12-1982

Effects of antimicrobial food additives on growth and ochratoxin A production by Aspergillus sulphureus and Pencillium viridicatum

Chao-Hsiang Tong

To the Graduate Council:

I am submitting herewith a thesis written by Chao-Hsiang Tong entitled "Effects of antimicrobial food additives on growth and ochratoxin A production by Aspergillus sulphureus and Pencillium viridicatum." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

Frances Ann Draughon, Major Professor

We have read this thesis and recommend its acceptance:

P. M. Davidson, J. R. Mount

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a thesis written by Chao-Hsiang Tong entitled "Effects of Antimicrobial Food Additives on Growth and Ochratoxin A Production by Aspergillus sulphureus and Penicillium viridicatum." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.

Frances Ann Draughon
Frances Ann Draughon, Major Professor

We have read this thesis
and recommend its acceptance:

John R Mount

P Michael Davidson

Accepted for the Council:

L Evans Roth
Vice Chancellor
Graduate Studies and Research

4

EFFECTS OF ANTIMICROBIAL FOOD ADDITIVES ON GROWTH AND OCHRATOXIN A PRODUCTION BY *ASPERGILLUS SULPHUREUS* AND *PENICILLIUM VIRIDICATUM*

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Chao-Hsiang Tong

December 1982

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation and gratitude to Dr. Frances Ann Draughon for her advise, guidance, patience, understanding, friendship, and reviewing this thesis. Appreciation and gratitude are also extended to Dr. P. M. Davison and Dr. J. R. Mount for their assistance and reviewing this thesis as committee members.

Thanks are extended to the Department of Food Technology and Science staff for assistance and knowledge provided during the course of his graduate studies.

Finally, the author is very grateful to his parents for their love, encourgement, and financial support.

ABSTRACT

The effects of antimicrobial food additives on growth and ochratoxin A production by strains of *Aspergillus sulphureus* NRRL 4077 and *Penicillium viridicatum* NRRL 3711 were investigated. At pH 4.5, in order to obtain 100% inhibition of both mycelium and toxin production, 0.02, 0.067, 0.0667, and 0.2% of potassium sorbate, methyl paraben, sodium bisulfite, and sodium propionate were required, respectively. At pH 5.5, 0.134% of potassium sorbate and 0.067% of methyl paraben completely inhibited the growth and ochratoxin A production by both fungi. Sodium bisulfite at 0.1% and sodium propionate at 0.64% were found to inhibit fungal growth by the following percentages, respectively: *A. sulphureus* NRRL 4077, 45.45%, *P. viridicatum* NRRL 3711, 89.38%; *A. sulphureus* NRRL 4077, 79.91%, *P. viridicatum* NRRL 3711, 88.45%. For toxin inhibition, the following percentages were achieved according to their previous sequence: 97.17, 99.89, 99.40, and 100%.

TABLE OF CONTENTS

CHAPTER PAGE

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

CHAPTER I

INTRODUCTION

The ochratoxins comprise a group of chemically related secondary metabolites isolated from culture extracts of seven species of *Aspergillus* and seven species of *Penicillium*. Ochratoxin A, 7-carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3-R-methyl isocoumarin linked to L-phenylalanine, is the most important metabolite both in respect to occurrence and toxicity. Toxin formation is influenced by pH, temperature, incubation time, and water activity of the substrates.

The first detection of ochratoxin A as a natural contaminant was in a USDA-ARS survey of corn for aflatoxins, ochratoxins, and zearalenone. Since that time the mycotoxin has been found in wheat, barley, green coffee beans, peanuts, dried white beans, oats, grain sorghum, rye, rice, pecans, and mixed feed. The toxin is retained as a residue in the tissue of animals when contaminated feed is ingested. Therefore, transfer of ochratoxin A from animal feed to human food is possible.

Ochratoxin A has been found toxic to rats, chickens, swine, hens, turkeys, Japanese quails, ducklings, mice, Guinea pigs, dogs, cattle, goats, and fish. The toxicologic responses to ochratoxin A vary with the dose, duration of exposure, animal species, and age. Most alternations occur

in the liver, gut, lymphoid tissues, and especially in the renal tubules. Ochratoxin A is teratogenic in the mouse and chicken embryo, and is carcinogenic to trout when fed in conjunction with the co-carcinogen, sterculic acid.

Using chemicals to preserve foods is not new. Several food additives termed as GRAS (generally recognized as safe) have been shown to prevent mold grow at certain concentrations and pH values. However, specific studies of the chemical inhibition of ochratoxin A are not available. This experiment was undertaken to determine the effects of four antimicrobial food additives on ochratoxin A production and mycelial formation by A. sulphureus NRRL 4077 and P. viridicatum NRRL 3711.

CHAPTER II

REVIEW OF THE LITERATURE

I. CHEMISTRY OF OCHRATOXINS

Chemical Structures and Properties

The ochratoxins constitute a group of structurally related, secondary metabolites produced by several species of Aspergillus and Penicillium. The basic structure of these toxins is shown in Figure 1. Ochratoxin A, 7-carboxyl-5-chloro-8-hydroxyl-3,4-dihydro-3-R-methyl isocoumarin linked to L-β-phenylalanine, was the first toxin to be discovered in this group (van der Merwe et al., 1965). Ochratoxin B, C, D, β, and the methyl and ethyl esters of ochratoxin A and ochratoxin B have all been isolated from fungal cultures under laboratory conditions (Steyn, 1977).

Ochratoxin α is a mammalian metabolic product of ochratoxin A that can be found in the intestine, liver, urine, and feces of rodents (Hult et al., 1976). It also can be produced by acid hydrolysis of enzymatic reaction of ochratoxin A. By means of thin layer chromatography on silica gel and spectrophotometric methods, Pitout (1969) reported that ochratoxin A is hydrolyzed by carboxypeptidase and chymotrypsin to form ochratoxin α. However, under natural, non-laboratory conditions, only ochratoxin A and very rarely, ochratoxin B have been encountered. Therefore,

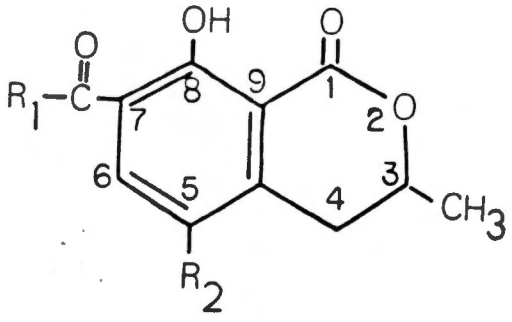


Figure 1. The chemical structure of the ochratoxins.

	R_1	R_2
Ochratoxin A:	$C_6H_5CH_2CH(COOH)NH$-	-Cl
Ochratoxin B:	$C_6H_5CH_2CH(COOH)NH$-	-H
Ochratoxin C:	$C_6H_5CH_2CH(COOC_2H_5)NH$-	-Cl
Ochratoxin D:	4-hydroxyochratoxin A	
Ochratoxin α:	-OH	-Cl
Ochratoxin β:	-OH	-H
Methyl ester of OA:	$C_6H_5CH_2CH(COOCH_3)NH$-	-Cl
Methyl ester of OB:	$C_6H_5CH_2CH(COOCH_3)NH$-	-H
Ethyl ester of OB:	$C_6H_5CH_2CH(COOC_2H_5)NH$-	-H

for all pratical purposes, only ochratoxin A is a natural contaminant of foods and feedstuffs (Shimoda, 1979). Some of the most important properties of ochratoxins are summarized in Table 1.

Stability of Ochratoxin A

Ochratoxin A is a very stable compound. It does not decrease substantially in ethanol even after one year of storage in the refrigerator (Chu and Butz, 1970). In cereal products some degradation occurs with time. Thus, Trenk et al. (1971) recovered only 47-48% and 36-47% of ochratoxin A added to oatmeal and rice and stored for 12 weeks at 4°C and 28°C, respectively. When cereals containing ochratoxin A were autoclaved for up to three hours, 12.5-31.5% still survived depending upon the amount of water in the cereals (Trenk et al., 1971). In the processing of beans by canning, 11% of ochratoxin A was destroyed after one hour of heating at 121°C compared to 34% reduction in ochratoxin A content when canned moldy beans were heated for four hours (Harwig et al., 1974).

The stability of ochratoxin A in brewing of beer has also been investigated. Krogh et al. (1974a) observed that during malting and brewing, ochratoxin A was completely degraded from moderately contaminated barley lots. The use of highly contaminated barley resulted in transmission of ochratoxin A into the beer, but only 2-7% of the initial

Table 1. Chemical Properties of Ochratoxins.

Name	Formula	Molecular Weight	Melting Point (°C)	Absorption Maxima (nm)[a] and Molar Absorptivity (ε x 10^3)
Ochratoxin A	$C_{20}H_{18}ClNO_6$	403	169(xylene)[b] or 89-95(benzene)[b]	213-216 (34.00-39.80); 332-334 (5.90-6.50); or 330-333(2.40-4.10) and 378-380 (2.70-3.30)[c]
Ochratoxin B	$C_{20}H_{19}NO_6$	369	220-221(MeOH)[b]	218(33.60-37.20); 318(6.50-6.90)[c]
Ochratoxin C	$C_{22}H_{22}ClNO_6$	431	-	213-214(30.00-32.70); 333(6.50-7.00); or 331(4.10) and 378 (2.05)
Ochratoxin D (4-hydroxy-ochratoxin A)	$C_{20}H_{18}ClNO_7$	419	216-218 (benzene)[b]	213(32.50); 314(6.40)
Ochratoxin α	$C_{11}H_9ClO_5$	256	229; 233-234; 246-249 (acetone-benzene)	212-214(28.00-32.00); 336-338 (5.40-5.60); 217(32.09);336 (6.44)
Ochratoxin β	$C_{11}H_{10}O_5$	222	223	218(32.00); 322(3.00)
Methyl ester-ochratoxin A	$C_{21}H_{20}ClNO_6$	417	-	Identical to ochratoxin C, 333(6.50)
Methyl ester-ochratoxin B	$C_{21}H_{21}NO_6$	383	134-135 (benzene)[b]	Similar to thyl ester of ochratoxin B
Ethyl ester-ochratoxin B	$C_{22}H_{23}NO_6$	397	102-103(ether)[b]	218(32.00); 318(6.70); or 318 (5.20) and 364(1.25)

[a]Determined in EtOH.

[b]Solvents used in the crystallization.

[c]Depends on pH values.

Source: Chu, 1974b

content was detected. Chu et al. (1975) found that ochratoxin A was partially lost during brewing with most of the loss occurring during the malt mash, boiled wort, and final fermentation stages. About 25% of added mycotoxin was recovered from completed beer at a dose of 10 μg/g of cereal. Nip et al. (1975) studied the fate of ochratoxin A in brewing by adding [^{3}H]ochratoxin A to the raw material at 1- and 10- μg/g levels during mashing in a conventional microbrewing process. Large portions (28-39%) of the added toxin were recovered in spent grain, with less recovery in the yeast (8-20%) and beer (14-18%).

II. NATURAL OCCURRENCE

Ochratoxin-producing Fungi

Aspergillus ochraceus Wilhelm, a species of the A. ochraceus group, was first found to be an ochratoxin A producer. According to Raper and Fennell (1965), this group includes the species A. sulphureus (Fres.) Thom & Church, A. sclerotiorum Huber, A. alliaceus Thom & Church, A. auricomus (Gueguen) Saito, A. melleus Yukawa, A. ochraceus Wilhelm, A. ostianus Wehmer, A. elegans Gasperini, and A. petrakii Voros. Lai et al. (1968, 1970) A. melleus Yukama and A. sulphureus (Fres.) Thom & Church to be ochratoxin A producers. Ciegler (1972) found that A. ochraceus Wilhelm, A. sclerotiorum Huber, A. alliaceus Thom & Church, A. ostianus Wehmer, A. melleus

Yukawa, and A. sulphureus (Fres.) Thom & Church produced not only ochratoxin A, but also penicillic acid on liquid media and in cereal grains. Extensive studies by Hesseltine et al. (1972) indicated that ochratoxin A and ochratoxin B production is common to seven species in the A. ochraceus group.

In addition to the fungi in the A. ochraceus family, Penicillium viridicatum Westling was discovered to be a source of ochratoxin A (van Walbeek et al., 1969). Scott et al. (1972) isolated both ochratoxin A and citrinin from grains with P. viridicatum Westling or P. palitans Westling.

To date, seven species of Aspergillus and seven species of Penicillium have been reported as ochratoxins producers (Table 2). However, not all strains of the species mentioned are capable of ochratoxin A production.

Natural Occurrence of Ochratoxin-producing Fungi

Most of the ochratoxin-producing fungi are widespread in nature and have been characterized as the storage fungi. A. sclerotiorum was isolated by Huber (1933) from surface washing of normal apples. A. alliaceus was originally discovered as a wound parasite of onions (El-Helaly et al., 1962). A. alliaceus and A. petrakii are known to have been isolated from dead insects (Raper and Fennell, 1965). The ability of members of the A. ochraceus group to withstand high osmotic pressure is reflected by their frequent

Table 2. Producers of Ochratoxins.

Penicillium Link	
Monoverticillata:	
P. frequentans series:	_P. purpurrescens_ Sopp
Asymmetrica-Lanata:	
P. commune series:	_P. commune_ Thom
Asymmetrica-Fasciculate:	
P. viridicatum series:	_P. viridicatum_ Westling _P. palitans_ Westling
P. cyclopium series	_P. cyclopium_ Westling
Biverticillata-Symmetrica:	
P. purpurogenum series:	_P. variabile_ Sopp
P. funiculosum series:	_P. verruculosum_ Peyronel
Aspergillus Micheli	
Aspergillus ochraceus group:	_A. sulphureus_ (Fres.) Thom & Church _A. sclerotiorum_ Huber _A. alliaceus_ Thom & Church _A. melleus_ Yukawa _A. ochraceus_ Wilhelm _A. ostianus_ Wehmer _A. petrakii_ Voros

Source: Bullerman, 1979; Hesseltine et al., 1972; Krogh, 1978; Shimoda, 1979.

occurrence in the Orient on dried and salted fermented fish products. A. ochraceus, A. melleus, A. ostianus, and A. sulphureus have been isolated from such foods (Hesseltine et al., 1972). A. sulphureus and A. melleus also have been found in Brazil nuts (van Walbeek et al., 1969) and soybeans (Mislivec and Bruce, 1977).

A. ochraceus is the most commonly occurring fungus in the A. ochraceus group. It has been frequently isolated from stored grain (Wallace and Sinha, 1962), peanuts (Doupnik and Peckham, 1971), soil, insects, decayed vegetables (Raper and Fennell, 1965), damaged tobacco (Welty and Lucas, 1968), sorghum (Christensen et al., 1968), brewery hops (van Walbeek et al., 1969), black and red peppers (Christensen et al., 1967), rice (Yamazaki et al., 1970), corn (Chu, 1974b), pecans (Doupnik and Bell, 1971), country cured ham (Escher et al., 1973), soybeans (Mislivec and Bruce, 1977), dried beans (Mislivec et al., 1975), flour and bread (Bullerman and Hartung, 1973), wheat, rye (van der Merwe et al., 1965), and poultry feed (Bacon et al., 1973).

P. viridicatum and P. cyclopium are the two most common species in the genus of Penicillium which can produce ochratoxin A. Both of them have been isolated from beans (Mislivec et al., 1975), wheat grain, oats, rye, barley, peanuts, mixed feed (Scott et al., 1972), soil, vegetables (Raper and Thom, 1949), soybeans (Mislivec and

Bruce, 1977), fruit-filled pastries (Kuehn and Gunderson, 1962), and corn (Koehler, 1938; Davis et al., 1975). P. viridicatum was also found in country cured ham (Escher et al., 1973). It is the chief mold which develops in corn and grains stored at low temperatures (Scott et al., 1972; Semeniuk and Barre, 1943). P. cyclopium is not only widely distributed, but also commonly causes a bulb rot in liliaceous plants (Raper and Thom, 1949). P. palitan has been discovered in fruits, cheddar cheese, coconut oil (Raper and Thom, 1949), fruit-filled pastries (Kuehn and Gunderson, 1962), mixed feed (Scott et al., 1972), and inshell pecans (Schindler et al., 1974). P. commune, P. purpurrescens, P. variabile, and P. verruculosum appear to be comparatively rare in nature. They were mainly isolated from soil (Raper and Thom, 1949).

Natural Occurrence of Ochratoxin A

Ochratoxin A was first reported as a natural contaminant by Shotwell et al. (1969) in a USDA survey of corn for aflatoxins, ochratoxins, and zearalenone. Since that time the mycotoxin has been found in a variety of plant products in countries of North America and Europe (Table 3 and Table 4). Although ochratoxin A mainly occurs in cereals, it can be isolated from animal sources. When farm animals are exposed to feed containing ochratoxin A, a portion of the ingested toxin will be retained as residues in the

Table 3. Natural Occurrence of Ochratoxin A in Foods of Plant Origin.

Commodity	Country	No. of Samples Analyzed	Percentage Contaminated	Range of Ochratoxin A Level (μg/kg)	Reference
Corn	USA	164	1.3	110-150	Shotwell et al. (1969)
Corn	USA	293	1.0	83-166	Shotwell et al. (1971)
Corn	France	463	2.6	15-200	Krogh (1978)
Corn	France	461	1.3	20-200	Krogh (1978)
Corn	Yugoslavia	542	8.3	6-140	Pavlovic et al. (1979)
Wheat (hard red winter)	USA	291	1.0	5-35	Shotwell et al. (1976)
Wheat (hard red spring)	USA	286	2.8	15-115	Shotwell et al. (1976)
Wheat	Yugoslavia	130	8.5	14-135	Pavlovic et al. (1979)
Wheat bread	Yugoslavia	32	18.8	"[a]	Pavlovic et al. (1979)
Green coffee beans	USA	267	7.1	20-400	Levi, et al. (1974)
Beans	USA	58	35.0	20-1000	Wilson et al. (1976)
Barley	USA	127	14.2	10-40	Krogh (1978)
Barley	USA	180	13.0	10-37	Fischbach and Rodvicks, (1973)
Barley	Yugoslavia	64	12.5	13.5-25.6	Pavlovic et al. (1979)
Malting barley	USA	180	1.0	"	Fischbach and Rodvicks, (1973)
Malting barley	Yugoslavia	50	6.0	9-189	Krogh (1978)

[a]Not available

Table 4. Natural Occurrence of Ochratoxin A in Feeds of Plant Origin.

Commodity	Country	No. of Samples Analyzed	Percentage Contaminated	Range of Ochratoxin A Level (μg/kg)	Reference
Barley,wheat oats,rye corn	Poland	150	5.3	50-200	Krogh (1978)
Mixed feed	Poland	203	4.9	10-50	Krogh (1978)
Mixed feed	Canada	13	16.0	20-530	Scott et al. (1972)
Corn	Yugoslavia	191	25.7	45-5125	Shimoda (1978)
Barley,oats	Denmark	33	57.6	28-27500	Krogh et al. (1973)
Barley	Sweden	84	6.0	16-410	Krogh et al. (1974b)
Wheat,oats barley,rye	Canada	32	56.3	30-27000	Scott et al. (1972)
Oats	Sweden	84	2.4	29-77	Krogh et al. (1974b)
Wheat	Canada	95	6.4	30-6000	Prior (1976)
Wheat	Canada	"[a]	"	20-100	Scott et al. (1970)
Hay	Canada	95	1.0	30	Prior (1976)
Dried white beans	Canada	"	"	50-2100	Scott et al. (1972)
Peanuts	Canada	"	"	4900	Scott et al. (1972)

[a]Not available.

tissues with increasing concentrations from fat, muscle, liver to kidney (Krogh et al., 1974c; Krogh et al., 1976a; Krogh et al., 1976b; Krogh et al., 1976c). According to Krogh et al. (1976b), ochratoxin A disappears from tissues exponentially, with RL_{50} values in the range of 3-5 days. In a survey of slaughtered pigs, ochratoxin A was found in 18 out of 19 pigs delivered from a farm where ochratoxin A-contaminated barley was used, with residue levels up to 67 µg/kg (Krogh, 1978). Residues of ochratoxin A have also been observed in kidney, liver, and muscular tissue of slaughtered poultry, at concentrations between 4.3 to 50 µg/kg (Elling et al., 1975; Krogh et al., 1976a). Recently, Hult et al. (1979, 1980) determined ochratoxin A in blood of slaughtered pigs in Sweden. From blood samples of 279 randomly selected pig herds, 47 herds were found to contain more than 2 ng of ochratoxin A per milliliter of blood. Blood from 14 herds contained more than 10 ng of ochratoxin A per milliliter of blood. The highest value was 280 ng/ml. The concentration of ochratoxin A in the blood can act as an index to calculate the amount of ochratoxin A in the feed given to the pigs. Because the only observable lesion which developed in animals was kidney damage, the remaining parts of the carcasses might pass meat inspection. Therefore, transfer of ochratoxin A from animal feed to humam food is possible.

III. TOXIN FORMATION

Ochratoxin A production is determined by several factors: incubation time, pH, temperature, water activity, (Aw), substrates, and amount of aeration.

Incubation Time

The optimum time for ochratoxin A formation is in the range of 6 to 14 days with incubation temperatures of 22 to 30°C. Sansing et al. (1973) found that the peak production of ochratoxin A by A. ochraceus occurred at 25°C after 10 and 12 days incubation on a nutrient solution, but the yields were not significantly different from those obtained at 8 and 14 days. Usually higher incubation temperature requires less time for maximum toxin production. Therefore, they also found that ochratoxin A production at 30°C after 6 and 8 days incubation was in the range of peak ochratoxin A production at 25°C, but dropped off rapidly with continued incubation. Studying the conditions for production of ochratoxin A by Aspergillus species in a synthetic medium incubated at 22 to 27°C, Lai et al. (1970) found that A. sulphureus produced maximum amounts of ochratoxin A within 8 to 10 days, and the amounts were reduced only slightly by incubating cultures for as long as 20 days. Trenk et al. (1971) reported the optimum time for ochratoxin A production ranged from 7 to 14 days at 28°C in different cereal products. Lillehoj et al. (1978) examined the kinetics of

toxin accumulation in submerged culture of A. sulphureus in modified Czapek solution and found the highest ochratoxin A levels occurred on day 11 and day 12 of incubation at 28 and 22°C, respectively. However, they concluded that a marked reduction in toxin levels at both incubation temperatures occurred during the period after maximum ochratoxin A accumulation (Figure 2). A longer period of 19 to 21 days of incubation at 22°C to give a maximum yield of ochratoxin A was also recorded (Schindler and Nesheim, 1970). The decrease of ochratoxin A after maximum accumulation might due to two reasons:

1. degradation by light.
2. autolysis of cells to release some proteolytic enzymes which can break down ochratoxin A.

pH

Not much research has been done on the relationship between pH and ochratoxin A production. Lai et al. (1970) studied the effect of pH on ochratoxin A production and growth of A. sulphureus in a synthetic medium buffered with either sodium phosphate or citric acid-sodium citrate plus Na_2HPO_4 and incubated at 22 to 27°C for 8 days. They found that maximum ochratoxin A production was attained at pH 6.0 to 6.3 within the range of pH 3.0 to 7.8. The optimum pH for mycelial growth was around pH 5.0 (Figure 3). Using a nutrient solution of 4% sucrose and 2% yeast extract with

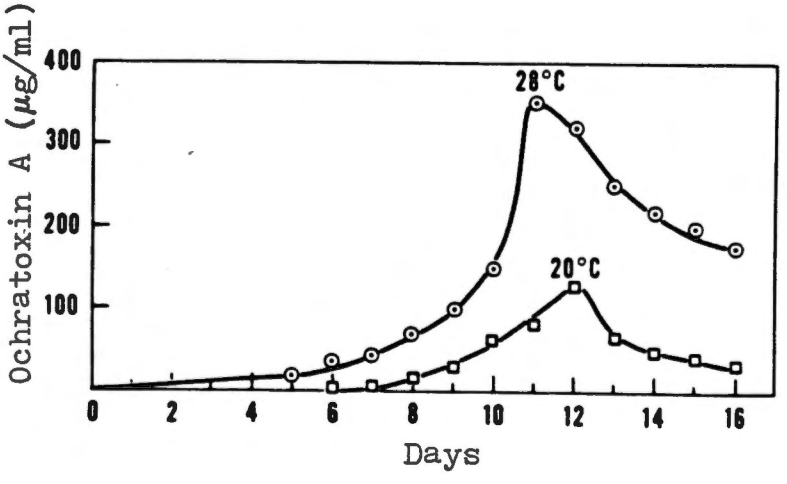


Figure 2. Production of ochratoxin A by a strain of A. ochraceus in a modified Czapek solution incubated at 20 and 28°C (Lillehoj et al. 1978).

