AN INVESTIGATION INTO THE METABOLISM OF THE IMPORTANT MYCOTOXINS PRODUCED BY ASPERGILLUS SPECIES

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A C K N O W L E D G E M E N T S

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For those who contributed to this work in some way I express my thanks - to Bill Porter and colleagues (mass specs.) and Dr John Peberdy (protoplasts) of Nottingham University, Dr B. Davis (Hepes buffer), Sheffield Polytechnic; also, Dr Y. Hatsuda, Tottori University, Japan; Dr P. S. Steyn, National Chemical Research Laboratory, Pretoria, South Africa; Dr Y. Berger, University of Liege, Belgium; Dr H. W. Schroeder, U.S. Dept. Agriculture, Texas, U.S.A.; Dr J. S. F. Holker, University of Liverpool; Dr J. A. Donkersloot, National Institute of Health, Maryland, U.S.A.; Dr J. W. Bennett, Tulane University, Louisiana, U.S.A.; Dr A. F. Schindler, Bureau of Foods, Washington D.C.; Dr K. E. Papa, University of Georgia, U.S.A.; Dr R. W. Detroy, U.S. Dept. of Agriculture, Illinois; Dr D. P. H. Hsieh, University of California, Davis, U.S.A.; Dr L. S. Lee, U.S. Dept. of Agriculture, New Orleans, Louisiana, for their culture samples, authentic metabolites

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C O N T E N T S

I N T R O D U C T I O N

History

The outbreak of a disease amongst turkey poults in parts of England in 1960 and the eventual implication of the aflatoxins (Sargeant et al 1961) awoke international interest in mycotoxins.

The aetiological agent of "Turkey X" disease was found to be a groundnut meal imported from Brazil. Examination of the meal led to the isolation and identification of a toxin-producing organism, namely a saprophytic mould Aspergillus flavus Link ex Fries (Sargeant et al 1961). Elucidation of the toxins produced by this organism demonstrated that they could be separated chromatographically into four distinct compounds named aflatoxins B_1, B_2, G_1 and G_2 (Nesbitt et al 1962, Sargeant et al 1961, Van der Zidjen et al 1962). The name "aflatoxin" was given to these components to designate their generic origin, whilst delineation of the four substances was made on the basis of their fluorescent colour under U.V. light (blue = B, green = G). The subscripts (B_1, B_2) relate to their relative chromatographic mobility. An historical précis of the aflatoxins has been reviewed by Moss (1972), sic.

1960 "Turkey X" disease was described and rainbow trout hepatomas discovered.

1961 It became evident that other animal diseases may have been caused by the same agent as that

which caused Turkey X disease. This agent was shown to be a fungal metabolite, 20µg of which was lethal to ducklings in 24 hours (Sargeant et al 1961). It was also demonstrated that this same agent was carcinogenic to rats (Lancaster et al 1961).

1962 Some of the physicochemical properties and analytical techniques for studying the aflatoxins were described. It was now realised that a family of compounds were involved and that they were photosensitive in hydroxylic solvents (Van der Zidjen et al 1962).

1963 The chemical structures of the aflatoxins were described (Asao et al 1963).

1964 X-ray crystalographic confirmation of the structures was reported (Cheung and Sim 1964).

1965 The aflatoxins were associated with the disturbance of events in the cell nucleus (Legator et al 1965).

1966 The total synthesis of racemic aflatoxin B_1 was described (Buchi et al 1966).

Occurrence

Aspergillus flavus (Link ex Fries) is not only a widespread saprophytic mould and an occasional pathogen of

animals, but it is also very closely related to strains of Aspergillus extensively used in the production of foods and beverages in the Far East and other parts of the world (Hesseltine 1965). Other fungi, including A. niger, A. ruber, A. wentii, Penicillium citrinum, P. frequentans, P. variable and P. puberulum have been reported to produce aflatoxins, but confirmatory studies are lacking (Kulik and Holaday 1966).

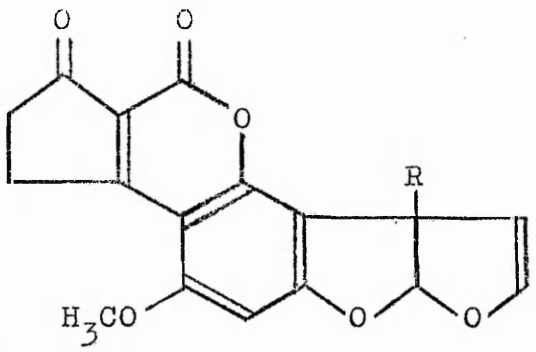
The natural occurrence of aflatoxins in various foods and feeds has been widely investigated. Toxin-contaminated commodities include corn, barley, cassava, cottonseed meal, peanuts, peanut meal, peas, rice, soybeans, wheat and sorghum seed (Wilson 1973). Toxicogenic isolates are components of the soil, air, seed and forage microflora throughout the world.

Chemistry and classification of aflatoxins and related metabolites

The aflatoxins are a family of "secondary metabolites" (Bullock 1965) characterized by the presence of a fused bisfuran ring system attached to a substituted coumarin nucleus. From A. flavus to A. parasiticus a number of metabolites have been isolated containing the fused bisfuran moiety; these include the four original aflatoxins B_1(I), B_2(II), G_1(III), G_2(IV) and the five hydroxylated forms GM_1(IIIB), B_{2a}(V), G_{2a}(VI), M_1(VII) and M_2(VIII); aflatoxin B_3 (IX) was also isolated (Heathcote and Dutton 1969) or

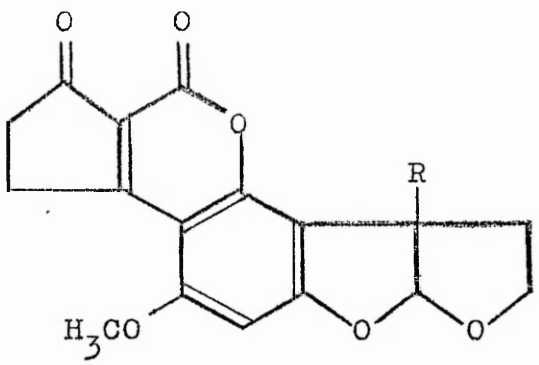
parasiticol (Stubblefield *et al* 1970). In 1968, two more compounds were isolated from *A. flavus* in which the fused bisfuran ring system was attached to a xanthone nucleus. These two metabolites, O-methyl sterigmatocystin (X)(Burkhardt and Forgacs 1968) and aspertoxin (XI)(Rodricks *et al* 1968) are both derivatives of the metabolite sterigmatocystin (XII) which was first isolated from *A. versicolor* by Hatsuda and Kuyama (1954) and described by Bullock *et al* (1962). A family of xanthone metabolites possessing the fused bisfuran ring have been isolated from *A. versicolor*, i.e. 5-methoxy-sterigmatocystin (XIII)(Holker & Kagal 1968), 6-methoxy-sterigmatocystin (XIV)(Bullock *et al* 1963) and demethyl-sterigmatocystin (XV)(Elsworthy *et al* 1970).

Another extensive group of metabolites isolated from both *A. versicolor* and *A. parasiticus* are anthraquinones, which are classified into two groups. The first group belongs to the versicolorin series containing a fused bisfuran ring attached to an anthraquinone nucleus. This includes versicolorin A (XVI), B (XVII) and C (XVII) (Hamasaki *et al* 1965) and various derivatives such as 6-deoxyversicolorin A (XVIII)(Elsworthy *et al* 1970) and aversin (XIX)(Bullock *et al* 1963). Versiconol (XX)(Hatsuda *et al* 1969) can also be included in this group, even though it does not possess the fused bisfuran ring, because it does contain a C_4 side chain, unlike the metabolites of the second group which all possess a C_6 side chain attached to an anthraquinone nucleus. This group includes averufin (XXI)(Donkersloot *et al* 1972) and derivatives such as deoxyaverufinon (XXII), dehydroaverufin



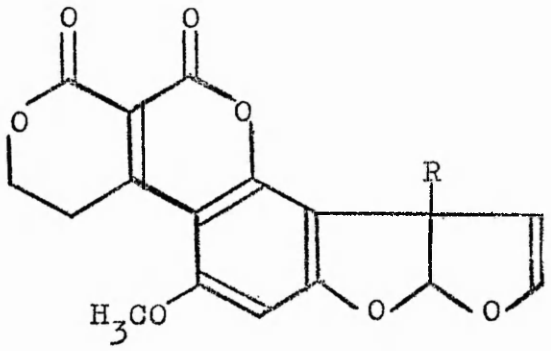
(I) Aflatoxin B_1 R = H

(VII) Aflatoxin M_1 R = OH



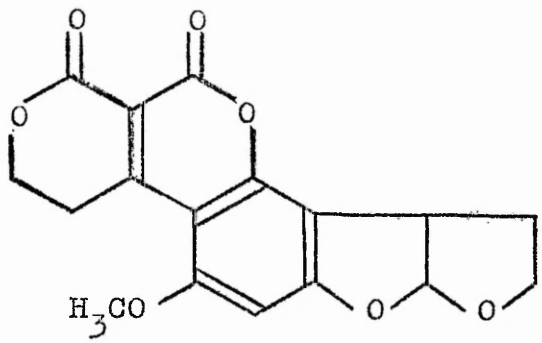
(II) Aflatoxin B_2 R = H

(VIII) Aflatoxin M_2 R = OH

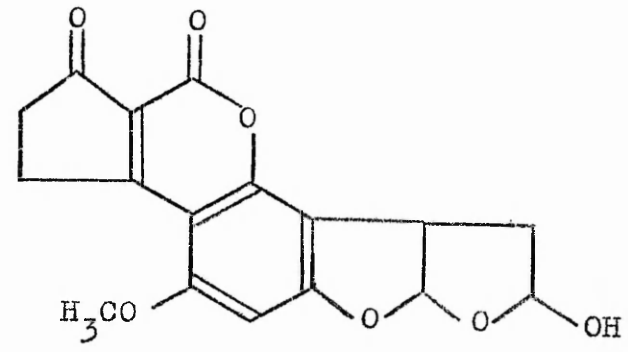


(III) Aflatoxin G_1, R = H

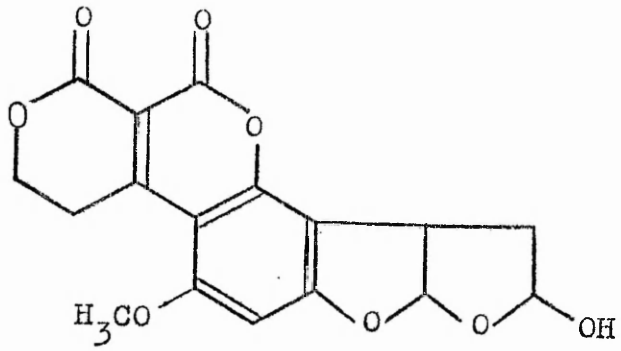
(IIIB) Aflatoxin GM_1, R = OH



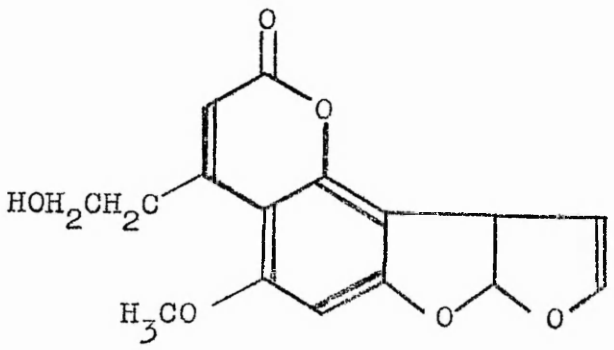
(IV) Aflatoxin G_2



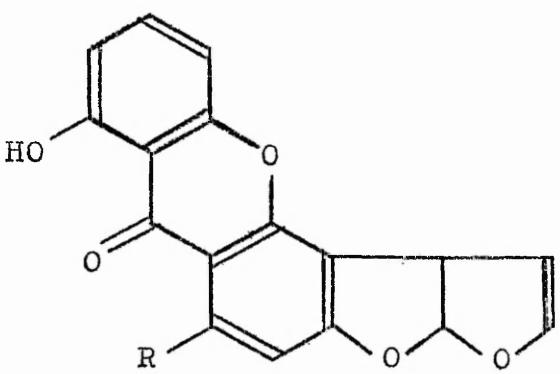
(V) Aflatoxin B_{2a}



(VI) Aflatoxin G_{2a}

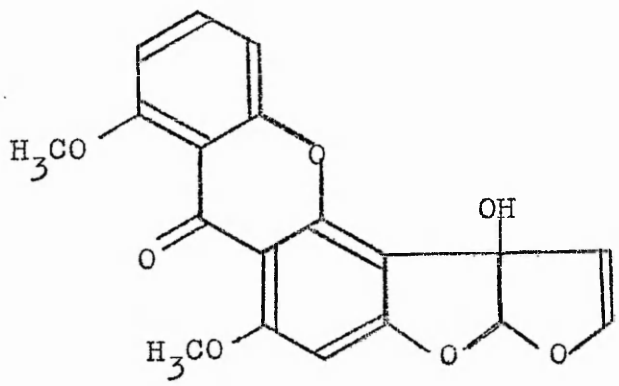


(IX) Aflatoxin B_3 (Parasiticol)

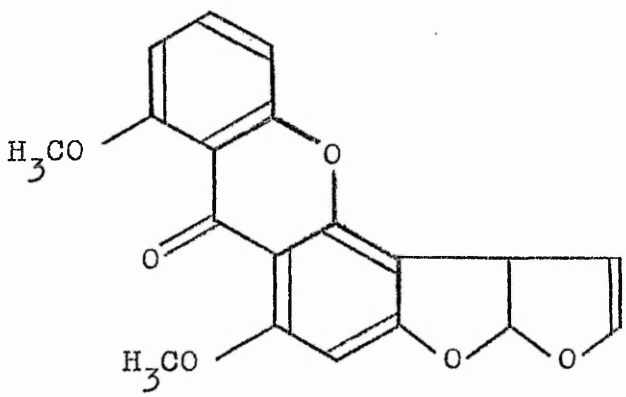


(XII) Sterigmatocystin, R = OCH_3

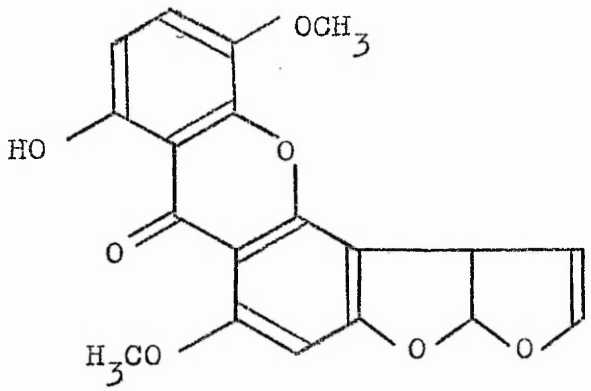
(XV) Demethylsterigmatocystin, R = OH



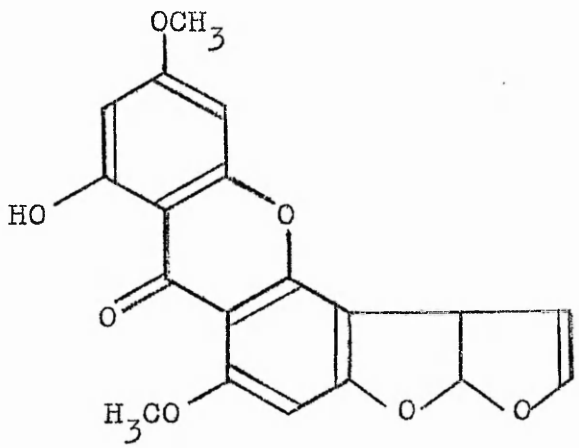
(XI) Aspertoxin



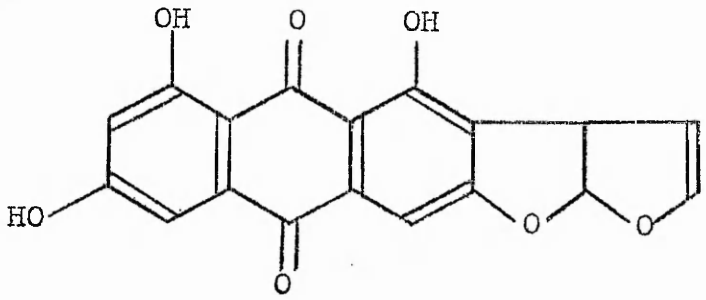
(X) O-methyl sterigmatocystin



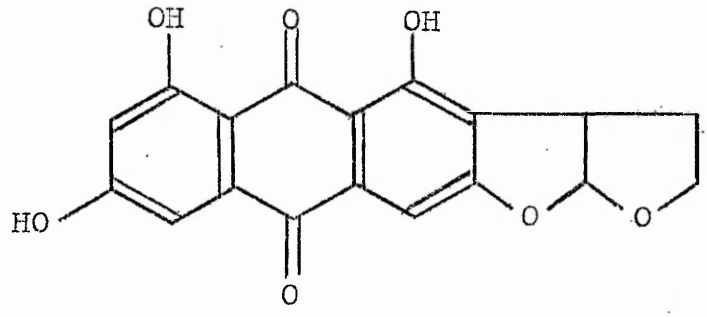
(XIII) 5-methoxysterigmatocystin



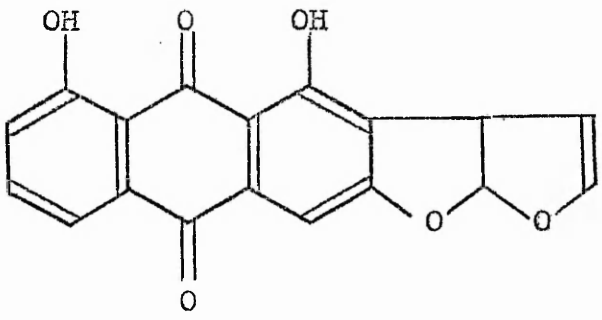
(XIV) 6-methoxysterigmatocystin



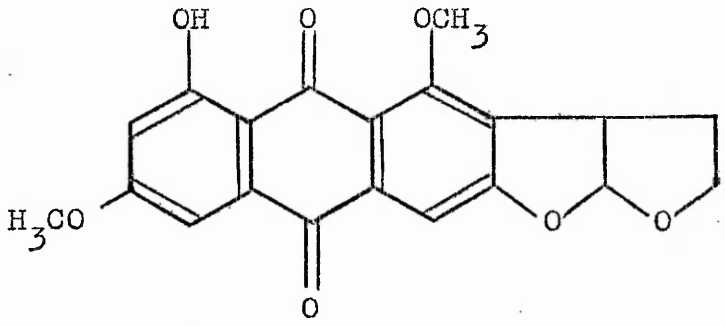
(XVI) Versicolorin A



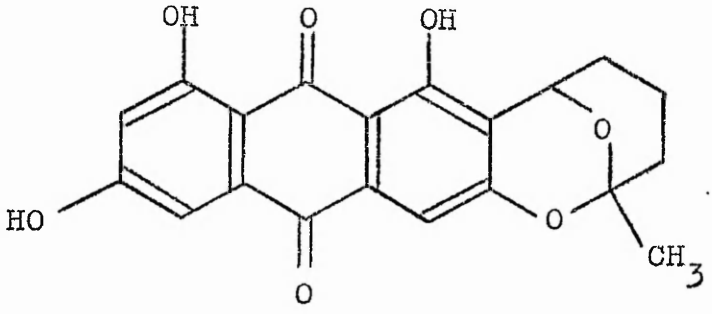
(XVII) Versicolorin B
Racemic form = versicolorin C



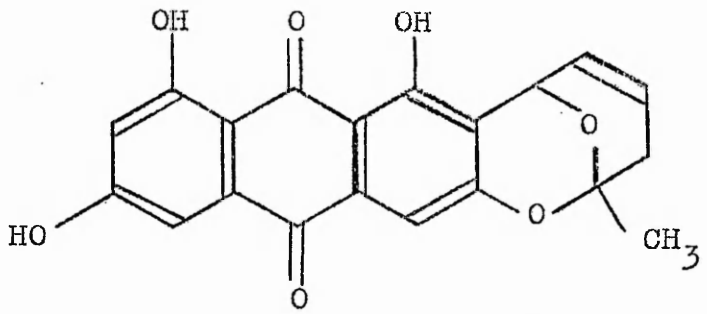
(XVIII) 6-deoxyversicolorin A



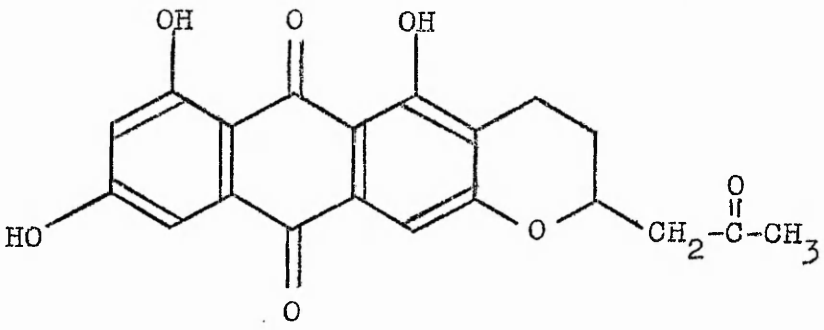
(XIX) Aversin



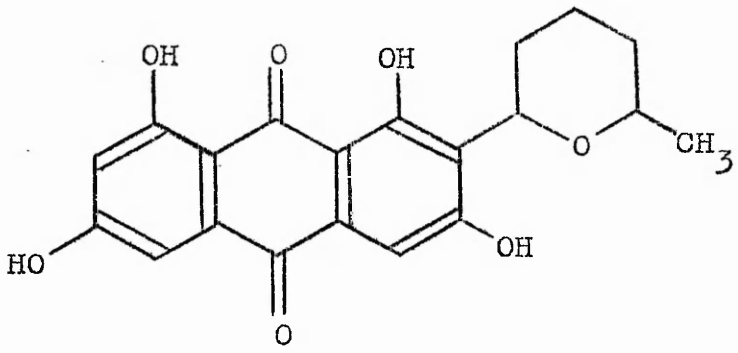
(XXI) Averufin



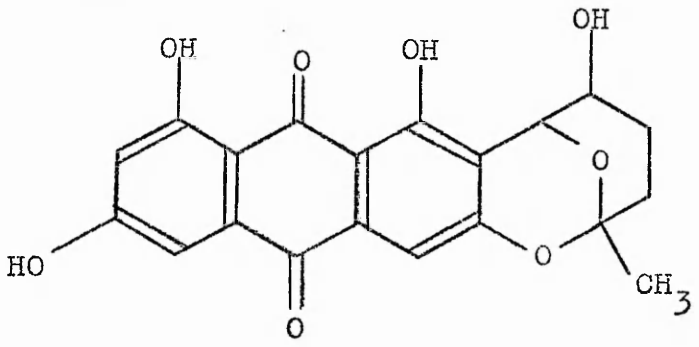
(XXIII) Dehydroaverufin



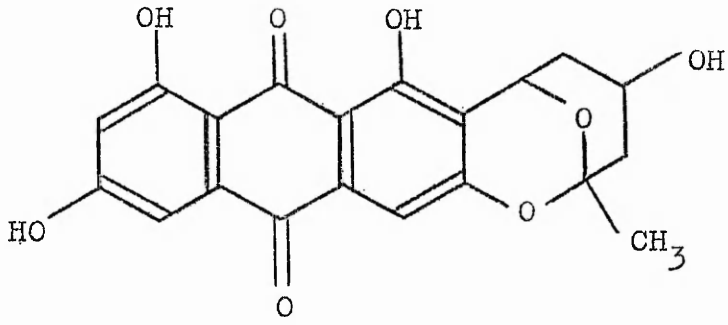
(XXII) Deoxyaverufinon



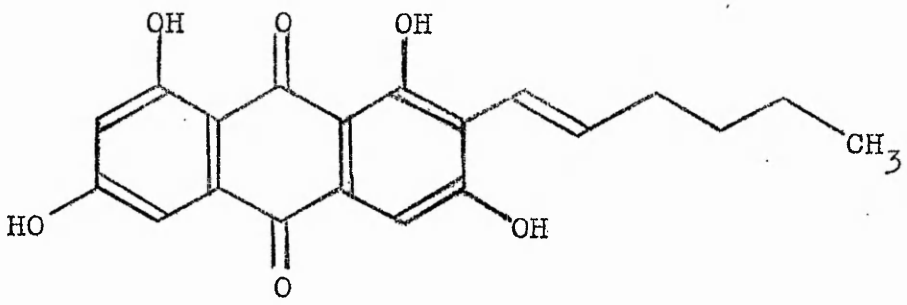
(XXIV) Averufanin



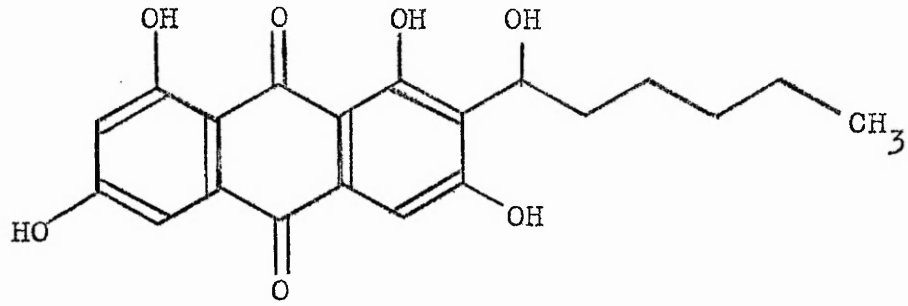
(XXV) Nidurufin



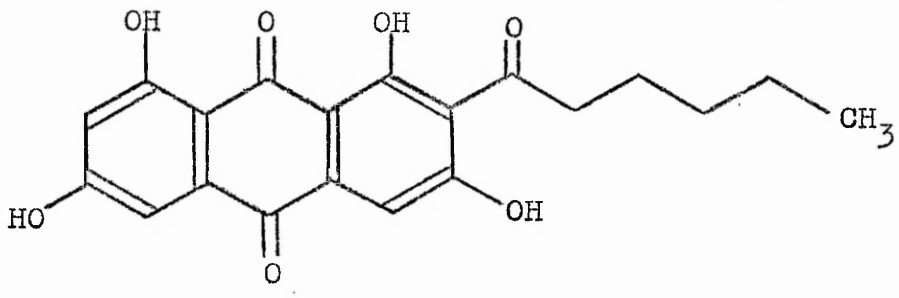
(XXVI) Hydroxy-4, averufin



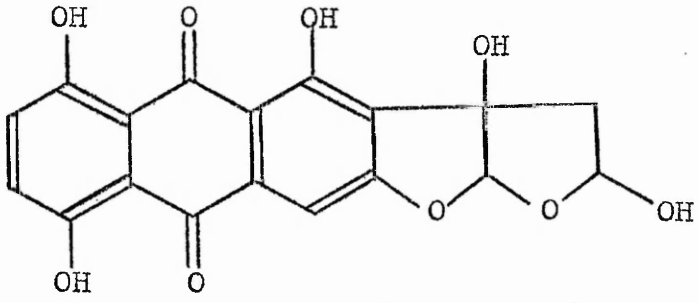
(XXVII) Averythrin



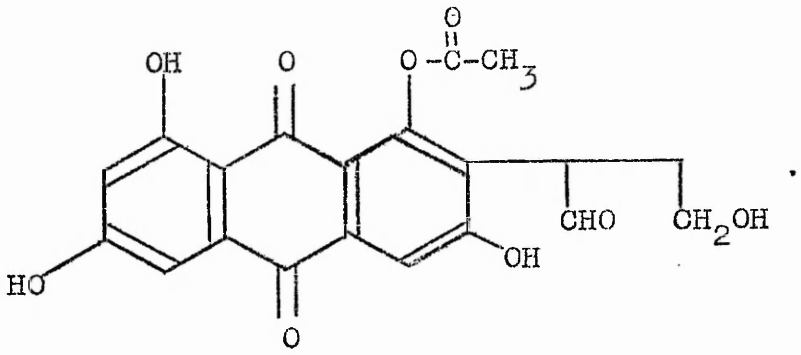
(XXVIII) Averantin



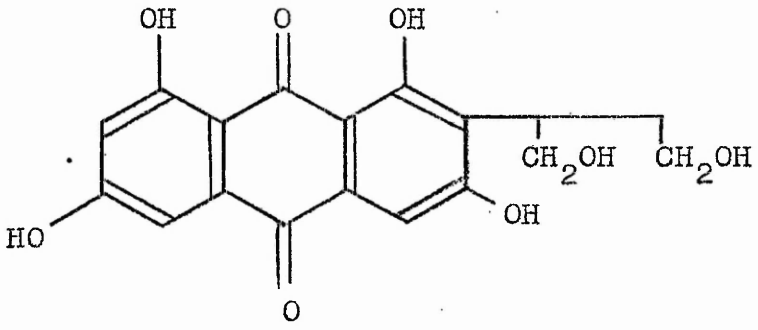
(XXIX) Norsolorinic acid



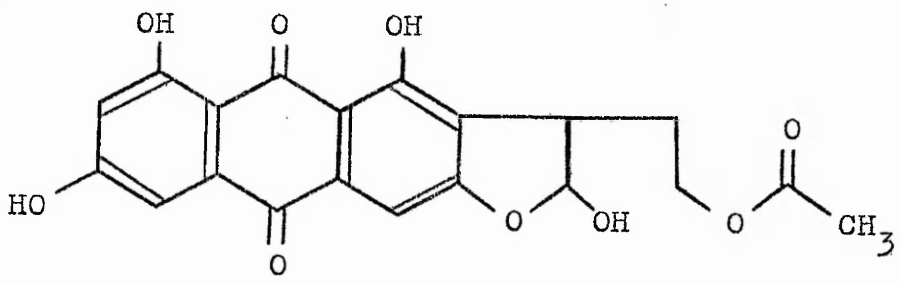
(XXXI) Dothistromin



(XXX) Versiconal acetate
after Schroeder et al 1974



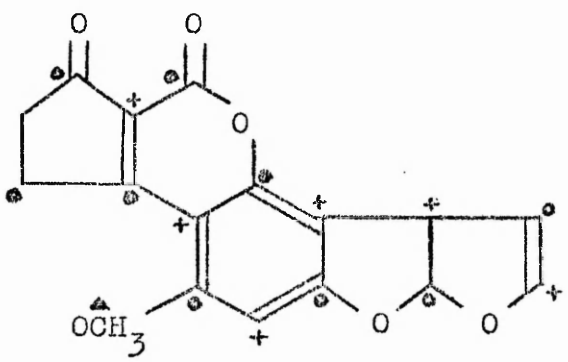
(XX) Versiconol



(XXXII) Versiconal hemiacetal acetate

after Fitzell *et al* 1977

Fig. 1



$^{+}CH_3^{\circ}COOH$

$^{\blacktriangle}CH_3SCH_2CH_2CH(NH_2)COOH$

Labelling pattern of Aflatoxin B_1 derived from acetate and methionine (Biollaz et al, 1970)

