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DEVELOPMENT OF A VERSICOLORIN A ANTIBODY AND APPLICATION TO ENZYME-LINKED IMMUNOSORBENT ASSAY; AND PREPARATION OF AN IMMUNOGEN FOR ERGOSTEROL

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DEVELOPMENT OF A VERSICOLORIN A ANTIBODY AND APPLICATION TO ENZYME-LINKED IMMUNOSORBENT ASSAY; AND PREPARATION OF AN IMMUNOGEN FOR ERGOSTEROL

By

Gwen Natalie Reynolds

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An immunogen was prepared for the primary fungal metabolite, ergosterol, to produce specific antibody for measuring fungal contamination in food products. Ergosterol (ERG) was converted to a hemisuccinate derivative and conjugated to ovalbumin (OA), thyroglobulin (TG), and keyhole limpet hemocyanin (KLH) using a N-hydroxysuccinimide ester method. Twenty-four BALB/c female mice were immunized with a combination of intraperitoneal and subcutaneous injections of ERG-TG or ERG-KLH conjugates. Two booster injections were administered. Competitive indirect ELISA was used to analyze antisera. Although titers of 3200 were detectable by ELISA, antibody was not specific for ERG since free hapten did not inhibit binding of antisera to the solid phase. Antibody produced against ERG-KLH immunogen did show specificity for ERG-OA solid phase when compared to OA only.

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fr

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Pr

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VA

mol

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ABSTRACT

DEVELOPMENT OF A VERSICOLORIN A ANTIBODY AND APPLICATION TO ENZYME-LINKED IMMUNOSORBENT ASSAY

By

Gwen Natalie Reynolds

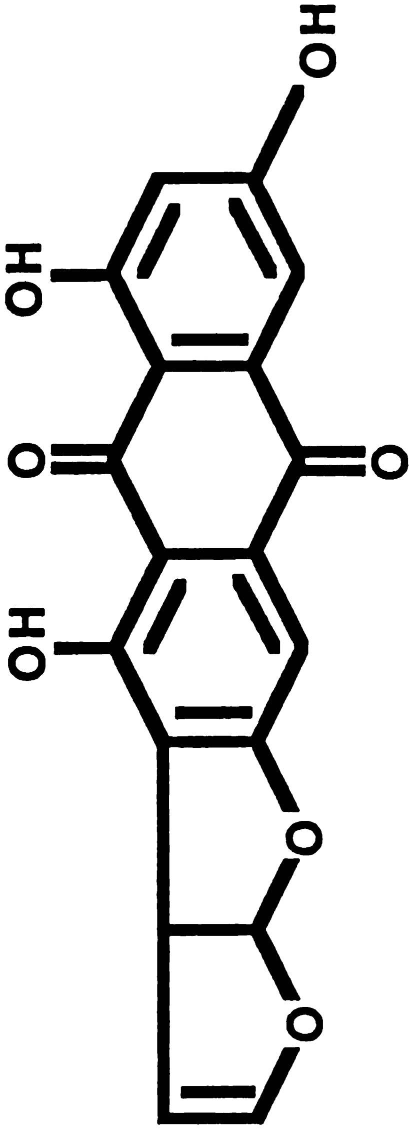
Methods were developed to purify versicolorin A (VA) from *Aspergillus parasiticus* ATCC 36537 cultures by semi-preparative high performance liquid chromatography (HPLC). Purity of crystalline VA was determined by HPLC. Identity was confirmed by mass spectrometry and UV absorption spectrum. Hemiacetal derivatization and conjugation by reductive alkylation were used to produce immunogen against VA with specificity toward the anthraquinone portion of the molecule. Results indicated that 16 moles of VA were conjugated to 1 mole of carrier protein. Final titers of 2000 to 4000 were detected in rabbits immunized with VA conjugate. All precursors in the aflatoxin biosynthetic pathway that were analyzed showed cross-reactivity with the VA antibody. The antibody was applied to direct competitive ELISA and used to detect and quantitate VA production by ATCC 36537 cultures.

INTRODUCTION

RATIONALE

Aflatoxins are a group of highly toxic secondary metabolites frequently detected in agricultural commodities such as corn, peanuts, cottonseed, cassava, sorghum, and treenuts. Aflatoxin B_1 (AFB_1) is the most potent, naturally occurring hepatocarcinogen known. Versicolorin A (VA) (Figure 1) is the first biosynthetic precursor in the AFB_1 pathway to exhibit the characteristic difuran structure associated with the potent biological activity of AFB_1. Insight into the molecular basis for biosynthesis of VA and its control is thus fundamental in understanding regulation of aflatoxin biosynthesis. Characterization of VA biosynthesis requires an efficient method for monitoring production of this compound. Current methods for quantitative and qualitative measurement of VA production generally include thin layer chromatography and high performance liquid chromatography. These methods have the disadvantages of requiring time-consuming extensive extraction and cleanup. Immunochemical assays are now commonly being used for detection of mycotoxins. This study was focused on the development of immunochemical approaches for evaluation of VA production in *A. parasiticus* strains.

Figure 1. Chemical structure of versicolorin A. This compound may be the most critical of all aflatoxin precursors because it is the first to contain the difuran moiety essential for toxicity. Though accumulated by a blocked strain of *Aspergillus parasiticus*, levels are still relatively low and difficult to detect and quantitate.



VERSICOLORIN A

REVIEW OF THE LITERATURE

OVERVIEW OF AFLATOXIN

In 1960, over 100,000 young turkeys and tens of thousands young pheasants and ducklings in England died after consumption of a mold-contaminated Brazilian peanut meal. The condition, labeled as "Turkey-X disease" was determined to be caused by a toxic metabolite of the fungal strain *Aspergillus flavus* Link ex Fries (Sargeant, 1961). Aflatoxin ("*A. flavus* toxin") was purified and identified as the causative agent of Turkey-X disease by two groups of researchers in 1962 (Van der Zijden et al., 1962; Nesbitt et al., 1962).

Nesbitt et al. (1962) and Hartley et al. (1963) determined that the toxic extract could be further subdivided into four main components when chromatographed on thin layer silica gel plates and developed in chloroform-methanol. Two components fluoresced blue under ultraviolet light and were named aflatoxin B_1 (R_f=0.4) and aflatoxin B_2 (R_f=0.36). Two other compounds fluoresced green under ultraviolet light and were designated aflatoxin G_1 (R_f=0.34) and aflatoxin G_2 (R_f=0.31). Structures of these compounds were elucidated by Asao et al. (1963). Austwick and Ayerest (1963) reported that the fungal strain *Aspergillus parasiticus* Speare also produced aflatoxins.

In retrospect, it is now generally agreed upon that the Turkey-X outbreak was probably not the first reported epidemic of aflatoxin poisoning (Heathcote, 1978). A disease in dogs referred to as "hepatitis X", recognized in 1952, was traced to a contaminated peanut meal. Toxic cultures of *A. flavus* were isolated from moldy corn implicated in a fatal swine and cattle disease in 1957. An outbreak of hepatoma in trout hatcheries throughout the Western United States occurred at nearly the same time as the Turkey-X disease epidemic. Aflatoxin contamination of cottonseed meal was determined to be the causative agent (Sinnhuber et al., 1965).

Not all wild-type *Aspergillus parasiticus* and *A. flavus* can produce aflatoxins. Summarizing numerous literature surveys, Bennett (1982) reported that 56% of *A. flavus* and *A. parasiticus* strains isolated from foodstuffs were aflatoxigenic. *A. parasiticus* isolates are generally highly toxigenic and produce aflatoxin B_1 (AFB_1; Figure 2a), aflatoxin B_2 (AFB_2; Figure 2b), aflatoxin G_1 (AFG_2; Figure 2c), and aflatoxin G_2 (AFG_2; Figure 2d) while *A. flavus* isolates include a lower percentage of toxigenic strains and produce only B aflatoxins.

Aflatoxin contamination can occur during crop growth, harvest, transportation, and storage. Moisture in, or relative humidity surrounding the substrate is the most important factor in growth and toxin production by *A. flavus* or *A. parasiticus* (CAST, 1979). Commodities which are

Figure 2. Structures of the four major naturally occurring aflatoxins. (a) Aflatoxin B_1, (b) Aflatoxin B_2, (c) Aflatoxin G_1, and (d) Aflatoxin G_2

frequently contaminated with aflatoxin include corn, peanuts, cottonseed, cassava, copra, and many types of tree nuts (Davis and Diener, 1988). Climates with warm temperatures and high humidity generally favor increased aflatoxin production. In the South, aflatoxin has been a common problem in inadequately dried corn in storage and in over matured corn left in the field. Stress caused by drought conditions can result in damaged corn husks and kernels that are subject to invasion by insects that carry aflatoxigenic spores (CAST, 1979). Recently, the severe lack of precipitation and hot weather experienced throughout the Midwest during the summer of 1988 promoted extremely high levels of aflatoxin contamination in the corn supply.

Milk, milk products, and animal tissues may also become contaminated as a result of ingestion of aflatoxin tainted feed. Aflatoxin M_1 (AFM_1), a metabolite of AFB_1, can be found in milk of animals which have ingested AFB_1. The United States Food and Drug Administration (FDA) has established action levels for total aflatoxins in foods and feeds based upon risk to human health from consuming contaminated foods. Milk and dairy products are regulated at a 0.5 ppb level for AFM_1 based upon risk to infants and young children that may consume large amounts of milk. The FDA action level for total aflatoxins is 20 ppb for all non-dairy foods and cottonseed meal intended for dairy cattle feed. Other feeds are regulated at a 100, 200, and 300 ppb level depending upon the age and species of the animal.

Aflatoxins are a small part of a larger group of compounds known as mycotoxins which are defined as toxic metabolites produced by fungi. Poisoning which is caused by ingestion of a food or feed that contains one or more mycotoxins is referred to as mycotoxicoses. Of numerous mycotoxins which have been identified over the past forty years, aflatoxins are of greatest concern due to their toxicity, carcinogenicity, mutagenicity, and teratogenicity (Busby and Wogan, 1979). Aflatoxin B_1 is the most potent, naturally occurring liver carcinogen known. LD_{50}s range from 0.3 to 40 mg/kg depending on the species (Pestka, 1986). Order of toxicity for the major aflatoxins is as follows: $B_1 > G_1 > B_2 > G_2$. Microsomal mixed-function oxidases, found in highest concentration in the liver, are primarily responsible for metabolism of aflatoxin (Bennett and Christensen, 1983).

Documented cases of human deaths due to consumption of aflatoxin contaminated foods have been reported. In 1974, a large outbreak of acute toxic hepatitis resulted in approximately 100 deaths in India following consumption of *Aspergillus flavus* contaminated corn (Krishnamachari et al., 1975). Stored grain maintained under high moisture conditions appeared to be cause of an acute hepatitis outbreak in Kenya during 1982 which led to 12 deaths. Aflatoxins at 39 ppb and 89 ppb were detected in two liver samples at autopsy (Ngindu et al., 1982). Epidemiological investigations have shown a positive correlation between high levels of aflatoxin consumption and high incidence of liver cancer in areas of Southeast Asia, China, India, and Africa (Shank, 1978). Primary liver cancer in Kenya, Swaziland, Mozambique, and Thailand (0.7 to 25.4 cases/10^5/year) could be correlated with daily AFB_1 intakes of 3.5 to 222.4 ng/kg body weight/day (Stark, 1980). This correlation, however, has not been seen in the United States. Intake of aflatoxin in the Southeast is about nine times higher than in the country as a whole, however, deaths attributed to liver cancer in the Southeast are proportionally lower (CAST, 1979).

Adverse biological effects in domestic and experimental animals of chronic aflatoxin exposure include liver damage, reduced growth rate and immunosuppression (CAST, 1979). Studies have indicated that some animals are more susceptible than others. The rainbow trout (Salmo gairdneri) is the experimental animal most sensitive to the carcinogenic effects of AFB_1 (Lee et al., 1968). The embryo exposure technique with rainbow trout was used by Hendricks et al. (1980) to test the carcinogenicity of aflatoxin B_1, and two aflatoxin intermediates, sterigmatocystin and versicolorin A. Relative potency of AFB_1 to sterigmatocystin was approximately 4:1 and to versicolorin A was approximately 50:1. The Salmonella typhimurium mutagenesis assay developed by Dr. Bruce Ames was used by Wong et al. (1977) to assess fungal metabolites related to aflatoxin biosynthesis on the basis that these compounds would occur concomitantly with aflatoxins and be ingested along with them. Versicolorin A and sterigmatocystin, containing the dihydrofuran moiety, were the only compounds found to demonstrate mutagenic activity. Relative to AFB_1, versicolorin A and sterigmatocystin mutagenicity were 5.83 % and 10.66 %, respectively.

It has been well established that the chemical site responsible for biological activities of aflatoxins and related compounds is the C_2-C_3 double bond in the dihydrofuran moiety of these molecules (Stark, 1980). Formation of a reactive epoxide at this site in AFB_1 and subsequent covalent binding to nucleic acid is responsible for carcinogenicity and mutagenicity (Essigmann et al., 1982). Reduction of this bond to form AFB_2 results in a 150 fold decrease in carcinogenicity and a 200-500 fold decrease in mutagenicity.

AFLATOXIN BIOSYNTHESIS

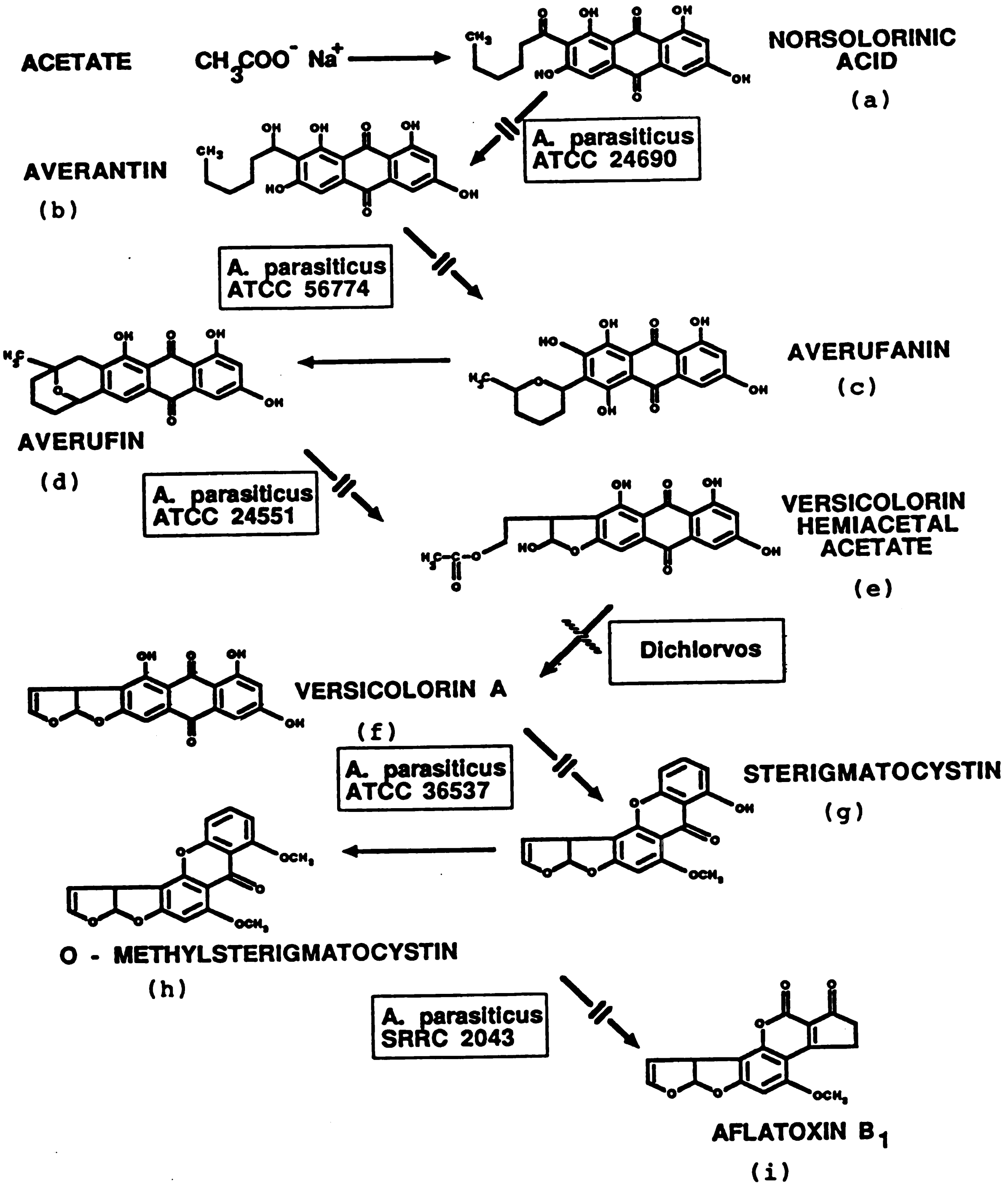
Research on aflatoxin biosynthesis has focused primarily on production of aflatoxin B_1 by _Aspergillus parasiticus_ strains because B_1 is the most abundant and biologically potent aflatoxin. A partial putative pathway generally accepted for aflatoxin biosynthesis is: acetate -> norsolorinic acid -> averantin -> averufanin -> averufin -> versiconal hemiacetal acetate -> versicolorin A ->

sterigmatocystin -> O-methylsterigmatocystin -> aflatoxin B_1 (Figure 3). Biotransformation experiments with blocked mutants, radiotracer and stable isotope studies with [^{14}C] and [^{13}C] labeled intermediates, respectively, have been essential in construction of this pathway.

Mycotoxins are classified as secondary metabolites which are defined as "natural products that have a restricted taxonomic distribution, possess no obvious function in cell growth and are synthesized by cells that have stopped growing" (Weinberg, 1970). As products of secondary metabolism, starting blocks are derived from products of primary metabolism. Acetate-malonate-derived compounds constitute the largest group of secondary metabolites. Polyketide synthesis is a common pathway for many fungal secondary metabolites, including aflatoxins. Polyketide formation involves condensation of enzyme-bound acetyl CoA as the chain initiator with a variable number of malonate units derived from malonyl CoA to allow for chain elongation (Birch, 1967). Concomitant decarboxylation, subsequent aromatization, and various modifications produce the numerous diverse compounds. The polyketide pathway is analogous to fatty acid synthesis but does not have systematic dehydration and reduction steps.

Biollaz et al. (1968) established the polyketide origin of the highly substituted coumarin, aflatoxin B_1 (Figure 3i), by adding [^{14}C] acetate to _A_. _flavus_ cultures to produce radioactive AFB_1. Degradation of the aflatoxin

Figure 3. Putative pathway for biosynthesis of aflatoxin. Arrows may represent more than one step. -¦¦- metabolic block, -¦- metabolic enzyme inhibitor.


ACETATE
CH$_3$COO$^-$ Na$^+$
NORSOLORINIC ACID
(a)
A. parasiticus ATCC 24690
AVERANTIN
(b)
A. parasiticus ATCC 56774
AVERUFANIN
(c)
AVERUFIN
(d)
A. parasiticus ATCC 24551
VERSICOLORIN HEMIACETAL ACETATE
(e)
Dichlorvos
VERSICOLORIN A
(f)
A. parasiticus ATCC 36537
STERIGMATOCYSTIN
(g)
O - METHYLSTERIGMATOCYSTIN
(h)
A. parasiticus SRRC 2043
AFLATOXIN B$_1$
(i)

molecule demonstrated that the carbon skeleton of AFB_1 was completely derived from C-1 and C-2 of acetate while the methoxy group carbon indicated a [methyl-^{14}C] methionine origin. Biollaz et al. suggested involvement of C_{18} compounds in biosynthesis of AFB_1 though later findings support theories based upon a C_{20} precursor (Pachler et al., 1976).

The xanthone sterigmatocystin (STG) (Figure 3g) was first recognized as the main metabolite of Aspergillus versicolor (Hatsuda and Kuyama, 1954) and later found to be produced in trace quantities by aflatoxigenic A. parasiticus strains (Schroeder and Kelton, 1975). Direct evidence that sterigmatocystin was derived from the same acetate-malonate pathway as AFB_1 was obtained by Holker and Mulheirn (1968). Distribution of labeled acetate that became incorporated into sterigmatocystin indicated that STG and AFB_1 had the same absolute configuration. Hsieh et al. (1973) provided direct experimental evidence that STG was an aflatoxin intermediate by demonstrating that [^{14}C] STG was efficiently converted to aflatoxin B_1 by the resting mycelium of aflatoxigenic A. parasiticus. Using cell-free mycelial preparations, Singh and Hsieh (1976) showed that this same conversion could be accomplished enzymatically.

Early theories regarding aflatoxin biosynthesis included hydroxyanthraquinones as intermediates (Thomas, 1965). The first experimental evidence for existence of these compounds in aflatoxigenic Aspergilli was obtained by

Heathcote and Dutton (1969) who isolated both dihydroaverufin and versicolorin C from *A. flavus*. Little support for involvement of specific anthraquinones existed until averufin was established as an aflatoxin precursor.

Donkersloot et al. (1972) produced an averufin-accumulating mutant (ATCC 24551) by treating conidia of *Aspergillus parasiticus* ATCC 15517 with nitrosoguanidine. Single colony isolates were grown in yeast extract-sucrose medium and tested for aflatoxin production. The mutant produced less than 2% aflatoxin relative to the parent strain. Analysis of purified compound matched properties specified for averufin (AVR) (Figure 3c), also a minor metabolite in *A. versicolor* (Pusey et al., 1963). Lin et al. (1973) demonstrated incorporation of AVR into AFB_1 using ATCC 24551. Radiolabeled AVR was fed to resting parent strain (ATCC 15517) cells which resulted in radioactivity detectable in AFB_1. Lin et al. also found trace amounts of AVR produced by the parent strain. Data obtained regarding AVR provided further evidence to support the theorized sequence of the polyketide synthetic pathway: anthraquinones -> xanthones -> coumarins.