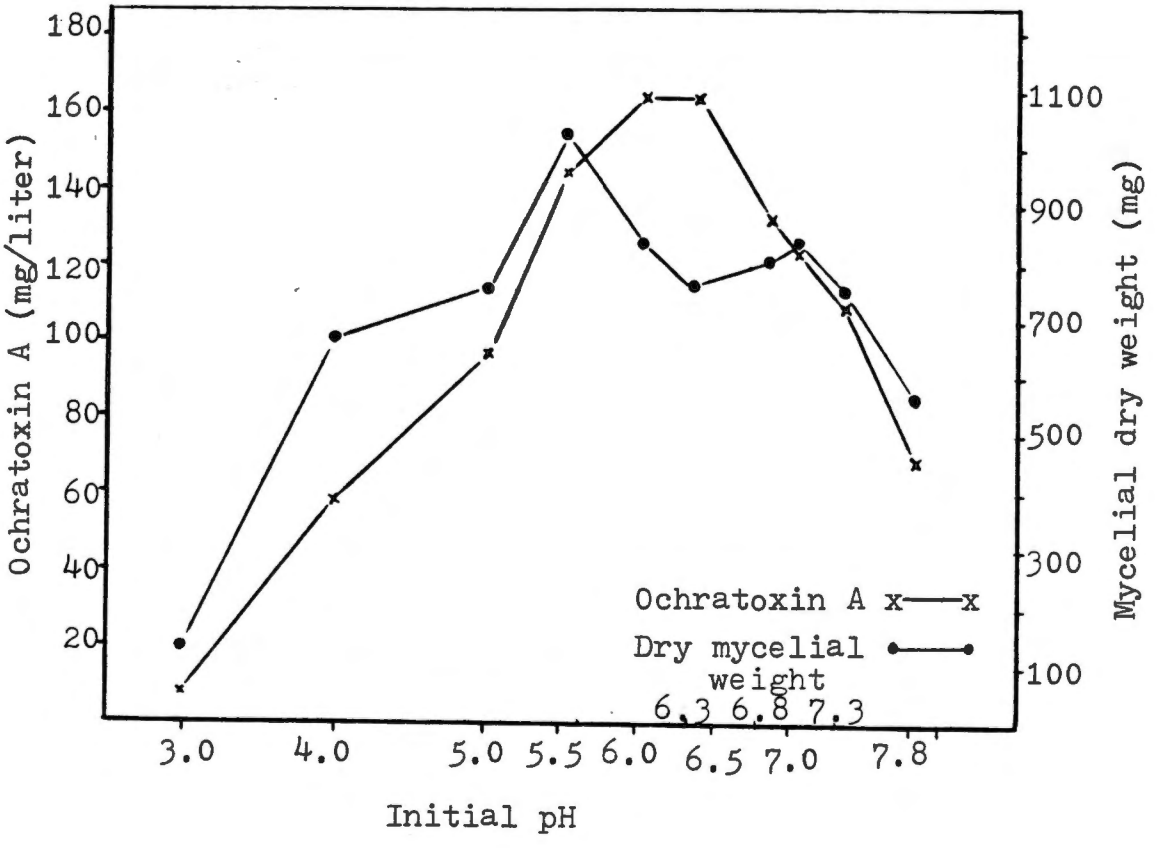


Figure 3. Effect of pH on ochratoxin A production and growth of Aspergillus sulphureus NRRL 4077 in a synthetic medium for 8 days at 22 to 27°C. Medium was buffered with 0.15 M sodium phosphate (pH 5.0 to 7.8), or with 0.1 M citric acid-sodium citrate (pH 3.0 and 4.0) plus 1.5 g of Na_2HPO_4 per liter (Lai et al., 1970).

an initial pH of 5.4 Sansing et al. (1973) reported that high pH values of 7.6 to 8.2 coincided with high ochratoxin A production at 25°C for 8 to 14 days incubation.

Temperature and Water Activity

Temperature and water activity are the two most important factors for ochratoxin A formation. Generally, they are affected by one another. At optimum Aw, the temperature range for ochratoxin A production by A. ochraceus is 12 to 37°C with the optimum temperature around 25 to 31°C (Bacon et al., 1973; Northolt et al., 1979; Sansing et al., 1973; Trenk et al., 1971), whereas that of P. cyclopium and P. viridicatum is 4 to 31°C with the optimum temperature around 24°C (Harwig and Chen, 1974; Northolt et al., 1979). However, very low levels of ochratoxin A, produced by A. ochraceus, were detected in corn, rice, and wheat bran at 4°C (Trenk et al., 1971). Studying a strain of A. ochraceus which can produce both penicillic acid and ochratoxin A on poultry feed, Bacon et al. (1973) reported that a combination of low temperature and low Aw favors the production of penicillic acid; high temperature and Aw favors the production of ochratoxin A. Ochratoxin A began to accumulate at an Aw of 0.85, whereas maximum production occurred at an Aw of 0.95 at 30°C. Harwig and Chen (1974) found that an Aw of 0.90 to 0.93 is most favorable for production of toxin by P. viridicatum

in wheat and barley at 25°C. Northolt et al. (1979) adjusted the Aw of agar media by addition of sucrose or glycerol, and determined that the minimum Aw values for ochratoxin A production by A. ochraceus, P. cyclopium, and P. viridicatum were between 0.83-0.87, 0.87-0.90, 0.83-0.86, respectively. They also found that 0.99 is the optimum Aw value for toxin formation by A. ochraceus, and that 0.95-0.99 are the best Aw values for P. viridicatum and P. cyclopium at 24°C. The conditions for growth and ochratoxin A production by some species are illustrated in Figure 4, Figure 5, and Figure 6.

Natural Substrates

Most commodities from which the ochratoxin-producing fungi were isolated are good substrates for ochratoxin A production. Trenk et al. (1971) studied the effects of natural substrates on ochratoxin A production by A. ochraceus. Chopped corn was found to be the best substrate for production of toxin. Polished rice and wheat bran yielded comparable amounts of toxin, but required longer incubation. Bleached flour did not support the production of large amounts of ochratoxin A.

Synthetic and Semi-synthetic Media

Synthetic and semi-synthetic media have been developed. MYE (mycological broth + 0.5% yeast extract) supports toxin formation by A. ochraceus (van Walbeek et

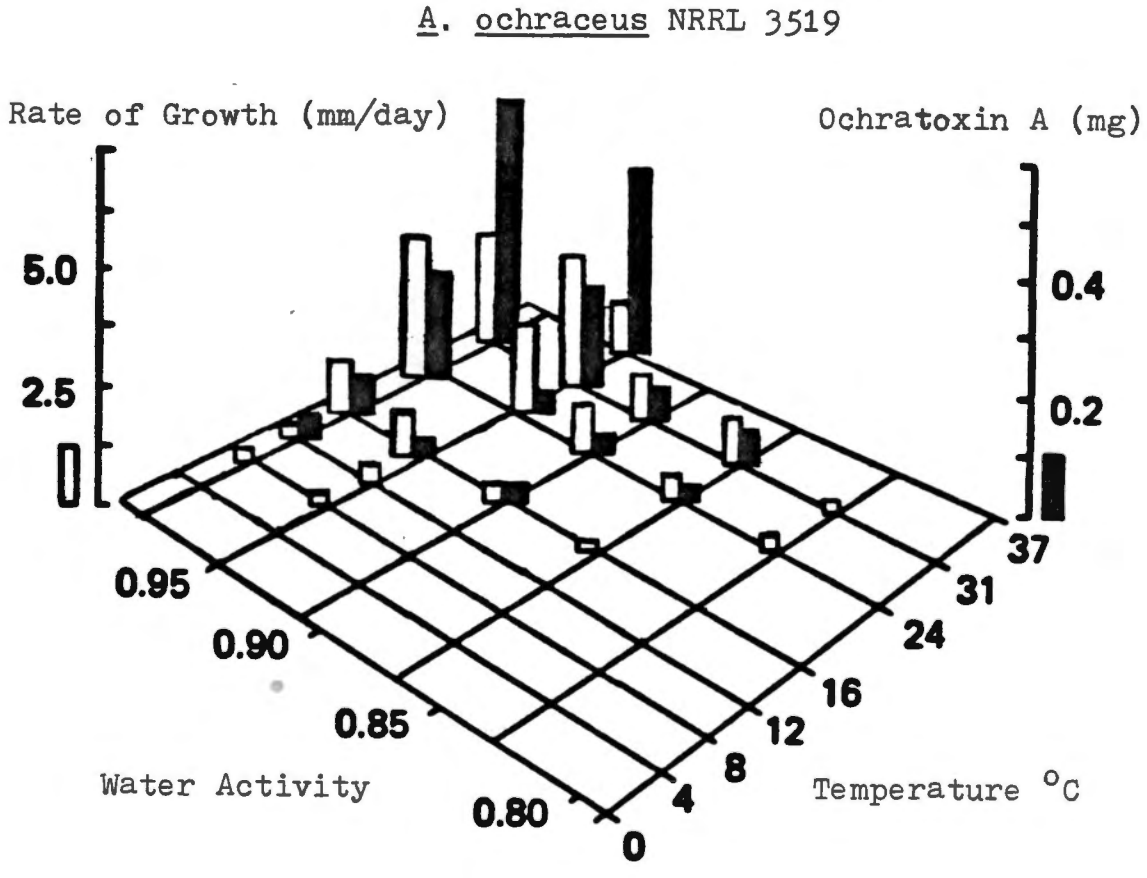


Figure 4. Growth and ochratoxin A production by one strain of Aspergillus ochraceus on malt extract sucrose agar (MES) under various conditions of water activity and temperature (Northolt et al., 1979).

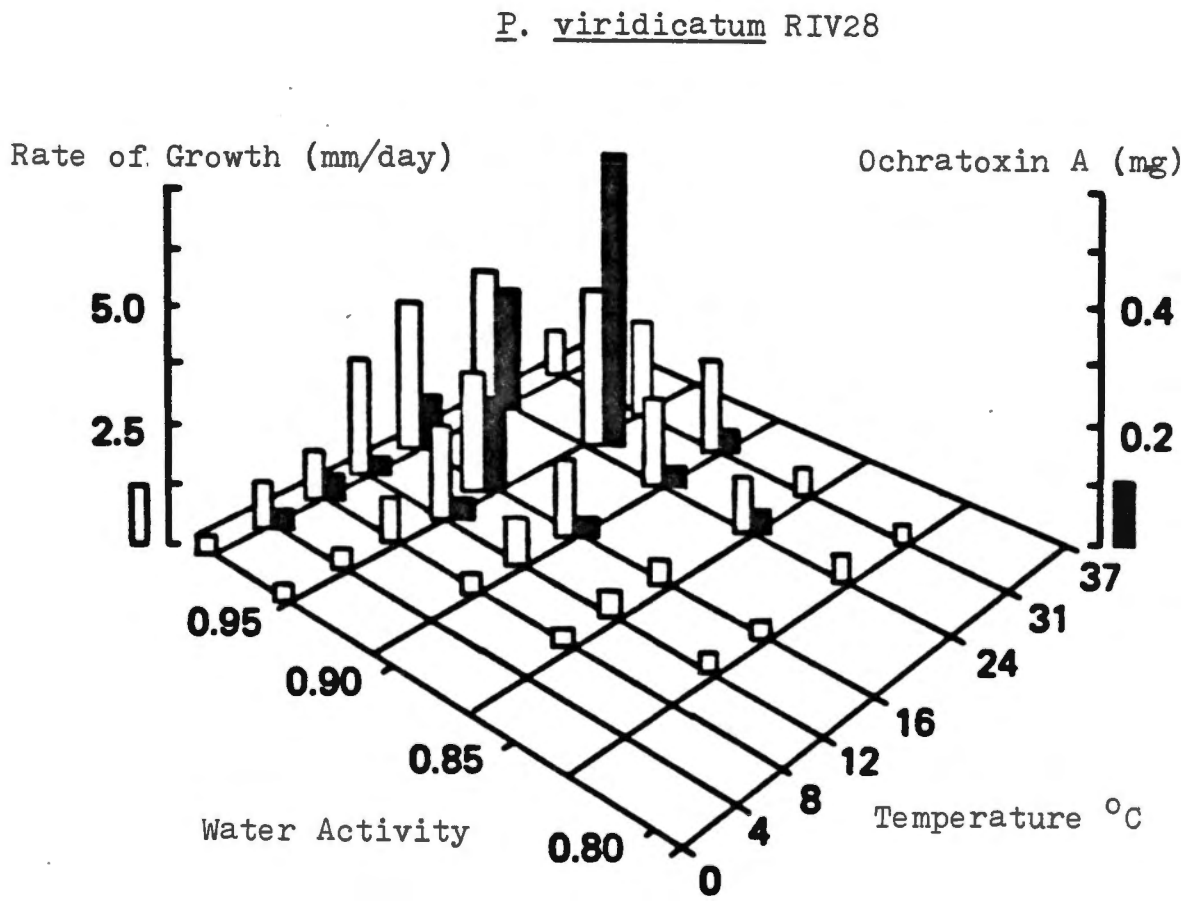


Figure 5. Growth and ochratoxin A production by one strain of Penicillium viridicatum on Czapek maize extract sucrose agar (CMS) under various conditions of water activity and temperature (Northolt et al., 1979).

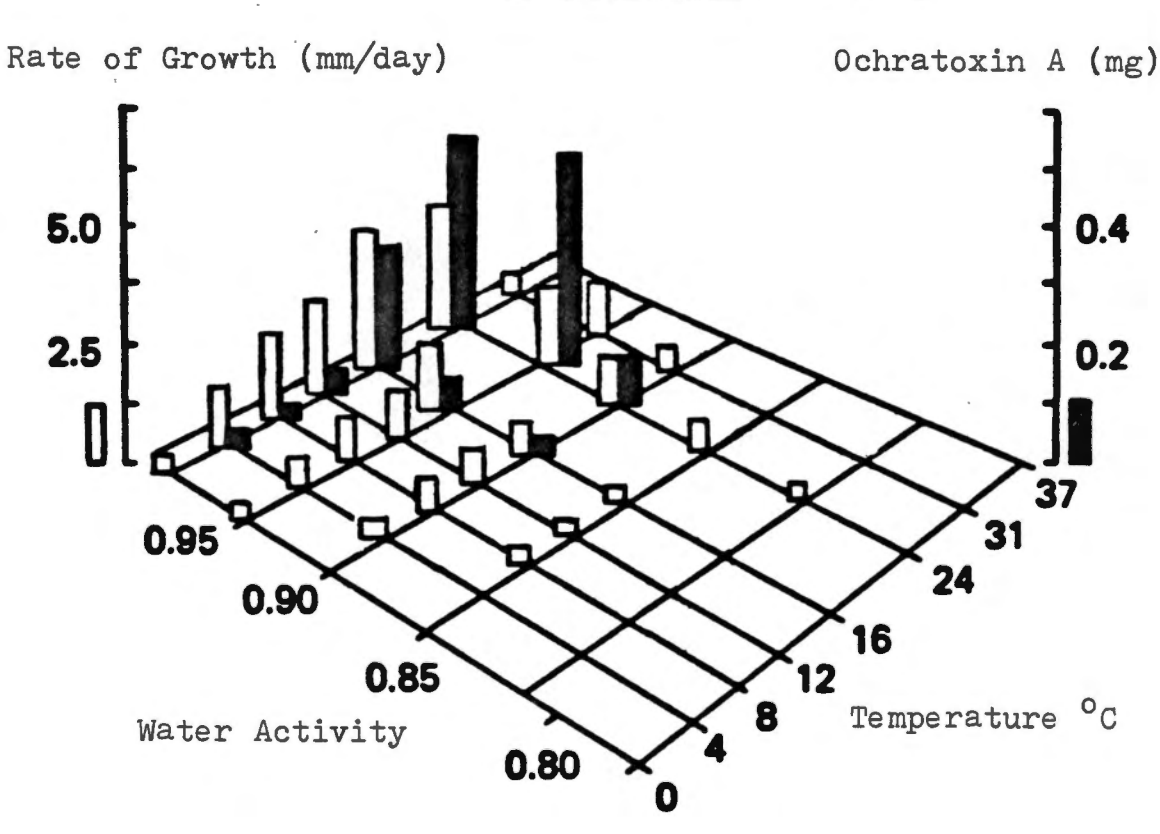


Figure 6. Growth and ochratoxin A production by one strain of Penicillium cyclopium on Czapek maize extract glycerol agar (CMG) under various conditions of water activity and temperature (Northolt et al., 1979).

al., 1968). YES (2% yeast extract and 15% sucrose) is good for ochratoxin A production by both A. ochraceus and P. viridicatum (van Walbeek et al., 1968 and 1969). More specific studies for the production of ochratoxin A by A. ochraceus in YES media were performed by Davis and associates (1969). By varying the sucrose concentrations from 0 to 32%, they reported that peak ochratoxin A production (29 mg/100 ml) occurred in 4% sucrose and 2% yeast extract medium. The fungal growth continued to increase directly higher concentration of sucrose from 4 to 32% without a corresponding increase in ochratoxin A production. Ochratoxin B was produced only when the sucrose concentration was 8% or higher. Among many nitrogen sources tested, glutamic acid and proline were best for toxin production. After adding 1% L-phenylalanine to a nutrient solution with 4% sucrose, 2% yeast extract, and some trace elements, Yamazaki et al. (1970) found significant increase in ochratoxin A production. Lai et al. (1970) investigated culture conditions for ochratoxin A production by species of Aspergillus with different sugars. The most toxin was produced by A. sulphureus from sucrose and glucose and decreasing amounts from maltose, mannose, galactose, xylose, and arabinose. The fungus did not grow on lactose. Trace elements Fe^{3+}, Zn^{2+}, Cu^{2+}, B^{3+}, and Mo^{6+} were not needed for good growth and ochratoxin A production by A. sulphureus, but they were required by A. ochraceus and A. melleus.

However, for maximum production of ochratoxin A by A. sulphureus, 100 mg potassium/liter, 25 mg phosphorus/liter, and 300 mg magnesium sulfate/liter were necessary. The effect of zinc, copper, and iron levels on the production of ochratoxin A by A. ochraceus in a synthetic medium in a shake culture was also determined by Steel et al. (1973). Optimum concentrations of $ZnSO_4$, $CuSo_4$, and $FeCl_3$ were 0.055 to 2.2 mg/liter, 0.004 to 0.04 mg/liter, and 1.2 to 24 mg/liter, respectively.

Amount of Aeration

Sansing et al. (1973) found that maximal amounts of ochratoxin A were produced by A. ochraceus in 25 ml of medium/125 ml flask and 75 ml of medium/500 ml flask at 25°C in stationary culture in 8 days. No ochratoxin A was produced in shake culture. The medium used was a nutrient solution of 4% sucrose and 2% yeast extract.

IV. BIOSYNTHESIS OF OCHRATOXIN A

The present evidence indicates that ochratoxin A is synthesized by an enzyme reaction directly linking the L-β-phenylalanine and dihydroisocoumarin moiety (ochratoxin α). Although the biosynthesis of phenylalanine via the shikimic acid pathway has not actually been demonstrated during ochratoxin A biosynthesis, its wide-spread occurrence in other species and forms of life makes it

reasonable to assume its functioning (Lehninger, 1975). Searcy et al. (1969) investigated the biosynthesis of ochratoxin A by A. ochraceus Wilhelm using phenylalanine-1-^{14}C and sodium acetate-2-^{14}C. Results showed that phenylalanine was incorporated unaltered into the phenylalanine moiety of ochratoxin A. They also found that the isocoumarin moiety was mostly derived from acetate condensation. Studying the biosynthesis of ochratoxin A by A. ochraceus using phenylalanine-U-^{14}C, sodium acetate-1-^{14}C, and methionine-$^{14}CH_3$, Steyn et al. (1970) concluded that the isocoumarin moiety was formed by the condensation of five acetate units in a head-and-tail fashion and the carboxy carbon of the amide group was derived from the C_1 pool. The involvement of the C_1 unit in ochratoxin A formation was confirmed by Yamazaki et al. (1971) using ethionine competitive inhibition and ^{13}C-NMR techniques. Sodium ^{13}C-formate was incorporated solely into the carboxy-carbon of isocoumarin moiety.

Still provoking question about the biosynthesis of ochratoxin A is the stage of chloronation in the molecule. Some evidences indicate that chlorination may occur at an early stage along the biosynthetic path (Wei et al., 1971). According to Huff and Hamilton (1979), the chlorination can be accounted for by assuming the intervention of an enzyme called chloroperoxidase. While ochratoxigenic fungi have not been examined for chloroperoxidase, chloroperoxidase

occurs widely in plants, animals and microorganisms and represents the agent invoked in the biosynthesis of over 200 naturally occurring halogenated compounds (Sinda et al., 1973).

$$X^- + HA + H_2O_2 \xrightarrow{\text{chloroperoxidase}} AX + H_2O + OH^-$$

X^- = Halogen doner

HA = Halogen acceptor

A proposed scheme for the biosynthesis of ochratoxin A is summarized in Figure 7 (Huff and Hamilton, 1979).