(XXIII), averufanin (XXIV), nidurufin (XXV), hydroxy-4, averufin (XXVI) (Berger and Jadot 1976). Also included are averythrin (XXVII) (Roberts and Roffey 1966), averantin (XXVIII) (Roffey *et al* 1967) and norsolorinic acid (XXIX) (Lee *et al* 1971).

Two other anthraquinones should also be mentioned in this section. Versiconal acetate (XXX) first described by Schroeder *et al* (1974), but later renamed versiconal hemiacetal acetate (XXXII) by Fitzell *et al* (1977) and secondly, dothistromin (XXXI), a compound which also possesses a fused bisfuran moiety, has been isolated from *Dothistroma pini* (Bassett and Buchanan 1970) a fungus totally unrelated to *Aspergillus* species.

Biosynthesis of the aflatoxins

It was initially thought that shikimic acid was involved in the biosynthesis of the aflatoxins (Adye and Matales 1964) while Moody (1964) implicated mevalonate in their biosynthesis.

The polyacetate hypothesis for aflatoxin biosynthesis is strongly supported by the incorporation of labelled acetate into the aflatoxins by the resting cell cultures of *A. flavus* used for preparing aflatoxins with high specific activity (Donkersloot *et al* 1968). Subsequent chemical degradation studies of the radioactive aflatoxin B_1 suggested that nine labels in the aflatoxin B_1 molecule were derived from

(1-^{14}C) acetate and seven labels from (2-^{14}C) acetate (Biollaz et al 1968 a, b, 1970). The methoxy carbon of aflatoxin B_1 was found to be derived exclusively from the methyl group of methionine (Fig. I). The activity of the labelled carbon atoms is equal throughout the molecule, suggesting the intermediacy of a single polyacetate chain. A hypothetical pathway for the biosynthesis of the aflatoxins consonant with the labelling pattern was proposed by Biollaz et al (1970) and Hsieh (1969). Here an acetate-polymalonate derived polyhydroxynapthacene underwent oxidation, rearrangement and isomerization to produce versicolorin A (XVI), methylation of which gave aversin (XIX). Oxidative cleavage of aversin gave sterigmatocystin, which then methylated through O-methylsterigmatocystin (XII) and demethylation to aflatoxin B_1 (I). However, findings by Lin & Hsieh (1973) show that averufin (XXI), a C_{20} polyketide, can be readily converted into aflatoxin B_1 by A. parasiticus, indicating that the biosynthesis of aflatoxin B_1 involves a C_{20} rather than a C_{18} intermediate. Evidence for the biogenetic origin of the bisfuran ring structure using ^{13}C NMR analysis of aflatoxin B_1 derived from ^{13}C averufin by Hsieh et al (1976a) implies that averufin is a direct precursor of aflatoxin B_1. Five hypothetical mechanisms explaining the conversion of averufin into aflatoxin B_1 have been proposed to date. Thomas (1965) proposed a pathway in which sterigmatocystin is derived from averufin and subsequently converted to aflatoxin. However, he revised this scheme (reviewed by Moss 1972) deriving the C_4 bisfuran ring from averufin

through an acetylated furan intermediate (Fig. II). Tanabe et al (1976) suggest a Favorskii rearrangement involving a cyclopropanone intermediate (Fig. III). Gorst-Allman et al (1977) using ^{13}C averufin propose the involvement of an epoxide intermediate which undergoes rearrangement to versiconal hemiacetal acetate (Fig. IV). Kingston et al (1976) suggest that the key rearrangement step involves a pinacol-type rearrangement of the open chain form of nidurufin (Fig. V). In their identification of versiconal hemiacetal acetate Fitzell et al (1977) suggest removal of two terminal carbons from a rearranged open chain form of averufin; this is followed by addition of an acetyl group (Fig. VI).

Berger et al (1976 a) have implied averufin, versicolorin A and sterigmatocystin to be involved in aflatoxin biosynthesis, while Berger et al (1976 b) have isolated dehydroaverufin (XXIII) which, along with hydroxy-4 averufin (XXVI), they have implicated in the biosynthesis of averufin.

Conversion of certain radioactively labelled intermediates by resting cell cultures has provided evidence for an "orange pigment tentatively identified as versiconal acetate" (XXX) (Yao & Hsieh 1974), versicolorin A (Lee et al 1976) and sterigmatocystin (Hsieh et al 1973a) being involved in the biosynthesis of the aflatoxins. Singh & Hsieh (1977) have used this technique, involving several mutants and the inhibitor dichlorvos, to verify these results (Fig. VII).

Fig. II

A possible mechanism for deriving the difuran ring system from the C_6 side chain in Averufin (Moss, 1972)

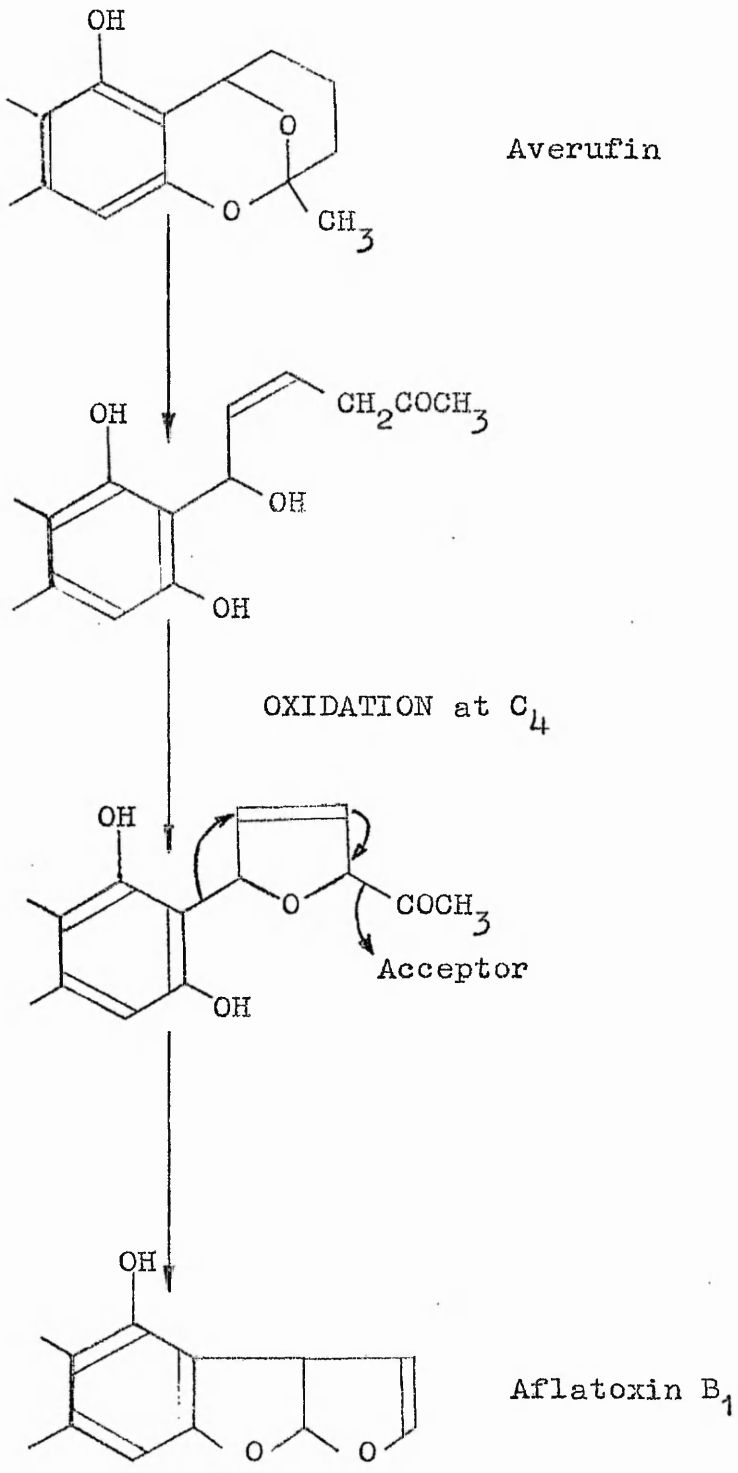
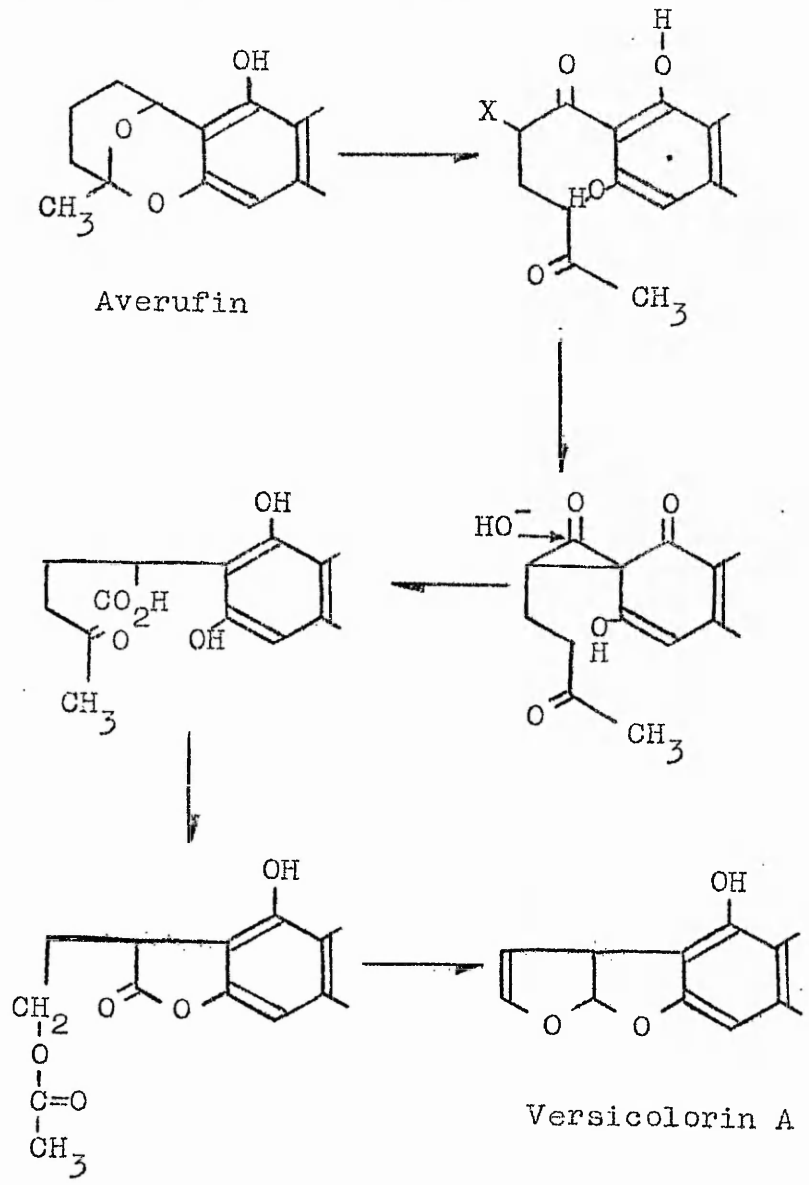


Fig. III

Versicolorin A formation from Averufin (Tanebe et al 1976)



X = Pyrophosphate

Fig. IV

Versiconal acetate formation from Averufin (Gorst-Allman et al 1977)

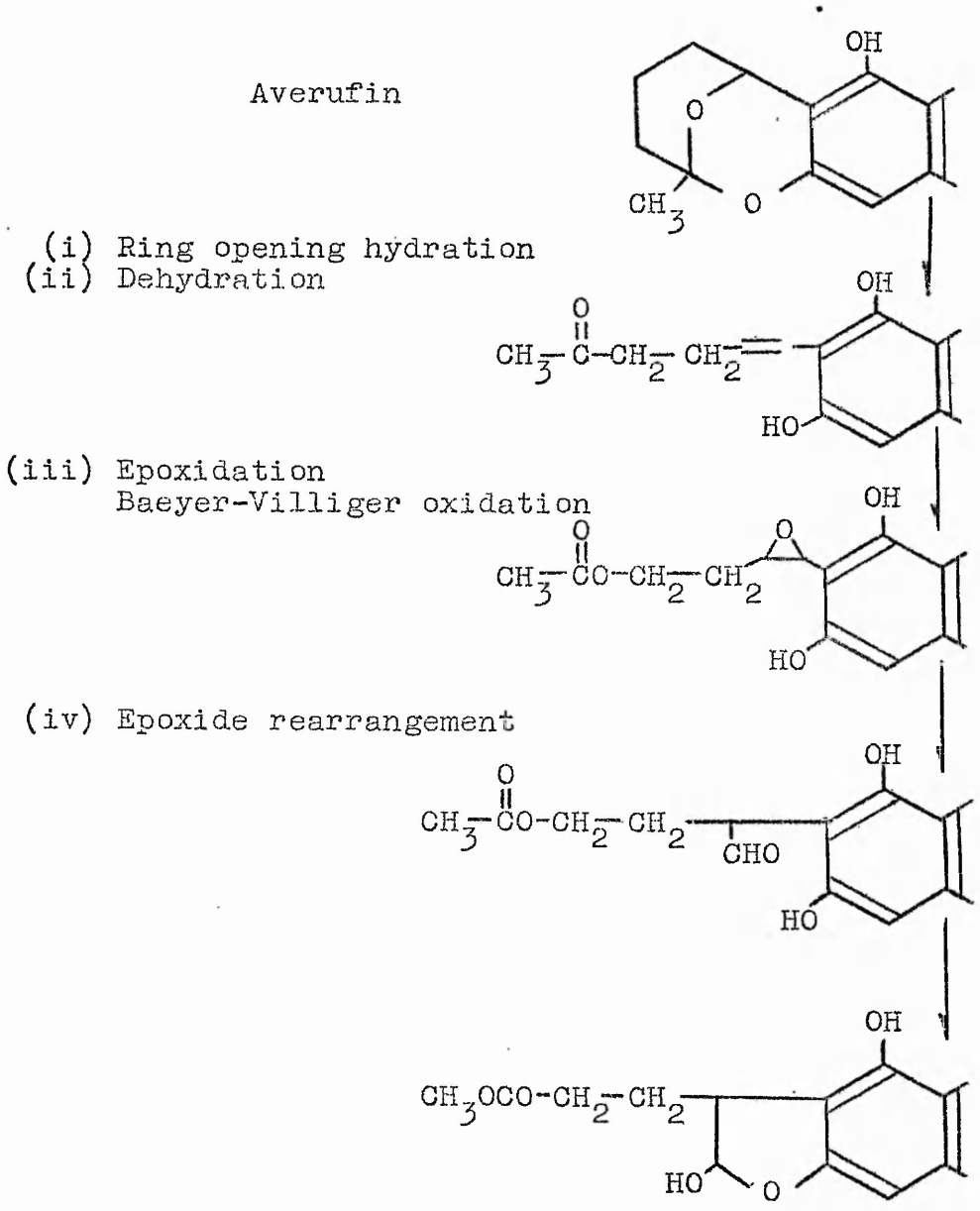
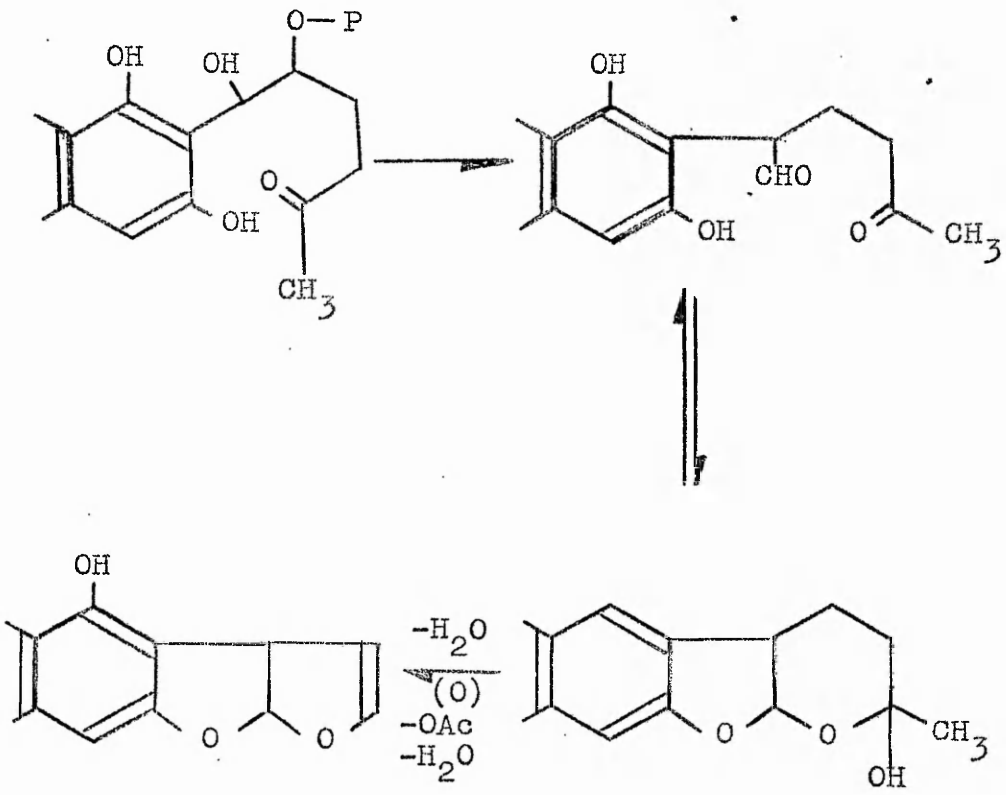


Fig. V



Aflatoxin B_1 formation from Nidurufin

(Kingston et al 1976)

Fig. VI

Versiconal hemiacetal formation from Averufin (Fitzel et al 1977)

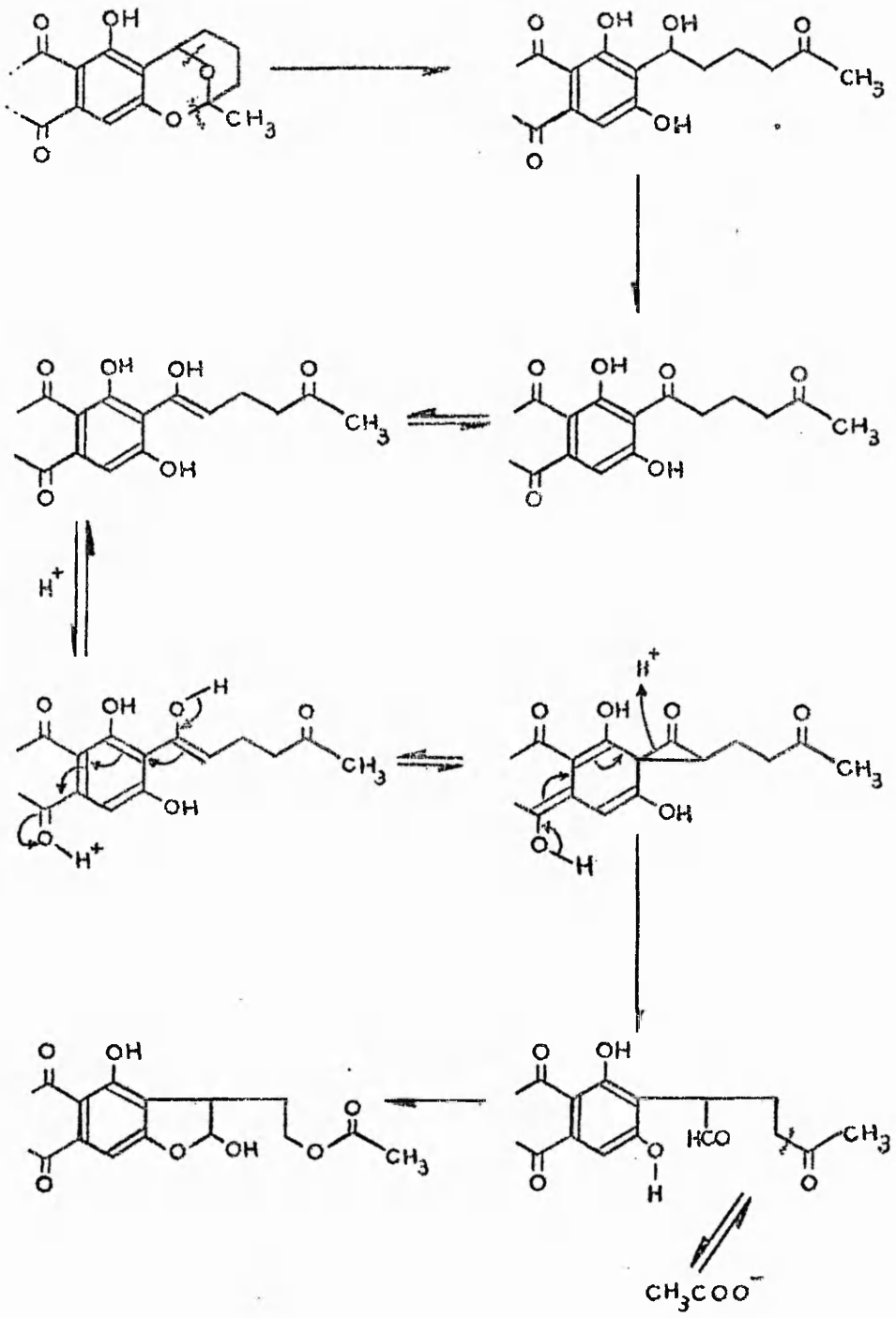
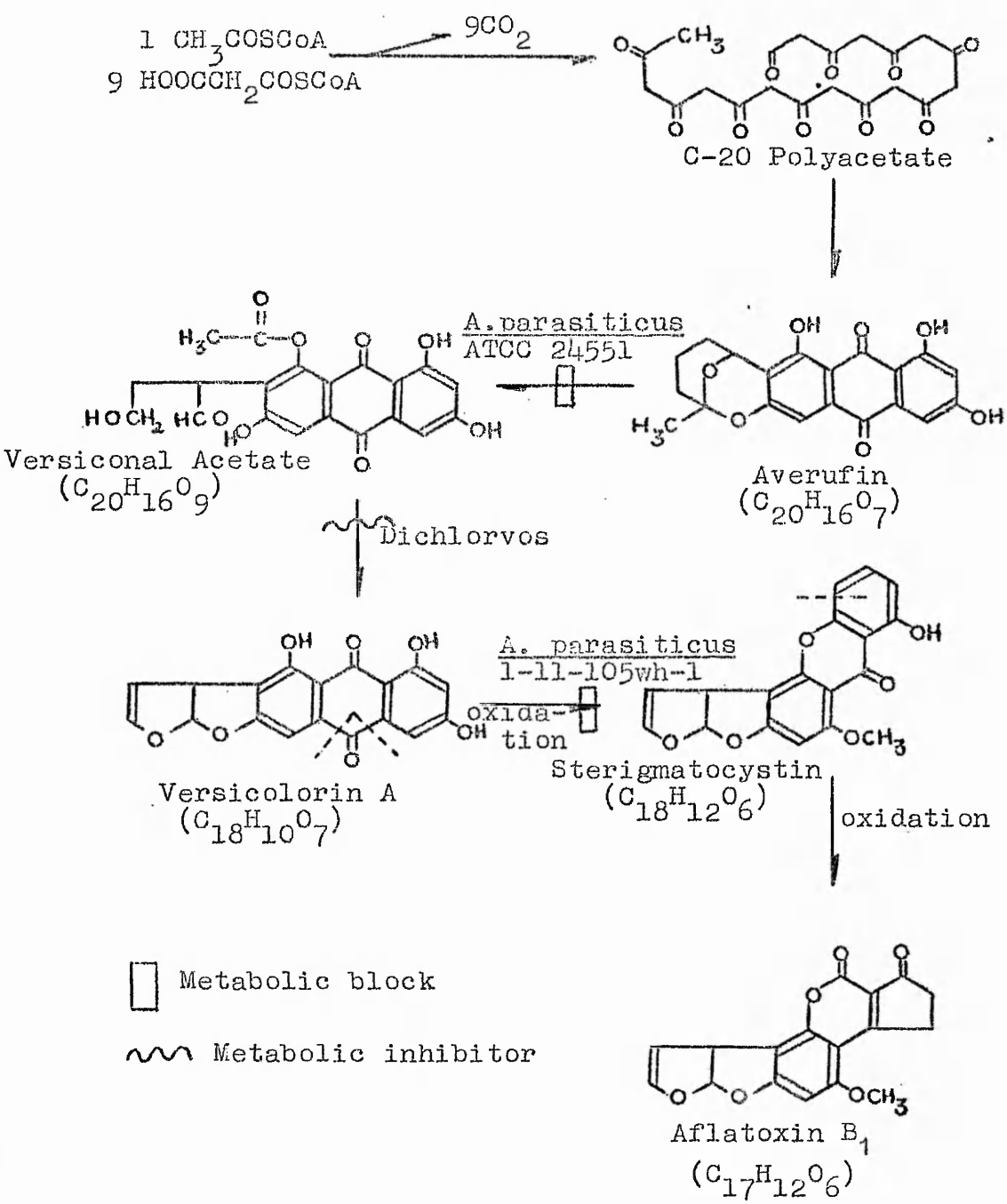


Fig. VII



Aflatoxin formation using replacement cultures

Singh & Hsieh (1977)

The biosynthesis of secondary metabolites using a cell-free system

Although many cell-free systems have been successfully employed in the studies of primary metabolic processes, excellent reviews of which are given by Gunsalus (1955), Stine *et al* (1964) and Sansing *et al* (1975), similar techniques of preparation have found limited application in the study of secondary biosynthetic systems. A considerable amount of work has been done on the preparation of cell-free extracts from fungi with an aromatic synthetic capacity; this has generally centred around *P. patulum*, an organism which excretes 6-methylsalicylic acid and related substances. Lynen and Tada (1961) obtained a soluble extract that was effective in synthesizing 6-methylsalicylic acid from acetyl coenzyme A and malonyl Co A in the presence of NADPH. A close analogy to fatty acid synthesis, therefore, led Lynen to speculate that a multi-enzyme complex, to which the intermediates would be bound by thioester linkage, was involved (Lynen 1961). From the same organism has been isolated a hydroxylase capable of hydroxylating m-hydroxy-benzyl alcohol to gentisyl alcohol (Murphy & Lynen 1975), while Scott & Beadling (1974) have isolated a dehydrogenase and a dioxygenase capable of catalysing the conversion of gentisyl alcohol through its aldehyde to patulin. Cell-free systems have been isolated from *Penicillium lilacinum* capable of transforming steriods by side chain cleavage oxidoreductases (Carlstrom 1974 a) and acyl transfer esterases

(Carlstrom 1974 b). Attempts have also been made to compare electrophoretically the esterase complement of aflatoxin and non-aflatoxin producing strains of A. flavus. Other investigators (Gatenbeck and Hermodsson 1965, Gaucher and Shepherd 1968) have succeeded in isolating cell-free systems capable of synthesizing orsellinic acid and alternariol, phenols derived exclusively from acetate.

