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Title: ACUTE TOXICITY AND CARCINOGENIC ACTIVITY OF OCHRATOXIN IN RAINBOW TROUT (SALMO GAIRDNERI)

Abstract approved: Redacted for Privacy
Russell O. Sinnhuber

The acute toxicity of two metabolites of Aspergillus ochraceus, ochratoxins A and B plus their dihydroisocoumarin derivatives, ochratoxins a and b were determined using rainbow trout (Salmo gairdneri) as a test animal. Also, a 12 month feeding trial was conducted in which trout were fed three levels of ochratoxin A in an effort to determine the possible carcinogenic activity of this toxin.

Ochratoxins A and B were isolated from A. ochraceus cultures grown on shredded wheat, then purified by column chromatography and crystallization. The dihydroisocoumarin derivatives (a and b) were prepared by acid hydrolysis of the respective ochratoxins.

Ochratoxin A was the only compound found lethal to trout at the levels dosed. The ten-day LD_{50} of I. P. dosed ochratoxin A in six-month-old trout was 4.67 mg/kg body weight. The toxin was found to produce hepatic parenchymal cell degradation including nuclear swelling and cytoplasmic and nuclear lipid vacuolation. Several possible

mechanisms are proposed to account for the hepatic steatosis. Ochratoxin A produced necrosis in proximal tubules, hematopoietic tissue, and glomeruli of kidneys. Also noted were pycnotic nuclei, cast formation, and lipid vacuolation in renal tubules. It was suggested that animals metabolizing ochratoxin A to ochratoxin a are performing a detoxification mechanism whereby the toxic parent compound is converted to a non-toxic water-soluble polar product which is readily excreted.

Ochratoxin A failed to induce hepatoma in trout at levels up to 64 ppb in a semi-synthetic diet fed for 12 months. Pathological changes noted in livers from trout fed diets containing ochratoxin A included those which typically precede hepatomagenesis. These changes included parenchymal cell degeneration, nuclear swelling, and bile duct proliferation. Foci of lymphocytes presumably attacking hepatic parenchymal cells were a common occurrence. Also noted in some cases was a mild arterial hyperplasia.

Acute Toxicity and Carcinogenic Activity of Ochratoxin in Rainbow Trout (Salmo gairdneri)

by

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ACUTE TOXICITY AND CARCINOGENIC ACTIVITY OF OCHRATOXIN IN RAINBOW TROUT (SALMO GAIRDNERI)

INTRODUCTION

Interest in the mycotoxins has been stimulated by numerous reports on the toxic and carcinogenic effects of the most thoroughly investigated mycotoxins, the aflatoxins, and by suggestions that these toxins offer a reasonable explanation for the high incidence of liver cancer in Africa and comparable regions (60). It is becoming increasingly apparent that mold metabolites besides aflatoxin cause significant pathological changes in experimental animals.

Metabolites of the Aspergilli molds have probably received the greatest attention since they elaborate extremely toxic and powerful carcinogens. Among the Aspergilli is the mold Aspergillus ochraceus which was the first mold found to produce metabolites called ochratoxins, some of which have been shown to be toxic when fed to laboratory test animals. Subsequently ochratoxin was found to be produced by two other Aspergilli and a Penicillium species. Since these metabolites have been found in certain agricultural commodities, they are potential hazards to animal and human health.

Studies of the toxic effect of ochratoxins have been limited to the two major ochratoxins, A and B, plus their methyl and ethyl esters. Thus far, only the ochratoxin A compounds have been found to be toxic.

In addition, while the ochratoxins possess structural features found in many active carcinogens (25, 26), none have been reported to possess carcinogenic activity.

Therefore, the purpose of this study was to determine the toxicity of four related ochratoxin compounds designated A, B, a, and b, and to determine the carcinogenic activity of the most thoroughly studied compound in this group, ochratoxin A. The test animal used in this study was the rainbow trout (Salmo gairdneri).

Ochratoxins A and B are the two parent mycotoxins most commonly found in A. ochraceus culture extracts while ochratoxins a and b are the corresponding dihydroisocoumarin derivatives of ochratoxins A and B, respectively. Ochratoxin a has been found to be excreted by rats dosed with the parent ochratoxin A (49) and it is likely that ochratoxin B would similarly be metabolized by rats and other animals to ochratoxin b. Additionally, ochratoxin b is structurally and probably biogenetically closely related to 3-methyl-4, 8-dihydroisocoumarin (4-hydroxy mellein), also a metabolite of A. ochraceus (21). Thus, ingestion of food contaminated with A. ochraceus and other ochratoxin producing molds could ultimately result in exposure to all four compounds used in this study.