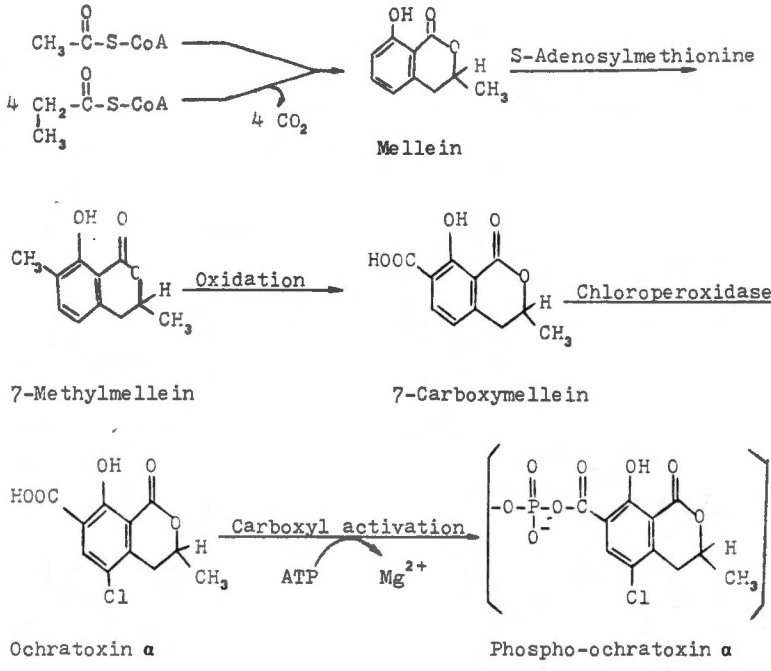
V. CHEMICAL ANALYSIS

General Considerations

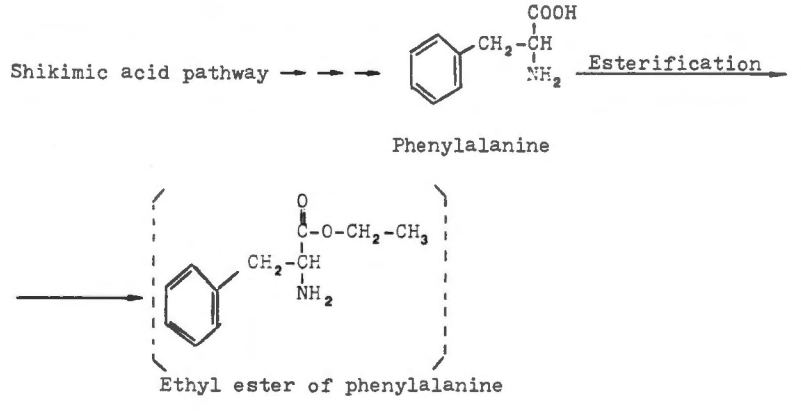
Ochratoxins exhibit fluorescence when exposed to ultraviolet light. This phenomenon is the basis for chemical analysis of ochratoxin A. In order to get the best and most accurate results, however, some factors must be taken into account:

1. Interaction of ochratoxin A with benzene. When ochratoxin A is crystallized from benzene, the product usually contains one mole of benzene. In the making of standard solutions of ochratoxin A by weight, this factor must be considered (Chu, 1974b).
2. Effects of pH and solvent on fluorescence properties and molar absorptivity. Golinski and Chelkowski (1978) studied the spectral behavior of ochratoxin A in different solvents. They reported that solvent polarity greatly

1) polyketide



2) shikimic acid pathway



3) synthesis

Phospho-ochratoxin α + Ethyl ester of phenylalanine $\xrightarrow[\text{Esterase}]{\text{Synthetase}}$ Ochratoxin A

Figure 7. Biosynthesis of ochratoxin A.

influences the fluorescence spectra. The fluorescent excitation and emission maxima under different conditions are summarized in Table 5. The molar absorptivity of ochratoxin A was also found to be solvent and pH dependent (Table 1; Golinski and Chekowski, 1978).

3. Photodecomposition by ultraviolet light exposure. Neely and West (1972) observed that ochratoxin A was photodecomposed upon prolonged exposure to ultraviolet light. Therefore, analysis of ochratoxin A should be carried out under subdued light (Chu, 1974b).

Analytical Methods

Thin layer chromatography (TLC) is a frequently used method for ochratoxin identification and quantitation. Ochratoxin A is seen as a green fluorescent spot under longwave ultraviolet light on TLC plates. The sensitivity can be increased by treating the TLC plates with ammonia fumes which change the fluorescence of ochratoxin A to blue with a greater intensity (Trenk and Chu, 1971). The applications are shown in Table 6. A spectrophotometric method for the quantitative measurement of ochratoxin A was reported by Hult and Gatenbeck (1976). In the method described, ochratoxin A is cleaved into ochratoxin α and phenylalanine, using carboxypeptidase A. Detection is based on the difference in the fluorescent excitation spectra of ochratoxin A (380 nm, maxima) and ochratoxin α (340 nm,

Table 5. Fluorescent Properties of Ochratoxin A under Different Conditions.

Conditions of Determination	Excitation Wavelength (nm)	Emission Wavelength (nm)
Acidic, aqueous or TLC plate	330-340	465 and 475
Basic, aqueous or TLC plate exposure to NH_3	360-390	427-450
1.2×10^{-4} - 1.5×10^{-6} in EtOH	330-340	465
1.5×10^{-6} - 8.0×10^{-9} in EtOH	376	426

Source: Chu, 1974.

Table 6. Methods for The Determination of Ochratoxin A in Foods Based on TLC.

Food(s) Analyzed	Extraction Solvents	Cleanup	Recovery (%)	Detection Limit (μg/kg)	Reference
Corn, barley	Chloroform 1[a] Methanol 1	$NaHCO_3$ solution	-	100, 10-20	Steyn and van der Merwe (1966)
Cereal products	Methanol-water (55:45) + hexane	Partition into chloroform-hexane (1:1) on Celite	80-100	25	Scott and Hand (1967)
Various foods	Chloroform-water (10:1)	Silica gel, elute benzene-acetic acid (9:1)	40-60	50 (corn)	Eppley (1968)
Grains, peanuts	Chloroform 8 Methanol 2 Hexane 1	Silica gel, elute benzene-acetic acid (9:1)	80-100	20	Vorster (1969)
Cereal products	Hexane, chloroform-methanol (1:1)	$NaHCO_3$ solution; partition into chloroform	85-96	10-50	Chu and Butz (1970)
Grains	Acetonitrile, aqueous KCl	Isooctane; partition into chloroform	86-100	45-90	Stoloff et al. (1971)
Grains, beans	Methanol-water (55:45) + hexane	Partition into chloroform	68-100	-	Scott et al. (1972)
Cocoa beans	Methanol-$AgNO_3$ solution (55:45) + hexane	Partition into chloroform	68-100	20	Scott (1973)
Barley	Chloroform, 0.1 M H_3PO_4 solution	$NaHCO_3$ on Celite	112[b]	12	Nesheim (1973)
Green coffee beans	Chloroform-water (10:1)	$NaHCO_3$ on Celite	83.5	20	Levi et al. (1974)
Barley	Chloroform 10 H_3PO_4 solution 1	$NaHCO_3$ solution; silica gel	60-100	12-64	Hald and Krogh (1975)

Table 6. Continued.

Food(s) Analyzed	Extraction Solvents	Cleanup	Recovery (%)	Detection Limit (μg/kg)	Reference
Various foods	Methanol-water (8:2)	$ZnSO_4$, phosphotungstic acid; benzene; acidified florisil minicolumn	-	8	Holaday (1976)
Wheat	Chloroform	$NaHCO_3$ solution; partition into chloroform	-	-	Krogh et al. (1976b)
Corn, peanuts, dried beans	Chloroform-0.5N phosphoric acid (10:1)	$NaHCO_3$ on Celite	55-81	20-40	Wilson et al. (1976)
Corn	Acetonitrile-water (90:10)	$NaHCO_3$ solution; partition into chloroform	87	40	Balzer et al. (1978)
Various foods	Acetonitrile-4% KCl (9:10)	Isooctane; $NaHCO_3$ solution; partition into chloroform	92-95	140-145	Gimeno (1979)
Rice, corn, wheat, peanuts	20% H_2SO_4 2 4% KCl 20 Acetonitrile 178	Isooctane; partition into chloroform; silica gel elute benzene-acetone-acetic acid (75:20:5)	39-97	40-60	Takeda et al. (1979)

[a]Volumetric ratio.

[b]Determined by collaborative study.

maxima) in an alkaline buffer solution of pH 7.50. The quantitation of ochratoxin A can be achieved by measuring the loss of fluorescent intensity at 380 nm. The sensitivity was 4 µg/kg in barley and barley meal with an average recovery of 90%. Recently, high pressure liquid chromatography (HPLC) has been developed for the identification and quantitation of ochratoxin A in foods, using the extraction solvents and cleanup procedures in Table 5. HPLC has the advantages of good resolution, high degree of precision, reproducibility, and sensitivity (Hsieh et al., 1976; Pons, 1976). Hunt et al. (1978) determined a sentivity of 12.5 µg/kg with recoveries ranged from 60 to 95% in different foodstuffs. Josefsson and Moller (1979) studied high pressure liquid chromatographic determination of ochratoxin A in cereals. A detection limit of 1-5 µg/kg and recoveries of 54.7-92.3% were observed. Moreover, they found the adjustment of pH of mobile solvents to 4.5 is necessary to get a sharp peak of ochratoxin A. The best sensitivity of 0.04 nanograms was reported by Engostrom and associates (1977) with a HPLC system including one µBondapak/C_{18} column and a solvent system of acetonitrile:water:acetic acid 55:45:2 (v/v/v). A purification method for ochratoxin A, using liquid-liquid extractions and a final cleanup by HPLC, was also described (Peterson and Ciegler, 1978).

Biological Assay

Several biological assay systems are available for ochratoxin A quantitation by utilizing microorganisms (Broce et al., 1970), brine shrimp (Brown, 1969), chicken embryos (Choudhury and Carlson, 1973), day-old chickens (Chu et al., 1972), tracheal organ cultures (Cardeilhoc et al., 1972), and zebra fish larvae (Abedi and Scott, 1969). Biological assay can be used not only to supplement the results of chemical detection and confirmation of the presence of ochratoxin A in foodstuffs, but also to determine the biological activities. Usually an acute or inhibitory dose is used as the indicator for quantitation. However, the dose is greatly affected by the individual differences among testing species. Therefore, it is required to have large sample sizes in order to get a good reproducibility.

Several immunologic assays have also been developed to detect low concentrations of ochratoxin A in small samples of blood, urine, kidney, tissue, biopsy etc. Chu et al. (1976) studied the production of antibodys against ochratoxin A after repeated injection of different protein-ochratoxin A conjugates to rabbits. Bovine serum albumin-ochratoxin A was found to be the best antigen. The IgG antibody was specific for ochratoxins A and C, but not specific for ochratoxins B, α, and other coumarin derivatives. The percentage of binding between ^{3}H-labeled

ochratoxin A and IgG was reduced by unlabeled ochratoxin A. The sensitivity for ochratoxin A detection was in the range of 0.5 to 10 ng/0.5 ml sample. Aalund et al. (1975) reported a radioimmunoassay for ochratoxin A. Ochratoxin A was covalently coupled to electrophoretically pure bovine IgG for antigen formation. The antigen was injected into rabbits at a dose of 1.0 mg conjugate protein. Rabbits produced anti-ochratoxin A antibody and the antisera was used with ^{125}I-egg albumin-ochratoxin A as the radioactive antigen. Free ochratoxin A inhibited immunoprecipitation of the radioactive antigen. The procedure showed a lower limit of detection at 20 ppb of ochratoxin A.

VI. TOXICOLOGY

Toxicity of Ochratoxins

In addition to being associated with spontaneous nephropathies in chickens (Elling et al., 1975; Krogh et al., 1976a) and in swine (Krogh et al., 1976b,c), ochratoxin A has been found toxic to many test animals, including hens (Choudhury et al., 1971), turkeys (Prior et al., 1976), ducklings (van der Merwe et al., 1965; Pruchase and Nel., 1967), chicks (Chu and Chang, 1971; Huff et al., 1974; Galtier et al., 1976), Japanese quails (Prior et al., 1976), rats (Purchase and Theron, 1968; Galtier et al., 1974; Kanisawa et al., 1977; Hayes et al., 1977), mice

(Sansing et al., 1976), Guinea pigs (Thacker and Carlton, 1977), dogs (Szczech et al., 1973a,b), cattle (Ribelin et al., 1978), brine shrimp (Brown, 1969), swine (Szczech et al., 1973c), and bacteria (Singer and Roschenthaler, 1978). The toxicological responses to ochratoxin A vary with the dose, duration of exposure, animal species, age, and route of administration of the toxin. The acute dose of ochratoxin A for various biological system is listed in Table 7.

Clinical signs of ochratoxin A also vary with animal species, dose, duration of administration, age, and reproductive stage of the test animal. While the main pathological changes may occur in the liver, gut, and lymphoid tissues, the most consistent alternation is renal tubular damage (Shimoda, 1979).

Structure-toxicity Relationships

Chu et al. (1972) studied the structural requirements for ochratoxins intoxication. They reported that the phenolic hydroxyl group in the dissociated form is necessary for toxicity by ochratoxins. This was demonstrated by the fact that ochratoxin C failed to induce toxin effect after the phenolic hydroxy group was chemically modified. They also found that the toxicity of ochratoxins is closely related to the acid dissociation constant of the phenolic hydroxyl group. Ochratoxin A and ochratoxin C,

Table 7. Acute Dose of Different Organisms to Ochratoxins.

Organism	Test system	Toxin	LD_{50}	References
Bacteria				
B. megaterium	In culture medium	OA	4 μg/ml[a]	Chu, 1974b
B. subtilis	In culture medium	OA	12 μg/ml[a]	Singer and Roschenthaler, 1978
B. cereus mycoides	In disc (agar gel)	OA	1.5 μg/disc[a]	Broce et al., 1970
B. cereus mycoides	In disc (agar gel)	OB	3 μg/disc[a]	Broce et al., 1970
Chicken embryos	Zero day injection	OA	5-10 μg/egg	Chu, 1974b
	5-day-old egg, observed 48 hr after injection	OA	16.95 μg/egg	Chu, 1974b
	8-day-old egg (allantoic sac injection), observed after 1 week	OA	7 μg/egg	Chu, 1974b
	8-day-old egg	OB	25 μg/egg	Chu, 1974b
Chicks	Day-old, oral intubation	OA	135-166μg/chick	Chu and Chang, 1971; Peckham et al., 1971
	"	OA-M	216 μg/chick	"
	"	OB	1890 μg/chick	"
	"	Oα	None toxic at 1000 μg/chick	"
	Day-old ckicks, oral intubation	OA	2.14 mg/kg body weight	Huff et al., 1974
	3-week-old chicks, oral intubation	OA	3.60 mg/kg body weight	Huff et al., 1974
	10-day-old Leghorn chicks, oral intubation	OA	10.7 mg/kg body weight	Galtier et al., 1976
Beagle dogs	Feeding daily,	OA	0.2-3.0 mg/kg body weight	Szczech et al., 1973a,b
Ducklings	Day-old, feeding	OA	25 μg/duckling	van der Merwe et al., 1965
	Day-old, feeding	OA	150 μg/duckling	Purchase and Nel, 1967
Japanese quails	Day-old, feeding	OA	16.5 mg/kg body weight	Prior et al., 1976
Guinea pigs	Administrated by gastric intubation	OA	8.1-9.1 mg/kg body weight	Thacker and Carlton, 1977

Table 7. Continued.

Organism	Test system	Toxin	LD_{50}	References
Mice	Female, i.p. injection	OA	22-44.7 mg/kg body weight	Lindenfelser et al., 1973; Sansing et al., 1976; Galtier et al., 1974
	Swiss mouse, feeding	OA	58.3-62.4 mg/kg body weight	Galtier et al., 1974
	Swiss mouse, intravenous route	OA	25.7-33.9 mg/kg body weight	Galtier et al., 1974
Rats	Oral-stomach tubing, assay on approximately 80 g Wistar rats	OA	20-22 mg/kg body weight	Purchase and Theron, 1968
	Wistar rats, feeding	OA	21.4-30.3 mg/kg body weight	Galtier et al., 1974
	Wistar rats, i.p. injection	OA	12.6-14.3 mg/kg body weight	Galtier et al., 1974
	Male Wistar rats, gastric intubation	OA	28 mg/kg body weight	Kanisawa et al., 1977
Turkeys	Day-old Nicholas broad breasted white turkeys	OA	5.9 mg/kg body weight	Prior et al., 1976
Cattle	5-week-old Holstein calves, oral-stomach tubing	OA	11-25 mg/kg body weight	Ribelin et al., 1978
Goats	American La Mancha female goats, oral-stomach tubing	OA	3 mg/kg body weight	Ribelin et al., 1978
Fish	6-month-old Mt. Shasta rainbow trout, i.p. injection	OA	4.67 mg/kg body weight	Doster et al., 1972
Swine	Feeding daily	OA	1-2 mg/kg body weight	Szczech et al., 1973c
Zebra fish larvae	Toxins dissolved in aqueous solution	OA	1.7 μg/ml	Abedi and Scott, 1969
Brine shrimp *Artemia salina*	Toxin dissolved in aqueous solution	OA	10.1 μg/ml	Abedi and Scott, 1969

[a]Minimum dose showing inhibiting effect.

with a pK near neutral pH 7.05 to 7.10, were determined the most toxic. The acid dissociation constant of ochratoxin B is ten times smaller than that of ochratoxin A, whereas the toxicity of this toxin is about ten times less.

Teratogenesis

Treatment of pregnant mice with ochratoxin A dissolved in propylene glycol at a dose of 5 mg/kg body weight by intraperitoneal injection on gestation days 7-12 resulted in increased prenatal mortality, decreased fetal weight, and various fetal malformations (Hayes et al., 1974). Anomalies of the head, eyes, face, digits and tails were the most common defects and these included exencephaly, anophthalmiamicrophthalmia, cleft lip, median facial cleft, short jaws, syndactyly, and polydactyly. The malformation rate was greatest in fetus from mothers injected on gestation day 8.

Still et al. (1971) administered ochratoxin A dissolved in 0.5M sodium bicarbonate to bred female Sprague-Dawley rats on gestation day 10 by oral intubation at doses of 6.25, 12.5, and 25 mg/kg body weight. Ochratoxin A at all levels induced fetal death and resorption. Brown et al. (1976) administered ochratoxin A orally to bred Sprague-Dawley-derived young adult rats at doses of 0.25, 0.50, 0.75, 1, 2, 4, or 8 mg/kg body weight on days 6 through 15 of gestation. They observed that at

doses greater than 1 mg/kg acute ochratoxicosis A and nearly 100% loss of litters resulted. Doses of 0.75 mg/kg were embryotoxic and teratogenic and doses of 0.25 and 0.50 mg/kg were principally teratogenic. Gross abnormalities included subcutaneous edema, short snout, open eyes, and shortened body. Visceral abnormalities included small left atrium of the heart. Skeletal malformations consisted of wavy ribs, agenesis of sternebrae, and asymmetry of sternebrae.

Gilani et al. (1978) conducted experiments to evaluate the teratogenicity of ochratoxin A given to developing chick embryos. Ochratoxin A was dissolved in propylene glycol and injected into embryonating chicken eggs at doses ranging from 0.0005 to 0.007 mg/egg. The injections were made into the air sacs of eggs after 48, 72, and 96 hours of incubation. All embryos were examined at day 8. Short and twisted limbs, short and twisted neck, microphthalmia, exencephaly, everted viscera, and reduced body size were detected.

The teratogenic potential of ochratoxin A has also been studied in the hamster. Hood et al. (1976) administered by intraperitoneal injection ochratoxin A dissolved in 0.1N sodium bicarbonate to pregnant golden hamsters on one of gestation days 7 to 10 at doses of 2.5 to 20 mg/kg body weight. The largest dosages when given on

day 7, 8, or 9 increased prenatal mortality and on day 9 diminished fetal growth. Most frequently observed malformations were micrognathia, hydrocephalus, short tail, oligodactyly, syndactyly, cleft lip, micromelia, and heart defects.

Tumorogenesis

Ochratoxin A was found to be carcinogenic to rainbow trout when fed at a dose of 20 µg/kg of diet together with the cocarcinogen sterculic acid. On the other hand, administering 10 µg ochratoxin A/mouse suspended in 0.1 ml peanut oil to male and female CBA mice twice weekly for 36 weeks, Dickens and Waynforth (1968) reported that ochratoxin A was not tumorogenic in mice. Purchase and van der Watt (1971) administered ochratoxin A by subcutaneous injection or by oral intubation to male and female Wistar-derived rats to study the long-term toxicity of ochratoxin A to rats. The results showed that ochratoxin A did not cause tumors in the rats. The International Agency for Research on Cancer concluded in 1976 that all ochratoxin A studies were inadequate in terms of the number of animals used and survival rates, and that no evaluation of ochratoxin A as a chemical carcinogen could be made (Krogh, 1978).

VII. METABOLISM AND BIOCHEMICAL EFFECTS

Metabolism

Studying the metabolism of ochratoxin A in male Wistar rats given an intraperitoneal injection of 10 mg/kg of the toxin dissolved in 0.1N sodium bicarbonate, Nel and Purchase (1968) detected ochratoxin A in the blood, liver, and kidney within 30 minutes of dosing. Ochratoxin A and ochratoxin α were also found in the urine and feces.

In a quantitative analysis of the excretion of ochratoxin A after feeding 500 µg ochratoxin A daily to male Wistar rats for 6 days, van Walbeek et al. (1971) reported that about 10% of ochratoxin A consumed was excreted in the urine and feces. A portion of the ochratoxin A was metabolized to ochratoxin α and excreted mainly in the urine.

Doster and Sinhuber (1972) studied the rates of hydrolysis of ochratoxin A and ochratoxin B in vitro by bovine carboxypeptidase A and by enzymes in crude extracts of rat tissues. With rat tissue extracts, ochratoxin B was hydrolyzed at a rate 6 to 8 times faster than ochratoxin A. Ochratoxin B was also hydrolyzed much faster than ochratoxin A using carboxypeptidase.

Galtier (1974a) studied the transport of ochratoxin A in male Wistar rats fed a single dose of 10 mg/kg body weight ochratoxin A. Considerable amounts of ochratoxin A were detected in the plasma where the maximal concentration was obtained 8 hours after administration. Further studies

of the distribution in the tissues and the elimination of ochratoxin A and ochratoxin α in male Wistar rats under same conditions were conducted (Galtier, 1974b). The data showed that ochratoxin A was absorbed by the stomach and that it was distributed particularly to the liver, kidney, and muscle. Ochratoxin α was recovered in the cecum and the large intestine indicating hydrolysis of ochratoxin A by microbial flora.

Patterson et al. (1976) studied the metabolism of ochratoxin A and ochratoxin B in the pigs. A mixture of the two mycotoxins (0.38 and 0.13 mg/kg body weight) was fed to pigs daily for 8 days during early pregnancy. Compared with ochratoxin A, ochratoxin B was poorly absorbed and preferentially hydrolyzed in the intestinal tract. As a result, ochratoxin A and its hydrolysis product ochratoxin α were excreted in the feces, but ingested ochratoxin B was not found in the feces since it was completely hydrolyzed to ochratoxin β. Ochratoxin A and the two metabolites, ochratoxin α and ochratoxin β, were also excreted in the urine. Neither toxin crossed the placenta to the fetus.

Chang and Chu (1977) investigated the fate of ochratoxin A in male Sprague-Dawley rats given a single intraperitoneal injection of 1 milligram ochratoxin A and ^{14}C-ochratoxin A. Radioactivity in the serum reached a maximum within 30 minutes of injection of the toxin and

the concentration was equivalent to about 90% of the ^{14}C dose. During the first 8 hours, 70 to 80% of the radioactivity was in unaltered ochratoxin A whereas after 24 hours 56% of radioactivity was ochratoxin A. Thirty minutes after injection, the liver and kidney each contained 4.5 and 4.4% of the injected dose, respectively. The activity decreased thereafter and 1 to 2% of the injected radioactivity was found in the liver or kidney 24 hours after injection. Ochratoxin A was excreted primarily in the urine, either as the unchanged toxin or its metabolite, ochratoxin α. Excretion in the feces was less significant and was mainly in the form of unchanged toxin.

