transfer of substances from the mucosal to the serosal side of the gut wall (Barry et al., 1961; Clarkson and Rothstein, 1961; Crane and Mandelstam, 1960; Chain et al., 1960; Elsenhans et al., 1983; Hoben et al., 1958). This technique has the advantage that eversion exposes more of the intestinal active mucosa to the oxygenated suspension medium and also ensures contact between

it and the substrates in the medium. Furthermore, various sections of intestine may be placed in a single suspension medium thereby creating uniform experimental conditions. The internalized serosal solution may easily be removed from the sac for analysis at the completion of the experiment. This method could be applicable to investigating the uptake of natural toxins being studied by our group, including the mycotoxins aflatoxin B_1 (AFB_1) (Fig. 1A) and the plant toxin atractyloside (ATR) and its carboxylated derivative carboxy-atractyloside (CATR) (Fig. 2).

Aflatoxin B_1 is one of a family of aflatoxins biosynthesized by *Aspergillus flavus* and *Aspergillus parasiticus*. It is highly toxic and carcinogenic to most animals and, therefore, poses a problem in human foods (Diener, 1987) and animal feeds (Connaught, 1989). Although its *in vivo* and *in vitro* effects have been extensively studied (Heathcote and Hibbert, 1978), little work has been done on its uptake from the digestive tract. This also applies to aflatoxin B_{2a} (AFB_{2a}) (Fig. 1B), a water adduct, which arises when AFB_1 encounters acid conditions, such as in the stomach. The fact that AFB_{2a} is relatively nontoxic (Dutton and Heathcote, 1968) may be of significance in this context.

Atractyloside was originally isolated from the Mediterranean thistle, *Atractylis gummifera*, and selectively inhibits the ADP/ATP antiporter in mito-chondria. Later ATR was isolated from *Callilepis laureola* (Wainwright et al., 1977), a plant used by the rural Negroid population in South Africa, as a herbal medication. This has been implicated in cases of toxicosis (Bhoola, 1983) involving both kidney and liver damage. Again, little is known with regards to the absorption of ATR from the alimentary tract; uptake from the colon is of particular importance, as preparations from *C. laureola* are often administered as enemas.

The work reported here is an investigation into the passage of AFB_1 and AFB_{2a} and ATR across the rat alimentary canal wall using the everted sac technique to demonstrate the permeability of the gut to these toxins and the applicability of the technique to such studies.

MATERIALS AND METHODS

Chemicals

Oxygen/carbon dioxide (95%/5%) mixture was purchased from Air Products Ltd. Salicylic acid was obtained from Maybaker (SA) Ltd., the potassium salt of ATR and CATR from Sigma Chemical Co. (St. Louis), anisaldchyde from Merck (SA) and AFB_1 from Makor Chemicals Ltd. (Jerusalem). Aflatoxin B_{2a} was prepared from AFB_1 by cold treatment with 2M sulphuric acid (Dutton and Heathcote, 1969). All other reagents were of an analytical grade.

Preparation of the Everted Sac

Male Wistar rats were obtained from the Biomedical Resource Centre, University of Durban-Westville, Natal, and maintained in an animal house with free access to water and Epol (SA) laboratory rat food. Rats were euthanized using carbon dioxide and the intestines were rapidly removed and transferred to Krebs-Ringer physiological medium (Dawson et al., 1986) at 37°C. Segments 15 cm long were everted as described by Wilson and Wiseman (1954) with the order of eversion for particular parts of the tract being varied from experiment to experiment to ensure randomization of results (Barry et al., 1961). Each sac contained 5 ml Krebs-Ringer medium. All experiments were conducted in triplicate. The everted sacs were placed in 100 ml of well-gassed (95% O_2/5% CO_2) Krebs-Ringer solution at pH 7.2, unless otherwise specified, containing

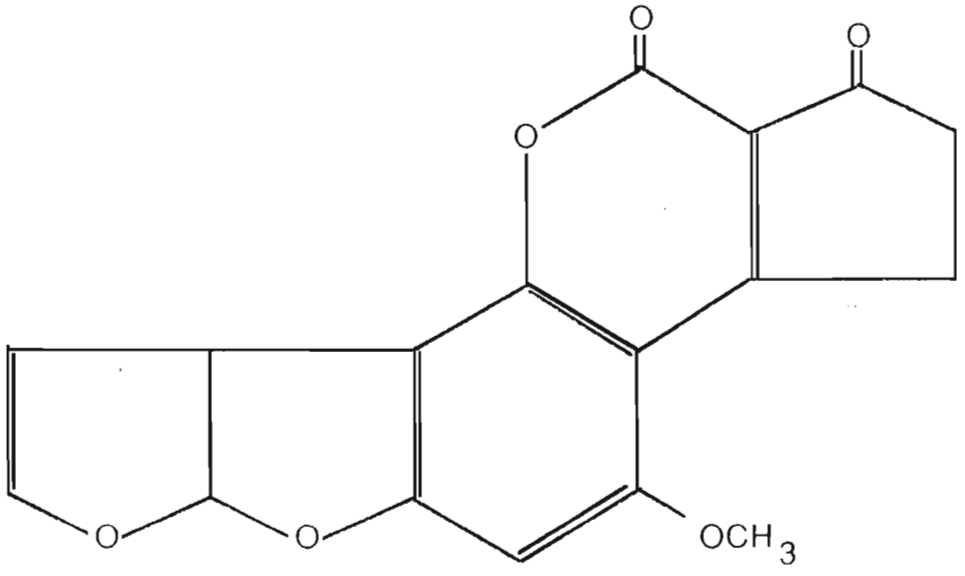

Figure 1A. Structure of aflatoxin B_1.

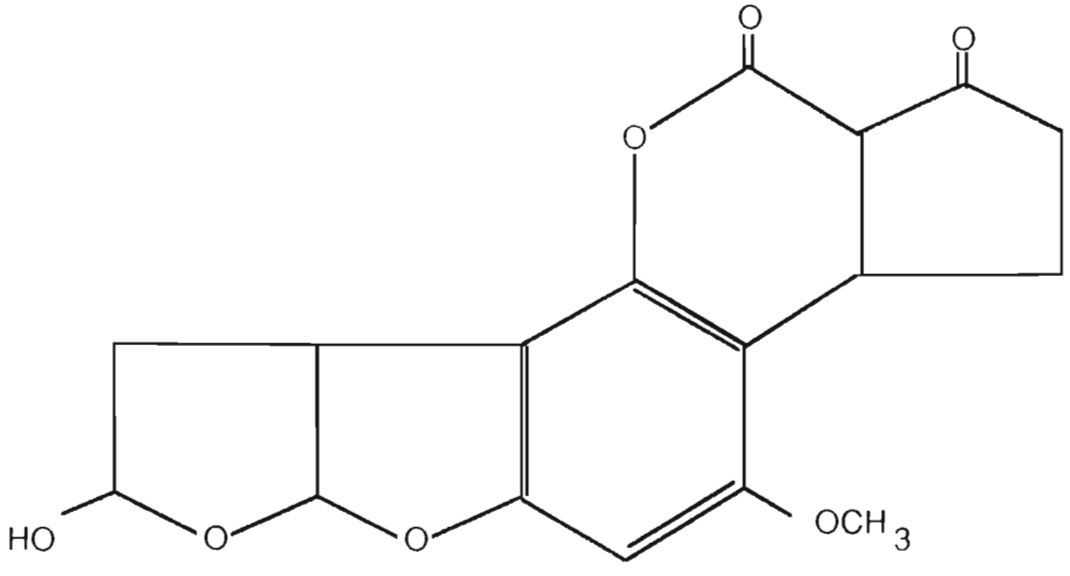

Figure 1B. Structure of aflatoxin B_{2a}.

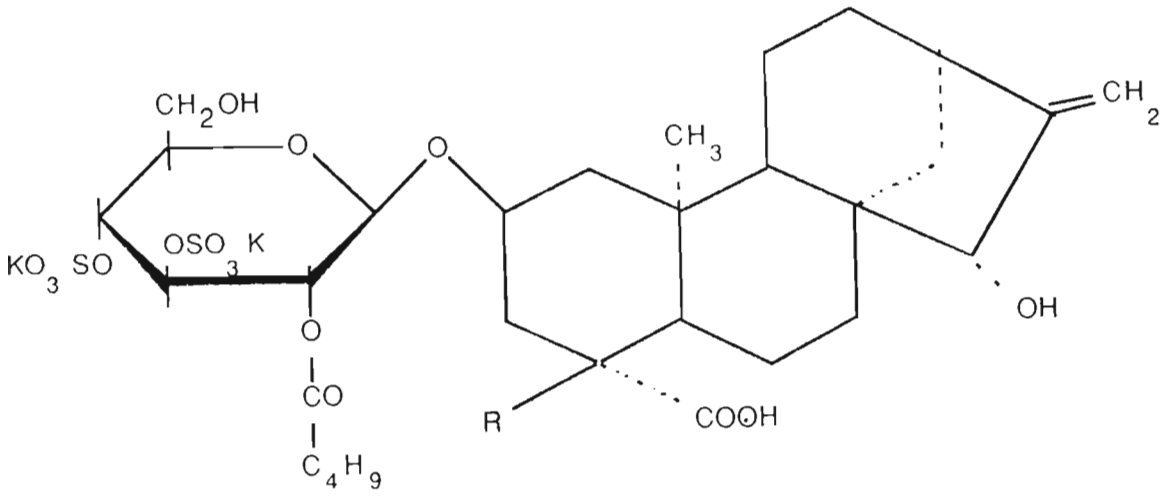

Figure 2. Structure of atractyloside (R = H) and carboxyatractyloside (R = COOH) (potassium salt).

measured amounts of toxin or control substances. Agitation was maintained by the gassing procedure for the whole of the incubation period of 1 hour in duration. At termination, sacs were removed, blotted, and the serosal solution was released for analysis by cutting the sac open.

Detection and Analysis of Test Substances

Salicylic acid was used as a control compound and was assayed by the method of Keller (Varley, 1967).

Atractyloside and CATR were detected by spotting 20 μl samples of the serosal solution on the origin of an aluminum-backed Keiselgel 60 thin-layer chromatography (tlc) plate (Merck). Standards and controls (20 μl) were spotted alongside, i.e., Krebs-Ringer solution, standard ATR (0.25 mg/ml) and CATR (0.25 mg/ml) solutions and a serosal solution devoid of toxin. The plates were developed in butanol/acetic acid/water/chloroform (6:2:2:1 v/v/v/v) air dried, sprayed with anisaldehyde/sulphuric acid reagent (Brookes, 1979; Wilson and Kazyak, 1957), and heated at 120°C for 2-3 minutes. A pink-purple spot is positive for ATR and other diterpene glycosides.

Similar tlc plates were prepared to detect aflatoxins. Serosal solution samples (20 μl) were spotted onto the origin alongside standards of AFB_1 and AFB_{2a} (0.1 mg/ml), and the plates were developed with either chloroform/ethyl acetate/propan-2-ol (90:55 v/v/v) or toluene/ethyl acetate/formic acid (6:3:1 v/v/v). Aflatoxins were detected by their fluorescence under ultraviolet light.

Aflatoxins were quantitated by high performance liquid chromatography (hplc); the serosal solutions were extracted

thrice with two volumes of ethyl acetate. Extracts were dried by passage through a small amount of anhydrous sodium sulphate and pooled. The ethyl acetate was evaporated under a stream of dry nitrogen and the residue dissolved in methanol (0.5 ml). Samples (20 μl) were injected into a Waters HPLC equipped with a Novapak C18, 4 μ column (Millipore Waters) and a 490 programmable multi-wavelength detector set at 365 nm. The aflatoxins were separated using a binary isocratic eluting solvent system consisting of methanol:1% acetic acid (55:45) v/v) at a flow rate of 1 ml/min.

RESULTS AND DISCUSSION

The uptake of salicylic acid in controls was reproducibly 60% (+/- 5%) from an initial mucosal concentration of 0.4 mg/ml, which is in agreement with reported literature values (Hoben et al., 1958). This indicated that the preparations were viable and capable of absorption.

The absorption of ATR in the alimentary canal was shown to occur along the length of the small and large intestine and colon. Unfortunately, no accurate method of quantitating ATR exists at the present so estimates of uptake had to be made by visual comparison of the spots with standards on tlc. The amount entering the serosal side of the wall was about 30% of that added to the mucosal side. A diagrammatic representation of the chromatogram is given in Fig. 3A, demonstrating that a secondary spot with a slightly higher RF value than ATR appears. This did not correspond to CATR, and it was conjectured that it may be a desulphated derivative (not yet substantiated). Another faint-staining spot of unknown origin appeared near the solvent front. Its absence in control experiments, including preparations with salicylic acid and from the ATR standard, show that it was not an artifact. Carboxyatractyloside appeared at a similar concentration to that of ATR in the serosal solution and apparently behaves in the same way as ATR.

Variation in the pH of the mucosal solution had a marked effect on ATR uptake. Figure 3B shows very little uptake of ATR at pH 9 and above and a falling off below pH 5. Thus, the everted sac technique does not exactly mimic normal gut conditions as the pH in a zone at the intestinal barrier is more acidic than the solution within the gut lumen (Hoben et al., 1958; Schanker et al., 1958; Wilson and Kazyak, 1957). Clearly, from these experiments ATR uptake is optimum somewhere between pH 5 and 7. Therefore, in the rat, ATR should be actively taken up in both the small intestine and the colon, which are reported to have pH values of between 3-6 and 6-7, respectively.

Results from tlc showed that AFB_1 accumulated in the serosal solution at about 20% of that initially added. There was no evidence, however, of AFB_{2a} in the serosal solution, indicating that AFB_{2a} is not readily transported across certain cell membranes. High performance liquid chromatography supported these findings. Aflatoxin B_1 was found in the serosal solution in a range from 18% to 21% of that added on the mucosal side (Table 1), whereas no AFB_{2a} was detected in the serosal solution under any of the conditions used. Since the initial volumes of solution on the mucosal and serosal sides of the sac were 100 ml and 5 ml, respectively, it can be seen that AFB_1 is actively accumulated on the serosal side, i.e., a final concentration of 0.43 ng/ml AFB_1 is actively accumulated on the serosal side, i.e., a final concentration of 0.43 ng/ml AFB_1 as compared with 0.08 ng/ml in the mucosal solution.

These results have an important bearing on the intoxication of animals, including humans, by both ATR and aflatoxin. If human alimentary tract wall behaves like that of the rat, then ATR and aflatoxin can penetrate intact and enter the portal blood supply and poison the major organs of the body. This could explain

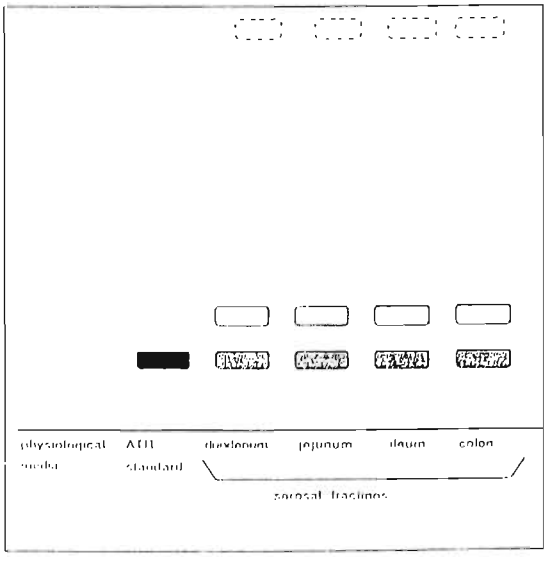

Figure 3A. Diagrammatic representation of a typical thin-layer chromatography plate obtained from an everted sac preparation. The plates obtained clearly showed the absorption along the length of the intestine with associated metabolites (Rfs 0.47 and 0.9) in all serosal fractions. The shading represents the amount of atractyloside/metabolite absorbed relative to the standard mucosal solution (0.25 mg/ml Atractyloside in Krebs-Ringer physiological media).

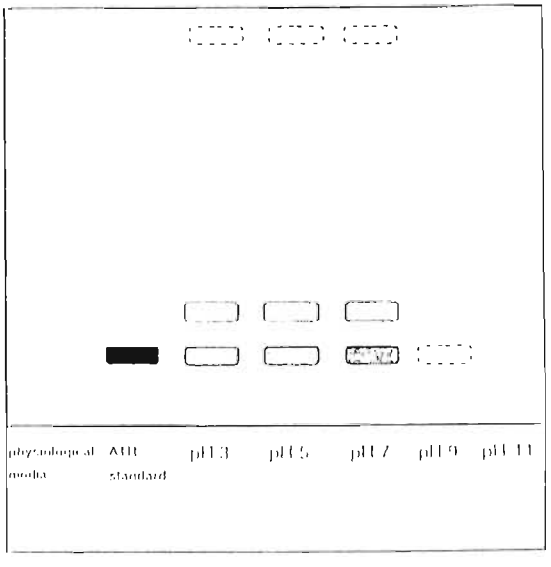

Figure 3B. Diagrammatical representation of a typical thin-layer chromatography plate obtained from pH tests on everted sacs. The plates showed no absorption of Atractyloside at pH 11 and minimal at pH 9. The most absorption of Atractyloside occurred at pH 5 and 7.

Table 1. The percentage absorption of AFB1 in everted sac preparations.

Concentration of AFB1		Total concentration (ng)	% Absorption
Mucosal solution (ng)	Serosal solution (ng)		
8.17	2.15	10.30	20.9
6.94	1.60	8.54	18.6
9.12	2.13	11.25	18.9

Average percentage - 19.5 ± 1%

cases of fatal herbal poisoning amongst rural black patients and the kidney damage observed in animals given ATR and plants containing it (Boohla, 1983).

As ATR is highly polar and has formal negative charges in its structure by virtue of the sulphate moieties, it could be concluded that some form of transport system is present in the gut wall. It seems likely that this is an active process, because the final concentration of ATR in the serosal solution was approximately ten times that in the serosal.

The situation with AFB_1 is not the same, as this molecule does have some lipid solubility and could passively diffuse through the wall in addition to being transported. Passive diffusion is supported by the fact that AFB_{2a} does not penetrate; its extra hydroxyl group increases polarity, reducing its lipid solubility and making this mechanism unlikely for this compound, although its total exclusion from the serosal side of the sac indicates that it is not transported by any other means. The lack of AFB_{2a} penetration may in part reduce the toxicity of ingested AFB_1, because acid conditions in the stomach will tend to convert it to AFB_{2a}, which will then not be taken up. It appears that this is not a major modification in rat, as orally administered AFB_1 can be recovered from rat liver unchanged or metabolized to substances other than AFB_{2a} (Butler and Clifford, 1965). In species where generation of AFB_{2a} in the digestive tract is optimal, however, it would be inconsequential as to whether AFB_{2a} has toxic properties similar to AFB_1, and this may have a bearing on the controversy surrounding the role of aflatoxin in human hepatocarcinoma (Stoloff, 1989).

ACKNOWLEDGEMENTS

Thanks are due to Ommesh Sutan and Ron Berry for technical assistance and to the University of Natal and Foundation of Research and Development and Foundation of Research and Development, CSIR, South Africa, for financial assistance to MT.

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Annals of Tropical Paediatrics (1992) 12, 241–247

Aflatoxins and kwashiorkor in Durban, South Africa

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(Received 3 October 1991)

Summary The present investigation has indicated that maize seeds stored under various simulated seasonal conditions show a spectrum of fungi that appear as a succession. The aflatoxin-producing fungus, *Aspergillus flavus*, is favoured by storage conditions of high temperature and humidity (summer and autumn seasons). This coincides with the more frequent admission of children suffering from kwashiorkor at King Edward VIII Hospital in Durban. Aflatoxin analysis was undertaken on 74 children diagnosed at King Edward VIII Hospital in Durban as cases of kwashiorkor, marasmus or underweight (Wellcome classification). The control group consisted of 35 age-matched patients with no symptoms of protein energy malnutrition. Aflatoxins were detected in serum and/or urine from all groups, including the controls. The serum/urine ratio was significantly higher in the kwashiorkor group than in the other groups. The control group, however, had a higher proportion of urine aflatoxins than the kwashiorkor group. These findings were interpreted in terms of impaired liver function in kwashiorkor. Aflatoxins may have a rôle in the pathogenesis of kwashiorkor, although the present findings do not indicate that they are a causal factor.

Introduction

Malnutrition is widespread and is one of the most important factors contributory to the high incidence of illness and death in developing countries. Protein energy malnutrition (PEM), which includes the conditions of kwashiorkor, marasmus and marasmic kwashiorkor, embraces all disorders attributable to deficiency of protein in the diet.[1]

Kwashiorkor was first described in the 1930s.[2] However, the precise aetiology of the disease still remains obscure. Recent reports have suggested that aflatoxins, which are mycotoxins produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, may have a rôle in the pathogenesis of kwashiorkor.[3–5] Aflatoxins are primarily metabolized in the liver by the microsomal mixed function oxidase system and bind covalently to nucleic acids and proteins, which is probably the basis of their toxic and carcinogenic effects.[6]

The *Aspergillus flavus* spp. are reported to contaminate many food commodities in tropical climates.[7–9] Maize is the dietary staple of most of the black population in South Africa, including the greater Durban area, which has a high relative humidity and temperature during summer and autumn. Such conditions are conducive to rapid fungal contamination and/or proliferation of fungal contaminants already present in poorly stored feed and foodstuffs. It is during these seasons that children suffering from kwashiorkor are seen most frequently at King Edward VIII Hospital in Durban. The

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TABLE I. Storage of maize seeds under various simulated seasonal conditions

Simulated seasonal conditions	Relative humidity (%)	Day temp. (°C)	Night temp. (°C)
Summer	90	25	25
Autumn	90	25	18
Natal Winter	90	18	8
Transvaal Winter	65	18	8

children suffering from kwashiorkor usually come from rural areas and from low income families.

The present study was initiated because it had been found that autopsy liver samples from children in Durban suffering from kwashiorkor showed the presence of aflatoxins.[10] This study was aimed at ascertaining whether PEM in children could be correlated with evidence of aflatoxin consumption. In addition, a pilot study was undertaken to examine the local seed storage mycoflora (*A. flavus* in particular) under various simulated seasonal conditions of storage, particularly in the Natal province.

Methods

Seed storage Mycoflora pilot study

Newly harvested maize seeds were stored in sterilized, sealed containers under various conditions of temperature and relative humidity (RH) to simulate the seasons of autumn and summer in Natal, Transvaal winter (TW) and Natal winter (NW) (Table I). Seeds were tested for fungal contamination and moisture content (MC) at the outset and after 3, 6, 9 and 12 weeks of storage.

Patients

Children between the ages of 6 months and 2 years with malnutrition were selected from the Paediatric Outpatient Department of King Edward VIII Hospital, Durban, South Africa. Having obtained ethical and parental consent for the study, patients were classified as cases of kwashiorkor, marasmus or underweight (Wellcome classification). Well nourished age-matched children who had arrived at the hospital and were being investigated for other conditions were taken as controls. For every one or two malnourished patients a control child of the same age was selected. Altogether, 109 consecutive patients were admitted to the study.

Blood for aflatoxin analysis was taken in the Outpatients Department on the day of admission. Collection of a urine sample, however, was not always possible. All samples were kept frozen prior to analysis.

Aflatoxin analysis was carried out by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) using fluorescence detection.[11] Analysis of serum was performed after hexane partitioning, chloroform extraction and silica-column clean-up. Detection of major aflatoxins B_1, B_2, G_1, G_2 and M_1 was facilitated using the above methodology. The lower limits of detection for aflatoxins (pg/ml) were as follows: serum: B_1, 25; B_2, 1; G_1, 50; G_2, 1; M_1, 5; urine: B_1, 25; B_2, 10; G_1, 25; G_2, 10; M_1, 5.

Statistical analysis

Chi-square was used with Yates' correction. If an expected cell value was <5 then Fisher's Exact test was used.

Results

Seed-storage mycoflora (Figs. 1a, 1b, 2a, 2b)

Fungal contamination prior to storage (0 time) showed that a mixed mycoflora comprising *Fusarium* spp. (100%) and *Penicillium* spp. (94%) dominated the seeds' internal environment.

Proliferation of *Aspergillus* spp. generally was evident throughout the storage period. However, under summer and autumn conditions (Figs. 1a, 1b) proliferation of *A. flavus*

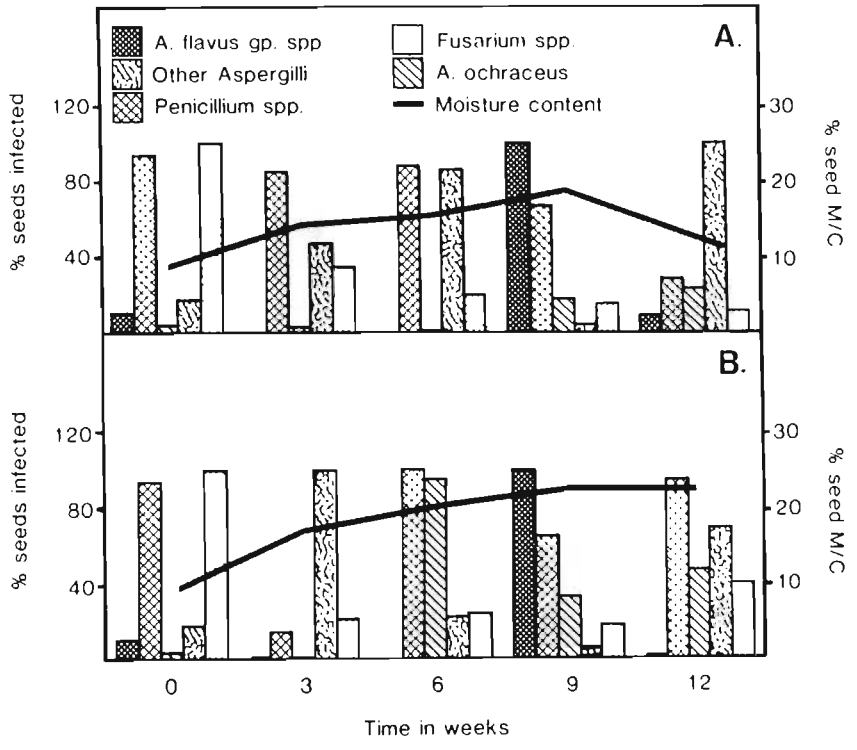

FIG. 1. Storage of maize seeds. A. Summer, B. Autumn.

and *A. parasiticus* (presently considered together as both are group species of *A. flavus*), which was negligible after 3 and 6 weeks of storage, peaked at 100% at 9 weeks, declining to an insignificant degree thereafter. These storage conditions were: 25°C and 90% RH, and alternating between 25°C by day and 18°C at night, also at 90% RH, respectively. However, storage under generally cooler conditions (NW [Fig. 2a]) and (TW [Fig. 2b]) was correlated with a low but persistent (especially under the latter conditions) incidence of *A. flavus* spp., although moisture content was relatively high. Under NW conditions, seed moisture was not consistently at a high level, permitting *A. flavus* spp. to proliferate,[12] which might explain its more erratic isolation compared with that of seeds from the TW regimes.

Other fungal species of *A. ochraceus*, *A. glaucus* and *A. tirreus* (classified as 'other Aspergilli'), *Penicillium* spp. and *Fusarium* spp. were also predominant under all storage conditions (Figs. 1a, b and 2a, b).

Results

Serum analysis

Serum analysis for aflatoxin was carried out on controls (35), marasmic (13), underweight (16) and kwashiorkor (45) children ranging in age from 6 months to 2 years. Aflatoxins were detected in the serum of 56% of both kwashiorkor and underweight cases, 31% of those with marasmus, and 49% of control infants. There were no significant differences among the various groups in the percentage of aflatoxin-positive results (Table II).

Individual serum aflatoxins

Prevalence. Serum from the kwashiorkor group showed a greater prevalence of the individual aflatoxins than that from the other groups, aflatoxin B_1 (18%), aflatoxin B_2 (27%) and aflatoxin G_2 (20%) being detected more frequently. There was a consistent presence of aflatoxin M_1 in all groups except the marasmic. The mean percentage

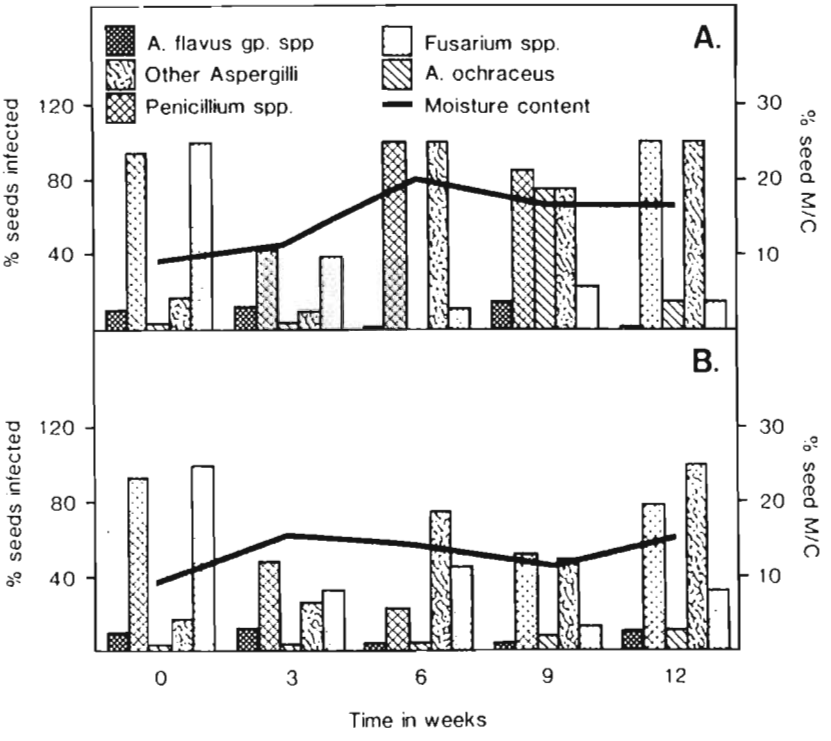

FIG. 2. Storage of maize seeds. A. Natal Winter, B. Transvaal Winter.

TABLE II. Aflatoxin detection in the different groups

Groups	Blood No. tested	Blood No. (%) positive	Urine No. tested	Urine No. (%) positive
Controls	35	17 (49)	16	4 (25)
Marasmus	13	4 (31)	10	1 (10)
Underweight	16	9 (56)	—	—
Kwashiorkor	45	25 (56)	24	4 (16)

of aflatoxins was slightly higher in the kwashiorkor group. The results are shown in Table III. Concentrations of aflatoxins B_1 and M_1 were considerably higher in the kwashiorkor group than in the other groups. However, no statistically significant difference was found. The results are shown in Table IV.

Urine analysis

The data are presented in Table II. Urine aflatoxins were detected in 16% of the kwashiorkor, 25% of the controls and 10% of the marasmic infants. There was no significant difference in the percentage of aflatoxin-positive results among the various groups.

Individual urine aflatoxins

The control group was characterized by levels of the various aflatoxins in the urine higher than in the kwashiorkor group (Table III).

TABLE III. Number of individuals in the different groups showing serum and/or urine aflatoxins

	Control ($n=35$)	Marasmus ($n=13$)	Underweight ($n=16$)	Kwashiorkor ($n=45$)
Serum:				
AFB_1	5 (14)	0	2 (13)	8 (18)
AFB_2	3 (9)	3 (23)	2 (13)	12 (27)
AFG_1	2 (6)	1 (8)	0	3 (7)
AFG_2	5 (14)	0	1 (6)	9 (20)
AFM_1	6 (17)	0	4 (25)	8 (18)
Urine:				
AFB_1	3 (19)	0	—	3 (13)
AFB_2	1 (6)	0	—	1 (4)
AFG_1	0	0	—	0
AFG_2	2 (13)	0	—	0
AFM_1	2 (13)	1 (2)	—	0

Percentages indicated in parentheses.

Serum vs urine aflatoxins

In the control and marasmic groups there was no obvious relationship between the serum and urine aflatoxins (Table II). However, in the kwashiorkor group, there was a significant change between the numbers with serum and urine samples testing positive for aflatoxins (McNemar's test: $\chi^2 = 10.67$, $p = 0.001$) (Table III). These results suggest that there is an abnormality in the metabolism of the toxins in the kwashiorkor infants. This view was further strengthened when serum and urine samples, taken from an additional 11 children suffering from kwashiorkor, indicated the presence of aflatoxins in all of the sera but in only four of the urine samples (Table V).

Discussion

The pilot study on seed storage mycoflora clearly demonstrates the preference for high temperature and humidity (summer and autumn conditions) of the *A. flavus* group species, although the decline of this species after 9 weeks is not pertinent to the present study.

The aflatoxin-producing species of the *Aspergillus flavus* group are known to proliferate under seasonal conditions similar to those experienced by the majority of our kwashiorkor cases. However, other mycotoxigenic fungi such as *Fusarium moniliforme*, *Aspergillus ochraceus*, and *Penicillium*, which are known to elaborate a variety of potent toxins, were frequently observed.[13,14] It is possible that the diet of children in the greater Durban area includes such miscellaneous toxins which may well act synergistically on the liver. However, this is at present speculative, and continuing research into the dietary contaminants of such children is required.

All members of the population in the greater Durban area may be exposed to dietary aflatoxins, and the metabolism of these toxins in children suffering from kwashiorkor may differ from that of apparently normal and marasmic children. Aflatoxins were found most frequently and at higher concentrations in the sera of kwashiorkor children than in the sera of other nutritional groups, although the differences were not significant. On the other hand, in the urine, aflatoxins were detected less frequently in the kwashiorkor group than in the control groups. There

TABLE IV. Mean individual serum aflatoxin concentration (pg/ml) in the different groups

	No. positive	Geometric mean	Standard deviation
Control			
$n = 35$			
AFB_1	5	161.2	15.52
AFB_2	3	27.5	2.82
AFG_1	2	685.4	6.23
AFG_2	5	243.2	6.94
AFM_1	6	462.6	3.27
Marasmus			
$n = 13$			
AFB_1	0	—	—
AFB_2	3	100.1	11.15
AFG_1	1	200.0	—
AFG_2	0	—	—
AFM_1	0	—	—
Underweight			
$n = 11$			
AFB_1	2	160.2	10.68
AFB_2	2	84.6	1.27
AFG_1	0	—	—
AFG_2	1	165.6	—
AFM_1	4	338.5	4.57
Kwashiorkor			
$n = 45$			
AFB_1	8	2192.7	2.79
AFB_2	12	47.6	3.61
AFG_1	3	58.2	2.59
AFG_2	9	148.4	3.88
AFM_1	8	835.8	3.99

TABLE V. Serum and urine samples from additional kwashiorkor children

Total serum and urine samples	Serum ($n = 11$) No. positive	Urine ($n = 11$) No. positive
11	11	4

was a significant difference in the detection ratio between serum/urine aflatoxins in the kwashiorkor group ($p = 0.001$), whereas no significant difference for this correlation was detected in other groups. The presence of a high concentration of serum aflatoxins correlating with the low incidence of urine aflatoxins indicates toxin retention by the kwashiorkor infants. These results support the proposition that liver function is impaired in kwashiorkor.

From the present investigation, it can be deduced that the children in all the experimental groups and in the control group were exposed to dietary aflatoxins. It is reasonable to suggest that this might be a common situation in the black juvenile population in and around Durban. The ability to metabolize the toxin may be dependent on the type and degree of malnutrition, which is related to the quality and quantity of protein in the diet. In particular, kwashiorkor patients are distinct from other cases of PEM and from children not obviously malnourished in that the liver detoxification of aflatoxins seems to be impaired. In view of the prevalence of these toxins in the juvenile population as a whole (exemplified by all the groups in the present study), it is unlikely that the basic aetiology of kwashiorkor is a function of aflatoxin consumption. However, impairment of hepatic function, as regards the detoxifying metabolism of the aflatoxins, seems to be indicated as a distinct feature of kwashiorkor. This may be an inherent (genetic) condition leading to sensitivity to aflatoxins rather than a pathological change caused by extraneous factors. Brief analysis of clinical details indicates that aflatoxin-positive kwashiorkor patients stayed longer in hospital, with greater clinical complications, than those who were aflatoxin-negative (unpublished results).

Consumption of poorly stored maize is likely to be accompanied by ingestion of a particular mycotoxin (or combination thereof), depending on the origin of the seed, the geographical location and the climatic conditions of the storage area.

Acknowledgment

The work has been supported by grants from the University of Natal Research Funds and the Medical Research Council (GR). GR is in receipt of a University of Natal post-graduate assistant bursary.