Raj et al (1969) have reported acetate, mevalonate and leucine to be incorporated into aflatoxins by the mitochondrial fraction from Aspergillus flavus. Yao (1974), using a Polytron, suggested that the enzymes responsible for aflatoxin biosynthesis were located in the mitochondrial fraction of his cell-free extract. The polytron method of cell-free extract production enabled Yao to show active conversion of averufin, sterigmatocystin, acetate and malonate into aflatoxin B_1. Singh and Hsieh (1976) have prepared a cell-free extract capable of converting ^{14}C-sterigmatocystin into aflatoxin B_1 in the presence of NADPH, thus indicating the involvement of an oxygenase. Conversion of the sterigmatocystin occurred in the cytoplasmic fraction.

Protoplasts

The term protoplast has been used since 1953 by Weibull and other workers to designate the structure remaining when the cell wall was removed from a bacterial cell. Since then the word has been used to denote equivalent forms in fungi (McQuillen 1960). The accepted criteria for defining protoplasts are loss of rigidity of a fungal cell resulting in a spherical form which is osmotically fragile (Villanueva 1966).

The methods used for the isolation of fungal protoplasts can be divided into three major groups. The first involving the use of mechanical or autolytic methods; the second based on the use of enzymes to dissolve the cell wall and the third making use of specific inhibitors of cell wall synthesis. In recent years nearly all fungal protoplasts have been produced using enzymes to dissolve away the cell wall. These enzymes can be extracted from various organisms, e.g. Streptomyces sp. (Gascon & Villanueva 1963), Trichoderma sp. (DeVries & Wessels 1972).

Due to the lack of external protection, protoplasts will immediately lyse during the process of release unless the osmotic concentration is adjusted, consequently, a stabilizing medium has to be used in their preparation. The formulation, concentration and pH of various stabilizers differs from species to species and excellent reviews have been published

by Villanueva and Garcia (1971) and Peberdy *et al* (1976).

Protoplasts have been used to investigate cytoplasmic membranes (Garcia, Mendoza & Villanueva 1967), mitochondria (Duell *et al* 1964), cell wall regeneration (Svoboda *et al* 1969), fusion (Anne & Peberdy 1976) and mode of action of antibiotics (Rodriguez Aguirre 1965). Fawcett and co-workers (1973) have investigated protoplasts of *Penicillium chrysogenum* and *Cephalosporium acremonium* for the production of penicillin N and cephalosporin C. Their findings showed that antibiotic production by the protoplasts did not significantly differ from that of whole mycelium.

Lysates of protoplasts, produced by diluting the suspension medium, can be used for investigating sub-cellular particles and enzymes which sometimes cannot be detected in cell-free extracts prepared by other methods (Gascon *et al* 1965).

Aims of the Experimental Work

The major aim of this project is the preparation of "cell-free" systems from *Aspergillus* species capable of aflatoxin biosynthesis, and investigation of the enzymes involved in this synthesis. In order to attain this objective the experimental work was carried out in three phases:-

1. To isolate and identify intermediates of aflatoxin biosynthesis and to use them as substrates in subsequent enzymatic studies.

2. To investigate and develop various techniques for their ability to produce a cell-free extract capable of converting intermediates to the aflatoxins.

3. To select one specific technique and use it to prepare and study enzymes involved in aflatoxin biosynthesis.

It was intended that this programme of work would contribute to an increase in the understanding of the mechanisms of aflatoxin biosynthesis, as well as the development of a routine reproducible method of preparing enzymes suitable for studying fungal secondary metabolism in general.

E X P E R I M E N T A L

S E C T I O N I

General Methods

Materials (I i)

The following Analar grade chemicals were used throughout:-

Ammonium molybdate, Ammonium sulphate, Calcium chloride dihydrate, Chloroform, Copper sulphate quinhydrate, Disodium tetraborate decahydrate, Di Potassium hydrogen orthophosphate, 2,5-diphenyloxazolylbenzene (P.P.O.), Ethanol, Ferrous sulphate septahydrate, L-Asparagine, Magnesium sulphate septahydrate, Manganous chloride quadrahydrate, Manganous sulphate quadrahydrate, Methanol, Potassium dihydrogen orthophosphate, Potassium chloride, Sodium lauryl sulphate, Sodium sulphate anhydrous, Sucrose, Zinc sulphate septahydrate

All the above-named chemicals were supplied by BDH.

Other chemicals were technical grade:-

Acetone, Chloroform (containing ethanol about 2% v/v), Ethyl acetate, Methanol, Toluene. Bacto Agar (Difco 014001), Bactopeptone (Oxoid L37), Malt extract (Difco 018601), Potato dextrose agar (PDA) (Difco 001301), Yeast extract (Difco 012701). Keiselgel 'G' nach Stahl. Dichlorvos (50g/100ml) supplied by Bayer, U-^{14}C Sodium acetate 58mCi/mMol, 50uCi supplied by the Radiochemical Centre, Amersham.

Culturing methods (I ii)

The following fungi have been used in the present studies:-

Aspergillus flavus N1, a single spore isolate of CMI91019b, which is capable of producing aflatoxins B_1, B_2, G_1, G_2, M_1, M_2, GM_1 and GM_2. When the insecticide dichlorvos is added (20mg/l) to a growing culture of this strain, "versiconal acetate", versicolorin C and a "versiconol like" pigment are produced.

Aspergillus flavus W49, kindly supplied by Dr Donkersloot, which is capable of producing averufin.

Aspergillus flavus F72338, kindly supplied by Dr Schroeder, which is capable of producing O-methyl sterigmatocystin.

Aspergillus parasiticus J8E, kindly supplied by Dr Ciegler, which is capable of producing norsolorinic acid.

Aspergillus parasiticus 1-11-105 Wh1, kindly supplied by Dr Bennett, which is capable of producing versicolorin A.

Dothistroma pini WN196, kindly supplied by Victoria University, Wellington, New Zealand, which is capable of producing dothistromin.

Trichoderma viridae (CBS 354-33), kindly supplied by Dr J. Peberdy.

All cultures of Aspergillus species were maintained on PDA. Dothistroma pini was maintained on malt extract and sucrose (8% sucrose w/v). Trichoderma viridae was maintained on TLE medium containing glucose (4% w/v) and bactoagar (4% w/v).

Using the normally accepted aseptic techniques, the maintenance media (50ml* in a 250ml medical flat) were inoculated with spores of the required culture by means of a bacteriological loop. All cultures were maintained at 25° until sporulation had occured (3 to 5 days); after a further 5 days at 25° they were transferred to a cold room (4° to 8°) for storage until required.

Spore suspensions were prepared by adding a sterile 0.1% solution of sodium lauryl sulphate (10ml) to the required sporulated culture in its medical flat. Following shaking, the suspension was ready for use in experimentation.

Preparation of Chromatoplates for Thin Layer Chromatography(TLC) (I iii)

Chromatoplates (20 x 20cm and 40 x 20cm) were prepared by spreading with a slurry of Keiselgel 'G' (30g) and water (about 70ml). This amount of slurry coated either four 20 x 20cm or two 40 x 20cm plates at a thickness of 0.3mm. Plates were spread upon a TLC Coater (Baird and Tatlock (London) Ltd.)

Total Aflatoxin assay (I iv)

Culture fluid (50ml) was extracted in a 250ml separating funnel with chloroform (50ml). After shaking the two phases together, the chloroform layer was separated and collected; this process was then repeated using ethyl acetate (50ml).

* ml used in preference to cm^3 as glassware was graduated in ml.

The chloroform and ethyl acetate fractions were then dried over anhydrous sodium sulphate for 5 minutes, the solvent fraction being separated from the anhydrous sodium sulphate by passing the mixture through a sintered glass filter, (Pyrex porosity 3). The anhydrous sodium sulphate remaining in the filter was washed, firstly with chloroform (10ml), then with ethyl acetate (10ml) and finally with methanol (10ml) in order to remove any aflatoxins that may have adsorbed to the solid. The solvent fraction, containing the required aflatoxins, was then concentrated in vacuo until no solvent remained visible. Chloroform (5ml) was added to the solvent-free fraction and the optical density of this extract was then determined at 363nm in a 1cm silica-glass cell, the extract being suitably diluted with chloroform where the optical density was too high to record. The spectrophotometer used throughout for assay procedure was a Unicam SP500 which had been standardized against potassium chromate solution (see Appendix 1). The concentration of total aflatoxins was calculated from the optical density by using the molar extinction coefficient of aflatoxin B_1 (Nabney and Nesbitt 1965, ε = 22,000) and the following equation :- at 363 nm.

$$\frac{\text{absorbance.} \times \text{MWt}}{\varepsilon \times P} = \text{mg/ml}$$

where absorbance = Optical density

MWt = Molecular weight

ε = Molar extinction coefficient

P = Path length of light

Recovery experiments from uninoculated culture fluid with known amounts of added aflatoxin showed an accuracy of 95% (see Table I).

Assay of individual Aflatoxins (I v)

A known volume (0.5ml) of concentrated aflatoxin extract in chloroform was taken and 100µl of this spotted onto a chromatoplate (20 x 20cm) of Keiselgel 'G'. The chromatoplate was then developed in chloroform : methanol (97 : 3 v/v) in an unequilibrated TLC tank until the solvent front was 1cm from the top of the chromatoplate. After air drying, the chromatoplate was then viewed under ultra-violet light (253 and 365nm) and the fluorescent spots, due to the various aflatoxins, were located and marked with a scribe.

The spots of Keiselgel 'G', containing the different aflatoxins, were then individually scraped from the chromatoplate. Each aflatoxin was eluted from its Keiselgel 'G' with a small volume of methanol, by filtering through a sintered glass filter (porosity 3) and washing, also with methanol (total not exceeding 5ml). Each aflatoxin was then made up to a known volume and its optical density determined, from which was calculated the concentration of aflatoxin using the equation described in I iv.

TABLE IA

Recovery of Aflatoxin added to uninoculated culture medium*

Aflatoxin investigated	Experiment	Amount added per litre mg	Amount recovered mg	Recovery %
	A	4.0	3.8	95.0
B_1	B	10.0	9.5	95.6
	C	20.0	18.9	94.6

* anhydrous sodium sulphate fully washed

All results in this thesis are expressed as an average of two experiments, unless otherwise stated. (The "average" results are reproducible).

TABLE IB

Recovery of Aflatoxin added to uninoculated culture medium*

Aflatoxin investigated	Experiment	Amount added per litre mg	Amount recovered mg	Recovery %
	A	4.0	2.7	67.5
B_1	B	10.0	7.0	70.0
	C	20.0	13.6	68.2

* anhydrous sodium sulphate not washed

Precautionary Measures (I vi)

Any work involving aflatoxins, due to their highly toxic and carcinogenic properties, was carried out with the following safety measures:-

Solutions containing aflatoxins were never openly evaporated but always concentrated in vacuo.

Strong alkali (sodium hypochlorite) was always made available to eliminate any spilt aflatoxins.

During operations connected with aflatoxins rubber gloves were always worn.

No large quantity (greater than 1mg) of any pure aflatoxin was ever handled or stored.

TLC plates possessing aflatoxin "spots" were disposed of by soaking the silica off in a solution containing sodium hypochlorite (10%) for one week.

All glassware, whether contaminated with aflatoxin or not, was washed in a commercially available detergent "Serve". This is a quaternary ammonium compound and a strong alkali. Safety glasses and dust mask were also worn.

Cultivation of Aspergillus flavus N1 over 13 days in 250ml conical flasks containing a chemically defined medium (I vii)

A standard, chemically defined medium (Reddy et al 1971) was used to examine the production of mycelium and metabolites by A. flavus (N1).

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

The above chemicals were made up to 1 litre with distilled water.

The medium was dispensed in portions (49ml) into 250ml conical flasks, which were then plugged with cotton wool and sterilized by autoclaving (15psi 15 minutes). All flasks were inoculated with a spore suspension of A. flavus N1 (1ml - approximately 12 x 10^6 spores) and incubated on a rotary shaker (MkV Orbital shaker, L.H. Engineering Co., Stoke Poges, Bucks.) at 25° (150 revs/minute).

After various time intervals, the mycelia were harvested by filtration through a weighed filter paper on a Buchner funnel. After washing, the mycelium and filter paper were dried at 80° to a constant weight; subtraction of original weight of filter paper gave mycelial dry weight. The culture fluid was assayed for total aflatoxin content and the results are expressed in Fig. I and Table 2.

Cultivation of A. flavus (N1) over 55 hours in 250ml conical flasks containing a chemically defined medium (I viii)

Experiment a) was repeated over a different time period (55 hours) using a different spore inoculum (16×10^6 spores). The results are expressed in Fig. 2 and Table 3.

Fig. 1

Growth and aflatoxin production by Aspergillus flavus N1 over 13 days on Reddy's medium

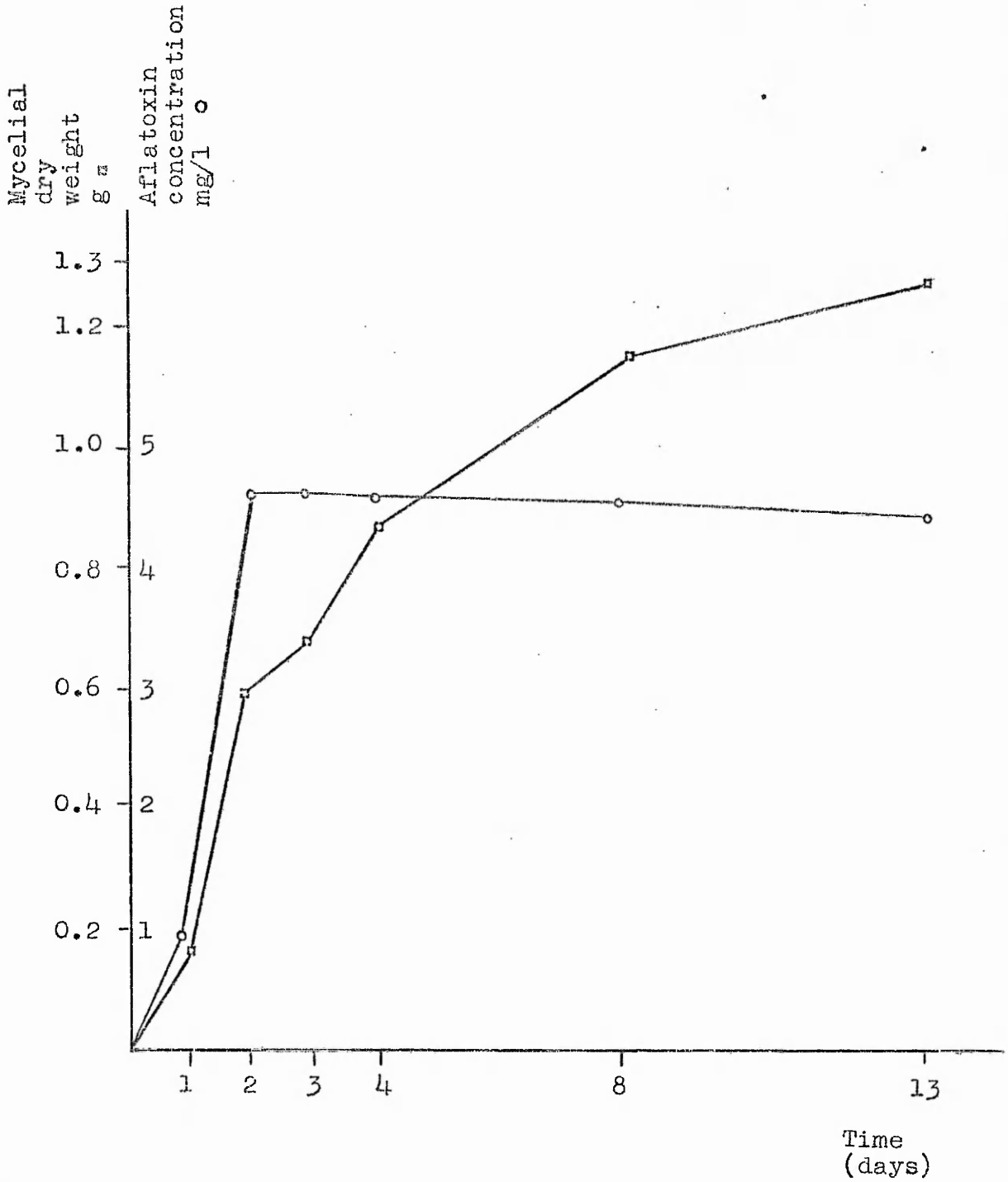


TABLE 2

Growth and Aflatoxin production by A. flavus N1 over 13 days on Reddy's medium

Incubation period (Day)	Mycelial dry weight (g)	Aflatoxin concentration (mg/l)
0	0	0
1	0.13	0.55
	0.14	0.55
	0.13	0.53
	0.14	0.55
	Mean = 0.135	Mean = 0.545
	S = 0.005	S = 0.01
2	0.529	4.55
	0.58	4.6
	0.58	4.6
	0.60	4.65
	Mean = 0.5875	Mean = 4.6
	S = 0.09	S = 0.04
3	0.67	4.59
	0.67	4.59
	0.67	4.59
	0.67	4.6
	Mean = 0.67	Mean = 4.5925
	S = 0	S = 0.005
4	0.87	4.6
	0.89	4.6
	0.88	4.6
	0.86	4.59
	Mean = 0.875	Mean = 4.5975
	S = 0.01	S = 0.005
8	1.09	4.55
	1.27	4.75
	1.06	4.5
	1.11	4.55
	Mean = 1.1325	Mean = 4.5875
	S = 0.09	S = 0.11
13	1.31	4.7
	1.27	4.5
	1.33	4.7
	1.12	3.8
	Mean = 1.2575	Mean = 4.425
	S = 0.09	S = 0.43

S = Standard Deviation

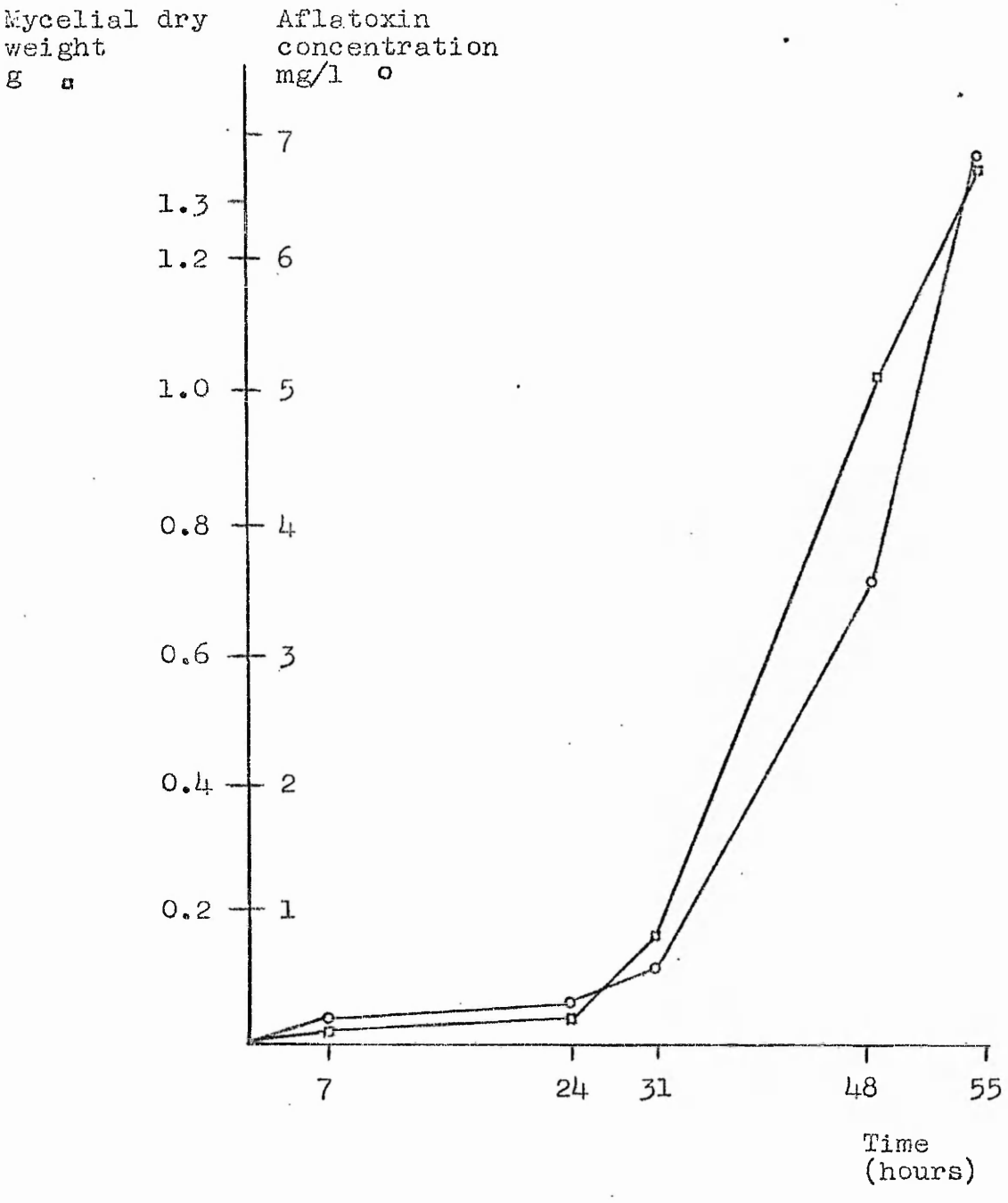
TABLE 3

Growth and Aflatoxin production by A. flavus N1 over 55 hours on Reddy's medium

Incubation period (Hours)	Mycelial dry weight (g)	Aflatoxin concentration (mg/l)
0	0	0
7	0.009	0.16
	0.009	0.16
	0.009	0.16
	0.009	0.155
	Mean = 0.009	Mean = 0.159
	S = 0	S = 0.002
24	0.03	0.21
	0.03	0.21
	0.03	0.21
	0.028	0.202
	Mean = 0.0295	Mean = 0.208
	S = 0.001	S = 0.004
31	0.15	0.57
	0.16	0.575
	0.15	0.57
	0.15	0.57
	Mean = 0.1525	Mean = 0.571
	S = 0.005	S = 0.002
48	1.0	3.65
	1.0	3.6
	0.98	3.45
	1.03	3.7
	Mean = 1.002	Mean = 3.6
	S = 0.02	S = 0.11
55	1.3	6.65
	1.4	6.7
	1.2	6.63
	1.38	6.7
	Mean = 1.32	Mean = 6.67
	S = 0.09	S = 0.03

Fig. 2

Growth and aflatoxin production by Aspergillus flavus N1 over 55 hours on Reddy's medium



Isolation and characterization of metabolites (I ix)

A medium containing yeast extract (2% w/v), sucrose (10% w/v) and agar (0.1% w/v) in distilled water was prepared and portions (350ml) were dispensed into Roux flasks. The flasks were plugged with cotton wool and then sterilized by autoclaving (15psi, 15 minutes). After inoculation with a spore suspension (5ml) of the required strain of fungus, the flasks were incubated as static cultures for 14 days.

The mycelium was then filtered off and dried in an oven at 60^{o}, after which it was exhaustively extracted in a soxhlet apparatus with ethyl acetate. Culture fluids were bulked and extracted repeatedly with ethyl acetate until it was judged that no more metabolites were taken up into the solvent fraction from the aqueous layer. Ethyl acetate extracts were then combined and concentrated in vacuo at room temperature to a volume of about 5ml. The concentrate was then streaked in a line along the origin of a 40x20cm Keiselgel 'G' chromatoplate by means of a glass capillary tube, the line of origin being approximately 2cm from the bottom edge of the chromatoplate. Each chromatoplate was loaded with sufficient concentrate to deposit approximately 20mg of total solids, the ethyl acetate being removed from the base line by a gentle stream of cold air.