Using ultraviolet irradiation, Lee et al. (1971) isolated a mutant strain, NRRL A-17,996 (ATCC 24690) from an aflatoxigenic *A. parasiticus* strain. The mutant produced 80% less aflatoxin than the parent strain and accumulated an orange-red pigment later determined to be norsolorinic acid (NA) (Figure 3a). This compound was previously isolated from

A. versicolor and characterized by Hamasaki et al. (1967). Detroy et al. (1973) reported that the compound was also produced in trace quantities by the parent strain. Hsieh et al. (1976) used this mutant to prepare [^{14}C] NA from [^{14}C] acetate to test for incorporation into AFB$_1$ by the aflatoxigenic A. parasiticus strain ATCC 15517. Over 2% of radioactive label was found in aflatoxin. Efficiency of norsolorinic acid conversion into aflatoxin was less than that which had previously been obtained for averufin, versicolorin A, and sterigmatocystin placing the precursor earlier in the pathway than any of these intermediates. Resumption of aflatoxin synthesis by the NA-accumulating strain upon addition of these later precursors further provided evidence for placement in the pathway.

A mutant strain of Aspergillus parasiticus SU-1 which is impaired in aflatoxin biosynthesis and accumulates versicolorin A (VA) (Figure 3f) was produced by Bennett and Goldblatt (1973). The mutant strain (ATCC 36537), originally designated 1-15-105 wh-1, was produced by ultraviolet irradiation. Growth on de Vogel agar demonstrated altered fluorescence indicating reduced aflatoxigenicity. The strain yielded white spores with deep yellow mycelia in contrast with dark green spores and light yellow mycelia of the toxigenic wild-type strain. The mycelia contained four closely related yellow pigments. Lee et al. (1975) characterized the major yellow pigment to be VA based upon properties reported by Hamasaki et al. (1967) who isolated the compound from A. versicolor. Lee et al. (1975) also isolated this pigment from the parent strain SU-1 suggesting that VA was an aflatoxin precursor. Bennett et al. (1976) reported that the anthraquinone pigments versicolorin A, versicolorin C (VC), and averufin were produced by both the wild type and VA-accumulating mutant. The mutant produced approximately five times as much AVR, 100 times as much VA, and 15 times as much VC as the wild type. Trace levels of aflatoxin B_1 are also produced by the mutant (Bennett and Papa, 1987).

The role of versicolorin A as an aflatoxin intermediate was demonstrated by Lee et al. (1976). Sodium [^{14}C] acetate and conidia from the VA-accumulating strain were added to broth and incubated to produce [^{14}C] VA. Labeled compound was then added to a growing culture of wild-type Aspergillus parasiticus. Approximately 46% of [^{14}C] VA was incorporated into AFB_1 verifying the compound's precursor status. The relative specific activity, number of labels from precursor incorporated into product, for aflatoxin B_1 from versicolorin A was 0.475. This relatively high value suggested that VA was incorporated into AFB_1 directly without first breaking down into acetate units.

Dichlorvos (dimethyl 2,2 dichlorovinyl phosphate), a pesticide and cholinesterase inhibitor, at 10 ppm was found to inhibit over 90% of aflatoxin production while complete inhibition occurred with 20 ppm dichlorvos (Hsieh, 1973). The orange pigment which accumulated upon addition of dichlorvos was tentatively identified as "versiconal acetate" by Schroeder et al. (1974). Using dichlorvos to treat cultures of *Aspergillus parasiticus*, Yao and Hsieh (1974) found that sterigmatocystin conversion to AFB_1 was not affected but averufin was converted to the orange pigment. This indicated that dichlorvos blocked an enzymatic step in aflatoxin biosynthesis beyond averufin but before sterigmatocystin. Untreated cultures efficiently converted pigment into AFB_1. Additionally, Bennett et al. (1976) found that dichlorvos inhibited formation of versicolorin A along with accumulation of the orange pigment to more accurately locate the synthetic block prior to VA production by the wild-type strain. Results of nuclear magnetic resonance research by Fitzell et al. (1977) renamed the pigment as versiconal hemiacetal acetate (VHA) (Figure 3e). Results of assignments made from their analysis using [^{13}C] indicated acetate as the sole carbon precursor for VHA to provide additional evidence of this compound's relationship with aflatoxin.

Placement of versicolorin A in the aflatoxin biosynthetic pathway was established by Singh and Hsieh (1977). It was found that the VA-accumulating mutant efficiently converted [^{14}C] labeled acetate, averufin, and versiconal hemiacetal acetate into versicolorin A placing the precursor after these early intermediates. Aflatoxin production resumed when sterigmatocystin was added to resting cell cultures which indicated that the mutant was blocked at the enzymatic step catalyzing conversion of VA into STG. Approximately 54% of the radioactivity from STG was incorporated into AFB_1 by the VA-accumulating mutant.

A study reported by Singh and Hsieh (1977) provided additional insight toward placement of precursors in the aflatoxin pathway. Using mutant strains ATCC 24551 (averufin-accumulating) and ATCC 36537 (versicolorin A-accumulating); $[^{14}C]$ labeled acetate, averufin, versicolorin A, versiconal hemiacetal acetate, and sterigmatocystin; and the metabolic inhibitor, dichlorvos, Singh and Hsieh were able to propose this portion of the pathway : acetate -> averufin -> versiconal hemiacetal acetate -> versicolorin A -> sterigmatocystin -> aflatoxin B_1.

Bennett et al. (1980) treated the VA-accumulating mutant with nitrosoguanadine to produce a new mutant (ver-mu-39) ATCC 56774 that accumulated a pigment identical to one previously isolated from _A_. _versicolor_ and named averantin (AVN) (Figure 3b) by Bennett and Lee (1979). Over 15% of label from $[^{14}C]$ AVN prepared from $[^{14}C]$ acetate could be detected in AFB_1 after conversion by the wild-type strain. Dichlorvos blocked aflatoxin production placing the precursor before VHA in the pathway. When $[^{14}C]$ averantin was fed to resting cells of the norsolorinic acid-accumulating mutant and the averufin-accumulating mutant, no radioactivity was detected in norsolorinic acid. Averufin retained over 30% of the AVN label placing AVN after NA and before AVR in the biosynthetic pathway .

A non-aflatoxigenic isolate of Aspergillus parasiticus CP461 (SRRC 2043) was reported by Bhatnagar et al. (1987) to accumulate *O*-methylsterigmatocystin (OMST) (Figure 3h). This strain was used to identify OMST as an AFB_1 precursor and to place it in the pathway between sterigmatocystin and aflatoxin B_1. When STG was added to this isolate in a low-sugar medium, OMST accumulation increased without any detectable aflatoxin synthesis. When radiolabeled [^{14}C] OMST was fed to resting mycelia of an A. parasiticus mutant not capable of producing alflatoxin, sterigmatocystin, or *O*-methylsterigmatocystin, [^{14}C] AFB_1 and AFG_1 were produced; addition of OMST produced AFB_1 and AFG_1, and addition of STG produced AFB_1 and AFG_1 indicating that OMST was a true aflatoxin metabolite between sterigmatocystin and aflatoxins B_1 and G_1. Another chemically related compound, designated HOMST, was later reported to be accumulated by this strain and may be a precursor of AFB_2 (Cleveland et al., 1987).

Averufanin (AVF) (Figure 3c) is the most recent AFB_1 precursor to be verified. There are no reported mutant strains or metabolic inhibitors which promote accumulation of this compound. McCormick et al. (1987) isolated and identified averufanin as one of the yellow pigments contained in the mycelia of the wild-type Aspergillus parasiticus strain SU-1. Verification as an aflatoxin precursor was established by isolation of [^{14}C] AVF from cultures of the wild-type strain incubated with [^{14}C] acetate, and by conversion of [^{14}C] AVF to AFB_1 by resting

SU-1. [^{14}C] averufanin was fed to *A. parasiticus* mutants to determine correct placement of the precursor in the biosynthetic pathway. Feeding experiments showed that averufanin was converted into averufin, versicolorin A, O-methylsterigmatocystin, and aflatoxin B_1, but not averantin, placing averufanin after averantin and before averufin.

Hydroxyversicolorone has been proposed by Townsend et al. (1988) as an intermediate in the pathway between averufin and versiconal hemiacetal acetate. Mutagenesis by ultraviolet irradiation of an aflatoxigenic *A. parasiticus* strain (SU-7) resulted in a new blocked mutant (WE-47, hvn-1). Thin layer chromatography revealed an orange fluorescent spot that did not correspond in mobility to anthraquinones previously identified as aflatoxin precursors. Currently, neither biotransformation nor radiotracer studies have been carried out with this proposed precursor.

Genetic approaches to gain insight into aflatoxin biosynthesis are also being explored. Lack of a sexual stage in aflatoxigenic fungi has hampered genetic analysis of aflatoxin producing ability (Bennett, 1982). An additional problem is that wild type *Aspergillus flavus* and *A. parasiticus* have mulitinucleate conidia. Haploid, diploid, and heterokaryotic spores all have the same conidial diameter eliminating a tool to screen ploidy. Emphasis of genetic analysis by Bennett and Papa (1987) has concentrated on identification of linkage groups and parasexual recombinational studies in *A. flavus* and *A. parasiticus*.

Another genetic approach currently being investigated (J. Pestka, J. Linz, J. Horng, and C. Skory, personal communication) involves isolation and characterization of the genes involved in aflatoxin biosynthesis. This will be accomplished by genetic screening of a genomic library for complementation of mutations occurring at different steps in the synthesis of aflatoxin with initial emphasis on conversion of versicolorin A to sterigmatocystin. Development of an efficient transformation system is currently in progress.

VERSICOLORIN A

Heathcote et al. (1973) specifically proposed versicolorin A as an intermediate in aflatoxin biosynthesis due to presence of the difuran group, a characteristic virtually exclusive in natural products to mold metabolites. This same moiety is present in aflatoxin B_1, and in sterigmatocystin, a compound which had previously been linked to aflatoxin biosynthesis.

Lee et al. (1975) reported that purified crystals of versicolorin A, isolated from the blocked mutant produced by Bennett and Goldblatt (1973), demonstrated the following properties: melting point 287 - 288 C, molecular weight 338 daltons, infrared absorptions at 1625, 1679, and 3340 cm^{-1}; and ultraviolet absorption maxima in ethanol, nm (E), at:

222 (31488), 254 (15227), 265 ((17756), 290 (26547), 321 (12118), and 452 (8166). The pigment was soluble in hexane, acetone, ethanol, methanol, and chloroform; and insoluble in water and sodium bicarbonate. Solutions of sodium carbonate and sodium hydroxide turned purple when added to the pigment, a positive test for hydroxyanthraquinones. All properties agreed with those published by Hamasaki et al. (1967) who first characterized versicolorin A from mycelial extracts of *Aspergillus versicolor*. Comparisons of absorption data and of nuclear magnetic resonance spectra of trimethyl ether derivatives made from the pigments from *A. parasiticus* and *A. versicolor* further substantiated that both pigments were versicolorin A.

VERSICOLORIN BIOSYNTHESIS

A significant amount of research has been dedicated specifically to biosynthesis of versicolorin A by both wild-type strains and the VA-accumulating blocked mutant. Using a defined medium devised by Adye and Matales (1964) that has frequently been used for production of aflatoxins and aflatoxin precursors, Bennett et al. (1979) studied effect of trace elements in this medium on versicolorin A production by the VA-accumulating mutant. Various nitrogen sources were also tested. Versicolorin production was undetectable when zinc was omitted from the medium, omission of manganese slightly stimulated production. Zinc is also a key element in biosynthesis of aflatoxins and has been shown to be an absolute requirement for aflatoxin production (Venkitasubramanian, 1977). Experiments on nitrogen sources indicated that substituting NH_4NO_3 for $(NH_4)_2SO_3$ increased versicolorin production over fourfold though chromatographic profiles revealed two additional compounds in the crude extract.

Bennett et al. (1981a) examined effects of temperature, and continuous white light versus continuous darkness on production of aflatoxins, averufin, versicolorin A and versicolorin C. A small inhibitory effect of light on VA production was seen at 30 C while complete inhibition of growth and production occurred at 15 C. There was no inhibitory effect of light on production at 20 C and 25 C. Of the six temperatures analyzed, VA production was maximum at 20 and 25 C. Contradictory to this study, Coupland and Niehaus (1987) found no effect of light on versicolorin synthesis.

Niehaus and co-workers have investigated many aspects of versicolorin biosynthesis by the VA-accumulating blocked mutant, ATCC 36537. Initial work determined that production of VA required zinc concentrations in excess of that required for growth (Niehaus and Failla, 1984). Maximal growth for the blocked mutant occurred with 2 um zinc, while 5um zinc in the culture medium was required for optimal versicolorin production. From this same study, it was determined that maximum VA production occurred after rapid growth had ceased but that production was not dependent upon depletion of the carbon source.

Failla and Niehaus (1986b) then investigated regulation of Zn^{+2} uptake and VA synthesis. They found that age of the culture and Zn^{+2} concentration which the mycelia had previously been exposed to affected ability of mycelia to accumulate zinc which was added to the culture medium. Stimulation of VA synthesis occurred only if supplemental Zn^{+2} was added between 18 and 32 hr post inoculation. It was concluded that VA synthesis began about 50 hr after inoculation and was directly proportional to mycelial zinc content at 30 hr. Failla and Niehaus, 1986a reported that inclusion of Cd^{+2} in a Zn^{+2} - limiting growth medium allowed optimal Aspergillus parasiticus growth and stimulated full versicolorin synthesis. Like Zn^{+2}, Cd^{+2} stimulated VA synthesis only when added within the first 30 hr after conidial inoculation.

Based upon the concept that biosynthesis of secondary metabolites is often inhibited when orthophosphate concentration is increased, Niehaus and Coupland (1987) investigated the effect of this compound on VA synthesis. A fourfold increase in VA synthesis was observed by increasing the culture medium initial phosphate concentration from 0.4 to 0.8 mM. Mycelial phosphate content was highly regulated. Above 5mM concentration in culture medium had no further effect on VA synthesis. As with Cd^{+2} and Zn^{+2}, orthophosphate stimulated VA synthesis only when added within the first 30 hr after conidial inoculation.

Kojic acid is a glucose-derived, secondary metabolite synthesized in large amounts by ATCC 36537. Coupland and Niehaus (1987) studied effect of various cultural factors to compare conditions favoring VA synthesis with those favoring kojic acid synthesis. Kojic acid synthesis was much more sensitive to nitrogen excess in the culture medium. Synthesis of kojic acid was unaffected by changes in Zn^{+2}, an element VA production had previously been shown to be completely dependent upon. Both kojic acid and versicolorin A synthesis were almost completely inhibited by 0.8M NaCl or 0.05M NaOAc.

When grown in a shaking culture at 37 C and 23 C, ATCC 36537 did not produce VA at the higher temperature (Niehaus, 1989). Temperature upshift (23 C -> 37 C) suspended versicolorin synthesis while synthesis was resumed after a 6 hr lag following a temperature downshift from 37 C -> 23 C. Data obtained after addition of cycloheximide and actinomycin D at the time of temperature shift indicated that a transcriptional event required for versicolorin synthesis occurs only in presence of Zn^{+2} and at temperatures below 37 C.

Fanelli et al. (1989) added carbon tetrachloride (CCl_4) or linoleic acid hydroperoxides (LAH) to 5 d cultures of the VA-accumulating mutant both in presence and absence of cysteamine, a strong free radical scavenger. Analysis after

10 more days of incubation indicated significant increase in VA production with addition of CCl_4 (1526 ± 188 ug) or LAH (1282 ± 160 ug) without cysteamine compared to the control (19.9 ± 1.5 ug). This study indicated that lipid peroxidation plays an important role in biosynthesis of aflatoxin precursors.

Recent research by El-Nabarawy et al. (1989) implies that versicolorin A and averufin are accumulated by a wild-type aflatoxigenic A. parasiticus strain upon addition of 1% sodium bicarbonate to culture media. Their results are based upon thin layer chromatography of mycelial extracts of the wild-type control and the bicarbonate treated wild-type strain. The control produced no detectable AVR or VA. The bicarbonate treated strain produced fluorescent spots at R_f values indicative of AVR and VA.

There is still a great deal to be determined regarding enzymology and mechanistic details of aflatoxin biosynthesis. Both Anderson and Dutton (1980), and Wan and Hsieh (1980) have isolated an oxygen-requiring cell-free system that converts versiconal hemiacetal acetate to versicolorin A. Wan and Hsieh proposed that the first step in conversion involved hydrolysis of the ester bond in VHA followed by elimination of the acetyl group. Oxidation of the terminal hydroxyl group to the aldehyde form with subsequent ring closure forms a hemiacetal which loses water to form the stable vinyl ether system of VA.

Conversion of versicolorin A to sterigmatocystin begins with cleavage of the anthraquinone moiety adjacent to the carbonyl to form the hypothetical lactone 6-deoxy-VA lactone (Dutton, 1988). A minimum of three enzymatic conversions follow to complete the conversion. These include (1) spontaneous or lactonase-derived hydrolysis of the lactone, (2) loss of the exposed carboxyl by oxidative decarboxylation, and (3) methylation of the hydroxyl formed by lactone hydrolysis with the final product structure dependent upon the order of the last 2 steps. Ring closure then occurs to yield sterigmatocystin.

DETECTION AND QUANTITATION OF VERSICOLORIN A

Thin layer chromatography (TLC) has frequently been the method of choice for detection and quantitation of anthraquinone aflatoxin precursors. Benzene:acetic acid (Bennett et al., 1976; Bennett and Lee, 1979) and toluene:ethyl acetate:acetic acid (Bennett and Lee, 1979; Fanelli et al., 1989; and McCormick et al., 1987) are common solvent systems reported in the literature for these compounds. While a significant advantage of TLC is that it can be a very inexpensive technique, principal disadvantages of analysis are lack of automation potential and accurate quantitation. Visual comparison of fluorescent intensity of the sample to a standard is often basis for quantitation and is subject to a large amount of variation. Inherent errors in visual estimation of fluorescence for aflatoxin quantitation as high as 20-30% have been reported while use of fluorodensitometric measurements provided a measurement precision of $^{+}/-$ 2-4% for AFB_1 and AFG_1 when resolved on TLC plates (Pons et al., 1968). While use of densitometry can increase accuracy, it can significantly increase overall cost. Also, the similarity in chromatographic mobility of anthraquinone precursors frequently results in R_f values virtually indistinguishable without use of relatively expensive high performance TLC plates. Using a benzene:acetic acid (95:5) solvent system, Bennett et al. (1976) reported the following R_f values: averufin (0.45), versicolorin A (0.38), and versicolorin C (0.33). Bennett and Lee (1979) reported R_f values of 0.82, 0.79, and 0.70 for norsolorinic acid, averufin and versicolorin A, respectively, using toluene:ethyl acetate:acetic acid (80:10:10).

Niehaus and Failla (1984) developed a spectrophotometric assay for quantitation of versicolorin A. The method is based upon ionization of phenolic hydroxyl groups in VA upon addition of alkali. Comparison of VA's absorption spectrum from 220 -> 700 nm before and after addition of KOH revealed a shift in the yellow absorbance peak (445 nm) to the red absorbance peak (520 nm). An extinction coefficient of 2.9×10^3 M^{-1} cm^{-1} was calculated for 520 nm as basis for quantitation of VA. However, approximately 15% of alkali-generated red color from mycelial extracts was not due to VA decreasing specificity of this assay. Also, quantitation from single samples involved several tedious steps including lyophilization of mycelia followed by multiple extractions making the method impractical for analysis of large sample numbers.

Numerous high performance liquid chromatographic (HPLC) methods have been developed for purification and analysis of versicolorin A and related metabolites. Using normal phase HPLC and a solvent system comprised of hexane:ethyl acetate: acetic acid (83:17:1), Kingston et al. (1976) required a cyano-bonded solid phase to successfully separate VA from other hydroxylated anthraquinones extracted from Aspergillus versicolor cultures. At a flow rate of 2 ml/min, versicolorin A and versicolorin C retention times were unacceptably long - approximately 35 and 45 min, respectively. A reverse phase HPLC method was developed by Berry et al. (1984) for separation of aflatoxin biosynthetic intermediates. A gradient of acetonitrile:tetrahydrofuran: water (25:20:55) provided separation of aflatoxins, NA, VHA, VA, and AVR but could not resolve versicolorin A and versicolorin C. The procedure described required temperature control at 50 C.

McCormick et al. (1988) also developed a reversed phase HPLC procedure for anthraquinone separation using a C_{18} u-Bondapak 30 cm x 3.9 mm i.d. column. The method provided good resolution of all major anthraquinone metabolites in wild-type _A. parasiticus_. Retention times reported by McCormick et al. (1988) were as follows: versiconal hemiacetal acetate, 3.1 min; versicolorin C, 7.8 min; versicolorin A, 9 min; averufin, 19.7 min; averantin, 22.4 min; averufanin, 24 min; and norsolorinic acid, 37.5 min. A disadvantage of this procedure was that the solvent system required a binary step gradient consisting of "A" (methanol:tetrahydrofuran, 2:1) and "B" (0.1 M acetic acid), going from 67 to 79% A at 30 min. The procedure also utilized temperature control at 25C.

Billington and Hsieh (1989) described a reversed-phase HPLC procedure developed for analysis of VHA conversion in an _A. parasiticus_ cell-free extract. An isocratic HPLC solvent system consisting of methanol:acetonitrile:acetic acid (0.053 N glacial acetic acid in water) successfully separated VHA and VA; a second isocratic solvent system of acetonitrile and acetic acid without methanol was necessary to separate versicolorin A from versicolorin C.

While HPLC methods are highly sensitive and reproducible, they still exhibit some major disadvantages. Extensive cleanup of samples is required which can be very time consuming. Also, since only one sample at a time can be analyzed, it would be impractical to use HPLC for analysis of large sample numbers. Instrumentation and solvent costs are significant expenses. Additionally, technical expertise is required for HPLC analysis.

IMMUNOASSAYS FOR MYCOTOXINS

The potential hazard to human and animal health has caused concern over presence of mycotoxins in foods and feeds. Immunochemical assays were first suggested for detection of mycotoxins over ten years ago (Chu and Ueno, 1977). High specificity and sensitivity, ease of use, speed, and adaptability to automation make immunoassays preferable to other available methods. Immunoassays have been reported in the literature for many mycotoxins including aflatoxin B_1 (Candlish et al., 1985; Lawellin et al., 1977; Pestka et al., 1980), aflatoxin M_1 (Dixon-Holland et al., 1988; Pestka et al., 1981a), aflatoxin G_1 (Chu et al., 1985), sterigmatocystin (Li and Chu, 1984), deoxynivalenol (Casale et al., 1988; Xu et al., 1986), zearalenone (Dixon et al., 1987; Warner et al., 1986), and ochratoxin A (Morgan et al., 1983; Pestka et al. 1981b).