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Pharmacol Ther Vol. 70, No. 2, pp. 137–161, 1996

ISSN 0163-7258/96 $32.00
PII S0163-7258(96)00006-X

Associate Editor: K. D. Bhoola

Fumonisins, Mycotoxins of Increasing Importance: Their Nature and Their Effects

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ABSTRACT. The fumonisins (FBs) are a group of closely related mycotoxins that are prevalent in maize. They were isolated from strains of *Fusarium moniliforme* (Sheldon), which were implicated in the aetiology of human oesophageal cancer in the Transkei, South Africa. Their discovery explained the cause of equine encephalomalacia, or "hole in the head" syndrome, when it was found by feeding trials in horses that they elicited the disease. Subsequently, they were found to cause hepatic cancer in rats and pulmonary oedema in pigs, with most animal species tested showing liver and kidney damage. FB1 is the most important of the group and, although poorly absorbed from the gastrointestinal tract, its action is at the cellular level, affecting sphingolipid metabolism. Ceramides derived from sphingosine metabolism are cell regulatory factors affecting, among other things, DNA synthesis. Because FB1 has a close molecular resemblance to sphinganine, it interferes with ceramide biosynthesis and, hence, the processes that it regulates, which is thought to explain its carcinogenic properties. Studies on the FBs are still at a relatively early stage, but it is already clear that they play an important role in animal mycotoxicoses and, by implication, in human disease. A more positive aspect is that they will be used in elucidating the role of sphingolipids in cellular regulation. PHARMACOL. THER. 70(2): 137–161, 1996.

KEY WORDS. Fumonisin, *Fusarium moniliforme*, encephalomalacia, ELEM, liver cancer, maize, sphingolipid metabolism.

CONTENTS

ABBREVIATIONS. AAL, *Alternaria alternata*; ELEM, equine leucoencephalomalacia; FB, fumonisin; GIT, gastrointestinal tract; OC, oesophageal cancer; OPA, O-phthaldialdehyde.

1. INTRODUCTION

1.1. *Mycotoxins and Human Disease*

Mycotoxins, the poisonous products of fungi, have plagued humans since the commencement of agriculture. It is also probable that poisonings occurred before this development, due to toadstools and moulded materials being consumed by the uninitiated. The mass production of food and its storage, such as that described in the Jewish scriptures by Joseph, allowed for the infection of the stored produce by fungi, production of mycotoxins, and their distribution in food staples. The development of modern agricultural methods, together with large-scale processing, has exacerbated the problem, resulting in outbreaks of acute diseases termed mycotoxicoses (Newberne, 1974) and also chronic conditions, often not recognised as such (Campbell, 1990).

The effects of mycotoxins have been documented through-

out history; there is an Assyrian bas-relief describing what is possibly ergot (Berry, 1988), and ergotism was reported throughout the middle ages (Robbers, 1979), but it was not until the beginning of the 1960s that mycotoxins were widely recognised as potential causes of disease. The reason for this sudden notoriety was the outbreak of "Turkey X disease" in Britain in 1961 and the discovery of its putative cause, aflatoxin (Sargeant *et al.*, 1961).

Soon after the discovery of the aflatoxins, a whole range of fungi were screened for toxic metabolites, and past literature was scoured for documented evidence of mycotoxins and their effects. Both approaches yielded results and, by 1984, over 240 fungal metabolites were reported to have toxic properties towards animals and animal cell cultures (Cole and Cox, 1981). At this stage, the general feeling was that the golden age of mycotoxin discovery was over. Those that posed a problem by their ubiquitous nature, e.g., the aflatoxins, and those that were more restricted to specific crops and regions, such as *Alternaria* (AAL) toxins in infected fruit, were thought to be understood and, if not completely eliminated from the first world food chain, could be reduced by vigilant screening and legislative control (Van Egmond, 1989). As is the case in the affairs of mankind, problems have a habit of resurfacing, and this has happened with the discovery of the fumonisins (FBs).

1.2. *Historical Background*

Although the FBs are a more recent addition to the mycotoxins, for at least this century, their effects have been observed in a sporadic fatal condition in horses and related members of the *Equidae*, called equine leucoencephalomalacia or ELEM. An early description of the disease was given by Butler in 1902, who called the disease leucoencephalomalacia and produced the symptoms in a test animal with mouldy feed. Other names used to describe the condition have been: blind staggers, foraging disease, corn stalk disease, mouldy corn poisoning, leucoencephalitis, and cerebritis.

From the work of Butler and many others, the causative agent of ELEM was well understood for most of this century as feed infected with species of the genus *Fusarium* (Wilson and Maronpot, 1971). The source of the fungus is maize, which is well known as being susceptible to *Fusarium* infections (Rheeder *et al.*, 1990a; Voss, 1990), although other cereals have also been implicated in ELEM, e.g., oats (Wilson *et al.*, 1985c). FB has been found in forage grass in New Zealand (Mirocha *et al.*, 1992b), in laboratory animal feed (Thigpen *et al.*, 1992), and a FB-producing strain of *Fusarium moniliforme* (Sheldon) was isolated from jimsonweed (Abbas *et al.*, 1993b). In particular, *F. moniliforme* was implicated (Kellerman *et al.*, 1972; Wilson and Maronpot, 1971), but this was not a very useful observation, because this fungal species produces a range of toxins including fusaric acid, moniliformin, and fusarin C (Bruckner *et al.*, 1989).

It is not easy to define a mycotoxicosis, such as ELEM, when the diagnosis depends upon mycological identification of species, rather than the mycotoxin itself. Confusion can arise by attaching a label to a condition, such as "mouldy corn poisoning," because that could cover a range of related fungi, if not unrelated ones. Furthermore, the identification of a specific species of *Fusarium* is notoriously difficult (Nelson *et al.*, 1983). Consequently, it is not always clear from earlier literature whether the disease is truly ELEM, a different mycotoxicosis altogether, or a combination of different mycotoxicoses. The example of "Turkey X" disease is a case in point, because it now seems that the symptoms of this disease are more consistent with cyclopiazonic acid poisoning, rather than that of aflatoxin (Bradburn *et al.*, 1994), both toxins being produced by members of the *Aspergillus flavus* group.

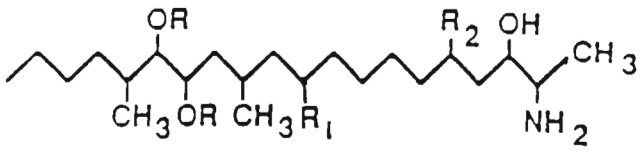
The origin of ELEM disease was solved when, in 1988, Marasas' group [Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC)] in South Africa, discovered the FBs (Bezuidenhout *et al.*, 1988; Anon, 1989). Several workers over the preceding decade, e.g., Gelderblom *et al.* (1983), had been attempting to isolate the causative agent of ELEM from fungi known to be involved with this and associated diseases. An example is when a strain of *F. verticillioides* (now described as *F. moniliforme*) caused acute toxicity with pulmonary oedema when fed to pigs; this also appears to have a long history (Tibor, 1995) of severe nephrosis and hepatosis in sheep, and hepatic cirrhosis and cardiac thrombosis in rats (Kriek *et al.*, 1981).

In South Africa, however, an additional line of investigation had been pursued where the high prevalence of oesophageal cancer (OC) in certain areas of the Transkei (a region on the eastern seaboard) was being studied. A difference between the fungal flora in OC areas compared with similar nonOC areas in the region was observed (Marasas *et al.*, 1979, 1980). Fungal isolates, in particular *F. moniliforme*, were found to be toxic and carcinogenic to rats (Gelderblom *et al.*, 1988a,b). Using the rat, various carcinogenic fractions from toxigenic strains of *F. moniliforme* were detected (Bezuidenhout *et al.*, 1988). From these, two toxic metabolites were purified and given the trivial names, FB1 (isolated and called macrofusine by Laurent *et al.*, 1989b) and FB2. Subsequently, other related compounds were isolated from both *F. moniliforme* and *Alternaria* spp. (Cawood *et al.*, 1991; Chen *et al.*, 1992).

Feeding trials on two horses, using partially purified and purified FB1, elicited typical ELEM symptoms, including necrotic brain lesions (Kellerman *et al.*, 1990). Further studies on other species revealed that FB1 caused, in addition, liver cancer in rats and lung oedema in pigs (Haschek *et al.*, 1992; Osweiler *et al.*, 1992). Despite extensive studies, however, none of the FBs have been shown to produce OC in any species tested (Diaz and Boermans, 1994), including primates (Fincham *et al.*, 1992).

1.3. *Characteristics*

The FBs are a group of related, polar metabolites originally isolated from *F. moniliforme* (Bezuidenhout *et al.*, 1988; for a review, see Scott, 1993). Their structures are based on



R = $COCH_2CH(COOH)CH_2COOH$

FB1 R_1 = OH, R_2 = OH
FB2 R_1 = OH, R_2 = H
FB3 R_1 = H, R_2 = OH
FB4 R_1 = H, R_2 = H

FIGURE 1. Structure of FBs.

long hydroxylated hydrocarbon chain (pentahydroxyeicosane) containing methyl and either amino (B_1 and B_2) or acetyl amino groups (A_1 and A_2). Two hydroxyls are esterified to two propane-1,2,3-tricarboxylic acid molecules trivially named tricarballylic acid. FB1 differs from FB2 in that it has a extra hydroxyl at position 10 (Fig. 1), the backbone of the FB1 molecule being chemically converted to FB2 by two methods (Badria *et al.* 1995). FB3 and FB4 were isolated later (Gelderblom *et al.*, 1992a; Plattner *et al.*, 1992), and were found to differ from the others in hydroxylation pattern (Fig. 1). A further addition was FBC1, which lacked the terminal methyl group adjacent to the amine (Branham and Plattner, 1993b).

The mass spectra of the FBs and associated metabolites was studied by Caldas *et al.* (1995) using electrospray injection, and agreed with the accepted structures. Because of the four free carboxyl groups and the amine group, the compounds are water soluble, but not soluble in nonpolar organic solvents. The lack of solvent solubility of the FBs explains, in part, the delay in their discovery, because isolation of most mycotoxins depends upon solvent extraction, which avoids contamination of extracts with materials such as carbohydrates and amino acids. Consequently, methods of purifying FBs are long and tedious (Cawood *et al.*, 1991), often resulting in products that are not completely pure.

The relative and absolute stereochemistry of FB1 (ApSimon *et al.*, 1994; Hoye *et al.*, 1994; Poche *et al.*, 1994; Blackwell *et al.*, 1995; Shier *et al.*, 1995) and FB2 (Harmange *et al.*, 1994) have been investigated using NMR spectroscopy. It was found that the C2 and C3 stereo configuration is threo and opposite to that of sphingosine. Overall, it is: 2S, 3S, 5R, 10R, 12S, 14S, 15R, 16R, which is consistent with the stereo structure found for FB2. The absolute configuration of the chiral centres for the tricarballylic acid moieties of FB2 was found to be R (Boyle and Kishi, 1995a), which is in contradiction to that proposed for FB1 and AAL toxin (Shier *et al.*, 1995). This anomaly was studied further, and both FB1 and AAL toxin T_A were also found to be R (Boyle and Kishi, 1995b), although it was pointed out that different strains of fungi were used as a source of the toxins.

The total stereochemical structure of the FBs is important to their biological effects, but it may not have optimal activity, as chemically synthesized analogues of FB1 with an extended chain were more toxic when evaluated by cell cultures (Kraus *et al.*, 1992). Three-dimensional minimum energy conformations for all four FBs were calculated (Beier *et al.*, 1995), and it was found that the amine and carboxylic acid groups are spatially related, suggesting that they have chelating properties and, hence, could cause membrane ion leakage, as found in treated plants (Abbas *et al.*, 1992a).

1.4. *Detection and Quantitation*

1.4.1. Isolation and thin layer chromatography. After characterization had been achieved, methods of detection and quantitation for the FBs could be devised. Of these, TLC is the simplest but, like all other analytical procedures, depends upon their efficient extraction and "clean-up." The lack of a suitable chromophore in the molecule means that the metabolites must be derivatised with reagents to allow detection.

Extraction is achieved using aqueous methanol mixtures after acidification of the material (Cawood *et al.*, 1991). Clean-up can be done with either reverse phase cartridges, C_{18} (Rottinghaus *et al.*, 1992), or ion-exchange cartridges (strong anionic exchanger) (Sydenham and Thiel, 1992*; Stockenstrom *et al.*, 1994), the latter authors claiming that ion exchange was superior to C_{18}. More recently, affinity columns have become available and have been evaluated (Ware *et al.*, 1994). These are effective because of their high affinity for the test molecule; the only drawback seems to be that they become saturated at higher levels of FB concentrations, which have to be diluted before use.

Cawood *et al.* (1991) used silica gel G plates with two developing solvents, chloroform/methanol/water/acetic acid (55:36:8:1) and chloroform/water/acetic acid (6:3:1), FBs being visualized with anisaldehyde spray reagent or ninhydrin (0.2 g in ethanol) after heat at 120°C. This system was used with effect to optimize the purification of the four FBs in their quantitative isolation. FB1 gave an R_f value of 0.23 in the first system and 0.15 in the second; FB2 gave one of 0.30 in the first and 0.2 in the second. Ackermann (1991) used chloroform/methanol/acetic acid (60:35:10) with silica gel G plates, which separated FB1 from FB2 (R_f values of 0.32 and 0.52, respectively) and methanol/water (8:2) using octadecyl silica plates (R_f values 0.61 and 0.47). Plattner *et al.* (1990) also used silica gel and chloroform/methanol/acetic acid (6:3:1), which gave an R_f value of 0.24. A reverse-phase C_{18} TLC plate was used by Rottinghaus *et al.* (1992), who developed the plates with methanol:1% aqueous potassium chloride (3:2). The FBs were detected by spraying with fluorescamine solution and buffered acetonitrile, which gave a bright yellowish fluorescence under UV light at R_f values of FB1, 0.10 and FB2, 0.50. Because of low solvent solubility, the preparation of standard FB solutions can be a problem, especially as methanolic solutions can generate the methyl

* Sydenham, E. W. and Thiel, P. G. (1992) Training Course 2: Fumonisins. In: Eighth International IUPAC Symposium on Mycotoxins and Phycotoxins, Mexico City, 6–13 November 1992, pp. 1–10.

esters.* Acetonitrile/water mixtures are recommended, because the FBs were found to be stable in this solvent for up to 6 months.

1.4.2. High-performance liquid chromatography. After the FBs had been discovered, worldwide interest was aroused with respect to their contribution to disease and occurrence. Before useful surveys could be conducted, accurate and sensitive methods of detection and measurement were needed and, soon after their discovery, a method employing HPLC was reported from the PROMEC laboratory (Sydenham *et al.*, 1990a; Alberts *et al.*, 1993a). Commodities are extracted with aqueous methanol and cleaned by use of an Amberlite XAD2 column, then separated by HPLC on a reverse-phase column, using methanol/phosphate buffer as the running solvent. In this method, maleic anhydride was used to make the maleyl derivative that was detected by absorption at 250 nm, whereas Vesonder *et al.* (1990) used a refractometer (RI detector) as an alternative means of detection.

Although the maleyl derivatization method was adequate for determining the FBs in cultures of *F. moniliforme* and heavily contaminated feedstuffs, i.e., levels >10 mg/kg (Sydenham *et al.*, 1990a), it was not sensitive enough for the lower levels found in foods and physiological fluids and tissues. For this purpose, a method using a fluorescent derivatizing reagent, O-phthaldialdehyde (OPA), was devised and subsequently widely used (Shephard *et al.*, 1990; Sydenham *et al.*, 1992b; Stack and Eppley, 1992; Thiel *et al.*, 1993). This system allows levels as low as 50 μg/kg of FB1, FB2, and FB3 to be measured and has been specifically modified for FB2 measurements (Shephard *et al.*, 1995a). Visconti *et al.*† reported an inter-laboratory comparative trial on this method and recommended attention to certain aspects, such as reagent purity, to optimise reproducibility.

Many other HPLC methods have been developed. They are all essentially the same, but using different derivatizing agents, including 4-fluoro-7-nitrobenzofurazan (Scott and Lawrence, 1992); fluorescamine (Holcomb *et al.*, 1993a), although this reagent has been criticised because it produces two derivatives (Ware *et al.*, 1993); 9-(fluorenylmethyl)chloroformate, which was preferred by Holcomb *et al.* (1993b), who criticised other derivatives, including OPA, because of instability; naphthalene-2,3-dicarboxyaldehyde (Ware *et al.*, 1993; Bennett and Richard, 1994); 4-(N,N-dimethylaminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole) (Akiyama *et al.*, 1995); and 6-aminoquinolyl N-hydroxysuccinimidylcarbamate (Velazquez *et al.*, 1995).

Other variations on detecting the FBs were: electrochemical and fluorescence of the OPA/2-methyl-2-propanethiol derivative (Holcomb *et al.*, 1994); fast atom bombardment mass spectrometry (Holcomb *et al.*, 1993a); thermospray, fast atom bombardment, and electrospray mass spectrometry (Korfmacher *et al.*, 1991); and evaporative light scattering detection after reverse phase HPLC for all four FBs (Wilkes *et al.*, 1995).

1.4.3. Capillary electrophoresis. More recently, capillary electrophoresis has been developed as a complementary method of analysis to HPLC. This depends upon a high voltage, typically 20–30 kV, being applied down a fused silica capillary, diameter 1 μm, filled with a suitable buffer, and cooled to avoid heating effects. Not only ionised molecules of the correct charge pass down the capillary, but also uncharged and opposite charge due to an ion flow created in the buffer. FBs, having ionisable groups, would seem to be highly suitable for this method, which has the advantage of a very high resolving power although, at the moment, the sensitivity is not as good as fluorescence detection used with HPLC. A method using this instrumentation has been developed by Maragos (1995), which also employs a derivatizing reagent, fluorescein isothiocyanate, and another with electrospray ms as the detection method (Hines *et al.*, 1995).

1.4.4. Other methods. Gas chromatography with mass spectrometry detection has been used to quantitate the FBs (Plattner *et al.*, 1990, 1991; Thiel *et al.*, 1991a; Pestka *et al.*, 1994) through the de-esterified long chain. Although this method is acceptable, it does have the disadvantage that it requires expensive instrumentation and a hydrolytic pre-treatment (Shephard *et al.*, 1992b).

A sensitive and specific method of analyzing for mycotoxins is the use of immunoassay, which can be simple with less costly equipment, but it does depend upon the quality of an antibody raised against the mycotoxin. Both monoclonal (Azcona-Olivera *et al.*, 1992b; Fukuda *et al.*, 1994) and polyclonal antibodies (Azcona-Olivera *et al.*, 1992a; Usleber *et al.*, 1994) have been developed against the FBs as well as anti-idiotype antibodies (Chu *et al.*, 1995). The monoclonal antibody was found to effectively measure total FBs (Shelby *et al.*, 1994), giving a higher level of FB concentration than by TLC.

An assessment of an ELISA based on a polyclonal antibody (Pestka *et al.*, 1994) vs. gas chromatography-mass spectrometry and HPLC, indicated that it could assay FB in foods as effectively as the other methods. A further study by this group (Tejada-Simon *et al.*, 1995) compared an ELISA against HPLC for the detection of FB1 and used the antibody in an immunocytochemical study to localise FB1 in *Fusarium* cultures. Abouzied and Peska (1994) devised a multimycotoxin immunoblot based on monoclonal antibodies immobilised on nitrocellulose. This was claimed to be capable of detecting FB1, aflatoxin B_1, and zearalenone simultaneously. Schneider *et al.* (1995) have developed a dipstick that can detect FB1 down to levels of 40–60 ng/g of corn-based foods. Clearly these methods will be useful where rapid tests are required as primary screens to check the safety of food and legislated tolerances.

*Visconti, A., Doko, M. B., Schurer, B. and Broenke, A. (1993) The stability of fumonisins in methanol and acetonitrile/water. In: Proceedings of the United Kingdom Workshop on the Occurrence and Significance of Mycotoxins, London, 21–23 April 1993, pp. 196–199. Skudamore, K. A. (ed.) MAFF, London.

†Visconti, A., Doko, M. B., Schurer, B. and Broenke, A. (1993) Intercomparison study for the analysis of fumonisins B$_1$ and B$_2$ in an unknown solution. In: Proceedings of the United Kingdom Workshop on the Occurrence and Significance of Mycotoxins, London, 21–23 April 1993, pp. 200–202. Skudamore, K. A. (ed.) MAFF, London.

1.5. *Occurrence*

Many mycotoxins have limited distribution, either being restricted because of climatic conditions, e.g., citrinin in temperate zones, or type of commodity, e.g., *Alternaria* toxins in fruit. There are notable exceptions; the aflatoxins are one, having been found in most food commodities at some time and in all parts of the world (CAST Report, 1989). FBs, although mainly restricted to maize, are also becoming regarded as a global problem. They are produced by members of the genus *Fusarium* in the section Liseola, including *F. moniliforme* (Sheldon), *F. proliferatum*, *F. nygamai* (Thiel *et al.*, 1991a; Nelson, 1992; Norred, 1993; Bullerman and Tsai, 1994; Meireles *et al.*, 1994), *F. anthophilum*, *F. dlamini*, and *F. napiforme* (Nelson *et al.*, 1992), *F. oxysporum var redolens* (Abbas *et al.*, 1995c), and are also related to the 'A' mating population of *Gibberella fujikuroi* (Leslie *et al.*, 1992a,b; Desjardins *et al.*, 1992, 1994, 1995). Such mating populations were found in Mexican maize, and it was concluded that the potential for the production of FB existed (Desjardins *et al.*, 1994). More recently, FB1 was detected in cultures of *F. polyphialidicum*, isolated from seeds of *Pinus strobus* (Abbas and Ocamb, 1995).

Because *F. moniliforme* is widely distributed in the world, it is not surprising to find global surveys reporting the presence of fungal strains capable of producing the FBs (Nelson *et al.*, 1991), in addition to the presence of the toxins themselves (Table 1). In South Africa, Sydenham *et al.* (1990a) made the first conclusive report of FB1 in maize that was obtained from the Transkei. In a further survey (Sydenham *et al.*, 1991) of maize and maize products destined for human consumption, the high mean levels of FB1 (2380 μg/kg) and FB2 (595 μg/kg) were found in Egyptian maize meal. Other samples that were found to be positive came from South Africa, Peru, the United States, and Canada. Surveys of commercially grown maize in South Africa* (Rheeder *et al.*, 1995) showed that FB was the main mycotoxin present, although the levels were relatively low compared with other areas in the world. As in other studies, "screenings" or "fines" removed from grain by sieving were found to be relatively higher in FB1 than grain, and it was suggested that their removal from bulk grain would significantly lower the FB levels (Sydenham *et al.*, 1994).

Maize from Iowa, Wisconsin, and Illinois, USA, 1988–1989 (Murphy *et al.*, 1993) and Indiana, USA (Binkerd *et al.*, 1993) was found to have FB1, FB2, and FB3. The highest levels were found in maize screenings at 37.9 mg/kg in the 1989 material. A surveillance programme for mycotoxins in 1991 feed (Price *et al.*, 1993) showed a mean level of 12.1 mg/kg FB1 in United States maize screenings. Food products, including maize meal, canned corn, dog, and cat food purchased in Iowa, USA were examined for FBs. Some of the foods had up to 4140 μg/kg FB1/FB2, and tortilla and canned yellow corn had hydrolysed FB1 present (Hopmans and Murphy, 1993). A survey of corn from Georgia, USA showed the presence of both FB and aflatoxin in 23 samples from a total of 28 analyzed (Chamberlain *et al.*, 1993). More recently, using a modified HPLC method devised for canned and frozen sweet corn, Trucksess *et al.* (1995) found that 35 of 97 commercial samples taken from all over the United States contained FB1 with maximum levels of 235 μg/kg and 350 μg/kg in canned and frozen corn, respectively. Sydenham *et al.* (1991) found FB1 and FB2 in 16 maize meal and 10 grit products destined for human consumption in the United States at mean concentrations of FB1, 1048 μg/kg and FB2, 601 μg/kg. Of the Canadian samples tested, only one showed detectable levels of FB1.

FBs were first found in Brazilian maize-based feeds associated with mycotoxicoses affecting horses, pigs, rabbits, and poultry (Sydenham *et al.*, 1992a). Levels of FB1 ranged from 0.2 to 38.5 mg/kg in naturally contaminated materials. Sydenham *et al.* (1993) also examined maize samples from Argentina and found that the bulk of them, 16 of 17, had combined FB levels in excess of 2000 μg/kg. One of four samples of Peruvian maize was found to contain FB1 at a level of 660

TABLE 1. Examples of the Occurrence of FB1 in Maize in Different Countries

Location	Number of samples (positive/total)	Level (μg/kg)[1]	Reference
Argentina	16/17	2000	Sydenham *et al.*, 1993
Brazil	20/21	38,500	Sydenham *et al.*, 1992a
Benin	9/11	2310	Doko *et al.*, 1995
Canada	1/2	50	Sydenham *et al.*, 1991
China	5/20	1732	Yoshizawa *et al.*, 1994
Croatia	11/19	70	Doko *et al.*, 1995
Egypt	2/2	2380	Sydenham *et al.*, 1991
France	95/100	50,000	Le Bars and Le Bars, 1995
Honduras	24/24	6555	Julian *et al.*, 1995
Hungary	36/56	334,000	Fazekas and Tothe, 1995
Italy	All	5310	Doko *et al.*, 1995
Japan	14/17	2600	Ueno *et al.*, 1993
Korea	5/12	1327	Soo *et al.*, 1994
Nepal	12/24	4600	Ueno *et al.*, 1993
Peru	1/4	660	Sydenham *et al.*, 1991
Poland	2/7	30	Doko *et al.*, 1995
Portugal	9/9	3450	Doko *et al.*, 1995
Romania	3/6	30	Doko *et al.*, 1995
Sardinia	6/6	250,000	Bottalico *et al.*, 1995
South Africa	10/10	1890	Sydenham *et al.*, 1994
	187/249	7100	Rheeder *et al.*, 1995
Spain	8/50	80	Sanchis *et al.*, 1994
Switzerland	44/120	790	Pittet *et al.*, 1992
United States	35/97	350	Trucksess *et al.*, 1995
	25/26	1048	Sydenham *et al.*, 1991
Georgia	23/28	321	Chamberlain *et al.*, 1993
Iowa	44/160	37,900	Murphy *et al.*, 1993
	15	1410	Hopmans and Murphy, 1993
Zambia	20/20	1710	Doko *et al.*, 1995

[1] Highest concentration recorded.

* Viljoen, J. H., Marasas, W. F. O. and Thiel, P. G. (1993) Fungal infection and mycotoxin contamination of commercial maize. In: Proceedings of the ICC International Symposium: Cereal Science and Technology; Impact on a Changing Africa, Pretoria, 9–13 May 1993, pp. 837–853, Taylor, J. R. N., Randall, P. G. and Viljoen, J. H. (eds.) CSIR, Pretoria.

μg/kg (Sydenham *et al.*, 1991). Preharvest and farm-stored maize in Honduras was examined for fungi and mycotoxins (Julian *et al.*, 1995). *F. moniliforme* and *F. subglutinans* were found to be predominant, and FB1 was found in all of the 24 samples tested at levels between 68–6555 μg/kg.

Of maize-based food and feed bought in Switzerland, about one third had FB1 ranging from 55 to 790 μg/kg (Pittet *et al.*, 1992). Eight out of 50 samples destined for human consumption in Spain were found to be contaminated, albeit at a low level of a mean of 80 μg/kg. A survey of samples, mainly from European countries (Visconti and Doko, 1994; Doko *et al.*, 1995), discerned a group of countries with high FB levels (1710–3310 μg/kg), these being: Italy, Portugal, Zambia, and Benin. The second group, Croatia, Poland, and Romania, showed positive, but lower, levels (<70 μg/kg). An earlier study on Italian corn products gave similar results, with puffed corn having the highest level of FB1 at 6100 μg/kg* (Doko and Visconti, 1994). Hungarian maize was also found to be contaminated with FBs, with a mouldy sample having 33.4 mg/kg FB1 (Fazekas and Tothe, 1995). A study of the toxigenicity of *F. proliferatum* (Logrieco *et al.*, 1995) derived from Italian maize revealed the production of several mycotoxins, with FB1 being the most prevalent, at levels of up to 2250 mg/kg.

Ninety-five percent of the strains of *F. moniliforme* isolated from maize seeds harvested by hand in France showed levels of 5 mg/kg and over, when grown on sterile maize after 28 days of incubation (Le Bars and Le Bars, 1995). In a study on maize destined for human consumption in Spain, 8 of 50 samples were found to contain FB1 at low levels, a mean of 80 μg/kg (Sanchis *et al.*, 1994). Of 6 selected samples of preharvest maize ear rot collected in Sardinia, all were found to contain FBs, up to 250 mg/kg, and 4 of these were also found to contain another *Fusarium* toxin, beauvericin (Bottalico *et al.*, 1995).

Soo *et al.* (1994) analysed Korean maize, and FB1 was found in 5 of 12 samples, at levels ranging from 53 to 1327 μg/kg. Tseng *et al.* (1995) showed that 66% of the *F. moniliforme* strains isolated in Taiwan were able to produce FBs. A study (Ueno *et al.*, 1993) was done of maize and maize products imported into Japan; 8 of 9 maize samples were positive for FB1, and all the gluten-based feeds contained FB1. Of the samples of maize originating in Nepal, 12 of 24 samples were positive for FB1. Similar results were found in imported Chinese maize. Other studies in Southeast Asia also showed the presence of FBs (Yamashita *et al.*, 1995).

One survey for the presence of FB in milk was done (Maragos and Richard, 1994), and of the 165 samples analyzed, only one was positive at a low level, 1.29 μg/L of FB1, suggesting that contamination of raw milk is possible, although dairy cattle dosed with FB1 p.o. (1 and 5 mg/kg bodyweight) or by i.v. (0.05 and 0.20 mg/kg) had no detectable residues in milk (Scott *et al.*, 1994).

* Doko, M. B. and Visconti, A. (1993) Fumonisin contamination of maize based foods in Italy. In: Proceedings of the United Kingdom Workshop on the Occurrence and Significance of Mycotoxins, London, 21–23 April 1993, pp. 49–50, Skudamore, K. A. (ed.) MAFF, London.

1.6. *Production*

Because of the perceived importance of the FBs and their effects, methods were developed to prepare purified quantities. Production of natural material is necessary, as total chemical synthesis would be too costly and tedious. The production of the FBs by *Fusarium* spp. depends on parameters such as substrate, temperature, aeration, and water activity. *F. moniliforme* was grown on maize grain at different water activities (Cahagnier *et al.*, 1995), which is critical in the formation of most mycotoxins. When the water activity was dropped from 1.0 by 5%, growth rate remained unchanged, but there was a 3-fold drop in FB1 production. A further lowering of 5% (aw 0.9) caused a 20-fold loss of growth rate and 300-fold loss of FB1 production, with total cessation of growth and metabolic activity at aw 0.85.

The PROMEC group preferentially used the *F. moniliforme* strain MRC 826 in their investigations, and this is the basis for the commercial production of the FBs in South Africa. Focus was mainly on solid substrates and, in an early investigation of the effects of temperature, it was found that incubation at 25°C for 11 weeks gave a yield on moist maize of 16.5 g/kg FB1 (Alberts *et al.*, 1990). Production of FBs by liquid culture was best with a chemically defined medium with glucose as the carbon source, but a solid medium of maize flour with water in the form of a "patty" in a petri dish produced the highest yields at 1–3 g/kg (Jackson and Bennett, 1990; Alberts *et al.*, 1994). More recently, FB1 has been produced in 10-L stirred liquid cultures, where yields comparable to solid substrate cultures were claimed with simpler extraction and cleanup procedures (Miller *et al.*, 1994).

Most approaches made to produce radiolabelled FBs depend on the addition of labelled methionine to fungal cultures, which labels the methyl groups on the FB backbone; deuterium was introduced into FB1 using this method (Plattner and Shackelford, 1992). A similar method was used to introduce methyl groups labelled with ^{14}C by Alberts *et al.* (1993b). More recently, Blackwell *et al.* (1994) added labelled ^{14}C acetate to liquid cultures and obtained FB1 labelled throughout the molecule. The use of this method with ^{13}C-labelled additives indicated the involvement of glutarate and that the backbone of the molecule was polyketide derived.

The biosynthesis of the rest of the FB molecule is not clear, apart from the demonstration that alanine was the precursor of the nitrogen-containing end of the molecule, analogous to the way that serine is incorporated in sphingosine (Branham and Plattner, 1993a). These workers, however, were not able to demonstrate the incorporation of stearic acid into FB1 in place of palmitate utilized in sphinganine biosynthesis.

1.7. *Related Metabolites*

All mycotoxins belong to a group of compounds known as secondary metabolites. These differ from the compounds that are essential for the functioning and growth of a normal living cell, and are characterised by their exclusive nature.

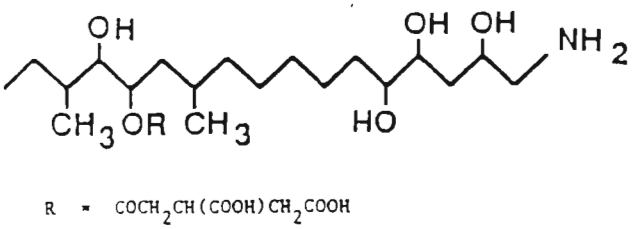


FIGURE 2. General structure of AAL toxin.

This explains why various fungal species tend to produce specific mycotoxins and no others. If one looks at secondary metabolite production in general, however, the ideal criterion is not met, in that several species of a genus might produce the same toxins. Often these are closely related, e.g., *Aspergillus flavus* and *Aspergillus parasiticus* both producing aflatoxin B_1. In some cases, they may not be closely related, e.g., cyclopiazonic acid produced by members of the *Aspergillus* and *Penicillium* genera. There are several explanations for this phenomenon, including convergent evolution, direct transfer of genetic information between organisms, and taxonomical classification based on morphology rather than genetic principles. Whatever the reason for lack of strict exclusivity, the FBs are no exception to this, and members of the genus *Alternaria* produce compounds very similar to the FBs and, in at least one case, actually produce FB itself (Chen *et al.*, 1992).

Four compounds were isolated from cell-free filtrates of *Alternaria alternata* f.sp. *lycopersici* and were found to cause necrotic symptoms in tomatoes characteristic of the producing fungus (Bottini *et al.*, 1981). These compounds were isolated and found to be two pairs of structural isomers with a tricarballylic acid esterified at either the C_{13} or C_{14} of an amino polyol chain, and were named AAL toxins (see Fig. 2 for general structure), TA1, TA2, TB1, and TB2 (Bottini and Gilchrist, 1981; Bottini *et al.*, 1981). Thus, they have a structural relationship to the FBs, exhibit similar biological activities (Mirocha *et al.*, 1992a), and have the same stereochemistry, i.e., 2S, 4S, 5R, 11S, 13S, 14R, and 15R (Oikawa *et al.*, 1994). Subsequently, strains of *Alternaria alternata* were found to produce three new sets of stereoisomers designated AAL toxins TC, TD, and TE (Caldas *et al.*, 1995) and FB1 (Chen *et al.*, 1992).

Aspergillus fumigatus was found to produce a group of metabolites named sphingofungins because of their structural similarity to sphingosine and, hence, the FBs, but without esterified tricarballylic acid moieties (Van Middlesworth *et al.*, 1992). These compounds were shown to have limited antifungal properties. It is probable that further related metabolites will be isolated as investigations proceed.

2. DISEASE AND EFFECTS

2.1. Equidae

2.1.1. Horses. The earlier documented symptoms of ELEM in horses are variable and, in more temperate climates, such as those found in the United States, the disease tends to be seasonal, being most prevalent from late autumn to early spring (Buck *et al.*, 1979). This latter observation can now be rationalised on the basis of the fungus having had time to grow on feedstuffs and to produce a toxin during this period. The literature on ELEM can be divided into phases, i.e., where it was understood that ELEM was caused by the contamination of feedstuffs with certain toxigenic strains of *F. moniliforme*, and where it was known that the causative agent was, in fact, FB, produced as a toxic metabolite of the fungus.

FIGURE 3. Brain lesion in a horse suffering from ELEM (T. S. Kellerman, personal communication).

In earlier accepted cases of ELEM* (Wilson and Maronpot, 1971; Kellerman *et al.*, 1972; Marasas *et al.*, 1976; Buck *et al.*, 1979; Pienaar *et al.*, 1981; Wilson *et al.*, 1985b; Domenech *et al.*, 1984a,b), common elements are observed as follows: the animal (1) develops inappetence after a period of eating contaminated feed; (2) becomes lethargic; and (3) as neurotoxic effects become apparent, develops uncoordinated movement and aimless walking with blindness and violent blunderings into the front of stalls and walls. In general, the animal will become difficult to handle and ill-tempered. In some cases, death can occur without any nervous symptoms and, in others, liver-related symptoms are seen, such as swelling of the lips and nose, severe icterus, petechial haemorrhages in the mucous membranes, and cyanosis.

Two main types of histopathological signs are found on autopsy. The first being those involving the brain, where oedema in the form of an accumulation of clear fluid under the meninges is seen and liquefaction of areas within the cerebral hemispheres is found, causing lesions that might range in size from microscopic to one occupying most of the lobe. In severe cases, there may be a large liquified cavity within the white matter of the right cerebral hemisphere, with the cerebrum posterior to the cavity enlarged, oedematous with congested blood vessels (Fig. 3).

The other organ affected is the liver, which often shows a mild swelling with a colour change to yellow-brown. In

* Kriek, N. P. J., Marasas, W. F. O. and Kellerman, T. S. (1981) Aspects of equine leukoencephalomalacia. In: Proceedings of the Veterinary Pathology Group of the South African Veterinary Association, Pretoria, 1980, p. 75. South African Veterinary Association.

more severe cases, gross liver lesions may be seen with fibrosis of the centrilobular area. Hepatocytes on the edge of the fibrotic area have large fatty globules in their cytoplasm.