Once loaded, the chromatoplates were then developed in a solvent system of ethyl acetate : toluene (1 : 1 v/v) in

unsaturated Shandon chromatotanks until the solvent front almost reached the top of the plate. The chromatoplates were then removed from the tanks and air dried in order to remove solvents. The Keiselgel 'G' zone with the required metabolite, was then scraped from the chromatoplate and the metabolite eluted from the Keiselgel 'G' with ethyl acetate and then acetone. Any other metabolites visible on the remaining Keiselgel 'G' were also scraped off the chromatoplate, eluted and preserved separately for further investigation.

Fractions containing the desired metabolite were then concentrated in vacuo and re-chromatographed using a different solvent system (chloroform : methanol, 97 : 3 v/v) until it was chromatographically pure (usually requiring a further two runs). The relatively pure metabolite, thus obtained, was tested chromatographically for purity using a small sample, the test spot being compared with the Rf value of pure metabolite. A sample of prepared metabolite (2mg) was then examined by mass spectrometry and compared with a spectrograph of the known metabolite.

After comparison of mass spectrometry data of the resultant pure compound with that of authentic compound (data given in Tables 4-11) the pure sample was stored for further experimentation.

TABLE 4

Mass spectral data of prepared Versicolorin A and authentic Versicolorin A

	m/e for the ions produced from prepared Versicolorin A	*m/e for the ions of authentic Versicolorin A
Parent ion	338 S	338 S
Fragmentary ions	325 M/S 310 S 309 S 281 S	

Relative intensities of ions:- S = strong
M = medium

*Lee et al (1975)

TABLE 5

Mass spectral data of prepared Norsolorinic acid and authentic Norsolorinic acid

	m/e for the ions produced from prepared Norsolorinic acid	*m/e for the ions of authentic Norsolorinic acid
Parent ion	370 S	370
Fragmentary ions	327 S 314 S 300 S 299 S 272 M/S	

Relative intensities of ions:- S = strong
M = medium

* Lee et al (1971)

TABLE 6

Mass spectral data of prepared O-me-sterigmatocystin and authentic O-me-sterigmatocystin

	m/e for the ions produced from prepared O-me-sterigmatocystin	* m/e for the ions of authentic O-me-sterigmatocystin
Parent ion	338	338
Fragmentary ions	-	-

* Burkhardt & Forgacs (1968)

TABLE 7

Mass spectral data of prepared Versicolorin C and authentic Versicolorin C

	m/e for the ions produced from prepared Versicolorin C	* m/e for the ions of authentic Versicolorin C
Parent ion	340 S	340 S
Fragmentary ions	325 S	325 S
	311 S	311 S
	297 S	297 S

Relative intensities of ions:- S = strong
M = medium

* Dutton (1969)

TABLE 8

Mass spectral data of prepared Versiconal acetate and authentic Versiconal acetate

	m/e for the ions produced from prepared Versiconal acetate	*m/e for the ions of authentic Versiconal acetate
Parent ion	382 M/W	382 M
Fragmentary ions	340 S	340 S
	322 S	322 S
	312 S	312 S
	311 S	311 S
	297 S	297 S

Relative intensities of ions:- S = strong
M = medium
W = weak

* Schroeder et al (1974)

TABLE 9

Mass spectral data of prepared Averufin and authentic Averufin

	m/e for the ions produced from prepared Averufin	* m/e for the ions of authentic Averufin
Parent ion	368 S	368 S
Fragmentary ions	350 S	350 S
	325 S	325 S
	311 S	311 S
	310 S	310 S
	297 S	297 S

Relative intensities of ions:- S = strong
M = medium

* Donkersloot et al (1972)

TABLE 10

Isolation of Metabolites from
Aspergillus flavus M1 plus Dichlorvos

m/e for parent ion	metabolite
370	averufanin
342	unknown (A_2)
368	averufin
340	versicolorin C
382	versiconal hemiacetal acetate
384	unknown (VAc II)
360	"versiconol-like pigment"

TABLE 11

Isolation of Metabolites from
Aspergillus parasiticus 1-11-105 Whl

m/e for parent ion	metabolite
338	versicolorin A
407	unknown (V_1)
410	unknown (V_2)
356	unknown (V_3)

Preparation of radioactively labelled compounds (I x)

A spore suspension (1ml) of the required strain of fungus was inoculated into a 250ml conical flask containing a sterile solution of Reddy's chemically defined medium (49ml). The flask was then incubated at 25° on a rotary shaker (150 rev/min) for 48 hours. The mycelium was then harvested and transferred to a 250ml conical flask containing sterile resting culture medium which was comprised of:-

Potassium dihydrogen orthophosphate	5.0g
Magnesium sulphate septahydrate	0.5g
Potassium chloride	0.5g
Disodium tetraborate decahydrate	0.7mg
Ammonium molybdate	0.5mg
Copper sulphate quinhydrate	0.3mg
Ferrous sulphate septahydrate	10.0mg
Manganous sulphate quadrahydrate	0.11mg
Zinc sulphate septahydrate	17.6mg

The above chemicals were made up to 1 litre with distilled water. (Yao 1974)

U-^{14}C sodium acetate was added to the resting culture medium (1ml = 5μCi). The flask was then re-incubated at 25° on a rotary shaker (100 rev/min) for 72 hours, after which time the mycelium was harvested. The required metabolite was then extracted as described in General Methods section I vii and the pure metabolite obtained by repeated TLC using two

solvent systems, namely ethyl acetate : toluene (1 : 1 v/v) and chloroform : acetone (85 : 15 v/v). The activity of the pure metabolite was then determined by taking a known amount of metabolite (determined spectrophotometrically using the known wavelength and extinction coefficient for that metabolite, followed by evaporating the solvent to dryness in vacuo). See appendix 4 and 5.

The metabolite was then dissolved in scintillation fluid (10ml) and counted in a Tricarb 3300 liquid scintillation counter. Absolute counts were determined using the channels ratio method and corrected for background counts.

S E C T I O N I I

"Cell-free" extracts of Aspergillus flavus

Materials (II i)

In addition to those materials previously described in Sections I and II the following chemicals were used:-

ATP	Supplied by Sigma Chemical Company
NADPH	"
NADH	"
Coenzyme A	"
Sodium acetate	Supplied by BDH
Glucose	"
Methionine	"

Preparation of "cell-free" extracts of Aspergillus flavus N1 (II ii)

In all experiments to prepare a "cell-free" extract, the mycelium of a 3 day old culture of Aspergillus flavus N1 grown in Reddy's chemically defined medium was removed by filtration. The mycelium was washed with buffer (0.2M phosphate buffer pH 7.6) and damp dried by blotting with filter paper. Following preparation of the "cell-free" extract, a protein determination was made on each sample using the Biuret method (see Appendix 2). Results are expressed in Table 1.

To each sample of "cell-free" extract (20ml) was added a "Basal Cofactor Medium" consisting of:-

NADH	2mg	Glucose	50mg
NADPH	2mg	Sodium acetate	50mg

ATP	10mg	Methionine	10mg
Coenzyme A	5mg	(In a final volume of 20ml)	

After addition, the extract was incubated at 25° on a rotary shaker (100 rev/min.). Portions of extract (2ml) were withdrawn at various times and the aflatoxins were extracted as described in Section I. The aflatoxins were estimated spectrophotometrically and by TLC.

Experiment I

Preparation of a "cell-free" extract utilizing liquid Nitrogen

A sample of damp mycelium (10g fresh weight) was placed in a large cold mortar, liquid nitrogen (approximately 100ml) was then poured into the mortar. The frozen mycelium was crushed with the pestle until a white powder was obtained; at no time during the process did the temperature rise above -10°.

The white mycelial powder was removed from the mortar and placed in a cold 100ml beaker. Cold (0°) phosphate buffer (0.2M; pH 7.6 containing glycerol 10% v/v) (20ml) was added to the powder in the beaker and, after mixing, mycelial debris was filtered off using glass wool and then centrifugation (4,000g, 30 minutes).

The resultant supernatant was used as the "cell-free" extract and incubated with basal cofactor medium. Results are expressed in Fig 1.

TABLE I

Protein release from 'cell-free' extracts produced by various techniques

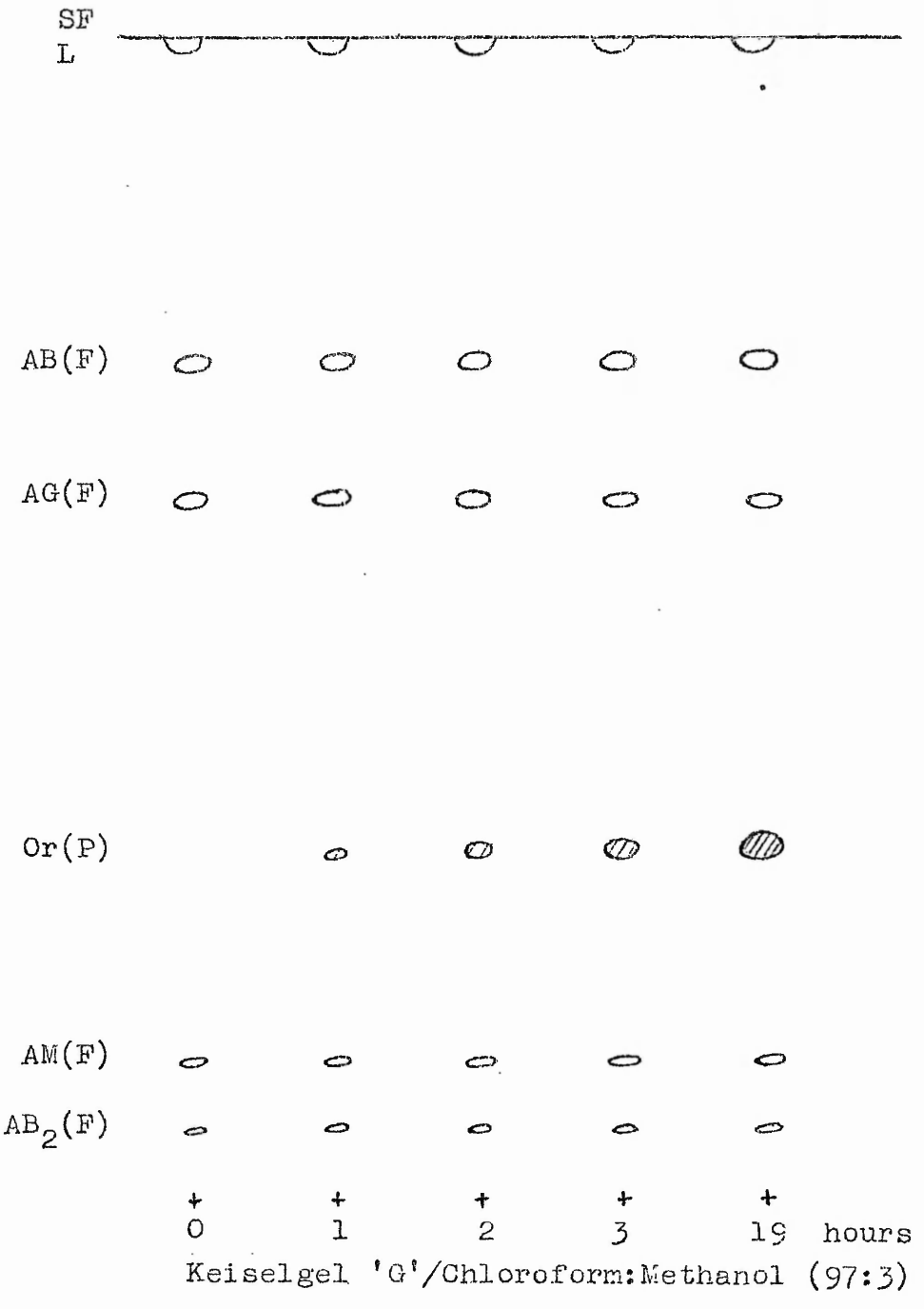
Technique	Protein Release mg/ml
Liquid nitrogen	0.8
Lypholization	0.6
Sonication	0.1
Homogenization	0.1
Stomacher	2.2
Ultraturrax (60 seconds)	1.3

KEY TO CHROMATCGRAPHY DIAGRAMS

AB	=	Aflatoxin B
AB_2	=	Aflatoxin B_{2a} or Aflatoxin M_2
AG	=	Aflatoxin G
AM	=	Aflatoxin M_1
B	=	Blue
(F)	=	Fluorescent under ultra violet light
L	=	Lipids
Or	=	Orange
(P)	=	Stained with Pauly's reagent
SF	=	Solvent front
VAc	=	Versiconal acetate
VC	=	Versicolorin C

Fig. 1

Liquid nitrogen "cell-free" preparation incubated for various time intervals with Basal Cofactor Medium



Experiment II

Preparation of a "cell-free" extract utilizing ultrasonic disintegration

A sample of damp mycelium (10g fresh weight) was suspended in cold (0^{o}) phosphate buffer (0.2M; pH 7.6 containing glycerol 10% v/v) (20ml) and then sonicated (MSE 100 watt Ultrasonic disintegrator) for various time intervals at 8 microns peak to peak using a pointed probe with an end ratio of 7.6 : 1. After sonication, the mycelial debris was filtered off using glass wool and then centrifugation (4,000g for 30 minutes). The resultant supernatant was used as the "cell-free" extract and incubated with basal cofactor medium.

Experiment III

Preparation of a "cell-free" extract utilizing homogenization

A sample of damp mycelium (10g fresh weight) was suspended in cold (0^{o}) phosphate buffer (0.2M; pH 7.6 containing glycerol 10% v/v) (5ml). This mixture was then placed in a cold mortar and to it was added cold, acid washed sand (100g).

After grinding with a pestle, the mixture was filtered through glass wool and centrifuged (4,000g for 30 minutes) in order to remove mycelial debris. The resultant supernatant was used as the "cell-free" extract and incubated with basal cofactor medium.

Experiment IV

Preparation of a "cell-free" extract utilizing a Stomacher

A sample of damp mycelium (10g fresh weight) was suspended in cold (0^{o}) phosphate buffer (0.2M; pH 7.6, containing glycerol 10% v/v) (10ml) and this was then placed in a stomacher bag (50ml). The bag containing mycelial suspension was then placed in a "Stomacher" (A. J. Seward) and "stomached" for 2 minutes. After "stomaching", the contents of the bag were removed and suspended in phosphate buffer (10ml). Mycelial debris was then filtered off using glass wool followed by centrifugation (4,000g for 30 minutes) and the resulting supernatant was used as the "cell-free" extract and incubated with basal cofactor medium.

Experiment V

Preparation of a "cell-free" extract utilizing lypholization

A sample of damp mycelium (10g fresh weight) was placed in a round bottom flask (50ml) which was then fitted onto a freeze drier. The mycelium was freeze dried for 6 hours and the resultant powder removed and dissolved in cold (0^{o}) phosphate buffer (0.2M; pH 7.6, containing glycerol 10% v/v) (20ml). Mycelial debris was then filtered off using glass wool followed by centrifugation (4,000g for 30 minutes). The resulting supernatant was used as the "cell-free" extract and incubated with basal cofactor medium.

Experiment VI(a)

Preparation of a "cell-free" extract utilizing an Ultraturrax for various time periods up to 90 seconds

A sample of damp mycelium (10g fresh weight) was suspended in cold (0^{o}) phosphate buffer (0.2M; pH 7.6, containing glycerol 10% v/v) (20ml). The mycelial suspension was then treated with an Ultraturrax (U.T. Typ 18-10, Janke & Kunkel KG., IKA WERK, Staufen, Breisgau) for various timed intervals.

Following treatment, mycelial debris was filtered off using glass wool followed by centrifugation (4,000g for 30 minutes). The resulting supernatant was used as the "cell-free" extract and protein determination was made on each timed extract. Results are expressed in Table 2 and Fig. 2.

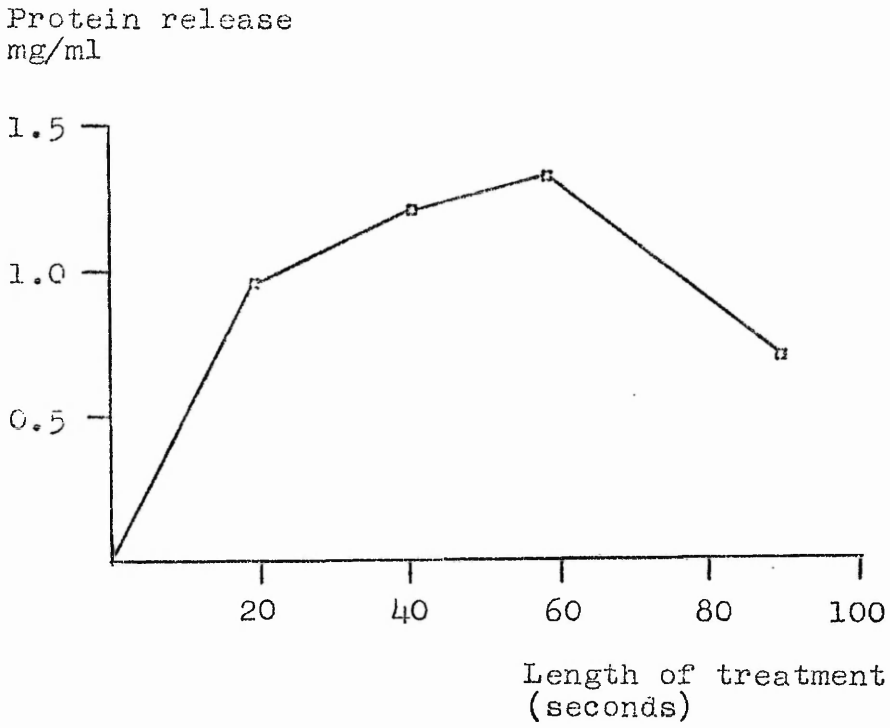
TABLE 2

Protein release from Ultraturrax treatment on a 3 day old mycelium of A. flavus N1

Length of treatment (seconds)	Protein release mg/ml
0	0
20	0.95
40	1.20
60	1.30
90	0.70

Fig. 2

Protein release from Ultraturrax treatment on a 3-day-old mycelium of Aspergillus flavus N1



Experiment VI (b)

Preparation of a "cell-free" extract utilizing an Ultraturrax for various ages of mycelium

Samples of damp mycelium (10g fresh weight) from mycelia of various ages were suspended in cold buffer (20ml) as in Experiment VI (a). Samples underwent ultraturrax treatment for 60 seconds and mycelial debris was filtered off using glass wool followed by centrifugation (4,000g for 30 minutes). The resulting supernatants were used as the "cell-free" extracts and protein determinations were made on each separate extract. Results are expressed in Table 3 and Fig. 3.

TABLE 3

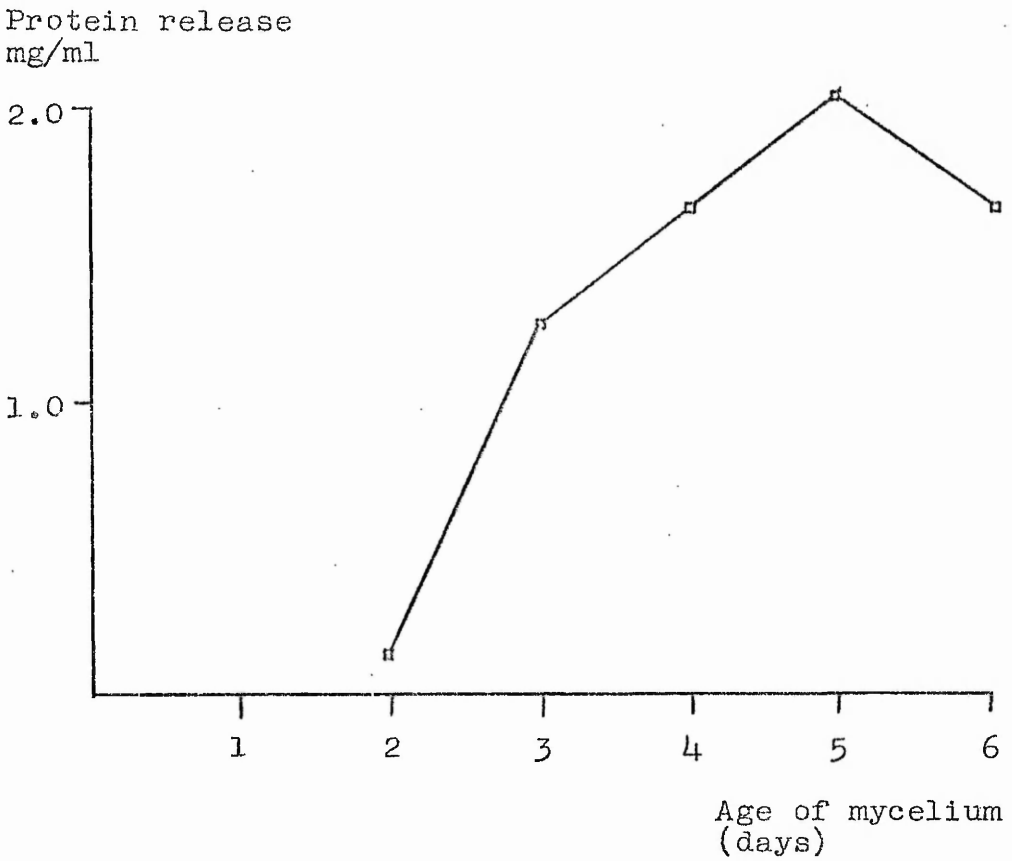
Protein release from Ultraturrax treatment (60 seconds) on mycelium of various ages from A. flavus N1

Age of mycelium Days	Protein release mg/ml
0	-
1	-*
2	0.15
3	1.30
4	1.70
5	2.05
6	1.70

* Not estimated, insufficient material

Fig. 3

Protein release from Ultraturrax treatment (60 seconds) on mycelium of various ages from Aspergillus flavus M1



Experiment VI (c)

Preparation of a "cell-free" extract utilizing an Ultraturrax on a 3 day old mycelium

A "cell-free" extract was prepared as in Experiment VI (a) (60 seconds ultraturrax treatment on a 3 day old mycelium) and this was then incubated with basal cofactor medium. The experiment was repeated twice more substituting "versiconal acetate" and secondly, versicolorin C for the basal cofactor medium. Results are expressed in Figs. 4 & 5.

Fig. 4

Ultraturrax "cell-free" preparation incubated for various time intervals with Versiconal acetate

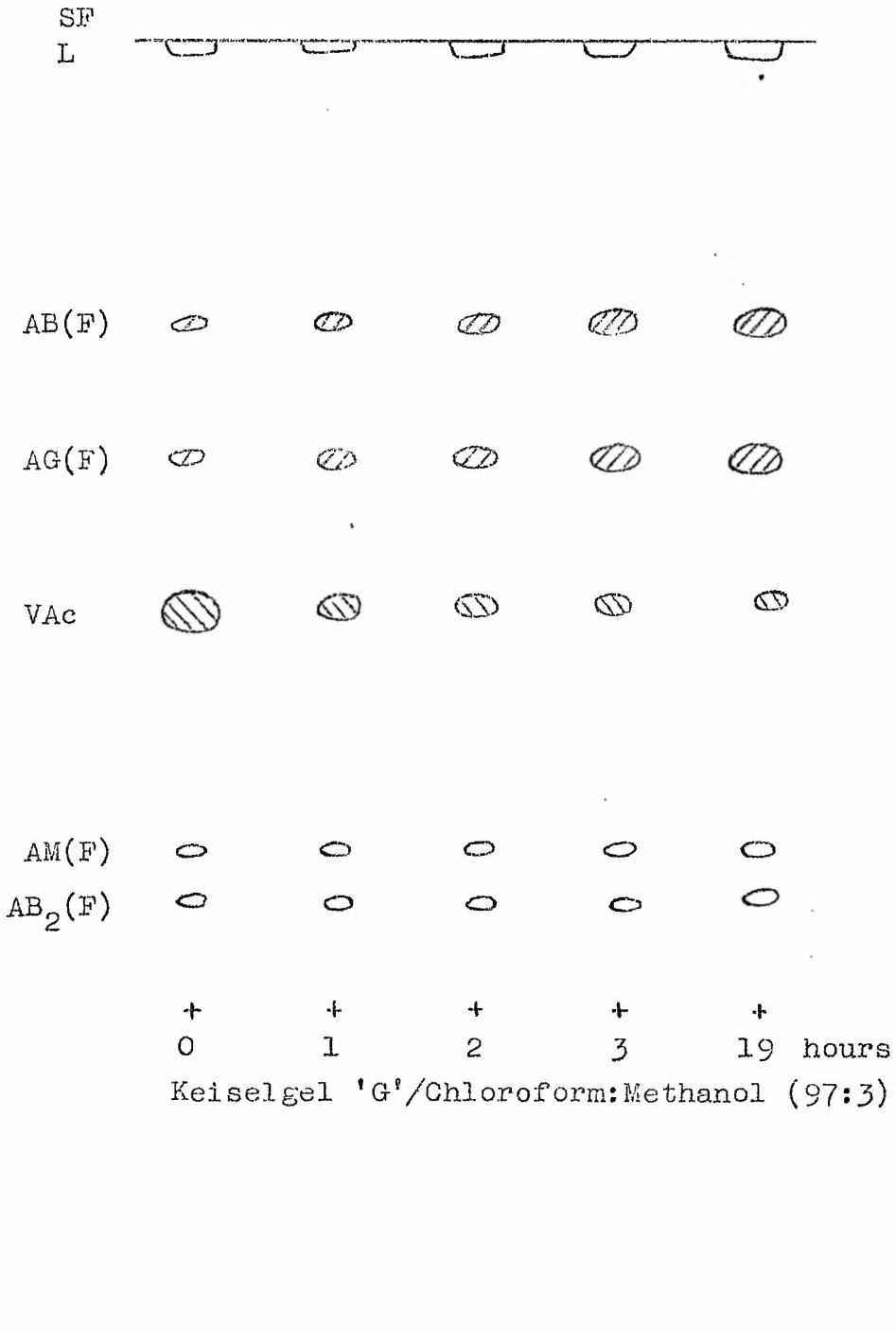
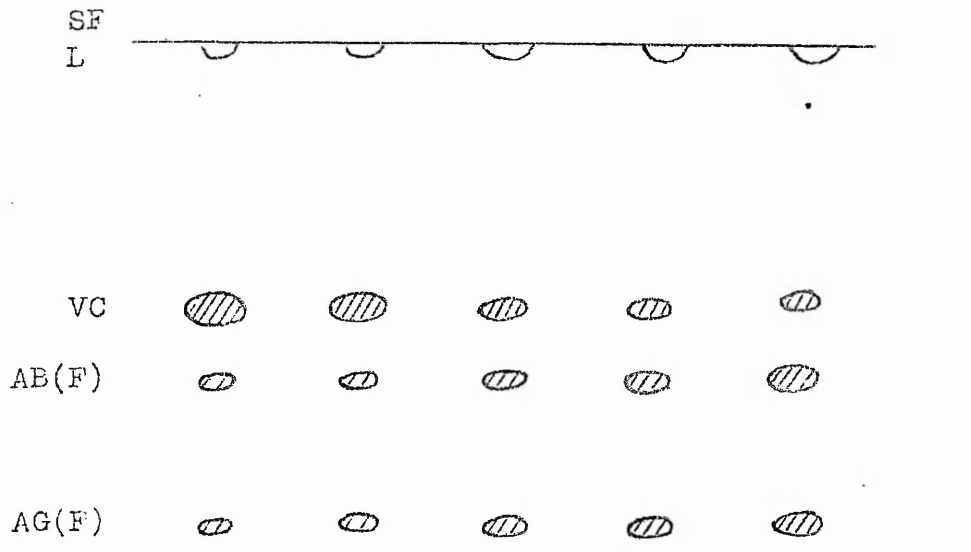


Fig. 5

Ultraturrax "cell-free" preparation incubated for various time intervals with Versicolorin C



Section II

Results from Experiment II (ultrasonic disintegration), III (homogenization), IV (stomacher) and V (lypholization) were negative for all techniques using the basal cofactor medium. Only Experiment I (liquid nitrogen) gave any positive results with the basal cofactor medium. Similarly, Experiment I and VIc (ultraturrax) gave positive results for various metabolites added.