The basis for these immunoassays is competition between labeled mycotoxin and unlabeled or "free" mycotoxin for antibody binding sites. An antibody is defined as protein which is produced in response to stimulation by foreign material called an antigen or an immunogen. Affinity constants for antibody-antigen interactions often exceed 10^{-8} (Pestka, 1988) which allows for the high sensitivity of immunoassays. High specificity is due to ability of antibodies to differentiate very minute chemical dissimilarities between antigens. The radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are the most common immunoassays used for mycotoxins. RIA uses a tritium labeled mycotoxin; ELISA uses an enzyme labeled mycotoxin. The RIA has several major disadvantages including radioactive waste disposal, requirement of a scintillation counter, difficult and expensive labeling of toxin with tritium, and gradual degradation of toxin due to isotope emission. These concerns favor use of ELISA for detection of mycotoxins.

The first step in development of an immunoassay is production of antibodies against the mycotoxin of interest. Molecules with a molecular weight less than 10,000 generally cannot elicit an immune response and are called haptens. Mycotoxins normally range in molecular weight from 300 - 400 daltons and are immunologically classified as haptens. To be rendered immunogenic, mycotoxins must be conjugated to a larger carrier protein. Bovine serum albumin (BSA), and ovalbumin (OA) are commonly used carrier proteins for mycotoxins. Conjugation of mycotoxin to carrier protein is complicated by functional groups present. Most mycotoxins, including aflatoxins and aflatoxin precursors, lack a reactive group and must first be derivatized prior to conjugation.

Carboxymethyloxime or hemisuccinate/hemiglutarate are common functional moieties that are introduced to provide a reactive group for conjugation (Chu, 1986). Mycotoxins with a carbonyl group are usually conjugated to protein after formation of a carboxymethyloxime. Chu and Ueno (1977) converted aflatoxin B_1 to AFB_1-O-carboxymethyloxime by refluxing with ethylhydroxylaminohemi-HCl for 4 hr in presence of pyridine to produce a reactive group for conjugation. Similar procedures were used for derivatization of T-2 toxin by Gendloff et al. (1986) and for zearalenone by Dixon et al. (1987). Bifunctional acid anhydrides such as hemisuccinate and hemiglutarate are frequently used to introduce a carboxylic acid into mycotoxins with one or more reactive hydroxyl groups. Deoxynivalenol was converted to 3-O-hemisuccinyl-deoxynivalenol by Casale et al. (1988) to produce a carboxylic acid group for carrier protein conjugation. Presence of three hydroxyl groups on deoxynivalenol required protection of two groups with a cyclic boronate ester, esterification at the desired hydroxyl position, followed by removal of the boronate ester. Extensive purification was needed to purify the derivative.

Methods for conjugation of mycotoxin to carrier protein include mixed anhydride, carbodiimide, and hydroxysuccinimide (Pestka, 1988). Conjugation for immunogen preparation, conjugation of mycotoxin to the immunoassay enzyme marker, and conjugation of mycotoxin to solid-phase bound protein can generally be preformed by the same method. The mixed anhydride method of Lau et al. (1981) was used by Pestka et al. (1985) to conjugate zearalenone-6'-carboxymethyloxime to BSA. The derivative was dried, dissolved in tetrahydrofuran, and cooled to -5 C followed by addition of triethylamine and isobutylchloroformate. This mixture was then added at 4 C to BSA dissolved in water and pyridine. The carbodiimide method was used by Chu and Ueno (1977) to couple AFB_1-O-carboxymethyloxime to BSA by combining derivative and protein for 48 hr at room temperature in a solution containing 1-ethyl-3,3-dimethylaminopropylcarbodiimide. Conjugation by the hydroxysuccinimide method of Kitagawa et al. (1981) was used by Gendloff et al. (1986) to conjugate AFB_1-O-carboxymethyloxime and T-2 hemisuccinate to protein. The derivatives were mixed with dicyclohexylcarbodiimide and N-hydroxysuccinimide at room temperature. The precipitate was washed, dissolved in dimethylformamide, and added dropwise to a buffered protein solution.

Reductive alkylation is an alternative conjugation procedure which has been used for aflatoxins and related metabolites that possess a dihydrofuran moiety. (Li and Chu, 1984; Gaur et al., 1981; Morgan et al., 1986; Pestka and Chu, 1984). Pohland (1968) reported that aflatoxin B_1 was converted to the hemiacetal form by addition of a water molecule to the vinyl ether double bond of the B_1 terminal ring under mild acidic conditions. Ashoor and Chu (1975a, 1975b) provided evidence that aflatoxin B_1 hemiacetal, also called aflatoxin B_{2a}, formed a phenolate ion with two aldehyde groups generated at neutral and alkaline pH. The phenolate ion could interact with amino acids or proteins to form an unstable Schiff base. Reduction by sodium borohydride led to formation of a stable protein conjugate. Reductive alkylation has been used by Pestka and Chu (1984) to conjugate AFB_1-diol and by Li and Chu (1984) to conjugate sterigmatocystin hemiacetal.

Determination of resulting antibody specificity is highly dependent upon site of mycotoxin conjugation to carrier protein (Pestka, 1988). Largest antibody response is generally initiated by the molecule portion positioned furthest from the site of conjugation, termed the "immunodominant" portion. Chemical structures similar to the distant moiety are likely to cross-react with antibodies produced. Gaur et al. (1981) demonstrated this principle when comparing cross-reactivity of AFB_1-oxime and AFB_{2a} antisera with various aflatoxins. The cyclopentenone ring of AFB_{2a} was immunodominant; minimal cross-reactivity of antisera was seen with aflatoxin G_1 and G_2 which lack this moiety. Opposite results were demonstrated with AFB_1-oxime antisera where the difuran ring of AFB_1 was immunodominant.

Mycotoxin antibodies are generally produced using multiple-site immunization of rabbits (Chu, 1986; Pestka, 1988). Mycotoxin - carrier protein conjugate ranging from

100-1000 ug and complete Freund's adjuvant are used for initial injection followed by booster injections at 5 - 6 week intervals using conjugate and incomplete adjuvant. Usable antisera is typically available after 3 to 4 months. Resultant antisera are classified as "polyclonal" due to immunoglobulin origin from multiple lymphocyte clones and inherent variability in affinity and specificity. Alternatively, monoclonal antibodies, which are homogenous antibodies produced by stable hybridoma cell lines, have been used for mycotoxin immunoassays. Monoclonal antibodies demonstrated more highly reproducible results and a lower degree of interassay variation when utilized in enzyme-linked immunosorbent assays for zearalenone (Dixon et al, 1987).

Both indirect and direct ELISAs have been used for mycotoxin detection. In a typical competitive indirect ELISA, mycotoxin conjugated to a carrier protein (different from the protein used for the immunogen) is bound to a solid phase, for example, a microtiter well. Free mycotoxin and antibody specific for the mycotoxin are added at the same time to the well. Free mycotoxin and the solid phase conjugated mycotoxin compete for antibody sites. A second anti-species antibody conjugated to an enzyme marker, generally horseradish peroxidase, is then added to determine total bound antibody. After addition of an appropriate enzyme substrate, color will develop relative to amount of total bound antibody. This color can be used for visual estimation or it can be objectively measured as end product absorbance by spectrophotometry. Free mycotoxin concentration will be inversely related to amount of absorbance. Using a known concentration of mycotoxin, a standard curve can be plotted with increasing toxin concentration versus decreasing end product absorbance. In a typical competitive direct ELISA, mycotoxin-specific antibody is bound to the solid phase. Free mycotoxin and mycotoxin conjugated to the enzyme marker incubated over the solid phase compete for antibody sites. Addition of an appropriate substrate will result in color development dependent upon concentration of bound mycotoxin-enzyme conjugate. As in the indirect assay, free mycotoxin concentration will be inversely related to end product absorbance or color development. A similar standard curve can be plotted (Pestka, 1988).

RESEARCH OBJECTIVES

The research described here was aimed at developing immunochemical approaches for evaluation of VA production in *Aspergillus parasiticus* strains. The experimental approach consisted of three steps: (*1*) production and purification of VA, (*2*) production of VA immunogen, and (*3*) application of antibody to ELISA. For the first step, the research objective was to develop a procedure for large-scale laboratory production of VA and to develop a method for crystalline purification of the compound at quantities required for immunogen production and immunoassays. The objective of the second step was to produce an immunogen through hemiacetal derivatization and reductive alkylation conjugation for antibody specificity directed towards the anthraquinone portion of the molecule. For the third step, the objective was to develop an ELISA using antibody produced against the immunogen. Immunoassays would be used to characterize antibody for determination of sensitivity and specificity. Antibody demonstrating high affinity for VA would be utilized in development of competitive, direct ELISA for detection and quantitation of VA production in *A. parasiticus* cultures.

MATERIALS AND METHODS

Inoculum Preparation

Spores from Aspergillus parasiticus ATCC 36537, a versicolorin A-accumulating mutant, were suspended in 0.1 % sterile peptone, spread plated onto potato dextrose agar (Difco, Detroit, MI) plus 0.5 % yeast extract (Difco) (PDYA), and incubated at 28 C for 48 hr. An inoculating needle was used to select spores from an isolated colony on a plate containing less than ten colonies. Spores were transferred to the center of several 150 cm petri plates containing PDYA and incubated at 28 C for 2 weeks, or until plates were densely covered with growth.

A small volume of a sterile 0.01 % Triton X-100 (Research Products International Corp., Elk Grove Village, IL) solution was poured onto each plate's surface and spores were dislodged using a glass hockey stick. Suspensions were transferred to a Buchner funnel lined with several layers of cheese cloth and filtered by gravity to remove mycelia. Filtrates were combined and centrifuged at 1900g for 15 min. The supernatant was removed, spores were resuspended in distilled water, and centrifuged as previously described. The supernatant was again removed and spores were resuspended in distilled water. Spore concentration was determined by counting on a hemocytometer (American Optical, Buffalo, NY) and adjusted to approximately 1 x 10^7 conidia/ml. Glycerol was added to a final concentration of 15 % (v/v). Spores were dispensed in 1 ml portions into 1.5 ml microcentrifuge vials and stored at -80 C.

In one instance, a spore suspension made by washing growth from a slant culture of ATCC 36537, was not filtered but was immediately diluted and plated onto PDYA. This improper technique resulted in a morphological variant.

Culture Media

A defined aflatoxin producing medium developed by Adye and Matales (1964), as modified by Bennett et al. (1979), was used for large volume cultivation of ATCC 36537. The culture media was prepared by adding the following compounds to distilled water on a per liter basis: NH_4NO_3, 8.0 g; KH_2PO_4, 10.0 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; and sucrose, 75.0 g. The solution was mixed until all chemicals were completely dissolved before micronutrients were added. Stock solutions of micronutrients were made in 1000x concentrations and added to culture media on a 1 ml per liter basis. The following amounts of micronutrients per 100 ml volume were used to prepare stock solutions: $Na_2B_4O_7 \cdot 10H_2O$, 1.4 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.0 g; $Fe_2(SO_4)_3 \cdot 6H_2O$, 20.0 g;

$CuSO_4 \cdot 5H_2O$, 0.6 g; $MnSO_4$, 0.22 g; and $ZnSO_4 \cdot 7H_2O$, 3.52 g. Media was dispensed into flasks which were plugged with cotton and covered with foil prior to autoclaving at 121 C for 20 min.

Extraction of Versicolorin A

Preparation of crude mycelial extracts were based upon the procedure described by Lee et al. (1975). After appropriate incubation, mycelia were strained through cheese cloth and rinsed twice with 100 ml distilled water. Vacuum filtration was used to remove as much water and culture broth as possible. Mycelia were extracted with acetone by blending at high speed for 15-30 seconds in a explosion resistant blender (Waring 707SB, New Hartford, CT) in a fume hood. The mycelial slurry was passed through Whatman No. 4 filter paper, then returned to the blender and exhaustively blended with acetone until mycelia and/or extracts were virtually colorless. A protective mask was worn during blending and filtering mycelia to prevent inhalation of particles.

Extracts were filtered through anhydrous sodium sulfate to remove water, combined, and evaporated under vacuum to dryness. The residue was washed with a small volume of petroleum ether. The extract was resuspended in 500 ml 30% acetone in water and transferred to a 1 liter separatory funnel. Versicolorin A was partitioned from the acetone and water solution with 100 ml portions of hexane. This process was repeated until hexane extracts were colorless. Hexane extracts were dried under vacuum and resuspended in a minimal amount of acetone.

Thin Layer Chromatography of Anthraquinone Standards

Spotting standards of versicolorin A, versicolorin C, and averufin (three anthraquinones accumulated by ATCC 36537) were obtained from Dr. Louise Lee (USDA Agriculture Research Service, New Orleans, LA). Versicolorin A and C were in a mixture which could be separated on silica gel TLC plates using toluene:ethyl acetate:acetic acid (TEA) (50:30:4, v/v/v) as the solvent system (McCormick et al., 1987). The VA and VC mixture was also developed with BA on 10 X 10 cm high performance TLC (LHP-K Linear K, Whatman Chemical Separation Inc., Clifton, NJ) (HPTLC) plates.

A solvent system described by Heathcote and Hibbert (1975) for separation of versicolorins was also analyzed using HPTLC plates. This system consisted of toluene:ethyl acetate (TE) (8:1, v/v).

Two dimensional TLC was used to analyze anthraquinone standards to further assess their purity. The VA and VC mixture was spotted on a HPTLC silica gel plate and developed first in TEA. After solvent had evaporated, the plate was turned 90° counterclockwise and developed in benzene:acetic acid (BA) (95:5, v/v). It was very important to use fresh solvent and to equilibrate the TLC tank when using the BA solvent system. Benzene is highly volatile and tended to evaporate quickly upon opening and closing the TLC tank. This led to poor separation of the three anthraquinone pigments and R_f values that were not reproducible.

Two dimensional TLC was utilized in an attempt to purify the anthraquinone standards. The first dimension was TE followed by BA as the second solvent system. The VA and VC mixture was spotted 5 ul at a time on a 5 cm square HPTLC plate and successively developed in the two systems. Individual spots were scraped into separate vials. A total of approximately 100 ul was spotted. Pigments were eluted with acetone and dried. Individual spots were analyzed at the Michigan State University Mass Spectrometry Facility. A direct insertion probe was used with an electron impact ionization mode.

Fluorodensitometry

Fluorodensitometry was used to compare VA production in shaking versus stationary incubation of cultures. High performance TLC separated anthraquinones while fluorescence measured by densitometry provided a more reliable quantitation. The time course for VA and mycelial dry weight accumulation during shaking versus stationary incubation was quantitated.

Culture media, as previously described, was dispensed in 100 ml volumes in 500 ml Erlenmeyer flasks which had been coated with Prosil 28, an organosilane surface treating agent (PCR Inc., Gainesville, FL). Without this treatment, under shaking conditions, spores would adhere to sides of flasks resulting in atypical growth that did not accumulate versicolorin A. Each flask was inoculated with 10^7 ATCC 36537 conidia. Cultures were incubated at 28 C in the dark for 4, 5, 6, 7, and 8 days. One set of flasks was incubated under stationary conditions; the other set was agitated on a rotary shaker (200 rpm).

After appropriate incubation, mycelia were separated from culture filtrate by vacuum filtration through filter paper and then rinsed twice with 250 ml distilled water. Excess water was removed by vacuum filtration. Rinsed mycelial pads were dried overnight at 50 C and then weighed to determine dry weight. Mycelia were then soaked overnight in acetone and subsequently blended into a macerated slurry. The slurry was strained through a filter paper lined funnel and then blended repeatedly with acetone until extracts were colorless.

The procedure used for quantitative TLC was based upon the method described by Pons et al. (1968) and the Official Methods of Analysis prescribed by the A.O.A.C. (1984). Preliminary thin layer chromatography was performed to approximate VA concentration to determine if extracts required dilution. Aliquots of 10 ul from each crude extract were spotted on a 10 x 10 cm high performance TLC plate (LHP-K) and developed in benzene:acetic acid (95:5, v/v) (BA) in a foil covered TLC tank. Plates were viewed under longwave ultraviolet light. Fluorescence at the appropriate R_f was compared to a VA standard for quantitative estimation. Crude extracts were diluted, if necessary, to contain approximately 10 ug VA per ml.

For densitometric measurement, aliquots of 5 ul and 10 ul from each crude extract were dried, resuspended in benzene:acetonitrile (98:2), and spotted exactly 10 mm apart on a 10 x 10 cm high performance TLC plate along with 5, 10, and 15 ul of a 10 ug/ml VA standard. It was noted that commercially available TLC spotting guides frequently varied by 1-2 mm in distance between lanes. Precise spacing between lanes was necessary for accurate automated scanning when using the densitometer. Plates were developed in BA, dried in a 150 C oven for 2 min, and placed in a desiccator shielded from exposure to light. Quantitation was performed within 20 min. Plates were linearly scanned on a Dual Wavelength TLC scanner (Model CS-930, Shimadzu Scientific Instruments, Inc., Columbia, MD) using a 365 nm irradiation source (mercury). Emission was observed for at 420-460 nm. Area of the spot from each extract at the corresponding R_f was compared to areas obtained for standards to determine amount of VA present in the crude extract. The following equation was used:

ug versicolorin A = (B x C x F)/(D x E)

where B = average amount of versicolorin A spotted in ng; C = area of crude extract; D = average area of versicolorin A standard; E = volume of crude extract spotted in ul; and F = total volume of extract in ml.

Derivatization of Crude Versicolorin Extract

Because it was desired to produce antibodies specific for VA and with cross-reactivity with anthraquinone precursors, hemiacetal derivatization was chosen. This reaction was described in detail by Li and Chu (1984) for derivatization of sterigmatocystin.

Initially, semi-purified VA extract was used to perform hemiacetal derivatization. Ten mg VA extract were dissolved in 10 ml acetone and refluxed in a small reaction vessel with 50 ul 10% sulfuric acid for 2 hr. It was noted that the water bath needed to be at 63 C or more to maintain refluxing conditions. The hemiacetal mixture was dried under vacuum and then resuspended in 10 ml acetone. Ten ml water were then added and acetone was removed by drying under vacuum. The mixture was transferred to a 50 ml separatory flask and extracted with 5 ml volumes of chloroform until chloroform extracts were colorless.

The hemiacetal mixture was loaded onto a 900 mg silica gel cartridge (Alltech Maxi-Clean, Alltech Assoc., Deerfield, IL) connected to a 5 ml glass syringe. Unreacted VA eluted with chloroform. Additional unreacted VA eluted with 50% acetone in chloroform just prior to elution of hemiacetal which was evident as an orange band.

Preparative Thin Layer Chromatography

Purification of versicolorin A from a crude extract was first attempted by scraping the appropriate zone from a preparative TLC plate. Using benzene:acetic acid (BA) (95:5, v/v) as the solvent system and 2 mm preparative silica gel G TLC plates (Redi-Plates, Fisher Scientific Co.), several trials were made to separate yellow pigments in the crude extract. After numerous developments, a thick yellow band with no separation remained. Silica gel containing this band was scraped from the plate and pigments were eluted with acetone. HPTLC was used to analyze the eluted material.

High Performance Liquid Chromatography (HPLC)

Reverse phase HPLC was used to assess procedures reported in the literature for separation of aflatoxin precursors. The solvent system described by Berry et al.

(1984) consisting of acetonitrile:tetrahydrofuran:water (25:20:55, v/v/v) was analyzed using both an analytical column and a semi-preparative column. For analytical HPLC, a Model 2300 HPLC pump and V^4 variable wavelength absorbance detector (5 mm flow cell) (ISCO, Lincoln, NE) was used. The system was equipped with RP-18 Spheri-10 MPLC analytical (22 cm x 4.6 mm i.d.) and guard (3 cm x 4.6 mm i.d.) cartridges (Brownlee Lab, Inc., Santa Clara, CA). Flow rate was 1 ml/min. and detection was performed at 325 nm and 0.01 a.u.f.s.. Prior to injection onto the column, extracts were dried, resuspended in the mobile phase, and passed through 0.45 um HPLC filters (Micron Separations Inc., Westboro, MA). Ten microliter samples were analyzed. Peak areas were determined by a Hewlett-Packard 3392A Integrator (Hewlett Packard Co., Avondale, PA). Peaks were collected manually at the detector outlet and analyzed by TLC.

A semi-preparative HPLC was constructed using a Model 396-89 mini-pump (Laboratory Data Control, Milton Roy Corp., Riviera Beach, FL) attached to a Valco C6U sample loop injector (Valco Instruments Co., Inc., Houston, TX) and Partisil 10 ODS Magnum 9/25 prepacked 25 cm x 9.40 mm i.d. column (Whatman Chemical Separations, Inc.) connected to a guard column (CSK I, Whatman) packed with Pellicular ODS (Whatman). A 2000 ul sample loop was attached to the injector. Samples were prepared in the same manner as described for analytical HPLC. Semi-preparative column flow rate was adjusted to approximately 3 ml/min to compensate for a larger internal diameter (9.4 mm) compared with the analytical column (4.6 mm). TLC and analytical HPLC, as previously described, were used to monitor fractions.

Purification of Versicolorin A

Modifications of the procedure described by McCormick et al., (1988) for separation of aflatoxin precursors were used for purification of VA. The solvent system was modified from a gradient to an isocratic system using methanol: tetrahydrofuran:0.1 M acetic acid (4:2:1, v/v/v) as the mobile phase. While McCormick et al. used analytical HPLC, a semi-preparative HPLC system (as described in the previous section) was utilized for VA purification.

To prepare samples for injection onto the semi-preparative column, approximately 0.5 - 1.0 mg crude VA extract was dried, resuspended first in 1125 ul methanol, then mixed with 575 ul tetrahydrofuran, and finally added to 300 ul 0.1 M acetic acid. This technique allowed maximum versicolorin A solubility in the mobile phase. Three ml fractions were collected. Purification of versicolorin A was continued until approximately 10 mg were collected.

Analytical HPLC, as previously described, was used with this solvent system to analyze crude VA and VA hemiacetal. Peaks were detected at 313 nm and a flow rate of 1 ml/min was used. Peaks were collected manually at the detector outlet and analyzed by TLC for identification of compounds.