These observations, in general, are supported by field outbreaks of ELEM where FBs were determined in the feed (Pellegrin *et al.*, 1990; Wilson *et al.*, 1990a,b; Thiel *et al.*, 1991b; Ross *et al.*, 1991a,b, 1992; Wilson *et al.*, 1991; Caramelli *et al.*, 1993; Wilkins *et al.*, 1994) or by experiments using FBs in added cultured material (Kellerman *et al.*, 1988; Ross *et al.*, 1994), or administered as purified compounds (Marasas *et al.*, 1988b; Laurent *et al.*, 1989a; Kellerman *et al.*, 1990).

In addition to ELEM, the role of *F. moniliforme* cultures in duodenitis and proximal jejunitis in horses was studied (Schumacher *et al.*, 1995) because it was thought that this condition, which is characterized by a copious reflux of gastric juice, did not have a bacterial cause and might be related to FB1 ingestion. Plumlee and Galey (1994) have also reviewed the effects of FB1 and four other mycotoxins that cause neurotoxicological symptoms and concluded that, for a correct diagnosis, the specific mycotoxin has to be isolated and identified from the feed.

2.1.2. Donkeys. In examining the effects of maize inoculated with a toxigenic strain of *F. moniliforme* (derived from an outbreak of ELEM) on 3 horses and 3 donkeys, Kellerman *et al.* (1972) observed varying results. One horse and 1 donkey were unaffected, 1 donkey developed a transient pruritus, and the remaining animals died. Observations on autopsy of the animals were similar, the gross lesions being severe cardiac haemorrhages, oedema, icterus, and liver damage. No brain lesions, other than small perivascular haemorrhages, were found. With hindsight, it seems very likely that these effects were caused by a combination of *Fusarium* toxins.

Other studies involving feeding donkeys with infected material were done by Badiali *et al.* (1968), Wilson and Maronpot (1971), Wilson *et al.* (1973) and Haliburton *et al.* (1979). In all of these studies, the animals were found to develop ELEM in varying degrees.

2.1.3. Ponies. Ponies were given feed containing a maize culture of a strain of *F. moniliform* associated with an outbreak of ELEM (Brownie and Cullen, 1987). Of the five treated ponies, one developed clinical signs of toxicity and, on autopsy, was found to have brain lesions consistent with ELEM.

Following their observation that FBs can alter sphingosine metabolism (see Section 2.5), Wang *et al.* (1992) gave ponies feed containing 15–44 mg/kg FB1. In addition to liver cytotoxicity, encephalopathy, and death, they found that free sphingosine and sphinganine were elevated. The latter effects appeared rapidly, and it was suggested that such measurements could be used as early markers for exposure to FB. Similar symptoms were also observed by Ross *et al.* (1993) in a feeding trial with FB1 in 4 ponies.

Another study was done by Wilson *et al.* (1992) to determine the minimum toxic dose of FB1 on ponies. A group of 4 animals were fed rations with 1–22 mg/kg and a second group of 5 animals, 8 mg/kg. One pony in the first group died with ELEM after 225 days of feeding (the last 22 days of the diet contained 22 mg/kg), the other animals having various degrees of lesions mainly associated with liver and cerebral hemispheres. The second group of ponies showed mild transient clinical signs, and autopsy revealed mild brain lesions.

2.2. *Pigs*

An outbreak of disease in pigs in Georgia, USA in 1989 was investigated by Harrison *et al.* (1990). On autopsy, the animals had pulmonary oedema and hydrothorax, with the thoracic cavities being filled with a yellow liquid. The problem was traced to maize screenings, and *F. moniliforme* was isolated, leading to the suspicion that a mycotoxicosis was involved. Four pigs were injected in the cranial vena cava, with various amounts of FB1 and FB2. The one that received the highest dose, 0.4 mg/kg body weight for 4 days, a total of 11.3 mg, died on the fifth day and exhibited similar lesions to the pigs involved in the field outbreak.

Another outbreak of disease in the United States in pigs during 1988 and 1989 was also reported, which was characterized by foetal mortality and respiratory disorders in older animals, and was referred to as "mystery swine disease" (Bane *et al.*, 1992; Osweiler *et al.*, 1992). In light of the outbreak in Georgia, USA, samples of feed were taken from outbreaks in Illinois and Iowa, USA, analyzed for FB, and found to be positive. FB1 was found to range from 20 to 330 mg/kg. Several studies link what has been called porcine pulmonary oedema syndrome with outbreaks of ELEM (Ross *et al.*, 1990, 1991a), and it soon became accepted that the two diseases had a common cause, i.e., FBs.

Studies involving the dosing of pig with FBs used either feed naturally contamination with FBs at known levels (Casteel *et al.*, 1993, 1994; Haschek *et al.*, 1992; Motelin *et al.*, 1994; Osweiler *et al.*, 1992; Ross *et al.*, 1991b, 1992; Colvin and Harrison, 1992; Colvin *et al.*, 1993; Harrison *et al.*, 1990) or by injection with quantities of purified FB (Haschek *et al.*, 1992; Harrison *et al.*, 1990). In general, animals fed high levels of FB1 died with pulmonary oedema, and those surviving lower levels had evidence of subacute hepatotoxicosis. From the limited evidence, animals receiving pure toxin were more likely to develop pulmonary oedema, whereas those given naturally contaminated feed had both lesions. One exception was where 3 young pigs were given feed ranging from 100 to 190 mg/kg FB1 content, and developed nodular hyperplasia of the liver and changes in their distal oesophageal mucosa. These variations could be explained by the differences between acute and chronic poisonings; however, an intriguing alternative is the possibility that FB is somehow different in its action when administered either in a purified form or in combination with other metabolites in naturally contaminated material. Certainly, synergistic effects are known between other mycotoxins, including FB1 itself with fusaric acid (Bacon *et al.*, 1995).

Feeding FB1 to lactating sows did not affect suckling pigs and there was no evidence of the toxin present in the milk

(Becker *et al.*, 1995). This agrees with other studies on cows' milk (Scott *et al.*, 1994).

As with other animal studies, the uptake of ^{14}C-labelled FB1 administered intragastrically to pigs was poor, 3–6% and, in that given i.p., elimination was rapid, 80% of the dose being recovered within 3 days in the excreta, with the remainder being associated with liver and kidney tissue (Prelusky *et al.*, 1994). Riley *et al.* (1993a) found alterations in sphinganine-to-sphingosine ratios in pigs fed diets containing FB1 and FB2, and suggested this as an early biomarker for exposure to the toxin.

2.3. *Rodents*

2.3.1. Rat. Prior to the discovery of the FBs, several trials had been done where rats were dosed with feed infected with *F. moniliforme* related to outbreaks of ELEM (Wilson *et al.*, 1985a; Voss *et al.*, 1989) or to OC (Marasas *et al.*, 1984; Jaskiewicz *et al.*, 1987b,c,d; Gelderblom *et al.*, 1988a). In general, these studies indicated the presence of a hepatocarcinogen in these cultures with the development of hepatic nodules, adenofibrosis, hepatocellular carcinoma-ductular carcinoma, and cholangiocarcinoma.

The rat was used as a model to explore the carcinogenic potential of FB, in light of its connection with OC. Several studies on rats were conducted by the workers at PROMEC (Gelderblom *et al.*, 1991, 1992b; Shephard *et al.*, 1992a,b; Gelderblom *et al.*, 1994; Shephard *et al.*, 1994a) and FB2 (Shephard *et al.*, 1995b). These took two forms, those exploring the absorption and excretion of FBs and those examining its effect on the animal.

An initial experiment on clearance was done by dosing rats, both by i.p. injection and by gavage, with FB1 (Shephard *et al.*, 1992a). The injected doses were rapidly absorbed and reached a maximum in 20 min. Injected FB1 was cleared with a half-life of 18 min, 16% of the total dose being recovered from the urine in an unchanged form. In the case of administration by gavage, only 0.4% of the dose was recovered in the urine. Two points were raised by these results: where had the rest of the i.p. dose gone, and why was there such a low excretion from dosage by the p.o. route. Radiolabelled FB1 was used to explore this problem (Shephard *et al.*, 1992c) and, from i.p. administration of ^{14}C-FB1, 66% was found in the faeces, 32% in the urine, 1% in the liver, and traces in other tissues such as kidney. Of the material given p.o., 101% was found in the faeces. A further study (Shephard *et al.*, 1994a) clarified how FB1 ended up in the faeces of animals dosed i.p., because 67% of the dose in this latter study was found in the bile. In the animals dosed by gavage, only 0.2% ended there.

Evidently, only a small portion of the doses (<1%) are absorbed from the gastrointestinal tract (GIT), which is understandable considering their high polarity and presumable lack of membrane transport systems. This may explain why relatively high levels of contamination of feed are required, >5 mg/kg to produce symptoms in animals, but it also indicates that the FBs must be highly active once internalised.

Other work reported by the PROMEC group shows that the FBs are hepatotoxins and carcinogens in the rat. Culture material from *F. moniliforme* fed to rats produced micro- and macronodular cirrhosis in animals that died, cholangiofibrosis and primary hepatocellular carcinomas (Gelderblom *et al.*, 1988b, 1991), and adenofibrosis (Wilson *et al.*, 1985a). These effects were investigated further using purified FB1 and FB2 (Gelderblom *et al.*, 1992b), and with a comprehensive study into the cancer-initiating capability of FB1, FB2, and FB3 (Gelderblom *et al.*, 1993, 1994).

Investigations by other groups broadly support these observations (Voss *et al.*, 1990, 1993; Bondy *et al.*, 1995) but, interestingly, in addition to hepatic effects, tubular nephrosis was found in the renal cortex of animals fed material infected with *F. moniliforme* associated with outbreaks of ELEM (Voss *et al.*, 1989) and inhibition of protein synthesis in hepatocytes (Norred *et al.*, 1990). Both these materials were shown to contain FBs, but they were not quantitated. Later work, where purified FB1 was fed to rats, showed that sphingolipid metabolism in the kidney is more susceptible to the toxin than in the liver (Riley *et al.*, 1994). More recent work also showed that the kidney was more susceptible to p.o. doses of FB1 (Voss *et al.*, 1995a,b), and various markers of nephrotoxicity were measured in rats dosed with FB1 (Suzuki *et al.*, 1995). The use of ^{14}C-labelled FB1 as a single dose to rats killed at time intervals gave recoveries of the label in faeces and urine of over 80% (Norred *et al.*, 1993). The balance, however, remained in the blood, liver, and kidney for the duration of the experiment, i.e., up to 96 hr, which does not quite agree with the PROMEC results, in that larger quantities remained bound in the animal.

Other workers using rats provided additional data on organs other than the liver. Porter and co-workers (1990, 1993) investigated the effect of FB1 on neurotransmitters in the brain. The point of this investigation was to see if the imbalances in rat brain 5-hydroxytryptamine and 5-hydroxyindoleacetic acid, induced by feeding with *F. moniliforme*, was due to FB. Although changes in norepinephrine-to-dihydrophenylacetic acid(s) ratios were disturbed in rats fed 150 ppb FB1 for 4 weeks, nothing corresponding to the changes in 5-hydroxytryptamine/5-hydroxyindoleacetic acid ratios were observed, and it was concluded that other compounds in *F. moniliforme* cultures were responsible for the effect.

Lebepe-Mazur *et al.* (1995a,b) showed that FB1 effected the foetus in pregnant rats, causing low litter weights and foetal bone development, as compared with untreated controls, and found that placental glutathione S-transferase was a more useful marker of γ-glutamyl transferase in detecting hepatic cancer foci.

2.3.2. Other rodents. Pregnant mice fed culture material containing known amounts of FB were shown to have lowered body weights, increased morbidity and mortality in a dose–response way (Gross *et al.*, 1994). Other observations were liver damage and ascites and a reduction in the number of live offspring.

Similar results were found in another investigation that indicated that FB1 fed at <81 ppm to female mice caused hepatotoxicity, and doses at 27 ppm or less had no effect, which is in contrast to the rat where nephrosis was observed at 9 ppm (Voss *et al.*, 1995a). FB1 fed to Syrian hamsters at a rate of 18 ppm also resulted in increased foetal death, but without evidence of maternal intoxication (Floss *et al.*, 1994a,b). It may be that the rat is a better model for investigating the effects of the FB because of greater sensitivity compared with mice, which is the case for the aflatoxins.

2.4. *Other Species and Tissues*

2.4.1. Poultry. Considering the economic importance of chickens and their dependence on maize-based feeds, which are often contaminated with *F. moniliforme* (Bragulat *et al.*, 1995a,b), investigations using FBs only began in 1992, although earlier trials had been done with maize infected with *F. moniliforme* (Bryden *et al.*, 1987). Day-old broiler chicks were fed doses of FB1 ranging from 0 to 400 mg/kg for 21 days and 300 mg/kg for 2 weeks (Brown *et al.*, 1992; Ledoux *et al.*, 1992). Body weight gain was greatly reduced; hepatic necrosis, biliary hyperplasia, and thymic cortical atrophy were noted, along with diarrhoea and rickets. A further investigation (Weibking *et al.*, 1993a) revealed increased sphinganine and sphinganine:sphingosine ratios in young chicks treated with culture material containing FB1.

Another study where broiler chicks were fed with FB1 and FB2 in conjunction with moniliformin showed abnormal erythrocyte formation and lymphocyte cytotoxic effects (Javed *et al.*, 1993a; Dombrink-Kurtzman *et al.*, 1993). Chicken embryos exposed to FB1 showed a mortality of 100% when dosed at 100 μM. Pathological changes were found in liver, kidney, heart, lungs, musculoskeletal system, intestines, testes, and brain (Javed *et al.*, 1993b). Work by Espada *et al.* (1994) showed that pure FB1 fed at a rate of 10 mg/kg feed for 6 days was toxic to young chicks. A single dose of ^{14}C-labelled FB1 given to a laying hen (Vudathala *et al.*, 1994) gave similar results to those in the rat, with poor p.o. uptake and rapid excretion of the i.p. dose, although no FB1 residues were found in the eggs.

Chatterjee and co-workers (Chatterjee and Mukherjee, 1994; Chatterjee *et al.*, 1995) examined the effects of FB1 on chicken peritoneal macrophages, which exhibited breakdown of the nucleus with increasing numbers in a dose–response manner (6–18 μg/mL). This suggests impaired disease immunity in chickens exposed to FB1, which is in agreement with other work where extracts of three isolates of *F. moniliforme* were immunosuppressive (Marijanovic *et al.*, 1991) and organs involved in immunocompetence were affected (Martinova *et al.*, 1995). Vitamin A, an important factor in maintaining disease resistance in poultry, was depleted in the serum of broiler chicks fed both culture material containing FB1 and FB1 itself (Hall *et al.*, 1995).

Prior to the discovery of the FBs, cultured material from strains of *F. moniliforme* were fed to ducklings and 24 of 25 were found to be toxigenic (Jeschke *et al.*, 1987). Ducklings were used by Marasas (1982) and Vesonder *et al.* (1989) as a test animal to investigate material from an OC area a ELEM outbreak, respectively. Some of the isolates of *F. mon forme*, when grown and incorporated into feed, caused 10 mortality. These birds had slightly swollen and redden livers, with low body fat and loss in weight compared w controls. An outbreak of paralysis in quail was linked the presence of 17.7 mg/kg FB1 in their feed, but this co not reproduce the symptoms in a further feeding trial (Grin *et al.*, 1993).

Turkey poults were fed culture material at different lev of FB1 from three *F. moniliforme* strains that produced v ious amounts of the toxin (Weibking *et al.*, 1993b, 199 Variations in body and organ weight were observed, depe ing upon the treatment, with ratios of serum sphingani to-sphingosine increased in most birds, and generali hepatocellular hyperplasia in all the treated poults. Bili hyperplasia was found in turkeys fed FB1, 150–300 mg/ body weight, and FB was implicated in cases of turkey ce bral encephalomalacia (Ficken *et al.*, 1993). An investigati into the combined effects of aflatoxin and FB fed to turk poults was done by Weibking *et al.* (1994) and Kubena *al.* (1995a). There was variation in body and organ weig with increased sphinganine/sphingosine ratios, but no e dence of synergistic activity between the toxins, which, best, gave additive effects. A study with T-2 toxin in pl of aflatoxin showed similar results (Kubena *et al.*, 1995 although some of the various parameters measured w affected by the combination of toxin alone. It was conclud that a combination of toxins poses a greater problem to poultry industry than either of the toxins individually.

2.4.2. Other animals. Less work has been done on oth animals, presumably because of lack of exposure and la of intoxication in animals, such as ruminants. One case ELEM symptoms in white-tailed deer has been describ in the United States (Howerth *et al.*, 1989), which see to have been caused by the animal finding a source of infec maize. Calves given feed containing FB1, up to 148 mg/ were unaffected in terms of feed intake or weight ga although certain blood enzymes were elevated along w cholesterol (Osweiler *et al.*, 1993). Those fed at the high levels showed mild microscopic liver lesions, and it was c cluded that cattle are less susceptible to FB1 than oth animals.

Lambs were dosed intraruminally with FB-containing and showed evidence of liver and kidney damage and, those receiving 44.5 mg/kg total FB, death (Edrington *al.*, 1995). Liver and kidney damage were also observed mink given chronic doses of FBs (Restum *et al.*, 1995). Stud were done on catfish fed with cultured material contain FBs, and it was concluded that levels of 20 mg/kg or ab are toxic to both 1- and 2-year-old fish (Brown *et al.*, 19 Lumlertdacha *et al.*, 1995). FB1 was used by Strum *et* (1995) to investigate the role of ceramide in the meio cell cycle of *Xenopus laevis* oocytes. The results indica the potential use of FB1 in regulatory studies involv sphingolipids.

TABLE 2. Effects of FB1 on Various Species

Species exposed	Dose: route	Principal effect	Reference
Calf	148 mg/kg body weight: p.o.	Mild liver lesion	Osweiler *et al.*, 1993
Catfish	20 mg/kg feed: p.o.	Death, liver damage	Lumlertdacha *et al.*, 1995
Chicken	300 mg/kg body weight: p.o.	Liver damage	Ledoux *et al.*, 1992
Deer (Whitetail)	Unknown: p.o.	Brain lesion	Howerth *et al.*, 1989
Donkey	Unknown: p.o.	ELEM	Haliburton *et al.*, 1979
Duckweed (*Lemna minor*)	10 μg/mL medium	Reduced growth and chlorophyll, death	Vesonder *et al.*, 1992a
Horse	29.7 mg/kg body weight	ELEM	Kellerman *et al.*, 1990
Jimsonweed (*Datura stramonium*)	0.05 μg: leaf surface	Electrolyte leakage from cells	Abbas *et al.*, 1992a
Lamb	45 mg/kg: p.o.	Death, liver and kidney damage	Erdington *et al.*, 1995
Monkey (vervet)	3.3 mg/day: p.o.	Atherosclerotic response	Fincham *et al.*, 1992
Mouse	81 μg/kg body weight: p.o.	Liver damage	Voss *et al.*, 1995b
Pig	11.3 mg: i.v.	Death, lung edema	Harrison *et al.*, 1990
Pony	22 mg/kg/day: p.o.	ELEM	Wilson *et al.*, 1992
Rat	Feed total 13 mg/kg body weight: p.o.	Liver and kidney damage	Voss *et al.*, 1993
Turkey	Feed total 300 mg/kg body weight: p.o.	Liver and myocardium damage	Weibking *et al.*, 1993b
Yeast (*Saccharomyces* and *Pichia* spp.)	100 mg/L culture fluid	Sphingolipid metabolism affected	Wu *et al.*, 1995; Kaneshiro *et al.*, 1992

2.4.3. Plants. FB causes toxicity on contact with plant tissue and at sites removed from application, indicating mobility of the toxin within the plant xylem (for a review, see Abbas *et al.*, 1993a; Kuti and Abbas, 1993). An isolate of *F. moniliforme* found growing on jimsonweed *Datura stramonium*, was shown to produce a water-soluble material that caused mosaic-like patterns on leaves and inhibited growth when sprayed on the leaves (Abbas *et al.*, 1991, 1992a,b, 1993b). The major toxic component was found to be FB1. Further trials were done to investigate the phytotoxicity of FB1 and assess its potential as a weed control agent (Abbas and Boyette, 1992). A range of plants were shown to be sensitive, the minimum amount of FB1 administered to excised leaves to cause damage being 0.05 μg. The mycotoxin produced various symptoms, including chlorosis, necrosis, tissue curling, stunting, and defoliation. Leaf discs from this plant were further used in bioassay for the FBs and their metabolites (Abbas *et al.*, 1993a), as were jimsonweed seedlings (Abbas *et al.*, 1995a). As in other organisms, sphingolipid metabolism was affected (Abbas *et al.*, 1994, 1995b).

Vesonder and co-workers (1992a,b) examined the effect of FB1 on duckweed, *Lemna minor*, vs. other mycotoxins, including moniliformin, butenolide, and AAL toxin. Of these, FB1 was the most effective in reducing growth and chlorophyll biosynthesis, making it a suitable organism for bioassay (Tanaka *et al.*, 1993). Other plants found to be affected by the FBs are maize, with inhibition of seedling emergence (Doehlert *et al.*, 1994) and cell cultures (Van Asch *et al.*, 1992), and tomato, where leaf discs were affected in a similar manner to that promoted by AAL toxin (Gilchrist *et al.*, 1992). Excised shoots of certain cultivars of tomato were induced to produce roots earlier than controls, when treated with <0.5 mg/plant FB1, indicating that the toxin does affect differentiation of tissue, presumably via its interference with cellular control mechanisms (Bacon *et al.*, 1994).

The effects of FB1 on various species are summarized in Table 2.

2.4.4. Tissues. Laurent and co-workers (1989b, 1990) also isolated FB1 and called it macrofusin. They investigated its ability to cause heart failure in rats and horses by treating frog skeletal muscle with FB1 (Laurent *et al.*, 1989b), where it was found to cause a 50% reduction in sodium ion current at a concentration of 300 μM. It was suggested (Ecault *et al.*, 1990) that the calcium current was the main target in frog heart, and this was shown to be the case because calcium flux was blocked (Sauviat *et al.*, 1991), resulting in loss of heart muscle activity and a possible cause of reported heart failure in horses.

2.5. *Cell Cultures*

The FBs are cytotoxic (Norred *et al.*, 1991a) and the effects of FB1 and FB2 were tested on several mammalian cell lines, including rat hepatoma and dog kidney epithelial cells, which were found to be sensitive (Shier *et al.*, 1991). In an experiment evaluating the genotoxicity of several toxins to rat hepatocytes, however, Norred *et al.* (1992a) found FB1 at 0.5–250 μm to have no effect in the unscheduled DNA assay. Epidermal growth factor-induced DNA synthesis in rat hepatocyte was inhibited 90% by FB1 (Gelderblom *et al.*, 1995), which was reversed when the FB1 was removed. Labelling studies indicated that inhibition was not due to blockage of the primary receptor binding site; consequently, FB1 must interfere further down the cascade. In contrast, Vesonder *et al.* (1993) found that neither AAL toxin nor FB1 were cytotoxic to 5 mammalian cell lines at the level of 100 μg/mL culture medium.

On adding radiolabelled FB1 and FB2 to rat hepatocytes, it was found that FB2 had a higher cytotoxicity and specific binding than FB1, although at the effective dose level they

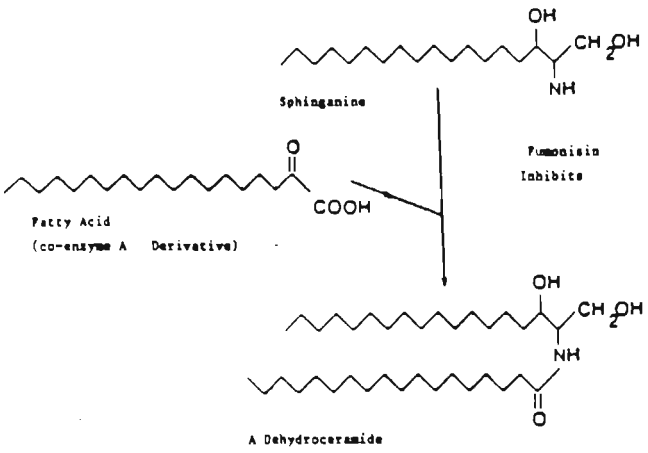
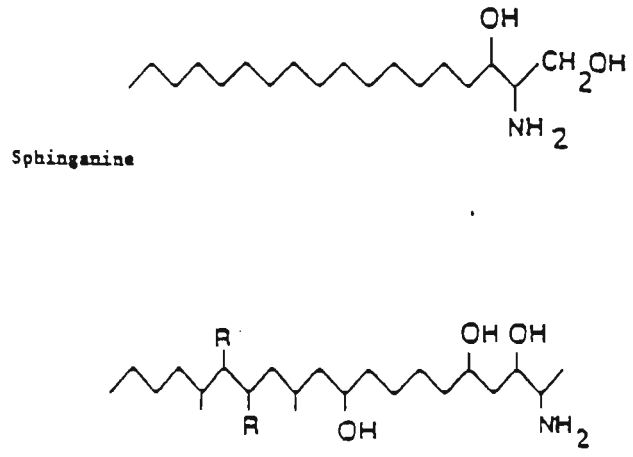


FIGURE 4. Step of ceramide biosynthesis inhibited by FB1.



FB1

FIGURE 5. Structure of sphinganine compared with th general structure of the FBs.

showed similar cytotoxicity (Cawood *et al.*, 1994). Very little of the FB bound to the hepatocytes (0.01%) and no catabolism of the molecule was detected, which agrees with *in vivo* rat experiments, where 67% of an i.p. administered dose of FB1 was excreted via the bile (Shephard *et al.*, 1994a). FB1 was also found to inhibit protein synthesis (Norred *et al.*, 1990) and inhibit the secretion of ceramide into very-low-density lipoprotein (Merrill *et al.*, 1995a).

An important investigation was that done by Wang *et al.* (1991), who showed that FB1 disrupted sphingolipid biosynthesis in rat hepatocytes. A further investigation using rat hepatocytes and pig kidney epithelial cells (Norred *et al.*, 1992b; Yoo *et al.*, 1992) confirmed that the FBs were potent inhibitors of sphingolipid biosynthesis in both types of cells, killing the renal cells after 3 days at a level of 70 μM of FB1. This effect was further studied in mouse cerebellar neurons (Merrill *et al.*, 1993a,b) and neuroblastoma cells (Rother *et al.*, 1992), where it was shown that FB1 specifically inhibited the conversion of sphinganine to dihydroceramides (Fig. 4). The inhibitory effect on neuron cultures of FB1 was demonstrated when treated hippocampal neuron axonal growth was completely suppressed (Harel and Futerman, 1993), as was short-term axonal branching (Schwartz *et al.*, 1995).

It was pointed out by Wang *et al.* (1991) that the structure of the backbone of the FB molecule was similar to the important phospholipid, sphingosine (Fig. 5) (Shier, 1992). Hence, it may exert its action by interfering with its metabolism and processes mediated by this type of molecule (Kim *et al.*, 1991), e.g., inhibition of protein kinase C (Hannun *et al.*, 1986) and via ceramides, regulation of cell growth, differentiation, and apoptosis (Huang *et al.*, 1994, 1995). Recently, Ramasamy *et al.* (1995) have shown that FB1 increases the rate of albumin transfer across endothelial cell monolayers derived from pulmonary artery, in addition to elevation of sphinganine levels, the latter possibly explaining the loss of endothelial barrier function.

Schroeder *et al.* (1994) (also discussed by Wolf, 1994) investigated the effect of FB1 on Swiss 3T3 fibroblasts and found that sphinganine accumulated, and was greatly promoted in the presence of insulin. These effects were paralleled b an increase in DNA synthesis. It was also shown that b using an inhibitor of sphingoid base biosynthesis, the increas in DNA synthesis is directly attributable to accumulatio of sphinganine. This result is important, not only becaus it suggests the use of FB1 as a tool to investigate the rol of free sphingoid bases as mitogens, but also gives a lea to the mechanism of tumour promotion by FB1 by stimu lation cell proliferation. In addition, the possible influenc of dietary sphingolipid and FB on the role of fat in diseas has been reviewed by Merrill *et al.* (1995b).

Chicken macrophages were exposed to up to 10 μg/m FB1, where cytotoxicity was observed (Qureshi and Hagler 1992). The results implied that exposure of chickens to FB increased their susceptibility to infection. Turkey lympho cytes were used in an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-based cytotoxicity assay by Dombrink Kurtzman *et al.* (1994), and the 50% inhibitory dose for FB or FB2 was 0.4–0.5 μg/mL medium. Cells exposed to th toxins exhibited vacuolization and were unable to proliferat Interestingly, FB2 was 3- to 4-fold more cytotoxic than FB Chicken chondrocytes in culture were exposed to FB1, whic was inhibited by levels as low as 25 μm, although a paralle study on growing broilers indicated that FB1, by itself, was nc sufficient to cause bone deformation (Wu, W. D. *et al.*, 1995

The yeasts *Saccharomyces cerevisiae* (Wu, W. I. *et al.*, 1995 *Pichia sydowiorum* (Kaneshiro *et al.*, 1993), and *Pichia ciferi* (Kaneshiro *et al.*, 1992), when treated with FB1, develope abnormalities in their sphingolipid metabolism, showing th lower organisms are equally affected by the toxin. The bi luminescent genotoxicity test using *Vibrio fischeri* reveale that FB1 was directly mutagenic without prior activatic by liver S9 fraction (Sun and Stahr, 1993). This was in co trast to the Ames test, because FB1 exhibited cytotoxici but no mutagenicity, against *Salmonella* tester strains (G derblom and Snyman, 1991), despite the mutagenic activi of *F. moniliforme* isolates (Bjeldanes and Thomson, 197

Other investigations using cell cultures included the treatment of green monkey kidney cells with FB1 to test the hypothesis that FB could inhibit protein kinase C (Huang *et al.*, 1995). The toxin was found to repress expression of protein kinase C, whereas it stimulated the promoter of a cyclic AMP receptor. These workers concluded that altered signal transduction pathway played a role in the carcinogenesis of the FBs. Similar results were found for baby hamster kidney cells treated with FB1 (Abeywickrama and Bean, 1992). The mechanism of action of several mycotoxins, including FB, have been reviewed by Coulombe (1993).

3. HUMAN INVOLVEMENT

3.1. *Disease*

3.1.1. Oesophageal cancer. The discovery of the FBs derived directly from the investigation by the PROMEC group of human OC in the Transkei in South Africa. OC occurs in geographical pockets around the world, e.g., China (Yang, 1980; Mingxin *et al.*, 1980), and was first reported in the Transkei in 1957 (Burrell, 1957), an observation that was amply confirmed (Jaskiewicz *et al.*, 1987a,b,d; Rose, 1973, 1982; Rose and McLashan, 1975; Van Rensburg, 1985). As in most rural communities in Southern Africa, the main staple of diet is maize. It was well established that maize grown in this region was susceptible to infection by several fungi, in particular *Fusarium* spp. (Marasas, 1982; Marasas *et al.*, 1979, 1980, 1988a) and, of these, *F. moniliforme* and *F. subglutinans* were the most prevalent, with *Diplodia maydis* being third. Because of the high incidence of these fungi together with their mycotoxins, they were investigated in outbreaks of OC.

A significant difference in the levels of FB in maize and maize used for making home-brewed beer (Rheeder *et al.*, 1992; Marasas *et al.*, 1993) between high and low OC areas was found. A similar distribution was found in China (Chu and Li, 1994; Yoshizawa *et al.*, 1994), where the incidence of FB in high-risk areas was twice that in low-risk areas.

3.1.2. Primate studies. In Section 2, the effects of the FBs on various animal species were considered. From the human point of view, it is disappointing that no species seemed to respond to FB in quite the same way and none developed OC. It seemed reasonable, therefore, that the best animal to model human response would be a primate, and several trials involving monkeys were done.

Prior to isolation of the FBs, as part of a study on the effect of toxic extracts of *F. moniliforme* on animals, baboons were found to develop hepatic cirrhosis and intraventricular thrombosis (Kriek *et al.*, 1981). In another investigation, 10 young vervet monkeys were fed *F. moniliforme* culture material (Jaskiewicz *et al.*, 1987a). This was found to cause a toxic hepatitis of varying degrees related to the amount fed, with the release of liver enzymes into the serum paralleling the liver damage. In another trial, four male and seven female vervet monkeys were fed cultured material containing FB1, FB2, and FB3 (Fincham *et al.*, 1992). Days of feeding ranged from 40 to 573 on a "high" dose regime (total FB ranging from 784 to 3257 μg/vervet/day) and 310 to 748 on a lower one (total FB ranging from 392 to 814 μg/vervet/day). The unexpected result was the appearance of cholesteroaemia, all treated animals having statistically significant increased serum cholesterol over that of controls and with raised plasma fibrinogen and activity of Factor VII, which would enhance atherogenesis. In addition, chronic hepatotoxicity was observed, which is in keeping with the findings in other animal species.

Further work on primates (Shephard *et al.*, 1994b) was aimed at investigating their absorption and excretion of FB, as previously studied in rats (Shephard *et al.*, 1992c). In this case, however, extraction from faeces was effected using EDTA, which presumably allowed the release of the FBs from their insoluble metal salts, which supports the idea of FBs as metal chelators. In one experiment, 2 vervet monkeys were given radiolabelled FB1 at a rate of 8 mg/kg body weight. The bulk of the radiolabel was found in the faeces and consisted mainly of unchanged FB1 and partially hydrolysed monoesterase derivatives.

In a later, more detailed, investigation using four vervet monkeys (Shephard *et al.*, 1994c), radiolabelled toxin was administered by gavage or i.p. injection. From 2 animals treated by i.p. injection, 47% of the dose was recovered in urine and faeces over 5 days as FB1 or its partially hydrolysed derivatives, whereas 61% was recovered from those dosed by gavage. A further 15% was accounted for in the latter animals, mainly as intestinal contents, with small amounts in skeletal muscle, liver, brain, kidney, heart, erythrocytes, and bile. This result is intriguing, as a quarter of the fed material could not be accounted for, which is in contrast to the experiment with rats.

3.2. *Exposure*

As already discussed in Section 1.5, FBs have been found wherever maize is grown. Systematic examination for these mycotoxins in commodities destined for human consumption does not seem to be in place yet, and such investigations are dependent upon the interest of independent investigators and agencies. Marasas *et al.* (1993) reviewed the situation in Africa, and this makes spartan reading, considering the importance of maize as a staple for many populations who live in this continent. The figures given are mainly for the Transkei, where work on OC was done.

FB1 and FB2 were found in "good" homegrown maize in both low and high OC areas, and were significantly different in the survey done in 1985, e.g., FB1 ranged from 0 to 550 μg/kg for low and 50 to 7900 μg/kg for high (Sydenham *et al.*, 1990b). In a 1989 survey, there was a higher trend for high OC areas, but this was not statistically significant. Nevertheless, FB1 ranged up to 5380 μg/kg for "good" maize in the high areas (Sydenham *et al.*, 1991; Rheeder *et al.*, 1992). If these figures are not bad enough, the levels in mouldy maize for this regions were greatly elevated in 1989 showing, in some cases, a 10-fold increase of FB1 in the high OC areas (up to 117,520 μg/kg) over low (11,340 μg/kg). The use of

mouldy materials in home-brewed beer making in these regions is common, and it could well be that FB needs other co-intoxicants, such as ethanol, to promote its action in OC initiation. There does seem to be a connection with the consumption of home-brewed beer and OC in other parts of South Africa (Segal *et al.*, 1988). Furthermore, it has been shown that fermentation of maize (Bothast *et al.*, 1992) and wort (Scott *et al.*, 1995) contaminated by FB does not cause appreciable FB1 breakdown. Other studies*† showed the presence of FB1 in locally grown maize in the KaNgwane district, which is in the northern part of South Africa, indicating the size of the problem in the rural populations.

The levels in commercially grown maize, although lower, do not encourage complacency. Maize meal from South Africa had levels of FB1 ranging from 0 to 475 μg/kg, and that from Egypt, 1780 to 2980 μg/kg (Sydenham *et al.*, 1991). The situation in the rest of the world is equally alarming, as summarized in Table 2 and by Sydenham *et al.*‡

3.3. *Control*

3.3.1. Risk assessment. From the above statistics, it is certain that people who eat maize are exposed to FBs. As with other intoxicants that occur in the environment, the risk to humans is difficult to ascertain. Analysis of food is one approach, but this, in itself, cannot give a definitive answer as to how much any individual might have ingested. With, for example, the aflatoxins (Groopman and Kensler, 1993) and ochratoxin A (Kuiper-Goodman and Scott, 1989), methods have been developed whereby levels of the toxins are measured in humans and animals, and this can be correlated to recent intake. Because FBs are not easily absorbed in animals and are rapidly excreted, their *in vivo* measurement as an index or marker of exposure is not practical. A more fruitful approach could be the measurement of sphingoid bases, as there is no reason to suppose that these are not affected, as in other animal species investigated (e.g., Riley *et al.*, 1993b).