It was decided that as the results obtained were not sufficient to extend research on these techniques that a new method of obtaining cell-free extracts would be attempted (see Section III).

S E C T I O N I I I

Protoplasts derived from Aspergillus flavus

Materials (III i)

In addition to those materials described in previous sections the following analar grade chemicals were used:-.

Supplied by Sigma -

Flavin adenine dinucleotide (FAD)

Hepes, N-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid

Dithiothreitol

NAD

NADP

p-nitrophenyl acetate

Supplied by BDH -

Ethylenediaminetetra acetic acid

Urea

N,N dimethyl formamide

Supplied by Dr J. S. Holker -

Sterigmatocystin

Preparation of a lytic enzyme for the purpose of protoplast isolation (III ii)

A medium containing the hyphal walls of Aspergillus flavus was used to produce lytic enzyme from Trichoderma viride.

Glucose	3.0g
Hyphal walls	5.0g dry weight (or 100g fresh weight)
Bactopeptone	1.0g
Urea	0.3g
Potassium dihydrogen phosphate	2.0g
Ammonium sulphate	1.4g
Magnesium sulphate	0.3g
Calcium chloride	0.3g
Trace elements	0.1ml Vogel's trace element solution

Made up to 1 litre with distilled water, (after Peberdy and Isaac 1976).

The medium was dispensed in portions (50ml) into 250ml conical flasks, which were then plugged with cotton wool and sterilized in an autoclave (15psi for 15 minutes). All flasks were then inoculated with a spore suspension (1ml) of Trichoderma viride CBS 354-33. The flasks were incubated at 25° on a rotary shaker (150 rev/min.) and after 7 days they were removed and the contents harvested. The culture fluid was separated from the mycelial debris by centrifugation (12000 rpm for 30 minutes). The culture fluid was then freeze dried down to 10% of the original volume and stored in a deep freeze (-20°) until required.

Preparation of Hyphal walls (III iii)

The hyphal walls, which were used in the preparation of lytic

enzyme, were isolated from various Aspergillus strains used in this project to obtain certain metabolites.

Once all the metabolites had been extracted from a culture (see General Methods I ix), the mycelium was placed in a Waring blender with an equal volume of water. Following homogenisation (3 mins.) the mycelium was "poured" into a Buchner funnel and the water filtered off. Water was then poured onto the mycelial mat in the Buchner funnel until the filtrate that was produced was clear, thus indicating the mycelial mat was "clean". The mycelium was then dried in an oven (80°C) for 48 hours. The dried mycelium was thus available, as a dark brown powder, for medium preparation.

Preparation of protoplasts from Aspergillus flavus N1 (III iv)

The mycelium of Aspergillus flavus N1, grown upon Reddy's chemically defined medium, was removed by filtration and washed with buffer-stabilizer (0.2M phosphate buffer, pH 5.8 plus 0.4M magnesium sulphate). The mycelium was damp dried with filter paper and then suspended in buffer-stabilizer (200mg mycelium/ml buffer stabilizer) plus an equal volume of lytic enzyme in a 250ml conical flask. This mixture was incubated at 30° on a rotary shaker (100 rev/min.) for 3 hours and after this time the resulting slurry was filtered through glass wool (twice) and a sintered glass filter

(Pyrex, porosity 1). The filtrate containing the protoplasts was then centrifuged (500g, 10 minutes), the supernatant being discarded. Sedimented protoplasts were then washed with buffer-stabilizer and re-centrifuged (500g, 10 mins.), this process being repeated twice more. The relatively "clean" protoplasts were then utilized in various experiments.

Experiment IA

Estimation of protoplasts isolated from Aspergillus flavus N1

A suspension of protoplasts, of various ages, in buffer stabilizer (10ml) was prepared as in the General Methods. The total number of protoplasts in this suspension was estimated using a calibrated haemocytometer, and the protein content of the suspension was then estimated using the Biuret method. Results are expressed in Table 1.

TABLE 1

Estimation of protoplasts

isolated from Aspergillus flavus N1

Age of mycelium days	Protein release from lytic digestion mg/g mycelium	Number of protoplasts released /ml
2	5.07	9.4×10^6
3	6.30	1.2×10^7
4	2.99	* -

* Too low to count

Experiment I

Conversion of Versicolorin A to Aflatoxins over 1 hour utilizing protoplasts of Aspergillus flavus N1

A suspension of protoplasts in buffer stabilizer (10ml) was prepared as in the General Methods, to which was added a solution (5 drops) of versicolorin A (100µg total) in N,N dimethyl formamide. The whole extract (in a boiling tube) was then incubated at 25° in a water bath. At various time intervals, samples (2ml) of suspension were removed and to them ethyl acetate (10ml) was added to stop any further reactions occuring in the protoplasts.

Aflatoxins were then extracted from the sample suspensions as described in the General Methods. Total extracts were spotted onto TLC chromatoplates and developed in ethyl acetate : toluene (1 : 1 v/v) solvent system. Once developed, the aflatoxin bands were eluted from the Keiselgel 'G' and their concentrations estimated as described in the General Methods (I v). Results are expressed in Table 2 and Fig. 1.

TABLE 2

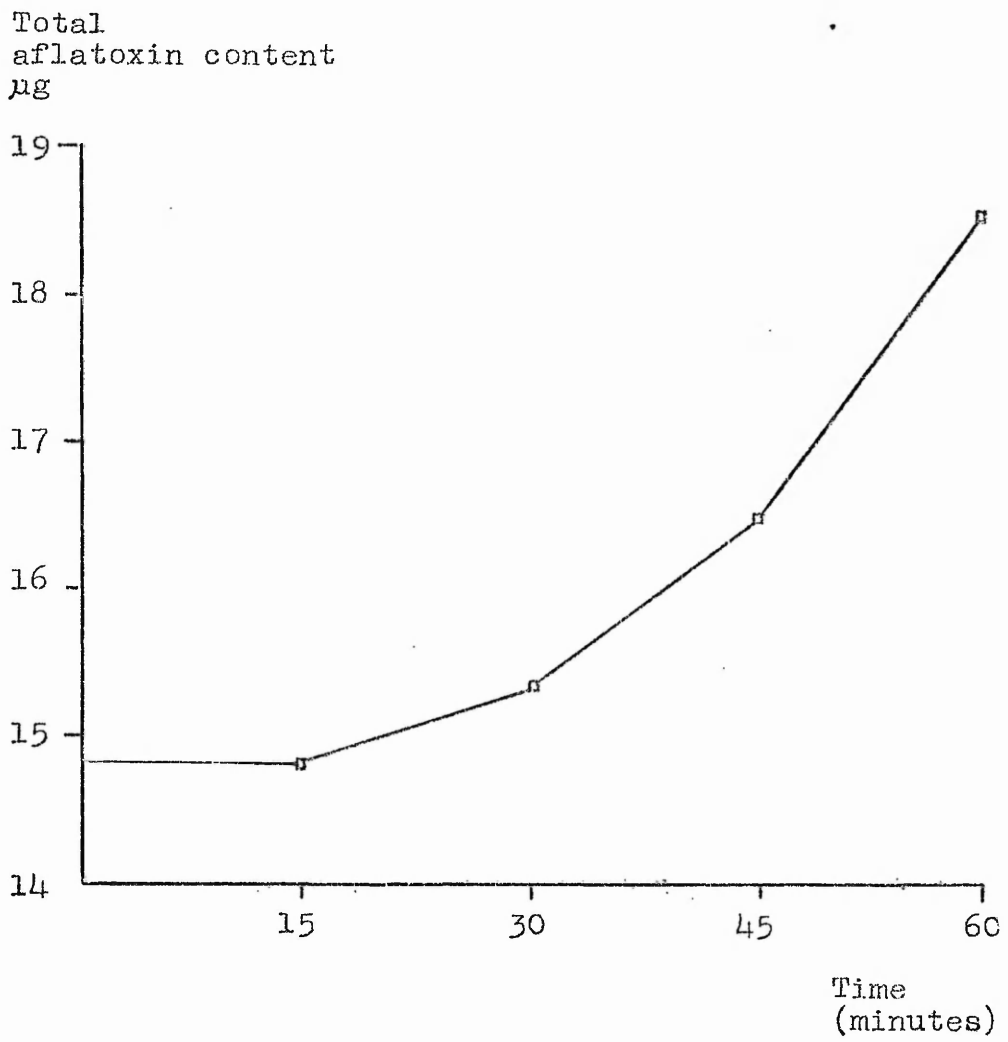
Conversion of Versicolorin A to aflatoxin over 1 hour utilizing protoplasts of Aspergillus flavus N1

Time of incubation minutes	Total aflatoxin* content µg with substrate	control no substrate
0	14.8	14.8
15	14.8	14.8
30	15.2	14.8
45	16.3	14.8
60	18.4	14.8

*Total estimated as Aflatoxin B_1 (see General Methods I v)

Fig. 1

Conversion of versicolorin A to aflatoxin over 1 hour utilizing protoplasts of Aspergillus flavus N1



Experiment II

Conversion of Versicolorin A to Aflatoxins over 18 hours utilizing protoplasts of Aspergillus flavus N1

Prepared protoplasts were incubated with versicolorin A (100µg total) as in Experiment I. At various time intervals, samples (2ml) of suspension were removed and centrifuged (1,000g, 10 minutes), thus separating the suspension into protoplast fraction and supernatant fraction. The supernatant fraction was pipetted off from the protoplast fraction, which was subsequently suspended in buffer stabilizer (2ml). The activity of both fractions was stopped by addition of ethyl acetate (10ml to each). Aflatoxins were then estimated as in Experiment I. Results are expressed in Table 3 and Fig. 2.

TABLE 3

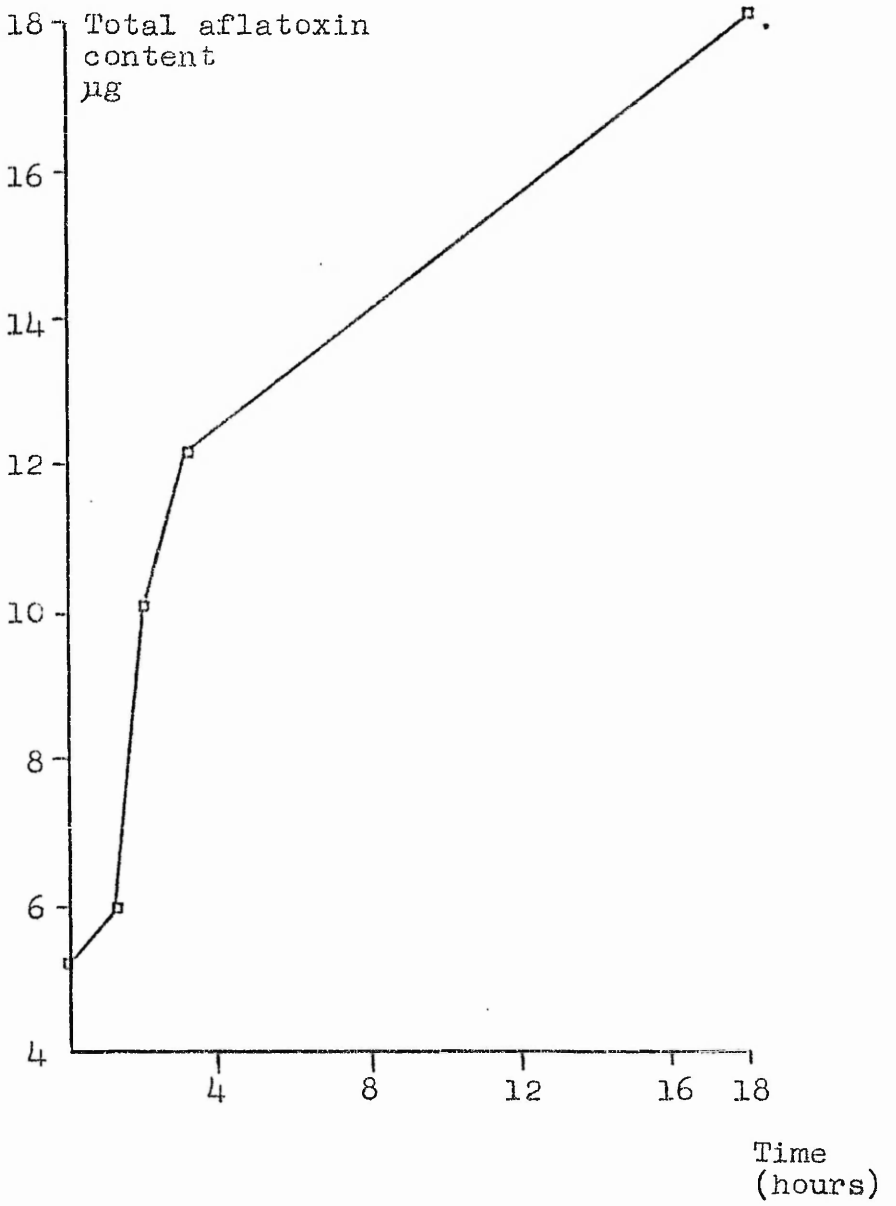
Conversion of Versicolorin A to aflatoxin over 18 hours utilizing protoplasts of Aspergillus flavus N1

Time of incubation hours	Aflatoxin* content of supernatant µg	Aflatoxin* content of protoplast fraction µg	Total aflatoxin* content µg	Control no substrate aflatoxin* content µg
0	-	-	5.1	5.1
1	1.3	4.9	6.2	5.1
2	2.1	8.0	10.1	5.2
3	2.8	9.3	12.1	5.2
18	2.8	15.3	18.1	5.2

* Total estimated as aflatoxin B_1 (see General Methods I v)

Fig. 2

Conversion of versicolorin A to aflatoxin over 18 hours utilizing protoplasts of Aspergillus flavus N1



Experiment III

Conversion of Sterigmatocystin to Aflatoxin over 18 hours utilizing protoplasts of Aspergillus flavus N1

Protoplasts were prepared as in Experiment I and to the suspension was added a solution (5 drops) of sterigmatocystin dissolved in N,N dimethyl formamide. Protoplasts were then incubated as in Experiment I and at various time intervals samples (2ml) of suspension were removed and treated as described in Experiment II. Aflatoxins were then estimated as in Experiment I. Results are expressed in Table 4 and Fig. 3.

TABLE 4

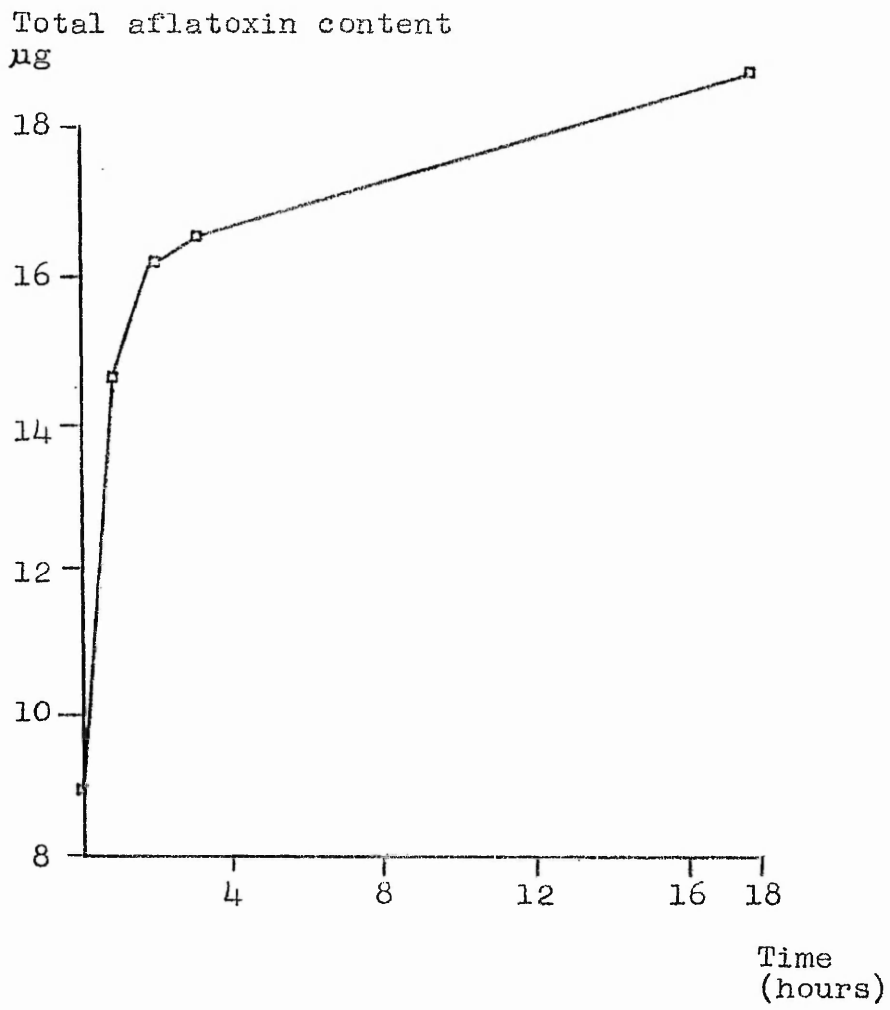
Conversion of Sterigmatocystin to aflatoxin over 18 hours utilizing protoplasts of Aspergillus flavus N1

Time of incubation hours	Aflatoxin* content of supernatant µg	Aflatoxin* content of protoplast fraction µg	Total aflatoxin* content µg	Control no substrate aflatoxin* content µg
0	5.2	3.8	9.0	9.0
1	8.4	6.2	14.6	9.0
2	8.9	7.4	16.3	9.1
3	8.2	8.4	16.6	9.1
18	10.1	8.5	18.6	9.3

* Total estimated as aflatoxin B_1 (see General Methods I v)

Fig. 3

Conversion of sterigmatocystin to aflatoxin over 18 hours utilizing protoplasts of Aspergillus flavus N1



Experiment IV

Conversion of "Versiconal acetate" to Aflatoxin over 1 hour utilizing protoplasts of Aspergillus flavus N1

Protoplasts were prepared as in Experiment I, and to the suspension was added a solution (5 drops) of "versiconal acetate" dissolved in N,N dimethyl formamide. Protoplasts were then incubated as in Experiment I and at various time intervals samples (2ml) of suspension were removed and treated as described in Experiment I. Aflatoxins were then estimated, also as in Experiment I. Results are expressed in Table 5 and Fig. 4.

TABLE 5

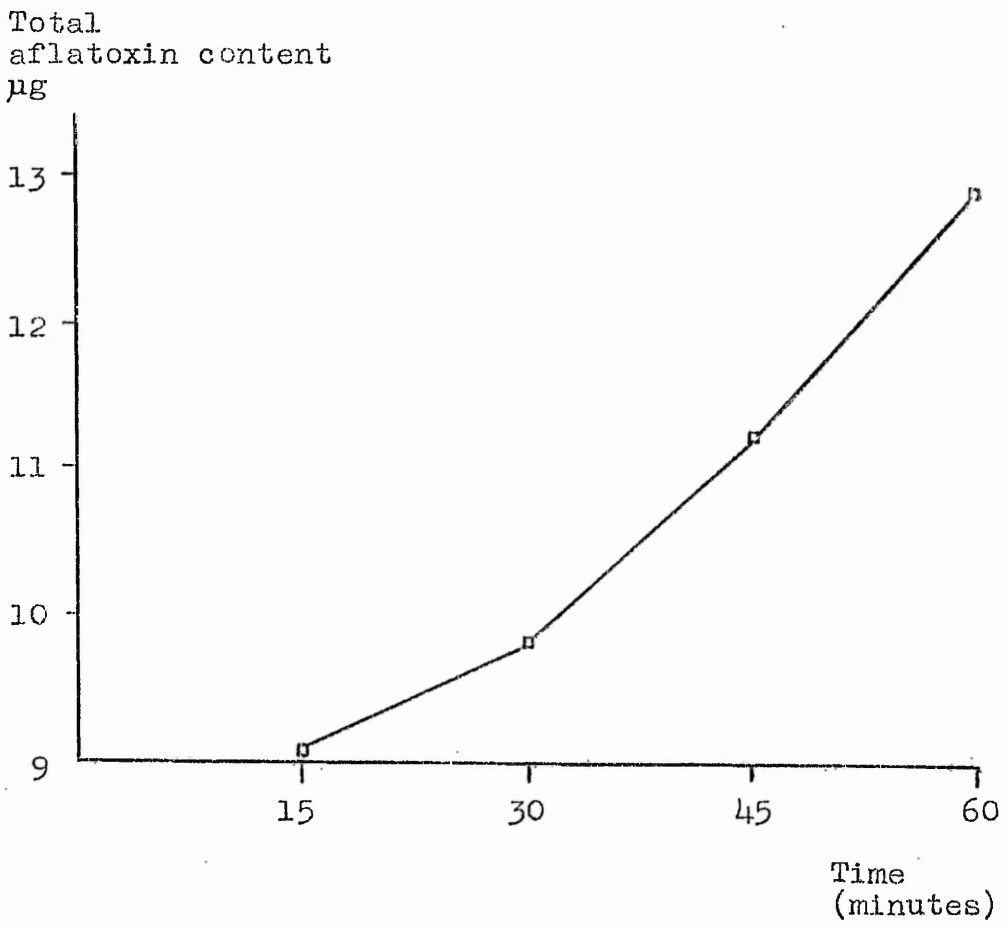
Conversion of "Versiconal acetate" to aflatoxin over 1 hour utilizing protoplasts of Aspergillus flavus N1

Time of incubation minutes	Total aflatoxin* content µg with substrate	control no substrate
0	9.0	9.0
15	9.0	9.0
30	9.7	9.0
45	11.1	9.0
60	12.7	9.0

*Total estimated as Aflatoxin B_1 (see General Methods I v)

Fig. 4

Conversion of versiconal acetate to aflatoxin over 1 hour utilizing protoplasts of Aspergillus flavus NI



Experiment V

Conversion of Versicolorin C to Aflatoxin over 1 hour utilizing protoplasts of Aspergillus flavus N1

Protoplasts were prepared as in Experiment I, and to the suspension was added a solution (5 drops) of versicolorin C dissolved in N,N dimethyl formamide. Protoplasts were then incubated as in Experiment I and at various time intervals, samples (2ml) of suspension were removed and treated as described in Experiment I. Aflatoxins were then estimated, also as in Experiment I. Results are expressed in Table 6 and Fig. 5.

TABLE 6

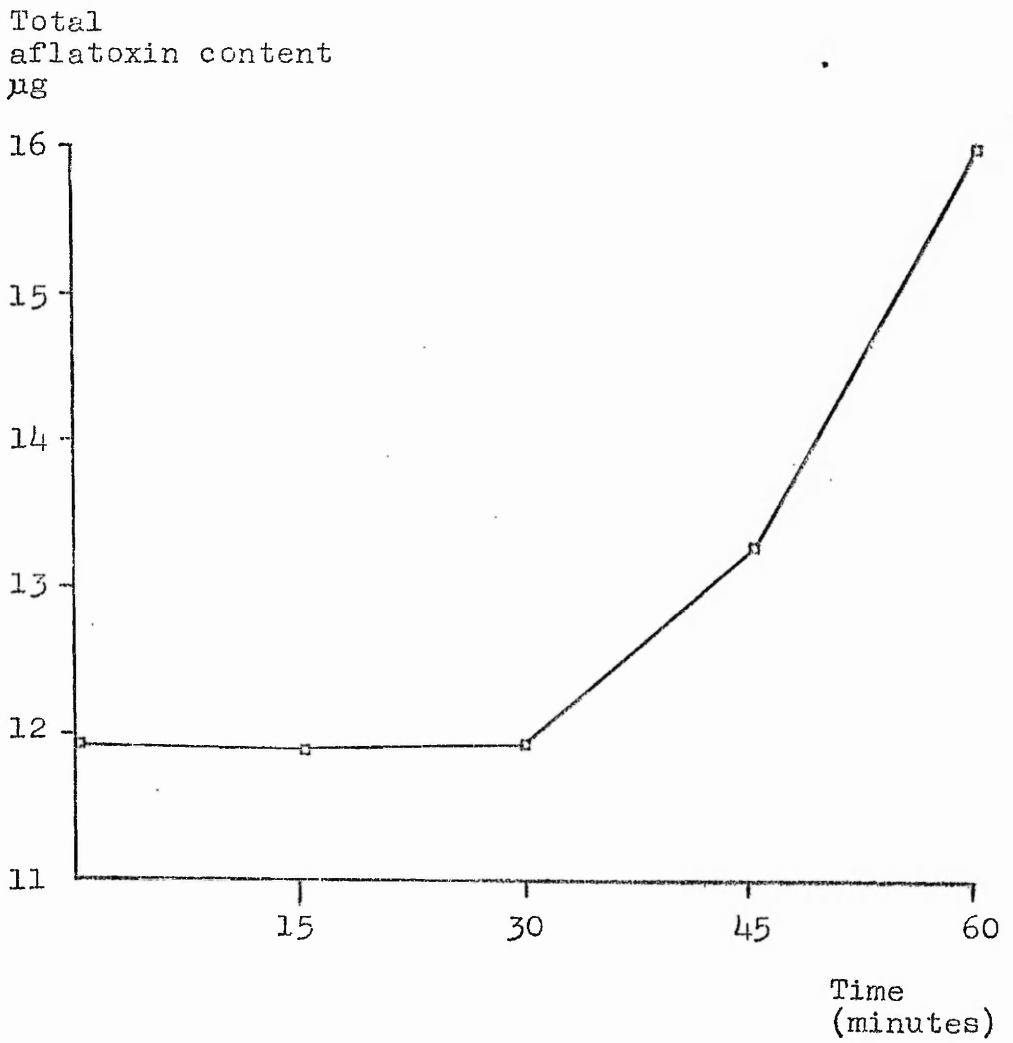
Conversion of Versicolorin C to aflatoxin over 1 hour utilizing protoplasts of Aspergillus flavus N1

Time of incubation minutes	Total aflatoxin* content µg with substrate	control no substrate
0	11.9	11.9
15	11.9	11.9
30	11.9	11.9
45	13.1	11.9
60	15.9	11.9

* Total estimated as Aflatoxin B_1 (see General Methods I v)

Fig. 5

Conversion of versicolorin C to aflatoxin over 1 hour utilizing protoplasts of Aspergillus flavus N1



Experiment VI

Attempted conversion of O-Methyl Sterigmatocystin, Averufin and Norsolorinic acid to Aflatoxin over 18 hours utilizing protoplasts of Aspergillus flavus N1

Protoplasts were prepared as in Experiment I, and to the suspension was added a solution (5 drops) of O-methyl sterigmatocystin dissolved in N,N dimethyl formamide. Protoplasts were then incubated as in Experiment I, and at various time intervals samples (2ml) of suspension were removed and treated as described in Experiment I. Aflatoxins were then estimated as in Experiment I.