LITERATURE REVIEW

Ochratoxin

Mycological Aspects

Aspergillus ochraceus has been isolated from a variety of agricultural commodities (15, 16, 17, 18, 65, 78). Symptoms of chronic or acute toxicity have been observed in experimental animals when Aspergillus ochraceus was grown in pure culture on corn, wheat, rye, sorghum, rice, buckwheat, soybeans, and peanuts and the molded substrates fed to the test animals (15, 16, 18, 28, 78). The toxic agent produced by the mold was first isolated and named ochratoxin by van der Merwe et al. (78, 79).

In 1969 Shotwell et al. (69) found ochratoxin A as a naturally occurring contaminant at levels up to 150 parts per billion (ppb) in sample grade corn. Scott et al. (67) surveyed grains and feeds associated with animal deaths and found ochratoxin A at levels up to 100 ppb.

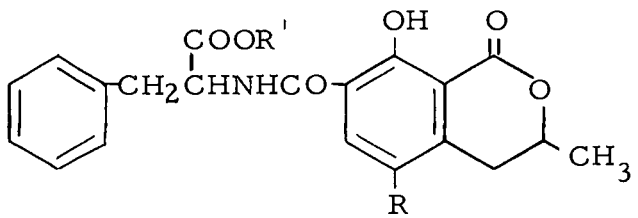
In several instances a toxic principle in corn produced by Penicillium viridicatum has been thought to be responsible for poisoning of animals. Marchionatto (46) found that corn moldy with P. viridicatum poisoned horses and pigs in Argentina, and Krogh and Hassellager (40) showed that barley inoculated with this fungus contained a toxic principle which caused kidney damage in rats and pigs.

Ochratoxin A has since been found to be produced by P. viridicatum in addition to A. melleus and A. sulphureus (41, 80). Van Walbeek (80) states that it is possible that ochratoxin A detected by Shotwell et al. (69) in moldy corn may have been produced by a Penicillium, since a high incidence of Penicillium species was found on culture plates of corn. Thus, ochratoxin A may be responsible for at least part of the disease syndrome caused experimentally by P. viridicatum in animals (12, 40).

Chemical Characteristics

Ochratoxin is a collective term used to describe six structurally related compounds of the isocoumarin type. The chemical structures were determined (73, 79) and verified by synthesis (61, 74). Ochratoxin A was shown to be 7-carboxy-5-chloro-8-hydroxy-3, 4-dihydro-3-methylisocoumarin, linked over its 7-carboxy group to L-β-phenylalanine by an amide bond. Ochratoxins B and C were characterized as the dechloro and ethyl ester derivatives of ochratoxin A respectively. The other three ochratoxins were found to be methyl esters of ochratoxins A and B, and the ethyl ester of ochratoxin B. These structures are shown in Figure 1.

Ochratoxins A and B are colorless crystalline compounds that fluoresce green and blue respectively under ultraviolet radiation at 366 nm. Ochratoxin C is amorphous and appears dull green as does



Ochratoxin A; R=Cl, R'=H

Ochratoxin B; R=H, R'=H

Ochratoxin C; R=Cl, R'=C_2H_5

Ochratoxin A methyl ester; R=Cl, R'=CH_3

Ochratoxin B ethyl ester; R=H, R'=C_2H_5

Ochratoxin B methyl ester; R=H, R'=CH_3

Figure 1. Structure of the ochratoxins.

the methyl ester of ochratoxin A. The methyl and ethyl esters of ochratoxin B both appear light blue under ultraviolet radiation (73). On holding thin layer chromatograms over ammonia fumes ochratoxins A, B, and C exhibit a bright blue fluorescence.

Chemical Reactions

The reactions involving ochratoxin that are significant to this study involve alteration of the phenylalanine moiety by either esterification or hydrolysis. These reactions are summarized in a modified scheme from Steyn and Holzapfel (73) shown in Figure 2.