Current evidence indicates that certain rural populations are exposed to high levels of FB. Thiel *et al.* (1992) estimated that people living in the Transkei had a daily intake of 0.014 mg/kg body weight from consuming so-called "healthy" maize, and this increased to 0.44 mg/kg for mouldy maize. These figures are alarming, and efforts must be made to minimise FB in food and to control permitted levels. Legislation of FBs levels in commercial maize-based foods is already being contemplated; a preliminary tolerance level for FBs in food of 1 mg/kg has been recommended in Switzerland (Zoller *et al.*, 1994). This has ramifications, not only for the development of sensitive routine analytical methods of analysis but also for maize producers around the world. Guidelines for safe levels of FBs in feeds have been adopted by individual states in the United States, and guidelines for human food in the United States and Canada are expected in the near future (Miller, 1995).

3.3.2. Limiting contamination. There are two broad strategies to be followed in limiting mycotoxins in the food chain, i.e., prevention and cure. Prevention can be assisted by developing crop cultivars that are resistant to infection with toxigenic strains of fungi. Such strains are being investigated (Rheeder *et al.*, 1990a,b; De Leon and Pandey, 1989). Another suggestion is the use of biocontrol agents, where maize kernels are treated at planting with antagonistic rhizobacteria (Bacon and Williamson, 1992).

Of more importance to the rural farmer is assistance with farming practice to limit mycotoxin formation during growing, harvest, and storage. Methods such as crop rotation, sun drying of crops, and construction of simple, but effective sealed storage facilities could minimise much of the spoilage that currently occurs.*

Cure in this context means detoxification, which has not been a commercially feasible operation for other mycotoxins to date. The FBs are resistant to breakdown in solution (Visconti *et al.*, 1994) and by heat§ (Alberts *et al.*, 1990; Dupuy *et al.*, 1993) and, therefore, are not eliminated by cooking. Ammoniation has been shown to degrade aflatoxins, and this has been attempted with FB-contaminated maize (Norred *et al.*, 1991b; Voss *et al.*, 1992), but with little success. However, at higher temperatures, Park *et al.* (1992) showed that FB1 was reduced by about 80%, with no mutagenic properties being elicited, and Scott and Lawrence (1994) obtained a similar result with higher pressures or temperatures in moist maize with no ammonia present. Alcohol fermentation is ineffective in removing FB1, with a 3–28% loss in wort spiked with levels up to 0.95 mg/L (Scott *et al.*, 1995).

Because no FB was found in tortilla flour made by treatment with calcium hydroxide (nixtamalization), it was suggested by Sydenham *et al.* (1991) that this process degrades FBs. Rats were fed maize that had been contaminated with FB1 from *F. proliferatum* cultures and then supplemented with nutrients or treated with calcium hydroxide (Hendrich *et al.*, 1993). Animals on the latter diet had much fewer adenomas, indicating that the treatment was effective, which was supported by the finding that almost all the FB1 had been hydrolysed. Curiously, supplementation of the diet with nutrients promoted adenoma formation. Maize treated with

* Dutton, M. F., Robertson, J., Mathews, C. and Beck, B. D. A. (1993) Occurrence of mycotoxins in maize in rural areas of South Africa. In: Proceedings of the United Kingdom Workshop on the Occurrence and Significance of Mycotoxins, London, 21–23 April 1993, pp. 56–63, Skudamore, K. A. (ed.) MAFF, London.

† Dutton, M. F., Robertson, J., Mathews, C. and Beck, B. D. A. (1993) Occurrence of mycotoxins in maize in a rural area in South Africa and methods of prevention of contamination and elimination. In: Proceedings of the ICC International Symposium: Cereal Science and Technology; Impact on a Changing Africa, Pretoria, 9–13 May 1993, pp. 823–835, Taylor, J. R. N., Randall, P. G. and Viljoen, J. H. (ed.) CSIR, Pretoria.

‡ Sydenham, E. W., Shephard, G. S., Gelderblom, W. C. A., Thiel, P. G. and Marasas, W. F. O. (1993) Fumonisins: their implications for human and animal health. In: Proceedings of the United Kingdom Workshop on the Occurrence and Significance of Mycotoxins, London, 21–23 April 1993, pp. 42–48, Skudamore, K. A. (ed.) MAFF, London.

§ Dupuy, J., Le Bars, P., Boudra, H. and Le Bars, J. (1993) Effect of thermal treatment of fumonisin B_1 in maize. In: Proceedings of the United Kingdom Workshop on the Occurrence and Significance of Mycotoxins, London, 21–23 April 1993, pp. 193–195, Skudamore, K. A. (ed.) MAFF, London.

0.1 M calcium hydroxide at room temperature for 24 hr lost the major part of their FB1 content (75% and above) as hydrolysed product (Sydenham *et al.*, 1995a) in a two-step sequential process via a mixture of the monoesters (Sydenham *et al.*, 1995b).

A method of reducing FB contamination is based on the observation that maize screenings generally contain the highest levels of FB (Colvin and Harrison, 1992; Wilson *et al.*, 1992; Murphy *et al.*, 1993). This is a problem in animal feed, but it can be used in flour milling to improve the quality of grain, as increased refinement reduces FB levels.

4. CONCLUSION

Current studies on the FBs cannot be described as preliminary, but are still at an early stage. Consequently, many questions and puzzles still remain, e.g.:

- Considering the polarity and presumable lack of uptake from the GIT and passage through membranes, how are FBs transported to exert their effect?
- The amount taken up from a single p.o. dose is <1%; is the toxin that potent?
- Where does the unaccounted 25% of the dose go in primates?
- Why have not incidences of OC been reported in test animals?
- Why are there considerable differences in the way various species respond to the toxin?
- What effect does FB have on humans, and at what level?

The question of absorption of FB1 from the GIT has not been resolved by p.o. administration of pure toxin, because the uptake is very small in all animal models, and there is no reason to suspect that humans are any different. Several possibilities present themselves: either the toxin is highly potent or its absorption is aided by other dietary factors, such as alcohol or fat. It seems unlikely that, in the digestive tract, the toxin is modified into a more accessible form, e.g., by esterification, or that there are transport systems that assist its passage, either present in an active or latent form. In their experiments with a horse given FB1 by gavage or i.p., Laurent *et al.* (1989a) concluded that other factors assisted absorption from the stomach during digestion.

The presence of other toxic fungal metabolites in naturally contaminated commodities does not help to clarify studies with unpurified materials, and reports from such investigations may be flawed because of synergistic action or unrelated toxic effects. *F. moniliforme* and *F. subglutinans* can produce several potent toxins in addition to FBs, and *Diplodia maydis*, which was found to infect 9.4% of maize seeds in the Transkei (Marasas, 1982), is known to have strains that produce metabolites that are highly toxic towards chickens. These possibilities, and the action of other dietary factors, such as alcohol, may explain the noninducement of OC in model animals.

The question of species response may not be as specific as it appears, because all animal models tested had evidence of liver and kidney damage. Effects such as brain damage and lung oedema may hinge on detoxification and excretion factors peculiar to the animals that exhibit them. Because of the distinct possibility of human intoxication with FBs, the problem of liver cancer in high-incidence areas, such as Southern Africa, should be reopened because they could well play a role alongside aflatoxins and hepatitis virus in the aetiology of this disease, in addition to other observed conditions such as idiopathic congestive cardiopathy (Campbell, 1990). Clearly, further work is needed to elucidate the specific action of the FBs. Diaz and Boermans (1994), for example, suggest that toxicokinetics, as well as toxicodynamics, of these metabolites need to be thoroughly investigated.

Several reviews, e.g., Nelson *et al.* (1993) and Riley *et al.* (1993b), draw attention to the concern that the effect of low levels of FBs in human food is unknown, as are safe levels for animal consumption, and pointing to the fact that humans are ingesting the toxin on a world-wide basis* (Coulombe, 1993). The problem is further compounded by the finding that "healthy" looking maize can be infected with *Fusarium* (Thomas and Buddenhagen, 1980) and have high levels of FBs (Thiel *et al.*, 1992). Infection with *Fusarium* does not affect the outward quality of the grain, whereas other moulds do, e.g., *Diplodia*, constituting "ear rot" (Rheeder *et al.*, 1990b).

Riley *et al.* (1993b) reported that the Mycotoxin Committee of the American Association of Veterinary Laboratory Diagnosticians recommends limits in feed (mg/kg) as: *equidae*, 5; pigs, 10; cattle, 50; and poultry, 50. These figures seem high and possibly are permitted because of the variation in levels of contamination on a day-to-day basis.

A problem is the lack of residual FB in treated animals, which does not allow for assessment of exposure by measuring *in vivo* toxin conjugates, as found with the aflatoxins (Gan *et al.*, 1988). An alternative approach to measure sphinganine and sphingosine levels, which are elevated using established methods (Merrill *et al.*, 1988), however, holds out some hope for exposure studies, but detailed work will be needed to correlate exposure levels with dose and time periods.

As with most mycotoxins, the FBs are regarded as dangerous and a problem, but there is a positive side. It has been suggested that they may serve as templates for the development of anti-atherosclerotic and anticancer drugs (Norred, 1993). They will be used to elucidate the role of sphingolipids in cell regulatory mechanisms (see Hanada *et al.*, 1993; Bose *et al.*, 1995; and an excellent review by Merrill *et al.*, 1993b).

*Marasas, W. F. O., Shephard, G. S., Sydenham, E. W. and Thiel, P. G. (1993) World-wide contamination of maize with fumonisins: foodborne carcinogens produced by *Fusarium moniliforme*. In: Proceedings of the ICC International Symposium: Cereal Science and Technology; Impact on a Changing Africa, Pretoria, 9–13 May 1993, pp. 791–805. Taylor, J. R. N. Randall, P. G. and Viljoen, J. H. (eds.) CSIR, Pretoria.

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Maize Storage and Health Related Problems for the Indigenous Rural Community: Challenges and Advances for South Africa

N. GQALENI, A.A. CHUTURGOON and M.F. DUTTON

Maize is the staple food for the rural African population of South Africa. At present, filamentous fungi (moulds) prevail in stored home-grown maize in rural areas. Some of these have been reported to produce mycotoxins which are health and environmental hazards. For example, the contamination of home-grown maize by mycotoxins, such as fumonisins and aflatoxins, has been shown to be correlated with the high incidence of oesophageal and liver cancer, and other disease conditions in rural communities. Mycotoxins are relatively stable. Thus, processing of maize will not easily destroy them. Ideally, proper drying of maize at harvest followed by dry and cool storage, and not any other treatment, would help inhibit both fungal growth and mycotoxin synthesis. Drying technologies exist which, when coupled with proper education, could contribute tremendously to the health and economy of these communities and also offer prospects for other tropical and sub-tropical countries.

Introduction

In South Africa maize (*Zea mays* L.) is the major part of the staple diet of the indigenous rural community and is used in a variety of ways. It is produced and utilised almost entirely at the village level and extensively used in food fermentations. Because of this it is reasonable to estimate that, following a good harvest, most people in rural areas will consume maize in at least two meals a day.

In the rural areas of South Africa, most of the methods used for maize storage and preservation have been practised for many years. The methods of storage used varies greatly and often depend upon tradition or custom and/or whatever facilities are conveniently at hand as in most other developing countries (FAO, 1985). The methods include structures made of corrugated iron, the roof area of

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Science, Technology & Development, Vol.15, Nos.2&3, August/December 1997, pp192-198
PUBLISHED BY FRANK CASS, LONDON

huts, maize cribs, metal bins, drums, sacks, or burying maize underground (Aidoo, 1993; Dutton *et al.*, 1993; FAO, 1984; Hall, 1970).

Like all food commodities, maize is susceptible to infection with *filamentous fungi* (moulds), some of which have the potential to produce toxic secondary metabolites known as mycotoxin, thus contaminating the grain. There are different ways of preserving rural maize, but traditional sun and field drying followed by storage is still the most commonly practised method. This may present health problems to the communities involved and requires urgent attention.

Fungi (Mould), Mycotoxin Occurring in Stored Rural Maize, and Health Problems

Climatic or geographical factors, insect damage and genetic make up of the plant generally dictate potential fungal contamination of the crop. Field fungi, several of which are toxigenic, normally exist on crops at the pre-harvest stages. Among these *Fusarium* species are notable for their occurrence in maize. In *particular F. moniliforme* is one of the dominant fungi found on maize throughout the world (Marasas, 1995). After harvest, the factor that plays the predominant role in the colonisation of maize by fungi is storage conditions. Among these the temperature and water availability, whether as grain water content or as free moisture surrounding the grain, are major factors that determine fungal growth and mycotoxin production (Gqaleni 1996; Gqaleni *et al.* 1996) resulting in variable levels of mycotoxin in natural samples (Smith *et al.* 1996).

Table 1 shows toxigenic fungi isolated from stored maize samples in different rural areas of South Africa. There are 14 toxigenic *Fusarium* spp. known to coproduce more than one mycotoxin (Frisvad and Samson, 1991). However, *F.*

TABLE 1
PRINCIPAL TOXIGENIC FUNGI DETECTED IN MAIZE SAMPLES FROM RURAL AREAS OF SOUTH AFRICA

Fungal species	*Mycotoxins produced*	*Range/level (mg/kg)*	*Analytical method*
Aspergillus flavus	Aflatoxin B_1 & B_2	50	HPLC
Aspergillus parasiticus	Aflatoxin B & G	100–120	HPLC
Diploidia maydis	'Diploidia toxin'	*	TLC
Fusarium spp.	Dexoynvalenol	>200	HPLC
	Fumonisins	0 –117, 520	HPLC
	T-2 Toxin	200–500	HPLC
	Zearalenone	200–800	TLC
Penicillium spp.	Penicillic acid	*	TLC

Source: Marasas 1995; Dutton *et al.*, 1993; Sydenham *et al.*,1990; Gleiderblom *et al.*,1988; Rabie, 1986; Marasas *et al.*, 1977; Marasas *et al.*, 1979.
* Qualitative results

moniliforme is one of the most prevalent seed-borne fungi associated with maize used predominantly for human consumption in South Africa (Marasas, 1995). This organism is known to produce a variety of mycotoxins such as fusarin C, fumonisins, and moniliformin (Frisvad and Samson, 1991). Of great concern are fumonisins, whose occurrence in mouldy home-grown maize used as the staple diet in the high oesophageal cancer areas of the Eastern Cape, South Africa, is alarmingly high (Rheeder *et al.*, 1992; Sydenham *et al.*, 1990). *F. moniliform* is usually considered a field fungus but can cause problems in storage when the water activity (aw) is high and temperatures low (Lacey and Magan, 1991). The IARC (1993) has *classed F. moniliforme* toxins as Group 2B carcinogens (possibly carcinogenic to humans). The occurrence of zearalenone, an oestrogenic agent, is also of concern because it is thought to be involved in gynaecomastia, 'apparent feminisation', of young black males (Campbell and Dutton, 1991) and swine (Sydenham *et al.*, 1988).

Aspergillus flavus and *A. parasiticus* are known for their ability to produce aflatoxins, the well-known acutely toxic, carcinogenic, and probably immunosuppressive secondary metabolites (IARC, 1993; Stoloff, 1989). Aflatoxin are considered to be ubiquitous (Heathcote and Hibbert, 1978). However, their occurrence in home-grown maize 'appeared to be sporadic' (Dutton *et al.*, 1993) and coincided with drought conditions in South Africa. They have been found to play a contributory, perhaps aggravatory role in the pathogenesis of kwashiorkor in black children exposed to these toxins in Natal (Ramjee *et al.*, 1992).

Maize Storage Conditions and Preservation

Maize requires to be properly dried soon after harvesting. Throughout South Africa the traditional method of drying grains is exposure to sun and wind in the field. Although this method is useful, grains are still susceptible to fungal spoilage and possible mycotoxin production during storage. Many tropical countries have improved grain drying by utilising purpose-built solar driers (Othieno *et al.*, 1981). However, to be effective, their performance requires careful monitoring and control to prevent cracking of grains, fungal growth and mycotoxin production. Improvements on grain drying by the driers include the development and use of low cost solid bentonite-$CaCl^2$ desiccants (Thoruwa *et al.*, 1996; Twidell *et al.*, 1993).

In some maize-growing rural areas of South Africa maize surpluses have been collected and stored by local millers in the last few decades (Dutton *et al.*, 1993). The mill acted as the maize bank and farmers could draw meal against the crop held by the mill, which in turn would take a percentage of the meal as payment. This removed from the farmer the potential risks of grain storage. However, many of the mills have closed due to financial difficulties, resulting in losses to these communities. Therefore, farmers had to fall back on their own methods of storage. In Mpumalanga (KaNgwane), three out of 85 farmers have

built maize cribs out of sticks and planks. After shelling the maize, grains are usually stored in metal drums. Later, the grain may be hand milled or pounded into a meal which is stored in metal bins, drums, trunks, sacks, or plastic buckets.

Control and Possible Elimination Strategies

Drying of maize grains before and during storage is essential to discourage fungal growth and mycotoxin formation and thus improve health of the people. Methods aimed at improving mycotoxin control have been attempted and can be classified as follows:

- plant breeding,
- reduction of fungal spores,
- detoxification, and
- dry storage.

Plant Breeding

Fungal-resistant maize cultivars have been selected and their potential use is currently under evaluation in field trials in the Eastern Cape (Rheeder *et al.*, 1992).

Reduction of Fungal Spores

In Mpumalanga, farmers' co-operatives are well-organised through which hybrid maize seeds dressed with pesticides can be purchased in addition to fertilisers (Dutton *et al.*, 1993). In KwaZulu-Natal, small seed samples may be hot-water treated to reduce the fungal inoculum but will not kill the seed with limited success (Berjak *et al.*, 1992).

Detoxification

Mycotoxins are very stable and, therefore, processing will not easily destroy them without making the food unpalatable. To date most attention has been paid to the management of mycotoxin by segregating fungal contaminated grains and chemical treatment. Fumonisins could be reduced by 95 per cent by treatment of contaminated maize and foods with $Ca(OH)^2$ (Sydenham *et al.* 1990). Campbell (1990) had earlier shown that this treatment did not alter the flavour and consistency of maize porridge. It is not certain whether this would be acceptable to these communities, as it turned the colour of the porridge yellow.

Dry Storage

This is an area that still requires further research in South Africa. Ideally, a solar drier should be able to be used in the subsequent storage of grains. The drier developed by Thoruwa (1996) and tested in Kenya has potential for application in this country.

Cytotoxicity Testing and Diagnosis

A great deal of time and effort has gone into detection and analysis of fungi and mycotoxins in rural maize. Subsequently, the impact of mycotoxin on the health of the South African population is now increasingly recognised. The detrimental effects mycotoxins exert on humans is manifested in a wide variety of clinical symptoms. These depend on the nature and concentration of the toxin, duration of exposure and the health status of the person. At the University of Natal, attention is focusing on establishing bioassays to monitor cancer-promoting or cytotoxic compounds from fungal cultures, utilising a modified MTT assay procedure (Visconti *et al.*, 1991). The procedure is both straightforward and rapid and involves use of tissue and cell culture. However, such an *in vitro* system cannot mimic the complex interactions of cell types in the body, although it is useful to alert to the presence of a wide range of toxins which would then be subjected to chemical analysis. This, coupled with the measurement of aflatoxin B_1-macromolecular conjugates (macromolecules like proteins and DNA), is useful in assessing the exposure of rural black populations and patients attending King Edward VIII hospital in Durban (Dutton *et al.*, 1996).

Conclusion and Recommendations

Mycotoxins are world-wide environmental hazards and will continue to be present in the stored maize of these communities. Research on mycotoxins in rural areas of South Africa has largely been in response to the incidence of various disease conditions observed in several local hospitals, which were suggestive of toxicoses. It is important that any subsequent programme of intervention should, among other things, be aimed at:

- reducing the levels of mycotoxin in the maize of these communities through improved storage, detection, and detoxification (where possible);
- improving their living conditions and nutritional status (supplementing diets with proteins, vitamins and trace elements); and
- disease conditions, including cancer, which need to be diagnosed early.

This would be a costly exercise to fund initially. However, it would improve the quality of lives as well as contributing to savings in the economy over the long term. In addition, proper education of the people about better maize drying and storage could make significant contributions to the management of mycotoxin and health of the rural community. Progress in South Africa would offer good prospects for other tropical and sub-tropical countries.

ACKNOWLEDGEMENTS

J. E. Smith of the Department of Bioscience & Biotechnology, University of Strathclyde, Glasgow, UK, and T. F. N. Thoruwa of the Appropriate Technology Centre, Kenyatta University, Nairobi, Kenya also have substantially participated in preparing this article.

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Mycotoxins and Phycotoxins - Developments in Chemistry, Toxicology and Food Safety, ed by M. Miraglia, H. van Egmond, C. Brera, J. Gilbert
Printed in the U.S.A., 1998

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The Detection and Measurement of Aflatoxin B_1 Adducts in Humans in Kwazulu-Natal, South Africa

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ABSTRACT

Samples of blood serum obtained from patients at two locations in South Africa representing rural and urban populations were examined for the presence of aflatoxin B_1 (AFB_1) both as a free and bound toxin. In addition tissue samples from patients having hepatocellular carcinoma were examined for the presence of bound AFB_1 by means of immunocytochemical techniques using gold probes.

The results show that a substantial proportion of all subjects examined, including controls, were positive for the presence of AFB_1 and, although the number of samples taken was too low for accurate statistical analysis, it is clear that the population of South Africa is exposed to aflatoxin in their diets.

The large number of HCC samples that gave positive labelling with the gold probe (14 from 15) indicates the importance of this toxin in aetiology of liver cancer in South Africa. Observation of label in the endoplasmic recticulum indicates the importance of this organelle in the activation of AFB_1 to its epoxide, whereas the presence of the probe in mitochondria is not as easily explained, unless mitochondrial cytochrome P_{450} activity or AFB_1-protein conjugate trafficking is involved.

1. INTRODUCTION

Aflatoxins are known to be prevalent in Southern Africa and are considered to be related to high levels of liver cancer [hepatocellular carcinoma (HCC)] in areas such as Mozambique and Swaziland (Peers *et al.*, 1976). In Africa south of the Sahara, the incidence of HCC is 15/100 000 per annum (Kew, 1994).

KEY WORDS: aflatoxin B_1, hepatocellular carcinoma, immunocytochemistry, cancer, liver, epoxide, adduct

The situation in KwaZulu-Natal, South Africa, is not very different in that the incidence of HCC is 6/100 000. Most of these patients with HCC present at King Edward VIII Hospital (KEH), Durban.

Possibly the *Fusarium* mycotoxins are more important in the health of rural peoples in Southern Africa, because of the use of maize as a food staple. This is, however, supplemented in many areas with crops such as groundnut and indeed, during periods of drought, deliberate attempts to inter-crop groundnut with maize has been attempted. Consequently the rural population is exposed to a range of mycotoxins, including the aflatoxins (AFs).

Ours (Dutton *et al.*, 1993) and other studies (Sydenham *et al.*, 1990) have indicated the routine presence of aflatoxin, deoxynivalenol, zearalenone and fumonisins in local food commodities. It is, however, only a hypothesis at present that the local populations have chronic disease conditions, such as idiopathic congestive cardiopathy (Campbell, 1990) or oesophageal cancer (Rheeder *et al.*, 1992) due to the ingestion of any particular toxin.

In order to assess the exposure of people to AFs attending KEH, it was decided to investigate the presence of free and conjugated toxins in these patients. An obvious starting point was to look for AFB_1 and its adducts, as the occurrence of this toxin as a protein adduct has been well studied (Wild *et al.*, 1990). Two approaches were used:

- The measurement of free AFB_1 and lysine-AFB_1 adduct in blood and blood albumin.
- The location of bound AFB_1 in tissue, particularly HCC samples, by immunocytochemical (ICC) techniques.

The results of this study are given in this presentation.

2. MATERIALS AND METHODS

2.1 Samples

Most of the samples were obtained from patients attending King Edward VIII Hospital which is located in Durban the principle port city of KwaZulu-Natal (KN). The population of KN, was mainly rural but is now rapidly urbanising with much informal settlement around the city. The principle staple of the inhabitants of these settlements is still maize supplemented with western style food and beverages such as wheat bread and soft drinks. For comparison studies, samples were also obtained from patients attending Themba hospital in KaNgwane, a rural area in the North East of South Africa. The main food staple of these patients is maize.

For ICC studies on HCC, twenty patients were used, 15 had liver cancer, 3 chronic hepatitis and 2 had died of respiratory failure and were used as controls.

Table 1. Free Aflatoxin B1 In Patient Serum Samples

SOURCE	TYPE	NUMBER	POSITIVE	RANGE (pg/ml)
Themba	Control	3	3	80-750
Themba	Paediatric	13	4	400-700
KEH	HCC	3	2	350-400

Table 2. Aflatoxin B1-Lysine Adduct in Serum Albumin*

SOURCE	TYPE	NUMBER	POSITIVE	RANGE (ng/ml)
Themba 1993	Control	3	0	-
Themba 1993	Paediatric	13	4	1.6-4.3
KEH 1993	Control Child	3	1	0.6
KEH 1993	Paediatric	15	5	0.8-3.7
KEH 1994	Control Child	2	2	1.2-3.2
KEH 1994	Paediatric	6	4	0.5-2.3
KEH 1994	Control Adult	1	0	-
KEH 1994	HCC	5	2	0.9-3.0

*Expressed as ng/ml of original serum
Paediatric = Kwashiorkor patients
HCC = Heptocellular carcinoma patients

significant. This result has, however, encouraged further investigations of these patients, which are now underway.

Table 2 shows that conjugated aflatoxin is also present in the blood protein of patients at both Themba and KEH. Surprisingly perhaps, there was a greater incidence of aflatoxin adducts in KEH patients as compared to Themba, who could be considered to be more rural but Themba patients showed higher levels, although this is not statistically significant, because of the limited numbers. Again some of the control children admitted to KEH had toxin adducts in their blood and this raises concern with respect to their diet. This aspect needs to be investigated further to identify the origin of the AFs, which from other studies is not likely to be maize (Rava *et al.*, 1996). In order to improve the statistical quality of our results, another 75 patients are being examined 40 of whom have kwashiorkor. The relationship between kwashiorkor and aflatoxin is still a vexed one but there is interest in these patients because they do tend to have higher levels of aflatoxin in their systems (Ramjee *et al.*, 1992).

Immunocytochemical investigation of liver tissue from HCC patients showed the presence of bound AFB_1 in most parts of the cell, including: the cytoplasm; swollen endoplasmic recticulum (ER), (Figure 1) which also involved cisternae and the ER membrane; mitochondria (Figure 2); ribosomes; and nuclei, which also involved the nucleolus (Figure 3).

The significance of these observation in the aetiology of the disease is difficult to assess but it does support the general view that AFB_1 does have a role in the disease, as all samples but one (14 from 15) showed positive labelling. It is interesting to note in this context that 14 out of the 15 HCC samples gave positive labelling for the surface antigen of hepatitis B virus.

Labelling within the ER was expected, as AFB_1 is converted to its active form, the epoxide, by cytochrome P_{450}, which is found primarily in the ER. The observation of AFB_1 bound in the mitochondrion is harder to explain. Two possibilities are that the mitochondrion itself has cytochrome P_{450} activity or that the AFB_1 is conjugated to nascent mitochondrial protein in the ER, the conjugate then being trafficked to the mitochondrion. These mechanisms are being currently investigated in our laboratory.

In conclusion it has been shown that both urban and rural populations in South Africa are exposed to aflatoxin. It is also highly likely that this aflatoxin has a role in liver carcinogenesis in these populations.

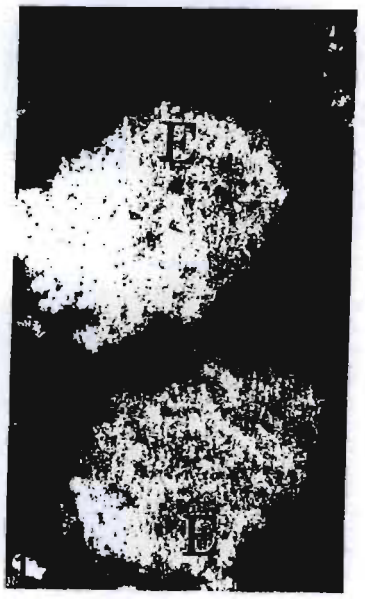

Figure 1: Immunogold labelled aflatoxin B_1 in swollen endoplasmic recticulum (E) of a hepatoma cell (x30000).

Figure 2: Immunogold labelled aflatoxin B_1 in a mitochondrion (M) and in the cytoplasm of a hepatoma cell (x 120000)

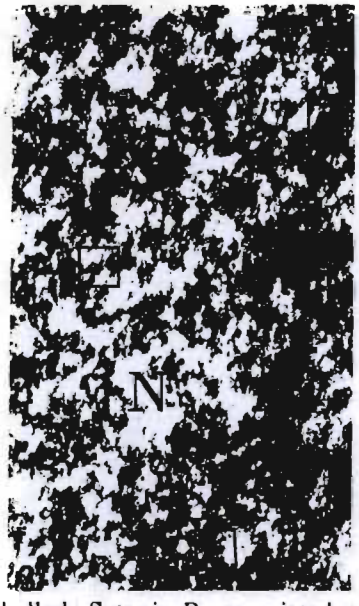

Figure 3: Immunogold labelled aflatoxin B_1 associated with the nucleolus (NL) of a liver cancer cell (x 120000)

4. ACKNOWLEDGEMENTS

Thanks are due to the University of Natal and the MRC South Africa for supporting this work and a bursary to SM.

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Analytical method for the determination of sphinganine and sphingosine in serum as a potential biomarker for fumonisin exposure

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ABSTRACT

The toxins produced by *Fusarium moniliforme,* which include fumonisins, are possible human carcinogens. Fumonisins are inhibitors of *de novo* sphingolipid biosynthesis. Alterations of the ratio of sphinganine (Sa) to sphingosine (So) in urine and serum has been proposed as a possible biomarker of exposure to this toxin. A new method was developed for their analysis in tissues and urine. This work describes the further adaptation of the method to the analysis of Sa and So in serum and its validation in sera of untreated and fumonisin B_1 (FB_1) treated rats and mice. No significant differences in the Sa/So ratios were observed in the FB_1 treated rats. In mice, the increase was only of marginal statistical significance. Determination of Sa/So ratios in human sera could readily be made in small volumes (from 0.3 to 0.5 ml) of serum.

Introduction

Fumonisins are mycotoxins produced by the fungus, *Fusarium moniliforme* and several other *Fusarium* species [1,2]. The mycotoxins were purified from cultured extracts of the *F. moniliforme* [3] and the chemical structures determined by Bezuidenhout *et al.* [4]. Contamination of animal feed and human foods by *Fusarium moniliforme* is widespread, the main substrate being corn (*Zea mays*) in which high levels of fumonisins have been detected [5,6] but also other foods may be contaminated e.g. beans [7]. Fumonisin B_1 (FB_1) induces leukoencephalomalacia in horses [8] and pulmonary oedema in pigs [9]. There is no evidence that fumonisins are genotoxic (for review, see [5]). FB_1 was shown to be hepatocarcinogenic in rats, [10] although most carcinogenicity testing was with *Fusarium moniliforme* cultures known to contain significant amounts of fumonisins. Based on these data an IARC working group concluded that there was sufficient evidence for the carcinogenicity of these cultures in animals [5].

A prominent biological effect of the fumonisins is the disruption of sphingolipid biosynthesis via the inhibition of sphinganine and sphingosine N-acyltransferase [11]. The interruption of sphingolipid biosynthesis was suggested to be an important mechanism through which the fumonisins could disrupt the regulation of cell growth and differentiation, subsequently leading to neoplastic transformation [12]. This effect was demonstrated in ponies [13], pigs [14], chickens [15], rabbits [16], rats [17] catfish [18], monkeys [19] and mink [20]. Various organs are affected including the liver, lung and kidney. The main target in rats is the kidney which is reflected by an altered urinary ratio of sphinganine to sphingosine (Sa/So) with a similar alteration in the

target tissues themselves and in blood [17,21]. A method to analyse sphingolipid bases has been reported [22,23] but involves numerous steps and consequently not ideally suited to analysis of large numbers of samples, as is often required in epidemiological studies. New methods have therefore been developed for the analysis of the Sa/So ratio in tissues as well as human and rat urine [24,25]. Ecological studies showed a positive correlation between dietary fumonisin levels and oesophageal cancer incidence rates in China and Southern Africa (for review see [5]). Epidemiological studies to further evaluate this possible relationship would be facilitated by specific biomarkers to monitor exposure of a population to the fumonisins at an individual level. The altered Sa/So ratio in the urine has been suggested as an appropriate biomarker of exposure to fumonisins. However, one problem encountered with the analysis of human urine was the extremely low level of Sa in males [24,25]. This would necessitate the use of large volumes of urine for exposure assessment in human populations. Shephard *et al.* [19] suggested that measurement of the Sa/So ratio in serum may be a more sensitive biomarker and therefore in the current study the method of Castegnaro *et al.* [24] was adapted to the analysis of the sphingoid bases in serum.

Experimental

Animal treatments

Eight week old male BDIV rats were treated by gavage with a daily dose (5 days a week) of 1 mg/kg body weight FB_1 over a period of 5 weeks. On the last day of the treatment the animals were killed, blood collected, the serum

prepared and frozen immediately in liquid nitrogen followed by storage at - 80 °C until analysis.

Hepatitis B virus (HBV) transgenic [26] and non-transgenic mice (C57 black) were treated by gavage with 16.8 mg FB_1/kg body weight 3 times a week for 63 weeks. These mice are part of a long-term study of the interaction between FB_1 and HBV that will be published elsewhere. The mice were bred in the IARC animal facility as described for previous studies [27]. Control animals from each group of mice received distilled water. The last day of the treatment, the animals were sacrificed and the sera prepared and stored as described above.

Human subjects

One set of 16 blood samples was collected by the "Centre de Transfusion Sanguine, Lyon, France" from healthy donors (10 females and 8 males).

A second set of 17 samples was obtained from South Africa. Four were from patients hospitalised with oesophageal cancer and 13 were healthy controls.

Chemicals

Dr J.D. Miller (Carleton University, Canada) kindly provided fumonisin B_1. C-20 sphinganine (C20 Sa) was a generous gift from Dr A.H. Merrill Jr (Emory University School of Medicine, Atlanta, Georgia). Sphinganine (Sa), sphingosine (So), mercapto-ethanol, *o*-phthaldiadehyde (OPA) and boric acid were purchased from Sigma (Saint-Quentin-Fallavier, France). Ethyl acetate (Merck LiChrosolv), potassium hydroxide, potassium hydrogen phosphate (analytical grade) were from Merck (Darmstadt, Germany). HPLC grade

methanol was from S.D.S. (Peypin, France) and pronase from Calbiochem (La Jolla CA, USA).

Stock solutions of Sa and So for HPLC analyses were prepared in ethanol at a concentration of 1 mM. The working standards were prepared by diluting the stock solution to 10 µM in ethanol. All the solutions were kept at -20 °C in the dark.

Equipment

An HPLC liquid chromatographic system (Perkin Elmer series 4) was coupled to an automatic injector (Spectra system AS 3000, Thermo Separation Products) maintained at 15 °C, a fluorescence detector (Perkin Elmer LS40) and a data computing system (Borwin V1.21). Separation of sphingolipids was on an Ultrabase column (Kromasil) C18, 5 µm (25 cm long x 4.6 mm) maintained at 35 °C.

A shaker with turning movement at a speed of 18 rpm (Amilabo AO 226244, Paris, France) was used for sphingolipid base extraction from the sera.

Determination of sphingosine (So) and sphinganine (Sa) in serum

Extraction procedures:

Ethyl acetate (as used by Castegnaro *et al.* [24]) and a mixture chloroform-methanol (3:1) (as used by Merrill *et al.* [22]) were compared as extraction solvents. In general ethyl acetate gave higher recoveries of Sa and So from the sera. In order to optimise the time of extraction, a series of experiments were performed by extracting the serum for 5, 20, 30 and 60 min with ethyl acetate.

In addition, several techniques were investigated in order to yield the

purest sphingolipid preparation for HPLC analysis. These included deproteinisation with a 10% salicylic acid solution, pronase to digest serum proteins, albumin extraction, and a pre-extraction with hexane. However, none of these approaches improved the quality of the sample preparations for HPLC analysis.

Detailed description of the optimised methodology :

Sphingolipid extraction

1.5 ml of 0.8 % potassium chloride solution, 50 µl 1 M potassium hydroxide and 5 µl of the C20 Sa standard solution were added to 0.5 ml of serum. The mixture was extracted with 4 ml of ethyl acetate by gentle rotation for 20 min and the phases were separated by centrifugation at 1100 x g. for 15 min. The organic phase was evaporated to complete dryness at 55°C under nitrogen.

OPA-derivatisation

The derivatisation mixture consisted of 12.5 mg OPA in 250 µl of ethanol containing 12.5 µl of mercaptoethanol and 3% boric acid solution adjusted to pH 10.5 with potassium hydroxide to obtain a final volume of 12.5 ml. This solution can be stored for a maximum of a week in a refrigerator at 4 °C in the dark. Care has to be taken to protect the mixture from light during derivatisation and to minimise the time kept at room temperature.

Following the ethyl acetate extraction the dried samples were dissolved by vortex shaking in 275 µl of a 0.07 M K_2HPO_4-methanol (1:9) solution and derivatised for at least 30 min by addition of 25 µl of the above OPA mixture.

HPLC analysis of the derivatives

The derivatives were analysed by HPLC with fluorescence detection (excitation wavelength of 340 nm, emission wavelength of 455 nm). The column was kept

in an oven at 35 °C and the flow rate maintained at 1 ml/min using the solvent gradient systems that are summarised in Table 1.