This experiment was then repeated substituting averufin and then norsolorinic acid for O-methyl sterigmatocystin. Results are expressed in Table 7.

TABLE 7

Addition of O-methyl sterigmatocystin averufin and norsolorinic acid to protoplasts of Aspergillus flavus N1 over an 18 hour incubation period

Metabolite added	Aflatoxin content zero time (µg)	Aflatoxin content after 18 hours incubation (µg)
O-me-sterigmatocystin	6.8	6.8
Averufin	7.4	7.4
Norsolorinic acid	5.3	5.3

Experiment VII

Production of aflatoxins by various ages of protoplasts from Aspergillus flavus N1 suspended in Reddy's chemically defined medium

A suspension of protoplasts, isolated from mycelium of various ages, in buffer-stabilizer (10ml) was prepared as described in the General Methods. The turbidity of this suspension was then recorded and adjusted appropriately to read 0.26 O.D. units* on an EEL spectrophotometer. When the turbidity had been corrected, the suspension (10ml) was centrifuged (500g, 10 minutes) in order to sediment the protoplasts and these were then re-suspended in buffer-stabilizer (1ml) and Reddy's chemically defined medium (9ml). The protoplast suspension was then incubated at 25° and at various time intervals portions (2ml) of suspension were removed and treated as in Experiment I. Aflatoxins were then estimated, also as in Experiment I. Results are expressed in Table 8 and Fig. 6.

* 0.26 O.D. units equivalent to 32mg dry weight/10ml suspension

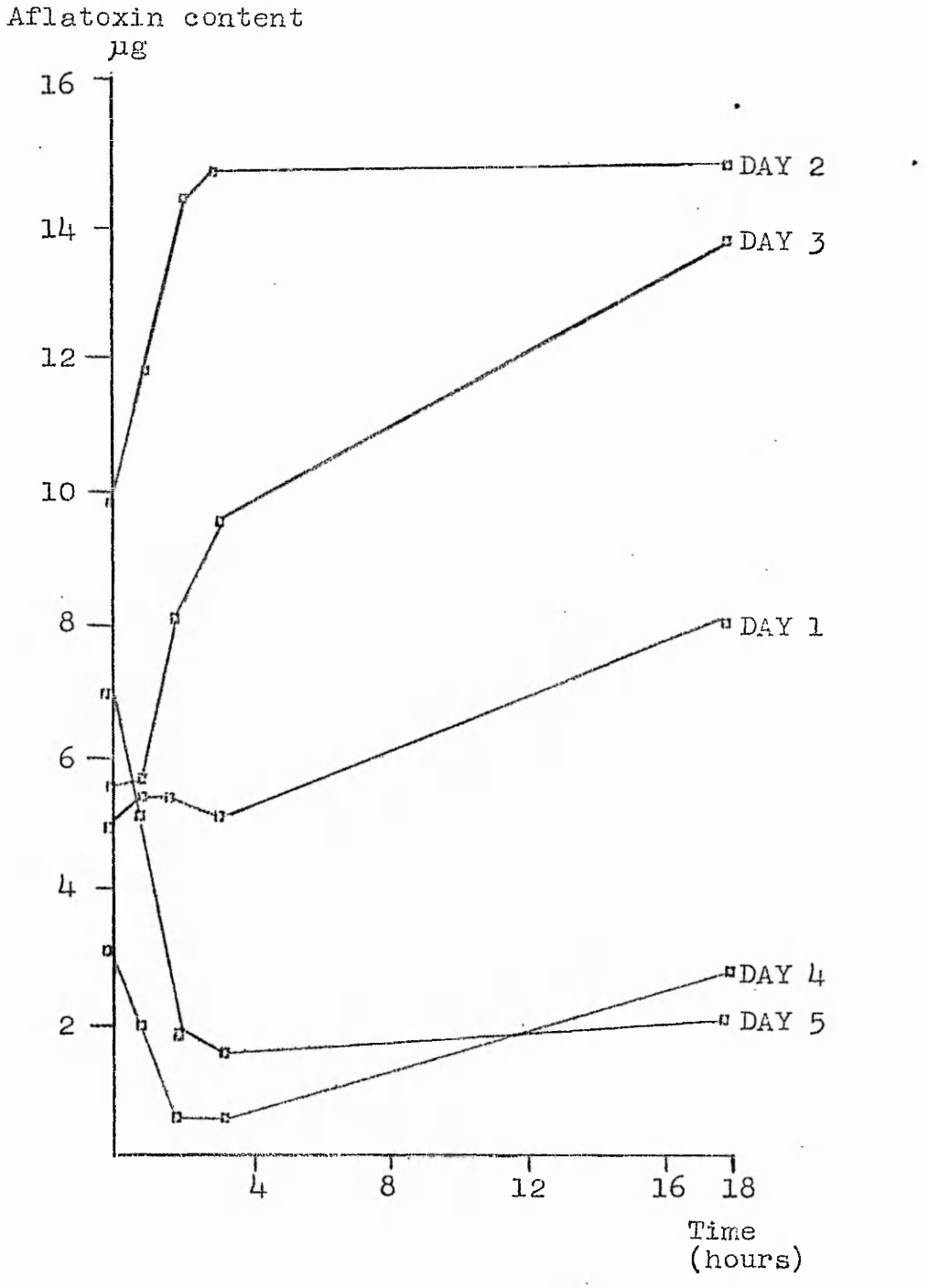
TABLE 8

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) total per 2ml suspension after				
	0 hours	1 hour	2 hours	3 hours	18 hours
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
Control	5.60	5.60	5.60	5.60	5.60

Fig. 6

Production of aflatoxins by protoplasts of various ages of Aspergillus flavus N1 mycelium suspended in Reddy's chemically defined medium



In all experiments using radioactive samples results are expressed as the amount of aflatoxin B_1 formed. This is calculated by subtracting the amount at the zero time reading from the amount formed at the final reading, thus negating the possibility of radioactive carrier substances which could have the same Rf as aflatoxin B_1 from being counted as an increase in aflatoxin B_1.

Experiment VIII

Conversion of U-^{14}C-acetate and G-^{14}C-versicolorin A to aflatoxin B_1 utilizing protoplasts of Aspergillus flavus N1

In order to substantiate the results of Experiments I, II and VII, protoplasts from Aspergillus flavus N1 were incubated with radioactively labelled compounds.

A suspension of protoplasts were prepared as in Experiment I and these were suspended in buffer stabilizer (1ml) and Reddy's chemically defined medium (9ml). To the suspension was added U-^{14}C-sodium acetate (0.21µCi, 234mCi/mole). The protoplast suspension was then incubated at 25^{o} and at various time intervals portions (5ml) of suspension were removed and treated as in Experiment I. Aflatoxins were then estimated , also as in Experiment I, after which the aflatoxin solution was evaporated to dryness in vacuo and then re-dissolved in scintillation fluid (10ml). Samples were then counted in a liquid scintillation counter and absolute counts estimated, determined using the channels ratio method.

This experiment was repeated substituting G-^{14}C-versicolorin A (0.0084µCi, 28.6mCi/mole) dissolved in N,N,dimethylformamide (5 drops) for the U-^{14}C-sodium acetate. Results are expressed in Table 9.

TABLE 9

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

Precursor Amount added			Product Aflatoxin B_1 Amount formed*		
	µCi	specific activity (mCi/mole)	µCi	specific activity (mCi/mole)	% conversion [a]
(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

$$a = \frac{\mu Ci \text{ product}}{\mu Ci \text{ precursor}} \times 100$$

* over 3 hour incubation period

Experiment IX

Respiration of protoplasts derived from various ages of mycelium of Aspergillus flavus N1

A suspension of protoplasts, isolated from mycelium of various ages, in buffer stabilizer (10ml) was prepared as described in the General Methods. The turbidity of this suspension was then recorded and adjusted appropriately to read 0.26 O.D. units on an EEL spectrophotometer. When the turbidity had been corrected the suspension was centrifuged (500g, 10 mins.) in order to sediment the protoplasts and these were then re-suspended in buffer stabilizer (0.4ml) and Reddy's chemically defined medium (3.6ml). The protoplast suspension was then incubated in a conventional warburg constant volume respirometer flask containing 10% potassium hydroxide in the centre well at 30°C.

The rate of oxygen consumption, after correction for the thermobarometer, was measured on a constant volume respirometer supplied by B. Braun (Apparatebau, Melsungen, W. Germany). Results are expressed in Table 10 and Fig. 7.

TABLE 10

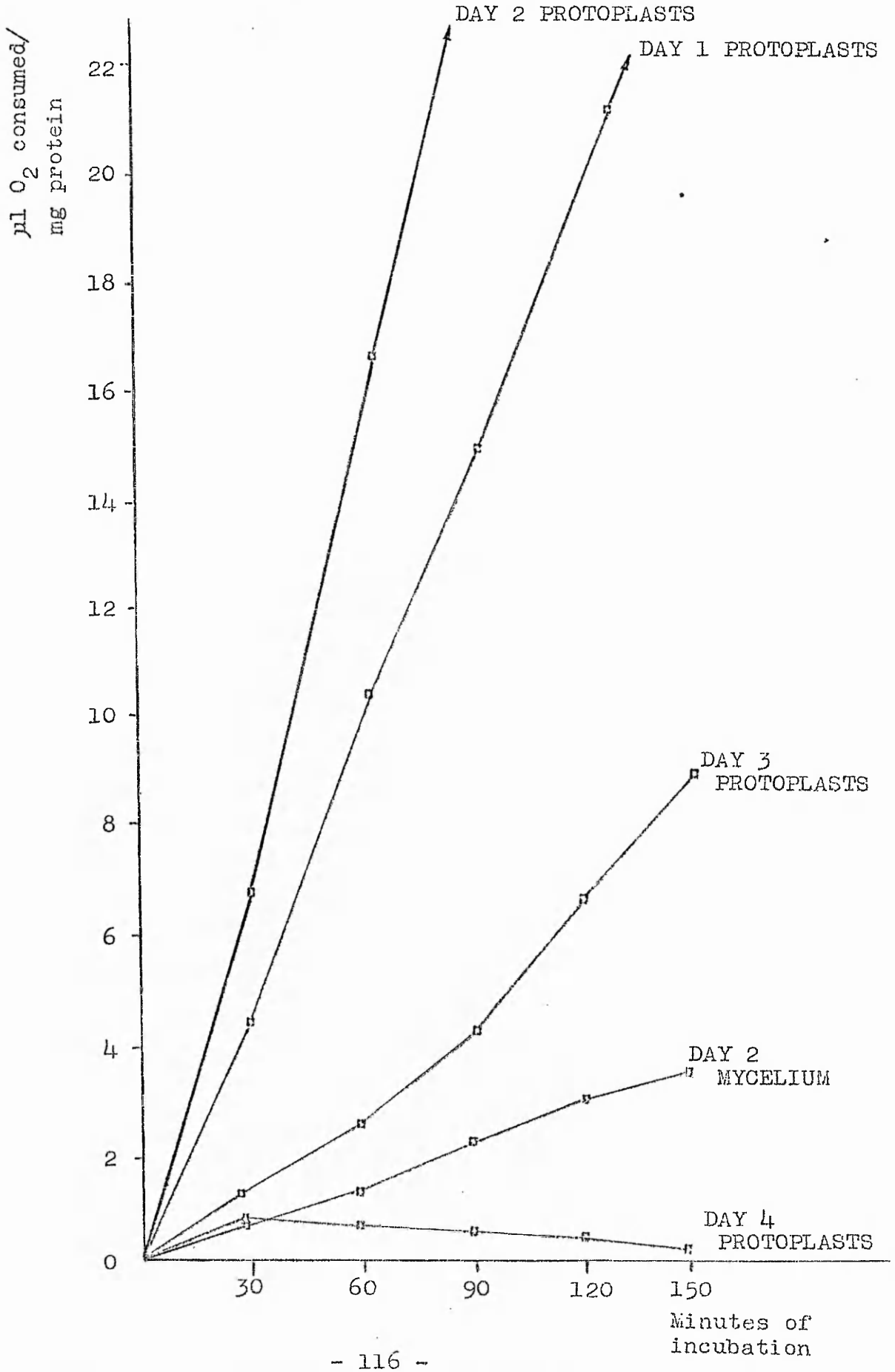
Respiration of whole protoplasts derived from various ages of Aspergillus flavus N1 mycelium

Protoplasts derived from mycelium of various ages (days)		1	2	3	4	2*	µl O_2 consumed/mg protein
Time (mins)	30	4.2	6.9	1.3	0.5	0.4	
	60	10.3	16.3	2.4	0.6	1.1	
	90	15.0	24.8	4.0	0.4	1.9	
	120	21.1	32.4	6.6	0.3	2.7	
	150	25.2	41.3	8.7	0.25	3.4	

* Whole mycelium only (µl O_2 consumed/mg mycelial dry weight)

Fig. 7

Respiration of whole protoplasts derived from various ages of Aspergillus flavus N1 mycelium



In all the following experiments 0.2M phosphate buffer was replaced by Hepes buffer (0.2M pH 5.8), B. Davis (1977). Using this as buffer, no insoluble precipitate ever formed when magnesium sulphate was added to make the buffer/ stabilizer.

Cell-free extracts derived from protoplasts

Experiment X

Conversion of G-^{14}C-versiconal acetate to aflatoxin B_1 by a supernatant fraction isolated from lysed protoplasts of Aspergillus flavus N1

A suspension of protoplasts in buffer stabilizer (10ml) was prepared as in the General Methods. The suspension was centrifuged (500g, 10 mins.) in order to produce a protoplast pellet, which was then re-suspended in cold (4°C) phosphate buffer (0.2M, pH 7.6) (4ml) and frozen for 30 mins (-10°C). Following freezing, the lysed protoplasts were thawed and the cell debris separated from the cytoplasmic content by centrifugation (10,000g, 30 mins.). Following estimation of the protein content (1ml for Biuret assay), the remaining suspension (3ml) was incubated in a standard warburg flask at 30°C with the following cofactors (total 1ml) added to give a final concentration of:-

Flavin adenine dinucleotide	10^{-6}M
Dithiothreitol	10^{-3}M
E.D.T.A.	10^{-3}M
Methionine	10^{-3}M
G-^{14}C-versiconal acetate	0.25µ mole
NADH	1µ mole
NADPH	1µ mole

At various time intervals samples (1ml) were removed and treated as in Experiment I. Aflatoxins were then estimated as in Experiment VIII to obtain their specific activity.

TABLE 11

Conversion of G-^{14}C-versiconal acetate to aflatoxin B_1 by a supernatant fraction isolated from lysed protoplasts of Aspergillus flavus

Experiment		Precursor added: Incubation time	Precursor added: µCi	Precursor added: specific activity mCi/mole	Aflatoxin formed: µCi	Aflatoxin formed: specific activity mCi/mole	Aflatoxin formed: % conversion *
A/	(i)	1 hour	0.0002	3.846	0.0000085	0.900	4.25
	(ii)	1 hour	0.0002	3.846	0.0000058	0.884	2.90
	(i)	18 hours	0.0002	3.846	0.000022	2.098	11.04
	(ii)	18 hours	0.0002	3.846	0.0000164	1.943	8.20
B/	(iii)	1 hour	0.0002	3.846	0.000014	1.769	7.04
	(iv)	1 hour	0.0002	3.846	0.0000165	1.963	8.28
C/	(v)	1 hour	0.0002	3.846	0.000001	-	0.1

* = $\frac{\text{µCi product formed}}{\text{µCi precursor added}}$

A = fraction derived from protoplasts of 3 day-old mycelium
B = fraction derived from protoplasts of 4 day-old mycelium
C = fraction derived from protoplasts of 4 day-old mycelium and no FAD in the incubation medium

This experiment was repeated using protoplasts derived from a different age of mycelium, and also with an incubation medium containing no FAD. Results are expressed in Table 11.

Experiment XI

Addition of G-^{14}C-versicolorin A to a supernatant fraction isolated from lysed protoplasts of Aspergillus flavus N1

A supernatant fraction of lysed protoplasts isolated from A. flavus N1 was prepared as in Experiment X.

Following estimation of the protein content (1ml for Biuret assay) the remaining suspension (3ml) was incubated in a standard Warburg flask at 30°C with the same cofactors (total 1ml) at the same concentration as in Experiment X. G-^{14}C-versicolorin A was added (0.25µM) as substrate in place of G-^{14}C-versiconal acetate.

At various time intervals, samples (1ml) were removed and treated as in Experiment I. Aflatoxins were then estimated as in Experiment VIII to obtain their specific activity. Results are expressed in Table 12.

TABLE 12

Addition of ^{14}C-versicolorin A to a supernatant fraction of lysed protoplasts derived from Aspergillus flavus N1

	Precursor added		Aflatoxin formed		
Incubation time	µCi	specific activity (mCi/mole)	µCi [a]	specific activity (mCi/mole)	% conversion [b]
A/1 hour	0.00136	24.5	-	-	-
1 hour	0.00136	24.5	-	-	-
18 hours	0.00136	24.5	-	-	-
18 hours	0.00136	24.5	-	-	-
B/1 hour	0.00136	24.5	-	-	-
1 hour	0.00136	24.5	-	-	-
18 hours	0.00136	24.5	-	-	-
18 hours	0.00136	24.5	-	-	-

a = never more than 0.000002µCi incorporated in any experiment
b = never more than 0.19% conversion in any experiment

Experiment XII

Comparative conversions of G-^{14}C-versicolorin A and G-^{3}H-versiconal acetate in a supernatant fraction derived from lysed protoplasts of Aspergillus flavus N1.

In order to verify the results obtained in Experiments X and XI, the incorporation of G-^{3}H-versiconal acetate and G-^{14}C-versicolorin A was estimated in the same supernatant fraction.

A supernatant fraction of lysed protoplasts from A. flavus N1 was prepared as in Experiment X. The supernatant fraction (3ml) was incubated with the same cofactors (1ml) at the same concentration as in Experiment X. G-^{14}C-versicolorin A (0.25µM) and G-^{3}H-versiconal acetate (0.25µM), kindly prepared by Dr. M. F. Dutton, were added as substrates.

At various time intervals, samples (1ml) were removed and treated as in Experiment I. Aflatoxins were then estimated as in Experiment VIII in order to obtain their specific activity. Results are expressed in Table 13.

TABLE 13

Addition of ^{14}C-versicolorin A and ^{3}H-versiconal acetate to a supernatant fraction of lysed protoplasts derived from Aspergillus flavus N1

Precursor added			Aflatoxin formed over 1 hr. incubation		
	µCi	specific activity (mCi/mole)	µCi	specific activity (mCi/mole)	% conversion
^{3}H-versiconal acetate	0.0039	15.6	0.00017	2.0	4.35
^{14}C-versicolorin A	0.00136	24.5	0.000002	0.2	0.15

Experiment XIII

Conversion of G-^{14}C-versiconal acetate to Versicolorin A in a supernatant fraction of lysed protoplasts derived from Aspergillus parasiticus 1-11-105 Whl

A suspension of protoplasts in buffer stabilizer (10ml) was prepared as in the General Methods, the origin of the protoplasts being the fungus Aspergillus parasiticus 1-11-105 Whl. A supernatant fraction of lysed protoplasts was prepared as in Experiment X.

Following estimation of the protein content (1ml for Biuret assay), the remaining suspension (3ml) was incubated in a standard Warburg flask at 30°C with the same cofactors (total 1ml) added to give the same concentration as those in Experiment X. G-^{14}C-versiconal acetate (0.25µM) was used as the substrate.

At various time intervals, samples (1ml) were removed and treated as in Experiment I. Versicolorin A was then estimated spectrophotometrically (321nM ϵ = 12118) before counting. Results are expressed in Table 14.

TABLE 14

The conversion of ^{14}C-versiconal acetate to versicolorin A in a supernatant fraction of lysed protoplasts derived from Aspergillus parasiticus 1-11-105 Whl

	Precursor added		Versicolorin A formed		
Incubation time	µCi	specific activity (mCi/mole)	µCi	specific activity (mCi/mole)	% conversion
1 hour	0.0002	3.846	0.0000061	0.572	3.03
3 hours	0.0002	3.846	0.000012	1.125	6.06
18 hours	0.0002	3.846	0.000018	2.423	9.09

Experiment XIV

The comparative conversion of G-^{3}H-versiconal acetate by a supernatant fraction of lysed protoplasts derived from Aspergillus parasiticus 1-11-105 Whl containing either NADP/NAD or NADPH/NADH

A supernatant fraction of lysed protoplasts derived from A. parasiticus, as described in Experiment XIII, was prepared. The fraction was divided into two halves; one with all the cofactors added as in Experiment XIII, the other with all the cofactors of Experiment XIII added but NADP and NAD (1µmole each) replacing the NADPH and NADH. G-^{3}H-versiconal acetate (kindly prepared by Dr. M.F. Dutton) (0.5 uM) was added to each fraction as substrate.

The fractions were incubated in standard Warburg flasks at 30°C and at various time intervals samples (1ml) were removed and treated as in Experiment XIII. Versicolorin A was then estimated as in Experiment XIII. Results are expressed in Table 15.

TABLE 15

Comparative conversion of ^{3}H-versiconal acetate by a supernatant fraction of lysed protoplasts derived from Aspergillus parasiticus 1-11-105 Whl containing either NADP/NAD or NADPH/NADH

	Precursor added		Versicolorin A formed over 1 hr. incubation		
	µCi	specific activity (mCi/mole)	µCi	specific activity (mCi/mole)	% conversion
+NADPH NADH	0.001	10.00	0.000026	6.30	2.6
+NADP NAD	0.001	10.00	0.000003	6.16	0.31

Experiment XV

The general esterase activity of supernatant fractions derived from lysed protoplasts of Aspergillus parasiticus 1-11-105 Whl

A supernatant fraction of lysed protoplasts derived from A. parasiticus 1-11-105 Whl was prepared as in Experiment XIII.

A sample of supernatant (1ml) was removed for protein estimation, a further sample (1ml) was used for an esterase assay. The remaining fraction (2ml) was further centrifuged (100,000 g x 60 mins.) to yield a microsomal supernatant fraction. This fraction was divided into two parts; one part (1ml) for protein determination and the other part (1ml) for esterase activity. Esterase activity was estimated using the method of Rahim and Sih (1969).

A sample of enzyme solution (1ml) was added to a solution of p-nitrophenylacetate (0.5ml, 0.1mg/ml in 0.1M phosphate buffer, pH 8.0). The optical density (X) of this solution was recorded, at 30°C, over a period of five minutes against a blank containing phosphate buffer (1ml, 0.1M, pH 8.0) in place of the enzyme solution.

Results are expressed as µmole p-nitrophenol produced per mg of protein per minute, see Table 16.

TABLE 16

General esterase activity of supernatant fractions derived from Aspergillus parasiticus 1-11-105 Whl

Fraction	Esterase activity (µM/mg/min)
10,000g	0.024
100,000g	0.064

Experiment XVI

The effect of dichlorvos on the general esterase activity of a supernatant fraction derived from lysed protoplasts of Aspergillus parasiticus 1-11-105 Whl

A supernatant fraction of lysed protoplasts, derived from A. parasiticus 1-11-105 Whl, was prepared as in Experiment XIII.

A sample of the fraction (1ml) was used for general esterase activity as described in Experiment XV. This was then repeated with the addition of dichlorvos (1µl, 5µg/µl) to the reaction cuvette. Further amounts (up to 10µl) of dichlorvos were added to the reaction cuvette to observe the effect of this insecticide on the general esterase activity. Results are expressed in Table 17.

TABLE 17

Effect of dichlorvos on the general esterase activity of a supernatant fraction derived from Aspergillus parasiticus 1-11-105 Whl

Fraction 10,000g	Esterase activity (µM/mg/min)
control	0.024
+ 5µg dichlorvos	0.02
+ 10µg dichlorvos	0.012
+ 15µg dichlorvos	0.008
+ 20µg dichlorvos	0.003
+ 50µg dichlorvos	0.001

Experiment XVII

Comparative conversions of G-^{14}C-versicolorin C and G-^{3}H-versiconal acetate in a supernatant fraction derived from lysed protoplasts of Aspergillus flavus N1.

A protoplast pellet of Aspergillus flavus N1 was produced as described in Experiment X. The pellet was resuspended in cold (4°C) phosphate buffer (0.2M; pH 7.6)(4ml) and homogenized in a Potter hand-held, ground glass tissue homogenizer for 5 minutes at 4°C (D. Wales 1977). The homogenized material was then centrifuged, as in Experiment X, and the supernatant fraction (3ml) incubated with the same cofactors (1ml) and at the same concentration as in Experiment X. G-^{14}C-versicolorin C (0.24µM) and G-^{3}H-versiconal acetate (0.24µM), (kindly prepared by Dr M. F. Dutton), were added as substrates.

At various time intervals samples (1ml) were removed and treated as in Experiment I. Aflatoxins were then estimated, as in Experiment VIII, in order to obtain their specific activity. Results are expressed in Table 18.

TABLE 18

Addition of ^{14}C-versicolorin C and ^{3}H-versiconal acetate to a supernatant fraction of lysed protoplasts derived from Aspergillus flavus N1

Precursor added				Aflatoxin formed over various time periods		
	µCi	specific activity mCi/mole	Incubation time	µCi	specific activity mCi/mole	% conversion
^{3}H-versiconal acetate	0.0014	3.16	1 hr	0.000046	0.7	3.3
			3 hr	0.000068	1.3	4.9
^{14}C-versicolorin C	0.00079	16.6	1 hr	ZERO	ZERO	ZERO
			3 hr	0.000023	5.1	2.9

Experiment XVIII

Comparative conversion of G-^{14}C-versiconal acetate and G-^{3}H-hydroxyversicolorin A in a supernatant fraction derived from lysed protoplasts of Aspergillus flavus N1

A supernatant fraction of lysed protoplasts was prepared as in Experiment XVII. This was incubated with the same cofactors (1ml) at the same concentration as in Experiment X. G-^{14}C-versiconal acetate and G-^{3}H-hydroxyversicolorin A (V_3), kindly prepared by Dr M. F. Dutton, were added as a double substrate.

At various time intervals samples (1ml) were removed and treated as in Experiment I. The total counts (dpm) in the aflatoxin fraction were estimated and the percentage conversion calculated. Results are expressed in Table 19.

TABLE IV

Conversion of ^{3}H-hydroxyversicolorin A and ^{14}C-versiconal acetate to aflatoxin B_1 by a supernatant fraction derived from lysed protoplasts of Aspergillus flavus N1

Precursor added			Aflatoxin B_1 formed over 1 hour incubation		
	uCi	specific activity mCi/mole	uCi	specific activity mCi/mole	% conversion
^{3}H-hydroxyversicolorin A	0.00068	17.0	0.00011	0.5	16.2
^{14}C-versiconal acetate	0.00007	1.4	0.00001	0.13	14.2

Experiment XIX

Comparative conversion of G-^{14}C-versicolorin A and G-^{3}H-hydroxy versicolorin A in a supernatant fraction derived from lysed protoplasts of Aspergillus flavus N1.