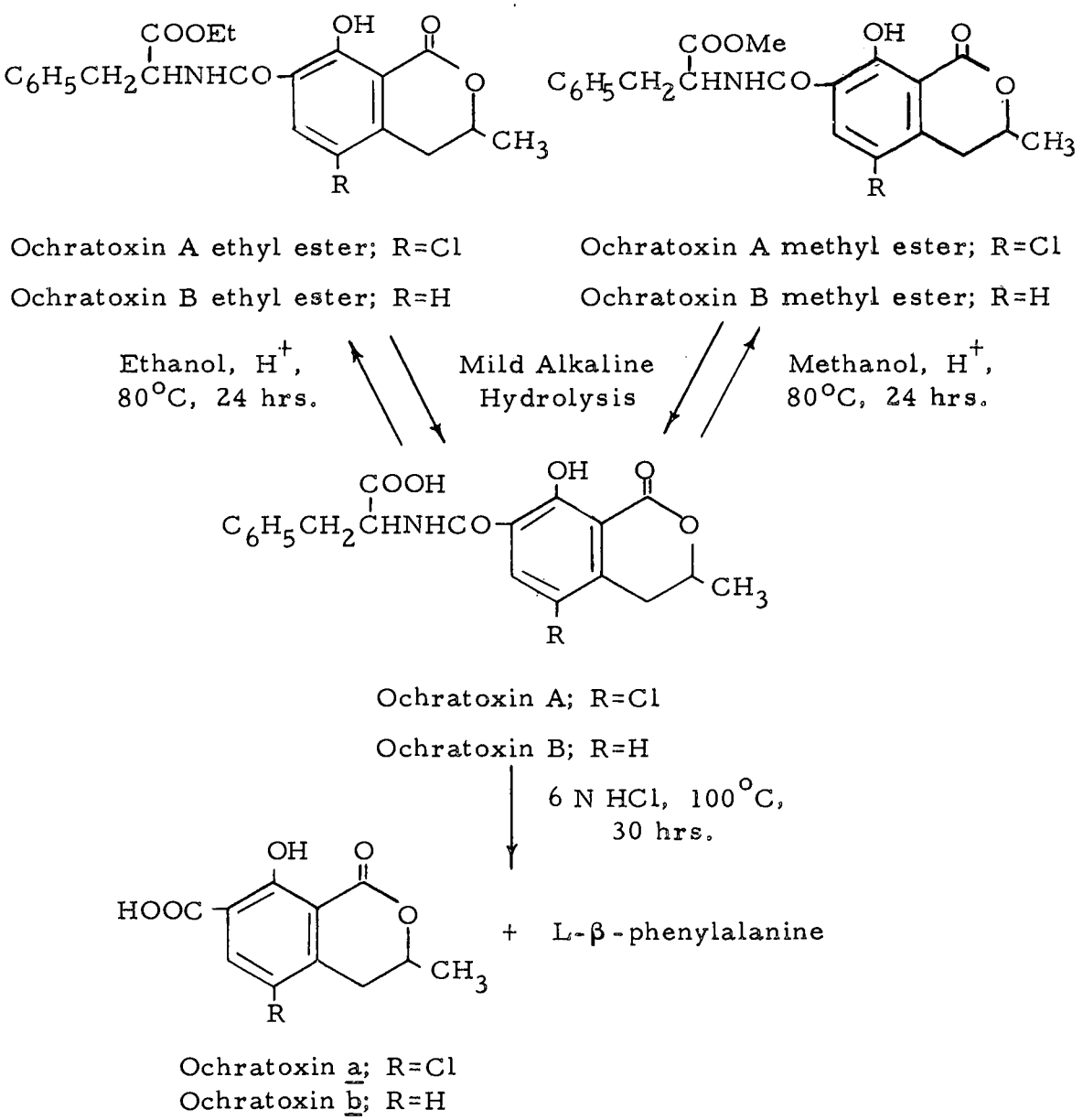


Figure 2. Chemical interconversions of ochratoxins.

Analytical Methodology

Preparation of Ochratoxins A and B. Natural ochratoxins A and B have been produced in highest concentrations by A. ochraceus on wet corn meal (79), rice (83), and shredded wheat breakfast cereal (52, 64). Davis et al. (22) used a semisynthetic medium containing 4% sucrose and 2% yeast extract that gave 29 mg ochratoxin A per 100 ml of medium. Ferreira (31) obtained yields of about 10 mg/100 ml synthetic nutrient solution in shaken flasks and 10-liter fermentors. In contrast, Schindler and Nesheim (64) produced an average of 239 mg of ochratoxin A per 100 g of shredded wheat. Ochratoxin B production was reported to be maximal under conditions favoring ochratoxin A production. Thus, yields from all media are much higher on solid substrates than liquid media.

Evidence seems to indicate that conditions favoring rapid growth and sporulation of A. ochraceus may not necessarily favor high toxin production. Toxin yields from all media seem to depend heavily on incubation time and temperature, moisture level, and strain of mold. Schindler and Nesheim (64) found that maximum yields of ochratoxin A were obtained with M-298 (American Type Culture Collection No. 18642) strain of A. ochraceus grown in Fernbach flasks containing 100 g of shredded wheat and 40 to 70 ml of water, and finally incubated at 22°C for 19 to 21 days.