Biochemical Effects

a. Protein Binding

Pitout and Nel (1969) studied the inhibitory effect of ochratoxin A on the hydrolysis of N-carbobenzoxyglycyl-L-phenylalanine by bovine carboxypeptidase A. Ochratoxin A was found to be a competitive inhibitor of carboxypeptidase A. The effect was about 70% after 30 minutes. No inhibitory effect of ochratoxin α on the enzyme was observed.

Chu (1971) investigated the interaction of ochratoxin A with bovine serum albumin by spectrophotometric, spectrophotofluorometric, equilibrium dialysis, and Sephadex gel filtration analyses. The absorption maximum

of ochratoxin A shifted to a longer wavelength after the interaction. The fluorescent properties of ochratoxin A were also altered in the presence of bovine serum albumin. One mole of bovine serum albumin bound 1.86 to 2.47 moles of ochratoxin A depending on the reaction temperature. No binding between ochratoxin A and γ-globulin was observed.

Chu (1974a) studied the interaction of different ochratoxins with bovine serum albumin by equilibrium dialysis, solubility, and spectrophotometric analyses. The absorption maxima shifted to longer wavelengths (365-377 nanometers for ochratoxin B and 380-390 nanometers for ochratoxin C) as a result of interaction, but the spectra of ochratoxin α and ochratoxin β were not altered. One mole of bovine serum albumin bound one mole of ochratoxin α and ochratoxin β, about two moles of ochratoxin B and about three moles of ochratoxin C. Both hydrophobic and ionic bonds were considered important in the binding.

b. Mitchondrial Effects

Moore and Truelove (1970) discovered that ochratoxin A and ochratoxin α at low concentration (1.0-4.3 mM) inhibited ADP-stimulated (state 3) respiration in mitochondria isolated from rat liver.

Meisner and Chan (1974) studied the effects of ochratoxin A on mitochondrial transport systems. Their results suggested that ochratoxin A acted to produce a

competitive type of inhibitory pattern of mitochondrial transport carriers located in the inner membrane. Respiration stimulated by ADP was progressively inhibited up to 0.4 mM ochratoxin A. The mycotoxin had a competitive type of inhibition with respect to the binding of dicarboxylic acids, adenine nucleotides and inorganic phosphate.

c. Carbohydrate Metabolism

Suzuki et al. (1975) studied the effect of ochratoxin A on carbohydrate metabolism in male Wistar rats. A marked depletion of glycogen in liver and an increase of serum glucose in ochratoxin A treated rats were noted. Glycogen synthetase activities were reduced whereas phosphorylase a activity was increased in the liver. However, according to the effects by Pitout (1968), Heller and Roschenthaler (1978), Huff et al. (1979), and Warren and Hamilton (1980), it is believed that ochratoxin A inhibits the phosphorylase enzyme system. Specifically, the binding of cAMP to protein kinase which subsequently activates the enzyme phosphorylase b kinase is inhibited by ochratoxin A. The inhibition is of the non-competitive type, at least up to a concentration of 100 µg/ml ochratoxin A (Heller and Roschenthaler, 1978). As a result, ochratoxin A produces accumulation of glycogen in rat and chicken livers.

VIII. CONTROL

As mentioned before, ochratoxin A is a very stable compound. It is almost impossible to get rid of all the mycotoxin once it is produced in foods or feedstuff. Prevention of fungal growth by proper drying appears presently to be the most efficient means of controlling contamination with ochratoxin A.

Chemical treatment can be used to reduce or prevent fungal growth and ochratoxin A production in grains and animal feeds. The effects of insecticides and fumigants on the production of mycotoxins in wheat were investigated by by Vandegraft et al. (1973a,b). None of the insecticides or fumigants reduced aflatoxins or ochratoxin A formation. Nevertheless, an organophosphate insecticide, dichlorvos, was found to inhibit ochratoxin A production by A. ochraceus in yeast extract sucrose medium and on corn (Wu and Ayres, 1974). The insecticide at the lowest concentration of 0.1 mg/100 ml of medium had no effect on fungal growth or ochratoxin A production. Production of ochratoxin A was reduced by 25% at a level of 1 mg/100 ml of medium. At a concentration of 10-30 mg/100 ml of medium, dichlorvos inhibited from 50 to 80% of ochratoxin A production. A similar inhibitory effect was also observed on corn.

Vandegraft et al. (1975) studied the effects of grain preservatives on production of aflatoxin and ochratoxin A

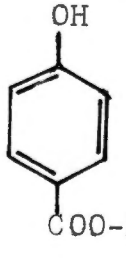
on corn. Corn was treated with either 2% ammonia or 1% propionic acid. Both ammonia and propionic acid significantly reduced mold growth and subsequent aflatoxin and ochratoxin A formation. Growth and the formation of mycotoxins were inhibited more by propionic acid than ammonia.

Specific studies of the chemical or physical reduction of ochratoxin A content and the detoxification procedures in relation to reduction of ochratoxin A concentrations in grains and other materials are not available.

IX. ANTIMICROBIAL FOOD ADDITIVES

Four groups of chemicals which are officially accepted as direct food additives by regulatory agencies of the United States and many other countries are discussed to reveal their properties, safety, regulatory status, antimicrobial activities, and uses.

Esters of p-Hydroxybenzoic Acid or Parabens



R = alkyl groups

The alkyl esters of p-hydroxybenzoic acid comprise a group of antimicrobial agents which have been used widely

in cosmetic and pharmaceutical products. All of the p-hydrobenzoic acid esters commonly used in foods are white, free-flowing powders. Methyl ester is odorless or has a faint characteristic odor and a slight burning taste according to Food Chemical Codex (1972). The solubilities of the esters are inversely related to the number of carbon atoms in the ester group (Furia, 1972). The parabens are stable against hydrolysis during autoclaving and resist saponification (Furia, 1972).

The acute toxicity of p-hydroxybenzoic acid esters declines with a reduction in the chain length. The LD_{50} of the methyl and propyl esters after being fed to mice is in the region of 8 g/kg body weight; while the respective figures for the sodium salts are 2 and 3.7 g/kg body weight (Lueck, 1980). The methyl, ethyl, and propyl parabens are listed in category A(1)* by the Joint FAO/WHO Expert Committee on Food Additives with an ADI up to 10 mg/kg body weight (FAO/WHO, 1979). p-Hydroxybenzoic acid esters are rapidly and completely absorbed from the gastro-intestinal tract and hydrolyzed to form p-hydrobenzoic acid which is excreted via the urine. No accumulation is likely to occur (Lueck, 1980).

---

*Category A(1) additives are those which have been fully cleared by the Joint FAO/WHO Expert Committee on Food Additives and have either been given an "acceptable daily intake" or have not been limited toxicologically.

Under U.S. Food and Drug Administration regulations, methyl and propyl parabens are "generally recognized as safe" (GRAS) when used as food preservatives with total addition limits of 0.1% (21 CFR 184.1490 and 184.1670).

The antimicrobial activity of p-hydroxybenzoic acid esters is proportional to the chain length (Furia, 1972). However, due to the low solubilities in water, lower esters are commonly used. The parabens destroy the cell membrane and denature protein in the interior of the cell. They also compete some coenzymes (Lueck, 1980). As p-hydroxybenzoic acid esters are non-dissociating compounds, their antimicrobial action is indepentent of the pH value of the medium to be preserved. They are effective up to pH 9 (Lueck, 1980). The parabens are most active against molds and yeasts but are less effective against bacteria especially Gram negative bacteria.

The methyl and propyl parabens are used in fat products, dairy products, meat and fish products, baked goods, beverages, beer, fruit products, jams and jellies, and syrups.

Sulfur Dioxide and Sulfites

Sulfur dioxide has been used in foods for many centuries. The earliest known use was the treating of wines in Roman times. Besides antimicrobial functions, various forms of sulfites have been used to prevent browning during

processing of light-colored fruits and vegetables (Furia, 1972). Sulfur dioxide is a colorless, non-flammable gas, with a suffocating odor. SO_2 is applied to foods as a gas or as a solution of the gas in water. The different sulfite salts, white powders with an odor of SO_2, offer the convenience of handling as dry chemicals. When dissolved in water, these sulfites form sulfurous acid (H_2SO_3), bisulfite ion (HSO_3^-) and sulfite ion ($SO_3^=$). The relative proportion of each form depends on the pH of the solution which is controlled by the starting materials or the addition of acid or alkali (Table 8).

The toxicity of sulfites was reviewed by the Institute of Food Technologists' Expert Panel on Food Safety and Nutrition and the Committee on Public Information (1975). The "no-effect level" for rats was 72 mg of SO_2/kg/day, and the "no-effect level" for pigs and Japanese quails was approximately 1.5 to 2 times higher, respectively than for rats. The ADI of sulfites, established by the FAO/WHO, is 0.7 mg of SO_2/kg body weight. The normal metabolic processes of the body convert sulfites to sulfates with the aid of an enzyme, sulfite oxidase. Sulfates are excreted in the urine.

Sulfur dioxide and sulfites are generally recognized as safe when used in accordance with good manufacturing practice except that they are not used in meats or in foods recognized as sources of vitamin B_1 (21 CFR 182.3616, 182.3637, 182.3739, 182.3766, 182.3798, and 182.3682).

Table 8. Undissociated Proportions of Preservative Acids at Various pH Values.

Preservative	Dissociation constant at 25°C	pK	Percentage of undissociated acid at pH[a] 3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0
Sulfurous acid	1.72×10^{-2}	1.76	5.8	1.8	0.58	0.18	0.058	0.018	0.006	0.002	0
Hydrogen sulfite	6.43×10^{-8}	7.19	100	100	99.9	99.8	99.4	98	94	83.1	60.9
p-Hydroxybenzoic acid	3.30×10^{-5}	4.48	97	91	75	49	23	9	2.9	1.0	0.3
Sorbic acid	1.73×10^{-5}	4.76	98	95	85	65	37	15	5.5	1.8	0.6
Propionic acid	1.32×10^{-5}	4.88	99	96	88	71	43	19	7.0	2.3	0.8

[a]Calculated from the equation of

$$\alpha = \frac{[H^+]}{[H^+] + D} \times 100$$

where α = amount of undissociated acid
$[H^+]$ = hydrogen ion concentration
D = dissociation constant

Source: Lueck, 1980.

Nevertheless, the use level is limited by the fact that, at residue levels above 500 ppm, the taste begins to be noticeable (Furia, 1972).

The pH value of the foods to be preserved is an important factor governing the antimicrobial effect of sulfites. The most powerful action is that of the undissociated sulfurous acid which is predominant at low pH values. Even though it is less effective, the bisulfite ion also has an antimicrobial action. The optimum pH for SO_2 and sulfites is between 2.5 and 5.0. The mechanism of action is based on the sulfurous acid blocking the enzymes of the microorganisms by reducing essential disulfide linkages (Furia, 1972; Lueck, 1980). Sulfurous acid and its salts are used mainly against bacteria, and are less effective against yeasts and molds.

SO_2 and sulfites can be applied to dehydrated fruits and vegetables, fruit juices, syrups, and wines.

Sorbic Acid and Its Salts

CH_3-CH=CHCH=CHCOOR R = H, Na, K

Sorbic acid is used both as a free acid and as its sodium and potassium salts. Sorbic acid is a white, free-flowing powder with a characteristic odor. It is only slightly soluble in water (0.16%). The alkali salts of sorbic acid have the advantage of good water solubilities. Potassium sorbate, the most widely used in food applications,

is a white, fluffy powder with a water solubility of 139.2 g/100 ml at 20°C (Sofos et al., 1979; Furia, 1972).

Sorbic acid was found harmless to rats and dogs when incorporated in their diets to the extent of 5%. Its toxicity is lower than that of sodium benzoate. The LD_{50} of sorbic acid is about 10 g/kg body weight in rats. The LD_{50} of sodium sorbate has been determined as 5.94 and 7.16 grams, respectively (Sofos et al., 1979; Lueck, 1980). The ADI accepted by FAO/WHO is 25 mg/kg body weight. Sorbic acid is utilized in the body in a way similar to other fatty acids except the first step of β-oxidation, α,β-dehydrogenation, is omitted. The half-life of sorbic acid in the body is 40-110 minutes, depending on the dosage. Under normal conditions of alimentation, sorbic acid is completely oxidized to CO_2 and H_2O (Sofos et al., 1979).

Sorbic acid and its salts are generally recognized as safe for use in foods (21 CFR 182.3089, 182.3225, 182.3640, and 182.3795). The maximum permissible quantity for most countries is between 0.1 and 0.2% (Lueck, 1980).

Sorbates inhibit growth of molds, yeasts and many bacteria, although their action against bacteria is not as comprehensive as that against molds and yeasts. The antimicrobial activity of sorbates depends on the pH of the substrates. At low pH values the amount of undissociated acid, the effective form, increases (Table 8). The

antimicrobial action of undissociated acid is based on the inhibition of various enzymes in the microbial cell. The optimum pH ranges is between 3.0 and 6.5 (Lueck, 1980).

Sorbic acid and its salts can be added to cheese and cheese products, meat products, baked goods, beverages, fruit juices, wines, fish products, vegetables, jams and jellies, salads and fruit cocktails, dried fruits, pickles and pickled products, and margarine.

Propionic Acid and Its Salts

CH_3-CH_2COOR R = H, Na or $(CH_3-CH_2COO)_2Ca$

Propionates have been used in the USA on a large scale in the preservation of bread and on a small scale to preserve cheese for over 40 years. Propionic acid is an oily liquid having a slightly pungent, rancid odor. It is miscible with both water and alcohol. The sodium and calcium salts are white, free-flowing, readily soluble powders. The sodium propionate is more soluble, dissolving at the rate of 150 g/100 ml of water at 100°C (Furia, 1972).

The LD_{50} of propionic acid for rats is between 2.6 and 4.3 g/kg body weight. The acute toxicity of sodium and calcium propionate is in the same order of magnitude. The administration of a diet comprising 75% bread with 5% sodium propionate to testing animals over one year produced no sign of organic damage (Lueck, 1980). Propionic acid and its salts are listed in category A(1) by FAO/WHO without

specified ADI. Propionic acid and propionates are readily absorbed by the digestive tract. There is no risk of their accumulation in the body as they are metabolized in the body in a similar manner as other fatty acids. The final metabolites are H_2O and CO_2 (Lueck, 1980).

Propionic acid, sodium and calcium propionates are considered GRAS (21 CFR 182.3081, 182.3784, and 182.3221). No upper limits are imposed except for bread, rolls and cheese which come under Standard of Identity. Sodium and calcium propionates are limited to 0.32% of flour in white bread and rolls and 0.38% in the corresponding whole wheat products. In cheese products they are limited to 0.3% (Furia, 1972).

The inhibitory action of propionic acid and its salts is due to the fact that they accumulate in the cell and block metabolism by inhibiting enzymes. Like other preservatives the pH values of the substrates are of great importance to the antimicrobial action because of the amount of undissociated form (Table 8). Propionates are active against molds, but have no activity against yeasts with the optimum pH ranges between 2.5 and 5.0 (Lueck, 1980).

Propionates are mainly used in baked goods and dairy products.

CHAPTER III

MATERIALS AND METHODS

I. TEST ORGANISMS

Aspergillus sulphureus NRRL 4077 and Penicillium viridicatum NRRL 3711, kindly offered by the Northern Regional Research Laboratory, were used in this study. A. sulphureus NRRL 4077 was grown on yeast extract sucrose agar (2% yeast extract, 4% sucrose, 1.5% agar) slants and P. viridicatum NRRL 3711 was grown on potata dextrose agar slants for 2 weeks at 32°C. The cultures were maintained at 4°C.

II. GROWTH MEDIUM

The fungi were grown in 500 ml Erlenmeyer flasks containing 100 ml autoclaved yeast extract sucrose broth (YES) which contained 20 g yeast extract and 40 g sucrose in 1 liter distilled water or equivalent.

III. ANTIMICROBIAL FOOD ADDITIVES

Table 9 shows the antimicrobial food additives and concentrations used. Methyl paraben was obtained from Ueno Fine Chemical Industries, Inc. (Osaka, Japan), sodium propionate and sodium bisulfite were purchased from Fisher Scientific Company, and potassium sorbate was supplied by Tri-K Industries, Inc. (Westwood, N.J.). Appropriate amounts

Table 9. Antimicrobial Food Additives and Concentrations Used in Growth Studies.

Antimicrobial food additive	Concentration (%)	[a]Amount of food additive/100 ml solvent (g)
Na propionate	0	0
	0.1000	10.00
	0.2000	20.00
	0.3200	32.00
	0.4800	48.00
	0.6400	64.00
Methyl paraben	0	0
	0.0111	1.11
	0.0222	2.22
	0.0333	3.33
	0.0670	6.70
Na bisulfite	0	0
	0.0167	1.67
	0.0334	3.34
	0.0500	5.00
	0.0667	6.67
	0.0834	8.34
	0.1000	10.00
	0.1200	12.00
K sorbate	0	0
	0.0110	1.10
	0,0220	2.20
	0,0440	4.40
	0,0670	6.70
	0.1340	13.40

[a]One ml of solvent was added after autoclaving to 99 ml of YES broth in each treatment.

of antimicrobial food additives were dissolved in 100 ml solvent (Table 9) and used in this experiment. For sodium propionate, sodium bisulfite, and potassium sorbate, the solvent used was distilled water. Methyl paraben was dissolved in 75% ethanol due to its low solubility in water. The solutions were filtered sterilized by using a Millipore filter with 0.45 μm filter paper and vacuum, sealed and stored at 4°C until needed.

IV. GROWTH STUDIES IN YES BROTH WITH FOOD ADDITIVES

Two pH values, 4.5 and 5.5, were evaluated. Sodium propionate and potassium sorbate are salts of strong bases and weak acids. When they are dissolved in water, the pH is expected in the basic range. On the other hand, the pH of 0.04N sodium bisulfite solution is 4.56 (Skoog and West, 1976). Methyl paraben is a non-ionic compound. The pH values of the YES medium were adjusted according to Table 10 using 6N and 1N hydrochloric acid before adding food additives. The medium was sterilized at 121°C for 15 minutes. Food addtives were added by transfering 1 ml of the solutions made in section III to 99 ml YES broth. Conidial suspensions were prepared by scraping spores from the surface of 14-day-old YES or potato dextrose agar slants into 5 ml aqueous, sterile solution of Triton X (0.005%). The spores were counted in a Petroff-Hausser counting chamber. Using an Eppendorf pipet 4700 (Brinkmann Instrument, Inc.), the liquid medium was

Table 10. pH Changes of Autoclaved YES Broth before and after Adding Different Levels of Antimicrobial Food Additives.

Food additive	Concentration (%)	pH 4.5[a] Before	pH 4.5[a] After	pH 5.5[a] Before	pH 5.5[a] After
Na propionate	0	4.50	4.50[b]	5.50	5.50
	0.1000	4.50	4.88[b]	5.22	5.50
	0.2000	4.50	5.12[b]	4.99	5.50
	0.3200	4.50	5.27[b]	4.79	5.50
	0.4800	4.50	5.42[b]	4.59	5.50
	0.6400	4.50	5.55[b]	4.37	5.50
Methyl paraben	0	4.50	4.50	5.50	5.50
	0.0111	4.50	4.50	5.50	5.50
	0.0222	4.50	4.50	5.50	5.50
	0.0333	4.50	4.50	5.50	5.50
	0.0670	4.50	4.50	5.50	5.50
Na bisulfite	0	4.50	4.50	5.50	5.50
	0.0167	4.50	4.50	5.56	5.50
	0.0334	4.50	4.50	5.57	5.50
	0.0500	4.50	4.50	5.58	5.50
	0.0667	4.48	4.50	5.58	5.50
	0.0834	4.48	4.50	5.63	5.50
	0.1000	4.47	4.50	5.73	5.50
	0.1200	4.47	4.50	5.76	5.50
K sorbate	0	4.50	4.50	5.50	5.50
	0.0110	4.47	4.50	5.48	5.50
	0.0220	4.45	4.50	5.47	5.50
	0.0440	4.40	4.50	5.43	5.50
	0.0670	4.35	4.50	5.40	5.50
	0.1340	4.20	4.50	5.33	5.50

[a]Determined by Model 12 Research pH meter (Corning Scientific Instrument).

[b]With sodium propionate at pH 4.5 only in order to bring the final pH of YES broth down to 4.5, 1 ml of 0.7N, 1.4N, 2.24N, 4.34N, and 6.0N hydrochloric acid was added to YES medium after adding 0.1000, 0.2000, 0.3200, 0.4800, and 0.6400% sodium propionate, respectively. This was due to the fact that if the pH values of the YES broth were brought down before adding sodium propionate to give final pH of 4.5, the desired values were below 4.0 which caused precipitation of yeast extract.

inoculated by adding 10^7 spores of each strain. Triplicate flasks were prepared for each concentration. The cultures were incubated at 25°C for 14 days. Experiments were replicated twice.

V. TOXIN ASSAY

Extraction

Ochratoxin A was extracted from the broth following the procedures of Scott et al. (1971). The chloroform extracts were combined and dried by filtering through anhydrous sodium sulfate to remove any water. The chloroform was evaporated at 60°C using a flash evaporator (Rotovap^R) to dryness. The residue was reconstituted with 5 ml chloroform. After filtering through a 0.45 µm millipore filter, the samples were sealed in vials and quantitated by HPLC for ochratoxin A.

High Pressure Liquid Chromatography

The liquid chromatograph used was a Waters Associates Model 6000A (Water Associates Inc.) equipped with a Model 440 UV detector operating at 340 nm, Model 420-C and 420-E fluorescence detector fitted with a 360 nm excitation filter and a 440 nm cut-off emission filter, M6000 pump, and U6K septumless injector. A µ-Porasil (Waters Associates Inc.) column, a solvent system of benzene-acetic acid-methanol (90:10:5 v/v/v), and a 25 µm sample injection syringe (Hamilton Inc.) were also used. Chromatograms were recorded

on a Omniscribe recorder (Houston Instrument) with the chart speed of 0.2 inch/minute. The average retention time for ochratoxin A was around 3 minutes and 40 seconds when the flow rate was 1 ml/minute. Figure 8 shows the representative chromatograms of ochratoxin A.

Thin-layer Chromatography

Qualitative analysis of ochratoxin A by thin layer chromatography was also performed if needed. Samples were spotted on Redi (Fisher Scientific Company) plates which were precoated with silica gel G and activated at 90°C for 30 minutes. The TLC plates were developed with benzene-acetic acid (90:10 v/v).

Calculation *

5 mg of pure crystalline ochratoxin A were purchased from Sigma Co. (St. Louis, Mo). It was dissolved in 50 ml benzene as standard solution. It was kept at 0°C and in the dark to avoid degradation. Before injecting samples into HPLC, the final volume in the vials was measured.

$$\text{Toxin (mg/100 ml YES)} = \frac{(\text{Conc}_{STD})(H_{SAM})(G_{SAM})(V_{i,STD})(\text{Vol}_{TOT})}{(H_{STD})(G_{STD})(V_{i,SAM})}$$

Where Conc_{STD} = concentration of standard solution in mg/ml.