A supernatant fraction of lysed protoplasts was prepared as in Experiment XVII. This was incubated with the same cofactors (1ml) at the same concentration as in Experiment X. G-^{14}C-versicolorin A and G-^{3}H-hydroxyversicolorin A (V_3), kindly prepared by Dr M. F. Dutton, were added as a double substrate.

At various time intervals samples (1ml) were removed and treated as in Experiment I. The total counts (dpm) in the aflatoxin fraction were estimated and the percentage conversion calculated. Results are expressed in Table 20.

TABLE 20

Addition of ^{14}C-versicolorin A and ^{3}H-hydroxyversicolorin A to a supernatant fraction derived from lysed protoplasts of Aspergillus flavus N1

Precursor added		Aflatoxin formed over 1 hour incubation	
	µCi	µCi	% conversion
^{14}C-versicolorin A	0.00009	ZERO	ZERO
^{3}H-hydroxyversicolorin A	0.00043	0.00007	16.3

Experiment XX

Conversion of G-^{3}H-sterigmatocystin to aflatoxin B_1 by a supernatant fraction isolated from lysed protoplasts of Aspergillus flavus N1

A supernatant fraction of lysed protoplasts was prepared as in Experiment XVII. This was incubated with the same cofactors (1ml) at the same concentration as in Experiment X. G-^{3}H-sterigmatocystin (kindly prepared by Dr M. F. Dutton) was added as substrate.

At various time intervals samples (1ml) were removed and treated as in Experiment I. The total counts (dpm) in the aflatoxin fractions were estimated and the percentage conversion calculated. Results are expressed in Table 21.

TABLE 21

Conversion of G-^{3}H-sterigmatocystin to aflatoxin B_1 by a supernatant fraction derived from lysed protoplasts of Aspergillus flavus N1

Precursor added		Aflatoxin formed over 1 hour incubation	
	µCi	µCi	% conversion
^{3}H-sterigmatocystin	0.00025	0.00007	28

D I S C U S S I O N

Cultivation of Aspergillus flavus N1 in a chemically defined medium

The aim of this experiment was to observe the growth characteristics of Aspergillus flavus N1, in Reddy's chemically defined medium, in relation to the production of aflatoxin by the mould.

Cultivation of the mould over the 13 day period showed a characteristic biphasic graph, designated tropophase and idiophase (Bu'lock 1965), (Fig I Page No. 47). There appeared to be a decrease in aflatoxin concentration (from day 4 to 13) after an initial stationary phase (from day 2 to 4) (Heathcote and Dutton 1969). The experiment was repeated over 55 hours in order to follow aflatoxin production over the tropophase/idiophase interface. Between 31 and 48 hours after spore inoculation there was a rapid increase in aflatoxin concentration from 0.57mg/l (28µg* total) to 3.6mg/l (180µg* total), corresponding to 0.2mg/l/hr. Similarly, there was a greater increase between 48 hours and 55 hours from 3.6mg/l (180µg* total) to 6.7mg/l (333µg* total), which corresponds to 0.44mg/l/hr. Thus between 31 and 55 hours after incubation starts, all enzymes responsible for aflatoxin production, as well as all the required nutrients, are likely to be present giving maximal aflatoxin yields. Differences in aflatoxin concentration observed between the 13 day and 55 hour experiments are probably due to the differences in spore viability

* Total in 50ml of medium

Thus this experiment has shown that Aspergillus flavus N1 behaves in a similar manner to other aflatoxin-producing strains of Aspergillus species used by workers in this field e.g. Detroy and Hesseltine 1970.

The isolation and characterization of metabolites

Various Aspergillus species produce several metabolites associated with aflatoxin biosynthesis (see Introduction). In order to determine the activity of "cell-free" extracts and protoplasts (Sections II and III), various metabolites were isolated and identified so that they could be used as potential substrates.

The isolation and characterization of metabolites from Aspergillus flavus N1

This mould, when grown on the yeast extract and sucrose medium plus dichlorvos (20 µg/ml) - an inhibitor of aflatoxin biosynthesis (Hsieh 1973b) - produces several metabolites. These have been identified by comparative TLC and mass spectrometry with authentic samples and data of the known compounds. Identification procedures reveal that on the addition of dichlorvos to the growth medium aflatoxin formation is greatly reduced, and the following metabolites are produced:- averufin, versicolorin C, averufanin and versiconal hemiacetal acetate. There are several unknown compounds also produced which have yet to be fully identified, they appear as orange/red/yellow spots on TLC plates. Mass spectrometry data reveals that these compounds have parent ions of m/e 342 (A_2), 384 (VAcII) and 360 (versiconol-like pigment.)

Preliminary NMR data of the "versiconol-like pigment" reveals that the basic structure of the molecule is an anthraquinone, having a substitution pattern characteristic of the anthraquinones associated with aflatoxin biosynthesis, i.e. two meta coupled positions in the A ring and one unsubstituted position in the C ring, see Fig. A.

At present, there is some difficulty in determining the actual structure of this "versiconol-like pigment"; NMR data reveals complex aliphatic protons around 1.5 to 2.5 It is highly probably that these may be lipid contaminants which have been carried through the TLC procedures by complexing with the phenolic - OH groups attached to the anthraquinone nucleus.

The two remaining unidentified pigments are possibly anthraquinones with C_4 or C_6 side groups in various stages of ring opening or ring closing. A_2 (m/e 342) may exist in any one of the three forms shown in Fig. B , but is more likely to be present as either structure B or C if derived from versiconal hemiacetal acetate. VAc II (m/e 384) is probably versiconal hemiacetal acetate which has lost - OH from the furan side group (dehydration and reduction), thus an overall loss of m/e 16 has occurred. This probably exists as the structure shown in Fig. C.

Fig. A

Versiconol

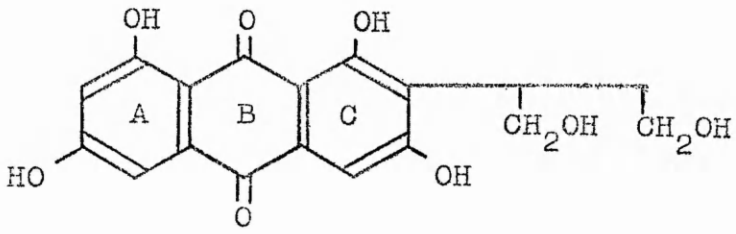
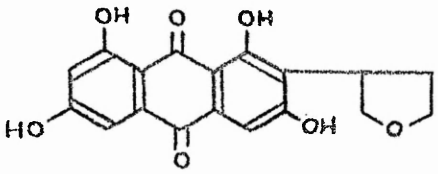
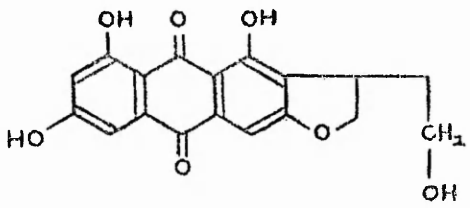


Fig. B

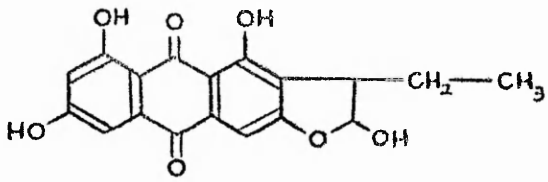
Possible structures for compound A_2 (m/e 542)



A



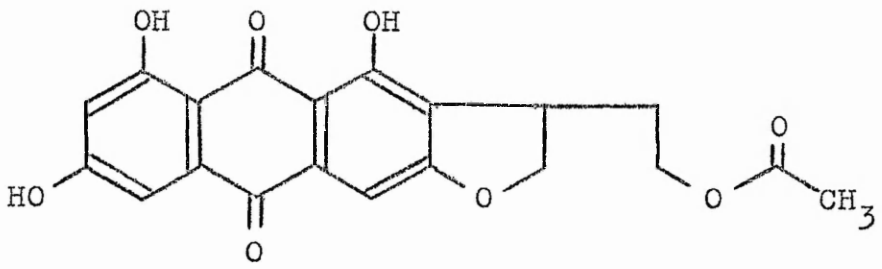
B



C

Fig. C

Possible structure for compound VAc II (m/e 384)



The isolation and characterization of metabolites from Aspergillus parasiticus 1-11-105 Whl

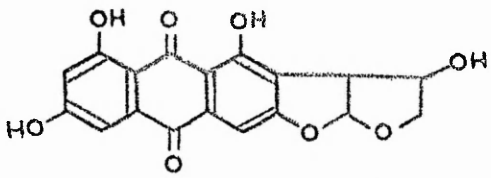
This mould, a U.V. mutant of an aflatoxin-producing strain, when grown on the yeast extract and sucrose medium produces large quantities of versicolorin A (100 mg/l). Three unidentified pigments (V_1, V_2 and V_3) were also produced, these have lower Rf values than versicolorin A, while mass spectra give parent ions at m/e 407, 410 and 356 respectively. From the mass spectra and relative Rf values on TLC, it would appear that V_3 (m/e 356) is a hydroxylated derivative of versicolorin A, similar to the hydroxylated form of aflatoxin B_1 ie. aflatoxin B_{2a}; here water is added across the vinyl ether double bond. Thus, V_3 probably exists as structure B in Fig. D.

V_2 (m/e 410) is probably a derivative of an anthraquinone with a C_6 side chain which is probably methylated, thus accounting for the higher molecular weight.

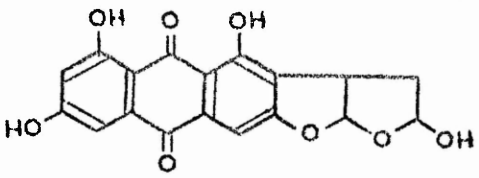
V_1 (m/e 407) may be a nitrogen containing anthraquinone due to its odd number parent ion. As all carbon compounds have an even number parent ion, it may be assumed that V_1 possesses a molecule requiring an odd number of bonds, e.g. a nitrogen atom. It is also possible that m/e 407 is not the parent ion. When cultures of the mould were treated with dichlorvos (20 µg/ml), versiconal hemiacetal acetate and the "versiconol-like pigment" were produced along with a decrease in

Fig. D

Possible structures for compound V_3 (m/e 356)



A



B

versicolorin A production. This indicates that the inhibitor blocks an enzyme step or steps prior to the formation of versicolorin A.

The isolation and characterization of metabolites from Aspergillus flavus 72338

This mould, when cultured on the yeast extract and sucrose medium, produces O-methyl sterigmatocystin (70 mg/l) and a blue grey fluorescent compound with a lower Rf value. This has a parent ion of m/e 336 which has yet to be fully identified as a related metabolite of sterigmatocystin.

The isolation and characterization of metabolites from Aspergillus flavus W49

This mould, a U.V. mutant, produces large quantities of averufin (120 mg/l), as well as trace amounts of aflatoxins. Thin layer chromatography, using ethyl acetate : toluene (1 : 1 v/v) as the solvent, separated averufin and aflatoxins sufficiently to be able to remove averufin in an almost pure state. A single spore isolate of a U.V. mutant of W49 has been isolated (U_2) which produces only averufin and no aflatoxins.

The isolation and characterization of metabolites from Aspergillus parasiticus J8E

This mould is a U.V. mutant which produces norsolorinic acid (80 mg/l) and trace amounts of aflatoxin. If dichlorvos (20 µg/ml) is added to this mould, the aflatoxins are no longer produced and the characteristic "versiconal acetate" is produced instead (as identified by Rf values). Norsolorinic acid and "versiconal acetate" production are inhibited with higher concentrations of dichlorvos (30 µg/ml), this indicates an inhibition of the polyketide synthetase as suggested by Schroeder et al 1974.

Preparation of "cell-free" extracts from Aspergillus flavus N1

Liquid Nitrogen

Table 1 shows the protein release from mycelium by this method (0.8mg/ml). Fig. 1 shows the results obtained when the "cell-free" extract, produced by this technique, is incubated with "basal cofactor medium".

Using "basal cofactor medium", a phenolic compound, observed on TLC plates, was produced after 1 hour's incubation, and increased in intensity with length of incubation; the phenolic compound was located by spraying the TLC plate with Pauly's reagent (see Appendix 3). Although this phenolic compound may not be involved in aflatoxin biosynthesis, it shows that the technique does not denature all enzymes in the mycelium. Sansing et al (1975) have found this technique useful in their work using Aspergillus ochraceus.

When a "cell-free" extract is incubated with "versiconal acetate" or versicolorin C there is no alteration of these compounds, as observed on TLC plates. This would indicate either the technique is ineffective in producing a "cell-free" extract capable of converting the anthraquinones to the aflatoxins, or possibly, both substrates are not intermediates in the biosynthesis of the aflatoxins. However, Yao and Hsieh (1974) have found "versiconal acetate" to

be converted to aflatoxin.

Ultraturrax

Yao (1974), using an instrument similar to the Ultraturrax (a Polytron), has shown encouraging results in obtaining a "cell-free" extract. The Ultraturrax preliminary results were discouraging in that the protein released from 60 seconds of treatment (1.3mg/ml) was much lower than the protein release quoted by Yao for 60 seconds of Polytron treatment (8.0mg/ml). However, versicolorin C and "versiconal acetate" were converted to aflatoxin (as measured by visual comparison on TLC plates).

Other methods

All other methods tested for production of a "cell-free" extract gave no conversion of acetate in the "basal cofactor medium". It is possible that these methods of cell disruption are either too drastic in bursting the cell or, they are not strong enough to burst the cell wall (as in homogenization) (Experiment III). Because of the lack of success of many workers in the field of secondary metabolite enzymology, it must be assumed that these enzymes are extremely labile and are probably only present in small quantities, thus making high percentage recovery a necessity, (Hsieh 1977).

Conclusions

"Cell-free" extracts, obtained by mechanical methods, do not give encouraging results in the hope of finding a suitable technique to isolate the enzymes which are involved in aflatoxin biosynthesis. It was decided that another method of obtaining a "cell-free" extract would be employed. This involves dissolving away the cell wall to yield osmotically fragile protoplasts which may be burst to yield a "cell-free" extract. This method was employed and is discussed in Section III.

Protoplasts

As shown in Section II, the use of mechanical means to disrupt the fungal mycelium has proved unsuccessful in yielding cell-free extracts capable of converting intermediates to the aflatoxins. Therefore, it was decided to dissolve away the cell wall by means of lytic enzymes prepared from culture extracts of Trichoderma viride. This method yields fungal protoplasts which are osmotically fragile and thus can be utilized to prepare cell-free extracts.

In all control experiments protoplasts derived from Aspergillus flavus N1 mycelium and suspended in buffer stabilizer, which did not contain a carbon source, were unable to biosynthesize aflatoxins de novo. Reddy's medium has been shown, in Section I, to stimulate growth and aflatoxin biosynthesis in A. flavus N1 cultures, therefore, it was used as a suitable chemically defined medium in the investigation of aflatoxin biosynthesis by whole protoplasts. Initially, protoplasts were derived from 3 day-old mycelium as this had given a "good" yield of protoplasts (Table 1 Page No. 90). In order to optimise aflatoxin formation, protoplasts from mycelium of different ages were investigated (see Table 8 Page No. 109).

The results show that aflatoxin formation is at an optimum in protoplasts derived from 3 day-old mycelium over the 18 hour period studied, although there is little difference from

those protoplasts derived from 2 day-old mycelium over the initial 3 hour period, (i.e. 1.3 µg/hr for the 3 day and 1.6 µg/hr for the 2 day). Protoplasts from 1 day-old mycelium seem to have little aflatoxin-producing capability over the initial 3 hour incubation period, but gain the ability to synthesize aflatoxins over the remaining 15 hours.

Protoplasts from 4 and 5 day-old mycelium appear to degrade aflatoxins over the initial 3 hour incubation period. This result has been observed using whole mycelium (Shih and Marth 1975), here a decrease in aflatoxin concentration with increasing age of mycelium is observed. Similarly, the growth experiment in Section I (Page Nos. 45 to 50) shows a slight decrease in aflatoxin concentration between the 8 day and 13 day samples. Protoplasts of 4 and 5 day-old mycelium appear to mirror this degradation of aflatoxin, the effect may be due to either a degradation enzyme(s) only, or a combination of degradation enzyme(s) and termination of synthesis.

It was noted that aflatoxins were already present in the protoplast preparations and, although this could be reduced by washing the preparation with buffer stabilizer, the protoplasts could not be prepared aflatoxin-free. Thus, protoplasts prepared from 2 and 4 day-old mycelium contained a larger aflatoxin concentration than the protoplasts derived from the 3 day-old mycelium. This may be an indication of

a better aflatoxin producing potential or, a greater ability to retain aflatoxins during the washing procedure which, in turn, may be due to a higher lipid content.

The difficulty with interpreting these results as a whole is that protoplasts are "naked" mycelium, devoid of a cell wall, and will probably revert fully to primary metabolism and resynthesize cell wall material when placed in the 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 hours but slows down over the remaining 15 hour period. Certainly, by the time 18 hours have elapsed mycelial cell walls are discernable in all protoplast preparations. It is important to note that the biosynthesis of the observed aflatoxins is a result of a number of enzyme activities and hence the graphs in Fig. 6 Page No. 110 are summations of these activities.

From these results it follows that added intermediates should be converted to aflatoxin by whole protoplasts providing the intermediates can penetrate the membrane. In order to study the feasibility of using whole protoplasts in this type of investigation, a number of proven and potential intermediates in aflatoxin biosynthesis were added to the protoplast preparations. Several of the intermediates e.g. versicolorins A and C, "versiconal acetate" and sterigmatocystin were converted to aflatoxins as judged by the formation of

aflatoxins over that in the controls. In control experiments protoplasts were suspended in the buffer stabilizer without a carbon source and no aflatoxin was produced (in the µg range) over the incubation period. This indicates little or no endogenous aflatoxin formation by the whole protoplasts.

The greatest rate of conversion was observed when versicolorin A was added (13.4 µg aflatoxin formed over 18 hours). In an attempt to increase the rate of formation of aflatoxins, excess versicolorin A was added to a protoplast preparation. The result of this was not an increase but rather an inhibition of aflatoxin production. This effect may be due to the presence of inhibitory amounts of the solvent NN dimethylformamide, indeed, when this is added to whole cultures of A. flavus N1 the production of aflatoxins is reduced by 76.5%.

In contrast, averufin, O-methyl sterigmatocystin and norsolorinic acid do not appear to be converted to aflatoxins over the 18 hour period of incubation. Both averufin and norsolorinic acid have been implicated as precursors in the biosynthesis of the aflatoxins (Hsieh et al 1976b), but results here suggest that over the 18 hour period of incubation they are not converted to aflatoxins. This may be due to their inability to pass through the cell membrane quickly enough, so that in whole protoplast experiments these metabolites may only just be emerging through the membrane

towards the end of the incubation period. It is also possible that they may not be true intermediates so that when they do enter the protoplasts, if at all, they will not be converted to aflatoxins.

The conversion of ^{14}C-versicolorin A and ^{14}C-acetate (Table 9 Page No. 113) further confirmed the ability of protoplasts to synthesize aflatoxins and that, not only were the enzymes involved in the latter stage of conversion present (versicolorin A to aflatoxin B_1), but also the initial synthetase as well (acetate to aflatoxin B_1). Conversion rates of whole protoplasts and whole mycelium reveal, that for the protoplasts there is a reduced ability to convert versicolorin A to aflatoxin B_1 over a three hour period (15.5%) compared with whole mycelium (between 22.4% and 44.8% depending on specific activity of the precursor, Lee et al 1976). This may indicate that protoplasts are incapable of prolonged conversion experiments, as they require a cell wall synthesis capability in order to function in a more 'normal' environment. Thus certain key co-metabolites e.g. NADPH, may be channelled off to sites of cell wall synthesis which, in turn, will "slow down" secondary metabolism. However, with the addition of acetate this channelling effect should not be so evident as the acetate can be metabolized through several pathways to provide not only secondary metabolites, but also necessary intermediates. This is evident by the 0.28% conversion over 3 hours observed in

whole protoplasts. This compares with a 0.94% conversion by whole mycelium over 24 hours (Singh & Hsieh 1977).

A final series of experiments, conducted on whole protoplasts, showed comparative respiration rates of protoplasts derived from various ages of mycelium (Table 10 Page No. 115). Here, protoplasts derived from 1 and 2 day-old mycelium respired at a higher rate than did 3 and 4 day-old material. This shows that protoplasts are good indicators of the metabolic state of the mycelium at any given time. In day 1 material, the mycelium will be rapidly growing and dividing and hence show a high respiration rate, thus, protoplasts derived from 1 day-old mycelium will reflect this and a similar high respiration rate will be observed. Similarly, in older material (4 day-old) large areas of the mycelium will be old and vacuolated, not rapidly dividing and thus, respiring at a reduced rate as compared with younger mycelium. This is again mirrored in the day 4 protoplast preparations where respiration is greatly reduced in comparison with younger material.

Having partially investigated whole protoplasts for their ability to synthesize aflatoxins, they were then used to obtain cell-free extracts capable of converting possible intermediates to the aflatoxins. The protoplasts were initially lysed using a double technique which relied on their fragility to osmotic tension. Following their preparation, protoplasts were collected by centrifugation

and the resultant pellet resuspended in cold ($4^{o}C$) buffer which did not contain the magnesium sulphate stabilizer. This caused the more fragile protoplasts (the large vacuolated types) to swell and burst. In order to cause further disruption of any unburst protoplasts the slurry was frozen for 30 minutes at $-10^{o}C$ (N. Ryder 1976); 95% of all protoplasts in a given preparation were burst using this double technique. Following lysis, the resulting slurry was centrifuged (10,000 xg for 30 mins.) in order to produce supernatant and residue fractions which were investigated for their ability to convert labelled intermediates to aflatoxin B_1.

Utilizing a supernatant fraction isolated from lysed protoplasts of Aspergillus flavus N1, it was shown that this cell-free extract was capable of converting ^{14}C-versiconal acetate to aflatoxin B_1 over various incubation times (Table 11 Page No. 119). There appeared to be a difference in percentage conversion by the 3 and 4 day-old material; 3.6% for the 3 day over 1 hour, and 7.6% for the 4 day over 1 hour. This indicates that there may be a higher concentration of enzymes involved in the latter part of the biosynthetic pathway in the 4 day-old material as compared with the 3 day-old material. It was also observed that there was an increase in percentage conversion in the 1 hour incubation sample as compared with the 18 hour incubation sample; 3.6% for 1 hour and 9.6% for 18 hour in the 3 day-old material. The residue fraction in all preparations did not convert the

^{14}C-versiconal acetate to aflatoxin B_1 and, as centrifugation was sufficient to remove mitochondria, it may be assumed that the enzymes responsible for the conversion of versiconal acetate to aflatoxin B_1 are present in the microsomal fraction. When this experiment was repeated omitting the FAD from the incubation medium, there was little incorporation of label from versiconal acetate to aflatoxin B_1, thus indicating the possible presence of an FAD linked oxygenase(s) in the biosynthetic pathway.

When ^{14}C-versicolorin A was added to a supernatant fraction as a substrate, there was little incorporation of label into aflatoxin B_1 (Table 12 Page No. 121), even over the 18 hour period studied incorporation never rose above 0.2%. In order to confirm this result, ^{14}C-versicolorin A and ^{3}H versiconal acetate were used as double substrates in a single supernatant fraction from lysed protoplasts of 3 day-old mycelium (Table 13 Page No. 123). When aflatoxins from the experiment were estimated, they were found to contain 4.4% of the ^{3}H label from versiconal acetate and 0.15% of the ^{14}C label from versicolorin A. This result appears to contradict the findings of various workers using replacement cultures (Lee et al 1976), and even the whole protoplast experiment using ^{14}C-versicolorin A as a substrate. These results indicate that versiconal acetate is a direct precursor of aflatoxin B_1, while versicolorin A is not. It is possible that there may be some mechanism whereby versicolorin A undergoes some form of activation process to

attach onto an enzyme or enzyme system; as this may not be available in cell-free extracts it will not be converted through to aflatoxin B_1.

Using a supernatant fraction of lysed protoplasts from A. parasiticus 1-11-105 Whl, ^{14}C-versiconal acetate was added as a substrate and was found to be converted to versicolorin A (3.03% conversion over 1 hour) (Table 14 Page No. 125). When this experiment was repeated using ^{3}H versiconal acetate as the substrate (Table 15 Page No. 127), a 2.6% conversion was obtained over the 1 hour incubation time. However, when NAD and NADP were substituted for the NADH and NADPH in the incubation medium, there was only a 0.31% conversion of the substrate to versicolorin A. This may indicate the presence of a nicotinamide linked oxygenase, and not a dehydrogenase, being involved in the final ring closure of the difuran group in versicolorin A. It has been shown (Dutton 1977) that alcohol dehydrogenase will not carry out this conversion; thus supporting the oxygenase hypothesis.

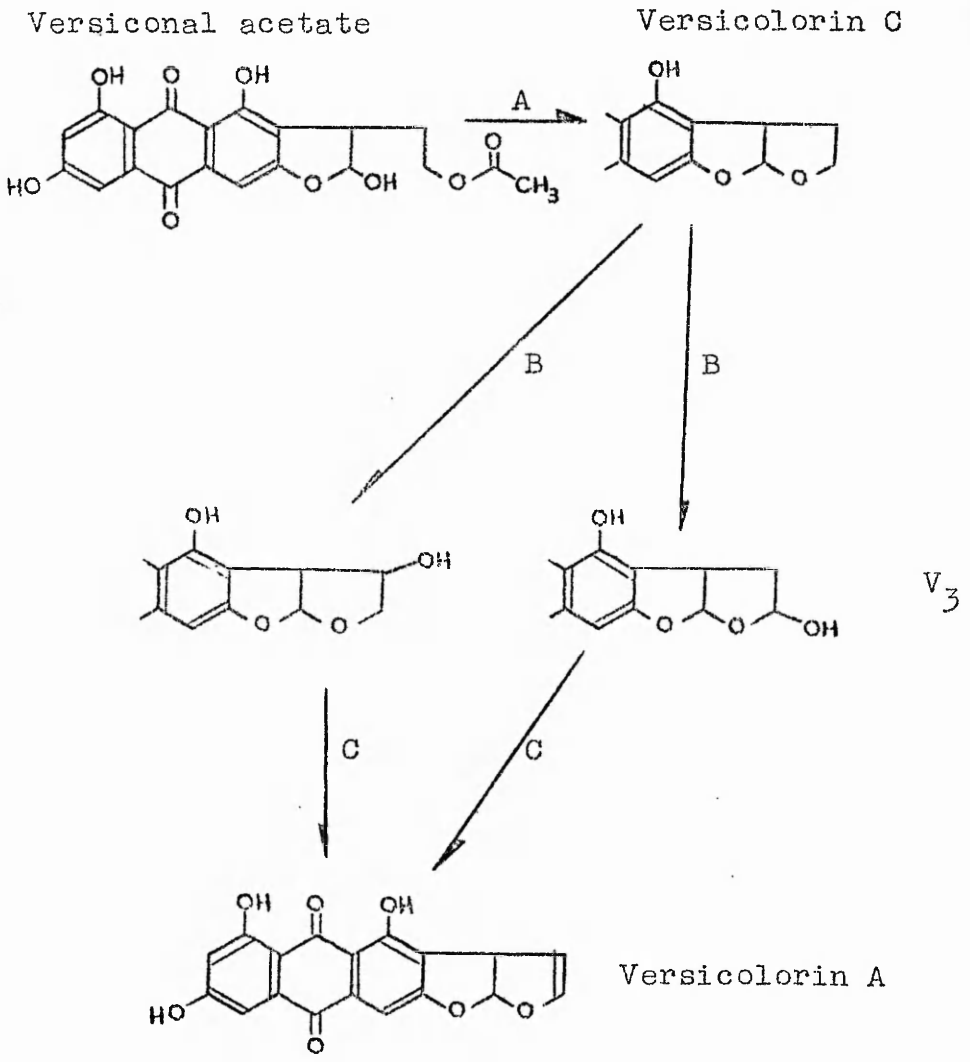
Dichlorvos (dimethyl 2,2 dichlorovinyl phosphate), an organophosphorous insecticide, has been shown to inhibit aflatoxin production (Rao and Harein 1972), however, when other organophosphates were added to aflatoxin-producing cultures there was little inhibition (Yao 1974). This would indicate that dichlorvos inhibition is dissimilar to acetylcholinesterase inhibition.