The current method is easy to perform and up to 50 samples can be analysed per week when using an automatic sample injector.

Statistical analyses:

All the data were analysed statistically by the non parametrical Kruskal-Wallis Test in order to determine if the mean values for Sa/So ratios differ significantly between groups.

Results and discussion

Determination of sphingosine (So) and sphinganine (Sa) in serum

Preliminary investigations:

As mentioned in "Experimental" several methods were evaluated for the extraction of Sa and So from serum and the purification of the extracts prior to HPLC. A mixture of chloroform-methanol (3:1) as extraction solvent resulted in lower recoveries of the sphingoid bases as compared to ethyl acetate (data not shown). With ethyl acetate, vigorous shaking led to formation of an emulsion from which it was difficult to separate sphingolipids. However, an alternative mild rotation on a shaker with a speed of 18 rpm resulted in an extraction without formation of an emulsion. Ethyl acetate was therefore selected for optimisation of the extraction.

Sphingoid bases recovery

When using the optimised method described above the recoveries of C20 Sa were generally between 50 and 70 % but with an overall range of 20 to 100% Tables 2, 3 and 4). It was therefore important to determine whether the

variation in C20 Sa recovery was associated with a parallel variability in Sa and So recoveries.

Comparative recoveries of Sa, So and C20 Sa were performed 6 times on the same spiked sample within the same experiment. The corresponding values, presented in Table 5A, indicated that the 3 sphingolipids are recovered to the same extent (96.5 ± 6.7; 90 ± 6.9; 96.5 ± 7.1). In addition, a series of different spiked samples were analysed whose results are presented in Table 5B. It would therefore appear that, although some variability in the recovery occurs between experiments this will have a minor impact on the Sa/So ratio and the Sa and So values which can be corrected by the C20 Sa recovery.

Sphingolipid levels and Sa So ratios in sera from rats, mice and humans

Typical chromatograms of serum extracts from a rat treated with 1 mg FB_1/kg body weight for 5 weeks and a corresponding untreated control is illustrated in Fig 1. No significant differences in Sa, So and the Sa/So ratio were detected between the control animals and the animals sacrificed immediately after the gavage treatments with FB_1 although the small numbers of animals should be noted (Table 2).

Figure 2 shows typical chromatograms of serum extracts from C57 BL and C57BL/-AlbHBV mice after gavage treatment for 63 weeks (16.8 mg FB_1/kg body weight 3 times weekly) and of the respective untreated controls that received distilled water. An increase in Sa/So ratio is observed after treatment with FB_1 when compared to the control values; 0.53 ± 0.24 *versus* 0.37 ± 0.11 although this is only of borderline statistical significance (p=0.09)

(Table 3). No significant changes were observed in the So concentration in the serum while the Sa concentration was slightly increased in the treated mice. The HBV status did not affect the Sa/So ratio.

Figure 3 shows chromatograms from a healthy blood donor and a patient with oesophageal cancer while the Sa, So values and C20 Sa recoveries are presented in Table 4. No significant difference was detected for the Sa/So ratio between the healthy South African control subjects and patients with oesophageal cancer. However the number of cancer patients is small (n=4). The patients are males and the controls female and in addition, data on the duration of hospitalisation prior to blood sampling is unknown. In rats, we have demonstrated that the altered Sa/So ratio returns to normal 2 weeks after cessation of the FB_1 treatment (IARC, unpublished data). One notable result is that the healthy females from South Africa have much higher serum So levels and lower serum Sa/So ratios than healthy female subjects from France; all the South African healthy female donors were of Asian origin. It is also worth noting the large interindividual variation in Sa/So ratios that is of a similar order of magnitude to that seen in human urine samples [24]. Clearly there are many factors, both environmental and genetic, which could influence this ratio in a given individual.

The above method provides a fast and sensitive tool for the analysis of the Sa/So ratio in serum of animals and humans. It will allow the investigation of the health effects of fumonisins in human populations and possibly other toxins that can modify sphingolipid biosynthesis such as AAL toxins produced by *Alternaria alternata* [12]. The method has the advantage of only requiring

small volumes of serum (0.3 to 0.5 ml), but clearly the sensitivity of the method in the case of human exposures needs to be established.

In the present study no increase of the Sa/So ratio has been detected in serum of rats following FB_1 treatment, while an alteration was detected in kidney, urine and liver of the same rats [21]. A recent study by Riley *et al.* [17] indicated that rats administered feed containing 150 µg FB_1/g for 4 weeks had significantly altered Sa/So ratios in the serum while those administered 50 µg FB_1/g had no alteration in ratio. As one rat consumes about 10 g feed/250g b.w./per day [28] the equivalent doses administered by Riley *et al.* [17] were about 6 and 2 mg/kg b.w./day for 4 weeks. Their data are therefore in agreement with our current observations since at a dose of 1 mg/kg b.w. for 5 weeks we did not see any significant changes.

A marginal significant (P=0.09) increase of the Sa/So ratio was detected when comparing FB_1 treated and untreated mice with a treatment dose >15 fold higher than in the rat study and a 12 fold longer treatment period.

While the methodology described above provides a convenient way of assaying changes in the Sa/So ratio, a considerable amount of research has to be performed to validate it as a possible marker of fumonisin exposure in human populations. Field studies in humans will be directed to investigate changes in the Sa/So ratios as a function of fumonisin exposure determined at an individual level. As mentioned above, it is currently unknown how sensitive the Sa/So ratio will be in human populations exposed environmentally to fumonisins.

Acknowledgements

The authors wish to thank Dr J.D. Miller for providing the fumonisin B_1 necessary for this investigation and Dr A.H. Merrill for the gift of C20 Sa. We also thank the "Centre de Transfusion Sanguine de Lyon" and Dr A. Hafferjee and V.S. Naidu (Durban hospital, South Africa) for providing us with randomly collected human blood samples and Mr Christophe Martire and Ms Stéphanie Riouffreyt (Trainees from the "Institut Universitaire Professionnel, Lyon"), for their technical assistance. C.P. Wild acknowledges the support of National Institute of Environmental Health Sciences grant No ES06052 in performing this work; M. Castegnaro, support from the French Ministries of "Affaires Etrangères" and "Education Nationale, Recherche et Technologie"; M. Dutton, Foundation for Research and Development for funding for UN group and (Maastricht University Center for International Cooperation in the Development of Education and; Paul Chelule, Moi University for a bursary.

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Table 1: Programme of gradient elution for HPLC

Time (min)	% solvent A	% solvent B
0	80	20
30	70	30
35	0	100
45	0	100
47	80	20
60	80	20

Solvent A: 0.07 M K_2HPO_4/ methanol (1/9)
Solvent B: Methanol.

Table 2 : Results from the analysis of Sa, So and C20 Sa in serum from FB_1 treated and untreated rats

Rat number	FB_1 treatment	So (ng/ml)	Sa (ng/ml)	C20 Sa recovery (%)	Sa/So
1	no	179.2	144.9	59	0.81
2	no	105.3	69.1	81	0.66
3	no	51.5	25.9	63	0.50
4	yes	104.4	36.9	41	0.35
5	yes	91.6	38.4	78	0.42
6	yes	147.9	61.3	62	0.41

Table 3 : Results from the analysis of Sa, So and C20 Sa in serum from FB_1 treated and untreated mice

Type of mice	FB_1 Treatment	So (ng/ml)	Sa (ng/ml)	C20 Sa recovery (%)	Sa/So
C57 BL	No	45	15	45	0.33
		40	13	50	0.33
		41	14	42	0.34
		10	6	98	0.6
C57BL/-AlbHBV		27	7	47	0.26
		112	51	67	0.46
		143	48	91	0.34
		93	26	97	0.28
C57 BL	Yes	91	75	67	0.82
		52	20	82	0.38
		101	21	100	0.21
		94	53	100	0.56
C57BL/-AlbHBV		16	13	51	0.81
		62	19	100	0.31
		22	16	97	0.73
		66	25	65	0.39

Table 4 : Results from the analysis of Sa, So and C20 Sa in human serum

Individuals	Sex	So (ng/ml)	Sa (ng/ml)	C20 Sa recovery (%)	Sa/So
Healthy controls from France	F	7	4	95	0.57
		13	7	47	0.54
		14	11	59	0.78
		18	7	68	0.39
		13	7	65	0.54
		32	11	37	0.34
		25	7	55	0.28
		22	4	35	0.18
		14	4	24	0.29
		2.5	n.d.	99	0
	M	14	4	58	0.29
		14	3	76	0.21
		18	8	69	0.57
		40	11	70	0.28
		14	4	74	0.29
		41	14	52	0.34
		22	8	76	0.36
		5.6	0.6	92	0.11
Healthy controls from S. A.	F	54	24	54	0.44
		153	29	47	0.19
		63	9	62	0.14
		36	8	67	0.22
		136	24	51	0.18
		54	12	39	0.22
		16	7	62	0.44
		41	7	52	0.17
		116	10	51	0.09
		65	10	34	0.15
		90	16	24	0.18
		160	22	44	0.14
		18	5	53	0.28
Cancer Patients	M	11	4	34	0.36
		22	4	69	0.18
		15	3	58	0.20
		55	9	53	0.16

Table 5: Comparative recovery of Sa, So and C20 Sa in serum samples

Sample number	So recovery (%)	Sa recovery (%)	C20 Sa recovery (%)
A			
1a	89	83	89
1b	96	89	98
1c	106	100	104
1d	104	96	105
1e	88	83	89
1f	96	89	94
Mean ± S.D.	96.5 ± 6.7	90 ± 6.9	96.5 ± 7.1
B			
2	76	76	69
3	67	66	63
4	68	72	70
5	72	77	67
6	46	45	37
7	45	39	45
8	72	77	67

Legends to Figures:

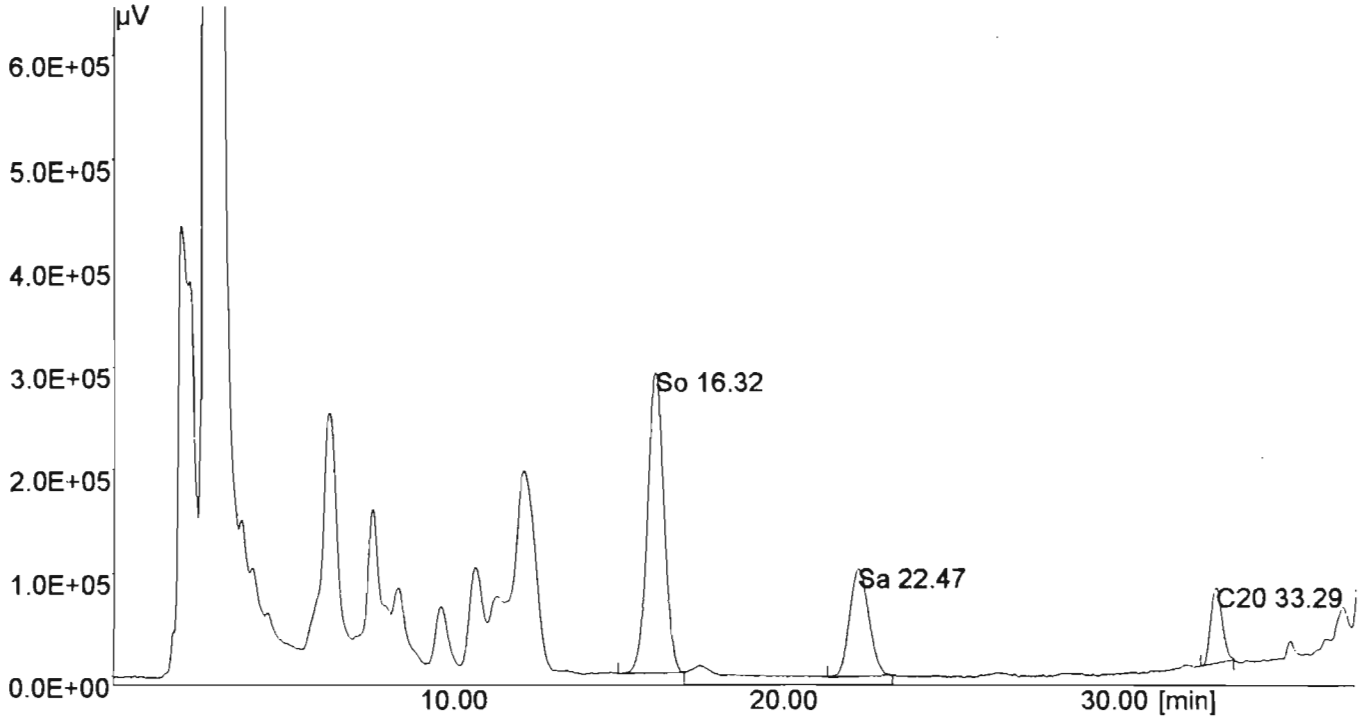
Figure 1: Typical chromatograms of sera from a rat treated for 5 weeks, 5 days a week, with 1 mg/kg body weight of FB_1 (a) and of the corresponding control. (b).

Figure 2: Typical chromatograms of sera from C57BL and C57BL/-AlbHBV mice treated for 68 weeks, 3 days a week, with 16.8 mg/kg body weight of FB_1 (a, c) and of the respective controls that received distilled water (b, d).

Figure 3: Typical HPLC chromatograms obtained from human blood. (a) healthy donor, (b) patient with oesophageal cancer.

Figure 1

(a)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3.0E+05
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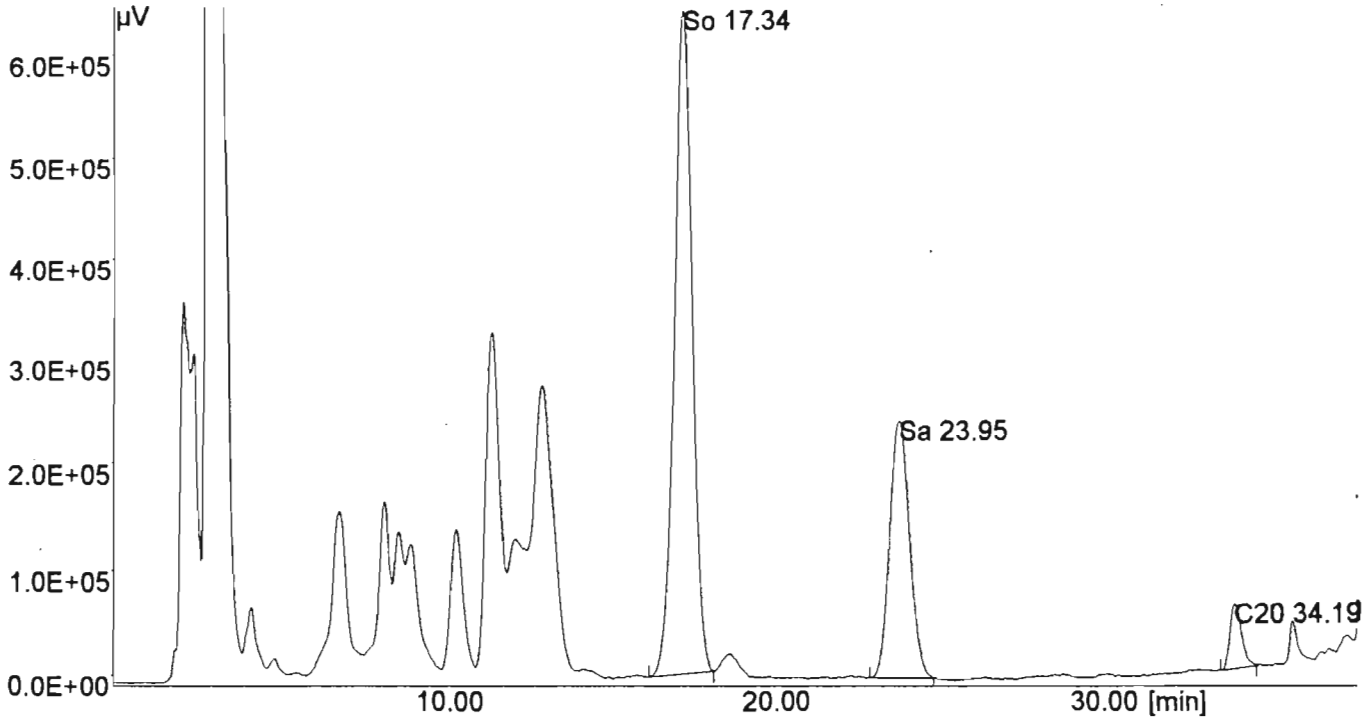
Sa 22.47
C20 33.29
10.00
20.00
30.00 [min]

(b)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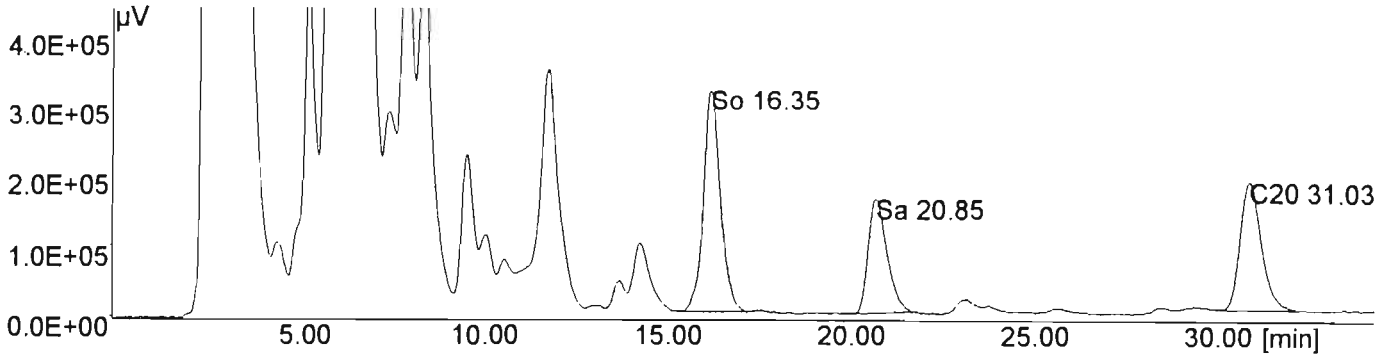
20.00
30.00 [min]

Figure 2

(a)



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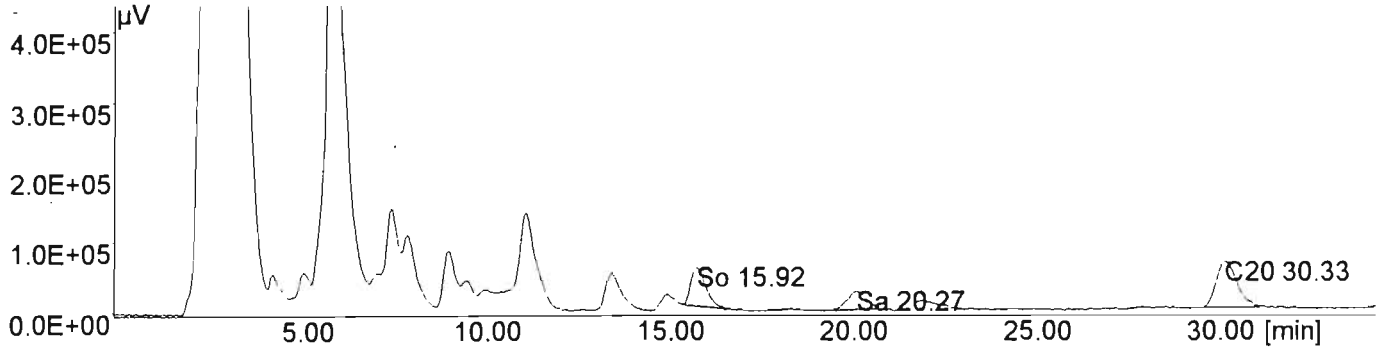
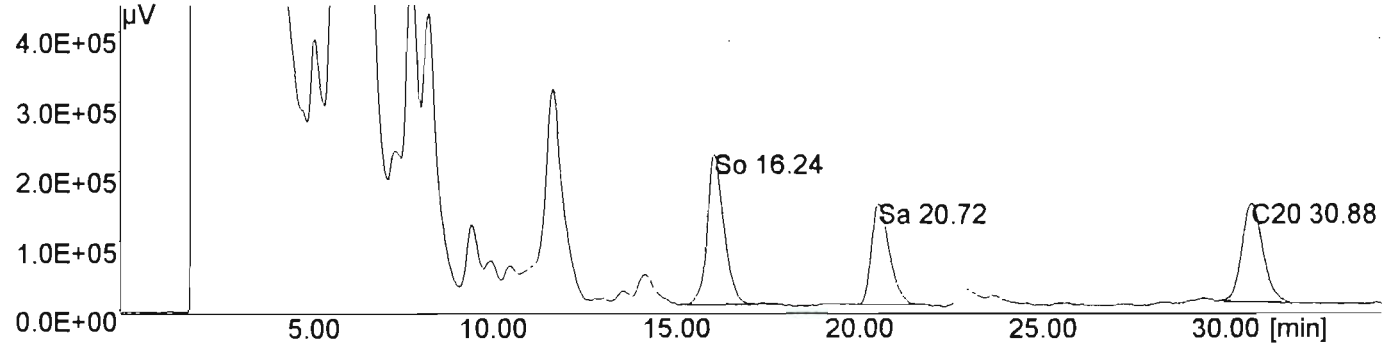


(c)



(d)

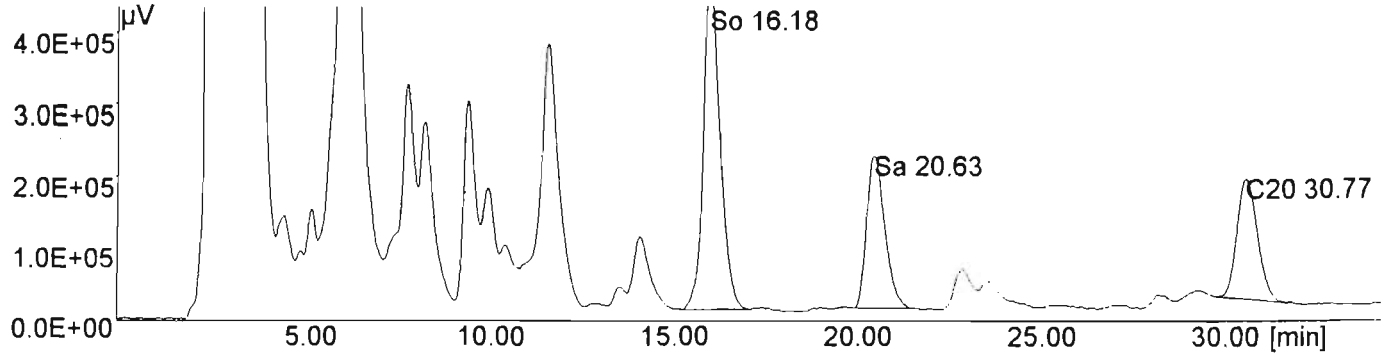
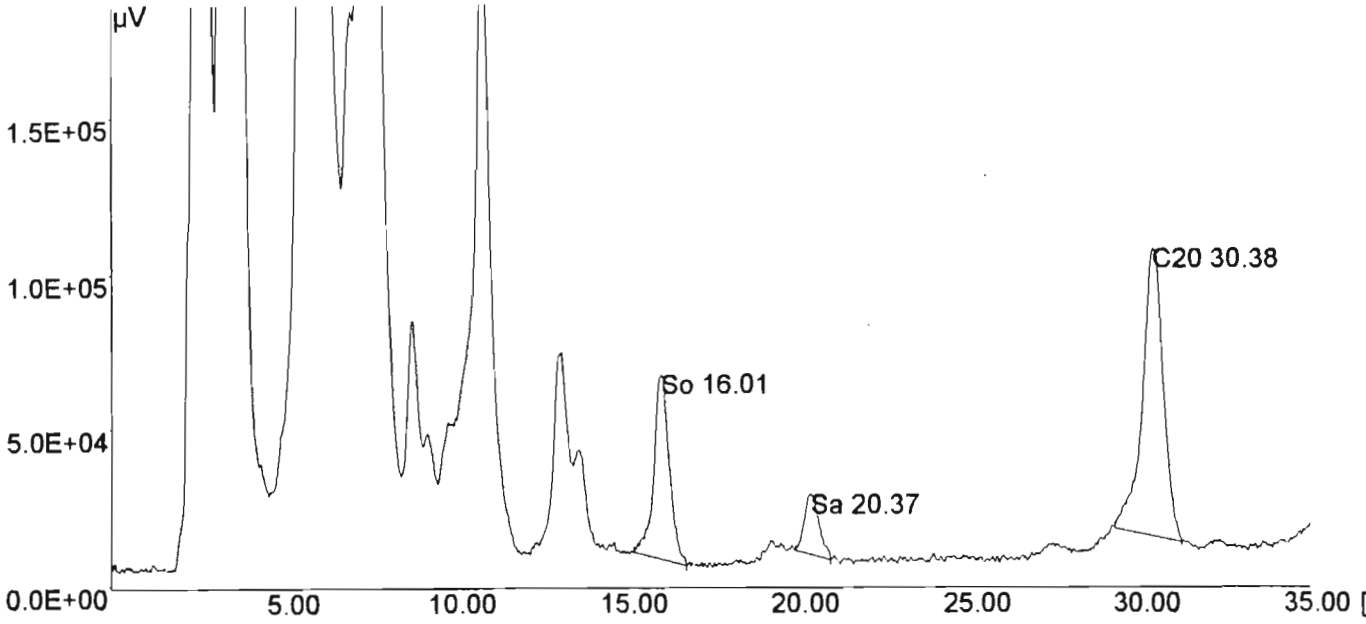


Figure 3

(a)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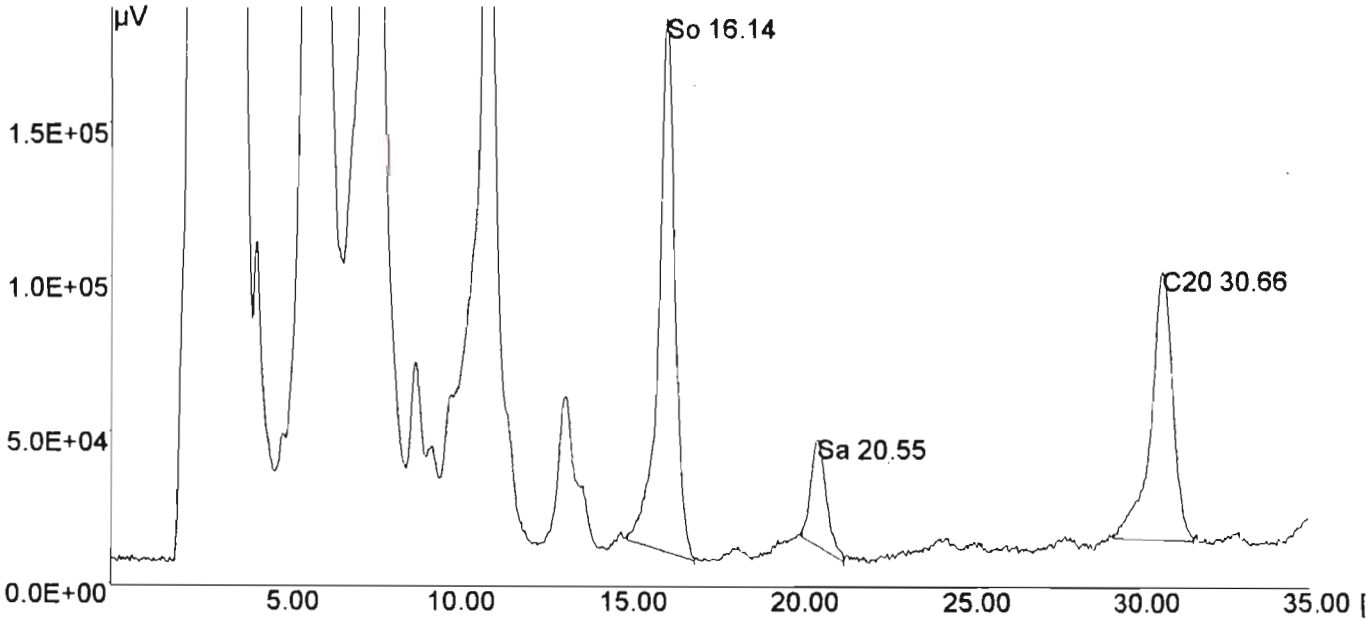
35.00

(b)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15.00
20.00
25.00
30.00
35.00

The determination of fumonisin B_1 in human faeces: a short term marker for assessment of exposure

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Running title: Fumonisin B_1 in human faeces

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Summary

Fumonisin B_1 (FB_1) is a compound that occurs frequently in rural foods and feeds creating health hazards. When ingested, FB_1 does not appear to change in structure and is mostly excreted unchanged in faeces within 24 hours. Twenty human stool samples obtained from rural school children of Vulamehlo, south of Durban (South Africa), were analyzed for FB_1, as well as twenty three samples obtained from various households within the Durban metropolitan area as urban controls. The samples were freeze-dried and ground to a fine powder. A fraction of each sample was extracted thrice with aqueous ethylenediaminetetraacetic acid at pH 5.2. The pooled extracts were purified using reversed phase C_{18} solid phase extraction cartridges. Analytical high performance liquid chromatography was used to quantitate the amount of FB_1 as an o-pthaldialdehyde (OPA) derivative in the extracts. The rural (35%) and the urban samples (9%) showed the presence of FB_1 ranging from 790 – 19560 ng/g of freeze dried stool. It was concluded that this method could be used as a routine biomarker for short term human exposure to FB_1 in contaminated food.

Keywords: Fumonisin B_1, faeces, biomarker, exposure

Introduction

Fumonisin B_1 (FB_1) is one of the related group of mycotoxins elaborated mainly by *Fusarium moniliforme,* which commonly occurs worldwide in maize based foodstuffs and feeds. Other prominent producers include: *F. proliferatum, F. nygamai,* and *F. napiforme.* Some members of the genus *Alternaria* are also known to produce FB_1 (Chen *et al.*, 1992). Fusaria infect maize and occasionally sorghum, millet and other grains grown throughout the world (Marasas *et al.*, 1984a). Several types of fumonisins have been isolated from *F. moniliforme* cultures: Fumonisins A_1, A_2, B_1, B_2, B_3, B_4 and C_1 (Gelderblom *et al,* 1992a; Branham and Plattner, 1993). Fumonisin B_1 is the most toxic and the most abundant representative of the known fumonisins. It has been associated with a number of animal diseases such as equine leukoencephalomalacia (ELEM) (Marasas *et al.*, 1988), porcine pulmonary oedema (PPE) (Harrison *et al.,* 1990) liver and kidney toxicity in rat (Riley *et al*, 1994a; Gelderblom *et al.,* 1996), human oesophageal cancer in South Africa (Myburg, 1998; Rheeder *et al.,* 1992) and China (Chu and Li, 1994) and immunosupression in chickens (Marijanovic *et al.,* 1991). There is no absolute evidence showing that FB_1 is carcinogenic although it has been immunolocalised in human eosophageal tumours (Myburg, 1998). However, it appears to be an initiator and promoter of carcinogenesis (Gelderblom *et al.,* 1992b) and has been classified as a "2B" carcinogen (IARC, 1993). Its major toxic effects are based solely on its disruptive effects on sphingolipid biosynthesis, accumulation of sphingoid bases leading to cell membrane damage, as seen, for example, in kidney cells (Yoo *et al.,* 1992).

The ubiquitous occurrence of FB_1 in maize and its products, sometimes at elevated levels, presents a potential threat to human and animal health and realistic tolerance levels need to be set. Hence, there is need to develop effective methods of

analysing food and to monitor human exposure using a suitable biomarker. The estimation of free FB_1 in blood is impractical due to its poor absorption and rapid excretion from the gut leading to low plasma levels (Prelusky *et al.*, 1994). Furthermore, FB_1 does not seem to readily form conjugates like aflatoxin B_1 (AFB_1) with either DNA or plasma proteins like albumin. Hence methods based on measurement of sphingoid bases, sphinganine (Sa) and sphingosine (So) which accumulate due to the action of the toxin, have been developed (Riley *et al.*, 1994). This approach appears to give some hope of appraising human exposure to FB_1, over a time scale approaching to that given by AFB_1 adducts but a disadvantage seems to be poor and erratic recoveries of the sphingoid bases from physiological fluids and tissues.

As the fumonisins, including FB_1, mainly pass straight through the digestive tract and what is absorbed is mainly excreted in bile (Shephard *et al.*, 1994a), it seems reasonable to look for FB_1 and its degradation products in faeces rather than in blood and urine. The added advantage of this, is that faecal samples are taken as a matter of routine in hospitals and do not require sick patients to undergo further invasive techniques. Fumonisin B_1 analysis in the faeces of non-human primates and other animals has been carried out previously (Shepard *et al.*, 1994; Prelusky *et al.*, 1994; Smith and Thakur, 1996). The results showed that when ingested, less than 1% of the administered dose is absorbed from the gastrointestinal tract. This may explain the high levels of contamination required (>5mg/kg) to produce symptoms of illness (Dutton, 1996). Fumonisin B_1 does not appear to undergo any significant biotransformation in the rat and is mostly excreted unmetabolized within 24 hours mainly in faeces (Shepard *et al.*, 1992c) at the rate of 80% with trace amounts in urine. However there is evidence that at least three decomposition products exist: one fully hydrolysed form (HFB_1), and two partially hydrolysed forms

of FB_1 ($PHFB_1$) (Figure 1) (Jackson *et al.*, 1996), which could be present in faeces. In this study, analysis of FB_1 in human faeces was carried out to monitor human dietary exposure to this toxin with a view of developing a routine short term marker.

Materials and Methods

Study area and population

A total of 43 faecal samples were processed and analysed for FB_1. Twenty of the samples were from Vulamelho, a rural district school south of Durban and 23 other samples were collected from the Durban metropolitan area. The samples were randomly selected from 200 samples which were collected in the ongoing study of the geohelminth infections in Kwazulu Natal. The geohelminth infection study was carried out at the MRC, Durban, South Africa. Samples for this study were selected after they were examined for helminthic infections. The selection of the samples did not take into consideration whether the included samples were positive or negative for the geohelmiths. A laboratory list of numbered samples was used for selection.

Reagents and materials:

All reagents were of analytical grade.

a) *SPE C_{18} cartridges:*10 ml capacity; (Varian Bond-Elut from Analytichem, Harbour City, CA 90710) containing 500mg sorbent.
b) *Mobile phase:* Methanol: 0.1M sodium dihydrogen phosphate (80:20,v/v) adjusted to pH 3.4 with orthophosphoric acid and pumped at a flow rate of 1 ml/min.
c) *o-Phthaldialdehyde (OPA) reagent:* 40 mg of OPA dissolved in 1 ml of methanol and diluted with 5ml of 0.1M sodium tetraborate and mercapthoethanol (50 ul).

d) *Solvents:* Acetonitrile/water (1:1, v/v), butanol, acetic acid and methanol, both obtained from BDH Chemicals, Poole, England.

e) o-*Phosphoric acid* (concentration >85%, obtained from BDH Chemicals, Poole, England).

f) *Anisaldehyde spray:* This was prepared by mixing 70 ml of methanol and 10 ml of acetic acid. Concentrated H_2SO_4 (5 ml) was added followed by 0.5 ml anisaldehyde.

g) *Fumonisin B_1 standard.* (1mg/ml and 50μg/ml) dissolved in acetonitrile/water (1:1v/v). The standard was obtained from PROMEC, Cape Town, South Africa.

h) *Silica 60 aluminium backed TLC plates:* Obtained from Merck, Darmstadt, Germany.

Extraction and cleanup of samples

Twenty faecal samples obtained from school children (aged between 6 and 12 years) of Vulamehlo, a rural district south of Durban (South Africa), were analysed for FB_1. The twenty three adult volunteers from the Durban metropolitan area were aged between 12 and 60 years. Frozen faecal samples were first lyophilized and then ground to a fine powder. A fraction (1.5g) of the sample was extracted thrice by vortexing for 1min in a capped tube with 15ml of 0.1M ethylenediaminetetraacetic acid (pH 5.2). The mixture was centrifuged at 2000g for 10min at 4°C, the supernatant removed and the extraction repeated a further 2 times. The supernatants were combined, acidified to pH 2.9-3.2 with 5M hydrochloric acid and centrifuged at 4000g for 10min. A supernatant aliquot of 10ml was applied to a Bond-Elut C_{18} cartridge previously conditioned with 5ml methanol and 5ml of water. The sorbent was firstly washed with 5ml water, followed by 5ml methanol:water (1:3, v/v) and finally with 3ml of methanol:water (1:1, v/v). Fumonisin B_1 was eluted with 15ml of methanol and the solvent evaporated under a stream of nitrogen at 60°C.

Standard Recoveries on spiked samples

Three stool samples (1.5g) which showed no detectable FB_1 were each spiked with 50µg of FB_1 in 3ml of methanol. They were left to dry overnight at room temperature in a fume cupboard and were then extracted as described above.

Thin Layer Chromatography (TLC)

The dried extracts were dissolved in 200µl of acetonitrile:water (1:1, v/v) and an aliquot of 20µl spotted on silica thin layer chromatography (TLC) plates (10x10 cm). Standards (10µl) of known concentration (5, 10 and 100µg/ml) were also spotted on the plate and developed unidimensionally in butanol:water:acetic acid (12:5:3, v/v); dried and sprayed with anisaldehyde. The plates were heated briefly for 3min at 110°C. Resolution factors (R_fs) were noted and the quantity of FB_1 in the extracts was deduced by comparing the intensity of the purple coloured spots of the samples to those of the known standards.