Analysis of Crystalline Versicolorin A

Purity of VA was verified by mass spectrometry, HPLC, and analysis of the UV absorption spectrum. Mass spectrometry was performed at the Michigan State University Mass Spectrometry Facility. A direct insertion probe was used with an electron impact ionization mode. The procedure developed for purification was used for analytical HPLC analysis of crystalline VA. The absorption spectrum of versicolorin A in absolute ethanol was determined from 210 to 500 nm (UV/VIS spectrophotometer, Lambda 4B, Perkin-Elmer).

PRODUCTION OF IMMUNOGEN

Hemiacetal Derivatization

Pure versicolorin A was used for derivatization to the hemiacetal form. The procedure was conducted as previously described with the following modifications. Briefly, 1.73 mg pure VA were dissolved in 2 ml acetone and refluxed with 10 ul 10% sulfuric acid for 2 hr. Thin layer chromatographic analysis of the reaction indicated that a large amount of VA remained unreacted after 2 hr. An additional 10 ul 10% sulfuric acid was added and refluxing was continued for another 60 min. The reaction was then terminated though TLC analysis showed that some VA remained unreacted. Two ml acetone and 2 ml water were added to the dried hemiacetal mixture.

Unreacted VA and purified VA hemiacetal were analyzed by spectrometry to determine percent recovery for the reaction. VA was measured at 452 nm (E = 8166) (Lee et al., 1975). VA hemiacetal was measured at 456 nm (E = 6100) (Chen et al., 1977).

Conjugation of Versicolorin Hemiacetal to Protein

Versicolorin hemiacetal was conjugated to bovine serum albumin (Sigma) and horseradish peroxidase (Sigma) by a reductive alkylation method described in detail for sterigmatocystin hemiacetal by Li and Chu (1984). This same procedure was used for conjugation of versicolorin hemiacetal with minor modifications. Bovine serum albumin (BSA) concentration was scaled down from 20 mg in 5 ml phosphate buffer to 5 mg BSA in 1 ml buffer. Versicolorin hemiacetal, 0.5 mg, was dissolved in 0.75 ml methanol and added dropwise to BSA in phosphate buffer while stirring slowly. The mixture was reacted in a small vessel immersed in a 37 C water bath. After all VA hemiacetal had been dissolved in the BSA solution, the reaction was allowed to continue for an additional 30 min. Sodium borohydride (50 ul) was added and the reaction was continued at 4 C as previously described. Hydrochloric acid (25 ul) was added to destroy excess sodium borohydride. The conjugate solution was transferred to dialysis tubing and dialyzed for 48 hr against 0.05 M phosphate buffer (pH 7.4). Conjugation to horseradish peroxidase (HRP), an enzyme marker for use in ELISAs, was conducted in a similar manner. A 50:1 molar ratio was used for conjugation.

Conjugation ratio of hapten to carrier was estimated spectrophotometrically at 456 nm. BSA was used as reference standard. The VA hemiacetal:BSA conjugate produced was used for all immunization procedures.

Immunization of Rabbits

Five albino male rabbits were injected to produce antibody against VA. The animals were supplied by Laboratory Animal Care Services (LACS), Michigan State University, and were housed in their facilities. LACS personnel performed all immunization and blood collection.

All five rabbits were subjected to the same immunization protocol. The immunization schedule and concentration of conjugate were the same as those commonly employed for mycotoxins. The initial injection consisted of 500 ug VA-BSA in 1 ml 0.9% NaCl emulsified with 2 ml Freund's complete adjuvant (Difco). Three ml of the mixture were injected intradermally at approximately 30 sites on the back of each rabbit.

Four weeks following this initial injection, a booster injection was made. VA-BSA (250 ug) in 0.5 ml 0.9% saline was mixed with 1 ml Freund's incomplete adjuvant (Difco). The injections were intramuscular with 0.75 ml injected into each hind leg of the rabbit. At week 18, a second booster injection was made. The immunogen was prepared and administered in the same manner as described for the first booster injection.

Rabbits were bled via the marginal ear vein at weeks 5, 7, 9, 12, and 20 for determination of antibody titers. Collection of blood at week 23 was achieved by cardiac puncture. Blood was allowed to clot overnight at 4 C and then centrifuged at 500 g for 15 min. The translucent supernatant containing antiserum was collected. Antibody was purified from serum by a modification of the ammonium sulfate precipitation method described by Hebert et al. (1973). Ammonium sulfate was added at a ratio of 3.5 g/ 10 ml serum (approximately 50 % saturation) in a 50 ml centrifuge tube. The compound was added all at once to the serum and immediately shaken gently until dissolved. Centrifuge tubes were placed on a platform shaker and slowly agitated overnight at 4 C. Tubes were then centrifuged for 15 min at 500g. The supernatant was removed and the pellet was resuspended to the original serum volume with phosphate buffered saline (PBS) (pH 7.5). Ammonium sulfate was added at the same concentration as previously used. Tubes were shaken overnight and centrifuged as previously described.

The supernatant was removed and the pellet was resuspended to one-half the original serum volume with PBS. Solutions were transferred to 12,000 - 14,000 molecular weight exclusion dialysis tubing (Spectrum) and dialyzed against PBS for 24 hr. at 4 C. After removal from dialysis tubing, antisera were resuspended to original serum volume with PBS, dispensed in microcentrifuge vials and stored at -20 C.

Development of Immunoassays and Characterization of Antibody

Determination of Antibody Titers

Antibody titers were determined by direct ELISA. In a typical experiment, 100 ul of antisera diluted in 0.05 M carbonate buffer (pH 9.6) were added to microtiter wells (Removawell Microtiter Strips, Dynatech Laboratories, Alexandria, VA) and incubated at 37 C in a forced air incubator. Unbound antibody was removed from plates with two 300 ul washes of 0.1 M PBS previously mixed with 0.20% (v/v) Tween 20 (PBS-Tween). Unbound sites were blocked by addition of 300 ul of 1% ovalbumin in PBS (OA-PBS) to each well followed by incubation for 30 min at 37 C. The plate was washed three more times with PBS-Tween. VA conjugated to HRP was diluted 1:500 in OA-PBS and added at 100 ul per well. Plates were incubated for 60 min at 37 C. Unbound enzyme conjugate was removed by washing 8 times with PBS-Tween. Bound peroxidase activity was determined using 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as described by Pestka et al. (1980): 100 ul substrate solution (0.4 mM ABTS, 1.2 mM hydrogen peroxide in 50 mM citrate buffer, pH 4.0) were added to each well. The reaction was stopped by adding 100 ul stopping solution (300 mM citric acid containing 15 mM sodium azide) to each well. Absorbance was read at 405 nm on an ELISA plate reader (Dynatech Laboratories, Alexandria, VA). Titers were defined as the highest dilution of antiserum resulting in absorbance greater than the control.

Determination of Sensitivity

Direct competitive ELISA was utilized to determine ability of the antisera to detect VA. Competitive ELISA was performed in the same manner as previously described for direct assays with the following modifications. After blocking plates with OA-PBS, 100 ul VA was added to each well immediately prior to addition of VA-HRP enzyme conjugate. VA at various concentrations was diluted in 20% methanol in PBS (MEOH-PBS). Control wells were filled with 100 ul MEOH-PBS. Antisera from the five immunized rabbits were analyzed at dilutions of 1:250, 1:500, 1:1000, and 1:2000 using 0.1, 1.0, 10, 100, 1000, and 10,000 ng/ml VA. VA-HRP concentration was increased to 1:250.

Determination of Cross-Reactivity

Direct competitive ELISA was used to analyze for cross-reactivity. Various aflatoxin precursors (averufin, averantin, averufanin, and norsolorinic acid) were obtained from Dr. Deepak Bhatnagar (USDA ARS, New Orleans, LA) to evaluate cross-reactivity between these structurally similar compounds and VA antibody. Versicolorin hemiacetal, sterigmatocystin (Sigma), and aflatoxin B_1 (Sigma) were also evaluated.

The assay was performed as previously described with the following modifications. A standard curve for VA at concentrations of 10, 50, 100, 500, and 1000 ng/ml was used for one set of wells. Various dilutions of precursors, dissolved in MEOH-PBS, were used for the other wells. All compounds were added at the step immediately prior to addition of VA-HRP.

Miniscale Production of VA

Direct competitive ELISA was used to determine the time course of VA accumulation by *A. parasiticus* ATCC 36537 when grown in miniscale cultures. Flasks (50 ml) were treated with Prosil 28 as previously described. and 10 ml culture media were added. Five 6 mm (diameter) glass beads were added to half of the flasks to reduce potential clumping of mycelia that tended to occur during incubation. Flasks were inoculated with 10^6 ATCC 36537 conidia, plugged with foam stoppers, covered with foil, and incubated in the dark at 28 C with continuous shaking (200 r.p.m.) for 1, 2, 3, 4, and 5 days.

The contents of individual flasks were transferred to a 20 ml plastic syringe containing a small amount of glass wool to separate mycelia from culture broth. (The syringe plus glass wool were weighed before adding culture). Five hundred mg Celite 545 (Fisher) were previously added to cultures incubated with glass beads to facilitate filtration. The syringe plunger was pushed in to expediate filtration and to extract as much culture broth from mycelia as possible. Culture filtrate was collected for pH determination on a pH meter. For extraction of VA, 10 ml methanol were added to mycelia by slowly pulling back on the plunger. The syringe was capped and attached to a rotating platform at 180 r.p.m. for 15 minutes. Extract was removed by pushing the plunger into the syringe. Extraction was repeated with an additional 10 ml methanol.

Following extraction of VA, mycelial dry weight was determined by placing the entire syringe plus mycelia in a 50 C incubator overnight. Previous weight recorded for the syringe plus glass wool, plus Celite (if applicable) was subtracted from the final weight obtained.

Direct, competitive ELISA, as previously described was used to determine VA content. Methanol extracts were analyzed at dilutions of 1:10, 1:100, 1:1000, and 1:10,000.

Laboratory Safety

All glassware, equipment, media, and miscellaneous materials utilized for VA, or any other toxic precursors were routinely soaked in commercially available 10% NaOCl solution for several hours followed by treatment with acetone and autoclaving to destroy the toxins.

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RESULTS AND DISCUSSION

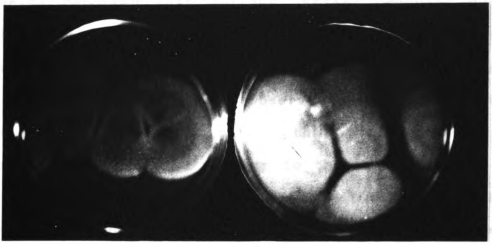
Isolation of a Morphological Variant

Direct plating onto PDYA of growth washed from a slant of ATCC 36537 without filtration of somatic hyphae resulted in a morphological variant. After incubation, it was noted that two distinguishable types of colonies appeared on plates. (Figures 4 and 5). Typical ATCC 36537 colonies produced abundant white sporulation on the surface with accumulation of deep yellow to orange pigment when viewed from the reverse side (Figure 4a, Figure 5a). Atypical colonies produced very few spores on the surface and also demonstrated a thinning of growth on the outer diameter of each colony that looked similar to a folding fan (Figure 4b). From the reverse side, very little pigment accumulation was apparent and deep furrowing growth into the agar was also evident (Figure 5b). Similar results with other aflatoxin biosynthetic mutants and aflatoxigenic strains have been reported in the literature. Bennett (1981) reported that transfers of ATCC 24690, an A. parasiticus that accumulates norsolorinic acid and low levels of aflatoxin, containing hyphae rather than conidia resulted in unpigmented, non-aflatoxigenic forms. Bennett et al. (1981) also reported that serial transfer of mycelial macerates, as opposed to conidial transfers, of aflatoxigenic strains of A. parasiticus produced two morphological variants which

Figure 4. Surface view of typical ATCC 36537 colonies and morphological variant. Cultures grown on PDYA (see Methods). (a) Typical growth represented by abundant white sporulation. (b) Morphological variant produced very few spores and demonstrated a thinning of growth on the outer diameter of each colony.

A B

Figure 5. Reverse view of ATCC 36537 colonies and morphological variant. Cultures grown on PDYA (see Methods) (a) Typical accumulation of deep yellow to orange pigment. (b) Morphological variant demonstrated very little pigment accumulation. Furrowing growth into the agar was also evident.



A
B

v

c

a

i

v

c

c

p.

mc

ac

were designated as "fan" and "fluff." "Fan" variants display flat growth and a gradient of sporulation density with heavy sporulation in the colony center but only slightly at the edges. These variants also show an absence of mycelial pigmentation and furrows on the reverse of the colony. "Fluff" variants demonstrate abundant aerial mycelium, sparse sporulation, absence of mycelial pigmentation and furrows on the reverse of the colony. While Bennett et al. (1981b) reported that neither variant produced detectable aflatoxins, further research indicated that the "fluff" variant was unstable and could revert to aflatoxigenicity (Bennett et al., 1986). Observation of the ATCC 36537 variant isolated indicated that the growth pattern demonstrated was most similar to that described by Bennett as "fluff." TLC analysis of mycelial extracts after 5 d incubation indicated no detectable VA. The unexpected variant produced demonstrated the importance that only conidial transfers, free of somatic hyphae, are used for culture propagation.

Versicolorin A Production

Cultivation of ATCC 36537, using a defined aflatoxin producing medium developed by Adye and Matales (1964) and modified by Bennett et al. (1979), resulted in mycelial accumulation of VA and was therefore utilized throughout this experiment. Bennett et al. (1979) substituted ammonium nitrate for ammonium sulfate as the nitrogen source in the formulation described by Adye and Matales (1964); this modification resulted in 4 times more VA production. Personal communication with Dr. Joan Bennett (Tulane University, New Orleans, LA) indicated that doubling the concentration of micronutrients in the media also increased VA production. Disavantages reported by Bennett et al. (1979) when using this formulation were that two additional compounds were detectable with thin layer chromatography in the crude extract. Higher yields of several other compounds previously seen in only trace amounts were also reported. Despite these disadvantages, this culture media was utilized on the basis of the increased VA yields reported.

Versicolorin A Extraction

Extraction of crude VA from ATCC 36537 mycelia was successfully achieved using a procedure similar to that described by Lee et al. (1975). The number of extractions required to remove pigment were significantly reduced by soaking mycelia in acetone overnight before blending. Caution was necessary to prevent formation of emulsions during extraction of VA from acetone/water with hexane.

sta
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aver
lowe
(365
deve
two,
syst
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0.3
VA

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tog
dev
a sh
orig
deve
aver
(0.36
1976)

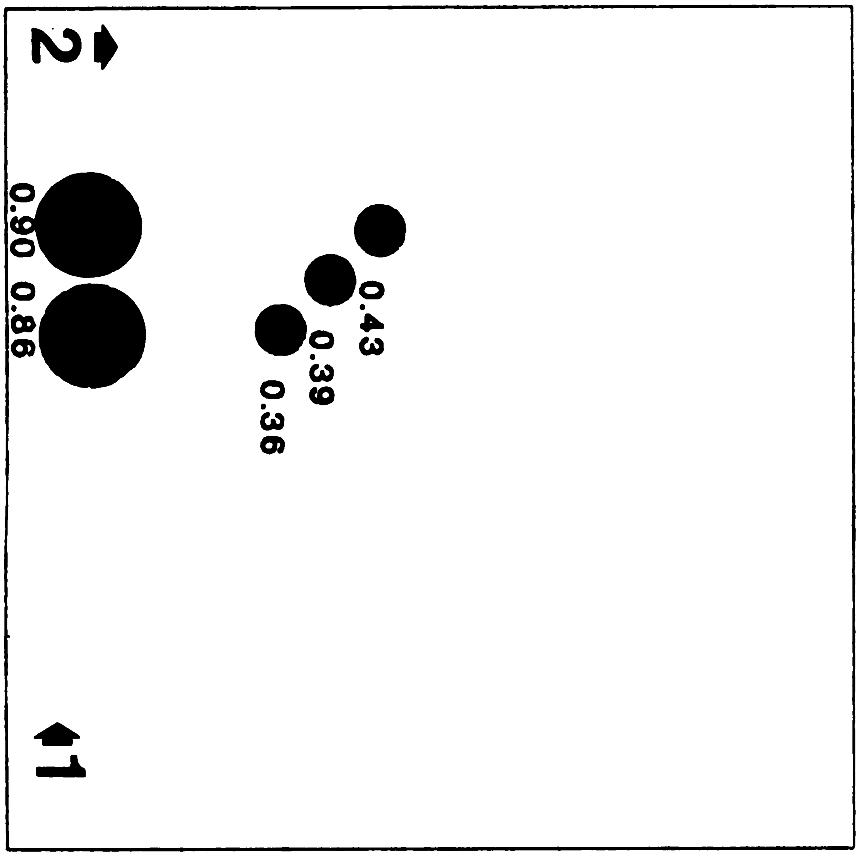
Thin Layer Chromatography of Anthraquinone Standards

Using TEA as the solvent system, anthraquinone spotting standards were not separable. The higher of the two R_f values for the versicolorin A and C mixture was the same for averufin (R_f=0.90). Versicolorin C should appear as the lower R_f yellow fluorescent spot when viewed under longwave (365 nm) ultraviolet light. When the VA and VC mixture was developed with BA on (HPTLC) plates; three, rather than just two, yellow fluorescent spots were detected. With the BA system, averufin again had the same Rf (0.43) as the leading spot in the VA and VC mixture.

The TE solvent system described by Heathcote and Hibbert (1975) for separation of versicolorins produced good resolution though separation between versicolorin A (R_f= 0.30) and versicolorin C (R_f=0.25) was better than between VA and averufin (R_f=0.34).

Following the first development (TEA solvent system) when using two dimensional TLC, only two spots very close together (R_f=0.90, 0.86) were apparent. After the second development (BA solvent system), three spots were present on a sharp diagonal indicating that they were a product of the original two spots (Figure 6). R_f's obtained from the second development agreed with values stated in the literature for averufin (0.43), versicolorin A (0.39) and versicolorin C (0.36) when using the BA solvent system (Bennett et al., 1976). Two dimensional TLC solvent verified that (1) the VA

Figure 6. Two dimensional HPTLC analysis of anthraquinone standards. 1. Development in the first direction used the solvent system toluene:ethyl acetate:acetic acid (50:30:4, v/v/v). 2. Second development used benzene:acetic acid (95:5, v/v).


2
1
0.90
0.86
0.43
0.39
0.36

and VC mixture also contained averufin and that (2) the toluene:ethyl acetate:acetic acid solvent system (50:30:4 v/v) did not sufficiently separate averufin, versicolorin A and versicolorin C and would not be useful for analyzing crude extracts from ATCC 36537.

Use of two dimensional TLC (TE, BA) was partially successful for purification of the anthraquinone standards. Results indicated that versicolorin compounds had apparently become contaminated during attempts to purify them and that only averufin (m.w. 368) could be positively identified (Figure 7) by comparison of results with mass spectra data published for the compound by Cole and Cox (1981). Not only was this attempt insufficient for purifying standards, it was readily apparent that this method would not be suitable for purifying versicolorin A to the purity and quantity necessary for specific antibody production.

Fluorodensitometry

Thin layer chromatographic separation on high performance silica gel plates with quantitation by fluorodensitometric scanning provided a convenient method for estimation of versicolorin A present in crude extracts. Results from this study are shown in Figures 8 and 9. Versicolorin A accumulation was greater when cultures were incubated under shaking conditions versus stationary

Figure 7. Mass spectrum of averufin. Material was analyzed as described in Methods.

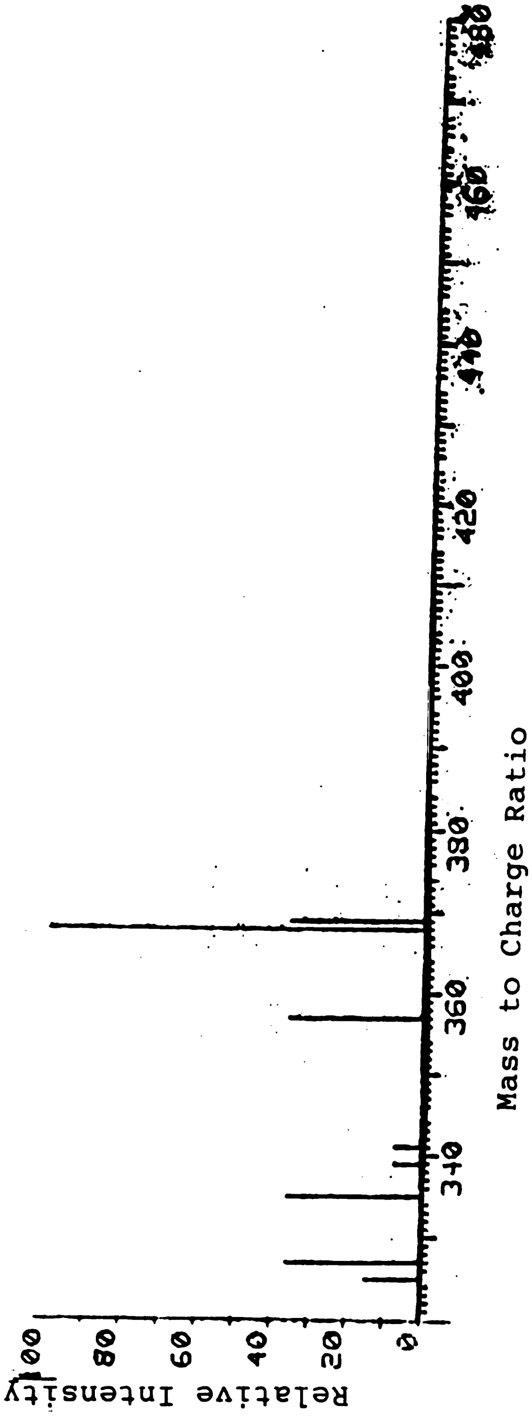

Relative Intensity
0
20
40
60
80
100
Mass to Charge Ratio
340
360
380
400
420
440
460
480

Figure 8. Time course of VA production by ATCC 36537 cultures during shaking vs. stationary incubation. Cultures were prepared and incubated as described in Methods. VA production was determined by TLC plus fluorodensitometry as described in Methods. Values are averages of 2 determinations.