H = peak height in millimeter.

G = GAIN for fluorescence detector

V_i = volume injected into HPLC in µL.

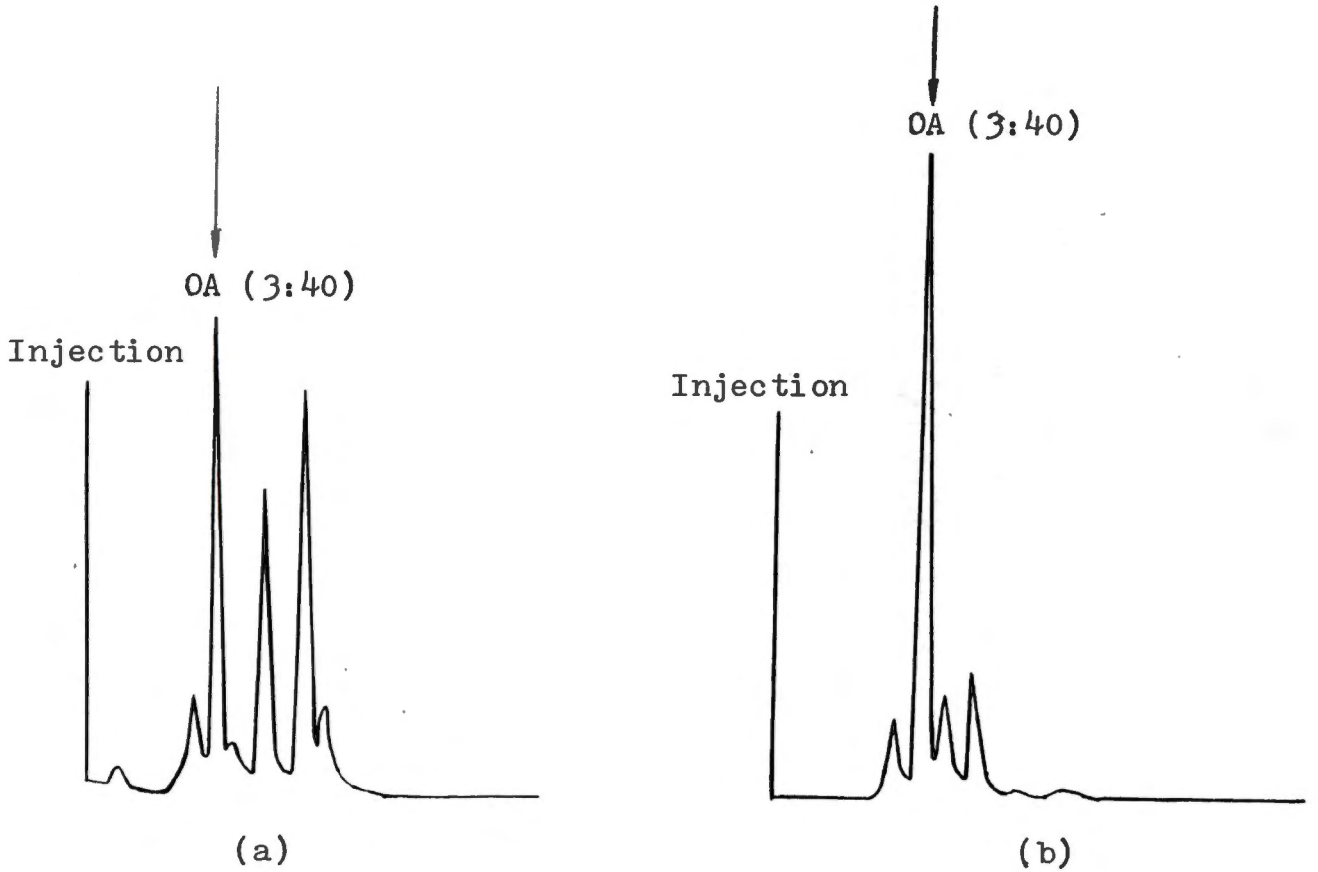


Figure 8. HPLC chromatogram of the metabolites of
(a) A. sulphureus NRRL 4077
(b) P. viridicatum NRRL 3711
grown in YES broth and extracted with chloroform.
Fluorescence detector was used.

STD = standard solution.

SAM = sample.

Vol_{TOT} = final volume in the vial in milliter.

VI. MYCELIAL GROWTH INHIBITION

After extraction, the mycelium was separated from the broth culture by filtering with a Buchner funnel using vacuum and Whatman #1 filter paper. The mycelial mat was transferred to a preweighed filter paper, dried at 100°C for 4 hours, and then weighed on a Mettler analytical balance to determine dried weight.

VII. STATISTICAL ANALYSIS AND EXPERIMENTAL DESIGN

An analysis of variance was performed to analyze data. The General Linear Model was used for the analysis of balanced or unbalanced data. When significant differences were found, the Duncan's Multiple Range Test was used to determine significant differences among means. The randomized block experimental design was used.

VIII. PRELIMINARY TESTS

Table 11 shows ochratoxin A production as affected by different levels of sugar. Sucrose was found a better source than glucose. 2% yeast extract and 4% sucrose was best for ochratoxin A production. Higher levels of glucose favored ochratoxin B production.

Table 11. Ochratoxin A and Ochratoxin B Production by Aspergillus ochraceus NRRL 3174 in Liquid Medium with 2% Yeast Extract and Different Levels of Sucrose and Glucose.

Concentration (%)	Sucrose*		Glucose*	
	Ochratoxin A (mg/100 ml)	Ochratoxin B (mg/100 ml)	Ochratoxin A (mg/100 ml)	Ochratoxin B (mg/100 ml)
4	0.33000	0.00173	0.04455	0.00131
6	0.05300	0.00185	0.01653	0.00124
8	0.01981	0.00196	0.00797	0.00269
10	0.01500	0.00207	0.00810	-
12	0.01808	0.00138	0.00860	0.00944
16	0.01519	0.00185	0.00655	0.01185
20	0.01885	0.00138	0.00422	0.01474

*The pH of the medium was 5.5. Incubation time = 14 days. Incubation temperature = 25°C.

Originally, the maximal levels for each food additive were going to be used in this study. Those were 0.1% for methyl paraben, 0.2% for potassium sorbate, 0.32% for sodium propionate, and 500 ppm for sodium bisulfite. Two more concentrations between controls and maximum permitted levels had also been picked up for each food additive. However, methyl paraben and potassium sorbate were found to completely inhibit fungal growth at a very low level. At pH 5.5, 500 ppm sodium bisulfite and 0.32% sodium propionate did not completely inhibit mold growth. Therefore, lower levels were chosen for methyl paraben and higher concentrations were tested for sodium bisulfite and sodium propionate (Table 9).

A study was performed to determine the reproducibility of the HPLC system used by injecting standard solution 10 times at concentrations of 0.1, 0.05, 0.01, 0.005, and 0.001 mg/ml.

CHAPTER IV

RESULTS AND DISCUSSIONS

I. PRELIMINARY TESTS

Growth Medium

As shown in a previous chapter (Table 11), ochratoxin A production depended upon level and type of sugar. It was found that peak ochratoxin A production occurred in growth medium containing 4% sucrose and 2% yeast extract. Sucrose supported ochratoxin A formation better than glucose. The amount of ochratoxin A produced was inversely proportional to sugar concentrations while ochratoxin B increased slightly with increasing sugars.

Another reason for choosing the 2% yeast extract and 4% sucrose as the growth medium was that Gooding et al. (1955) reported that salts and sugars have marked synergistic effect on sorbic acid fungistatis. In designing research to study the antifungal activity of potassium sorbate when applied to grains, lower sugar concentrations are preferred since most grains contain only low levels of sugar. Thus, the YES broth with 2% yeast extract and 4% sucrose used in this study provides a good model of a grain since the sugar level was low.

Separation, Resolution, and Reproducibility of Ochratoxin A by HPLC

Throughout this experiment, peak heights were used to calculate the concentration of ochratoxin A. It was important to get sharp, symmetric, and well-separated peaks for quantitative determination. Several combinations of solvent systems, columns and detectors were tested with the HPLC. Initially, a C-18 reverse phase column was tested with the mobile solvent of acetonitrile:water:acetic acid 55:45:2 (v/v/v). Both a UV detector operating at 340 nm and a fluorescence detector with a 360 nm excitation filter and a 440 nm emission filter were used. It was found that the fluorescence detector had higher sensitivity and better reproducibility than the UV detector. The fluorescence detector also had the advantage of omitting some interfering peaks because only substances which contain an electron donating group or multiple conjugated bonds would be picked up by fluorescence detector (Pomeranz and Meloan, 1978). However, there still were metabolites in the sample solution which resulted in incomplete separation of the toxin peak. On TLC plates, ochratoxin A was well-separated from other metabolites using the developing solvent of benzene:acetic acid 90:10 (v/v). The same solvent could also be feasible on HPLC. Therefore, it was decided to try a μ-Porasil polar phase column with benzene:acetic acid 90:10 as the mobile phase to elute the ochratoxin A. Because of its greater sentivity, the fluorescence detector was used. The standards were found to give sharper, better peaks with this system

when compared to the C-18 column acetonitrile:water:acetic acid 55:45:2 system. For samples, all peaks separated satisfactorily. Nevertheless, the retention time for ochratoxin A was 13 minutes and 15 seconds when the flow rate was 1 ml/minute. With this retention time, it could take more than 20 minutes for one injection. In order to decrease the retention time, the polarity of the mobile phase was increased by changing the ingredients to benzene:acetic acid:methanol 90:10:5 (v/v/v). Under this system, the peak for ochratoxin A was sharp and symmetrical. The retention time was changed to approximately 3 minutes and 40 seconds. Although the resolution also decreased with increasing polarity, the separation was still satisfactory for quantitation (Figure 8). Table 12 shows the reproducibility of the HPLC system used by injecting standard solutions of ochratoxin A at different concentrations 10 times. The coefficient of variation ranged from 2.4 to 6%.

II. MYCELIAL INHIBITION

Figures 9 to 12 show the effects of potassium sorbate, methyl paraben, sodium bisulfite, and sodium propionate on growth of A. sulphureus NRRL 4077 and P. viridicatum NRRL 3711 in YES broth at two pH values. The ability of the food additives to inhibit mycelium production varied widely. However, by analysis of variance, a significant decrease ($p<.05$) in mycelial weight was obtained with the addition

Table 12. Reproducibility of HPLC System with a μ-Porasil Column and a Solvent System of Benzene:Acetic Acid:Methanol 90:10:5 at Flow Rate of 1 ml/ Minute.

Concentration of standard solution (mg/ml)	Mean[a]	Standard deviation of mean	C.V.
0.1	219.5[b]	6.451	2.94
0.05	178.3	4.234	2.38
0.01	124.2	7.435	5.99
0.005	130.3	6.885	5.28
0.001	124.2	6.888	5.55

[a]Mean of peak height of 10 injections.

[b]The peak height is subject to change with changing the volume of injection and the GAIN of the fluorescence detector.

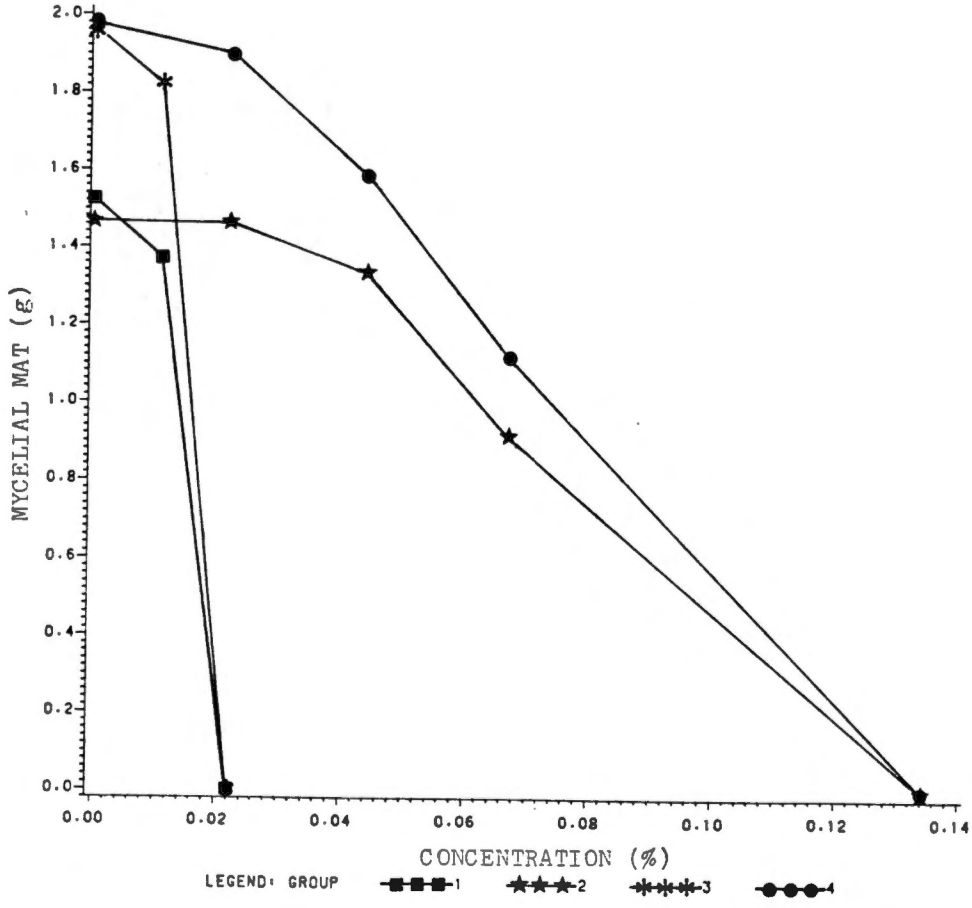


Figure 9. Effects of potassium sorbate on growth of A. sulphureus NRRL 4077 and P. viridicatum NRRL 3711 in 100 ml yeast extract sucrose broth incubated at 25°C for 2 weeks.

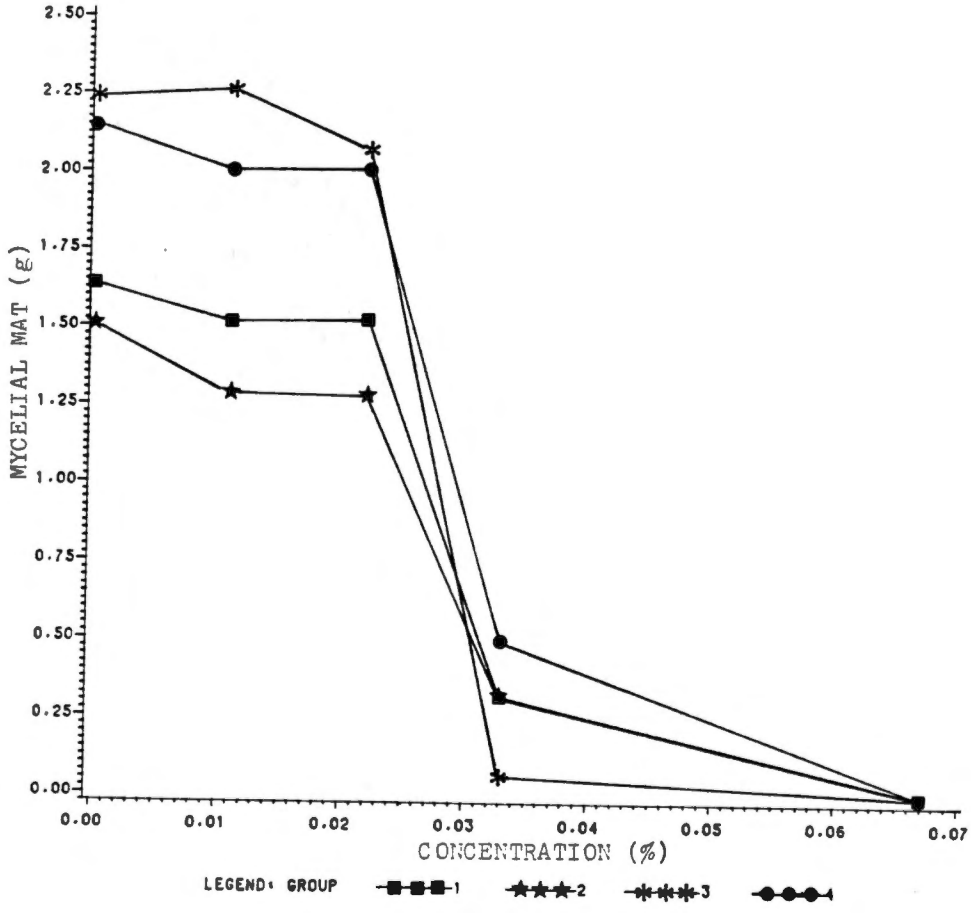


Figure 10. Effects of methyl paraben on growth of A. sulphureus NRRL 4077 and P. viridicatum NRRL 3711 in 100 ml yeast extract sucrose broth incubated at 25°C for 2 weeks.

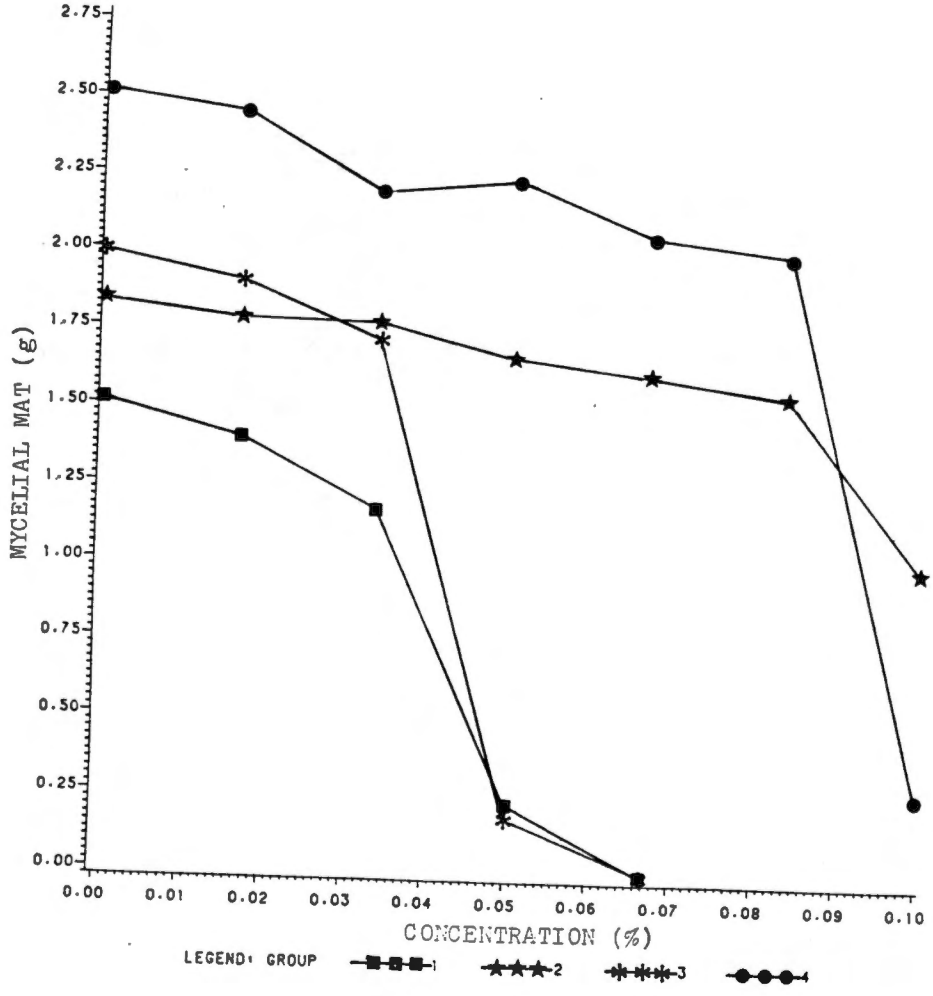


Figure 11. Effects of sodium bisulfite on growth of A. sulphureus NRRL 4077 and P. viridicatum NRRL 3711 in 100 ml yeast extract sucrose broth incubated at 25°C for 2 weeks.

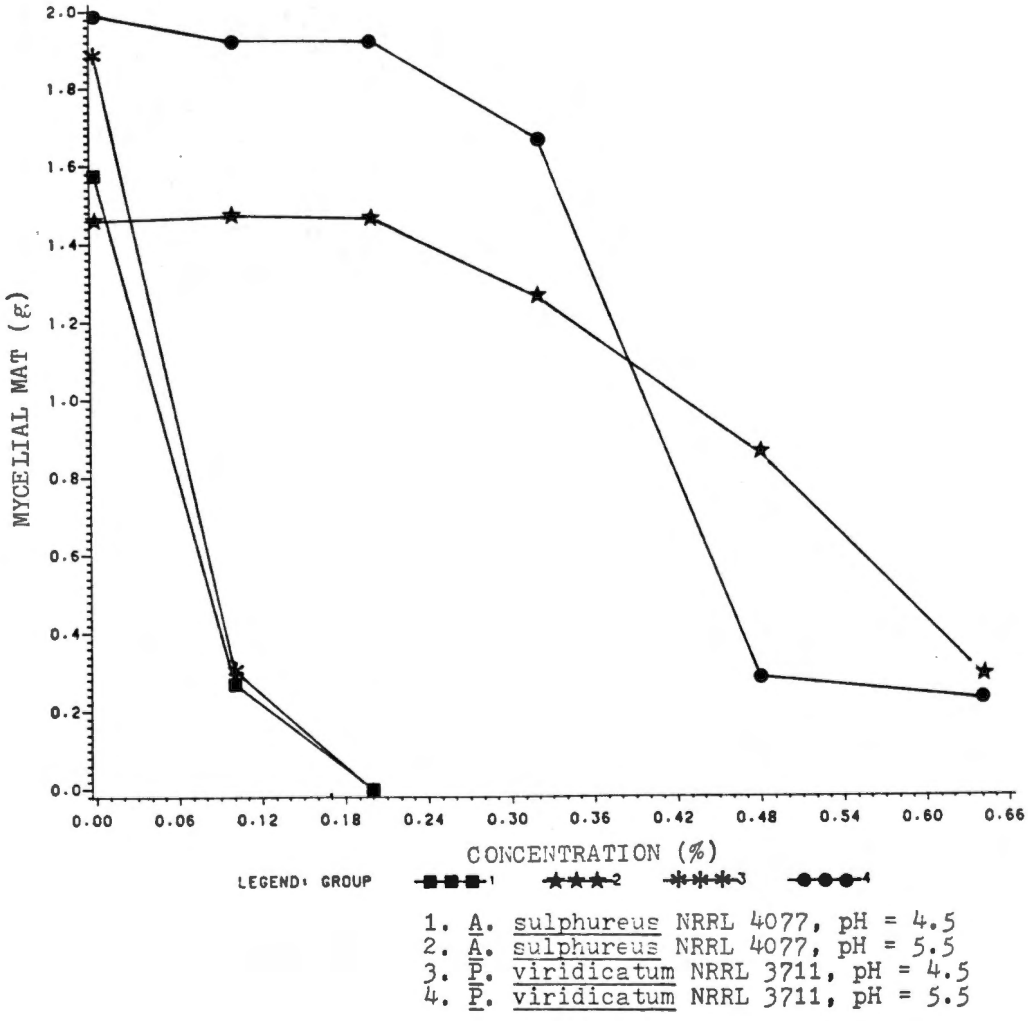


Figure 12. Effects of sodium propionate on growth of A. sulphureus NRRL 4077 and P. viridicatum NRRL 3711 in 100 ml yeast extract sucrose broth incubated at 25°C for 2 weeks.

of all four antimicrobials (Appendix Tables A-1 to A-4). Potassium sorbate was the most effective antimicrobial in this study for reducing fungal growth at lower pH values since only 1/10 of the maximum permitted level was required to get 100% inhibition. However, it was found to completely inhibit fungal growth at 0.134%, 2/3 of the regulatory level, when the pH of the broth was 5.5. Originally, 0, 0.022, 0.044, 0.067, 0.134% were studied for potassium sorbate. Nevertheless, mycelium production was not detected even at 0.022% at pH 4.5. Therefore, one additional level, 0.011%, was selected. At pH 4.5, most of the inhibition occurred between 0.011 to 0.022%. It was found that the effectiveness of potassium sorbate at pH 4.5 was approximately seven times higher than that of pH 5.5 according to level used and percentage inhibition.