5.2.5 High Performance Liquid Chromatography (HPLC)

Extracts were further analysed by high performance liquid chromatography (HPLC) system with Spectra SYSTEM P2000 manual injector pump, Nova-Pak 4um C_{18} reversed phase analytical column (150 x 3.9mm i.d., from Waters, Milford, MA, USA) Spectra SYSTEM FL2000 fluorescent detector. Detector excitation and emission wavelengths were set at 335 and 440 nm respectively.

A sample (25µl) or standards (50µg/ml) were pipetted into a tube and 225µl of OPA was added and mixed. An aliquot of 20µl of derivatized sample or standard was injected into the column within 1 min. of adding OPA. The mobile phase,

methanol:sodium dihydrogen phosphate (80:20, v/v), was run isocratically at the rate of 1 ml/min. Fumonisin B_1 was identified by its constant retention time. Quantities were deduced by comparing the peak areas of the standards to those of the samples.

Results

Thin Layer Chromatography

Fumonisin B_1 standards and spiked samples showed a purple visible colour with an R_f of 0.59. Some of the samples had purple spots at R_f 0.63. All the samples had yellow and brown pigments with R_f values of 0.75 and 0.78 respectively. Partial cleanup of FB_1 using C_{18} did not remove most of the impurities making the TLC readings difficult. Some samples which appeared positive on TLC were negative on HPLC. This could be due to anisaldehyde spray which is not specific for fumonisins. Because of these problems, sample analysis by TLC was not regarded as being reliable and was dis-continued.

High Performance Liquid Chromatography

High performance liquid chromatography results in Table 1 show that 7 out of 20 rural samples had varying levels of FB_1 from 6.0-19.56μg /g while the urban samples had only two positive samples (3.5 and 16.2μg /g). The detection limit of the method was 50pbb. The results whilst encouraging from the development of a biomarker point of view, are quite alarming. Thirty five percent of the rural samples were positive, although it might be argued that the highest concentration of about 20.0 μg/g of dry faeces is nothing to cause concern. What this represents in terms of dietary uptake, however, is unknown.

Discussion

This is the first report showing the presence of FB_1 in human faeces. The peak period of excretion is not known but it can be concluded that the amount of FB_1 excreted decreases with time. However, the amount of food taken and the frequency of bowel movements may play a role in transit time (clearance) of FB_1 from the body. It would not be possible therefore, to obtain the same concentration value of FB_1 from the samples collected at different times of the day. The HPLC method used in this investigation was developed by Shephard *et al.,* 1994 with minor modifications. In this procedure, monkey faeces required at least six extractions to obtain approximately full recovery (Shephard *et al.,* 1994) and nine extractions in case of rat and bovine faeces (Shephard *et al.*,1992c and 1995; Smith and Thakur, 1996). While high recovery is desirable, in this case it resulted in long extraction procedures and increased expense in both time and the amount of solvent used. In our study, extraction of spiked faecal samples showed that 74% of FB_1 could be recovered in three extractions (Table 2). These number of extractions were adopted for all the samples. This had the positive effect of minimising the amount of contaminating materials also being extracted. It was also observed that addition of methanol into the extraction solvent (Shephard *et al.,* 1995), did not improve the recovery while it extracted more impurities, making the analytical procedure difficult. Thin layer chromatography was found not to be very useful as a tool for FB_1 analysis in faeces since the extracts had bile pigments which could have reacted with anisaldehyde spray and had the same R_f as that of FB_1. This led to unnecessary confusion.

Attention has to be given to the possibility of the formation of fumonisin degradation products, which is likely due to the acidic nature of the stomach and the microbial activity of the lower gastrointestinal tract (GIT). Little at the moment is known about the gastrointestinal absorption of FB_1. A limited amount of FB_1 is

absorped into the bloodstream in its intact form. However when it loses the tricarballylic acid side chains by hydrolysis, absorption is enhanced (Hopmans *et al.,* 1997) and this may explain the greater toxicity of HFB_1 in comparison to FB_1. One school of thought is that FB_1 is polyanionic and may interfere with its own absorption by binding cations such as sodium, potassium, and and other large molecules which are required for active transport across the intestinal membrane. Polycations have been known to inhibit active transport of sugars and amino acids across the intestinal membrane in rats (Elsenhans *et al.,* 1983).

Although $PHFB_1$ and HFB_1 (FB_1 degradation products, figure 1) have been analysed alongside FB_1, in monitoring FB_1 exposure in animals (Shephard *et al.,* 1994, Smith and Thakur, 1996), their concentration in faeces is relatively small in comparison to that of FB_1 and their standards are not readily available. In addition, different chromatographic conditions from those used for FB_1 analysis in this study are necessary in order to separate $PHFB_1$ from FB_1 since they co-elute (Shephard *et al.,* 1994). These, render the technique unnecessarily lengthy. In this case, analysis of FB_1 alone was found to be satisfactory.

(This to the results section?) The results (Table 1) whilst encouraging from the development of a biomarker point of view, are quite alarming. Thirty five percent of the rural samples were positive, although it might be argued that the highest concentration of about 20.0 µg/g of dry faeces is nothing to cause concern. What this represents in terms of dietary uptake, however, is unknown. Clearly, the rural population have greater exposure as they consume locally produced maize which is often contaminated with FB_1 since maize and its products are not subject to any regulatory restrictions in South Africa at the moment. Considering the losses due to degradation during digestion, recovery, dilution effects and the time interval to when food was consumed, the quantities detected might be equivalent to considerable

amounts of fumonisins in the food. Clearly the children from the rural area, who were not regarded as suffering from any disease, are exposed routinely to FB_1, which is resident in their GIT for considerable periods of time, depending upon personal habits. This situation cannot be conducive to good health in later life.

An even more interesting result was found in the samples from the urban area in Durban. If positive results were to be found, it was predicted that these would be from the rural population since maize is the staple food. Suprisingly, 2 volunteer subjects (9%, Table 1) from the urban area were positive at levels in the higher range of contamination (3.5 and 16.2μg /g respectively). Whether this represents a real situation remains to be seen from further studies. It is possible that the positive volunteers could have travelled from the rural area to the city after ingesting contaminated food. It is usual, however, for urbanised black people to eat maize products, presumably from urban stores, and, therefore, may be complacent about its quality.

In conclusion, it would seem that the measurement of FB_1 in human faeces is the basis for a short term biomarker for exposure to FB_1. Ironically, it may very well become a way of determining the quality of maize being consumed by a particular population. Work currently being carried out in the rural Kwazulu Natal, which involves extensive sampling and comparison of biomarker methods may resolve many of the questions raised in this study.

Acknowledgments

Thanks are due to the FRD Quality Assurance programme for financial support; for MHO/NUFFIC Scholarship by University of Maastricht through Moi University, Kenya, for financial assistance to Paul Chelule; Celia Anderson, Thulani Dube, Annalies Gumede and Thandi Shange from MRC Durban for providing the samples analysed in this study. Technical assistance on HPLC by Arthur Peterson, is greatly acknowledged.

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Table1: Concentration of fumonisin B_1 detected by HPLC in feacal samples of rural schoolchildren from Vulamehlo (V1-V20) and samples collected from the Durban Metropolitan area (T1-T23).

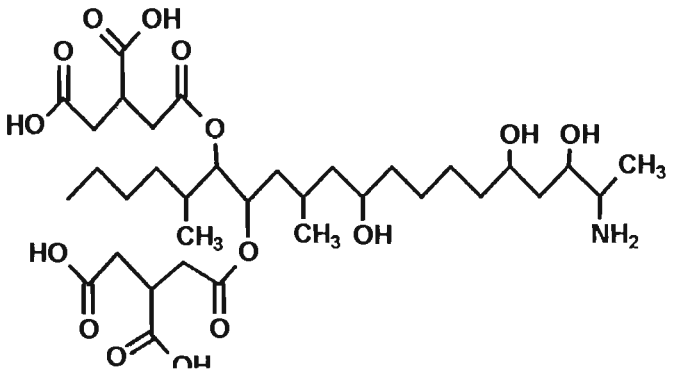
RURAL SAMPLES	[FB_1] µg /g	URBAN SAMPLES	[FB_1] µg /g
V1	ND	T1	ND
V2	ND	T2	16.2(±4.6)
V3	ND	T3	ND
V4	ND	T4	ND
V5	ND	T5	ND
V6	10.0(±4.0)	T6	ND
V7	11.0(±4.7)	T7	ND
V8	ND	T8	ND
V9	ND	T9	ND
V10	0.79(±0.2)	T10	ND
V11	ND	T11	ND
V12	18.4(±5.7)	T12	ND
V13	ND	T13	ND
V14	19.56(±2.8)	T14	ND
V15	ND	T15	ND
V16	14.7(±4.4)	T16	3.5(±0.9)
V17	0.6(±0.1)	T17	ND
V18	ND	T18	ND
V19	ND	T19	ND
V20	ND	T20	ND
		T21	ND
		T22	ND
		T23	ND

ND = Not Detected (below the detection limit of 50ng/g)

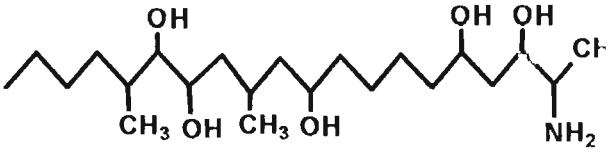
Table 2: Recoveries obtained from spiking stool samples with 50μg of Fumonisin B_1. Analysis was carried out using HPLC.

	Amount recovered (μg)	%Recovery
Extract 1 (3 times)	37.0 (± 2.3)	74
Extract 2 (6 times)	42.5 (± 3.6)	85
Extract 3 (9 times)	45.5 (± 6.2)	91

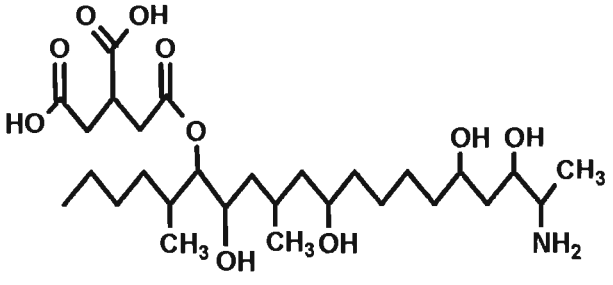
* three different samples

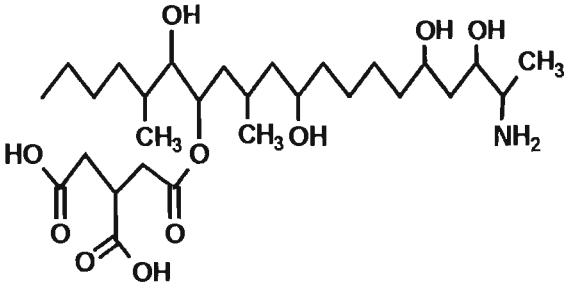


Fumonisin B_1



Hydrolyzed FB_1





Partially Hydrolyzed FB_1

Figure 1. Fumonisin B_1 and its hydrolyzed derivatives

Table 2: Recoveries obtained from spiking stool samples (1.5g) with Fumonisin B_1 (50μg). Analysis was carried out using HPLC.

Sample*	Amount recovered (μg)	%Recovery
Extract 1 (3 times)	37.0 (± 2.3)	74
Extract 2 (6 times)	42.5 (± 3.6)	85
Extract 3 (9 times)	45.5 (± 6.2)	91

* three different samples

Serum fumonisin B_1 levels in Black African women with pre-eclampsia

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Mycotoxin, fumonisin B1, found in sera of eclamptic women

Objective : To analyse sera of women with hypertension in pregnancy for the presence of fumonisin B_1 (FB1) using high- performance liquid chromatography (HPLC).

Design: A cross-sectional blinded analytical study was carried out involving HPLC analysis of sera.

Setting: A large urban referral hospital.

Population: Black African pregnant women with normal pregnancies, and pregnancies complicated by pre-eclampsia (PEC) and eclampsia (EC).

Hypothesis: That pregnant African women with PEC and/or EC would have detectable levels of FB1 in their serum.

Method: An HPLC procedure for quantitating FB1 was optimised using a commercial standard. The toxin was extracted from the serum using liquid -liquid extraction and was purified on Bond-elut SAX columns. Standards and serum samples were derivatised with o-phthaldialdehyde (OPA) and subjected to immediate HPLC analysis with fluorescence detection.

Main Outcome Measures: A significant difference in levels of FB1 in the PEC and the EC women, as compared to normal pregnant women, indicates that the mycotoxin may be a contributory factor to the onset of the disease.

Results: A significantly higher mean concentration of FB1 was found in the EC group ($P< 0.005$) as compared to the PEC and normal groups. The levels of toxin found in the EC group ranged from 0.069 to 4.865ng/ml of serum.

Conclusion The presence of FB1 in EC sera indicates that the toxin may have a role to play in eclampsia for which the aetiology is still unknown.

INTRODUCTION

Pre-eclampsia is a disease involving hypertension during pregnancy. It is associated with proteinuria and oedema usually occurring after the 20th week of gestation[1]. Eclampsia is the severest form of pre-eclampsia resulting in convulsions and may result in death of the patient if appropriate action is not taken. Pre-eclamptic hypertension has been found to complicate approximately 7% of all pregnancies in Kwazulu Natal and approximately eighteen percent of all admissions to the King Edward VIII Hospital Obstetric Unit, have some degree of hypertension (140/90 mmHg and higher when taken on two occasions of 6 hours apart) [1, 2].

Pre-eclampsia is a multi-system disorder, which can affect the maternal liver, kidneys, cardiovascular, central nervous, and clotting systems and can cause impaired placental function[1]. Currently the aetiology of pre-eclampsia is unknown, although various stresses on the patient, both physical and metabolic, have been hypothesised as contributing to the condition.

Maize is the staple food of most South African Black women and is often contaminated with fungal toxins derived mainly from *Fusarium* spp., such as the fumonisins. It was thus hypothesised that there might be an association between the incidence of ingested FB1 and eclampsia, particularly in the light of the high incidence of the disease in Kwazulu Natal and also of fumonisins in the locally grown maize[3]. This association could possibly be reflected in the levels of FB1 in the sera of PEC and EC patients.

Fumonisins are a group of structurally related mycotoxins produced by *Fusarium spp.* and contaminate maize worldwide [4]. Of the six known fumonisins , FB1 (Figure 1), is the most abundant in contaminated feeds and foodstuff and global levels range from 30 to 334 000 ug/kg [5]. Animal models have shown FB1 to be hepatotoxic and hepatocarcinogenic in rats[6], to cause leukoencephalomalacia (ELEM or 'hole in the head 'disease) in horses[7] and pulmonary oedema in pigs[8]. Statistical analysis has associated FB_1in home-grown maize among the rural population of the Transkei, South Africa, with oesophageal cancer[9].

Fumonisin B_1 is the diester of propane -1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydoxicosane, in which the C_{14} and C_{15} hydroxyl groups are esterified with one of the terminal carboxyl groups of the tricarboxylic acid [10]. The structure of the backbone of the fumonisin molecule was found to be similar to the important phospholipid, sphingosine (Fig.1) and animal cell culture experiments have shown that FB_1 are potent inhibitors of sphingolipid biosynthesis [11]. Fumonisin B_1 was found to cause an accumulation of sphinganine and a paralleled increase in DNA synthesis in Swiss 3T3 fibroblasts [12].

Common symptoms in animals appear to be oedema, lesions , haemorrhages and other cellular changes in the liver , kidney , brain , heart , musculosketal system and intestines[13]. Other studies showed that FB_1 affected the foetus in pregnant rats and mice causing low litter weights and poor

foetal bone development, increased morbidity and mortality in a dose response way [14], together with liver damage and increased foetal deaths.

Many sensitive analytical procedures have been developed to quantitate the fumonisins in feeds and foodstuff but preliminary studies using the Association of Official Analytical Chemists (HPLC) procedure for quantitating FB1 in biological material has proved reproducible and sensitive [15] . This method was used as the basis for quantitating FB1 in the sera of PEC patients. A double-blind study was carried out, which included sera of non-PEC (normal or control), PEC and EC patients of the King Edward V111 Hospital. Analysis was done in duplicate.

MATERIALS AND METHODS

Study population and sample collection

The study was conducted at the King Edward VIII Hospital, Durban, South Africa following institutional ethical approval. The sample, which were provided blind, comprised normal pregnant women (16), PEC women (17) and EC (18) women attending the obstetric clinic. The blood samples were collected and treated with EDTA and the resultant sera were stored in cryovials at -20°C.

Extraction and clean up of serum

Serum (2ml) was de-proteinised by centrifuging with methanol (0.4ml) at 1200xg for ten minutes at 20°C. The supernatant was collected and applied to a solid-phase extraction cartridge, *Bond-elut* strong anion exchange (SAX) cartridge from Varian. The cartridge was pre-conditioned with 5ml of methanol (HPLC grade from Merck) and 5ml methanol/water (3:1). The sample supernatant was then applied to the SAX cartridge at a flow rate of 1ml/minute. The cartridge was washed with 5ml of methanol/water (3:1) and 5ml methanol. The sample was eluted with 10ml of 5% acetic acid {Analar grade from Merck) in methanol at a flow rate of 1,0 to 1,5 ml/minute. The eluate was dried under N_2 at 60°C.

Derivatisation and HPLC analysis

Standard FB1 and sample residues were derivatised using o-phythaldialdehyde (OPA) reagent[15], A 50µg/ml FB_1 standard solution was prepared and 50 µl was transferred to an autosampler vial. The purified sample residue was re-dissolved in 200µl of acetonitrile/water (1:1) and 50µl of this

was added to a vial. Both the standard and the samples were each derivatised by adding 450µl of the OPA reagent. The solutions were mixed and subjected to immediate HPLC analysis on a Lichrosorb 5 µm C_8 reverse phase column (25 cm x 4mm) (Merck). Peaks were detected using a Lachrom variable fluorescent detector (Merck) set at 335nm (excitation) and 440 nm (emission). The mobile phase used was HPLC grade (Merck) methanol/0.1 M sodium dihydrogen phosphate (68+32), pH of 3.3 held at a flow rate of 1,2 ml/minute. Quantification of the FB1 content of serum sample, in duplicate, was calculated from the chomatographic peak areas of the treated samples and FB1 standards treated in identical manner.

Recovery and confirmatory Studies

Recovery studies were done where 1ml of control serum, shown to be free of FB1, was spiked with 2 to 8µl of a 50 µg/ml FB_1 standard solution and analysed by HPLC as described above. Selected positive samples were also spiked with FB1 to confirm the identity of the toxin in chromatograms.

Selected serum sample extracts (prepared as above) were applied to immuno-affinity columns (Vicam fumonitests) and washed with emthanol/water. The absorbed FB_1 was eluted with 2ml methanol/acetic acid 1% and the eluate was dried under nitrogen and analysed as previously described.

RESULTS

After determination of the FB1 content in the serum samples was completed, they were organised into 3 groups from patient records, i.e., normal (17) PEC (17) and EC(18). The data for these patients is given in Table 1 and the results of the FB1 analysis, including corrections for recovery, are presented in Table 2 and Figure 2. Figure 3 shows the mean values of FB1 (ng/ml) recovery of FB_1 for the three groups, i.e., control, PEC and EC patients. The FB_1 standard peak occurs at a retention time (RT) of approximately 7,5 minutes. In the EC sera 3 peaks were noted relating to FB_1 and its 2 hydrolysed products. The one at RT 7.04 being FB1 and RTs 8.14 and 9.33 probably the half ester and fully hydrolysed FB1, although these were not investigated further.

Non-parametric statistical tests indicated a significant difference between the means of the normal and the EC groups with p= 0,005. The histogram (Fig. 3) shows the difference in the means of the 3 groups of sera studied.

DISCUSSION

The results from this study are not only remarkable but unexpected in that animal studies have shown the absorbance of FB_1 from the diet is very low (<1.0%)[16] and blood levels return to near zero after 48hours of administration, in the rat. This trend seems to be similar in other species investigated, e.g., monkey[17], where it is rapidly excreted in the faeces together with unabsorbed material. In both monkey and rat the half life of FB1 in blood is of the order of hours, which shows a very short residency in free solution. If humans have a comparable absorption of FB_1 comparable to the rat, then the levels in the food (assumed to be principally maize) that the patient has consumed would have to be in the range of mg/kg (ppm). This does not allow for time between ingestion, presumably the last meal at home, and sampling of the blood, which was done in the hospital. Analysis done on rural and animal feed maize from Kwazulu Natal does indicate the ubiquitous occurrence of FB_1, which is in over 50% of the samples analysed. The highest concentrations measured were 120ppm which compares to that found by Sydenham *et al*[18] of 118ppm in mould maize for beer brewing in the Transkei, S.A., in 1989. Continual ingestion of maize with levels of FB_1 of this order, coupled with mal-nourishment and avitaminosis could account for the levels in serum found.

From the analytical point of view, the appearance of peaks in the chromatographs of serum extracts, subject to the assay method, with the RT of FB_1 is not conclusive that they can be assigned with absolute certainty as the toxin. Support of the identity of the peaks as being FB_1, was obtained in that spiked serum samples, showed that the added FB_1 was coincident in HPL chromatograms. In order to confirm the presence of FB_1 in the samples, some of the extracts were subject to an ELISA assay being developed in this laboratory. Although the ELISA method is not at a stage where quantitation of FB_1 in serum is not precise, the results did broadly agree with those derived by hplc. In addition serum samples were cleaned up using commercially available immunoaffinity columns. The eluted FB_1 fraction from these gave comparable results to those from SAX columns. As the immunoaffinity columns bind FB1 and its congeners specifically, this result serves to confirm the identity of FB_1 in the serum of the subjects investigated.

Recover experiments using spiked samples showed that the extraction of FB_1 from serum is very poor, particularly at lower levels (Table 2/Fig.2). The trend is to be expected, as whatever agent in serum is retaining FB_1, it would become increasingly saturated at higher concentration of the toxin. Considering that FB_1 can be found in members of the Black population in KwaZulu Natal,

it is now imperative that more reliable and effective routine methods of extraction and analysis are developed. Such methods are currently under study in our laboratories, e.g., the ELISA assay.

As the results show a statistical difference between controls, PEC and EC patients, it is tempting to speculate that FB_1 has some role to play in the aetiology of the disease. The finding of higher levels of the toxin in patients with the terminal condition, in itself, is not proof and other possibilities exist, not least that they are purely coincidental, e.g., a poor diet is the contributory factor and FB_1 is a marker of that. A major difficulty is to distinguish between cause and effect. Thus the results might be showing that patients with pre-disposition to eclampsia poorly excrete the toxin and it accumulates and circulates in the blood system. In animals a major route of excretion is via the bile and it is highly likely that malnourished mycotoxin-challenged pregnant women have impaired liver function.

If, however, it is accepted that FB_1 is implicated in the aetiology of the disease, then explanations for two questions are possible. The first one is why are pre-eclampsia and eclampsia so common in the eastern seaboard of South Africa? This is the area where maize is the main food staple and the conditions are right for extensive contamination of the crop by *F. moniliforme* and the production of FB_1 and other fumonisins. The second is that a standard treatment for pre-eclampsia is administration of magnesium sulphate. Fumonisin is known to be polycationic and binds calcium and sodium, which in fact contributes to its poor uptake in the GIT. It is likely that magnesium ions administered after FB_1 uptake, could sequester it in the blood, perhaps rendering it more amenable to excretion and also preventing it from binding to cells and being taken up by its normal sites of action.

On the assumption that FB_1 is a factor in South African eclampsia, then there is a need to speculate about its mode of action. Because the condition is relieved at birth and expulsion of the placenta, it may be speculated that the toxin interferes with sphingolipid biosynthesis, or signalling dependent upon sphingolipid in the placental tissue. Fumonisin is known to cause apoptosis in cells[19], which reflects interference in these mechanisms. Such activity within placental tissue would lead to pathological disturbances within the patient.

TABLE 2: DATA FOR FUMONISIN B_1 ANALYSIS OF PATIENT'S SERA

SERUM SAMPLE	AVERAGE AREA OF FB1 PEAK, (X 10 6)	CONCENTRATION OF FUMONISIN B1, (ng/ml)
1. H 138	4,3642	4,233
2. H 204	0,0054	0,005
3. H 121	0,0716	0,069
4. H 128	0,7389	0,717
5. H 125	0,0784	0,076
6. H 76	0,6240	0,605
7. H 282	0.0	0,0
8. H 126	0,072	0,070
9. H 29 3/6	4,3165	4,187
10. H 209 12/9	0,5077	0,492
11. H 201 10/9	0,2490	0,242
12. H 252 16/10	0.8378	0,813
13. H 225 udd	1,2163	1,180

SERUM SAMPLE	AVERAGE AREA OF FB1 PEAK, (X 10 6)	CONCENTRATION OF FUMONISIN B1, (ng/ml)
14. H 251 pd	0,1580	0,153
15. H 55 23/6	0,5162	0,501
16. H 210 pd	5,0159	4,865
17. H 31 3/6	0,9230	0,895
18. H 179 27/8	0,4932	0,478
19. H 15 24/5	0,4673	0,453
20. H 191 2/9	0,1198	0,116
21. H 214 ppd	0,0588	0,057
22. H 252 ppd	0,2753	0,267
23. H 191 pd	0,4892	0,474
24. H 167 ppd	1,2004	1,164
25. H 201 ppd	0,9566	0,928
26. H 88 16/9	0,5593	0,543
27. H 252 15/10	0,7969	0,773

SERUM SAMPLE	AVERAGE AREA OF FB1 PEAK, (X 10 6)	CONCENTRATION OF FUMONISIN B1, (ng/ml)
28. H 252 udd	1,2575	1,220
29. H 170 8/11	16,1178	15,634
30. H 185 30/8	0,7340	0,712
31. H 186 3/9	2,3763	2,305
32. H 60 29/6	4,3127	4,183
33. H 232	0,0	0,0
34. H 118	0,0340	0,033
35. H 117	0,8441	0,819
36. H 112	0,0	0,0
37. H 106	0,0560	0,054
38. H 105	0,6313	0,612
39. H 102	0,0014	0,001
40. H 101	0,5978	0,580
41. H 100	0,6833	0,663

SERUM SAMPLE	AVERAGE AREA OF FB1 PEAK, (X 10 6)	CONCENTRATION OF FUMONISIN B1, (ng/ml)
42. H 12	0,5300	0,514
43. H 18	0,1833	0,178
44. H 234 4/10	0,0010	0,001
45. H 235	0,0706	0,068
46. H 231	0,4538	0,440
47. H 241	0,3130	0,304
48. H 98	2,1353	2,071
49. H 43	0,0	0,0
50. H 233	0,0	0,0
51. H 237	0,0	0,0
52. H 234	0,0022	0,002

Combine mean values at the bottom of this table

Show in separate column or parenthesis which group patient belong to – get table on 1 page

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A study of the occurrence of fumonisin B_1 in a rural community in KwaZulu Natal, South Africa

By Paul K. Chelule[1], Nceba Gqaleni[1], Anil A. Chuturgoon[1], Jean J. Castegnaro,[2] and Michael F. Dutton[1]

A survey of households in a rural area of KwaZulu Natal, South African (S.A.) was done in order to assess the exposure of the inhabitants to fumonisin B_1 (FB_1), a mycotoxin produced by *Fusarium moniliforme*. This is important in Southern African regions, because of the use of maize as a staple food by the population and its ubiquitous infection by *F. moniliforme*. Furthermore, in other rural areas in S.A., high levels of FB_1 in maize have been associated with oesophageal cancer (OC). Assessment of exposure of the population was done at three levels, namely, the analysis of stored maize, plate ready food and faeces for FB1. Households were located and their position recorded using a Geographical Information System (GIS) for ease and accuracy of follow up. Other data recorded, *inter alia*, was weather patterns, disease incidence and source of water. It was found that of 50 rural households examined, 32% had stored maize with levels of FB_1 ranging from 92-22,225μg/kg and 29% were consuming maize (*phutu*) with FB_1 concentrations from 104 - 364μg/kg. The levels in faeces of FB_1 were 513-39,000μg/kg at an incidence of 33%. These correlates with the incidence in stored maize having a similar magnitude of FB1 concentration but have two magnitudes greater than phutu. As these levels are lower than those in high OC regions (117,520μg/kg,) it is concluded that the risk of OC is lower in the Tugela region than the Transkei and this is borne out by the statistics from the local hospital.

Introduction

Certain mycotoxins have been associated with disease conditions amongst rural populations around the world, e.g., aflatoxin B_1 and liver cancer (*1*). More recently, fumonisin B_1 (FB_1) has been associated with the aetiology of oesophageal cancer (OC) in South Africa (*2*) and this has been supported by the observations on the immunolocalisation of FB_1 in OC tissue (*3*). *Fusarium moniliforme* Sheldon, a producer of FB_1, has been identified, as a major fungus that infects maize especially in the home-grown crop intended for human consumption (*4*). Maize is the staple diet of the South African rural population, which, therefore, increases the their chances of being exposed to fumonisins. Studies carried out in four districts of Transkei, South Africa (S.A.) linked high OC rates in Butterworth and Kentani, to the consumption of maize contaminated with elevated levels of FB_1, 117,250 μg/kg in 1989, as compared to lower levels in the control areas (Bizana and Lusikisiki) of 11,340μg/kg (*5*).

It would be important to know whether other rural populations of S.A., are exposed to FB1 at the same level as in the Transkei, and, in addition, to investigate other parameters, such as disease incidence and climatic variation. This study reported here was, therefore, carried out in the districts of Mphise and Ngcolosi, a rural area near Kranskop, KwaZulu Natal, to determine the level of FB_1 in raw (stored) and cooked food. The analysis of

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[2] International Agency for Research on Cancer, 150 Cours Albert-Thomas, 69372 Cedex 08 Lyon, France

faecal samples was also carried out as a short-term marker for exposure to FB_1 (*6*).

The use of the Geographical Information System (GIS) in health has been applied to epidemiological investigations in S.A., including malaria (*7,8*). In this study GIS was used to locate and plot the rural dwellings for identification and follow-up sampling.

Materials and Methods

All chemicals unless otherwise specified were of Analar Grade. Ethical permission for the study was obtained from the Ethical Committee, University of Natal, Medical Faculty Ethical Committee (Ref: H194/97). All chemicals unless otherwise specified were of Analar Grade.

a) *SPE C_{18} cartridges:* 10ml capacity; (Varian Bond-Elut from Analytichem, Harbour City, CA 90710) containing 500mg sorbent.
b) *Mobile phase:* Methanol: 0.1M sodium dihydrogen phosphate (80:20,v/v) adjusted to pH 3.4 with orthophosphoric acid.
c) *o-Phthaldialdehyde (OPA) reagent:* 40 mg of OPA dissolved in 1 ml of methanol and diluted with 5ml of 0.1M sodium tetraborate and mercapthoethanol (50 µl).
d) *Solvents:* Acetonitrile/water (1:1, v/v), butanol, acetic acid and methanol, both obtained from BDH Chemicals, Poole, England.
e) o-*Phosphoric acid* (concentration >85%, obtained from BDH Chemicals, Poole, England).
f) *Fumonisin B_1 standard.* (1mg/ml and 50µg/ml) dissolved in acetonitrile/water (1:1v/v). The standard was obtained from PROMEC, Cape Town, South Africa.

Collection of samples

Two locations in the Tugela valley (Mphise and Ngcolosi villages) were identified for the study. Recruitment of families for the study, started by random sampling (choosing every fifth of those who volunteered to take part in the study). The recruited volunteers were visited in their households and their consent for sampling was obtained. In order to assess the home situation, family size, health history, food source and storage, a questionnaire was then administered in the local dialect (Zulu) through a trained interpreter. Processed foods (cooked maize, *phutu*, sour porridge, *amahewu* and local brew, *isizulu*) and unprocessed stored cereals, were collected. Faeces were collected the day following that of cooked food collection, from the same households and stored in a refrigerator at Ntunjambili hospital. Analysis of samples was performed at the University of Natal. The study area was mapped out using GIS, and climatic data was obtained from the Weather Bureau, Johannesburg, S.A.

A similar study was carried out within Durban Metropolitan area, as a control group. Black households were visited and the questionnaire survey carried out. The households varied from formal to informal settlements. Food and

faecal samples were also collected and analyzed as in the rural study. It was important to include this control group, because of its urban character and, therefore, people from this region consume a more mixed diet. Where maize is consumed, it is of commercial grade for human consumption, and more likely to be mycotoxin free.

Data analysis

The questionnaire survey data was analysed using Excel (Microsoft Corporation, NY) and SPSS statistical package (Microsoft Corporation, NY). Geographical data were processed and household locations displayed on a map using Mapinfo professional software package (Mapinfo Corporation, Troy, NY)

Analysis of maize Samples

Extraction and Clean-up

Ground maize samples (100g) were mixed thoroughly and a 25g sample was extracted with 100ml methanol/water (3:1 v/v) for 1 hour in a homogenizer as per the method of Sydenham *et al.,* 1992 (*9*). Liquid foods, *isizulu* and *amahewu*, were homogenised and 50 ml of each, was extracted with an equal volume of methanol/water (3:1 v/v). Samples (25g) of cooked food, *phutu*, were extracted using 100ml of 50% aqueous acetonitrile (adjusted to pH 2.7 using 0.1M HCl), as described above (*10*). The aqueous acetonitrile extracts were filtered, and 10ml aliquots were carefully dried by rotary evaporation under low heat (60°C). These samples were then reconstituted in 10 ml of methanol/water (3:1 v/v). The pH was adjusted to 6.5 and cleaned up using strong anion exchange (SAX) solid phase cartridges, previously conditioned by washing with 5ml methanol and 5ml methanol/water (3:1 v/v). The columns was then washed with 5ml of methanol/water 3:1 v/v, 3ml methanol and the FB_1 eluted at the rate of 1ml/min. with 1% acetic acid in methanol (10 ml). The eluates were dried under a stream of nitrogen at 60°C and stored at 4-8°C until further analysis. The methanol-water extracts were filtered, pH adjusted to 6.5 and 10ml aliquots were applied to the SAX cartridges for clean up repeated as described above.

Analysis and Recoveries of FB_1 in maize

Four samples of maize meal (25g) containing no detectable FB_1 in 250ml conical flasks, were throughly mixed with 10 ml of methanol containing 100μg of FB_1 and were left to dry overnight. Two of the spiked samples were used to prepare *phutu,* a common traditional Zulu dish, by adding boiling water (20ml) and a pinch of sodium chloride. The mixture was stirred to an even paste using a glass rod and heated to 93 °C with stirring. The temperature was then reduced to about 70°C and the samples left to simmer for a further 15 minutes. After cooling, the cooked samples together with the un-cooked spiked samples were each extracted with 100ml of 50% aqueous acetonitrile at pH 2.7. The samples were continuously homogenised for 1 hour, filtered, and cleaned up as described previously.

Analysis of Faecal samples

This was done as previously described (*6*). Briefly, faeces were lyophilised, and then ground to a fine powder. A fraction (1.5g) of the sample was extracted thrice by vortexing for 1min in a capped tube with 15ml of 0.1M EDTA (pH 5.2). The mixture was centrifuged at 2000g for 10min at 4°C, the supernatant removed and the extraction repeated a further 2 times. The supernatants were combined, acidified to pH 2.9-3.2 with 5M hydrochloric acid and centrifuged (4000g, 10min). A supernatant aliquot of 10ml was applied to a Bond-Elut C_{18} cartridge previously conditioned with 5ml methanol and 5ml of water. The sorbent was first washed with 5ml water, followed by 5ml methanol:water (1:3, v/v) and finally with 3ml of methanol:water (1:1, v/v). Fumonisin B_1 was eluted with 15ml of methanol and the solvent evaporated under a stream of nitrogen at 60°C.

Quantitation by high performance liquid chromatography

Quantitation of FB_1 was carried by out by HPLC *(9)* using a Spectra Physics SCM400 SYSTEM with a P2000 manual injector pump, Nova-Pak 4µm C_{18} reversed phase analytical column (150 x 3.9mm i.d., from Waters, Milford, MA, USA) and a Spectra SYSTEM FL2000 fluorescent detector. Detector excitation and emission wavelengths were set at 335 and 440 nm respectively. Serial dilutions of FB_1 standard were used to construct a calibration curve from which the concentration of FB_1 was determined (Figure 1). The detection limit of the method was 50 ng/g FB_1. Briefly, a sample (25µl) and FB_1 standard (50µg/ml) were pipetted into a tube and 225µl of OPA was added and mixed. An aliquot of 20µl of derivatized sample or standard was injected into the column within 1 minute of adding OPA. The mobile phase, methanol:sodium dihydrogen phosphate (80:20, v/v), was run isocratically at the rate of 1 ml/min. Fumonisin B_1 was identified by its constant retention time and its quantities deduced by comparing the peak areas of the standards to those of the samples.