Extraction and Purification. Several procedures have been

proposed for extraction and purification of ochratoxins (22, 29, 49, 64, 66, 73, 75, 79). Basically, the methods that are currently being used rely upon extraction of the toxins with a suitable solvent (usually chloroform), extraction of this extract with bicarbonate to yield the acidic ochratoxins A and B, and finally acidification and back extraction of the acidified aqueous phase with chloroform. The extracted toxins are then analyzed by spotting on thin layer plates which are developed in one of several benzene:glacial acetic acid mixtures (64, 73, 75, 80).

Extracted ochratoxins A and B have been purified by column or preparative thin layer chromatography using one of several adsorbents (9, 51, 73, 79). Since large amounts of pure ochratoxins are needed for toxicity and feeding trial studies, purification procedures which fulfill this need must be used. Of particular interest in this regard is a procedure described by Nesheim (51) in which large amounts of ochratoxin A and B mixtures were separated from each other by gradient silica gel chromatography using an acetic acid:benzene eluant.

Structural Confirmation of Ochratoxins A and B. Comparing R_f's of standard and unknown mycotoxin spots on thin layer plates has become a common practice as the sole criteria for structural confirmation. It is becoming increasingly apparent that this practice can possibly result in the misidentification of compounds having identical R_f's and fluorescent properties. In this regard, Eppley (29) reported

that both zearalenone and ochratoxin A had the same R_f when thin layer plates were developed in benzene:glacial acidic acid 9:1 v/v. In addition, several fluorescent impurities have been extracted in other mycotoxin studies (58, 71, 76). Therefore, only presumptive confirmation of mycotoxin structures can be obtained by TLC analysis.

A review of the literature reveals there are a number of methods which have been used to confirm the structure of ochratoxins. These methods include migration of thin layer plates using three different solvent systems (69), subjecting chromatograms to ammonia fumes and noting the change in color of fluorescing spots (69, 75), solubility in dilute bicarbonate (29), chemical derivative formation (9, 69), and various instrumental methods such as ultraviolet, infrared, nuclear magnetic resonance, and mass spectrometry (50, 51, 74, 79).

Biological Activity

Toxicity studies conducted thus far deal only with acute dosage experiments (49, 59, 60, 73, 77, 78, 79). Ochratoxin A has been shown to be toxic to rats, mice, and day-old ducklings (59, 73, 78), while ochratoxins B and C were originally reported to be non-toxic (79). Both ochratoxin C and the methyl ester of ochratoxin A have since been reported to be as toxic to day-old ducklings as ochratoxin A itself (73).

Ducklings. LD_{50} values of ochratoxin A have been reported

ranging from 135 to 170 μg per day-old duckling (60, 73). For comparison, the LD_{50} of aflatoxin B_1 is 18 μg per duckling (13). Ochratoxin B has been reported to be non-toxic to ducklings at a thousand-fold higher dose level than the toxic ochratoxin A level (79).

Theron et al. (77) reported that ultrastructural changes in livers from 50 g ducklings administered 100 μg of ochratoxin A show a close resemblance to those encountered in choline deficiency. The most prominent lesion reported by these authors was fatty infiltration of the hepatocytes of the liver. Also noted were swollen liver mitochondria and a decrease in the number of ergastoplasmic membranes and ribosomal granules. There was also an increase in the number of free ribosomes in the cytoplasm. Similarly, van der Merwe et al. (78) reported acute fatty infiltration of parenchymal liver cells in ducklings dosed with ochratoxin A at levels up to 75 μg per duckling. In contrast, however, these latter authors reported the unusual occurrence of intranuclear fat vacuolation. It is interesting to note that intranuclear fat inclusions have also been observed in choline-deficient rats (45). Theron et al. (77) did, however, report changes such as dilation of endoplasmic reticulum (ER) cisternae and focal degeneration of the liver cytoplasm which have not been encountered in choline-deficient rats.

Rats. Theron et al. (77) found that 60 g weanling rats given 100 μg ochratoxin A develop single-cell necrosis of the liver. Hypertrophy

of the smooth ER was the earliest ultrastructural change in liver cells of rats dosed with ochratoxin A. It was suggested that this represents an attempt by the liver cells to detoxify the ochratoxin since it is known that several enzymes that play a role in the metabolism of hepatotoxic drugs are localized in the smooth ER (34). Because of the fact that only damage and disorientation of the ER occurred and no alterations were found in liver mitochondria, Theron et al. (77) suggested that the primary site of action of ochratoxin A in the rat appears to be the ER.

Purchase and Theron (59) found that the oral LD_{50} values of ochratoxin A was 22 and 20 mg/kg for male and female Wistar rats respectively. This is about half that of aflatoxin B_1, the oral LD_{50} of which has been estimated as 7 mg/kg in male and 16 mg/kg in female Wistar rats (11).