Methyl paraben was also found 100% effective in inhibition of mycelial growth at 2/3 of the maximum permitted level, 0.1%. The growth inhibition patterns were similar with methyl paraben regardless of the pH values (Figure 10). A significant decrease in weight of mycelial mat began with 0.033% methyl paraben.

The levels studied for sodium bisulfite and sodium propionate were up to twice as much as the maximum permitted levels for foods due to their poor effectiveness at pH 5.5. The percentages of inhibition for sodium bisulfite at the maximum regulatory level at pH 5.5 were: A. sulphureus NRRL NRRL 4077, 8.95%; P. viridicatum NRRL 3711, 10.64%; those at 0.1% were: A. sulphureus NRRL 4077, 45.45%; P. viridicatum

3711, 89.38% (Table A-3). It was found that sodium bisulfite was the only food additive that did not completely retard fungal growth at the maximum permitted level, 500 ppm, at pH 4.5. The percentages of inhibition were 85.16% for A. sulphureus NRRL 4077 and 90.89% for P. viridicatum NRRL 3711. In order to obtain 100% inhibition, 667 ppm sodium bisulfite was required at pH 4.5. For sodium bisulfite, significant decreases in mycelial weight occurred between 334 to 500 ppm at pH 4.5 and 834 to 1000 ppm at pH 5.5, respectively (Figure 11). The reduced antifungal activity of sodium bisulfite at pH 5.5 vs. 4.5 is typical of antimicrobials which are most active in the undissociated form.

The practical use of sodium propionate as fungal inhibitor was significantly related to pH. At the maximum permitted level, there was only 13.31% inhibition for A. sulphureus NRRL 4077 at pH 5.5 compared to 100% at pH 4.5 and similiarly 16.04% compared to 100% for P. viridicatum NRRL 3711. At the concentration of 0.64% sodium propionate, 79.91 and 88.45% inhibition was achieved at pH 5.5 for A. sulphureus NRRL 4077 and P. viridicatum NRRL 3711, respectively (Table A-4).

P. viridicatum NRRL 3711 was found to produce more mycelium than A. sulphureus NRRL 4077. It was also found that both strains produced more mycelial mat at pH 4.5 than that at pH 5.5. Similiar results were reported by Lai et al. (1970). Generally, the inhibition patterns for antimicrobial

food additives were almost identical for A. sulphureus NRRL 4077 and P. viridicatum NRRL 3711 at pH 4.5. However, at pH 5.5 and at higher concentrations, the former was found more resistant to the antimicrobials than the latter Figure 9 to 12).

III. TOXIN INHIBITION

Figure 13 to 16 show the effects of antimicrobial food additives on ochratoxin A production by A. sulphureus NRRL 4077 and P. viridicatum NRRL 3711 in YES broth at two pH values. Again, by analysis of variance, the addition of food additives resulted in a significant decrease in ochratoxin A production when tested at the 5% significance level (Appendix Table A-5 to A-8). Actually, the ochratoxin A decrease followed a logarithmic scale. The major decreases occurred between controls and the lowest levels tested for each additive at both pH values except sodium bisulfite at pH 5.5. The overall percentages of inhibition at the maximum permitted levels levels were: potassium sorbate, 100% for both molds and pH values; methyl paraben, 100% for both fungi and pH values; sodium bisulfite, 99.78% for A. sulphureus NRRL 4077 at pH 4.5, 60.35% for A. sulphureus NRRL 4077 at pH 5.5, 99.06% for P. viridicatum NRRL 3711 at pH 4.5, and 58.95% for P. viridicatum NRRL 3711 at pH 5.5; sodium propionate, 100% for both molds at pH 4.5, 87.17% for A. sulphureus NRRL 4077 at pH 5.5, and 94.68% for

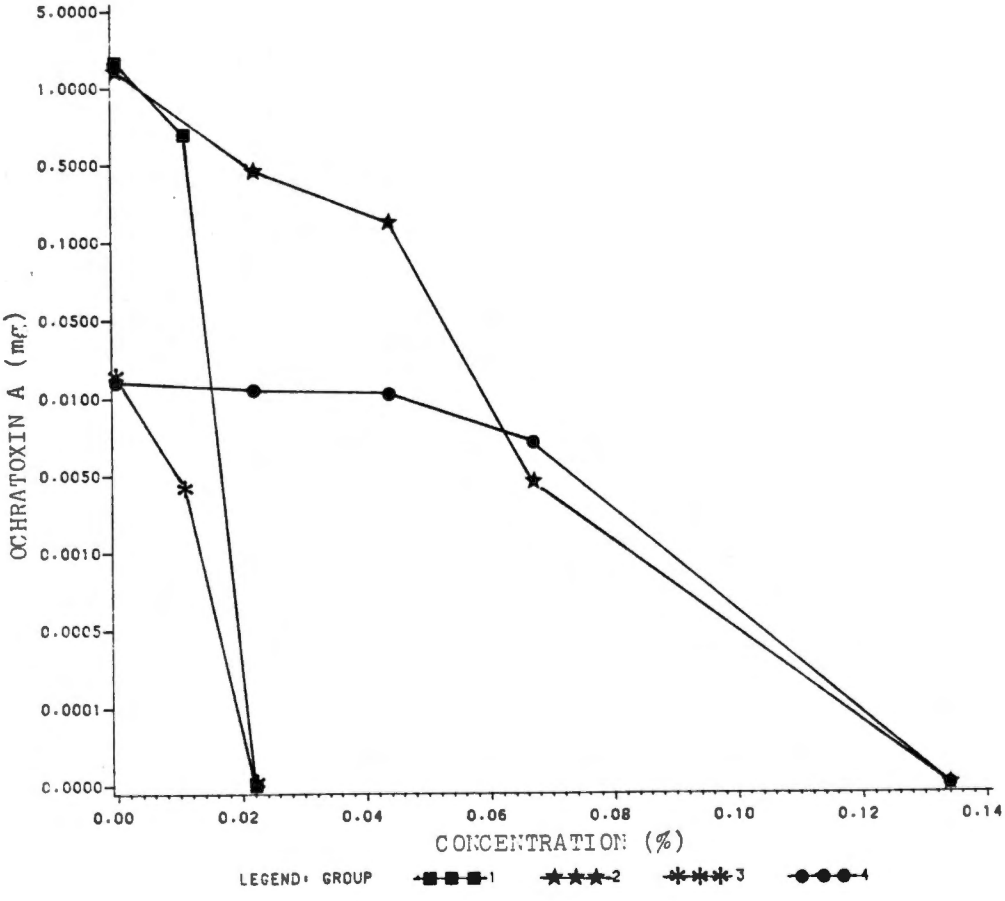


Figure 13. Effects of potassium sorbate on ochratoxin A production by A. sulphureus NRRL 4077 and P. viridicatum NRRL 3711 in 100 ml yeast extract sucrose broth incubated at 25°C for 2 weeks.

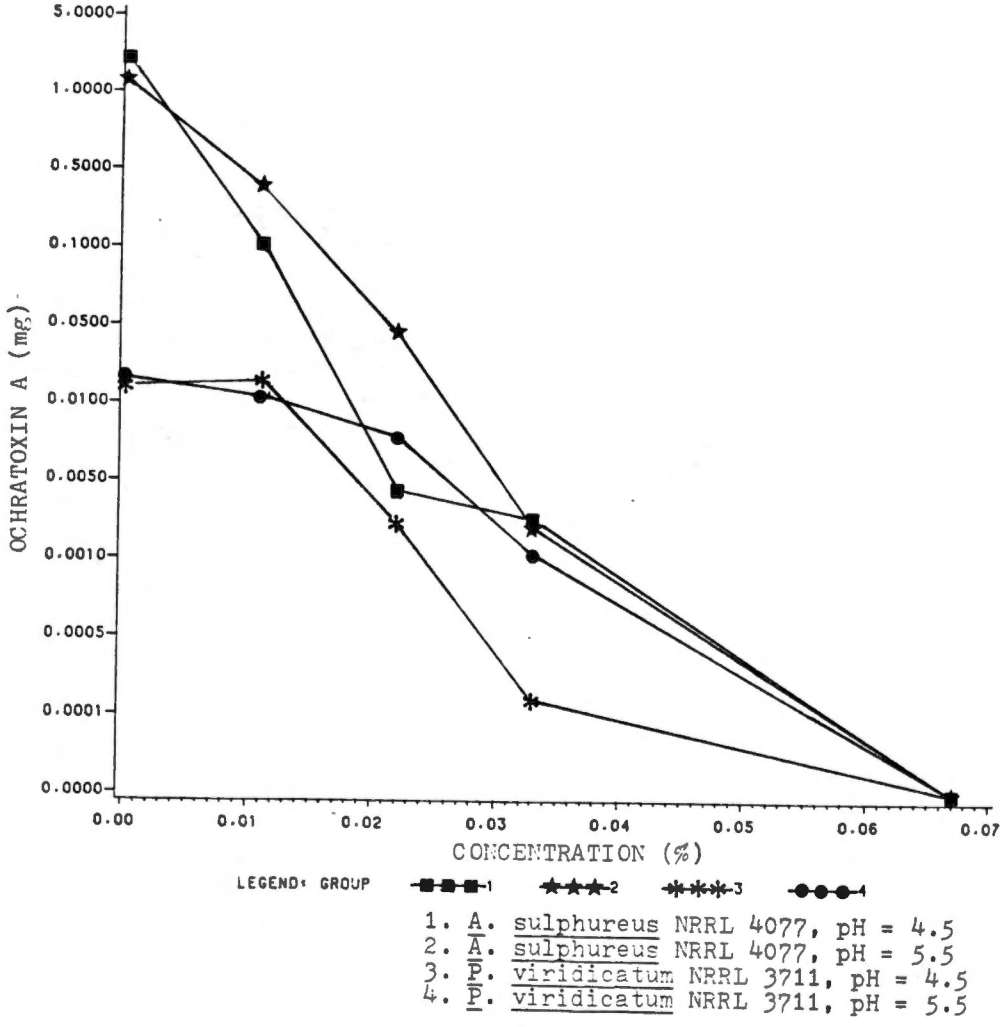


Figure 14. Effects of methyl paraben on ochratoxin A production by A. sulphureus NRRL 4077 and P. viridicatum NRRL 3711 in 100 ml yeast extract sucrose broth incubated at 25°C for 2 weeks.

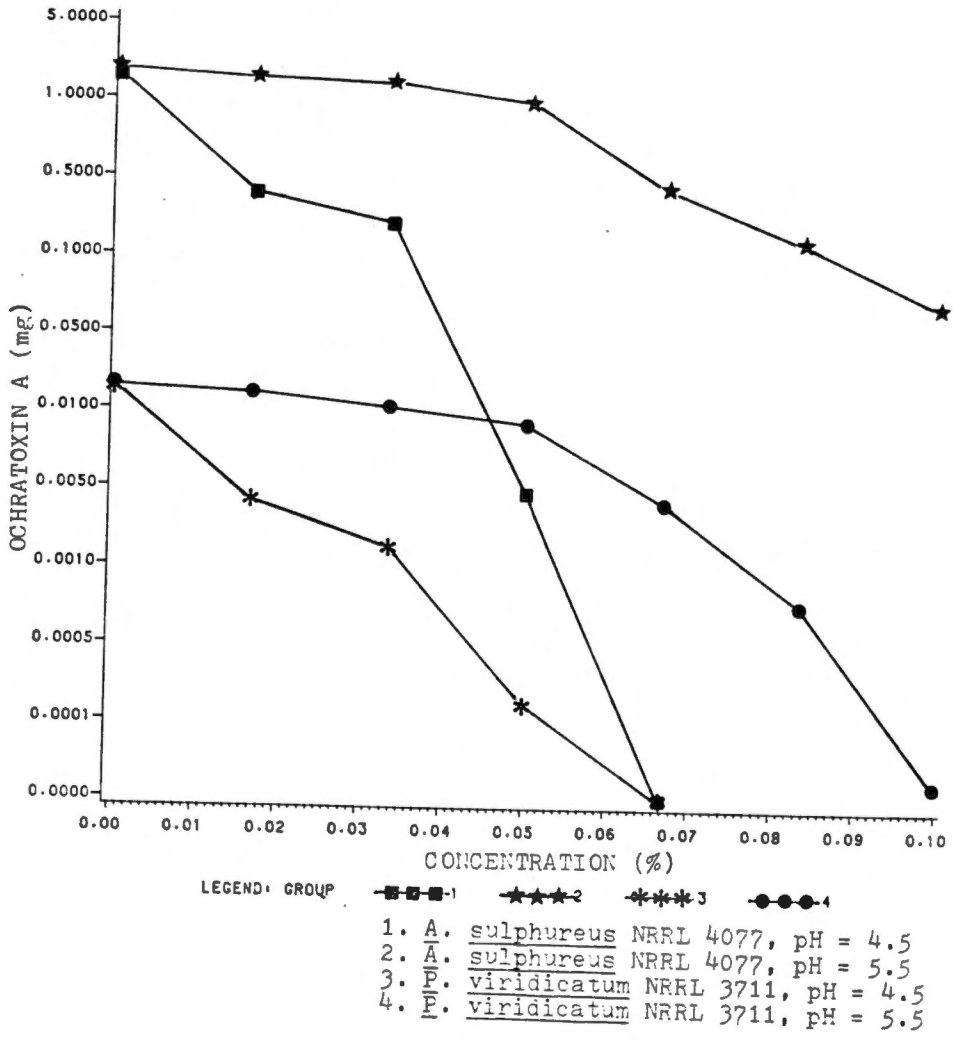


Figure 15. Effects of sodium bisulfite on ochratoxin A production by A. sulphureus NRRL 4077 and P. viridicatum NRRL 3711 in 100 ml yeast extract sucrose broth incubated at 25°C for 2 weeks.

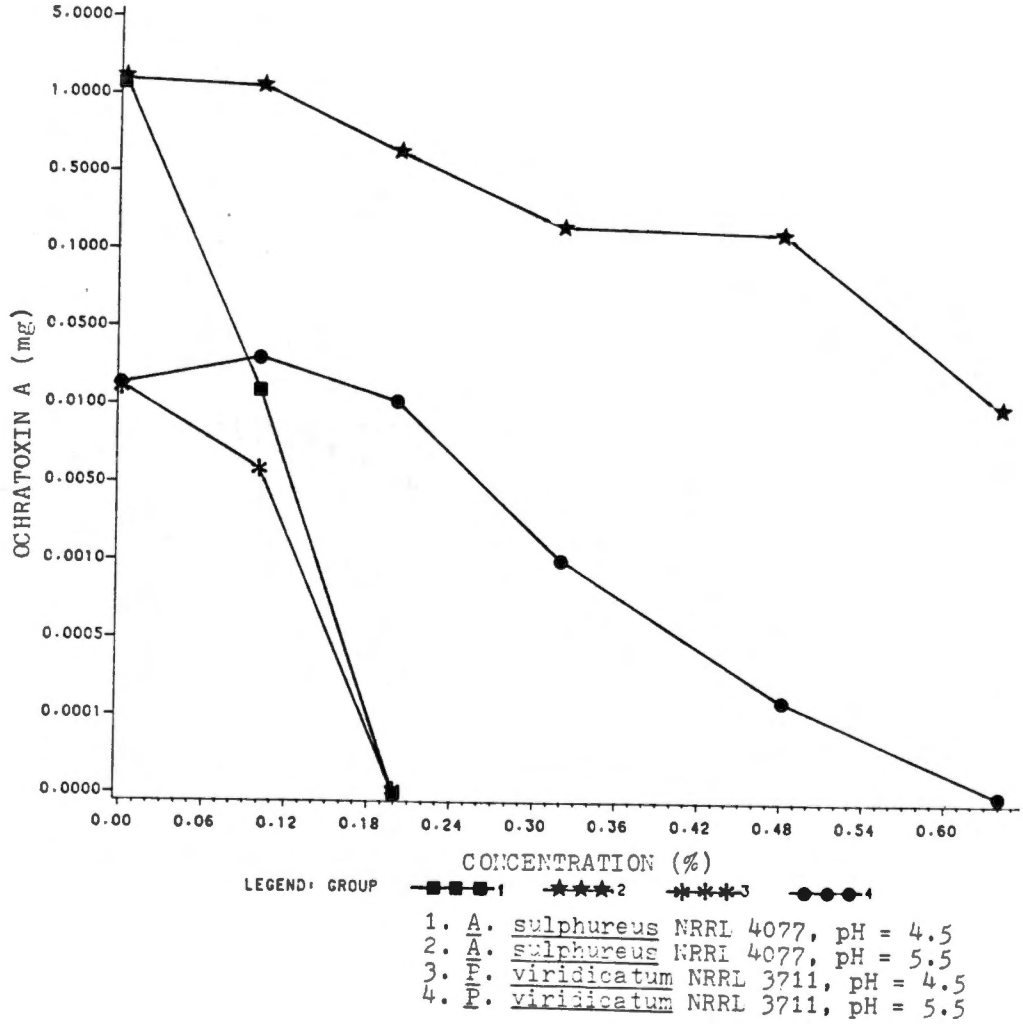


Figure 16. Effects of sodium propionate on ochratoxin A production by A. sulphureus NRRL 4077 and P. viridicatum NRRL 3711 in 100 ml yeast extract sucrose broth incubated at 25°C for 2 weeks.

P. viridicatum NRRL 3711 at pH 5.5 (Tables A-5 to A-8). By increasing the concentrations of sodium bisulfite and sodium propionate to 0.1 and 0.64%, more than 90% inhibition in ochratoxin A production was achieved for both strains and pH values.

Generally, toxin production decreased as mycelium formation decreased. However, inhibition of toxin was more significant than mycelial inhibition by antimicrobials. Although the reason for this has not been shown experimentally, it is a logical finding since toxin biosynthesis uses the excess acetate and malonate formed by primary biosynthesis. Thus, even small changes in primary biosynthesis can have large effects on secondary biosynthesis.

A. sulphureus NRRL 4077 produced about 100 times more ochratoxin A than P. viridicatum NRRL 3711 while the latter produced more mycelial weight than the former in YES broth without antimicrobial food additives. Therefore, it was not surprising to find that the overall percentages of inhibition for A. sulphureus NRRL 4077 were higher than those of P. viridicatum NRRL 3711. Lai et al. (1970) reported that pH 5.5 is better for ochratoxin A production than pH 4.5. Nevertheless, in this study, no significant difference in ochratoxin A formation was observed as a result of pH difference when control flasks were compared at pH 4.5 and 5.5. It was also found that the standard deviations of toxin means were much higher than the standard deviations of mycelium means. The

large variation for toxin could be due to the fluctuation of incubation temperature, extraction procedures, HPLC quantitation (Table 12), and biological differences. Despite a report by Chu and Butz (1970) that ochratoxin A is very stable when stored at refrigeration temperatures, the degradation of standard solution for HPLC determinations could also be a factor contributing to the variation.

IV. FINAL COMMENTS

Trenk et al. (1971) indicated that ochratoxin A can persist in foods even after 3 hours of autoclaving. The removal of ochratoxin A would be difficult once foods are contaminated and the best protection would be to prevent toxin formation through proper drying and addition antimicrobial agents. The effectivenesses of four antimicrobial food additives and the effects of pH values were evaluated in YES broth to provide future applications in grains.

It is well known that the activity of antimicrobial food additives, except for the parabens, is dependent upon pH. Usually, the lower the pH, the higher the effectiveness. The optimum pH range for sodium propionate and sodium bisulfite is between 2.5 to 5.0 and between 3.0 to 6.5 for potassium sorbate. Parabens are effective at above pH 7 (Furia, 1972). In this study, as expected, potassium sorbate and methyl paraben were effective for inhibiting fungal growth at low concentrations at both pH 4.5 and 5.5.

Although the level required to completely inhibit fungal growth and ochratoxin A production was same for methyl paraben at pH 4.5 and 5.5, the percentages of inhibition were still affected by pH. Sodium propionate failed to inhibit growth and toxin production by A. sulphureus NRRL 4077 and P. viridicatum NRRL 3711 at the maximum permitted level and twice the maximum regulatory level at pH 5.5. Vandergraft et al. (1975) reported that no ochratoxin A was detected in corn when the corn was treated with 1% propionic acid. The pH of corn is around pH 6.0 (Ockerman, 1978). A level between 0.64% and 1% is expected to have 100% in inhibition of ochratoxin A production. Sodium bisulfite can not feasibly be added into foods since its strong odor is detectable when the level exceeds 500 ppm. However, it could be applied to animal feeds to inhibit ochratoxin production because of low toxicity and low cost.

One thing that must be pointed out here is that in this experiment, the optimum conditions for mycelium growth and ochratoxin A production were actually used. They were:

(1) medium---YES broth with 2% extract and 4% sucrose (Table 11; Davis et al., 1969).

(2) incubation temperature---25°C (Bacon et al., 1973; Northolt et al., 1979; Trenk et al., 1971; Harwig and Chen, 1974).

(3) incubation time---14 days. Although 8 to 12 days were usually reported as the optimum time for ochratoxin A

formation, there was no significant drop when incubation was extended to 14 days (Sansing et al. 1973; Lai et al., 1970; Trenk et al., 1971). The antimicrobial action for most antifungal food additives is mainly fungistatic It may require longer incubation period to reach peak toxin production.

(4) pH---pH 4.5 and 5.5 were evaluated. As mentioned before, pH 5.5 is best for mycelial formation and is also good for ochratoxin A production (Lai et al., 1970).

(5) amount of inoculum---10^7 spores which was unusually high compared to natural occurrence of mycotoxin-producing fungi (Bothast et al., 1978).

For the above reasons, it can be assumed that if ochratoxin A and mycelial production were inhibited under optimum growth conditions, they can also be inhibited under other conditions.

Finally, there are several studies that need to be carried out to look at other aspects of this experiment. One study needs to increase the levels of sodium propionate and sodium bisulfite to get 100% inhibition in both mycelium and toxin production at pH 5.5. This same experimental design should be repeated using corn or grains as substrate to determine the difference between YES broth and natural substrates. The pH values for corn and some grains are in the range of 5.5 to 6.0 (Ockerman, 1978). To get 100% inhibition, higher levels of antimicrobials would probably

be required. The interactions of food additives with food components and the way to apply food additives into grains or foods should also be considered.