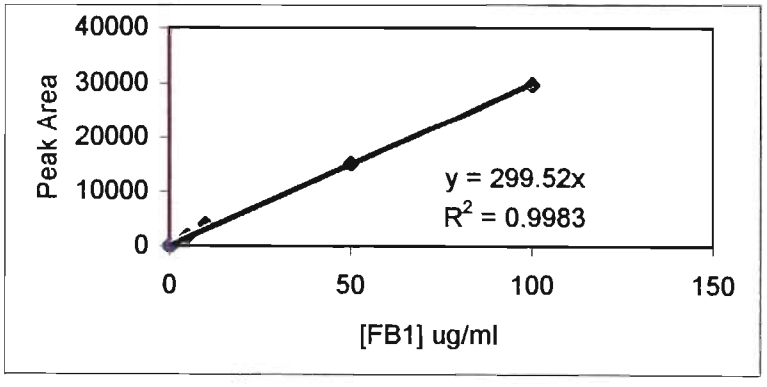

Figure 1: Calibration curve for the determination of FB_1

Results and Discussion

Questionnaires

In the Tugela Valley rural area, a total number of 47 households were initially recruited, seven later withdrew for various reasons from the study. The study was, therefore reduce to 40 dwellings with 50 persons involved. In the urban control area, 46 households were visited involving 50 household members. In the case of the urban group (UG), the various households were pinpointed using GIS and their locations are given in Figure 2. This tool proved highly valuable, as it avoided ambiguity, thus ensuring that repeated visits and follow-up could be easily done even with different workers in the field.

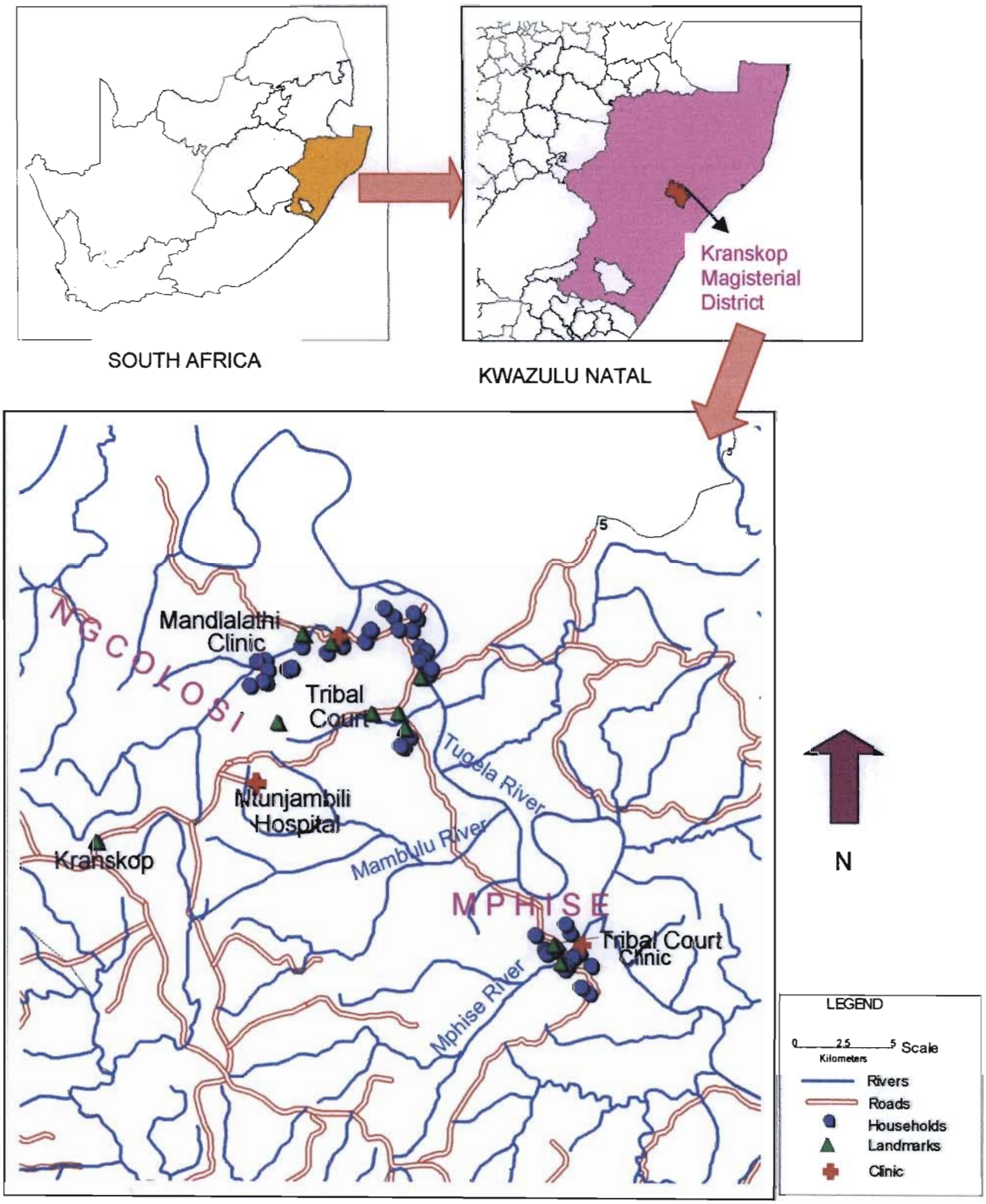

Figure 2: Plot of dwellings in the study area derived from Geographical Information System

The findings from the questionnaire are summarised in Table 1. Although the sample size is relatively small, there are trends that indicate, surprisingly that the consumption of home grown maize in the rural population group (RG) is low. This is the case even when considering households that use both home grown and commercially produced maize. From the results of the questionnaire, the incidence of smoking in the RG is much lower than for the urban population group (UG), which can be explained due to the greater affluence of the latter and access to tobacco products. It is highly likely, however, there is an incidence of smoking non-tobacco products (i.e., cannabis) in the RG, which would not be admitted to, because of legal implications of such practises.

Two statistics that are different, are those of alcohol consumption and source of water. The RG drink as a percentage, a far higher level of home brewed beer, which is a potential source of maize borne mycotoxins, in particular FB_1. The source of water for all uses, including drinking and cooking, is of some concern in the RG, as none of it is from quality controlled sources, whereas in the UG it is (80%). Not only is the likelihood of the RG being exposed to microbial pathogens greater but also dangerous ions, such nitrite, which has been implicated in OC induction (*11*).

Table 1: Summary of the questionnaire answered by householders indicating: demography, smoking status, and source of food and water

Subject/Activity	**Incidence (%)**	
	Rural Study Area	**Urban Reference Area**
Homes	40	46
Participants	50 (100)	50 (100)
Smokers/Non-smokers	18 (36)/32 (64)	47 (94)/3 (6)
Source of Food (Maize)		
Home grown	6 (12)	1 (2)
Shops	35 (70)	40 (80)
Home grown & shops	9 (18)	5 (10)
Source of alcohol		
Home brewed beer	39 (78)	6 (12)
Shop bought beer	4 (8)	16 (32)
Other	7 (14)	28 (56)
Source of water		
Tugela River	47 (94)	5 (10)
Rain (Storage Drum)	1 (2)	1 (2)
Tap	0	40 (80)
Well	2 (4)	0

Data Capture

Climatic Conditions

The rainfall and temperature patterns for the known OC districts in Eastern Cape (Butterworth and Lusikisiki with altitudes above sea level as 534 and 594m respectively) were compared to those of Kranskop, Mandini and Durban (Altitudes 1148 and 109m and 15m respectively, above sea level) (Figure 3). Climatic data from Mandini were used to represent Tugela Valley where the study was carried out for two reasons: there was no weather station within the study area and secondly, Mandini is the nearest station with the same altitude as Tugela Valley villages. Kranskop and Durban had the highest average annual rainfall, than the rest of the districts. There was no observed drastic change in average annual temperature pattern in the five regions (mostly over 20°C). Rainfall patterns were similar except for Durban and Lusikisiki, which had unusually heavy precipitation in the dry months of between May and September. Although Durban, the home of the urban group, had high annual rainfall in comparison with other areas, much of the food consumed in this region comes from the farms further inland, hence the observed weather data may not have any association with the type of food taken. Environmental and climatic factors like rainfall and soil types are among the factors, which play a role in OC endemic areas (*12*). The weather data gathered in this study show that the areas have comparative climatic conditions. However, they may not be used alone to predict the existence of OC in this area. Other environmental factors like soil type and minerals in water for drinking, should be analyzed as well.

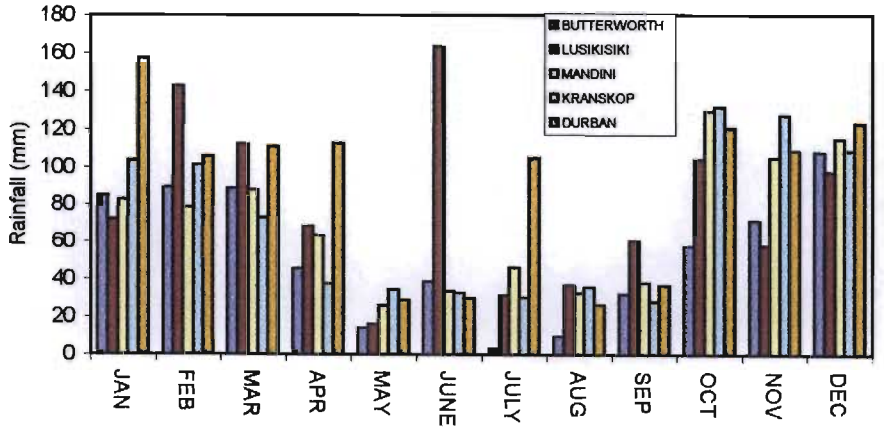

Figure 3a: Average annual rainfall (1993-97), for Butterworth, Lusikisiki, Kranskop, Mandini and Durban.
(Data obtained from South African Weather Bureau, Johannesburg).

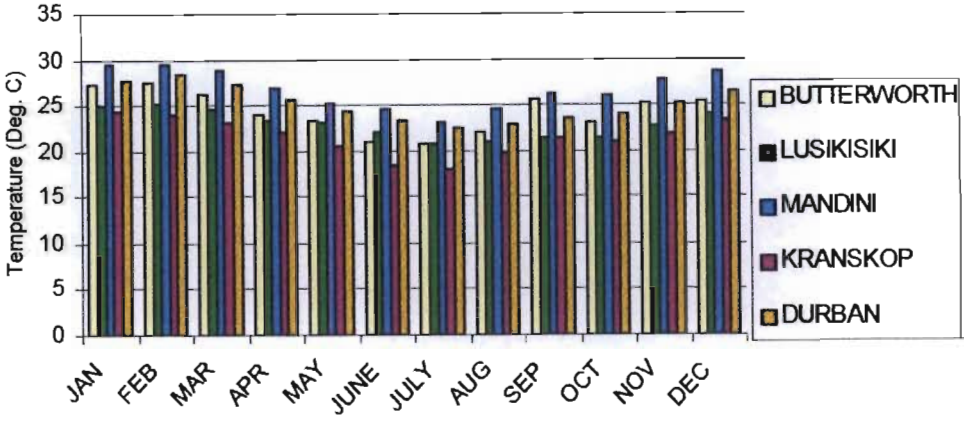

Figure 3b: Average annual temperature (1993-97), for Butterworth, Lusikisiki, Kranskop, Mandini and Durban.
(Data obtained from South African Weather Bureau, Johannesburg).

Clinical Conditions

The recorded incidence of OC in the local hospital serving the communities under study is given in Table 2. This statistic does compares with the national average of 4.8/100,000 females and 10.8/100,000 males respectively (17) and does not indicate the undue high incidence rates observed in the Transkei.

Table 2 Oesophageal cancer cases recorded at Ntunjambili hospital (1995 – September 1998), serving a population of 30000

Year	Male	Female
1995	9	5
1996	5	4
1997	4	4
1998	3	2

Chronic respiratory conditions (tuberculosis and asthma) were the major complaints in the RG (12 out of 50 (24%)) followed by heart related disorders mainly hypertension (6 out of 50 (12%)) (Figure 4). It was difficult to know what the main causes of the non-infectious disorders were but considering the type of dwelling and surrounding, it was likely that airborne particles derived from fungi were involved (*13*). Further investigations need to be carried out to confirm this hypothesis. Volunteers from the UG showed a similar trend of respiratory complaints as the RG for non-infectious conditions which were mostly asthmatic [9 cases out of 50 (18%)]. This is not unexpected, because most of the volunteers were from the informal and poorer housing sector where exposure to indoor fungal spores is high (*13*) and is the subject of another study.

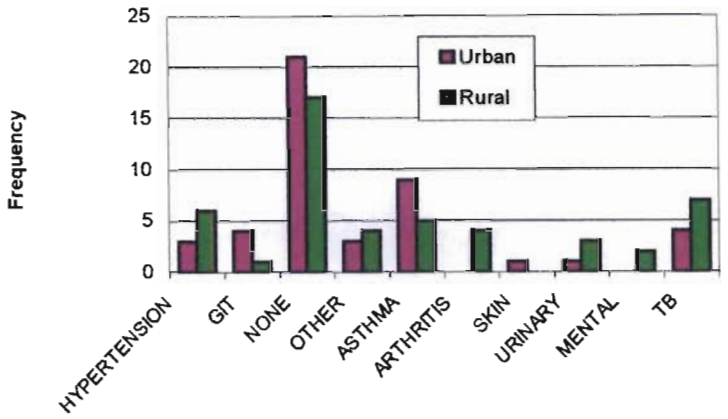

Figure 3 Urban control and rural study groups showing the distribution of the common medical complaints

Storage

The common storage containers in rural homes included: sacks (15, 35%), metal (10, 20%) and plastic drums (25, 50%). The majority of the households stored their food for 7-12 months (52%) (Table 3). This may increase the risk of food contamination by fungi if the storage environment is conducive. However, there was no correlation between the occurrence of FB_1 in stored cereals and type of storage. This may be explained by the fact that *F. moniliforme,* which produces FB_1, is mainly a field fungus. Other fungi could have been present but they were not investigated, as analysis of FB_1 was the main objective of this study.

Table 3. Duration of maize in storage in both rural and urban areas

Storage Period	Region	No.Dwellings*	Percentage
< 1month	Urban	43	93
	Rural	11	27.5
1-6months	Urban	3	6.5
	Rural	11	27.5
7-12 months	Urban	0	0
	Rural	26	65
> 1 year	Urban	0	0
	Rural	2	5

*n = 46 for Urban and 40 for Rural areas

Analysis of food

In order to get a better idea of FB_1 being ingested by the subjects in this study, plate ready food was analysed as well as raw maize. Because preparation and cooking could affect FB_1 levels, an experiment was done with FB_1 spiked maize, which was cooked in a traditional way as *phutu*. It was found that the pH of the material being extracted was critical. Without

modification of this only 36% recovery of FB_1 was made, whereas at pH 2.7 this rose to 89% (Table 4). It is important to adjust this extract as soon as possible to pH to 6.5 to avoid FB_1 hydrolysis and for the subsequent clean up. However, when FB_1 spiked samples were cooked, extraction with unmodified solvent (50% aqueous acetonitrile), gave 35.9%, while the modified extraction solvent (with pH of 2.7) gave a recovery of over 85% FB_1 (Table 4).

Table 4 Results of FB_1 recoveries from 25 g of cooked and uncooked maize based food on extraction with 50% aqueous acetonitrile ("M" for modified solvent pH 2.7 and "U" for unmodified solvent pH.)

Sample	Amount of FB_1 added	FB_1 Recovered*	% Recovery
FB_1 in phutu (M)	100μg	89μg (±3.4)	89
FB_1 in phutu (U)	100μg	36μg(±2.9)	36
FB_1 maize meal	100μg	97μg(±1.3)	97

* Recovery is a mean of two results

The summary of results on food analysis for FB_1 (Table 5) shows that maize from the rural area had the highest number of positives 15 from 47 samples (32%). The urban group had only three positives from 49 samples (6%). This difference in FB_1 distribution is noteworthy, as it shows that the rural populace is at a higher risk of FB_1-associated disorders. Cooked food- *phutu,* showed that 29% of rural samples were positive while there were none from the urban group. This clearly reflects and agrees with the incidence of FB_1 in the maize. As was shown in the recovery experiments, the preparation of *phutu* does not appreciably degrade FB_1. The high end of the range of over 22mg/kg is of some concern but is not as high as that found in the Transkei high OC regions of over 117mg/kg. This may explain the lack of elevated OC levels in the Tugela Valley.

Fermented foods and sorghum were all negative for FB_1 from both population groups. While the *F. moniliforme* mating population A is the main contaminant of maize, sorghum often gets infected by type F mating population, which is the lower producer of fumonisins (*14*). Although a limited amount of FB_1 is degraded during fermentation of contaminated maize (*15,16*) in these fermentations, i.e., *amahewu* and *isizulu*, fermented maize is used as a starter, with sorghum, the main component. This explains non-detectable levels of FB1 in the fermented products. From these results it seems that the rural population should be encouraged to consume traditional fermented products in preference to straight maize derived dishes.

Faecal analysis

Of the forty rural faecal samples analysed, 13 (33%) were positive for FB_1 (Table 5) while for the urban samples, only three samples out of 44 were positive (7%). This result mirrors the incidence of FB_1 in the maize consumed and is important in showing that analysis of faeces is a useful short term biomarker for FB_1 exposure, as the faecal samples were taken 24 hours after

maize consumption. It is tempting to correlate the range of RG intake of FB1 of 92 – 22,225ppb with the level found in stool of 513-39,000ppb but clearly, other factors are also of importance here. These include, personal habits, amount of food consumed, body weight, gut flora and body metabolism. However, it does not seem unreasonable to assume, as a working rule that in the RG, after 24 hours of ingestion, a similar magnitude of FB_1 will be found in the dried stool. A time course study after the ingestion of contaminated maize is required to evaluate the analysis of faeces as a practical marker of FB_1 exposure. In general, however, it is obvious that the RG is routinely exposed to FB_1 from their diet, a conclusion arrived at in a previous study (*6*).

Table 5. Analysis of cereals and faeces for fumonisin B_1 from Rural Group and Urban control group using hplc

Sample	Region	No. Samples	No. FB1 Positives	Range (pbb)	%positive
Maize	Rural	47	15	92-22,225	32
	Urban	49	3	205-476	6
Sorghum	Rural	13	0	-	-
	Urban	-	-	-	-
Amahewu	Rural	14	0	-	-
	Urban	-	-	-	-
Isizulu	Rural	11	0	-	-
	Urban	-	-	-	-
Phutu	Rural	28	8	104-364	29
	Urban	39	0	-	-
Stool	Rural	40	13	513-39,000	33
	Urban	44	3	607-16,200	6

Mean of 3 analyses; ppb = μg/kg; - = not detected, <50μg/kg

Acknowledgments

Thanks are due to the FRD-Franco fund, and CANSA South Africa for finance to carry out this study; the MHO/NUFFIC scholarship which enabled P.K. Chelule to study for his masters degree to the two chiefs (N.C. Cele and N.V. Bhengu for permission to work in the Mphise and Ngcolosi regions respectively. The superintendents of Untunjambili hospital, Dr Ghee (previous), and Dr Moongho (current), the local health workers, especially Mrs Yengwa for the provision of the OC data. Carrin Martin and the staff of the MRC who trained P Chelule in the use of GIS. South African Weather Bureau for the provision of the weather data; Geographical Information Management Systems (GIMS) for the for the roads and rivers data used to construct the map of the study area. Field helpers especially Mr S.E. Mcube. The people themselves.

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CHAPTER 6:

TOXINS FROM INDIGENOUS PLANTS

6.1 PAPERS AND STUDIES

Enzyme-linked immunosorbent assay for the detection of atractyloside in Impila (*Callilepis laureola*).
Paper 29 by Bye, Coetzer and Dutton

The inappropriate use of traditional medicines in South Africa
Paper 32 by Bye and Dutton

The isolation of a storage organelle of atractyloside in *Callilepis laureola*
Paper 33 by Bye, Dehrmann and Dutton

6.2 INTRODUCTION

Like fungi, higher plants produce secondary metabolites, some of which are toxin to animals. As with microorganisms, biologists argue that secondary metabolites confer an environmental advantage to higher plants. This contention is more tenable for higher plants, because secondary metabolites act in these organisms as: phytoalexins, pollinator attractants, i.e., pigments and perfumes, and some plant hormones. The fact that plants produce toxin metabolites could be interpreted that this is to protect them against herbivores. If this so it is not very effective, as only about 16% of all plant species do so and in many cases animals have adapted to overcome these toxicities. Where the toxicity factor seems to score is in areas of environmental pressure, such as deserts, where the number of toxic species increase.

Many plant secondary metabolites have physiological activity, which in many cases are useful to man. Currently plants from all over the world are being screened for useful pharmacological properties. In this drive, use is made of local knowledge and use of traditional medicinal plants, which is the case in South Africa. There is a huge informal trade in South Africa, which is controlled by the *izinyanga* who is the equivalent of the Western's medicine pharmacist, in contrast to the *izangoma* who is more like a doctor. The problems surrounding the trading of plants is beyond the scope of this work, suffice to say that the environment is being stripped of its useful plants. What is of importance though, is that all rural peoples are treated with traditional medicines at some time and this also extends to the urban Black population. Usually treatment with concoctions of various extracts and natural additives is safe, as the traditional healer is skilled in their use. However, this situation is rapidly changing, as unscrupulous people set themselves up as healers and prescribe dangerous potions.

As with all pharmacological preparations the boundary between a "safe" and toxic dose is often slim and, therefore, needs to be carefully controlled. In addition, with

medications prepared from natural sources, other toxic compounds may be present in the mixture, which have no beneficial effects. The results of "muthi" poisonings due to these causes are probably more common than is appreciated, as they are not easily proved by medical doctors, who in any case may never see many of these intoxicated patients. I have visited wards under the control of Dr G D Campbell, onetime co-worker on mycotoxicoses, and seen patients who are apparently suffering from such poisonings and invariably are in hypoglycaemic comas, which usually prove to be terminal. There are also many paediatric cases, because of the common practise of giving young children enemas to cleanse them. This is a particularly dangerous way of dosing, as compounds are readily absorbed from the colon and there is no rejection of the material, as may happen by vomiting. Often such children begin to haemorrhage from every body orifice with fatal results.

6.3 COMMENTARY

In 1987 Ms S Bye approached me with the purpose of registering for PhD under my supervision in the area of forensic science. On the advise of Professor Nel, Department of Forensic Medicine, Medical Faculty, University of Natal, it was decided to do a project on "muthi" poisoning due to his appraisal of the large number of patients presenting with this condition and the inappropriate use of tradition medications (Paper 32). Our choice of subject fell on the toxic principle of "impela" the dried tuber of *Callilepis laureola*, material widely used in Zulu traditional medicine. This was already know to contain the toxin atractyloside, which had been studied by Professor K Pegel and his co-workers, Chemistry Department, University of Natal. The main thrust of the work was to produce an antibody to atractyloside (ATR) which would be incorporated into an Elisa assay. This assay could then be used by physicians where "impela" poisoning was suspected, in order to confirm this or otherwise.

An Elisa was chosen, as the assay had to be specific (ATR would have to detected in serum) and sensitive (serum would have low levels of ATR). After much effort and many disappointments, such as assay was developed based on a polyclonal antibody produced in our laboratory (Paper 29). The difficulties were not simply with the Elisa itself but also in the development of an HPLC method to validate the Elisa itself. One of the major problems was the fact that ATR is high polar having two sulphate ester moieties. Chromatographic analysis was achieved by using the 9-anthryl diazomethane reagent (ADAM) which derivatises the free carboxyl group (PT4) .

Using these assay methods and the antibody as the basis for ICC, it was shown the *C.laureola* tubers contain ATR, which varies seasonally, as one might expect with a secondary metabolite. It was hoped that this information might be used to minimise the toxic effect of preparations contain *C.laureola* tubers but this unfortunately is not possible, as the tuber has to be harvested at certain times, determined by traditional usage. In order to find where the ATR was stored in the tuber another project was conducted whereby vacuoles from tuber cells were isolated and found to contain the bulk of the toxin (Paper 33). This is not unexpected as the vacuole is invariably the storage organelle of water soluble secondary metabolites, which also protects the producing plant from damage, as ATR is a specific inhibitor of the ATP/ADP antiport system in mitochondria.

Currently work is being done on medicinal plants by Mrs B Brookes to isolate metabolites with physiological activity, primarily in the area of uterine contraction. Several plants are used for such purposes and already 5 compound from three plant species with the required property (tested against guinea pig uterus in my laboratory) have been isolated and characterised (PT8 in preparation). In this case the compounds are tested for cytotoxicity using human cell lines, as described in Chapter 3.

Toxicon Vol. 28, No. 8, pp. 997–1000, 1990.
Printed in Great Britain.

0041-0101/90 $3.00 + .00

AN ENZYME IMMUNOASSAY FOR ATRACTYLOSIDE, THE NEPHROTOXIN OF *CALLILEPIS LAUREOLA* (IMPILA)

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(*Accepted for publication* 6 *March* 1990)

S. N. BYE, T. H. T. COETZER and M. F. DUTTON. An enzyme immunoassay for atractyloside, the nephrotoxin of *Callilepis laureola* (Impila). *Toxicon* **28,** 997–1000, 1990.—Tubers of *Callilepis laureola*, a traditional remedy, contain an inhibitor of oxidative phosphorylation; atractyloside. A "competitive" ELISA was developed, using the antiserum produced to an atractyloside–protein conjugate. An ovalbumin–atractyloside conjugate was adsorbed to microtitre wells and plates incubated with sample (atractyloside or tuber extract) and antiserum. After successive incubation with secondary antibody–enzyme conjugate and substrate, the absorbance was read at 405 nm. Antibody working dilution was low, but results, confirmed by thin layer chromatography, indicate the immunoassay has diagnostic potential.

THE ZULUS and other African people regard Impila, the rootstock of *Callilepis laureola* as a powerful medicament. Infusions of the tuber, administered orally or rectally, are reputed to ward off evil spirits (WAINWRIGHT *et al.*, 1977). The tuber is, however, highly toxic (BRYANT, 1909) inducing both liver and renal necroses, with often fatal results (BHOOLA, 1983, Thesis, University of Natal). The hepatotoxin is unknown, but atractyloside (Fig. 1) has been identified as the nephrotoxin (CANDY *et al.*, 1977). This diterpene glycoside competitively inhibits the transport of ADP across the inner mitochondrial membrane, so terminating oxidative phosphorylation (SANTI, 1964). Atractyloside has been isolated from the rhizomes of the Mediterranean thistle, *Atractylis gummifera* (SANTI and LUCIANI, 1978) and coffee beans (RICHTER and SPITELLER, 1978). Consequently, this glycoside has been implicated as a mediator of pancreatic cancer in coffee consumers (PEGEL, 1981). Atractyloside has been detected in plant extracts, using thin layer chromatography (BROOKES, 1979, Thesis, University of Natal) but diagnosis of atractyloside poisoning has been restricted to histopathological means, with inconclusive results. The aim of this study was to develop an enzyme immunoassay specific for atractyloside. The specificity of this assay makes it suitable for diagnostic purposes and may assist in confirming atractyloside-mediated deaths.

Atractyloside (potassium salt; Sigma, St Louis, MO) was coupled to free amino groups on bovine serum albumin (BSA; Boehringer Mannheim, Johannesburg, RSA) and ovalbumin (Grade III; Sigma, St Louis, MO) respectively, by a mixed anhydride reaction,

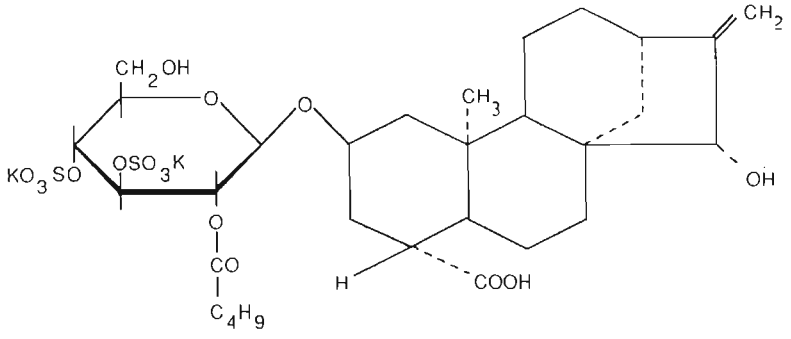

FIG. 1. THE STRUCTURE OF ATRACTYLOSIDE.

using the procedure of FUCHS and FUCHS (1969). Atractyloside (125 mg) was dissolved in dioxane (1.5 ml) and N,N'-dicyclohexylcarbodiimide (30 mg) added. The solution was stirred for 30 min at room temperature and centrifuged. The supernatant contains the acid anhydride and this was added dropwise to an ice-cold solution of protein (100 mg) in 0.1 M borate buffer, pH 8.5 (7 ml) and stirred overnight at 4°C. The conjugate was purified by dialysis for 16 hr against 0.1 M borate, pH 8.0 and 5 days against daily changes of distilled water (WEILER and WEICZOREK, 1981). The non-diffusible material was lyophilized.

Rabbits were immunized s.c. (1.0 ml) with a stable emulsion of atractyloside-BSA conjugate (2 mg/ml) triturated with equal volumes of physiological saline and Freund's adjuvant (Difco, MI). Boosters were administered a week later, twice fortnightly and thereafter monthly. Blood was collected from the marginal ear vein and immunoglobulin fractions were prepared by the method of POLSON (1977).

The "competitive" immunoassay was carried out with procedures modified from those of ROBINS (1986) and WEILER (1986). An atractyloside–ovalbumin conjugate (0.01 mg/ml) in 0.05 M carbonate buffer, pH 9.6, was adsorbed to NUNC microtitre wells (Maxisorp; Weil, Johannesburg, R.S.A.) for 16 hr at 37°C. Between all subsequent steps, plates were washed thrice with tap water (WEILER, 1986). Pure atractyloside, in incubation buffer (0.02 M Tris buffered saline, pH 7.4 containing 0.1% Difco-Bacto gelatin; w/v, 100 μl/well) and an equal volume of rabbit antiserum, diluted 1:10 in incubation buffer, were added to each well. Plates were incubated for 1 hr at 37°C, washed and incubated with a 1:3000 dilution of the goat anti-(rabbit IgG) IgG-alkaline phosphatase (EC 3.1.3.1) (Sigma, St Louis, MO) prepared in incubation buffer (200 μl/well) for 1 hr at 37°C. Freshly prepared enzyme substrate (1 mg/ml *p*-nitrophenyl phosphate; Boehringer Mannheim, Johannesburg, R.S.A.; in 0.1 M glycine buffer, pH 10.4, containing 0.001 M $MgCl_2$ and $ZnCl_2$) was added (200 μl/well) and the plates incubated for 1 hr at 37°C. The reaction was stopped by addition of 6 M KOH (50 μl/well) and the absorbance read at 405 nm, using a Biotek spectrophotometer.

Working dilution of primary antibody was notably low, but this phenomenon has been noted with toxic haptens. HUNTER *et al.* (1985) attribute the low working dilution of antiserum to T_2-toxin, to be the result of immunotoxicity. Using a Marbrook Chamber they demonstrated that T_2-toxin was released from a freshly dialysed T_2-toxin-BSA conjugate and inhibited protein biosynthesis in tissue cultures. Although atractyloside has a different inhibitory action, a similar situation may exist and is an area for future consideration.

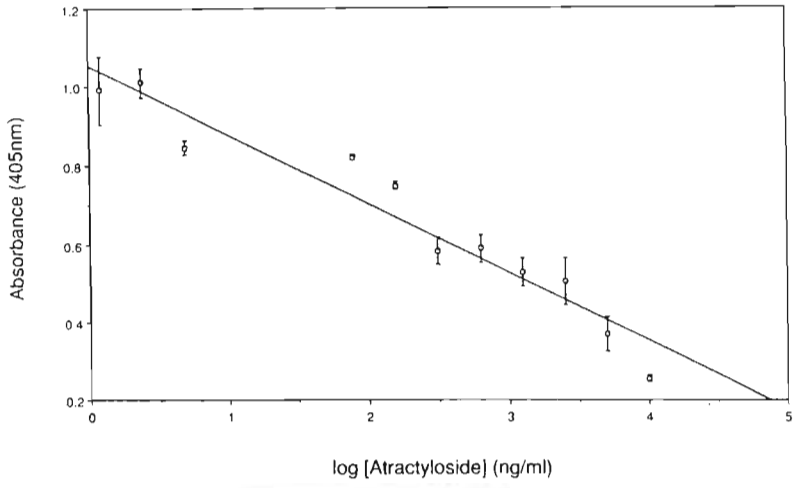

FIG. 2. STANDARD CURVE FOR THE DETERMINATION OF ATRACTYLOSIDE BY "COMPETITIVE" ELISA. Plates were coated with 0.01 mg/ml ovalbumin–atractyloside conjugate. To this was added the primary antibody and atractyloside. The correlation coefficient (r) was 0.97. Results represent the mean of three determinations on a single ELISA microtitre plate. Error bars represent the standard error. The coefficients of variations ranged from 3.1–13.4%. Toxin concentrations on the X axis are in log scale.

Throughout the procedure, suitable controls were included. Each step in the immunoassay was successively omitted, to determine non-specific binding and presence of endogenous phosphatase activity which could contribute to the final absorbance reading. In addition, the adsorbed conjugate was substituted with ovalbumin to determine non-specific binding by both primary and secondary antibodies. In all instances, readings were negligible, confirming the specificity of the assay. Antiserum was replaced with both pre-immune and inappropriate antiserum, but evidence of cross-reactivity was not apparent. The possible presence of coupling agent-modified residue antibodies was investigated (BRIAND *et al.*, 1985). Ovalbumin and conjugation reagents, excluding the hapten, were reacted and adsorbed onto microtitre plates. The immunoassay was carried out and absence of colour formation on addition of substrate, excluded the presence of coupling agent-modified residue antibodies. Furthermore, as atractyloside contains an ent-kaurene backbone (kaurenoic acid being a precursor to the gibberellin plant growth hormones) gibberellin GA_3 was tested, but cross-reactivity was negligible. From the results obtained it was concluded that the antiserum raised against atractyloside was specific for this compound. The results of the standard enzyme immunoassay are given in Fig. 2. The reactivity of the antibody examined by the immunoassay correlated with concentrations of atractyloside ranging from 10–10,000 ng/well. The assay was repeated on crude methanol extracts of the tuber. Atractyloside or related compounds were detected in the methanol extract and results were confirmed by thin layer chromatography. The presence of methanol did not appear to interfere with the assay. No attempt was made to quantify toxin levels in the samples. It has been suggested that the quantitative reporting of immunoassay results is inappropriate unless the assay has been verified by another method (BASELT, 1989). Investigations are underway to develop an alternative assay for atractyloside and to optimize detection of atractyloside in body fluids.

To our knowledge, this is the first report of the production of antibodies to atractyloside and although the immunoassay requires further development, the assay shows poten-

tial for diagnostic purposes and immunocytochemical investigations. The latter should reveal sites of storage of toxin in the tuber and the pathophysiology of the glycoside in humans.

Acknowledgements—The authors thank Mr R. K. BERRY, Mr L. JACKSON and STEVE WOZNIAK for their advice and assistance.

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Journal of Ethnopharmacology, 34 (1991) 253—259
Elsevier Scientific Publishers Ireland Ltd.

The inappropriate use of traditional medicines in South Africa

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(Accepted May 29, 1991)

Traditional medicines are culturally very important to the Zulu population of Southern Africa. The inappropriate use of herbs has however, resulted in numerous fatalities, invariably in children. This paper briefly summarises the belief of the Zulu population in traditional remedies, together with an outline of the problems at present being experienced in South Africa. A further note on the personal impressions and experiences of the authors and description of one such toxic herbal remedy, including use, toxic action and research carried out to date, are also given.

Key words: herbalism; Zulu medicine; atractyloside, nephrotoxin.

Introduction

Western ideology has largely underestimated the inordinate belief in traditional medicines by many people, particularly those from developing countries. The reliance of the Zulu population on traditional remedies is no exception (Wainwright et al., 1977; Ellis, 1986).

Geographically, the Zulus are concentrated in the north-east of South Africa, namely the Natal/KwaZulu region. Culturally, the Zulus believe disease to be a manifestation of disharmony between a person and the ancestors (Bryant, 1909). Sorcery and medicine are hence intrinsically linked and it is the function of the traditional healer, regarded as the protector of society, to act as diagnostician, apothecary and diviner (Krige, 1981).

The *Isangoma*, or diviner, is the diagnostician and augur of society and consults with the spirits to identify the source of an affliction. The herbalist, or *Inyanga*, claims no affiliation to the spirits, however, but functions as an apothecary, preparing and dispensing various herbal remedies (Kiernan, 1978). Conceivably, the link between magic and medicine has resulted in two distinct categories of traditional medicine. White medicine *(Umuthi omhlophe)* is used for the amelioration of physical afflictions, while black medicine *(Umuthi omynama)* is thought to exorcise evil spirits (Krige, 1981).

Despite the westernization of the Zulus through urbanization and education, the belief in traditional remedies and healers remains firm. A dire shortage of western doctors in the rural areas, estimated in 1982 to be in the ratio of one medical practitioner for every 17,500 people (Savage, 1985) has forced rural inhabitants to consult with traditional healers, if not by choice, then certainly by necessity. With the exponential population growth rate, the demand for traditional remedies is increasing at an insatiable rate, placing severe strain on already depleted natural resources (Cunningham, 1988).