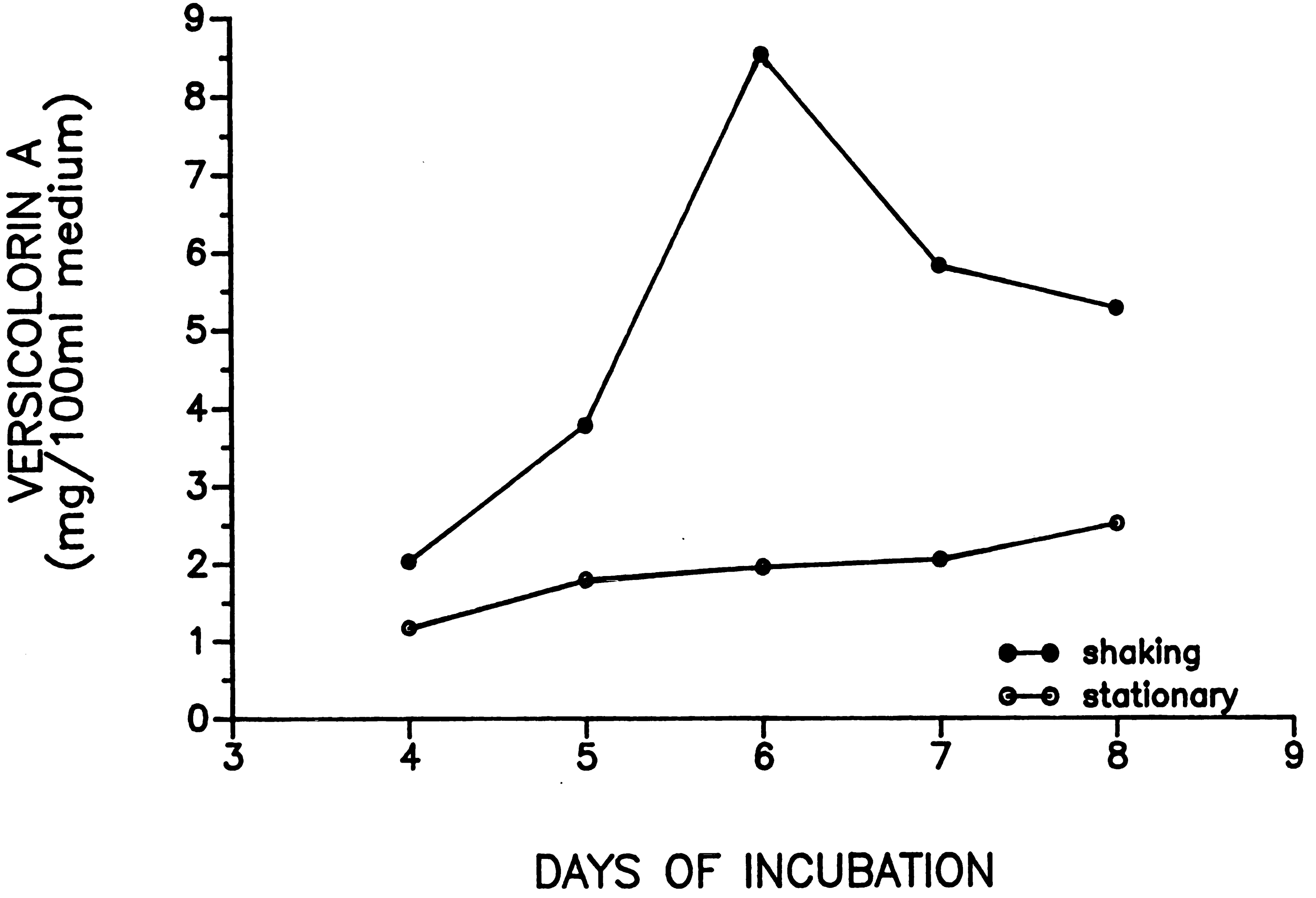
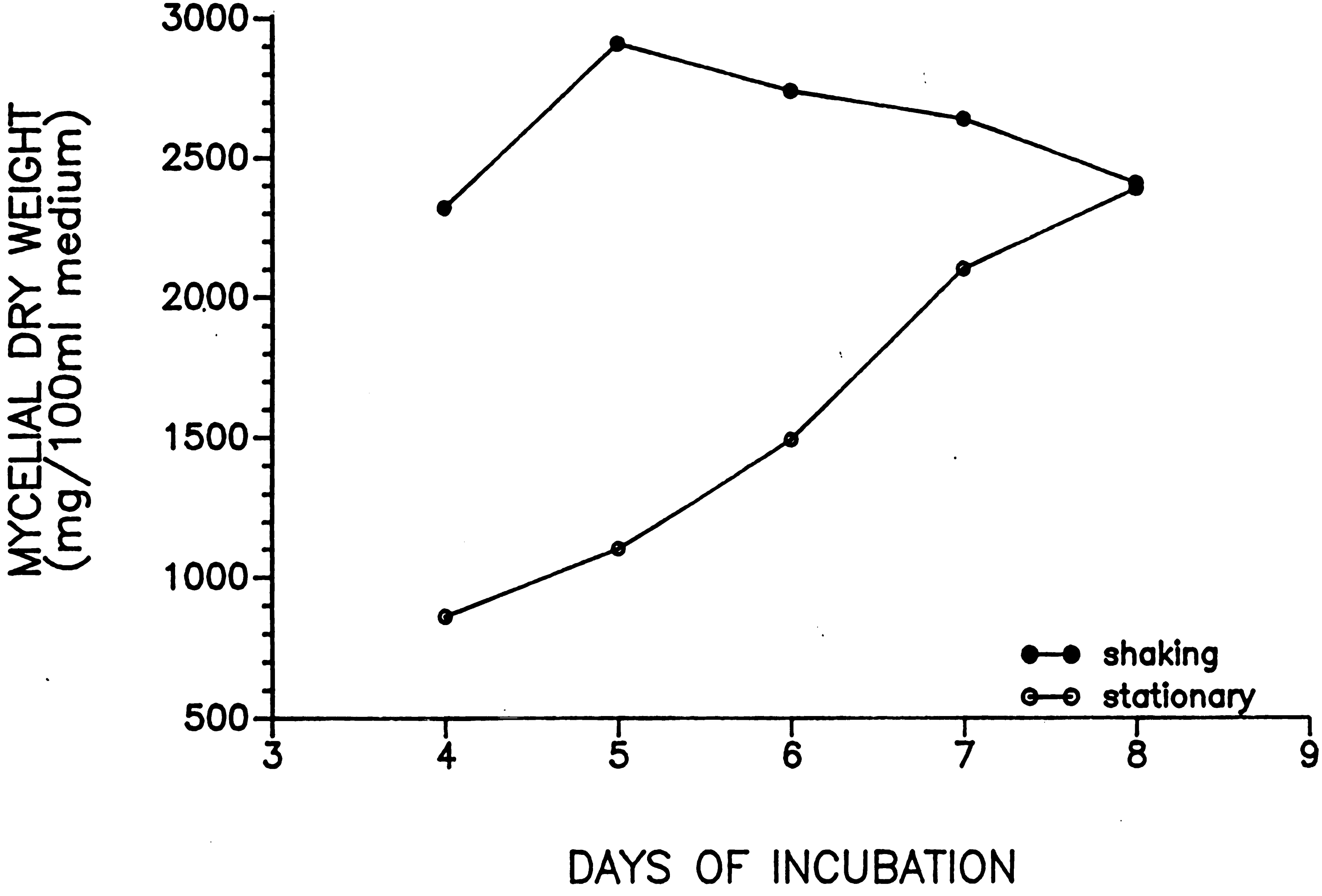

VERSICOLORIN A
(mg/100ml medium)
0
1
2
3
4
5
6
7
8
9
3
4
5
6
7
8
9
DAYS OF INCUBATION
●—● shaking
○—○ stationary

Figure 9. Mycelial dry weight accumulation by ATCC 36537 cultures during shaking vs. stationary incubation. Weight was determined by drying filtered and rinsed mycelia overnight at 50 C. Values are averages of 2 determinations.


MYCELIAL DRY WEIGHT
(mg/100ml medium)
3000
2500
2000
1500
1000
500
3
4
5
6
7
8
9
DAYS OF INCUBATION
shaking
stationary

conditions. Highest levels of the aflatoxin precursor were found in 6d (8.5 mg/100 ml) shaking cultures as compared to 4d (2.0 mg/100 ml), 5d (3.8 mg/100 ml), 7d (5.8 mg/100 ml), and 8d (5.7 mg/100 ml) cultures. Versicolorin A concentrations for stationary cultures were 1.2, 1.8, 2.0, 2.0, and 2.5 mg/100 ml for 4, 5, 6, 7, and 8d, respectively. Accumulation of VA in shaking cultures began to decrease after 6d in comparison with results from stationary cultures which indicated that VA concentration was continuing to increase by the end of the study.

Mycelial dry weights were greater with shaking incubation for 4d (2.3 g), 5d (2.9 g), 6d (2.7 g), and 7d (2.6 g) than stationary incubation (0.9 g, 1.1 g, 1.5 g, 2.1 g, respectively), but were essentially the same by 8d (shaking - 2.4 g, stationary - 2.4 g). With shaking incubation, mycelial dry weight peaked prior to versicolorin A peak accumulation. With stationary incubation, mycelial dry weight continued to increase by the end of the study. Based upon these results, it would be expected that VA accumulation at 9d for stationary conditions would be higher than reported for 8d.

It was noted that mycelial forms differed between the two types of incubation. Cultures incubated with stationary conditions produced mycelia that grew only on the surface of culture media. Mycelia produced during shaking incubation were in the form of small spheres with tightly wound hyphae. The enhanced growth and production of VA by shaking cultures may be attributed to the increased availability of nutrients to mycelia growing in the spherical form.

Extraction of mycelial pads following overnight drying at 50 C was very difficult and required extensive blending. It is recommended that extraction of pigment be done prior to mycelial dry weight determination. Weight of pigment extracted would be neglible and should not have a significant effect on total dry weight. Alternatively, a second set of flasks could be inoculated and cultured solely for the purpose of mycelial dry weight determination.

Based upon results from this study, conditions for cultivation of ATCC 36537 for optimum versicolorin A production were defined. Shaking incubation at 28 C for 6 days was used for the remaining production of VA for purification.

Derivatization of Crude Versicolorin Extract

In the procedure described by Li and Chu (1984), following reluxing of sterigmatocystin and sulfuric acid, the hemiacetal mixture was evaporated to dryness under reduced pressure and resuspended in 10 ml water. When the versicolorin hemiacetal mixture was dried under vacuum, it became difficult to resuspend in water. This may have been due to water insoluble impurities in the mixture. Addition of 10 ml acetone along with the water helped solubilize the mixture. Acetone has a lower boiling point than water and was quickly removed by drying under vacuum.

Li and Chu (1984) used three 5 ml chloroform extractions to remove sterigmatocystin hemiacetal. TLC analysis of the water phase following three 5 ml chloroform extractions of the versicolorin hemiacetal mixture indicated that a significant amount of hemiacetal remained. The procedure was modified so that the water phase was extracted until chloroform extracts were colorless.

TLC combined with fluorodensitometry demonstrated that the hemiacetal reaction was specific for VA and that major anthraquinones accumulated by ATCC 36537 would not react. Prior to refluxing, peaks were recorded for versicolorin C, versicolorin A and averufin. Following the reaction, peaks were recorded for only VC and AVR. Fluorescence was detected for a previously unrecognized compound at R_f 0.1 that was attributed to versicolorin hemiacetal.

Derivatization of crude versicolorin extract resulted in a mixture of compounds from which versicolorin hemiacetal could not be isolated. TLC analysis indicated that VA hemiacetal could not be separated from numerous other polar compounds that were present in the unreacted crude extract, or that may have been formed during derivatization. The entire derivatization procedure was repeated several times. Similar unsuccessful results were obtained. Collection of smaller fractions from the silica gel cartridge did not isolate VA hemiacetal, either. Purity of compounds is essential for production of specific antibodies. It was possible that these polar contaminants may produce undesirable side reactions during conjugation of VA hemiacetal to protein. It was concluded that hemiacetal derivatization should be performed with only highly purified versicolorin A.

Preparative Thin Layer Chromatography

Purification of versicolorin A using preparative TLC was unsuccessful. HPTLC analysis of the eluted material from the scraped preparative plate indicated that it contained not only VA, but many other compounds, also. Preparative TLC plating and scraping eliminated only a thin green band from the original crude mixture. This approach to purification was eliminated based upon poor results.

High Performance Liquid Chromatography

The HPLC solvent system described by Berry et al. (1984) for separation of aflatoxin precursors was not suitable for isolation of VA with the HPLC systems analyzed. Using an analytical column, TLC analysis of peaks collected indicated that versicolorin A and versicolorin C were not separable and had a retention time of approximately 5 min. Averufin had a retention time of approximately 10 min

(Figure 10). Using semi-preparative HPLC, only a very small amount of crude VA (0.25 mg) was soluble in the acetonitrile:tetrahydrofuran:water mobile phase. When higher concentrations were suspended in either acetonitrile or tetrahydrofuran, and then injected onto the column, the extract eluted immediately and was completely unresolved. It was apparent that this solvent system would not provide a purification method suitable for quantities of VA required for antibody production and subsequent ELISA research.

Purification of Versicolorin A

Modifications of the procedure described by McCormick et al. (1988) provided a successful procedure for purification of VA. Using semi-preparative HPLC, deep yellow pigment began eluting from the column at a retention time of 10 min. Unexpectedly, when fractions were stored at 4 C overnight, fine yellow needles crystallized from fractions containing high concentrations of versicolorin A. The needles were carefully collected by filtration and washed with cold benzene. Further crystallization occurred in some fractions following additional incubation at 4 C.

Using analytical HPLC, retention times were determined to be 10.30 min for versicolorin C, 11.86 min for versicolorin A, and 29.55 min for averufin (Figure 11).

Figure 10. Chromatogram from analytical reverse phase HPLC of semi-purified ATCC 36537 mycelial extract. HPLC was performed as described in Methods using a mobile phase (1 ml/min) of acetonitrile:tetrahydrofuran: water (25:20:55, v/v/v). Absorbance was monitored at 325 nm. VA = versicolorin A, VC = versicolorin C, AVR = averufin.

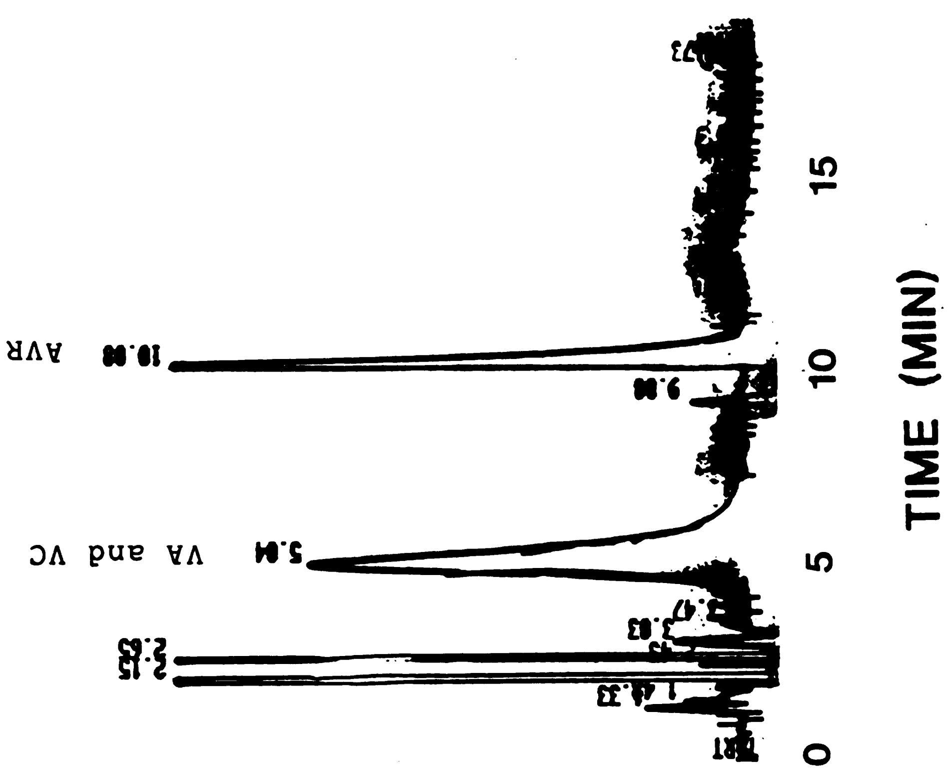
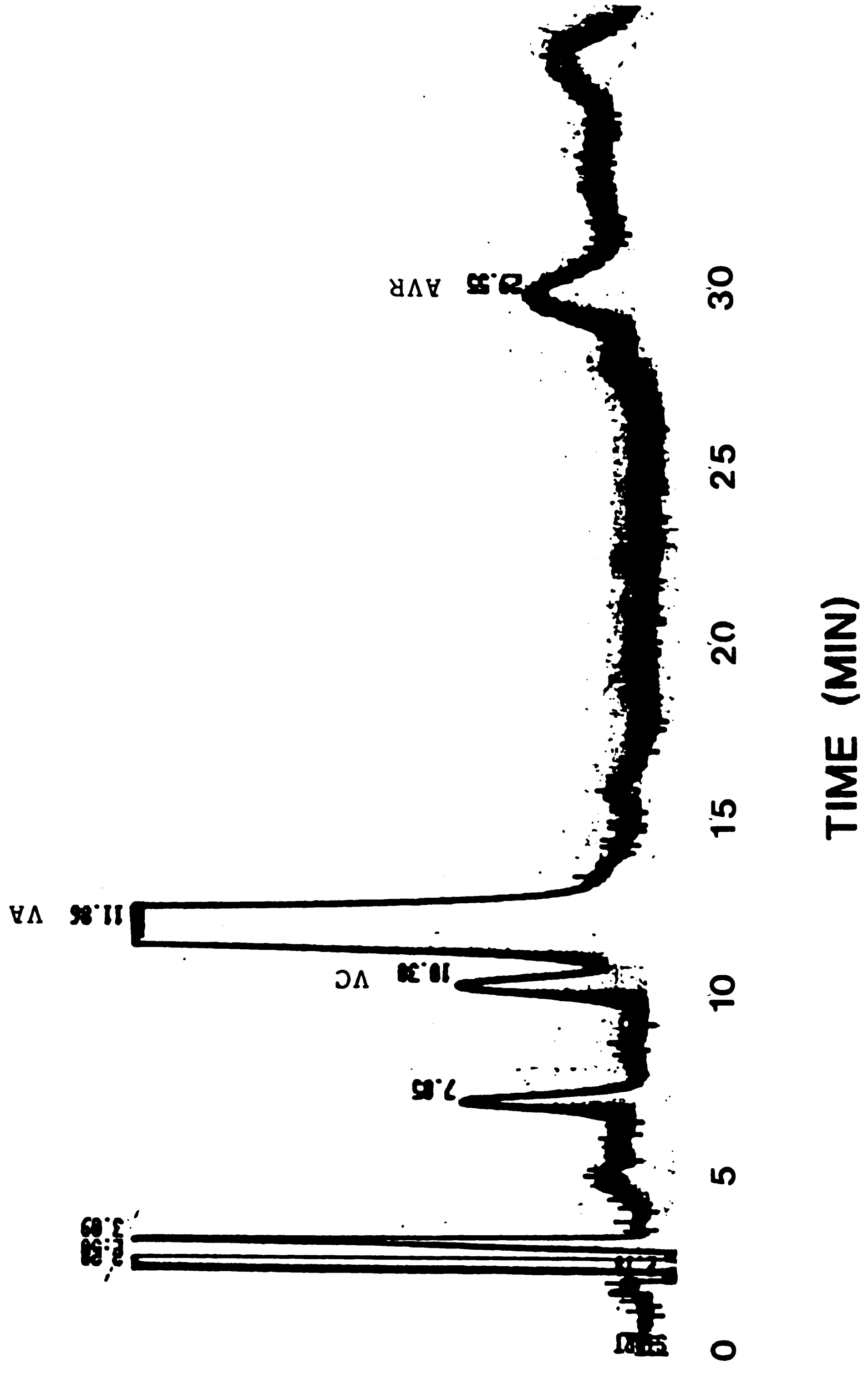

2.15
2.65
3.83
5.04
VA and VC
9.08
10.08
AVR
0
5
10
15
TIME (MIN)

Figure 11. Chromatogram from successful analytical reverse phase HPLC of semi-purified ATCC 36537 mycelial extract. HPLC was performed as described in Methods using a mobile phase (1 ml/min) of methanol:tetrahydrofuran:0.1 M acetic acid (4:2:1, v/v/v). Absorbance was monitored at 313 nm. VA = versicolorin A, VC = versicolorin C, AVR = averufin.


TIME (MIN)
0
5
10
15
20
25
30
7.65
10.30 VC
11.86 VA
AVR

Versicolorin hemiacetal eluted with a retention time of 5.46 min (Figure 12).

Analysis of Crystalline Versicolorin A

Results of mass spectrometry are shown in Figure 13. Purity was verified by comparing these results with published mass spectrometry data (Cole and Cox, 1981). Purity was also verified by analytical HPLC. The peak detected at 11.87 min for the crystalline compound (Figure 14) corresponds with the retention time previously determined for VA (Figure 11). Peaks for versicolorin C and averufin were not detected. The UV absorption spectrum recorded for VA (Figure 15) was identical to data published by Cole and Cox (1981).

Production of Immunogen

A summary of derivatization and conjugation is shown in Figure 16.

Final yield of recovery for VA hemiacetal was 83% (1.44 mg). Results of conjugation indicated a 16:1 molar ratio of VA hemiacetal:BSA. These results are comparable to the 74% recovery and 14:1 molar ratio reported by Li and Chu (1984)

Figure 12. Chromatogram from analytical reverse phase HPLC of semi-purified VA hemiacetal. HPLC was performed as described in Methods using a mobile phase (1 ml/min) of methanol:tetrahydrofuran:0.1 M acetic acid (4:2:1, v/v/v). Absorbance was monitored at 313 nm. VAH = versicolorin A hemiacetal, VA = versicolorin A, VC = versicolorin C, AVR = averufin.