CHAPTER V

CONCLUSIONS

This experiment has provided an initial evaluation of the effectiveness of potassium sorbate, methyl paraben, sodium propionate, and sodium bisulfite in inhibiting growth and toxin production by A. sulphureus NRRL 4077 and P. viridicatum NRRL 3711 in artificial culture medium. Both potassium sorbate and methyl paraben were found 100% effective against fungal growth and ochratoxin A production in YES broth below their maximum permitted levels in food when tested at pH 4.5 and 5.5. The effectiveness of sodium propionate was significantly affected by pH. It can be applied most effectively at low pH values. Sodium bisulfite appeared to be the least feasible for control of ochratoxin-producing fungi.

Antimicrobials were ranked as to effectiveness by comparing the level required for complete inhibition in ochratoxin A production to the maximal level permitted in food. At pH 4.5, the most effective one was potassium sorbate, followed by sodium propionate, methyl paraben, and sodium bisulfite, respectively. However, at pH 5.5, the most effective ones were methyl paraben and potassium sorbate, followed by sodium propionate and sodium bisulfite.

For foods having pH around 5.0, such as rye and sorghum, the antimicrobial of choice would be methyl paraben

or potassium sorbate. Whereas, corn silage and similar products which have pH around 4.5 could be treated with methyl paraben, potassium sorbate, or sodium propionate. Future studies should estimate the levels of the above antimicrobial food additives needed for complete inhibition of ochratoxin A production in grains.

For HPLC determination, it was found that the fluorescence detector had better reproducibility, sensitivity, and resolution. A μ-Porasil column and a benzene:acetic acid: methanol 90:10:5 (v/v/v) mobile phase at a flow rate of 1 ml/minute was used because this system resulted in good resolution and short retention time of ochratoxin A.

LITERATURE CITED

LITERATURE CITED

Aalund, O., K. Brunfeldt, B. Hald, P. Krogh, and K. Poulsen. 1975. A radioimmunoassay for ochratoxin A: A preliminary investigation. Acta Path. Microbiol. Scand. C 83:390-392.

Abedi, Z. H. and P. M. Scott. 1969. Detection of toxicity of aflatoxins, sterigmatocystin and other fungal toxins by lethal action on Zebra fish larvae. J.A.O.A.C. 52:963-969.

Bacon, C. W., J. G. Sweeney, J. D. Bobbins, and D. Burdick. 1973. Production of penicillic acid and ochratoxin A on poultry feed by Aspergillus ochraceus: Temperature and moisture requirements. Appl. Microbiol. 26:155-160.

Balzer, I., C. Bogdanic, and S. Pepeljnjak. 1978. Rapid thin layer chromatographic method for determing aflatoxin B_1, ochratoxin A, and zearalenone in corn. J.A.O.A.C. 61:584:585.

Bothast, R. J., L. R. Beuchat, B. S. Emswiler, M. G. Johnson, and M. D. Pierson. 1978. Incidence of airborne Aspergillus flavus spores in cornfield of five states. Appl. Environ. Microbiol. 35:627-628.

Broce, D., R. M. Grodner, R. L. Killebrew, and F. L. Bonner. 1970. Ochratoxin A and B confirmation by microbiological assay using Bacillus cereus mycoides. J.A.O.A.C. 53:616-619.

Brown, R. F. 1969. The effect of some mycotoxins on the brine shrimp, Artemia salina. J. Amer. Oil Chem. Soc. 46:119.

Brown, M. H., G. M. Szczech, and B. P. Purmalis. 1976. Teratogenic and toxic effects of ochratoxin A in rats. Toxicol. Appl. Pharmacol. 37:331-338.

Bullerman, L. B. 1979. Significance of mycotoxins to food safety and human health. J. Food Protect. 42:65-86.

Bullerman, L. B. and T. E. Hartung. 1973. Mycotoxin-producing potential of molds isolated from flour and bread. Cereal Sci. Today 18:346-347.

Cardeilhac, P. T., K. P. C. Nair, and W. M. Colwell. 1972. Tracheal organ cultures for the bioassay of nanogram quantities of mycotoxins. J.A.O.A.C. 55:1120-1121.

Chang, F. C. and F. S. Chu. 1977. The fate of ochratoxin A in rats. Fd. Cosmet. Toxicol. 15:199-204.

Choudhury, H. and C. W. Carlson. 1973. The lethal dose of ochratoxin for chick embryos. Poultry Sci. 52: 1202-1203.

Choudhury, H., C. W. Carlson, and G. Semeniuk. 1971. A study of ochratoxin toxicity in hens. Poultry Sci. 50:1855-1859.

Christensen, C. M., G. H. Nelson, C. J. Microcha, and F. Bates. 1968. Toxicity of experimental animals of 943 isolates od fungi. Cancer Res. 28:2293-2295.

Christensen, C. M., H. A. Fanse, G. H. Nelson, F. Bates, and C. J. Mocrocha. 1967. Microflora of black and red pepper. Appl. Microbiol. 15:622-626.

Chu, F. S. 1971. Interaction of ochratoxin A with bovine serum albumin. Arch. Biochem. Biophys. 147:359-366.

Chu, F. S. 1974a. A comparative study of the interaction of ochratoxins with bovine serum albumin. Biochem. Pharmacol. 23:1105-1113.

Chu, F. S. 1974b. Studies of ochratoxins. CRC Crit. Rev. Toxicol. 2:499-524.

Chu, F. S. and M. E. Butz. 1970. Spectrophotofluorodensitometric measurement of ochratoxin A in cereal products. J.A.O.A.C. 53:1253-1257.

Chu, F. S. and C. C. Chang. 1971. Sensitivity of chicks to ochratoxins. J.A.O.A.C. 54:1032-1034.

Chu, F. S., F. C. C. Chang, and R. D. Hinsdill. 1976. Production of antibody against ochratoxin A. Appl. Environ. Microbiol. 31:831-835.

Chu, F. S., I. Noh, and C. C. Chang. 1972. Structural requirements of ochratoxin intoxication. Life Sci. 11:503-508.

Chu, F. S., C. C. Chang, S. H. Ashoor, and N. Prentice. 1975. Stability of aflatoxin B_1 and ochratoxin A in brewing. Appl. Microbiol. 29:313-316.

Ciegler, A. 1972. Bioproduction of ochratoxin A and penicillic acid by members of Aspergillus ochraceus group. Can. J. Microbiol. 18:631-636.

Davis, N. D., J. W. Searcy, and U. L. Diener. 1969. Production of ochratoxin A by Aspergillus ochraceus in a semisynthetic medium. Appl. Microbiol. 17: 742-744.

Davis, N. D., R. E. Wagener, D. K. Dalby, G. Morgan-Jones, and U. L. Diener. 1975. Toxigenic fungi in food. Appl. Microbiol. 30:169-171.

Dickens, F. and M. B. Waynforth. 1968. Studies on carnogenesis of lactones and related substances. Brit. Empire Cancer Camp. 46:108.

Doster, R. C. and R. O. Sinnhuber. 1972. Comparative rates of hydrolysis of ochratoxin A and B in vitro. Fd. Cosmet. Toxicol. 10:389-394.

Doster, R. C., R. O. Sinnhuber, and J. H. Wales. 1972. Acute intraperitoneal toxicity of ochratoxin A and B in rainbow trout (Salmo gairdneri). Fd. Cosmet. Toxicol. 10:85-92.

Doupnik, B. Jr. and D. K. Bell. 1971. Toxicity to chicks of Aspergillus and Penicillium species isolated from moldy pecans. Appl. Microbiol. 21:1104-1106.

Doupnik, B. Jr. and J. C. Peckham. 1971. Mycotoxicity of Aspergillus ochraceus to chicks. Appl. Microbiol. 19:594-597.

El-Helaly, A. F., H. Elarosi, M. W. Assawah, and A. Kilani. 1962. Studies on fungi associated with onion crop in the field and during storage. Phytopathol. Medit. 2:37-45.

Elling, F., B. Hald, C. Jacobsen, and P. Krogh. 1975. Spontaneous toxic nephropathy in poultry associated with ochratoxin A. Acta Path. Microbiol. Scand. A 83:739-741.

Engstrom, G. W., J. L. Richard, and S. J. Cysewski. 1977. High-pressure liquid chromatographic method for detection and resolution of rubratoxin, aflatoxin, and other mycotoxins. J. Agric. Food Chem. 25:833-836.

Epply, R. M. 1968. Screening method for zearalenone, aflatoxin, and ochratoxin. J.A.O.A.C. 51:74-78.

Escher, F. E., P. E. Koehler, and J. C. Ayres. 1973. Production of ochratoxin A and B on country cured ham. Appl. Microbiol. 26:27-30.

FAO/WHO. 1979. Guide to Safe Use of Food Additives. Food and Agriculture Organization of the United Nations World Health Organization, pp. 63-65, Printed in Italy.

Fischbach, H. and J. V. Rodricks. 1973. Current effects of the Food and Drug Administration to control mycotoxins in food. J.A.O.A.C. 56:767-770.

Food Chemicals Codex. 1972. 2nd. edition. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection National Research Council, National Academy of Science, Washington, D. C.

Furia, T. E. 1972. Handbook of Food Additives. 2nd. edition. pp. 115-184, editor Chichester, D. F. and F. W. Tanner, CRC Press, Cleveland, Ohio.

Galtier, P. 1974a. The fate of ochratoxin A in the animal organism. I. Transport of the toxin in the blood of the rat. Ann. Rech. Veter. 5:311-318.

Galtier, P. 1974b. The fate of ochratoxin A in the animal organism. II. Distribution in the tissues and excretion in the rat. Ann. Rech. Veter. 5:319-328.

Galtier, P., J. More, and M. Alvinerie. 1976. Acute and short term toxicity of ochratoxin A in 10 day-old chicks. Fd. Cosmet. Toxicol. 14:129-131.

Galtier, P., J. More, and G. Bodin. 1974. Toxins of Aspergillus ochraceus Wilhelm. III. Acute toxicity of ochratoxin A in adult rats and mice. Ann. Rech. Veter. 5:233-247.

Gilani, S. H., J. Bancroft, and M. Reily. 1978. Teratogenicity of ochratoxin A in chick embryos. Toxicol. Appl. Pharmacol. 46:543-546.

Gimeno, A. 1979. Thin layer chromatographic determination of aflatoxins, ochratoxins, sterigmatocystin, zearalenone, citrinin, T-2 toxin, diacetoxyscirpenol, penicillic acid, patulin, and penitrem A. J.A.O.A.C. 62:579-585.

Golinski, P. and J. Chelkowski. 1978. Spectral behavior of ochratoxin A in different solvents. J.A.O.A.C. 61: 586-589.

Gooding, C. M., D. Melnick, R. L. Lawrence, and F. H. Luckmann. 1955. Sorbic acid as a fungistatic agent for foods. IX. Physiochemical considerations in using sorbic acid to pretect foods. Food Res. 20:639-648.

Hald, B. and P. Krogh. 1975. Detection of ochratoxin A in barley using silica gel minicolumns. J.A.O.A.C. 58:156-158.

Harwig, J. and Y. K. Chen. 1974. Some conditions favoring production of ochratoxin A and citrinin by Penicillium viridicatum in wheat and barley. Can J. Plant Sci. 54:17-22.

Harwig, J., Y. K. Chen, and D. L. Collins-Thompson. 1974. Stability of ochratoxin A in beans during canning. Can. Inst. Fd. Sci. Tech. 7:288-289.

Hayes, A. W., J. A. Cain, and B. G. Moore. 1977. Effects of aflatoxin B_1, ochratoxin A and rubratoxin B on infant rats. Fd. Cosmet. Toxicol. 15:23-27.

Hayes, A. W., R. D. Hood, and H. L. Lee. 1974. Teratogenic effects of ochratoxin A in mice. Teratology 9:93-98.

Heller, K. and R. Roschenthaler. 1978. Inhibition of protein synthesis in Streptococcus faecalis by ochratoxin A. Can. J. Microbiol. 24:466-472.

Hesseltine, C. W., E. E. Vandegraft, D. I. Fennell, M. L. Smith, and O. L. Shotwell. 1972. Aspergillus as ochratoxin producers. Mycologia 64:539-550.

Holaday, C. E. 1976. A rapid screening method for the aflatoxins and ochratoxin. J.A.O.A.C 53:603-605.

Hood, R. D., M. J. Naughton, and A. W. Hayes. 1976. Prenatal effects of ochratoxin A in hamsters. Teratology 13:11-14.

Hsieh, D. P. H., D. L. Fitzell, J. L. Miller, and J. N. Seiber. 1976. High-pressure liquid chromatography of oxidative aflatoxin metabolites. J. Chromatogr. 117:474-479.

Huber, G. A. 1933. Aspergillus sclerotiorum n. sp., and its relation to decay in apples. Phytopathology 23:306-308.

Huff, W. E. and P. B. Hamilton. 1979. Mycotoxins - Their biosynthesis in fungi: Ochratoxins - Metabolites of combined pathways. J. Food Protect. 42:815-820.

Huff, W. E., J. A. Doerr, and P. B. Hamilton. 1979. Decreased glycogen mobilization during ochratoxicosis in broiler chickens. Appl. Environ. Microbiol. 37: 122-126.

Huff, W. E., R. D. Wyatt, T. L. Tucker, and P. B. Hamilton. 1974. Ochratoxicosis in the broiler chicken. Poultry Sci. 53:1585-1591.

Hult, K. and S. Gatenbeck. 1976. A spectrophotometric procedure, using carboxypeptidase A, for the quantitative measurement of ochratoxin A. J.A.O.A.C. 59:128-129.

Hult, K., A. Teiling, and S. Gatenbeck. 1976. Degradation of ochratoxin A by a ruminant. Appl. Environ. Microbiol. 32:443-444.

Hult, K., E. Hokby, S. Gatenbeck, and L. Rutqvist. 1980. Ochratoxin A in blood from slaughter pigs in Sweden: Use in evaluation of toxin content of consumed feed. Appl. Microbiol. 39:828-830.

Hult, K., E. Hokby, U. Hagglund, S. Gatenbeck, L. Rutqvist, and G. Sellyey. 1979. Ochratoxin A in pig blood: Method of analysis and use as a tool for feed studies. Appl. Microbiol. 38:772-776.

Hunt, D. C., A. T. Bourdon, P. J. Wild, and N. T. Crosby. 1978. Use of high performance liquid chromatography combined with fluorescence detection for the identification and estimation of aflatoxins and ochratoxin in food. J. Sci. Fd. Agric. 29:234-238.

IFT Expert Panel & CPI. 1975. Sulfites as food additives. Food Technol. 29:117-120.

Josefsson, E. and T. Moller. 1979. High pressure liquid chromatographic determination of ochratoxin A and zearalenone in cereals. J.A.O.A.C. 62:1165-1168.

Kanisawa, M., S. Suzuki, Y. Kozuka, and Yamazaki. 1977. Histopathological studies on the toxicity of ochratoxin A in rats. I. Acute oral toxicity. Toxicol. Appl. Pharmacol. 42:55-64.

Koehler, B. 1938. Fungus growth in shelled corn as affected by moisture. J. Agr. Res. 56:291-307.

Krogh, P. 1978. Causal associations of mycotoxic nephropathy. Acta Path. Microbiol. Scand. A: Supplement No. 269, pp. 6-28.

Krogh, P., B. Hald, and E. J. Pedersen. 1973. Occurrence of ochratoxin A and citrinin in cereals associated with mycotoxic porcine nephropathy. Acta Path. Microbiol. Scand. B 81:689-695.

Krogh, P., B. Hald, P. Gjertsen, and F. Myken. 1974a. Fate of ochratoxin A and citrinin during malting and brewing experiments. Appl. Microbiol. 28:31-34.

Krogh, P., B. Hald, P. Englund, L. Rutqvist, and O. Swahn. 1974b. Contamination of Swedish cereals with ochratoxin A. Acta Path. Microbiol. Scand. B 82: 301-302.

Krogh, P., F. Elling, B. Hald, B. Jylling, V. E. Petersen, E. Skadhauge, and C. K. Svendsen. 1976a. Experimental avian nephropathy: Changes of renal function and structure induced by ochratoxin A-contaminated feed. Acta Path. Microbiol. Scand. A 84:215-221.

Krogh, P., F. Elling, B. Hald, A. E. Larsen, E. B. Lillehoj, A. Madsen, and H. P. Mortensen. 1976b. Time-dependent disappearance of ochratoxin A residues in tissues of bacon pigs. Toxicology 6:235-242.

Krogh, P., F. Elling, N. Gyrd-Hansen, B. Hald, A. E. Larsen, E. B. Lillehoj, A. Madsen, H. P. Mortensen, and U. Ravnskov. 1976c. Experimental porcine nephropathy: Changes of renal function and structure perorally induced by crystalline ochratoxin A. Acta Path. Microbiol. Scand. A 84:429-434.

Krogh, P., N. H. Axelsen, F. Elling, N. Gyrd-Hansen, B. Hald, J. Hyldgaard-Jensen, A. E. Larsen, A. Madsen, H. P. Mortensen, T. Moller, O. K. Petersen, U. Ravnskov, M. Rostgaard, and O. Aalund. 1974c. Experimental porcine nephropathy: Changes of renal function and structure induced by ochratoxin A-contaminated feed. Acta Path. Microbiol. Scand. A: Supplement No. 246, pp. 1-21.

Kuehn, H. H. and M. F. Gunderson. 1962. Psychrophilic and mesophilic fungi in fruit-filled pastries. Appl. Microbiol. 10:354-358.

Lai, M., G. Semeniuk, and C. W. Hesseltine. 1968. Nutrients affecting ochratoxin A production by Aspergillus spp. Phytopathology 58:1056.

Lai, M., G. Semeniuk, and C. W. Hesseltine. 1970. Conditions for production of ochratoxin A by Aspergillus species in a synthetic medium. Appl. Microbiol. 19:542-544.

Lehninger, O. L. 1975. Biochemistry. Worth Publishers, Inc., New York, N.Y.

Levi, C. P., H. L. Trenk, and H. K. Mohr. 1974. Study of the occurrence of ochratoxin A in green coffee beans. J.A.O.A.C. 57:866-870.

Lillehoj, E. B., O. Aalund, and B. Hald. 1978. Bioproduction of ^{14}C ochratoxin A in submerges culture. Appl. Microbiol. 36:720-723.

Lindenfelser, L. A., E. B. Lillehoj, and M. S. Milburn. 1973. Ochratoxin and penicillic acid in tumorigenic and acute toxicity tests with white mice. Ch. 41, Vol. 14, pp. 331-336, In "Developments in Industrial Microbiology" editor E. D. Murray. Garamond and Pridemark Press, Baltimore, MD, U.S.A.

Lueck, E. 1980. Antimicrobial Food Additives. Translated from the German by G. F. Edwards, Springer-Verlag, Berlin Heidelberg New York.

Meisner, H. and S. Chan. 1974. Ochratoxin A, an inhibitor of mitochondrial transport systems. Biochem. 13: 2795-2800.

Mislivec, P. B. and V. R. Bruce. 1977. Incidence of toxic and other mold species and genera in soybeans. J. Food Protect. 40:309-312.

Mislivec, P. B., C. T. Dieter, and V. R. Bruce. 1975. Mycotoxin-producing potential of mold flora of dried soybeans. Appl. Microbiol. 29:522-526.

Moore, J. H. and B. Truelove. 1970. Ochratoxin A: Inhibition of mitochondrial respiration. Science 168:1102-1103.

Neely, W. C. and A. D. West. 1972. Spectroanalytical parameters of fungal metabolites. III. Ochratoxin A. J.A.O.A.C. 55:1305-1309.

Nel, W. and I. F. H. Purchase. 1968. The fate of ochratoxin A in rats. J. Sou. Afr. Chem. Inst. 21:87-88.

Nesheim, S. 1973. Analysis of ochratoxin A and B and their esters in barley, using partition and thin layer chromatography. II. Collaborative study. J.A.O.A.C. 56:822-826.

Nip, W. K., F. C. Chang, F. S. Chu, and N. Prentice. 1975. Fate of ochratoxin A in brewing. Appl. Microbiol. 30:1048-1049.

Northolt, M., H. P. van Egmond, and W. E. Paulsch. 1979. Ochratoxin A production by some species in relation to water activity and temperature. J. Food Protect. 42:485-490.

Ockerman, H. W. 1978. Source Book for Food Scientists. AVI Publishing Company, Inc. Westport, Connecticut.

Patterson, D. S. P., B. A. Roberts, and B. J. Small. 1976. Metabolism of ochratoxin A and B in the pig during early pregnancy and the accumulation in body tissues of ochratoxin A only. Fd. Cosmet. Toxicol. 14:439-442.

Pavlovic, M., R. Plestina, and P. Krogh. 1979. Ochratoxin A contamination of foodstuffs in an area with Balkan nephropathy. Acta Path. Microbiol. Scand. B 87: 243-246.

Peckham, J. C., B. Doupnik Jr., and O. H. Jones Jr.. 1971. Acute toxicity of ochratoxin A and B in chicks. Appl. Microbiol. 21:492-494.

Peterson, R. E. and A. Ciegler. 1978. Ochratoxin A: Isolation and subsequent purification by high-pressure liquid chromatography. Appl. Environ. Microbiol. 36:613-614.

Pitout, M. J. 1968. The effect of ochratoxin A on glycogen storage in the rat liver. Toxicol. Appl. Pharmacol. 13:299-306.

Pitout, M. J. 1969. The hydrolysis of ochratoxin A by some proteolytic enzymes. Biochem. Pharmacol. 18:485-491.

Pitout, M. J. and W. Nel. 1969. The inhibitory effect of ochratoxin A on bovine carboxypeptidase A *in vitro*. Biochem. Pharmacol. 18:1837-1843.

Pons, W. A. Jr. 1976. Resolution of aflatoxins B_1, B_2, G_1, and G_2 by high-pressure liquid chromatography. J.A.O.A.C. 59:101-105.

Pomeranz, Y. and C. E. Meloan. 1978. Food Analysis: Theory and Practice. pp. 84-93. AVI Publishing, Inc., Westport, Connecticut.

Prior, M. G. 1976. Mycotoxin determinations on animal feedstuffs and tissues in Western Canada. Can. J. Comp. Med. 40:75-79.

Prior, M. G., C. S. Sisodia, and J. B. O'Neil. 1976. Acute oral ochratoxicosis in day-old white Leghorns, turkeys and Japanese quail. Poultry Sci. 55:786-790.

Purchase, I. F. H. and W. Nel. 1967. Recent advances in research on ochratoxin. Part 1. Toxicological aspects. In "Biochemistry of Some Foodborne Microbial Toxins" pp. 153-156, editors R. I. Mateles and G. N. Wogan, MIT Press, Cambridge, Mass.

Purchase, I. F. H. and J. J. Theron. 1968. The acute toxicity of ochratoxin A to rats. Fd. Cosmet. Toxicol. 6:479-483.

Purchase, I. F. H. and J. J. van der Watt. 1971. The long term toxicity of ochratoxin A to rats. Fd. Cosmet. Toxicol. 9:681-682.

Raper, K. B. and D. I. Fennell. 1965. The Genus Aspergillus. The Williams & Wilkins Co., Baltimore, Md.

Raper, K. B. and C. Thom. 1949. A Manual of the Penicillia. The Williams & Wilkins Co., Baltimore, Md.