The main pathological changes seen in ochratoxin A dosed rats by Purchase and Theron (59) were necrosis of renal tubules and periportal cells of the liver. Many pycnotic (or occasionally karyolytic) nuclei were seen in cells of the proximal convoluted tubules. No abnormal glomeruli were reported in kidneys. Vacuolation due to glycogen accumulation was also seen in periportal cells of the liver.

In a similar study Purchase and Nel (60) also reported a single-cell necrosis of the liver and a tubular necrosis of the kidney which, in the most severe cases, affected most of the cells in all tubules.

These authors also noted a slight vacuolation of the liver.

Biochemistry of Ochratoxins

Metabolism

Nel and Purchase (49, 60) investigated the metabolic fate of ochratoxin A in Wistar rats after intraperitoneal injection at a dose level of 10 mg/kg body weight. Extracts of ochratoxin from urine and feces contained unaltered ochratoxin A, ochratoxin a, and a second unidentified fluorescent metabolite. Ochratoxin A concentration was maximal at 6-18 hours in the urine and feces while the ochratoxin a concentration increased from six hours and was maximal at 30-42 hours. Ochratoxin A and minute quantities of ochratoxin a were also found in the blood, liver, and kidneys 30 minutes after intraperitoneal injection (49).

Since Purchase and Theron (59) found that administration of ochratoxin A to rats at a dose level of 10 mg/kg body weight caused accumulation of glycogen in the liver, Pitout (56) speculated that this probably results from ochratoxin interacting with nucleic acids and/or certain enzymes of carbohydrate metabolism. Pitout (56) subsequently found that ochratoxin A inhibits the hepatic phosphorylase enzyme system. The suggestion was advanced that the inhibitory effect of ochratoxin A on this system might be due to competition of the toxin

with 3'-5'-cyclic AMP for the enzyme phosphorylase b kinase. Phosphorylase b kinase catalyzes the activation of phosphorylase b to phosphorylase a, which in turn catalyzes the phosphorylytic cleavage of α-glucosidic 1,4-linkages of glycogen to α-glucose-1-phosphate. Pitout (56) reported no interaction of ochratoxin A with DNA, RNA, deoxyguanosine, deoxyadenosine, deoxycytidine HCl, thymidine, uridine, and 5-methylcytosine HCl. Also, no inhibitory effect was observed on the enzymes glucomutase, glucose-6-phosphate dehydrogenase, hexokinase, or phosphorylase a.

Moore and Truelove (47) found that ochratoxins A and a inhibited ADP-stimulated respiration when applied at low concentration to rat liver mitochondria. One hundred percent inhibition of respiration was noted at concentrations of 1×10^{-5} and 4.2×10^{-4} M for ochratoxins a and A respectively. Thus, the dihydroisocoumarin compound was more inhibitory than the parent compound.

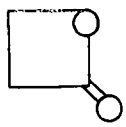
Carcinogenesis of Ochratoxin A

It is rather surprising that nothing has been reported concerning tests for ochratoxin carcinogenic activity. Therefore, the possibility that ochratoxin A possesses this activity can only be speculated. Certain unsaturated lactones having structural features similar to ochratoxin A have been reported to have a marked physiological activity in test animals (24, 25, 26, 27, 36). Dickens and Jones (24,

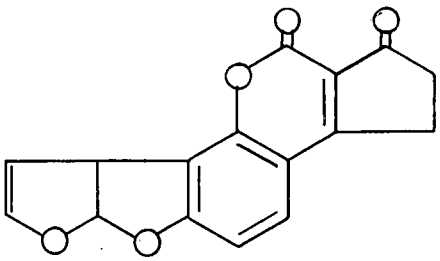
25, 26) studied the carcinogenic activity of four-, five-, and six-membered unsaturated lactones in rats and mice. They found that certain chemical features are prominent among the actively carcinogenic members of this series, including: (a) the presence of a four-membered heterocyclic ring such as β-propiolactone; (b) the presence of an α, β-unsaturated bond in a five or six-membered lactone ring; and (c) a cyclic anhydride such as maleic anhydride, which has α, β-unsaturation conjugated with two carbonyl groups in the anhydride ring. Structures of all compounds referred to in this section are shown in Figure 3.

Dickens and Jones (24) reported that carcinogenesis in four-membered ring lactones such as β-propiolactone appeared to be associated with highly strained rings. Roberts and Warwick, as cited by Dickens (27, p. 97), observed that β-propiolactone reacted with guanosine in the N-7 position to form 7-(2'-carboxyethyl) guanosine. This reaction could result in anomalous base pairing of the resultant ionized 7-alkylguanine with thymine instead of the usual guanine-cytosine pairing (10).