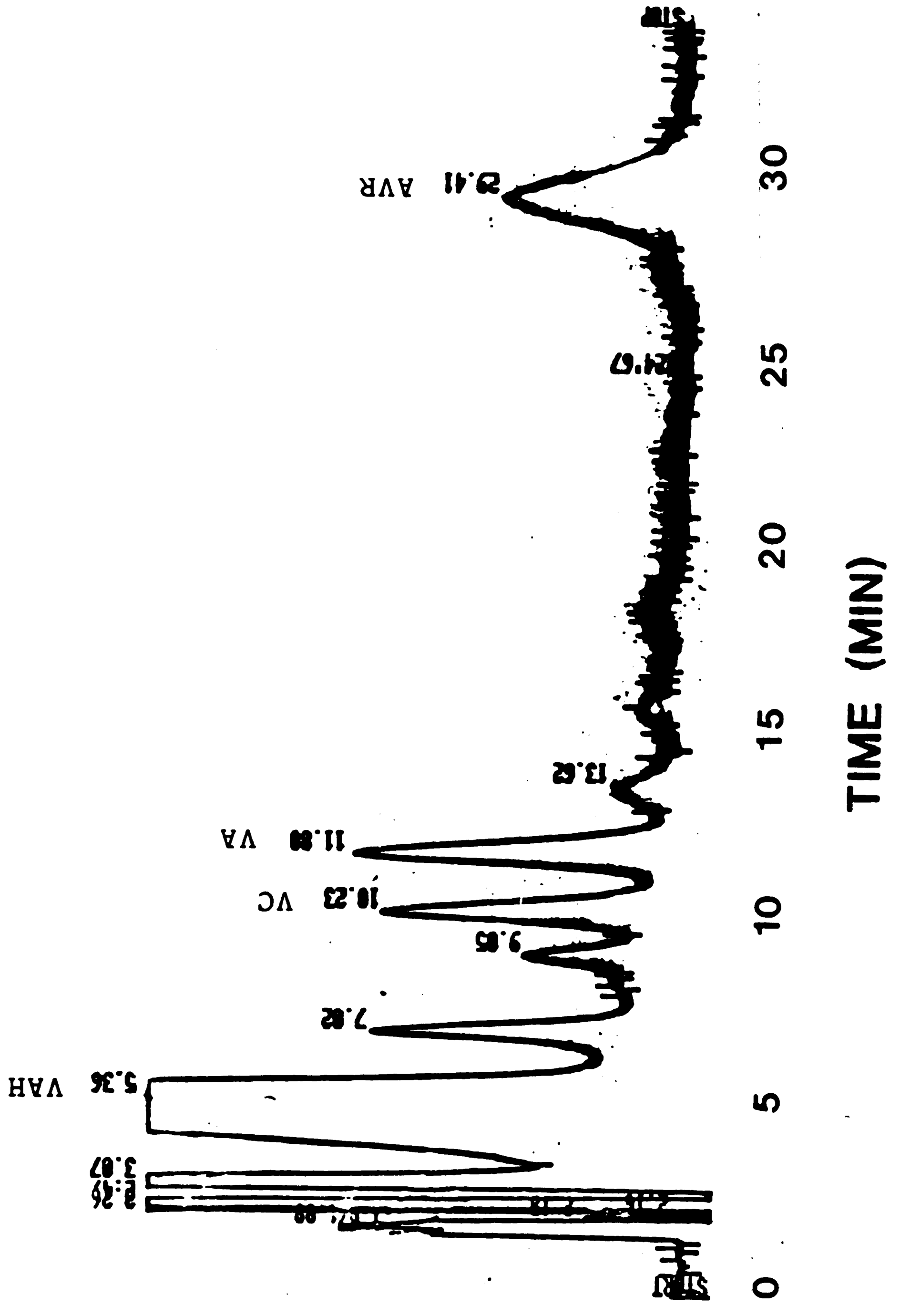

5.36 VAH
7.02
9.65
10.23 VC
11.80 VA
13.62
29.41 AVR
3.07
2.49
2.26
0
5
10
15
20
25
30
TIME (MIN)

Figure 13. Mass spectrum of versicolorin A. Material was analyzed as described in Methods.

FRN: 5031
SCAN NØ.: 199

TOT. PEAKS: 161
BASE PEAK: 309.3
REL.IONS.THRESH.(%):0

BASE PK. ABUND.: 528
BASE PK. % ABUND.: 9.13
TOT. IONS.: 5786

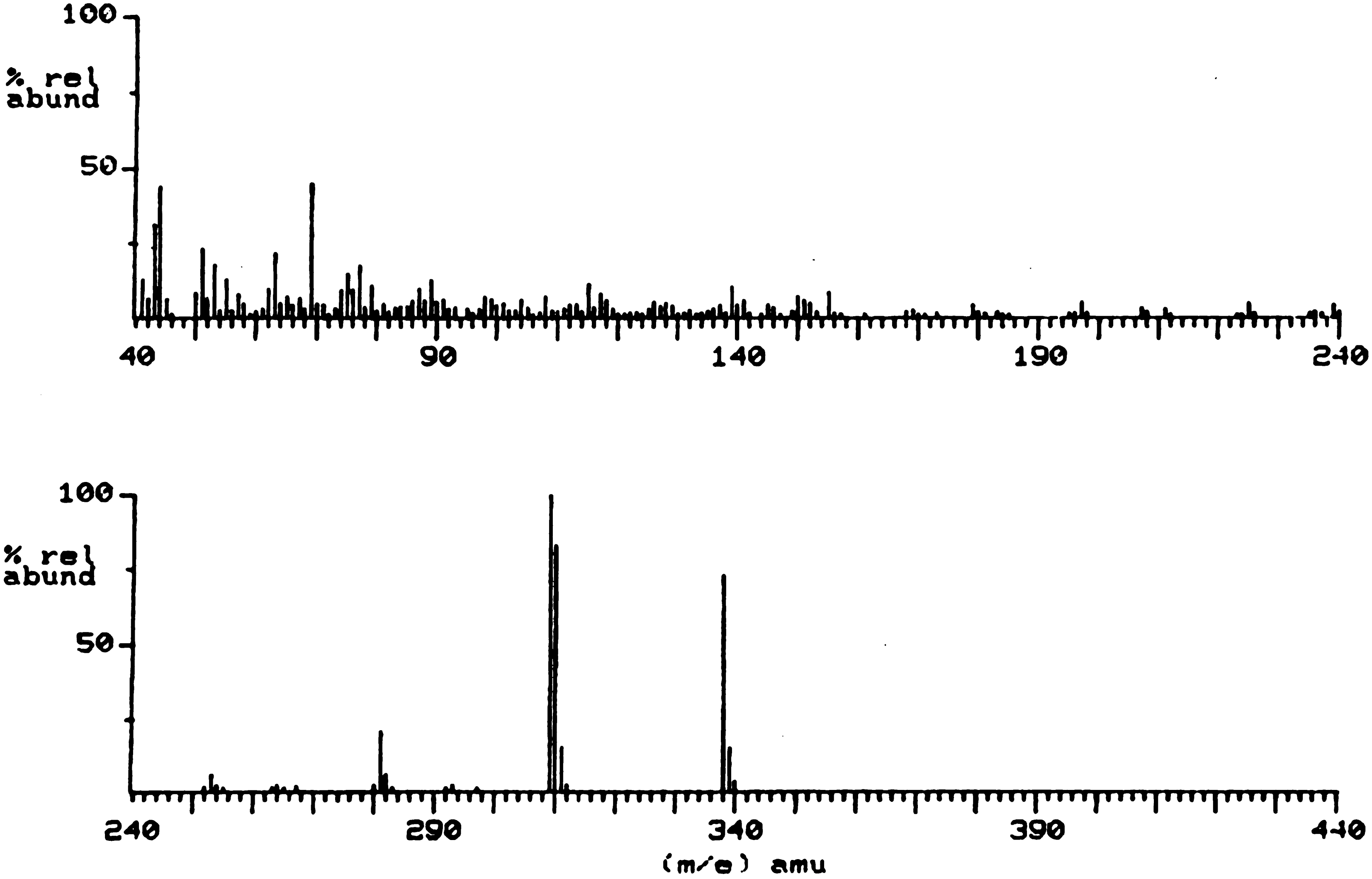


Figure 14. Chromatogram from analytical reverse phase HPLC of crystalline versicolorin A. HPLC was performed as described in Methods using a mobile phase (1 ml/min) of methanol:tetrahydrofuran:0.1 M acetic acid (4:2:1, v/v/v). Absorbance was monitored at 313 nm. VA = versicolorin A.


11.87
VA
.92
0
5
10
15
TIME (MIN)

Figure 15. UV/VIS absorption spectrum for versicolorin A in ethanol.

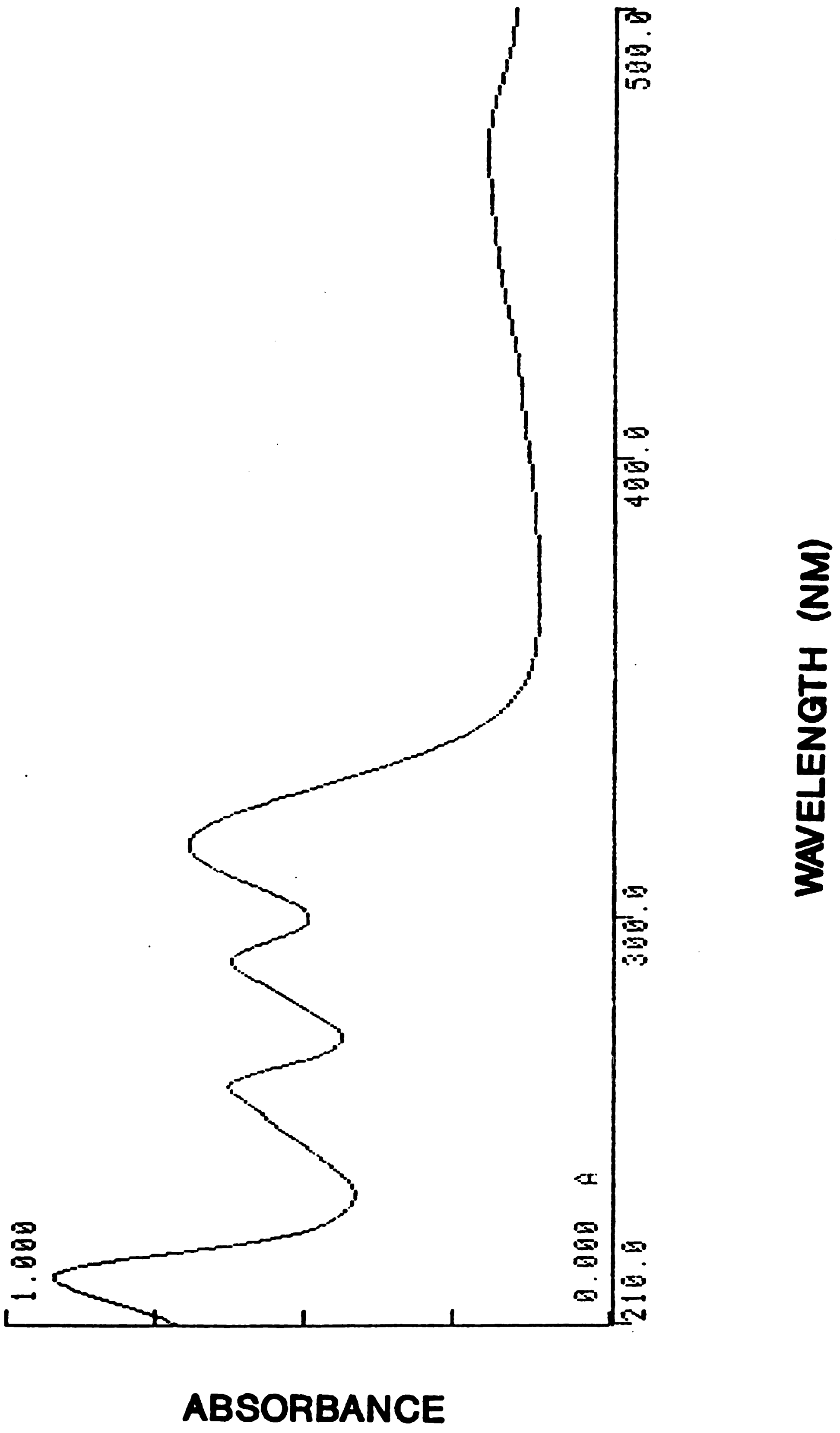
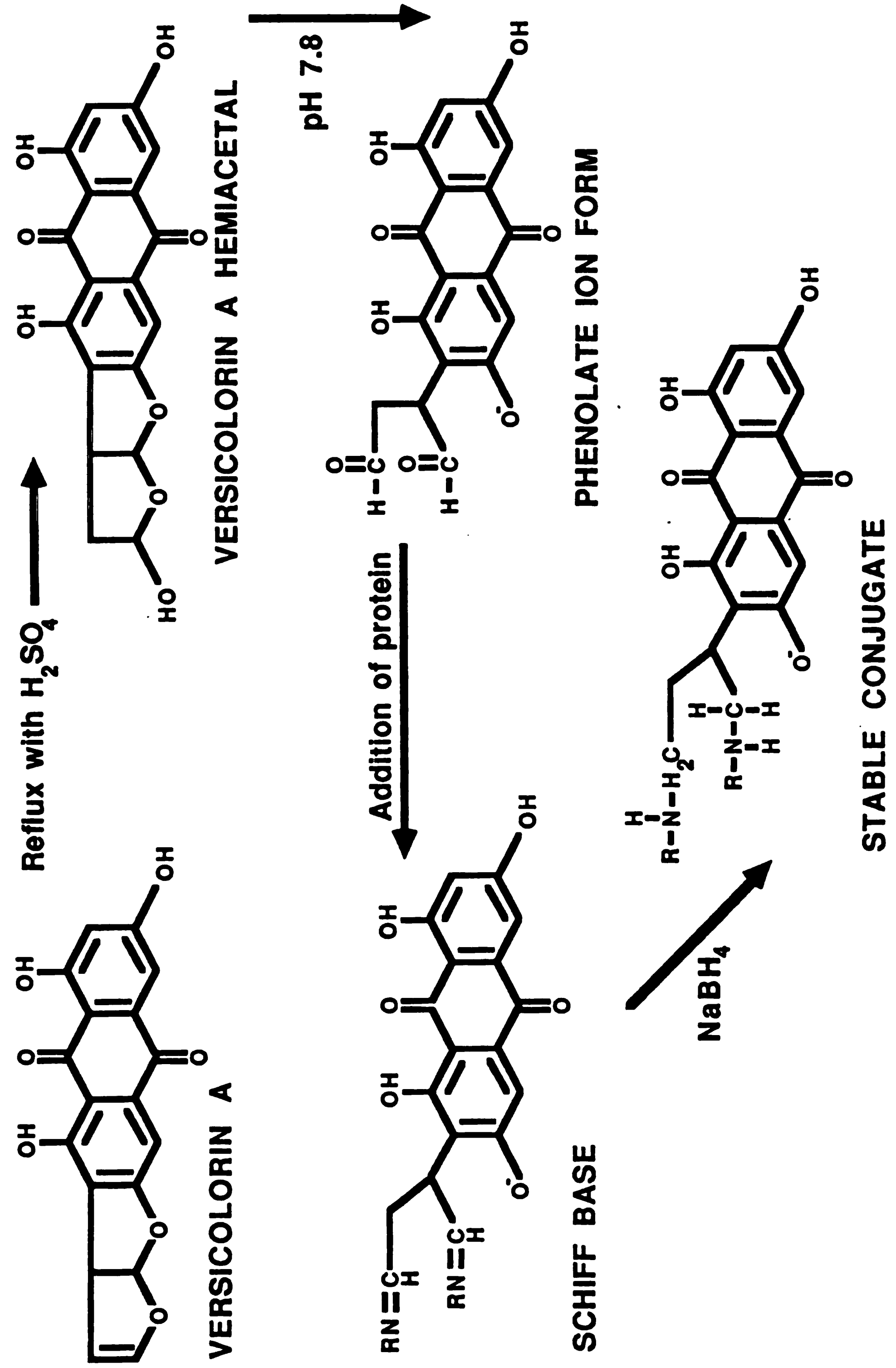

1.000
0.000 A
210.0
300.0
400.0
500.0
WAVELENGTH (NM)
ABSORBANCE

Figure 16. Preparation of versicolorin A - carrier protein conjugates. The reaction was performed as described in Methods. Versicolorin A was converted to the hemiacetal form by refluxing with dilute sulfuric acid. Raising the pH to 7.8 opened the difuran rings to a phenolate ion form. A Schiff base was formed upon addition of protein. Sodium borohydride reduced the double bonds to form a stable conjugate.


VERSICOLORIN A
Reflux with H_2SO_4
VERSICOLORIN A HEMIACETAL
pH 7.8
PHENOLATE ION FORM
Addition of protein
SCHIFF BASE
$NaBH_4$
STABLE CONJUGATE
OH
O
HO
O^-
H-C=O
RN=C-H
R-N-H
R-N-H_2C

for sterigmatocystin hemiacetal and STG-BSA conjugation, respectively.

The procedure described by Li and Chu utilized distilled water for dialysis of the STG-BSA conjugate. When distilled water was used for dialysis of VA-BSA conjugate, the conjugate precipitated out of solution. This may have been due to the low pH (approximately 5.5) of distilled water available from the laboratory at that time.

Determination of Antibody Titers and Specificity

Several trials were made before optimum conditions were determined for ELISAs. A major modification that improved results was substitution of carbonate buffer for dilution of antisera. Initial assays were carried out with PBS. Results were also improved when OA-PBS was not stored but prepared immediately before use. The assay was improved when freshly prepared ABTS was dispensed in 1 ml volumes in 1.5 ml microcentrifuge vials and stored at -20 C. Vials were thawed at 4 C prior to use. This practice reduced absorbance demonstrated by control wells.

Results of direct ELISA for antibody titers are shown in Table 1. Final titers ranged from 2000 to 4000. Similar titers (1280, 2560, and 5120) were reported by Gaur et al. (1981) when producing antisera against aflatoxin B_{2a} (hemiacetal form of AFB_1) using reductive alkylation for conjugation to carrier protein.

Table 1. ELISA titers for rabbits immunized with VA-BSA

Week post immunization[a]	ELISA titer in rabbit:				
	10A	12A	13A	14A	15A
5	1600	3200	1600	1600	800
7	1600	1600	1600	1600	800
9	1600	3200	1600	1600	800
12	3200	3200	3200	1600	1600
20	3200	3200	1600	1600	1600
23	4000	4000	3000	3000	2000

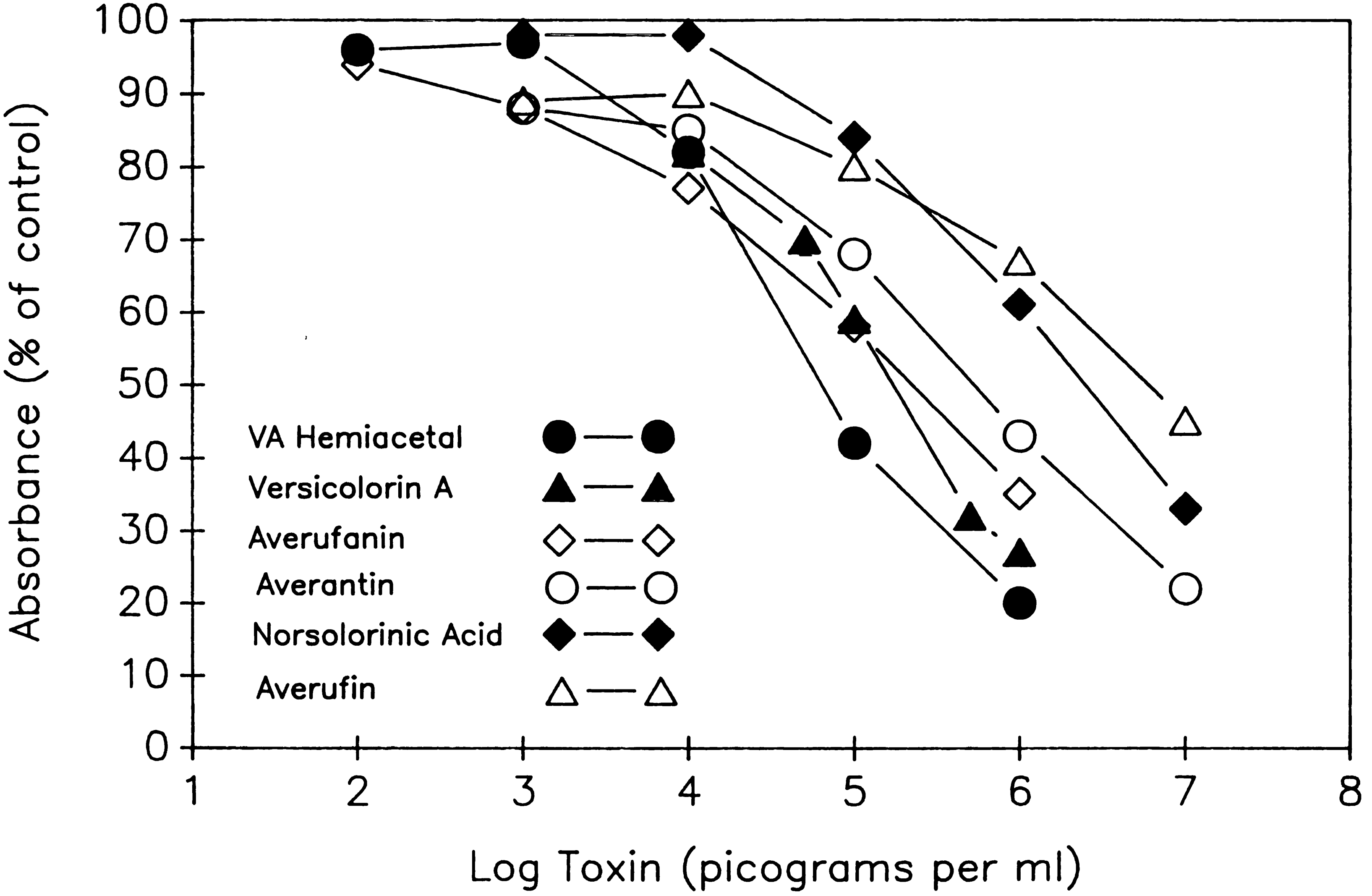
[a] Boosters given at weeks 4 and 18.

In direct competitive ELISA, inhibition of VA-HRP binding to the solid phase by free VA reduced end product absorbance compared to the control. Two of the 5 rabbits immunized produced antibodies with high specificity for VA. Fifty percent inhibition of end product absorbance for these antibodies occurred between 1000 and 10,000 ng/ml VA. The limit of dectection for VA was statistically determined to be 10 ng (a = .05).