Ribelin, W. E., K. Fukushima, and P. E. Still. 1978. The toxicity of ochratoxin to ruminants. Can. J. Comp. Med. 42:172-176.

Sansing, G. A., N. D. Davis, and U. L. Diener. 1973. Effect of time and temperature on ochratoxin A production by Aspergillus ochraceus. Can. J. Microbiol. 19: 1259-1263.

Sansing, G. A., E. B. Lillehoj, R. W. Detroy, and M. A. Miller. 1976. Synergistic toxic effects of citrinin, ochratoxin A and penicillic acid in mice. Toxicon 14:213-220.

Schindler, A. F. and S. Nesheim. 1970. Effect of moisture and incubation time on ochratoxin A production by an isolate of Aspergillus ochraceus. J.A.O.A.C. 53:89-91.

Schindler, A. F., A. N. Abadie, J. S. Gecan, P. B. Mislivec, and P. M. Brickey. 1974. Mycotoxins produced by fungi isolated from inshell pecans. J. Food Sci. 39:213-214.

Scott, P. M. 1973. Modified method for the determination of mycotoxins in cocoa beans. J.A.O.A.C. 56:1028-1030.

Scott, P. M. and T. B. Hand. 1967. Method for the detection and estimation of ochratoxin A in some cereal products. J.A.O.A.C. 50:366-370.

Scott, P. M., B. Kennedy, and W. van Walbeek. 1971. Simplified procedure for the purification of ochratoxin A from extracts of Penicillium viridicatum. J.A.O.A.C. 54:1445-1447.

Scott, P. M., W. van Walbeek, J, Harwig, and D. I. Fennell. 1970. Occurrence of a mycotoxin, ochratoxin A, in wheat and isolation of ochratoxin A and citrinin producing strains of Penicillium viridicatum. Can. J. Plant Sci. 50:583-585.

Scott, P. M., W. van Walbeek, B. Kennedy, and D. Anyeti. 1972. Mycotoxins (ochratoxin A, citrinin, and sterigmatocystin) and toxigenic fungi in grains and other agricultural products. J. Agr. Food Chem. 20:1103-1108.

Searcy, J. W., N. D. Davis, and U. L. Diener. 1969. Biosynthesis of ochratoxin A. Appl. Microbiol. 18: 622-627.

Semeniuk, G. and H. J. Barre. 1943. Molds in stored corn and their control. Ann. Rep. Iowa Corn Res. Inst. 8:55-57.

Shimoda, W. 1979. Conference of Mycotoxins in Animal Feeds and Grains Related to Animal Health. pp. 165-287. Sponsored by Bureau of Verterinary Medicine, Food and Drug Administration.

Shotwell, O. L., M. L. Goulden, and C. W. Hesseltine. 1976. Survey of U.S. wheat for ochratoxin and aflatoxin. J.A.O.A.C. 59:122-124.

Shotwell, O. L., C. W. Hesseltine, and M. L. Goulden. 1969. Note on the natural occurrence of ochratoxin A. J.A.O.A.C. 52:81-83.

Shotwell, O. L., C. W. Hesseltine, E. E. Vandegraft, and M. L. Goulden. 1971. Survey of corn from different regions for aflatoxin, ochratoxin, and zearalenone. Cereal Sci. Today 16:266-273.

Sinda, J. F. and J. F. DeBernardia. 1973. Naturally occurring halogenated organic compound. Lloydia 36: 107-143.

Singer, U. and R. Roschenthaler. 1978. Induction of autolysis in Bacillus subtilis by ochratoxin A. Can. J. Microbiol. 24:563-568.

Skoog, D. A. and D. M. West. 1976. Fundamentals of Analytical Chemistry. Hotl, Rinehart and Winston, Inc.

Sofos, J. N., F. F. Busta, and C. E. Allen. 1979. Botulism control by nitrite and sorbate in cured meats: A review. J. Food Protect. 42:739-770.

Steele, J. A., N. D. Davis, and U. L. Diener. 1973. Effects of zinc, copper, and iron on ochratoxin A production. Appl. Microbiol. 25:847-849.

Steyn, P. S. 1977. Ochratoxin and other dihydroisocoumaines, Chapter 2, pp. 179-205, In "Microbial Toxins" Vol. 6, "Fungal Toxins" editors A. Ciegler, S. Kadis, and S. J. Ajl, Academic Press, New York, N.Y.

Steyn, P. S. and K. J. van der Merwe. 1966. Detection and estimation of ochratoxin A. Nature 211:418.

Steyn, P. S., C. W. Holzapfel, and N. P. Ferreira. 1970. The biosynthesis of ochratoxins, metabolites of Aspergillus ochraceus. Phytochemistry 9:1977-1983.

Still, P. E., A. W. Macklin, W. E. Bibelin. and E. B. Smalley. 1971. Relationship of ochratoxin A to foetal death in laboratory and domestic animals. Nature 234: 563-564.

Stoloff, L., S. Nesheim, L. Yin, J. V. Rodricks, M. Stack, and A. D. Campbell. 1971. A multimycotoxin detection method for aflatoxins, ochratoxin, zearalenone, sterigmatocystin and patulin. J.A.O.A.C. 54:91-97.

Suzuki, S., T. Satoh, and M. Yamazaki. 1975. Effect of ochratoxin A on carbohydrate metabolism in rat liver. Toxicol. Appl. Pharmacol. 32:116-122.

Szczech, G. M., W. W. Carlton, and J. Tuite. 1973a. Ochratoxicosis in Beagle dogs. I. Clinical and clinicopathological features. Vet. Path. 10:135-154.

Szczech, G. M., W. W. Carlton, and J. Tuite. 1973b. Ochratoxicosis in Beagle dogs. II. Pathology. Vet. Path. 10:219-231.

Szczech, G. M., W. W. Carlton, J. Tuite, and R. Caldwell. 1973c. Ochratoxin A toxicosis in swine. Vet. Path. 10:347-364.

Takeda, Y., E. Isohata, R. Amano, and M. Uchiyama. 1979. Simultaneous extraction and fractionation and thin layer chromatographic determination of 14 mycotoxins in grains. J.A.O.A.C. 62:573-578.

Thacker, H. L. and W. W. Carlton. 1977. Ochratoxin A mycotoxicosis in the Guinea pig. Fd. Cosmet. Toxicol. 15:563-574.

Trenk, H. L. and F. S. Chu. 1971. Improved detection of ochratoxin A on thin layer plates. J.A.O.A.C. 54: 1307-1309.

Trenk, H. L., M. E. Butz, and F. S. Chu. 1971. Production of ochratoxins in different cereal products by Aspergillus ochraceus. Appl. Microbiol. 21:1032-1035.

Vandegraft, E. E., C. W. Hesseltine, and O. L. Shotwell. 1975. Grain preservatives: Effect on aflatoxin and ochratoxin production. Cereal Chem. 52:79-84.

Vandegraft, E. E., O. L. Shotwell, M. L. Smith, and C. W. Hesseltine. 1973a. Mycotoxin formation affected by fumigation of wheat. Cereal Sci. Today 18:412-414.

Vandegraft, E. E., O. L. Shotwell, M. L. Smith, and C. W. Hesseltine. 1973b. Mycotoxin production affected by insecticide treatment of wheat. Cereal Chem. 50:264-270.

van der Merwe, K. J., P. S. Steyn, L. Fourie, D. B. Scott, and J. J. Theron. 1965. Ochratoxin A, a toxic metabolite produced by Aspergillus ochraceus Wilh. Nature 205:1112-1113.

van Walbeek, W., P. M. Scott, and F. S. Thatcher. 1968. Mycotoxins from foodborne fungi. Can. J. Microbiol. 14:131-137.

van Walbeek, W., P. M. Scott, J. Harwig, and J. W. Lawrence. 1969. Penicillium viridicatum Westling: A new source of ochratoxin A. Can. J. Microbiol. 15:1281-1285.

van Walbeek, W., C. A. Moodie, P. M. Scott, J. Harwig, and H. C. Grice. 1971. Toxicity and excretion of ochratoxin A in rats intubated with pure ochratoxin A or fed cultures of Penicillium viridicatum. Toxicol. Appl. Pharmacol. 20:439-441.

Voster, L. J. 1969. A method for the analysis of cereals and groundnuts for three mycotoxins. Analyst 94: 131-137.

Wallace, H. A. H. and R. N. Sinha. 1962. Fungi associated with hot spots in farm stored grain. Can. J. Plant Sci. 42:130-141.

Warren, M. F. and P. B. Hamilton. 1980. Inhibition of the glycogen phosphorylase system during ochratoxicosis in chickens. Appl. Environ. Microbiol. 40:522-525.

Wei, R. D., F. M. Strong, and E. B. Smalley. 1971. Incorporation of chlorine^{36} into ochratoxin A. Appl. Microbiol. 22:276-277.

Welty, R. E. and G. B. Lucas. 1968. Fungi isolated from damaged flue-cured tobacco. Appl. Microbiol. 16: 851-854.

Wilson, D. M., W. H. Tabor, and M. W. Trucksess. 1976. Screening method for the detection of aflatoxin, ochratoxin, zearalenone, penicillic acid and citrinin. J.A.O.A.C. 59:125-127.

Wu, M. T. and J. C. Ayres. 1974. Effects of dichlorvos on ochratoxin production by Aspergillus ochraceus. J. Agr. Food Chem. 22:536-537.

Yamazaki, M., Y. Maebayashi, and K. Miyaki. 1970. Production of ochratoxin A by Aspergillus ochraceus isolated in Japan from moldy rice. Appl. Microbiol. 20:452-454.

Yamazaki, M., Y. Maebayashi, and K. Miyaki. 1971. Biosynthesis of ochratoxin A. Tetrahedron Lett. 25:2301.

APPENDIX

Table A-1. Effects of Potassium Sorbate on Growth of Ochratoxin A Producers in Yeast Extract Sucrose Broth Incubated at 25°C for 2 Weeks.

Strain	pH	Level (%)	Mycelium Mean* and Standard Deviation (g/100 ml broth)	Percent Inhibition
A. sulphureus NRRL 4077	4.5	0	1.52555 ± 0.05175a	
		0.011	1.37245 ± 0.04262b	10.04
		0.022	0 c	100
		0.044	0	
		0.067	0	
		0.134	0	
A. sulphureus NRRL 4077	5.5	0	1.46791 ± 0.03636a	
		0.011	- -	-
		0.022	1.46396 ± 0.05688a	0.27
		0.044	1.33525 ± 0.09770b	9.03
		0.067	0.91538 ± 0.08009c	37.64
		0.134	0 d	100
P. viridicatum NRRL 3711	4.5	0	1.96442 ± 0.05603a	
		0.011	1.82257 ± 0.06347b	7.22
		0.022	0 c	100
		0.044	0	
		0.067	0	
		0.134	0	
P. viridicatum NRRL 3711	5.5	0	1.97958 ± 0.03810a	
		0.011	- -	-
		0.022	1.89980 ± 0.05346a	4.03
		0.044	1.58818 ± 0.06958b	19.77
		0.067	1.11948 ± 0.14983c	43.45
		0.134	0 d	100

*Mean of 2 replications.

Values with the same letter are not significantly different at 0.05 level using Duncan's Multiple Range Test.

Table A-2. Effects of Methyl Paraben on Growth of Ochratoxin A Producers in Yeast Extract Sucrose Broth Incubated at 25°C for 2 Weeks.

Strain	pH	Level (%)	Mycelium Mean* and Standard Deviation (g/100 ml broth)	Percent Inhibition
A. sulphureus NRRL 4077	4.5	0	1.63808 ± 0.10506a	
		0.011	1.51713 ± 0.09464b	7.38
		0.022	1.52588 ± 0.07553b	6.85
		0.033	0.31858 ± 0.02186c	80.55
		0.067	0 d	100
A. sulphureus NRRL 4077	5.5	0	1.50951 ± 0.14117a	
		0.011	1.28823 ± 0.09275b	14.66
		0.022	1.28207 ± 0.07009b	15.07
		0.033	0.31202 ± 0.04878c	79.33
		0.067	0 d	100
P. viridicatum NRRL 3711	4.5	0	2.24060 ± 0.1373 ab	
		0.011	2.26580 ± 0.20255a	-1.12
		0.022	2.07848 ± 0.12802 b	7.24
		0.033	0.06007 ± 0.01471 c	97.32
		0.067	0 c	100
P. viridicatum NRRL 3711	5.5	0	2.15153 ± 0.03603a	
		0.011	2.00922 ± 0.02234b	6.61
		0.022	2.01602 ± 0.08845b	6.30
		0.033	0.49927 ± 0.03217c	76.79
		0.067	0 d	100

*Mean of 2 replications.

Values with the same letter are not significantly different at 0.05 level using Duncan's Multiple Range Test.

Table A-3. Effects of Sodium Bisulfite on Growth of Ochratoxin A Producers in Yeast Extract Sucrose Broth Incubated at 25°C for 2 Weeks.

Strain	pH	Level (%)	Mycelial Mean* and Standard Deviation (g/100 ml broth)	Percent Inhibition
A. sulphureus NRRL 4077	4.5	0	1.51593 ± 0.04904a	
		0.0167	1.40073 ± 0.07769a	7.60
		0.0334	1.16938 ± 0.15580b	22.86
		0.0500	0.22490 ± 0.24658c	85.16
		0.0667	0 d	100
		0.0834	0	
		0.1000	0	
A. sulphureus NRRL 4077	5.5	0	1.83440 ± 0.09295a	
		0.0167	1.78498 ± 0.07691ab	2.67
		0.0334	1.77405 ± 0.10273abc	3.29
		0.0500	1.67027 ± 0.05748 bcd	8.95
		0.0667	1.61872 ± 0.03732 cd	11.76
		0.0834	1.55703 ± 0.05959 d	15.12
		0.1000	1.00060 ± 0.28121 e	45.45
P. viridicatum NRRL 3711	4.5	0	1.99062 ± 0.06810a	
		0.0167	1.90433 ± 0.03073a	4.32
		0.0334	1.71927 ± 0.27233b	13.63
		0.0500	0.18131 ± 0.19872c	90.89
		0.0667	0 d	100
		0.0834	0	
		0.1000	0	
P. viridicatum NRRL 3711	5.5	0	2.51555 ± 0.12611a	
		0.0167	2.45100 ± 0.07090a	2.57
		0.0334	2.20242 ± 0.08173b	12.45
		0.0500	2.24972 ± 0.03330bc	10.64
		0.0667	2.06698 ± 0.09755 cd	17.83
		0.0834	2.01606 ± 0.10141 d	19.86
		0.1000	0.26703 ± 0.29308 e	89.38

*Mean of 2 replications.

Values with the same letter are not significantly different at 0.05 level using Duncan's Multiple Range Test.

Table A-4. Effects of Sodium Propionate on Growth of Ochratoxin A Producers in Yeast Extract Sucrose Broth Incubated at 25°C for 2 Weeks.

Strain	pH	Level (%)	Mycelial Mean* and and Standard Deviation (g/100 ml broth)	Percent Inhibition
A. sulphureus NRRL 4077	4.5	0	1.57653 ± 0.04266a	
		0.10	0.26990 ± 0.02431b	82.88
		0.20	0 c	100
		0.32	0	
		0.48	0	
		0.64	0	
A. sulphureus NRRL 4077	5.5	0	1.45913 ± 0.03030a	
		0.10	1.47358 ± 0.00982a	-0.99
		0.20	1.46573 ± 0.01932a	-0.45
		0.32	1.26497 ± 0.19570b	13.31
		0.48	0.85993 ± 0.11583c	41.07
		0.64	0.29310 ± 0.02736d	79.91
P. viridicatum NRRL 3711	4.5	0	1.88410 ± 0.05156a	
		0.10	0.30470 ± 0.01425b	83.83
		0.20	0 c	100
		0.32	0	
		0.48	0	
		0.64	0	
P. viridicatum NRRL 3711	5.5	0	1.99073 ± 0.07736a	
		0.10	1.92358 ± 0.05272a	3.37
		0.20	1.92218 ± 0.08934a	3.44
		0.32	1.67135 ± 0.05417b	16.04
		0.48	0.28710 ± 0.03877c	85.58
		0.64	0.22987 ± 0.11801c	88.45

*Mean of 2 replications.

Values with the same letter are not significantly different at 0.05 level using Duncan's Multiple Range Test.

Table A-5. Effects of Potassium Sorbate on Ochratoxin A Production in Yeast Extract Sucrose Broth Incubated at 25°C for 2 Weeks.

Strain	pH	Level (%)	Toxin Mean* and Standard Deviation (mg/100 ml broth)	Percent Inhibition
A. sulphureus NRRL 4077	4.5	0	2.29752 ± 0.44084a	
		0.011	0.69639 ± 0.08938b	69.69
		0.022	0 c	100
		0.044	0	
		0.067	0	
		0.134	0	
A. sulphureus NRRL 4077	5.5	0	1.91463 ± 0.37531a	
		0.011	- -	-
		0.022	0.47246 ± 0.13037b	75.32
		0.044	0.19637 ± 0.04971c	89.74
		0.067	0.00466 ± 0.00085c	99.76
		0.134	0 c	100
P. viridicatum NRRL 3711	4.5	0	0.02163 ± 0.00456a	
		0.011	0.00443 ± 0.00156b	79.62
		0.022	0 c	100
		0.044	0	
		0.067	0	
		0.134	0	
P. viridicatum NRRL 3711	5.5	0	0.01884 ± 0.00353a	
		0.011	- -	-
		0.022	0.01459 ± 0.00157b	22.34
		0.044	0.01241 ± 0.00112b	34.04
		0.067	0.00716 ± 0.00204c	61.70
		0.134	0 d	100

*Mean of 2 replication.

Values with the same letter are not significantly different at 0.05 level using Duncan's Multiple Range Test.

Table A-6. Effects of Methyl Paraben on Ochratoxin A Production in Yeast Extract Sucrose Broth Incubated at 25°C for 2 Weeks.

Strain	pH	Level (%)	Toxin Mean* and Standard Deviation (mg/100 ml broth)	Percent Inhibition
A. sulphureus NRRL 4077	4.5	0	2.73335 ± 0.45138a	
		0.011	0.11170 ± 0.04933b	95.91
		0.022	0.00454 ± 0.00258b	99.83
		0.033	0.00308 ± 0.00079b	99.89
		0.067	0 b	100
A. sulphureus NRRL 4077	5.5	0	1.59132 ± 0.33540a	
		0.011	0.40773 ± 0.22765b	74.38
		0.022	0.04919 ± 0.02762c	96.91
		0.033	0.00275 ± 0.00137c	99.83
		0.067	0 c	100
P. viridicatum NRRL 3711	4.5	0	0.01926 ± 0.00681a	
		0.011	0.02114 ± 0.02540a	-
		0.022	0.00278 ± 0.00252b	85.57
		0.033	0.00018 ± 0.00017b	99.07
		0.067	0 b	100
P. viridicatum NRRL 3711	5.5	0	0.02289 ± 0.00849a	
		0.011	0.01370 ± 0.00455b	40.15
		0.022	0.00779 ± 0.00388c	65.97
		0.033	0.00127 ± 0.00050d	94.45
		0.067	0 d	100

*Mean of 2 replications.

Values with the same letter are not significantly different at 0.05 level using Duncan's Multiple Range Test.

Table A-7. Effects of Sodium Bisulfite on Ochratoxin A Production in Yeast Extract Sucrose Broth Incubated at 25°C for 2 Weeks.

Strain	pH	Level (%)	Toxin Mean* and Standard Deviation (mg/100 ml broth)	Percent Inhibition
A. sulphureus NRRL 4077	4.5	0	2.18815 ± 0.22707a	
		0.0167	0.41628 ± 0.21090b	80.98
		0.0334	0.26435 ± 0.14446c	87.92
		0.0500	0.00479 ± 0.00527d	99.78
		0.0667	0 d	100
		0.0834	0	
		0.1000	0	
A. sulphureus NRRL 4077	5.5	0	2.51642 ± 0.29365a	
		0.0167	2.12951 ± 0.24822b	15.38
		0.0334	1.95018 ± 0.16693b	22.44
		0.0500	0.99764 ± 0.17313c	60.35
		0.0667	0.46043 ± 0.13625d	81.70
		0.0834	0.18639 ± 0.09821e	92.59
		0.1000	0.07118 ± 0.06437e	97.17
P. viridicatum NRRL 3711	4.5	0	0.02137 ± 0.00326a	
		0.0167	0.00440 ± 0.00152b	79.41
		0.0334	0.00204 ± 0.00131c	90.45
		0.0500	0.00020 ± 0.00024d	99.06
		0.0667	0 d	100
		0.0834	0	
		0.1000	0	
P. viridicatum NRRL 3711	5.5	0	0.02246 ± 0.00756a	
		0.0167	0.01939 ± 0.00268a	13.67
		0.0334	0.01210 ± 0.00213b	46.13
		0.0500	0.00922 ± 0.00162b	58.95
		0.0667	0.00437 ± 0.00267c	80.54
		0.0834	0.00078 ± 0.00036cd	96.53
		0.1000	0.00003 ± 0.00006 d	99.89

*Mean of 2 replications.

Values with the same letter are not significantly different at 0.05 level using Duncan's Multiple Range Test.

Table A-8. Effects of Sodium Propionate on Ochratoxin A Production in Yeast Extract Sucrose Broth Incubated at 25° C for 2 Weeks.

Strain	pH	Level (%)	Toxin Mean* and Standard Deviation (mg/100 ml broth)	Percent Inhibition)
A. sulphureus NRRL 4077	4.5	0	1.61143 ± 0.31108a	
		0.10	0.01761 ± 0.00545b	98.91
		0.20	0 c	100
		0.32	0	
		0.48	0	
		0.64	0	
A. sulphureus NRRL 4077	5.5	0	1.77079 ± 0.46931a	
		0.10	1.43048 ± 0.33076b	19.22
		0.20	0.63516 ± 0.26825c	64.13
		0.32	0.22728 ± 0.09986d	87.17
		0.48	0.20184 ± 0.24400d	88.60
		0.64	0.01059 ± 0.01144d	99.40
P. viridicatum NRRL 3711	4.5	0	0.01918 ± 0.00379a	
		0.10	0.00587 ± 0.00247b	69.40
		0.20	0 c	100
		0.32	0	
		0.48	0	
		0.64	0	
P. viridicatum NRRL 3711	5.5	0	0.02126 ± 0.00686ab	
		0.10	0.03471 ± 0.02468a	-
		0.20	0.01244 ± 0.00488ab	41.49
		0.32	0.00113 ± 0.00035 b	94.68
		0.48	0.00019 ± 0.00013 b	99.11
		0.64	ND b	100

*Mean of 2 replications.

Values with the same letter are not significantly different at 0.05 level using Duncan's Multiple Range Test.

VITA

Chao-Hsiang Tong was born in Taichung, Taiwan, Republic of China on November 12, 1956. He attended public schools in the Taichung area and graduated from First Tainan Senior High School in June, 1975. The following October he enrolled in National Chung-Hsing University, Taichung, Taiwan, Republic of China. He received a Bachelor of Science degree in Food Science in 1979.

In September, 1979, he entered the University of Tennessee, Knoxville and began study toward a Master's degree in Food Technology and Science. He is a member of Phi Tau Sigma, Gamma Sigma Delta, and Phi Kappa Phi.