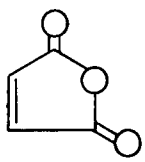
The presence of an α, β-unsaturated bond is reported to be a feature rendering five and six-membered lactones carcinogenic. For example, parascorbic acid, 2-hexenoic-γ-lactone and aflatoxin B_1 have been found to be active carcinogens (25). Lactones lacking the α, β-unsaturation have generally failed to produce tumors (27). An



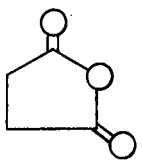
β- propiolactone



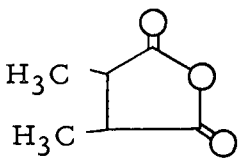
Aflatoxin B_1



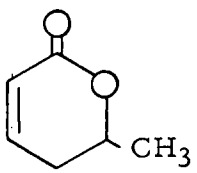
Maleic anhydride



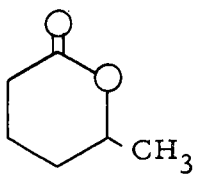
Succinic anhydride



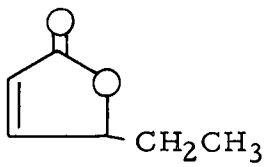
α, β- dimethyl maleic anhydride



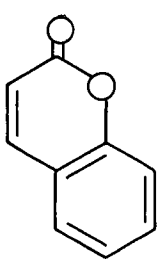
Parascorbic acid



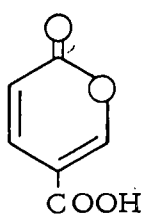
δ-valerolactone



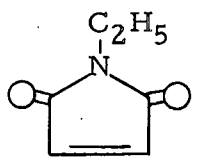
2-hexenoic-γ-lactone



Coumarin



Coumalic acid



N-ethyl maleimide

Figure 3. Structures of selected lactones.

example of this is δ-valerolactone the saturated counterpart of parascorbic acid.

The presence of α, β-unsaturation is not an absolute requirement for carcinogenicity, however, as pointed out by Dickens and Jones (26). These workers found that succinic anhydride and α, β-dimethyl maleic anhydride, which are two derivatives of α, β-unsaturated maleic anhydride, showed approximately the same carcinogenicity as maleic anhydride itself. It was pointed out, however, that anhydrides of this type are powerful acylating agents that react with sulfhydryl groups of cysteine in neutral aqueous solution. Therefore, these compounds might exert their effect on cellular proliferation mainly through reactivity with sulfhydryl groups essential to enzyme function (36).

The issue is further confused by the presence of α, β-unsaturation in some compounds not possessing carcinogenic activity. Dickens and Jones (26) found that no tumors were produced in rats treated twice weekly for 65 weeks with 2 mg of N-ethyl maleimide, coumalic acid, or coumarin, all of which possess α, β-unsaturation conjugated with a carbonyl group.

Therefore, it has generally been demonstrated that compounds having γ- or δ-lactones in which a carbonyl group is conjugated with one (e.g., parascorbic acid and 2-hexenoic-γ-lactone) or two (e.g., aflatoxin B_1 and maleic anhydride) double bonds are carcinogenic. The

ochratoxin molecule contains a six-membered unsaturated lactone ring which has a single double bond conjugated with the carbonyl group. It therefore possesses the structural features proposed by Dickens and Jones (25, 26) which are generally required for carcinogenesis.

EXPERIMENTAL

Preparation of Ochratoxins

Ochratoxins A and B were prepared by inoculating shredded wheat with Aspergillus ochraceus (American Type Culture Collection No. 18642)[1] and growing the mold for 19 days at 25°C. The crude ochratoxin was obtained by chloroform[2] extraction of shredded wheat. The chloroform extract was then fractionated into a sodium bicarbonate solution to remove ochratoxins A and B from their esters and less acidic or netural contaminants. After acidification to form the free acids, the ochratoxins were extracted from the aqueous phase with chloroform. Final purification was accomplished by column chromatography and recrystallization. Details of these procedures are reported in Appendices I through IX.

It should be emphasized that ochratoxin A has been reported to be unstable in daylight, especially in alkaline solutions (55). It was noted in the initial phases of this study that pure ochratoxins A and B, especially in the dry state, develop a yellowish-brown cast if exposed to daylight and/or strong incandescent light during handling. Therefore, all procedures were conducted in subdued light and

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[1] Obtained from A. D. Campbell, Food and Drug Administration, Washington, D. C.

[2] All solvents used in this study were USP or reagent grade, redistilled before use.

solutions of toxins were stored in the darkness at -15°C. By taking these precautions it was found that purified toxin solutions retained their purity and concentrations remained constant throughout the course of this study.