A series of experiments were conducted to estimate the general esterase activity of a supernatant fraction derived from lysed protoplasts of A. parasiticus 1-11-105 Whl (Table 16 Page No. 129). From the results, it can be seen that there is greater esterase activity in the supernatant fraction after 100,000 xg. The effect of dichlorvos on the general esterase activity of a supernatant fraction derived from lysed protoplasts of A. parasiticus 1-11-105 Whl was estimated (Table 17 Page 131). Results show that dichlorvos will inhibit the general esterase activity (50% inhibition when 10µg are added to 1ml of incubation mixture, and 96% inhibition when 50µg are added).

These results indicate that dichlorvos will inhibit esterase activity and that esterase activity is greater in a microsomal fraction. Thus, in the conversion of versiconal acetate to versicolorin A, it is possible that three enzymes are involved. An esterase may be involved in the removal of an acetate group resulting in the ring closed form of versicolorin C, this would then undergoe action by a nicotinamide-linked oxygenase to give a hydroxylated derivative of versicolorin C. This will have a m/e of 356, similar to V_3 found in A. parasiticus 1-11-105 Whl cultures; loss of water by a dehydrase will then result in versicolorin A formation (see Fig. E). Although it was evident that the freeze/thaw technique for protoplast lysis yielded cell-free extracts capable of converting certain substrates through to aflatoxin B_1, it was possible that the method

Fig. E

Proposed pathway of conversion of versiconal acetate to versicolorin A



A = esterase
B = nicotinamide linked oxygenase
C = dehydrase

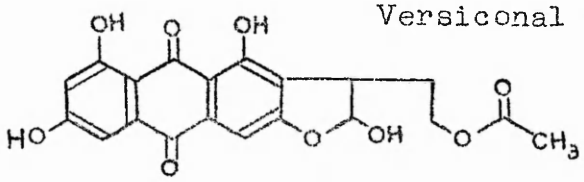
may have disrupted mitochondria, releasing their enzymes, and thus it was decided to use a different technique for bursting the protoplasts. The protoplasts were suspended in buffer containing no stabilizer and were homogenized for 3 minutes in a hand-held Potter tissue homogenizer (at $4^{o}C$). This burst the remaining whole protoplasts but not the mitochondria (Wales 1977).

This revised technique was used for obtaining cell-free extracts in order to study the anomaly of lack of incorporation of versicolorin A into aflatoxin B_1. It is possible that one of several schemes exists for the conversion of versiconal acetate through sterigmatocystin to aflatoxin B_1. Three such schemes are outlined in Figs. 1, 2 & 3. It has already been shown that the theoretically satisfactory scheme outlined in Fig. 1 does not occur to any extent, as versicolorin A is not incorporated into aflatoxin B_1 by cell-free extracts derived from protoplasts.

Using a double label experiment of ^{14}C-versicolorin C and ^{3}H-versiconal acetate, it can be seen that incorporation of both metabolites into aflatoxin B_1 does occur (Table 18 Page No.133). This suggests that the scheme in Fig. 2 may be possible; in this pathway, versiconal acetate is hydrolysed by an esterase producing versicolorin C, which will then undergo oxidation, methylation, decarboxylation and rearrangement to produce sterigmatocystin. However, Bennett et al (1976) have shown that inhibition of a

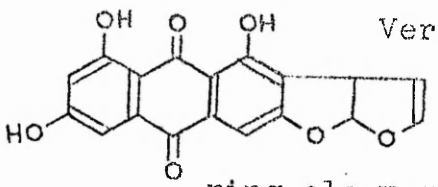
Fig. 1

Proposed pathway of aflatoxin biosynthesis involving Versicolorin A



Versiconal acetate

hydrolysis of acetate ester
ring closure
oxidation



Versicolorin A

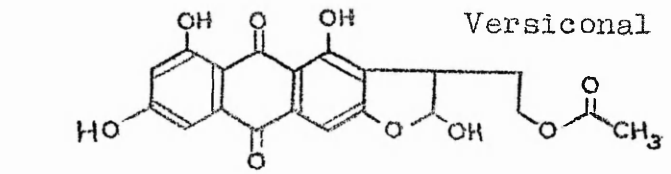
ring cleavage
methylation, decarboxylation,
rearrangement

Sterigmatocystin

Aflatoxin B_1

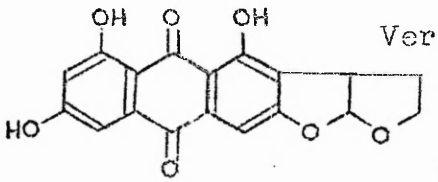
Fig. 2

Proposed pathway of aflatoxin biosynthesis involving Versicolorin C



Versiconal acetate

hydrolysis of acetate ester
ring closure



Versicolorin C

ring cleavage
decarboxylation,
rearrangement

methylation,
oxidation,

dehydrogenation

Versicolorin A

Sterigmatocystin

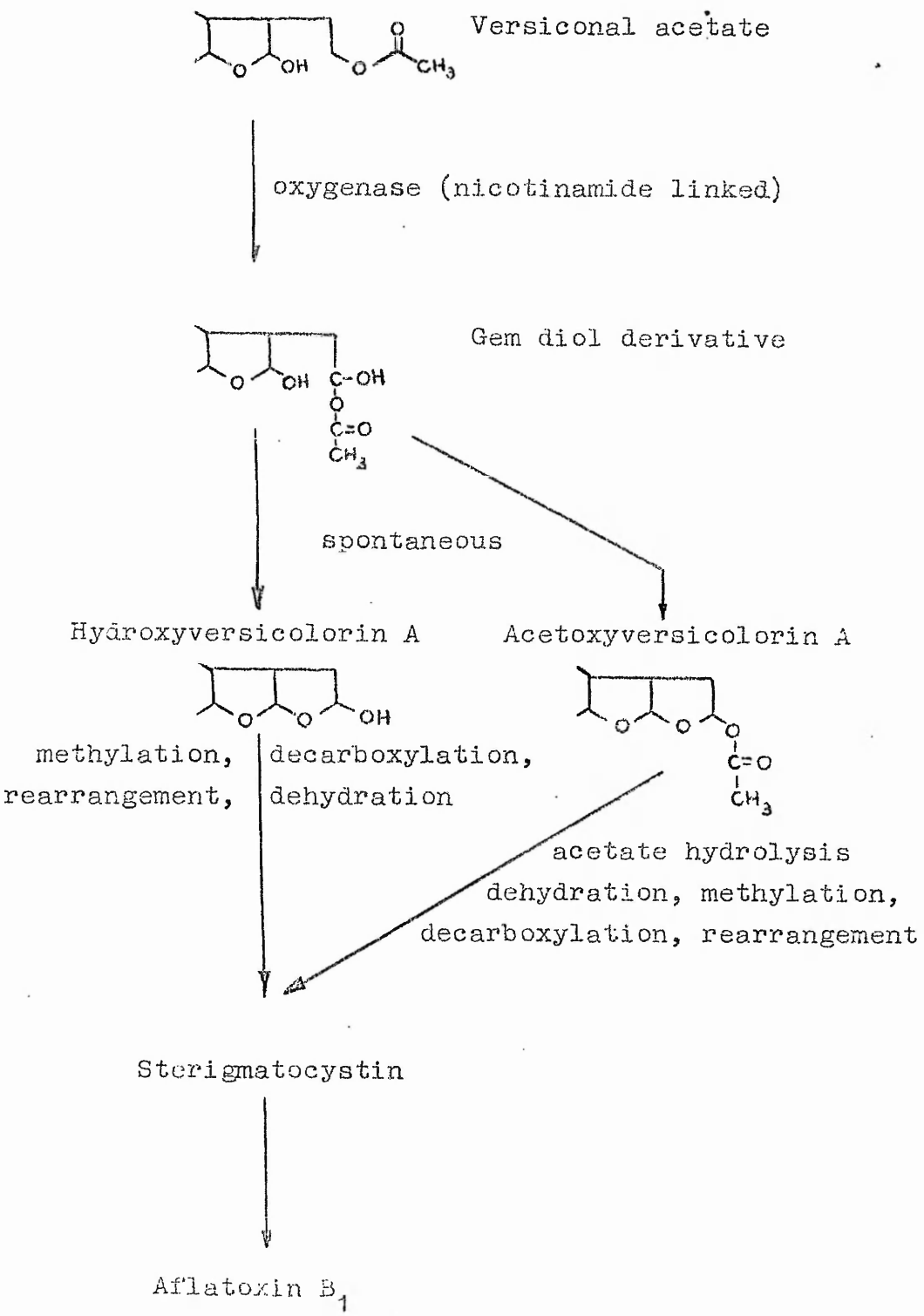
Aflatoxin B_1

versicolorin A-producing mutant of A. parasiticus by dichlorvos does not inhibit versicolorin C production. This may indicate that the scheme in Fig. 2 may possibly not occur or, at least, is not a main pathway.

Another series of experiments, utilizing the hydroxy derivative of versicolorin A (V_3), were performed to establish the validity of the scheme outlined in Fig. 3. The addition of ^{14}C-versicolorin A and ^{3}H-hydroxyversicolorin A in the same supernatant fraction yielded a 16% conversion of the hydroxyversicolorin A, but a zero percent conversion of the ^{14}C-versicolorin A. This further establishes that versicolorin A is not a true intermediate in aflatoxin biosynthesis, but that hydroxyversicolorin A is a likely intermediate. Thus, in scheme 3 (Fig.3) oxidation of versiconal acetate to the half ester of a gemdiol will be catalysed by an oxygenase, as evident by the requirement for NADPH/NADH (shown in Experiment XIV); as the gemdiol derivative will be unstable it may undergo spontaneous cleavage to hydroxyversicolorin A or ring closure may occur to produce acetoxyversicolorin C. In the latter case, hydrolysis of the acetate ester by an esterase, followed by oxidation, methylation, decarboxylation, rearrangement and dehydration, will result in sterigmatocystin. Hydroxyversicolorin A itself would undergo methylation, decarboxylation, rearrangement and dehydration to produce sterigmatocystin.

Fig. 3

Proposed pathway of aflatoxin biosynthesis involving hydroxyversicolorin A



The overall results from these experiments suggest a scheme as outlined in Fig. 4. It is apparent that versiconal acetate plays a key role as an intermediate in aflatoxin biosynthesis as it is produced in direct response to a specific inhibition.

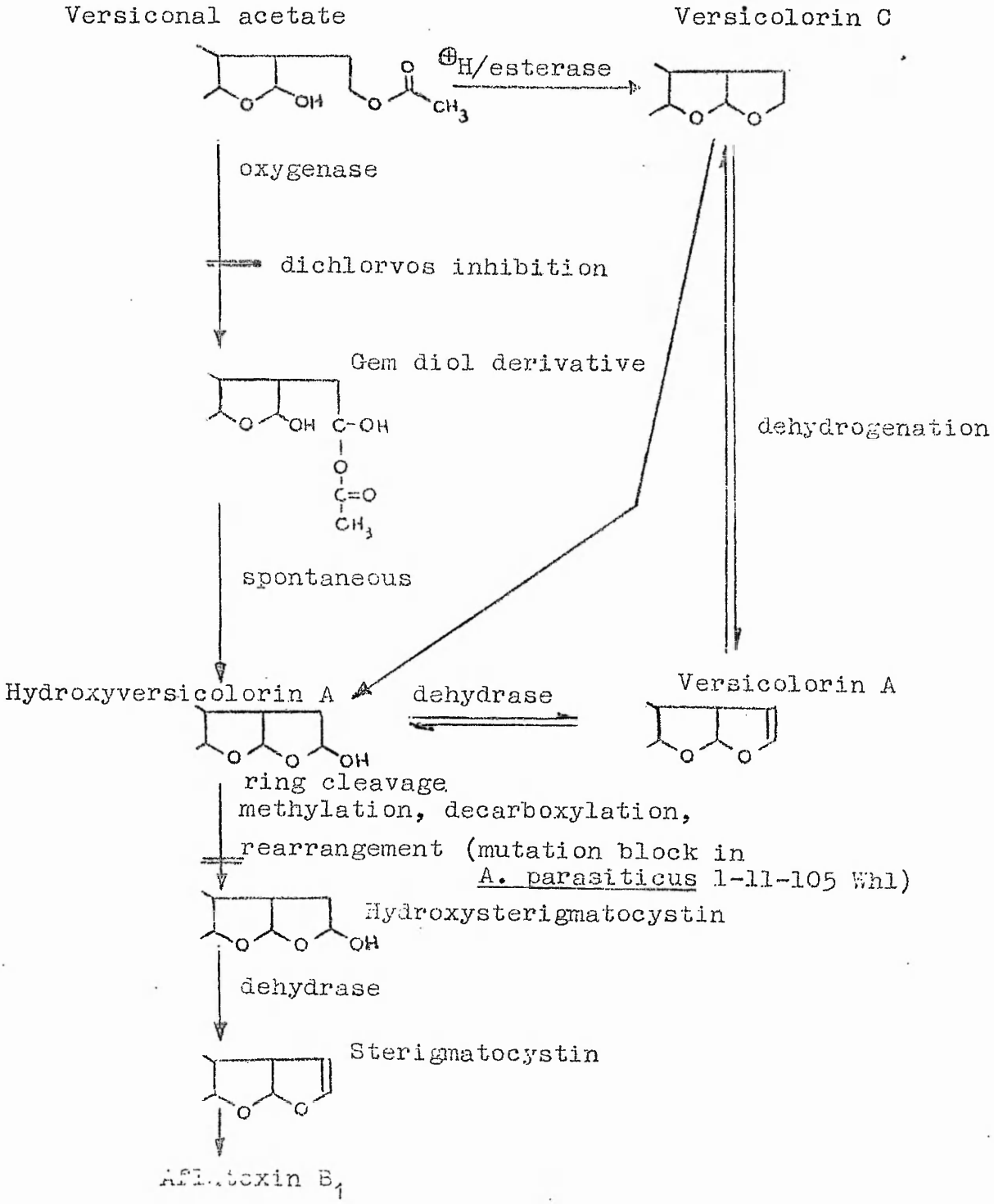
On addition of dichlorvos to the system, the enzymic conversion of versiconal acetate to its gem diol derivative is blocked and thus versiconal acetate will accumulate. It is therefore concluded that dichlorvos may act by, either blocking the oxygenase involved or, competing for an essential metal ion required by the enzyme.

The appearance of versicolorin C may be explained on the basis of the chemical addition of water in the culture when the pH drops to a sufficiently low level. However, as has been shown, versicolorin C may act as an intermediate in aflatoxin biosynthesis, and thus it seems probable that versicolorin C is converted to hydroxyversicolorin A, as catalysed by the oxygenase used to form the gem diol derivative. It seems likely that the difference in rates of conversion of versicolorin C and versiconal acetate is due to the difference in specificity of the enzyme for the two substrates.

The accumulation of versicolorin A in the mutant A. parasiticus 1-11-105 Whl may be explained on the basis that it is possible that the enzymes involved in the conversion of

Fig. 4

Proposed pathway of aflatoxin biosynthesis



hydroxyversicolorin A to (hydroxy)sterigmatocystin are no longer synthesized and that a dehydrase will convert the hydroxyversicolorin A to versicolorin A. This is supported by the fact that this versicolorin A-producing mutant also accumulates hydroxyversicolorin A.

A 16.2% conversion of ^{3}H-hydroxyversicolorin A and a 14.2% conversion of ^{14}C-versiconal acetate to aflatoxin B_1 further confirms the involvement of these two compounds in aflatoxin biosynthesis (see Table 19 Page No. 135). Thus, hydroxyversicolorin A appears to hold an important role as an intermediate of aflatoxin biosynthesis, and versicolorin A, long thought of as a model precursor of aflatoxins, is not in fact a true intermediate.

In order to confirm the involvement of sterigmatocystin in aflatoxin biosynthesis, ^{3}H-sterigmatocystin was added as a substrate (Table 21 Page No. 139) to the cell-free extract. The conversion of 28% to aflatoxin B_1 indicated that the preparation was highly active for the latter stages of aflatoxin biosynthesis and that sterigmatocystin is metabolically close to aflatoxin B_1.

S U M M A R Y

Various potential intermediates involved in aflatoxin biosynthesis have been isolated in preparation as substrates for cell-free extracts; several other compounds have also been isolated but their structures have yet to be fully confirmed.

Using a number of conventional techniques to obtain cell-free extracts, i.e. homogenization, sonication, liquid nitrogen and lypholization, it has been shown that the enzymes involved in aflatoxin biosynthesis are extremely labile and that a delicate method of cell-free extract isolation is required.

In order to obtain an active cell-free extract, protoplasts have been prepared by enzymically digesting away the cell wall. Whole protoplasts behave in a manner similar to the whole mycelium but with a reduced aflatoxin-producing potential, this is probably due to protoplasts in the idiophase reverting back to the tropophase. The whole protoplasts are capable of converting some potential intermediates into aflatoxins, e.g. versicolorin A and C, versiconal acetate and sterigmatocystin, but not averufin, O-methylsterigmatocystin or norsolorinic acid; this may indicate that none of these latter compounds are true intermediates. Lysis of the protoplasts, by osmotic tension and freezing, has yielded a cell-free extract capable of converting versiconal acetate, but not versicolorin A, to aflatoxin B_1. This suggests that versicolorin A is not a true intermediate in aflatoxin biosynthesis and that it may only be a "side-shunt" molecule.

Other results indicate that an FAD-linked oxygenase(s) is involved in aflatoxin biosynthesis. It is also shown that a nicotinamide-linked oxygenase is involved in versicolorin A production and that the general esterase activity of a cell-free extract from a versicolorin A-producing mutant is inhibited by dichlorvos. It is also suggested that a hydroxy derivative of versicolorin A is involved in aflatoxin biosynthesis and a biosynthetic pathway incorporating these results is outlined.

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A P P E N D I C E S

APPENDIX 1

Calibration of Optical Density Scale

The most convenient method of calibrating a spectrophotometer is to measure the transmittance of suitable standards. A solution of potassium chromate (0.004%w/v) in dilute sodium hydroxide solution (0.02M) has been carefully studied and is a suitable standard for the range λ = 220 nm to λ = 490 nm.

Wavelength (nm)	T	A	E
220	0.358	0.446	2167
225	0.601	0.221	1074
230	0.674	0.171	832
235	0.616	0.210	1022
240	0.512	0.295	1433
245	0.402	0.396	1922
250	0.319	0.496	2410
255	0.268	0.572	2778
260	0.233	0.633	3072
265	0.202	0.695	3374
270	0.180	0.745	3617
275	0.175	0.757	3676
280	0.194	0.712	3459
285	0.257	0.590	2866
290	0.373	0.428	2080
295	0.533	0.273	1327
300	0.709	0.149	726
305	0.834	0.079	383

Wavelength (nm)	T	A	E
310	0.895	0.048	234
315	0.900	0.046	222
320	0.864	0.064	308
325	0.804	0.095	460
330	0.710	0.149	722
335	0.600	0.222	1077
340	0.483	0.316	1535
345	0.373	0.428	2018
350	0.276	0.559	2715
355	0.199	0.701	3405
360	0.148	0.830	4030
365	0.116	0.936	4544
370	0.103	0.987	4795
375	0.102	0.991	4815
380	0.117	0.932	4526
385	0.150	0.824	4002
390	0.202	0.695	3374
395	0.294	0.532	2582
400	0.402	0.396	1922
410	0.632	0.299	968
420	0.751	0.124	604
430	0.824	0.084	408
440	0.882	0.054	265
450	0.927	0.033	160
460	0.960	0.018	86

APPENDIX 2

Biuret Method for Protein determination
(Gornall *et al* 1949)

Reagent:

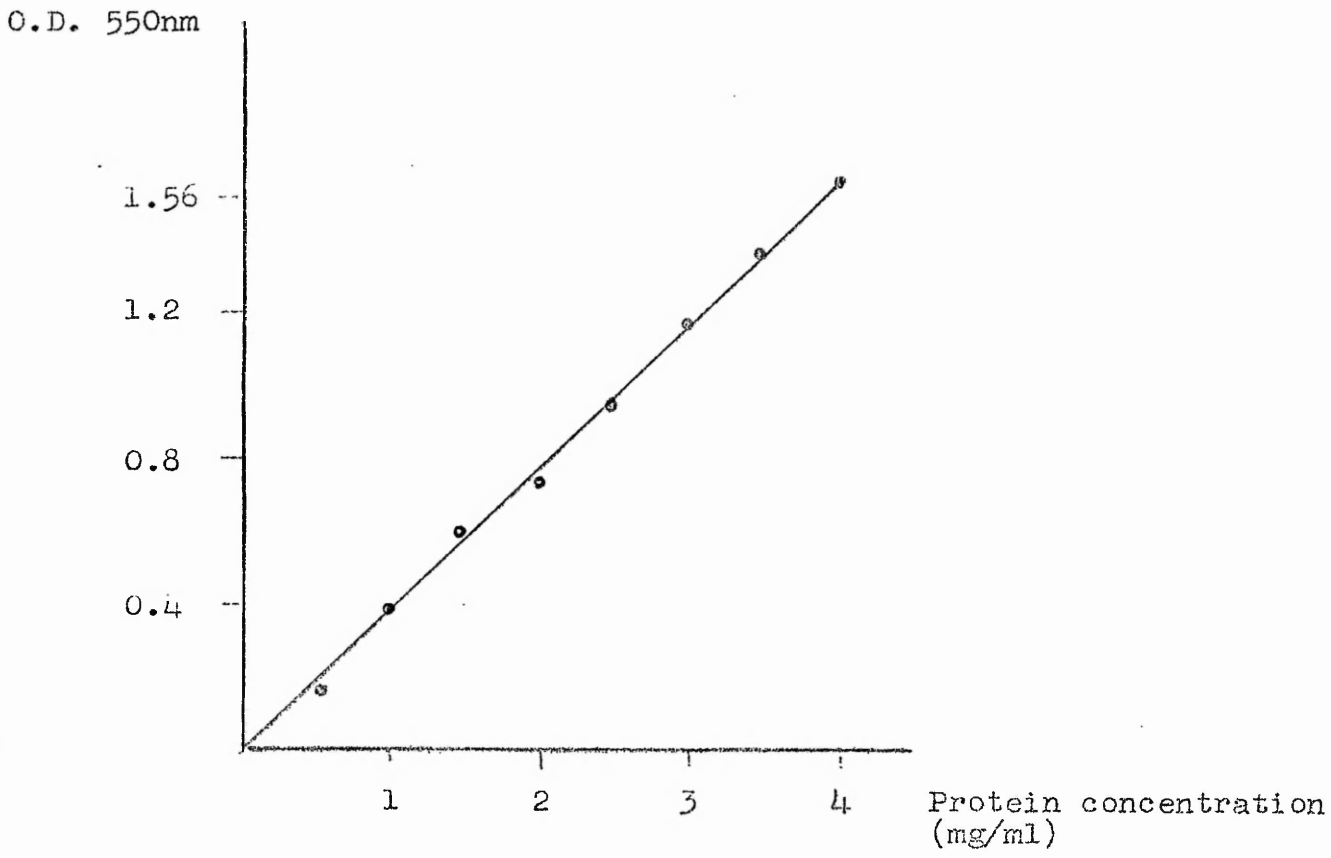
Dissolve copper sulphate (1.5g) and potassium sodium tartrate (6.0g) in water (500ml), add CO_2-free sodium hydroxide solution (10%, 300ml) and make up to 1 litre with water.

Test:

Take a sample (2ml) of solution to be tested and add Biuret reagent (8ml). Leave for 30 minutes and then record optical density at 550nm.

Protein Standard Curve using

the method of BIURET



APPENDIX 3

Spray Reagent for use on Chromatograms

Pauly's Reagent (Smith 1960)

Solution (1) Sulphanilic acid (9g) in concentrated HCl (90ml, water 900ml)

Solution (2) Sodium nitrite (5% w/v) in water

Solution (3) Sodium carbonate (10% w/v) in water

When required, solutions (1) and (2) were mixed in equal volumes and allowed to stand for 4 to 5 minutes at room temperature, two volumes of solution (3) were then added.

Physical properties of aflatoxins

Aflatoxin	Molecular Formula	Molecular Weight	Melting Point	U.V. absorption max, nm, in EtOH (Extinction Coefficient)		
B_1	$C_{17}H_{12}O_4$	312	268/9	233 (25,600)	265 (13,400)	360 (21,800)
B_2	$C_{17}H_{14}O_6$	314	286/9	222 (17,000)	265 (11,000)	362 (20,800)
G_1	$C_{17}H_{12}O_7$	328	244/9	243 (11,500)	264 (10,000)	362 (16,100)
G_2	$C_{17}H_{14}O_7$	330	237/240	214 (28,100)	265 (11,600)	362 (20,900)
M_1	$C_{17}H_{12}O_7$	328	299	226 (23,100)	265 (11,600)	357 (19,000)
M_2	$C_{17}H_{14}O_7$	330	293	221 (20,000)	264 (10,900)	357 (21,000)
B_{2a}	$C_{17}H_{14}O_7$	330	240	228 (17,600)	265 (10,100)	363 (20,200)
G_{2a}	$C_{17}H_{14}O_8$	346	190	223 (18,600)	262 (8,700)	365 (18,000)
GM_1	$C_{17}H_{12}O_8$	344	276	235 (21,200)	262 (16,300)	358 (12,000)
B_3	$C_{16}H_{14}O_6$	302	217	229 (10,000)	262 (7,550)	358 (9,350)

Physical properties of various anthraquinones

Compound	Molecular Formula	Molecular Weight	Melting Point	U.V. absorption max, nm, in EtOH (Extinction Coefficient)		
Versicolorin A	$C_{18}H_{10}O_7$	338	289	265 (17,756)	290 (26,547)	321 (12,118)
Versicolorin C	$C_{18}H_{12}O_7$	340	310	267 (20,400)	292 (28,800)	326 (10,000)
Versiconol	$C_{18}H_{16}O_8$	360	262/6	262 (7,900)	296 (9,400)	318 (11,000)
Versiconal Acetate	$C_{20}H_{16}O_9$	400	219/225	265 (14,000)	295 (23,000)	323 (11,300)
Averufin	$C_{20}H_{16}O_7$	368	283/9	265 (18,500)	294 (30,800)	319 (12,500)
Hydroxy-versicolorin A	$C_{18}H_{12}O_8$	356	269	266 (18,000)	291 (25,000)	317 (11,000)

Appendix 5