A plot of toxin concentration vs. absorbance relative to the control (expressed in percent) for VA, VA hemiacetal, norsolorinic acid, averantin, averufin, and averufanin is shown in Figure 17. Lower relative concentrations required for 50% inhibition indicated higher cross-reactivity. Results from this plot demonstrated that versicolorin hemiacetal required a lower concentration for 50% inhibition than VA. Similar results were reported by Li and Chu (1984) when producing antibody to sterigmatocystin through hemiacetal derivatization.

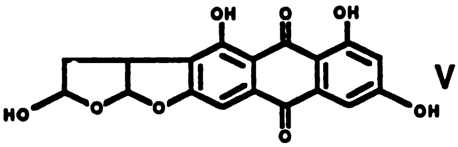
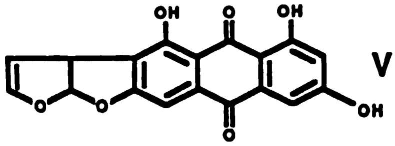
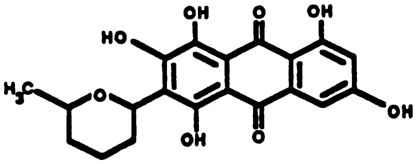
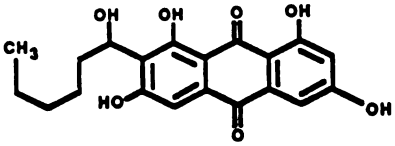
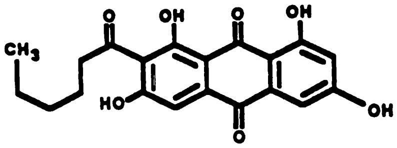
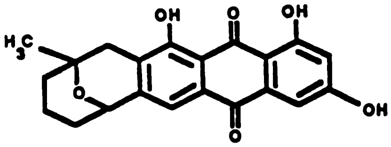
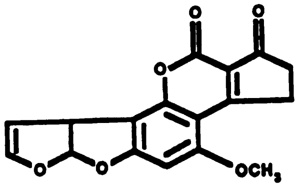
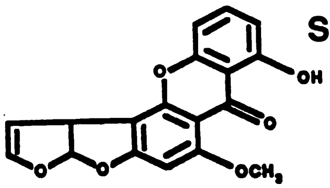
Absorbance values were converted to a logit/log form to determine relative cross reactivity (Figure 18). Concentrations required for 50% inhibition for VA and other compounds analyzed were compared to versicolorin hemiacetal (1.73×10^5 pg/ml). Cross reactivity of VA was 94% relative to VA hemiacetal. Averufanin, a precursor which also contains the anthraquinone moiety, showed a high degree of cross reactivity (80%). This higher cross-reactivity compared to other precursors may be due to structural

Figure 17. Cross-reactivity of VA analogs with VA antibody. Determined by direct competitive ELISA as described in Methods.



Absorbance (% of control)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
Log Toxin (picograms per ml)
VA Hemiacetal ●—●
Versicolorin A ▲—▲
Averufanin ◇—◇
Averantin ○—○
Norsolorinic Acid ◆—◆
Averufin △—△

Figure 18. Structures and cross-reactivities of VA and VA analogs. Determined by direct competitive ELISA as described in Methods.

		% CROSS REACTIVITY
	VERSICOLORIN A HEMIACETAL	100
	VERSICOLORIN A	94
	AVERUFANIN	80
	AVERANTIN	7
	NORSOLORINIC ACID	6
	AVERUFIN	2
	AFLATOXIN B_1	less than 1
	STERIGMATOCYSTIN	less than 1

similarity between averufanin's open ring and the open ring of the stable VA-BSA conjugate used for immunization. Averantin (7%), norsolorinic acid (6%), and averufin (2%) also demonstrated a significant amount of cross reactivity due to their anthraquinone structures. Sterigmatocystin and aflatoxin B_1 structurally share the difuran moiety with VA but showed <<<1% cross reactivity. These results correlate with the rationale used in selecting the difuran side of VA for the site of conjugation to carrier protein in order to produce antibodies with specificity for the anthraquinone portion of the molecule.

Application of ELISA to Miniscale Production of VA

Use of direct competitive ELISA was successful for detection and quantitation of miniscale VA production. Culturing of VA was scaled down to 10^6 conidia in 50 ml Erlenmeyer flasks containing 10 ml media to determine if VA could be detected by ELISA at these reduced levels of inoculum and media. Extraction of VA was simplified by the use of a syringe method.

Cultures of A. parasiticus ATCC 36537 grown with continuous shaking in more than 10 ml of culture media tended to produce large clumps of mycelia that produced greatly reduced levels of VA. When media volume was reduced to 10 ml, mycelia formed small spheres similar to those described when producing VA on a large scale. (Control cultures were grown under these conditions). Addition of glass beads to culture media also prevented formation of clumps. Mycelia produced by cultures grown with glass beads appeared as short threads of hyphae. Observation of the two mycelial forms under high power microscopy indicated that the glass beads severed hyphae into small pieces while control hyphae demonstrated typical fungal development.

Results of pH and mycelial dry weight determinations are shown in Figures 19 and 20. From an initial pH of 4.31, minimum values were recorded after 2 days of incubation (Figure 19). The pH increased to 6.0 and 7.6 for control and bead cultures, respectively by 6 d. Mycelial dry weights were higher for the bead cultures compared to the control until day 6 (Figure 20). Bead cultures demonstrated peak mycelial accumulation after 4 days of incubation.

Results of VA content determined by ELISA are shown in Figure 21. VA was not detected after 1 day of incubation. Bead cultures produced more VA than control cultures for the 2, 3, 4, 5, and 6 d incubation periods analyzed. These results indicate that addition of glass beads to culture media increases VA production. The levels determined by ELISA were comparable (based on VA concentration/ml culture medium) to levels determined by fluorodensitometry when analyzing large scale production of VA with shaking incubation (Figure 8).

Figure 19. Time course of pH changes in ATCC 36537 cultures. Flasks (50 ml) containing 10 ml culture media (see Methods) and five 6 mm (diameter) glass beads (BEADS) were inoculated with 10^6 conidia, plugged with foam, covered with foil and incubated in the dark at 28 C with continuous shaking. Control cultures were prepared in a similar manner without the addition of glass beads. Culture filtrates were used for determination of pH as described in Methods.

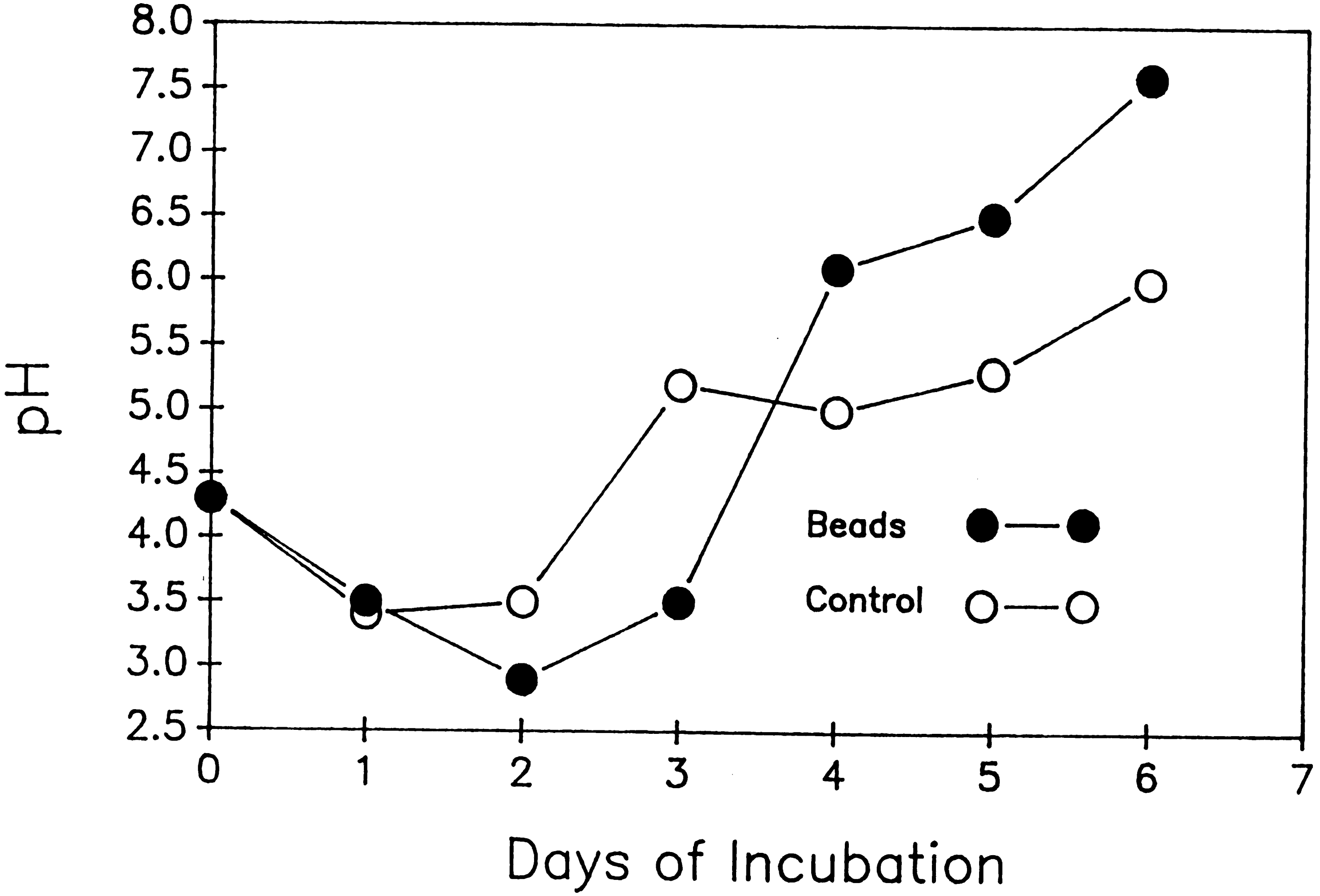

pH
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
0
1
2
3
4
5
6
7
Days of Incubation
Beads ●—●
Control ○—○

Figure 20. Time course of mycelial dry weight accumulation by ATCC 36537 cultures. Flasks (50 ml) containing 10 ml culture media (see Methods) and five 6 mm (diameter) glass beads (BEADS) were inoculated with 10^6 conidia, plugged with foam, covered with foil and incubated in the dark at 28 C with continuous shaking. Control cultures were prepared in a similar manner without the addition of glass beads. Weights were determined by drying extracted mycelia at 50 C overnight.

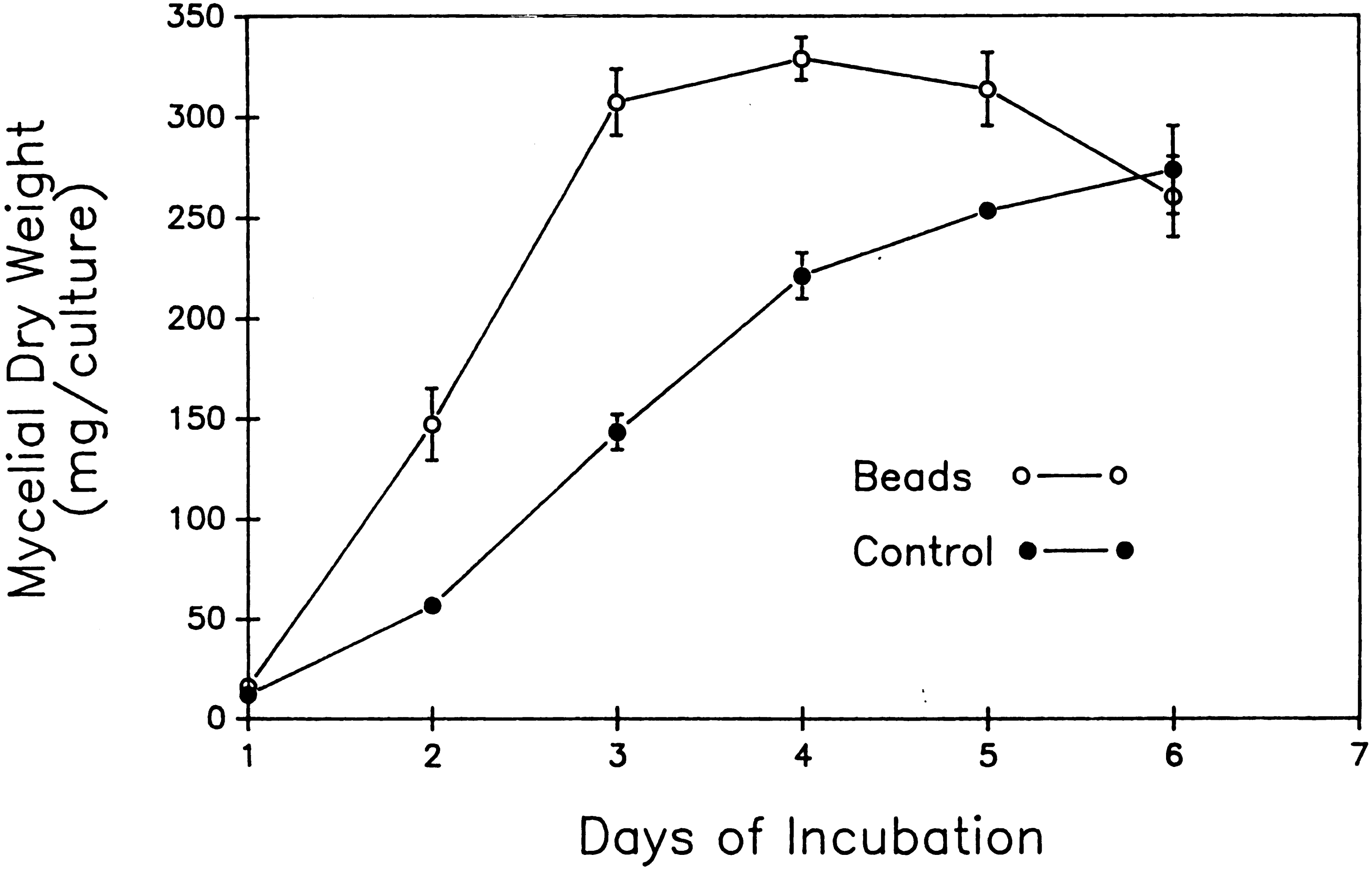
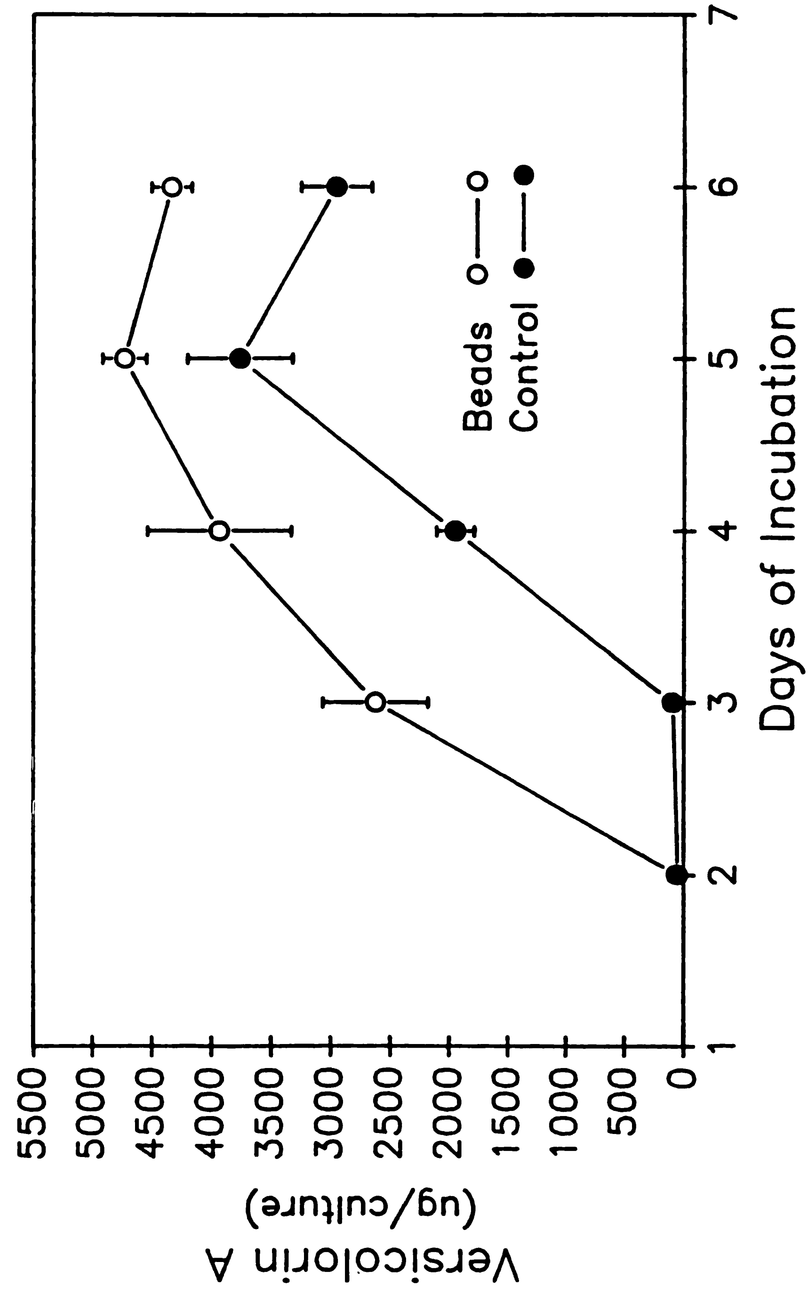

Mycelial Dry Weight
(mg/culture)
350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
Days of Incubation
Beads ○——○
Control ●——●

Figure 21. Time course of VA accumulation by ATCC 36537 cultures. VA content was determined by direct competitive ELISA as described in Methods. Flasks (50 ml) containing 10 ml culture media (see Methods) and five 6 mm (diameter) glass beads (BEADS) were inoculated with 10^6 conidia, plugged with foam, covered with foil and incubated in the dark at 28 C with continuous shaking. Control cultures were prepared in a similar manner without the addition of glass beads.


Versicolorin A
(ug/culture)
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
1
2
3
4
5
6
7
Days of Incubation
Beads ○—○
Control ●—●

CONCLUSIONS

The methods developed in the present study for the purification of VA by semi-preparative HPLC can provide quantities of crystalline VA needed for antibody production and subsequent immunoassays. Hemiacetal derivatization and conjugation by reductive alkylation produced antibody with specificity for the anthraquinone portion of the molecule. Therefore, the antibody produced in the present study could be used for detection of all aflatoxin precursors in the pathway up to VA. These compounds all contain the anthraquinone moiety and all showed some cross-reactivity with the antibody.

The antibody was applied to direct competitive ELISA for detection and quantitation of VA production. These assays have many advantages over the existing methods.

There are many potential applications for the VA antibody developed in this study. The antibody can be used in a complementary project currently under investigation in our laboratory that focuses on the isolation of genes responsible for aflatoxin production. Emphasis of this project is on the conversion of versicolorin A to sterigmatocystin. ELISA using the VA antibody would allow for detection and quantitation of VA by transformed *A. parasiticus* cultures. The antibody could also be used as a method to purify VA from mycelial extracts through the development of an affinity column. Several ATCC 36537 morphological variants have been isolated in our laboratory. ELISA utilizing the VA antibody could be used to investigate VA production by these cultures.

This is the first report of antibody produced against VA. Application of VA antibody to ELISA provides an effective tool for further investigation of aflatoxin biosynthesis.

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PART II

PREPARATION OF AN IMMUNOGEN FOR ERGOSTEROL

ABSTRACT

PREPARATION OF AN IMMUNOGEN FOR ERGOSTEROL

By

Gwen Natalie Reynolds

An immunogen was prepared for the primary fungal metabolite, ergosterol, to produce specific antibody for measuring fungal contamination in food products. Ergosterol (ERG) was converted to a hemisuccinate derivative and conjugated to ovalbumin (OA), thyroglobulin (TG), and keyhole limpet hemocyanin (KLH) using a N-hydroxysuccinimide ester method. Twenty-four BALB/c female mice were immunized with a combination of intraperitoneal and subcutaneous injections of ERG-TG or ERG-KLH conjugates. Two booster injections were administered.

Competitive indirect enzyme-linked immunosorbent assay (ELISA) was used to analyze antisera. Although titers of 3200 were detectable by ELISA, antibody was not specific for ERG since free hapten did not inhibit binding of antisera to the solid phase. Antibody produced against ERG-KLH immunogen did show specificity for ERG-OA solid phase when compared to OA only.

INTRODUCTION

Rationale

Molds are widely distributed throughout the environment and commonly isolated from foods. They can grow over a large range of pH, water activity, and temperature and are the primary type of spoilage in high acid fruits and vegetables. Detectable mold in processed food products is an indicator of poor sanitary practices and/or unacceptable raw ingredients. Current methods to detect mold lack the speed, simplicity, and sensitivity desirable for this important quality control indicator in the food industry.

Ergosterol is the principal sterol of most fungi and is either absent or detectable at minute levels in higher plants (Ness, 1977). High performance liquid chromatographic analysis of ergosterol has been utilized as an indicator of fungal contamination in grains (Seitz, 1977). Immunoassays are preferable to many available methods due to high specificity and sensitivity, ease of use, speed, and adaptability to automation. ELISAs for the fungal metabolites, mycotoxins, have been reported in the literature. Mycotoxins, like ergosterol, are small molecular weight fungal metabolites and they require special procedures to render them immunogenic. It is believed that the same methodology used for mycotoxins can be applied to ergosterol to develop an ELISA for ergosterol to measure fungal contamination in food products.

Methods for Detection of Molds in Foods

Detection and quantitation of mold contamination is essential to the food industry. Due to inherent presence of mold in production of agricultural commodities, the Food and Drug Administration has established Defect Action Levels for regulation of food quality under the adulteration clause, Section 402(a)4, of the Federal Food, Drug and Cosmetic Act. The Howard mold count (Howard, 1911), has been used since 1916 by the food industry and regulating agencies for detection of mold in foods. The procedure was originally developed to provide quality control for tomato products. It involves microscopic examination of a representative food sample portion which is placed in the specialized Howard cell. Mold filaments are counted and the proportion of positive fields compared to observed fields are noted (AOAC, 1984). This method has been criticized because it (1) requires special training of the microscopist for identification of mold fragments, (2) is subjective and often imprecise, (3) lacks sensitivity, and (4) is time consuming. Results may be affected by degree of communition during processing (Kallas, 1981), sample size, and level of training and eye strain of the microscopist (American Can Company, 1971). Insect contamination could also give rise to inaccurate results since chitin comprises a major component of the exoskeleton (Jarvis, 1977).