The dihydroisocoumarin derivatives of ochratoxins A and B were prepared by the acid hydrolysis method of van der Merwe (79). Since no procedures for purification of these compounds have been reported, all details of the methodology had to be determined. These derivatives were purified by silica gel column chromatography and recrystallization just prior to use. These procedures are found in Appendixes X through XV.

Ochratoxin Purity

Purity of each of the four ochratoxin compounds used in this study was verified in several ways. Thin layer chromatography[3] (TLC) using benzene:glacial acetic acid 9:1 v/v as the moving phase showed that only one fluorescent compound was present in the sample. The developed chromatograms were viewed in an ultraviolet viewing cabinet.[4] All compounds were spotted on thin layer plates at 8 μg/

[3] An MN-silica gel G-HR (Brinkman Instruments, Inc.) layer 250 microns thick, activated one hour at 110°C, was used throughout this study in an unequilibrated chamber unless otherwise stated.

[4] Chromato-vue cabinet, u.v., Model C-5, Ultraviolet Products, Inc., San Gabriel, California.

spot, developed, dried in air for 15 minutes, then scanned on a Photovolt densitometer[5] as described by Ayres and Sinnhuber (2). This indicated that only one fluorescent compound was present in each sample.

After scanning, each plate was placed in an iodine chamber for 30 minutes to detect possible non-fluorescent impurities. No such impurities were found.

Structural Confirmation

The structure of each of the four compounds studied was verified in several ways. Extracted ochratoxins A and B had the same R_f values as external standards of authentic ochratoxins A and B[6] when chromatographed on thin layer plates on silica gel in three different solvent systems (Table 1). Also, ethyl esters of ochratoxins A and B were prepared (Appendix XVII) and found to have R_f values identical to authentic ochratoxins C and B ethyl ester (identically prepared from authentic ochratoxin B) when chromatographed on silica gel in the three solvent systems shown in Table 1. Extracted ochratoxins A and B were also found to fluoresce bright blue on thin layer plates when exposed to ammonia fumes for five minutes.

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[5] Photovolt Corporation, New York, New York.

[6] Ochratoxin A, B, and C standards were obtained from A. D. Campbell, Food and Drug Administration, Washington, D. C.

Table 1. R_f values of ochratoxins in three solvent systems.

Ochratoxin	Solvent 1	Solvent 2	Solvent 3
A	0.50	0.67	0.73
B	0.29	0.56	0.66
C	0.70	0.92	0.88
B ethyl ester	0.55	0.68	0.73
a	0.34	0.26	0.69
b	0.24	0.23	0.62

Solvent 1: benzene:glacial acetic acid (9:1 v/v)
Solvent 2: benzene:methanol:glacial acetic acid (24:2:1 v/v/v)
Solvent 3: toluene:ethyl acetate:90% formic acid (5:4:1 v/v/v)

Ochratoxins a and b had the same R_f values as the dihydroisocoumarin derivatives prepared identically from authentic ochratoxins A and B when chromatographed in the three solvent systems shown in Table 1.

It should be pointed out that when developed chromatograms are observed under long wave ultraviolet radiation (366 nm), ochratoxins A, C, and a will fluoresce brightly. Under short wave ultraviolet radiation (254 nm) these compounds will retain their bright fluorescence. In addition, however, ochratoxins B, b, and B ethyl ester will now fluoresce brightly.

It was felt that there was a possibility that the severe acid hydrolysis procedure used in preparation of ochratoxins a and b might have altered the isocoumarin structure of the respective parent compounds.

In order to test this possibility ochratoxins A and B were hydrolyzed using a modification of the in vitro enzymatic hydrolysis procedure of Pitout (55). In this procedure 125 μg of ochratoxin A or B dissolved in 95% ethanol was placed in a small pear-shaped flask and evaporated to dryness using a rotary vacuum evaporator. The sample was dissolved in 2 ml of a 0.1 M NaCl-0.02 M Tris buffer, adjusted to pH 7.5 with HCl. Two microliters of carboxypeptidase A[7] was added and the hydrolysis was carried out in the darkness at 25°C for 24 hours.

Two microliters of the hydrolysates were spotted directly on three silica gel plates and the plates were developed in the three solvent systems shown in Table 1. The R_f's of the spots were compared with external standards of the parent ochratoxins and their corresponding dihydroisocoumarins prepared by acid hydrolysis. The R_f's of the enzyme-produced compounds had the same R_f values in all three solvent systems as the dihydroisocoumarin compounds produced by acid hydrolysis. Of academic interest, it was found that virtually all the ochratoxin B was hydrolyzed by the enzyme, while a substantial amount of ochratoxin A remained unaltered after 24 hours. The absence of the chloride on carbon five of ochratoxin B appears to allow this compound to be a better substrate for the enzyme.