Efforts are in progress to rationalise conservation policies regarding the gathering of herbs for medicinal purposes. Such local organizations as the KwaZulu Bureau of Natural Resources, Natal Parks Board, and the Durban Municipality, in conjunction with the Institute of Natural

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Resources (University of Natal, Pietermaritzburg), are investigating methods to overcome exploitation of flora and fauna for medicinal purposes, without inhibiting cultural practices. Commercial cultivation and inclusion of medicinal herbs as part of an annual crop by subsistence farmers, have been suggested as means of counteracting this problem.

The demand for herbs with the simultaneous decline in availability has served to increase the price of traditional remedies dramatically (Cunningham, 1988). Retail of these medicaments is, therefore, very lucrative, but some store proprietors claim to be ignorant of the beneficial properties of the herbs, rather relying on the knowledge of informed employees (Hutchings and Terblanche, 1989). The interior of a typical traditional remedy shop is shown in Fig. 1 (a and b).

By law all herbalists in KwaZulu are required to be registered, a prerequisite being that the person is "skilled in herbalism" (Zulu Law, Act 6 of 1981). To be registered as a herbalist, certain conditions must be met. The applicant must be black, have served an apprenticeship for seven years with a registered herbalist and be in possession of character references from the local Magistrate, Headman and a teacher or police officer. Application is then made to the Secretary for Health, KwaZulu, who in turn, notifies the Herbalist Association (Nyanga National Association, NNA). Officials from the NNA examine the applicant's knowledge of herbs and herbalism and if successful, the applicant pays a nominal fee for a permit, allowing the herbalist to practice his trade (Xaba, pers. commun., 1989). Registration, appears, however, to be relatively unsuccessful (Cunningham, 1988).

What then are the implications of ignorant people dispensing tribal medicaments? Despite routine use of some 400 herbs, the pharmacological properties are essentially unknown and many contain highly toxic principles (Gundidza, 1985). Undoubtedly, the use of herbal remedies has had fatal consequences, but the precise number of herb-induced fatalities is unknown. Suspected herbal-mediated deaths, primarily in infants, have done little to improve communications between doctors, traditional healers and patients alike (Hutchings and Terblanche, 1989). Many people are reticent to admit either using or administering traditional remedies. Some doctors, particularly paediatricians, express anger at the parents, who through ignorance, administer herbal remedies to healthy babies, resulting in the child's death.

The breakdown in communication is further aggravated by the fact that when the child enters the hospital, it is usually too late to respond to treatment and invariably dies. Therefore, the parents view western medical practices with suspicion, believing the doctors and not the herb potentiated the child's death. It was recounted that on one occasion a child was admitted to a rural hospital suffering from hepatitis and after intensive care treatment, the now fully recovered baby was discharged. A week later the child died as a consequence of the administration of a herbal remedy. The feeling of impotence was highlighted by the comment that the effort put into saving the child was in fact wasted.

Health officials working in these rural hospitals are all too aware of the problems of herbal intoxications, but at present are unable to alleviate the situation. It is indeed most sobering when visiting these rural hospitals to be greeted by a delegation of nursing sisters, who thank one in advance for coming and telling them "how to save the children", especially when you know that there are no answers at this time and the work you are covering is but the "tip of the iceberg". One is supposed to remain unemotional and clinical in science, but it is extremely difficult not to be affected when you are presented with such a situation.

Personally, one of the major hurdles to be crossed is convincing higher authorities of the problems. At present post mortems are not being carried out in some of these hospitals, unless unnatural cause of death is suspected. Herbal intoxications are not considered to be an unnatural cause of death, as if they were to be so, a murder docket has to be opened and a full police investigation mounted. Here one enters an ill-defined area, as can one be convicted of believing in one's tribal customs? This matter will remain unanswered, until such time as the precise number of herb-induced fatalities are known. With this information, authorities will have to take cognisance of these

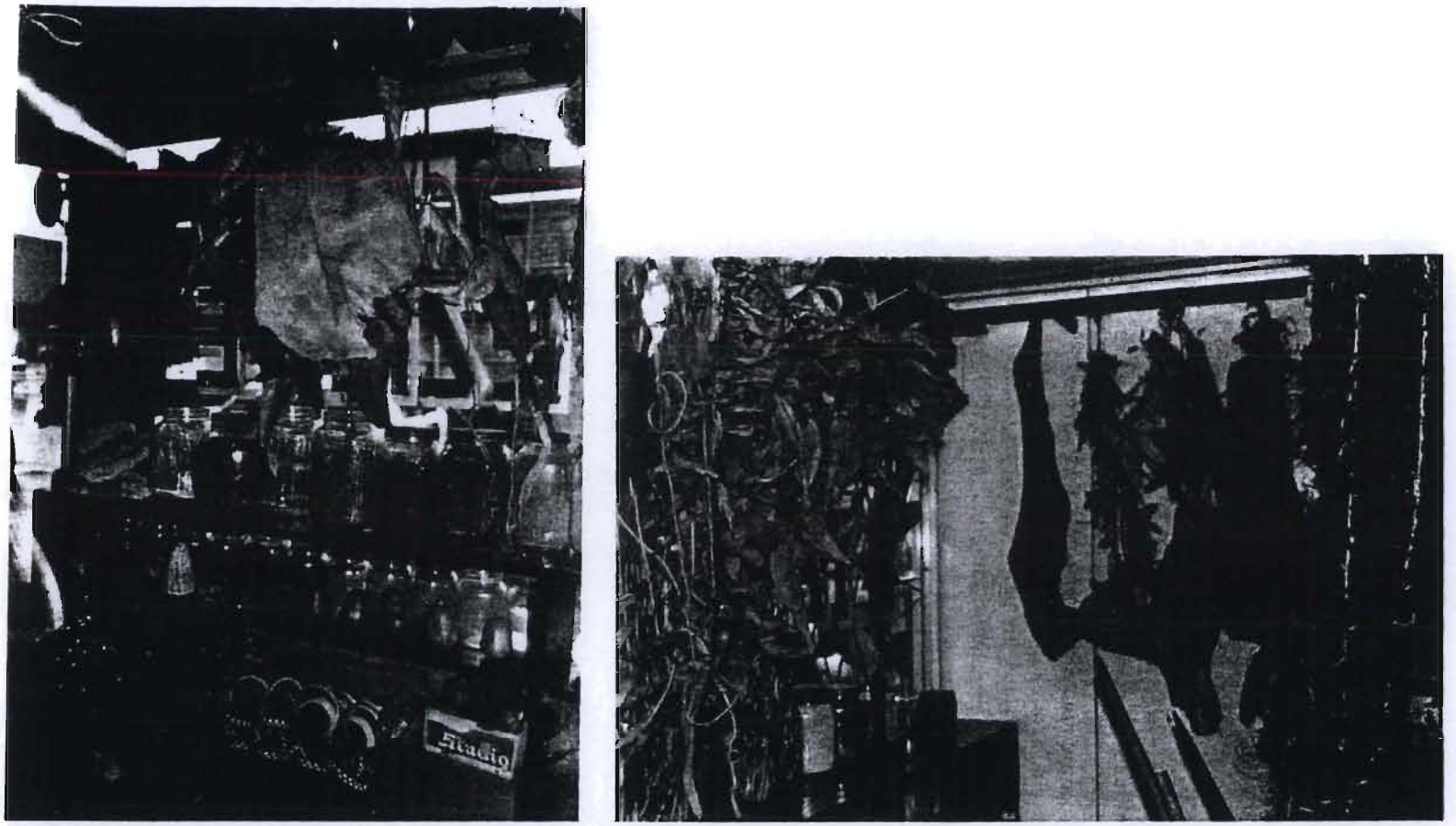

Fig. 1. Interior of a typical herbal medicine shop. (a) Window view, demonstrating the use of mixtures of milled plant material, stored in jars for distribution (b) Interior of store, demonstrating use of both animal and plant material for medicinal purposes (note: skins in background and bundles of reeds and tuberous plants in foreground).

deaths and decisions regarding the legal implications debated.

Precise analytical procedures will have to be developed to identify the type of poisoning and this will be concomittant to investigating the toxicity of popular medicinal herbs. In this manner it will be possible to accumulate data regarding the incidence of herbal intoxiciations in South Africa and hopefully identify which herbs are responsible. It has also been suggested that these toxic herbs be scheduled, on a system akin to that used for western pharmaceuticals (Cunningham, 1989). This information could be used to educate herbalists and rural inhabitants alike, and here cognisance will have to be taken of the cultural beliefs of the people. In addition, it may be possible to cultivate non-toxic forms of the plant and this will most likely present the most beneficial method. The depletion of natural resources may for once be a blessing in disguise, as only non-toxic strains may be cultivated by choice in commercial ventures.

These are obviously long term plans and the primary objectives at this time are to identify the toxic species and the toxic components. Suitable diagnostic assays will have to be developed, the toxicological action of these compounds investigated and an attempt made to devise antidotes for the compounds. At the same time the incidence of herbal poisonings can be obtained. With at least 88 toxic species to cover, this will require a great deal of time, energy and finance. Unfortunately, the latter is slow in forthcoming, but unless research of this nature is initiated, the indiscriminant deaths will continue unabated.

With this in mind, over the past three years, work has been initiated on one such toxic herb, namely *Callilepis laureola,* the idea being to perfect methodology on one such plant and then apply this to other toxic species. A member of the family Compositae, *C. laureola* is commonly known as the ox-eye daisy or *Impila* (Zulu = health). This perennial bears a tuberous root, likened to a sweet-potato (Wainwright et al., 1977, Brookes et al., 1983) and flowers from August to November, bearing solitary creamy-white flowers with a purple disc (Dyer, 1975; Hilliard, 1977; Palmer, 1985).

The Nguni people (Zulu, Bantu, Sotho) regard *Impila* as a valuable *muthi* (medicine) (Dlamini, pers. commun., 1987). The tuberous rootstock is used by the Zulus to prepare the potion (Debetto, 1978), although a leaf infusion is reputed to have limited curative properties (Bryant, 1909). The tuber is harvested in winter, dried and crushed (Bryant, 1909; Ellis, 1986). The resultant pulp is boiled for 30 min in a suitable volume of water and the decoction administered either orally or rectally (Insangoma, pers. commun., 1987).

Essentially a multi-functional *muthi, Impila* is taken to ameliorate stomach complaints, tapeworm infestations (Bryant, 1909), to induce fertility (Debetto, 1978), as a cough expectorant and whooping cough remedy (Maberley, 1906, cited by Watt and Breyer-Brandwyk, 1962). Undoubtedly though, the "protective powers" of this plant are its greatest attribute. A tuber buried in the vicinity of one's home is thought to intercept any evil directed towards the household (Dlamini, pers. commun., 1987), while an *Impila* tincture consumed prior to festivals is believed to offer protection from "those harboring evil intent" (Wainwright et al., 1977). Independent surveys indicate that the popularity of this herb cannot be underestimated (Bhoola, 1983; Ellis, 1986).

Why the scientific interest in *Impila*, when this herb is but one of 400 used and is by no means the most popular? In the mid-1970s, the high incidence of centrilobular liver necrosis in blacks residing in the Natal/KwaZulu regions, was cause for concern (Wainwright and Schonland, 1977). Invariably accompanied by renal necrosis, this hepatic damage accounted for 2% of all deaths at the King Edward VIII Hospital, Durban, over the period 1958—1977 (Bhoola, 1983). *Impila* was identified as the primary causative agent (Wainwright et al., 1977; Bhoola, 1983) and to date some 260 deaths, a third of these being in children of less than five years of age, have been attributed to use of this herb (Brookes, 1979; Bhoola, 1983). In view of the popularity of this herb and the unavailability of a specific assay to determine *Impila* poisoning, these figures are deemed conservative (Brookes, 1979).

The reluctance of the black population to admit using herbal remedies is highlighted by the fact that in only 20% of suspected herbal intoxications was use of traditional remedies conclusively pro-

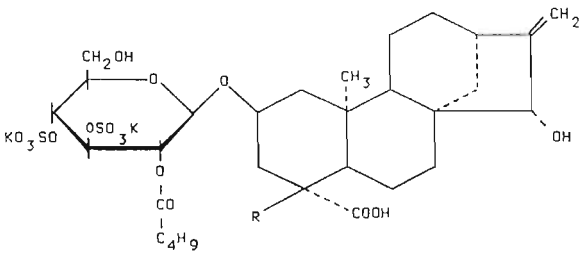

Fig. 2. The structure of atractyloside (R = H) and carboxy-atractyloside (R = COOH).

ven. Nevertheless, significant recurrent clinical and biochemical changes were noted in patients exhibiting centrilobular liver necrosis and of these, 59% were suspected to be *Impila* poisonings. Vomiting, jaundice, abdominal pain and convulsions were commonly seen. Hypoglycaemia, uraemia and evidence of disturbed liver function were presented. Clinical records showed that the duration of the illness was short and in 91% of cases, resulted in death. In summary, patients with suspected *Impila* poisoning demonstrated renal and hepatic necrosis, with associated hypoglycaemia, which usually culminated in death (Bhoola, 1983).

The major toxic principles, extracted from the tuber of *C. laureola* are atractyloside (ATR) and carboxy-atractyloside (CATR) shown in Fig. 2 (Brookes, 1979). Atractyloside was first isolated from the Mediterranean thistle *Atractylis gummifera*. This plant is still used for the treatment of syphilitic ulcers, for inducing abortions, and for bleaching teeth, but intoxication by this plant is not uncommon (Georgiou et al., 1988). Hydrolysis products of ATR consist of 1 mol of glucose, 2 mol sulphuric acid and 1 mol each of isovaleric acid and atractyligenin, respectively. Atractyligenin is an acidic diterpene with a basic 18-nor(−)kaurene skeleton (Ghisalberti et al., 1968) the latter being a precursor to the gibberillins (Takahashi et al., 1986). Subsequently, CATR was extracted from *A. gummifera* tubers. Identical to ATR, apart from a second carboxyl group on C_4 of the diterpene ring, CATR is restricted to fresh tubers and thermally decomposes to ATR (Bombardelli et al., 1972).

Compounds related to ATR are not restricted to *A. gummifera* and *C. laureola*, since an ATR-analogue has been isolated from coffee beans. In this derivative, the glucose moiety is replaced by glucuronic acid and is devoid of the sulphate and isovaleric esters. This compound is excreted in the urine of habitual coffee drinkers at the levels of 0.001 mg/ml (20—40 mg/day) (Obermann and Spiteller, 1976). It has been suggested that the sub-lethal but chronic administration of this compound may mediate pancreatic cancer in coffee drinkers (Pegel, 1981). As no suitable analytical test is available, levels of this toxin in coffee beverages are not being monitored and implications of this could be far reaching.

Atractyloside is the nephrotoxic agent (Bhoola, 1983) and its renal toxicity is well documented (Carpenedo et al., 1974). Furthermore, ATR is a potent hypoglycaemic agent (Luciani et al., 1978). Carboxyatractyloside, though inherently more toxic than ATR, is devoid of nephrotoxicity. This is either because CATR induces death before cellular damage is visible, or the increased polarity of CATR impedes access of the molecule to tubular cells. While the renal toxin of *Impila* has been conclusively identified, the hepatotoxin is as yet unknown.

Mitochondria have been identified as the target organelles of ATR (Vignais et al., 1962) and ATR has been conclusively demonstrated to inhibit oxidative phosphorylation and thus energy transduction (Santi, 1964; Bruni et al., 1964). The most active intramitochondrial reaction is ATP synthesis, whereby ADP is phosphorylated by ATP synthase, to produce energy in the form of ATP. By virtue of the high turnover rate of this reaction, transport of substrates such as ADP, P_i and ATP, associated with oxidative phosphorylation, must be both active and efficient (Klingenberg, 1978). These compounds are, however, hydrophilic and cannot traverse the inner mitochondrial membrane, unless facilitated by protein carriers. Two carriers are involved, one transporting P_i and the other, a protein translocase, importing ADP and exporting ATP to and from the matrix (Nicholls, 1982). Atractyloside competitively inhibits the transport of endogenous (cytosolic) ADP across the inner mitochondrial membrane, by binding to

the ADP-binding site on the intermembrane side of the protein translocase. This prevents passage of ADP across the inner membrane, ATP cannot be synthesized and without usable energy, the cell dies (Chappell and Crofts, 1965).

The toxicity of *Impila* cannot be refuted, and it was the initial aim to develop a diagnostic assay for use in both hospitals and forensic laboratories. Thereafter, it was decided to investigate storage sites of ATR in the plant and target sites of the toxin in mammals. Antibodies were raised to ATR, for use in both immunoassays (Bye et al., 1989) and immunocytochemical studies. In this manner it will be possible to detect ATR and ATR-analogues in biological fluids, both plant and animal. In addition, the availability of anti-(ATR) antibodies will provide visual evidence of storage sites of the toxin in the plant, target mammalian organs and organelles and hence subcellular binding sites of the toxin in animal tissue. Immunocytochemical location of the toxin in the tuber has been carried out and though requiring further refinement, may be applied to mammalian systems.

We have been accused of merely presenting the "bad side of the coin" and certainly, all the herbal remedies which are used with success are not seen by either ourselves or the doctors. Undoubtedly many traditional remedies are beneficial and do have a definite role to play in rural health, but what is of concern is that many of the potentially toxic herbs are in fact used for their "magical properties" and are doing little to prevent disease. The purpose of this paper was to give a brief resumé of work being carried out on toxic Zulu herbal medicaments. No attempts have been made to delve into the pharmacological potential of the plethora of herbs used by the Zulus and nor has the work being carried out in other scientific institutions been considered. The objective of the research being carried out at this University is, as mentioned, to develop assays for the toxic herbs and accumulate data regarding the incidence and type of medicinal herbs being used. In addition, this data can be used to inform both authorities and rural inhabitants alike of the dangers of these remedies and attempt to terminate the senseless deaths of black children.

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Journal of Ethnopharmacology, 34 (1991) 247–251
Elsevier Scientific Publishers Ireland Ltd.

The isolation of a storage organelle of atractyloside in *Callilepis laureola*

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(Accepted May 28, 1991)

A method has been developed for the preparation of protoplasts from both the leaves and tubers of *Callilepis laureola*, a plant used extensively as a medicament by black people in South Africa. The cellular vacuoles from these protoplasts were isolated and tested for the presence of the nephrotoxic substance, atractyloside, by thin layer chromatography and immunoassay. Both methods indicate that the vacuole of *C. laureola* is the primary site of storage for atractyloside in the cells of the tuber.

Key words: protoplasts; vacuoles; nephrotoxin; hepatotoxin; ELISA.

Introduction

The herbaceous plant *Callilepis laureola* is used extensively by the black tribes of South Africa as a herbal medicament. Its toxicity, however, was initially documented at the turn of the century (Bryant, 1909). An extraction prepared from the tuber of this plant is called *Impila* by the black population and, although it has use as a vermifuge and decongestant, the herb is primarily administered to ward off evil (Wainright et al., 1977). Infusions of the tuber, administered orally or rectally, induce both liver and renal necroses, with associated hypoglycaemia (Watson et al., 1979; Bhoola, 1983).

The diterpene glycoside, atractyloside, extracted from the tuber, has been identified as the nephrotoxin (Candy et al., 1977) but the hepatotoxin remains undetermined. Atractyloside (ATR) (Fig. 1) was first isolated from the rhizomes of the Mediterranean thistle, *Atractylis gummifera* (Santi and Luciani, 1978) and an associated metabolite has been extracted from coffee beans (Richter and Spiteller, 1978).

Atractyloside inhibits the transport of adenine nucleosides across the inner mitochondrial membrane and this inhibitory action is not restricted to mammalian cells but has also been demonstrated in cauliflower (Jung and Hanson, 1973). This suggests that *C. laureola* must possess an efficient transport and storage system, whereby intoxication of the plant is avoided. Atractyloside contains an ent-kaurene moiety, which is known to be the precursor to the gibberellins. As these growth hormones are stored in the vacuole and this organelle is responsible for their metabolic sequestration and storage (Wagner, 1982) it may well also store ATR.

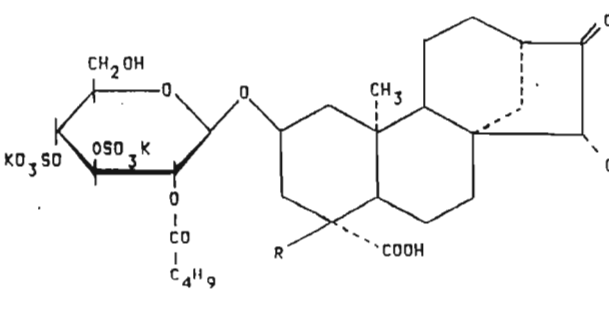

Fig. 1. The structure of atractyloside.

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0378-8741/$03.50

The aim of the work reported here was to establish and optimise a protocol for the isolation of vacuoles from the tubers of *C. laureola*, via the intermediate step of forming protoplasts; and to identify the vacuole as a possible storage organelle of ATR in this plant.

Materials and Methods

Materials

C. laureola specimens were collected from Eshowe, Natal. The pectinase Macerozyme and cellulase Onozuka RS enzymes were purchased from Yakult Honsha (Japan). Mannitol, ficoll, goat anti-(rabbit IgG) IgG-alkaline phosphatase conjugate and ATR (potassium salt) were obtained from Sigma Chemical Co, St Louis, U.S.A. Rabbit anti-atractyloside antibodies were prepared as described by Bye et al. (1990). Anisaldehyde was purchased from Merck (R.S.A.) and NUNC microtitre plates were obtained from Weil (Johannesburg, R.S.A.). All other reagents were of analytical grade.

Protoplast production

Fresh *C. laureola* tuber (1 g) was chopped (± 2 mm^3) and surface sterilized in sodium hypochlorite and Tween 80 (1% and 0.1%, respectively) solution for 10 min at room temperature. Sterilized tissue pieces were washed thoroughly with basal medium (0.5 M mannitol, 1 mM each of magnesium chloride and potassium dihydrogen phosphate and 0.002 g/ml each of citric acid and ascorbic acid, pH 5.6) to remove all traces of sterilants. The washed tissue was incubated for 6 h at 26°C at 70 strokes/min in 20 ml of basal medium containing 2% Macerozyme and 4% Onozuka RS (w/v), respectively. The solution was filtered through a 220-μm mesh filter and pooled with the initial filtrates. Aliquots (0.5 ml) of this suspension were added to equal volumes of Evan's blue dye (2% w/v in basal medium) and average yields determined by counting on an Improved Neaubauer Haemocytometer. Centrifugation of the filtrate at 100 × *g* for 10 min caused viable protoplasts and cellular debris to sediment. The supernatant was discarded, the pellet resuspended in basal medium and overlayed onto a 0.6 M sorbitol solution containing 25 mM potassium dihydrogen phosphate and titrated to pH 5.5 with citric acid. Centrifugation at 100 × *g* for 3 min allowed intact protoplasts to band at the interface and purity was evaluated microscopically.

Vacuole isolation

Various protocols were tested but that of Grandstedt and Huffaker (1982) proved most reproducible. Purified protoplasts from the tuber were removed from the interface with a pasteur pipette and resuspended in 6 equal volumes of 12% ficoll (w/v) containing 60 mM potassium dihydrogen phosphate (pH 8.0) and 1 mM dithiothreitol (DTT). Repeated inversions of the tube for 5 min caused protoplasts to lyse and the solution was overlayed with 2 volumes of 0.6 M mannitol solution with 50 mM potassium dihydrogen phosphate (pH 7.0) and 1 mM EDTA (sodium salt). Centrifugation at 1000 × *g* for 10 min caused vacuoles to migrate to the top 1—2 volumes (0% ficoll fraction) while cellular debris sedimented. Vacuoles were visualised with acidophilic neutral red dye.

The 0% ficoll phase, interphase and pellet fractions were removed for analysis by thin layer chromatography and immunoassay.

Thin layer chromatography

Samples of the resuspended pellet, interface and 0% ficoll fractions (20 μl) were spotted onto the origins of two-dimensional thin layer chromatography (TLC) plates (10 × 10 cm aluminium backed silica gel G 60, Merck). Suitable controls, i.e., a standard ATR solution (1 mg/ml), and a standard sugar solution containing equal amounts of basal medium, sorbitol solution and vacuole isolating medium, were also subjected to the same TLC system. All the chromatograms were developed in chloroform/methanol (3:2 v/v) in the first dimension and butanol/acetic acid/water/chloroform (6:2:2:1 v/v/v/v) in the second dimension. The chromatograms were air dried, sprayed with anisaldehyde reagent (Brookes, 1979) and heated at 120°C for 3 min.

Enzyme immunoassay

A competitive immunoassay was carried out, as

described by Bye et al. (1990). Briefly an ovalbumin-atractyloside conjugate (0.01 mg/ml in 0.05 M carbonate buffer, pH 9.6) was adsorbed on microtitre wells for 16 h at 37°C. The plates were washed three times with tap water and pellet, interface and vacuole fractions (undiluted; 1:10; 1:100; and 1:1000, diluted in 0.02 M tris buffered saline, pH 7.4 with 0.1% Difco-Bacto gelatin) were added to wells (100 μl) followed by an equal volume of rabbit anti-atractyloside-IgG, diluted 1:20. Plates were incubated for 3 h at 37°C, washed and the secondary antibody-enzyme conjugate (diluted 1:3000) added (200 μl/well). The plates were incubated for 1 h at 37°C, washed and enzyme substrate added (1 mg/ml *p*-nitrophenyl phosphate, in 0.1 M glycine buffer, pH 10.4 containing 0.001 M magnesium chloride and zinc chloride). The plates were incubated for 1 h at

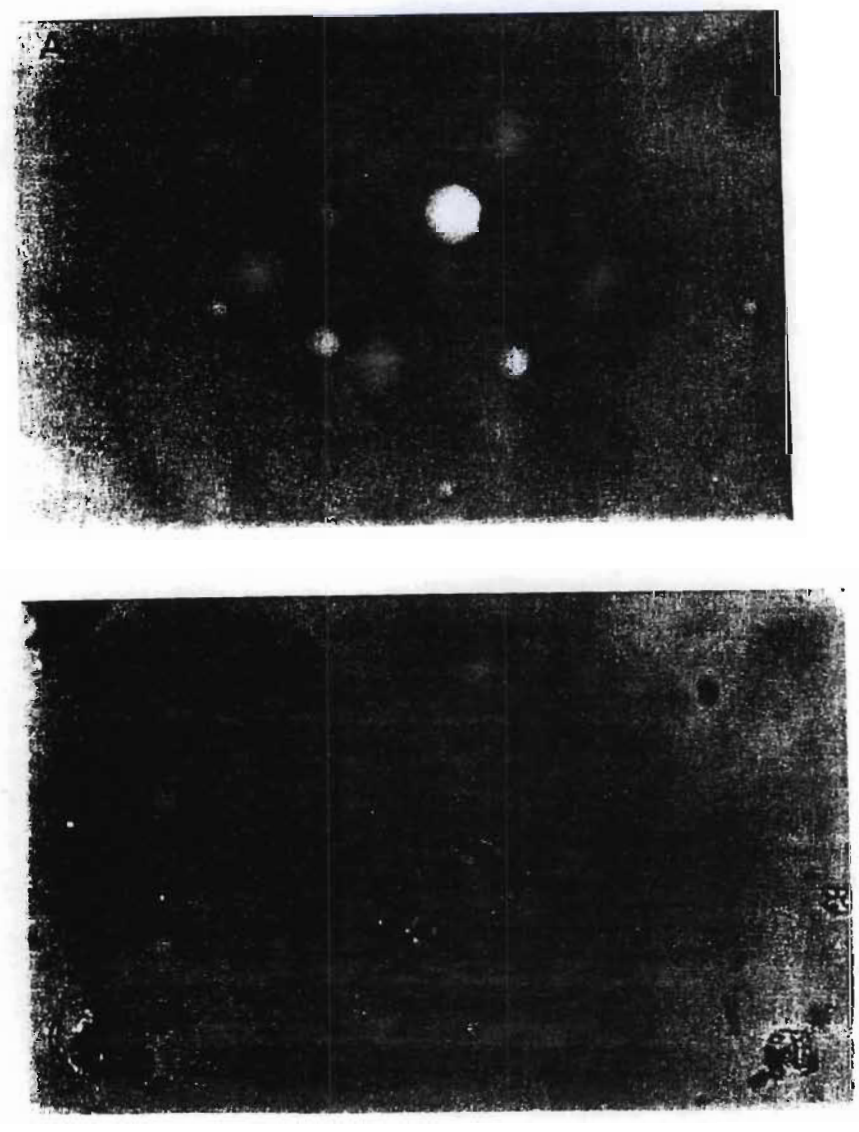

Fig. 2. (A) Protoplasts derived from the tuber of *Callilepis laureola* contrasted by staining the background with Evan's blue. (B) Protoplasts dervived from leaf tissue of *Callilepis laureola*.

37°C and the reaction was stopped by the addition of 6 M potassium hydroxide (50 μl/well) and the absorbance read at 405 nm, using a Biotek spectrophotometer.

Results and Discussion

Optimisation of protoplast production

Protoplasts were isolated from the tuber of *C. laureola* (Fig. 2A) but initial yields were low (4×10^3 to 4×10^4 protoplasts/ml). This in part was attributed to the presence of plant phenolics and to negate the effects of these, citric and ascorbic acids (0.002 g/ml) were added. This, together with the omission of the purification step using the sorbitol gradient, resulted in improved yields of $1—2 \times 10^6$ protoplasts/ml.

An additional optimisation involved the determination of the most effective sterilant concentration, which was found to be 1% sodium hypochlorite and 0.1% Tween 80 and became the treatment of choice.

Reduction of Onozuka RS (cellulase) from 4% to 2% (w/v) using the same protocol as applied to tuber tissue, enabled the production of protoplasts from leaf tissue at a level of $4—5 \times 10^6$ protoplasts/ml (Fig. 2B).

Subcellular fractionation and vacuole isolation

Samples of the vacuole fraction stained with equal volumes of neutral red solution, enable visualisation of vacuoles as bodies stained dark red. Microscopic examination revealed that the vacuoles were free from other plant material and debris and, therefore, constituted a pure fraction. Yields were low ($3—4 \times 10^2$ vacuoles/ml) due in part to the passage of neutral red dye into the vacuole upsetting the osmotic balance and causing the vacuole to collapse.

Location of atractyloside in the vacuole

Examination of the various fractions by TLC, showed that the vacuole fraction gave a pink spot with the anisaldehyde reagent at R_f values of 0.36 and 0.74 in the first and second dimensions, respectively. These values coincided with those obtained for standard ATR and were not observed on any of the other control or pellet and interface fraction chromatograms.

The results from the immunoassay indicated that ATR was present in all three fractions, i.e., vacuole, interface and pellet. Although the immunoassay is only semi-quantitative it did show, however, that the levels of ATR in the vacuole were much higher than in the other two fractions. This result was expected, as the immunoassay is much more sensitive than the TLC-spray reagent and ATR must be present in the other fractions, if only from vacuoles ruptured in the isolation procedure. It can be concluded that ATR is present in far higher concentrations in the vacuole than other parts of the cell and this, therefore, appears to act as the storage organelle in the plant.

This is the first report of the determination of the sub-cellular localisation of ATR in the vacuole of *Callilepis laureola*. Although more work is required to optimise the isolation of viable vacuoles in higher yields, their production from tubers and, in particular, leaves of the plant is reproducible and should, therefore, allow the mechanism of concentration of the toxin in the organelle to be elucidated.

Acknowledgements

The authors wish to thank Dr P. Watt and Mr R. Berry, University of Natal for their assistance and time; and the University of Natal for funds to support the work.

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CONCLUSION

The work descibed here and the publications springing from it, spans over 36 years, from when I commence a fourth year BSc project on amino acids present in a toxigenic strain of *Aspergillus parasiticus*. The person who inspired this initial sally into the wonderful world of mycotoxins was Dr J J Child who was a mycologist interested in the genus *Aspergillus*. He was supported by Dr J .G. Heathcote, Reader in Biochemistry, University of Salford who eventually became my supervisor for a PhD in this area. I have always been grateful to these academics and circumstances that gave me my chance to work with toxic natural products, which are my first love.

The journey, as we have seen, has moved through aflatoxin biosynthesis, onto mycotoxin analysis and occurrence and thence to medical aspects, picking up something of plant toxicology on the way. I have been advised to the point of reprimand many times, not least by the FRD (major funding organisation in S.A.) assessors, to focus my efforts and cut down on the width of study to ensure more in depth results. Being a stubborn Yorkshireman and being a dilitante at heart, I have always resisted this directive. As might be imagined, I regret the passing of broadly based research scientist into modern specialisations. I suspect that eventually there with be a journal for DNA sequences from sub-species of sea cucumbers with an impact rating of 50 and readership of two, including the editor. I have always drawn my ideas for research project from reading widely. The "New Scientist" is far more inspiring than "Mycotoxin Research".

I was asked recently at a meeting involving presentations by some of my students and colleagues, working on mycotoxin, how one could defend all the effort being made in this area, where there was so many other things to be tackled, especially in Africa. I concur that looking in the literature, a disproportionate time has been globally spent on mycotoxins, considering the fact that first world countries can hardly claim that mycotoxicoses are rife within their populations, especially where the health index of diet is of so much interest, even to the point of being a fad.

In our case, however, I make two strong points in favour of the broad study of mycotoxins by postgraduate students. From this work at can easily be seen that such studies cover a wide array of disciplines, ranging from analytical chemistry, through mycology to medicine. Postgraduate students entering my group are exposed to all these disciplines and their attendant methodologies and can specialise in any one or several of them. More importantly, it can be seen from Chapter 5 and 6 that the cosy situation in first world countries, with regards to diet and food safety, do not appertain in any country in sub-Saharan Africa and probably not in Mediterranean Africa ones either. It might be argued that where people do not get enough to eat why worry about mycotoxins. Where people do not have access to safe drugs, why not let then take a chance with "cowboy" traditional preparations. I strongly repudiate that type of thinking.

By applying our scientific training and knowledge to these problems, we can start to improve the quality of food and medical treatments. Simple pieces of advice on how to store cereals, what not to eat, when to collect medicinal herbs and how to

prepare them**, in consultant** with the local population, are practical steps that can be taken. Anybody who feels that this is a waste of time, can explain their view to the Black person with terminal oesophageal or liver cancer or to the mother whose child is in a hypoglycemic coma with multi-haemorrhages.

"knowledge is always preferable to ignorance but also engenders responsibility"

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APPENDIX: LIST OF CONFERENCE PROCEEDINGS AND THESES SUPERVISED

PAPERS AND POSTERS PRESENTED AT SOUTH AFRICAN NATIONAL CONFERENCES (NCON #)

(1) JEENAH MS and DUTTON MF. 1981.
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(2) DUTTON MF and WESTLAKE K. 1981.
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(3) DUTTON MF. and WESTLAKE K. 1981.
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(4) DUTTON MF. and JEENAH MS. 1981.
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(5) WESTLAKE K and DUTTON MF. 1982.
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(7) DUTTON MF and JEENAH MS. 1983.
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(17) DUTTON MF. 1986.
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(18) BERRY RK and DUTTON MF. 1986.
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(19) WILLIAMS RK and DUTTON MF. 1986.
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(20) CHUTURGOON AA, BERRY RK and DUTTON MF. 1986.
Enzymatic conversion of norsolorinic acid to averantin. S.A. Biochem. Congress, Johannesburg, p265.

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The role of aflatoxin in kwashiorkor. S.A. Nutrition Congress, Durban.

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(38) RAMAN J, CHUTURGOON AA and DUTTON MF. 1991.
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(39) SUTAN OS and DUTTON MF. 1991
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(40) SUTAN OS, TOWSHEND DJ, DUTTON MF and BERRY RK. 1991.
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(42) CHUTURGOON AA and DUTTON MF. 1992.
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(46) CHUTURGOON AA, MAHARAJ S and DUTTON MF. 1992.
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(47) CHUTURGOON AA, RAMAN J and DUTTON MF. 1992.
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(58) MOSONIK JS and DUTTON MF. 1995. Evaluation of a multi-mycotoxin screen for analyzing agricultural commodities. South African Biochemical Society, Thirteenth Congress, Bloemfontein, p149.

(59) PILLAY D, CHUTURGOON AA, BUX S and DUTTON MF. 1995. The cytotoxic evaluation of mycotoxins using a rapid colorimetric assay. South African Biochemical Society, Thirteenth Congress, Bloemfontein, p151.

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(65) GQALENI N, CHUTURGOON AA, DUTTON MF, SMITH JE and THORUWA TN. 1996.
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(72) MYENI SS, DUTTON MF, GENGAN R and CHUTURGOON AA. 1997.
Reaction of selected N-acetyl amino acids with aflatoxin B_1 epoxide. S.A. Society of Biochemistry and Molecular Biology 14th Congress Grahamstown, 20th-23rd January, SL46.

(73) REDDY L, ODHAV B and DUTTON MF. 1997.
Production, purification and detection of fumonisins. S.A. Society of Biochemistry and Molecular Biology 14th Congress Grahamstown, 20th-23rd January, p13.

(74) MYENI SS, DUTTON MF, GENGAN R and CHUTURGOON AA . 1997.
Synthesis of aflatoxin B_1 lysine adduct for use as a bio-marker for toxicity. S.A. Society of Biochemistry and Molecular Biology 14th Congress Grahamstown, 20th-23rd January, p54.

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