Several methods have been suggested as options to the Howard mold count. Detection of chitin, a major constituent of fungal cell walls, has been the primary focus in development of an alternative method for quantitation of mold contamination. Acid, alkaline, or enzymatic hydrolysis of chitin releases glucosamine which can be measured colorimetrically. Applications of fungal chitin colorimetric methods have been reported for agricultural commodities such as corn and soybeans (Donald and Mirocha, 1977), barley (Whipps et al., 1980), oats (Zak, 1976), and onions (Becker and Gerdemann, 1977).

Ride and Drysdale (1972) developed a method which used alkaline deacetylation of chitin to chitosan, followed by deamination of glucosamine residues to an aldehyde (2,5 - anhydromannose) which can be measured colorimetrically. Jarvis (1977) reported high positive correlation with the Howard mold count when using this procedure for analysis of non-homogenized tomato juices and purees. Bishop et al. (1982) used alkaline hydrolysis for estimation of fungal contamination in tomato products. Cousin et al. (1984) reported results from a study to determine if alkaline hydrolysis of tomato and fruit products followed by a colorimetric assay of glucosamine was suitable for detecting mold in processed foods. Using minor modifications of procedures described by Jarvis (1977) and Ride and Drysdale (1972), tomato and fruit purees and nectars were spiked with several common spoilage molds and then analyzed for glucosamine. Cousin et al. (1984) reported the assay to be dependent upon many variables such as product, quantity of mycelium, hydrolysis time, potassium hydroxide concentration, age and species of mold. Mold concentration was linearly correlated to glucosamine, but not to Howard mold count, when processed foods inoculated with molds were analyzed. The method also has the disadvantage of being time consuming and not applicable for quality control purposes.

Lin and Cousin (1985) developed a high performance liquid chromatographic method based upon detection of glucosamine for analysis of degree of mold contamination in processed fruit and vegetable products. To increase sensitivity of the assay for analysis in the picomole range, glucosamine was derivatized by *o*-phthalaldehyde. A linear relationship was observed between added mold concentration and glucosamine, however different species of mold produced different amounts of glucosamine.

Notermans et al. (1985, 1986), Lin et al. (1986), and Lin and Cousin (1987) have developed ELISAs for detection of mold in food. Antigens used by Notermans et al. consisted of water soluble fractions of Penicillium verrucosum var. cyclopium, Mucor racemosus and Fusarium oxysporum.

Immunoglobulin produced against *P. verrucosum* var. *cyclopium* antigen was reported to be specific for both *Penicillium* and *Aspergillus* species, however other species tested (*Mucor* and *Fusarium*) were not detectable. Due to the specificity of these antibodies, this assay would not be suitable for quantitation of all molds. Notermans et al. (1986) suggested that this immunoassay could be used for screening samples to be tested for mycotoxins since antigen used for antibody production was present in all food samples containing aflatoxin B_1. Lin et al. (1986) produced *Alternaria alternata*, *Geotrichum candidum*, and *Rhizopus stolonifer* antigens from lyophilized boiled molds. Antisera produced was relatively species specific with cross-reactivity less than 10%. Positive relationships were reported between ELISA readings and amount of mold added to tomato puree. When antibodies were used to detect these same species in processed fruit and vegetable products, bread, and cottage cheese (Lin and Cousin, 1987), positive correlation with ELISA readings and added molds were again reported. The ELISA method also gave results that correlated with those of the Howard mold count. While some cross-reactivity was seen with other species tested, the assay again was relatively specific and not suitable for quantitation of total mold content.

An alternative approach to quantitation of mold contamination in agricultural commodities is based upon ergosterol content. Ergosterol has long been recognized as the principal sterol in fungi and an important component of cell membranes. It was first isolated from ergot (*Claviceps purpurea*) by Tanret in 1908 and has since been found in a variety of fungi (Bills and Honeywell, 1928). Ergosterol is either absent or detectable at minute levels in higher plants (Ness, 1977).

Ergosterol determination as an indicator of fungal invasion in grain was described by Seitz et al. (1977). High pressure liquid chromatography (HPLC) using methylene chloride:isopropanol (99:1, v/v) as the mobile phase and u-Porasil as the stationary phase was used to measure ergosterol. Ergosterol levels in sorghum, wheat, and corn correlated with degree of fungal invasion when compared to results from whole-seed plating controls. The assay detected both viable and nonviable fungal biomass. This same solvent system, using a u-Bondapak C_{18} column (reverse phase), was applied to growth of *Alternaria* and *Aspergillus* species on milled rice (Seitz et al., 1979) and was found to be more sensitive, rapid, and convenient when compared to a chitin assay. Major problems with this methodology include extensive clean-up and extraction steps which were necessary prior to analyzing ergosterol by HPLC.

Research Objectives

The intent of this research was to produce antibodies with high sensitivity and specificity for the primary fungal metabolite, ergosterol. The experimental approach consisted of three steps: preparation of immunogens, mouse immunization, and application of competitive indirect ELISA.

Successful completion of these objectives was aimed towards production of a specific monoclonal antibody to ergosterol. This antibody would be used in development of a simple, rapid ELISA for ergosterol to assess utilization of the assay (1) for detection of mold contamination in foods, and (2) as an alternative to the Howard mold count.

An earlier attempt to produce antibody to ergosterol using bovine serum albumin as the carrier protein resulted in nonreactive antisera. Two different carrier proteins, thyroglobulin and keyhole limpet hemocyanin, were utilized for this experiment. Since previous work in our laboratory has indicated that immunization of a large number of mice will increase the chance of obtaining high affinity antibody producers that can be used for monoclonal antibody production, two dozen mice were used for this experiment.

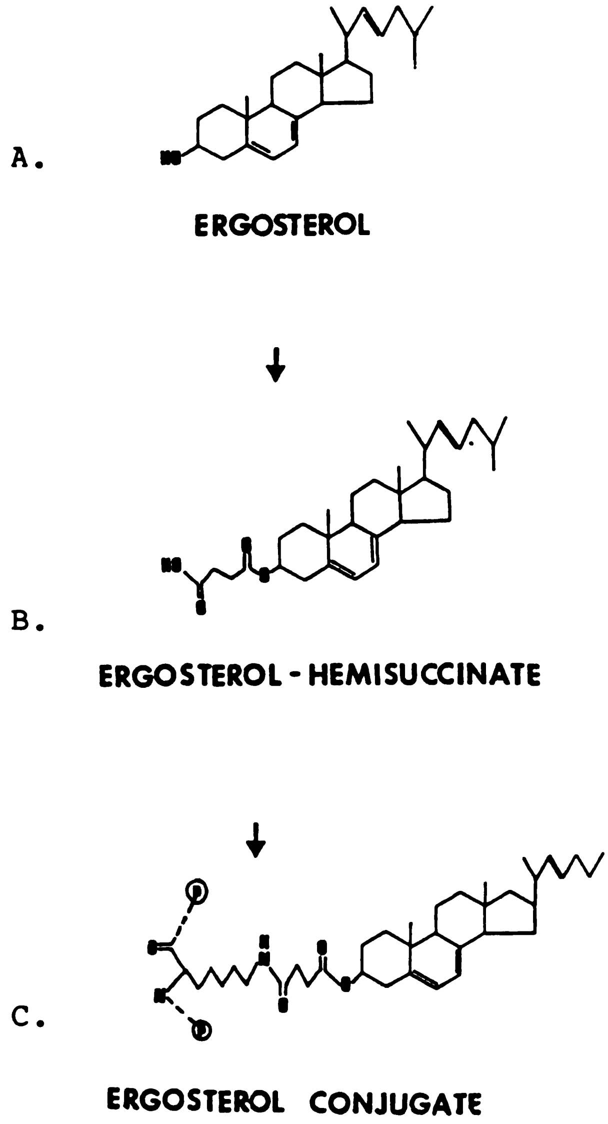
MATERIALS AND METHODS

Production of Ergosterol Immunogen

Ergosterol Derivatization

Ergosterol, with a molecular weight of 398, is too small to elicit an immune response and had to be conjugated to a larger carrier protein. Because it lacks a reactive group, ergosterol required derivatization prior to conjugation. Hemisuccinate derivatization was chosen due to presence of a hydroxyl group on the terminal portion of the ergosterol outer ring (Figure 1a). The procedure described by Wei et al. (1971) was the basis for derivatization of ergosterol to ergosterol hemisuccinate (ERG-HS) (Figure 1b). Succinic anhydride, when reacted with ergosterol, opened to form a hemisuccinate chain attached to the former hydroxyl site. Pyridine acted as an electron acceptor in the reaction. A carboxyl group on the terminus provided a reactive group for conjugation. In a typical experiment, 252 mg (2.5 mM) succinic anhydride (Sigma, St. Louis, MO) was briefly mixed until dissolved with 800 ul pyridine (Sigma) in a small reaction vessel. Ten mg (25 uM) ergosterol (Sigma) were added and the mixture was refluxed for 60 min, then allowed to cool for approximately 5 min. Pyridine was

Figure 1. Preparation of ergosterol - carrier protein conjugates. Ergosterol (a) was converted to a hemisuccinate derivative (b) and conjugated to protein (c) using a N-hydroxysuccinimide ester as described in Methods.


A.
ERGOSTEROL
B.
ERGOSTEROL - HEMISUCCINATE
C.
ERGOSTEROL CONJUGATE

removed by submerging the reaction vessel in a boiling water bath and applying a stream of nitrogen. Pyridine has a distinctive odor which aided in determining when the compound had completely evaporated.

Excess succinic anhydride was removed by partitioning the mixture in equal volumes of water and chloroform. Ergosterol hemisuccinate partitioned into the chloroform phase, succinic anhydride partitioned into the water phase. ERG-HS was dried under nitrogen and dissolved in 0.5 ml benzene:acetone (70:30, v/v).

Ergosterol Hemisuccinate Purification

ERG-HS was separated from unreacted ERG by low pressure chromatography on silica gel (Adsorbosil, 200/425 mesh, Anspec, Ann Arbor, MI). A glass chromatographic column, fitted with a safety shield, Teflon end fittings, connectors, and tubing, was packed with approximately 20 g silica gel which was slurried in solvent (benzene:acetone, 70:30, v/v) and poured into the column. A pump (Model RP-SY-1CSC; Fluid Metering Inc., Oyster Bay, NY), coupled to a pulse dampener, was used at high pressure (90 p.s.i.) to tightly pack silica gel by passing solvent for several minutes through the column. Pressure was lowered to approximately 60 p.s.i. (7 ml/min) before loading the reacted mixture. Because ERG-HS is more polar than ERG, free ERG eluted first. Fractions were spotted on silica gel TLC plates and developed in benzene:acetone (70:30). Compounds were visualized by spraying with sulfuric acid:acetic acid (50:50). Fractions containing ERG-HS (R_f=0.2) were pooled and dried under nitrogen. ERG-HS concentration was determined by absorbance in absolute ethanol at 282 nm (E = 11,500). Typical recovery of ERG-HS from the original amount of pure ERG was approximately 70%.

Conjugation to Protein

Ergosterol hemisuccinate was conjugated to protein (Figure 1c) via an activated N-hydroxysuccinimide ester (Bauminger et al., 1973). Carboxyl-containing hapten, dicyclocarbodiimide and N-hydroxysuccinimide (molar ratio 1:1:2) were reacted in dimethyl formamide (DMF). Dicyclohexylurea (DCC), a byproduct of the reaction, precipitated and was removed by centrifugation. N-hydroxysuccinimide (NHS) esters bind only to amino groups, avoiding cross linking of protein (via carboxyls) when carbodiimide alone is used. Two-fold excess of NHS insured complete reaction of DCC to avoid cross linking. In a typical experiment, 2.86 mg DCC (13.9 uM, 206 m.w.) and 3.19 mg NHS (27.8 uM, 115 m.w.) were mixed together in 100 ul DMF and stirred at room temperature for several minutes. After drying under nitrogen, 6.7 mg ERG-HS (13.9 uM, 497 m.w.) were mixed with 100 ul DMF. ERG-HS in DMF, and DCC and NHS in DMF were added together and mixed for an additional 30 min at room temperature. DCC was allowed to settle (or centrifuged briefly, if necessary). Supernatant was slowly added dropwise to each of two vials containing 2.5 mg protein dissolved in 200 ul 0.1 N sodium bicarbonate. Thryroglobulin (TG) (Sigma) and keyhole limpet hemocyanin (KLH) (Sigma) were used as the carrier proteins. Solutions were stirred for 2 hr at 4 C and then dialyzed against 50 mM sodium phosphate buffer (pH 8.0) for 24 hr. This same procedure was used for conjugation of ERG-HS to ovalbumin (Grade VII, Sigma) (OA) for later use in analyzing sera for ergosterol antibodies. Ergosterol-thryoglobulin (ERG-TG) and ergosterol-keyhole limpet hemocyanin (ERG-KLH) conjugates were adjusted with 0.9% NaCl to a concentration of 1 ug protein/ul based upon results from the Bradford protein assay (Bradford, 1976).

Immunization of Mice

Twenty-four BALB/c female mice (Charles River Laboratories), 8 - 10 weeks of age, were divided into groups of six. Individual mice within each cage were identified by clipping a digit on the posterior left foot. For initial injection, intraperitoneal injections with one of the following protein conjugates were given to all six mice within a cage: 100 ug ERG-TG, 250 ug ERG-TG, 100 ug ERG-KLH, or 250 ug ERG-KLH. All conjugates were mixed 1:1 (v/v) with Freund's complete adjuvant (Difco, Detroit, MI).

Booster injections were made three weeks later. Half of the mice were given intraperitoneal injections (100 ug or

250 ug ERG-TG or ERG-KLH) of conjugate mixed with Freund's incomplete adjuvant (Difco). The other half were given subcutaneous injections (250 ug or 500 ug ERG-TG or ERG-KLH) of conjugate mixed with 0.9% saline. A second booster injection was administered to all mice after three additional weeks. Each mouse received an identical injection as previously described for the first booster with exception that all conjugates were mixed with 0.9% saline for both subcutaneous and intraperitoneal injections.

Mice were bled via the retrobulbar plexus approximately 10 days following the third injection. Blood was incubated at 4 C overnight to allow clotting and then centrifuged to facilitate collection of serum. Sera was transferred to microcentrifuge vials and stored at -20 C.

Competitive Indirect ELISA

Sera was analyzed by competitive indirect ELISA. Briefly, 100 ul of 10 ug/ml ERG-OA dissolved in 0.05 M carbonate buffer (pH 9.6) was added to half of the wells in a 96-well microtiter plate (Removawell Microtiter Strips, Dynatech Laboratories, Alexandria, VA) and incubated at 4 C overnight. One hundred ul (10 ug/ml) OA, dissolved in carbonate buffer, were added to remaining wells. Unbound conjugate or protein was removed from plates with two 300 ul washes of 0.1 M phosphate-buffered saline (PBS, pH 7.4) previously mixed with 0.20% (v/v) Tween 20 (PBS-Tween).

Unbound well sites were blocked by incubation with 300 ul of 1% OA in PBS to each well for 60 min at 37 C. The plate was washed three more times with PBS-Tween. In duplicate, 50 ul 20% methanol in PBS were added to half of the wells originally coated with OA only and half coated with ERG-OA. Fifty microliters of 10 ug ERG/ml in 20% methanol in PBS were added to each of the remaining OA coated and ERG-OA coated wells. Sera were diluted 1:50 in PBS and added to each well. Plates were incubated for 60 min at 37 C. Unbound antibody was removed by washing 3 times with PBS-Tween, then 100 ul goat anti-mouse peroxidase conjugate (1:500 in 1% OA-PBS) was added to each well. Plates were incubated for 30 min at 37 C and then washed 8 times with PBS-Tween. Bound peroxidase activity was determined using 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as described by Pestka et al. (1980): 100 ul substrate solution (0.4 mM ABTS, 1.2 mM hydrogen peroxide in 50 mM citrate buffer, pH 4.0) were added to each well. The reaction was stopped by adding 100 ul stopping solution (300 mM citric acid containing 15 mM sodium azide) to each well. Absorbance was read at 405 nm on an ELISA plate reader (Bio-Tek EL-308, Burlington, VT).

RESULTS AND DISCUSSION

Purity of ergosterol was a concern throughout the experiment. The first compound used showed two additional spots when viewed under long wave ultraviolet light; one fluoresced blue, and the other spot fluoresced yellow. Both spots had a lower R_f (0.62 and 0.56 for the blue and yellow spots, respectively) than ergosterol (R_f=0.73) when developed in benzene:acetone (70:30). From personal communication with Dr. Gary Mills (Michigan State University), it was suggested that the blue fluorescent compound may have been ergosterane, an ergosterol derivative lacking the terminal hydroxyl group. It was later discovered that the compound had been improperly stored, without desiccation, and that breakdown products could have been formed.

An unopened bottle of ergosterol was then observed and found to have a deep yellow color and needle-like consistency. Upon personal communication with technical personnel from the supplier (Sigma), it was determined that the ergosterol in question had been prepared 5 years ago; and although the bottle had remained unopened and properly stored, the compound had probably oxidized. Recommended proper storage is at 0 C in a desiccator. The compound should be flushed with nitrogen, wrapped in parafilm and overwrapped with foil. A new 5 g bottle of ergosterol (Sigma) was used for preparation of ergosterol hemisuccinate and all ergosterol protein conjugates. However, personal communication with a technical representative from Sigma indicated that the ergosterol was only 94% pure by gas chromatographic analysis and that two or three spots were visible by thin layer chromatography. Undesirable reactions may have occurred with these impurities. It may have been advisable to further purify the ergosterol before preceding with derivatization.

The basic scheme for ELISA screening of serum was designed to determine specificity. Two different coating solutions were used: ovalbumin alone and ergosterol conjugated to ovalbumin. Binding of antisera produced against ERG should be greater in ERG-OA coated wells than in OA coated wells. Higher or equal binding in OA coated wells would indicate nonspecific antibody production. Addition of ergosterol along with antisera was also used to help determine specificity. Free ergosterol would inhibit binding of specific antibody detectable by decrease in absorbance compared to controls.

Results from competitive indirect ELISAs indicated that mice immunized with ERG-KLH demonstrated much higher absorbance values when ERG-OA was used as the solid phase compared to the OA solid phase. Typical ELISA results from ERG-KLH immunized mice are shown in Table 1. Mice immunized

Table 1. Absorbance values for ERG-KLH antisera as determined by indirect competitive ELISA[a]

	Solid Phase	
Sample No.	OA	ERG-OA
10	.39	1.26
11	.54	1.20
12	.16	.99
13	.33	1.13
14	.41	1.10
15	.83	.99
20	.92	.84
21	.07	.31
22	.45	.72
23	.10	.38
24	.69	.90

[a] Average of 8 wells, antisera were diluted 1:50 in PBS

with ERG-TG demonstrated absorbance values that differed very little depending on the solid phase (Table 2). Inhibition of binding by free ergosterol (10 ug/ml) was not evident for any of the antisera. The highest titers detected by ELISA were 3200. Interwell variability among duplicate samples analyzed by ELISA tended to be rather high, ranging from 8 - 25%.

Even though inhibition of binding was not detectable by free ERG at 10 ug/ml, the enhanced binding to ERG-OA indicated that ERG-KLH immunogen produced antibodies with some degree of specificity for ergosterol. Polyclonal antibodies are derived from various lymphocyte clones in vivo, therefore, an immunogen may produce antibodies with various specificities (Pestka, 1988). Immunogens prepared with ERG may produce antibodies with specificity for moieties such as ERG, ERG-HS, ERG-KLH or ERG-TG, the bridge between ERG-HS and the carrier protein backbone, ERG-HS plus the bridge, or for KLH or TG only. ERG-HS was conjugated to carrier protein via free lysine groups. Antibodies produced against ERG-KLH immunogen may have been specific for ergosterol bound to lysine (ERG-HS plus the bridge between this compound and the carrier protein backbone). This moiety would be common to both ERG-KLH and ERG-OA but not OA alone. This would explain the increased binding to the ERG-OA solid phase and the inability of free ERG to inhibit binding. It is also possible that concentrations higher than 10 ug/ml free ergosterol may have caused inhibition.

Table 2. Absorbance values for ERG-TG antisera as determined by indirect competitive ELISA[a]

	Solid Phase	
Sample No.	OA	ERG-OA
30	.84	.70
31	.75	.66
32	.18	.16
33	1.42	1.41
34	1.58	1.49
35	1.48	1.46
40	.59	.53
41	.17	.15
42	.42	.57
43	1.56	1.43
44	1.16	1.11
45	.77	.82

[a] Average of 8 wells, antisera were diluted 1:50 in PBS

To determine if antisera were specific for ERG-HS, 10 ug/ml free ERG-HS were used in competitive indirect ELISA in place of ERG. Again, no inhibition of binding was evident when comparing addition of free ERG-HS with control wells.

Nonspecificity of antisera produced against ergosterol may have been the result of alteration of compounds during derivatization and/or conjugation. Ergosterol is a compound very susceptible to decomposition. It is affected by air and light. Exposure to oxygen leads to formation of peroxides. When irradiated with ultraviolet light, ERG develops powerful vitamin D_2 activity. Bills and Honeywell (1928) reported that boiling ERG in such a low boiling solvent as acetone results in some decomposition. It has been reported that ergosterol peroxide, a compound undetectable on TLC plates under visible or UV light, may form from ERG spontaneously unless low level illumination is used when working with the compound (Davis et al., 1986). It is possible that the ERG decomposed during one or more of the numerous steps during derivatization and conjugation. Undesirable reactions may have occurred with breakdown products, for example, cross linking to the carrier protein. Antisera produced may have been more specific for these compounds than for ERG. ELISAs utilizing common derivatives of ERG for competition of binding may have helped define antisera specificity.

CONCLUSION

Production of antisera without specificity for ergosterol resulted in termination of this project. Procedures used in this research were those commonly employed for mycotoxins which are generally very stable compounds. The instability of ergosterol may require specially designed derivatization and conjugation procedures.

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