Further structural confirmation was obtained using ultraviolet

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[7] Bovine Carboxypeptidase A, two times crystallized, purchased from Sigma Chemicals Co., St. Louis. Concentration = 50 mg/ml.

and mass spectrometry. The ultraviolet spectra of all four compounds used in this study were consistent with published data (50, 51, 74, 79). Mass spectra of ethyl esters of the four ochratoxins were found to be consistent with the structures involved. Mass spectra of ochratoxin ethyl esters were determined since it was found that the free acid forms of the toxins decarboxylate when subjected to the conditions used, thus making the molecular ion imperceptible. Appendix XIX contains details of the mass spectral analysis.

Quantitation

Quantitation of ochratoxins A and B was carried out using the proposed official first action procedure outlined by Nesheim (50). Ochratoxins a and b were quantitated spectrophotometrically using ultraviolet absorbance and molar extinction coefficients reported by Steyn and Holzapfel (74). Details of these procedures are reported in Appendix XVI.

Great care was taken to assure complete removal of benzene from all four ochratoxin compounds used in toxicity and feeding trial studies because of the possible hepatotoxic effects of benzene. This was done by recording the ultraviolet spectrum from 230 to 360 nm of each compound dissolved in 95% ethanol. Benzene has five major absorption peaks in the region 235 to 275 nm and a toxin solution containing benzene will show signs of these peaks. It was found that this technique

will detect about 1.0 ppm benzene in 95% ethanol.

Preparation of Diets for Ochratoxin A Feeding Trial Study

The levels of ochratoxin A incorporated into diets that were fed to rainbow trout are listed in Table 3. Ethanol solutions of ochratoxin A were quantitated so that 1 ml of the toxin solution could be added to 300 g salmon oil for preparation of Diets 68, 69, and 70. The salmon oil containing the toxin was incorporated at a 10% level into a semi-synthetic ration with composition shown in Appendix XVIII. Also fed was a control diet (Diet 1) containing 10% salmon oil incorporated into the semi-synthetic ration.

Ochratoxin A Feeding Trial

Rainbow trout (Salmo gairdneri) eggs of the Mt. Shasta strain were spawned and hatched in our laboratory facility[8] and held on Diet 1 for 30 days before initiation of experimental diets. Groups of 80 trout fingerlings were randomly selected and placed in 150 gallon fiber-glass tanks with a flow rate of four gallons per minute at a temperature of 12°C with an oxygen content of 8.5 to 9.5 ppm.

Duplicate groups of trout received one of the four diets listed in Table 3. After 4, 8, and 12 months, trout were taken from each tank

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[8] Food Toxicology and Nutrition Laboratory, Department of Food Science, Oregon State University, Corvallis, Oregon.

with a net designed for random sampling. Ten trout were sampled from each tank at four months. At eight months, a number was taken to reduce the total number of trout remaining in the tank to 50. Forty of the remaining trout were sampled at 12 months. Fish were killed with tricaine methanesulfonate, weighed, and explored for gross abnormalities. The livers from all samplings were examined for possible gross surface hepatoma nodes then preserved in Bouin's fixative. The livers from the 8 and 12 month samplings were hand sectioned in 1 mm slices and further examined for nodes. Several of the livers from trout fed each diet were microtome sectioned and stained with hematoxylin and eosin (38, p. 130) for microscopic examination. Results are reported in Table 3.

Ochratoxin Toxicity Experiments

All four ochratoxins tested in this study were found to be quite soluble in dilute sodium bicarbonate. Therefore, organic solvent vehicles to which rainbow trout are sensitive could be avoided. Since Bauer et al. (4) found that rainbow trout readily regurgitated orally dosed dimethylformamide solutions of aflatoxin B_1, it was decided to dose trout intraperitoneally first to establish toxic levels before attempting oral dosage.

Six-month old rainbow trout, reared on Diet 1 and weighing an average of 30 g, were fasted 48 hours then given a single dose of

ochratoxin delivered by intraperitoneal injection. The purified toxins dissolved in 95% ethanol were quantitated as in Appendix XVII immediately before dosing. After quantitation the ethanol was completely removed using a rotary vacuum evaporator. The dry toxin was dissolved in 0.1 N sodium bicarbonate to give the following concentrations of each toxin: ochratoxin A, 1.2 mg/ml; ochratoxin B, 5.0 mg/ml; ochratoxin a, 4.2 mg/ml; and ochratoxin b, 4.0 mg/ml. Dilutions were made from these solutions to give lower dose levels for the toxicity experiments. Control trout were dosed with 0.1 N sodium bicarbonate. The amount of each solution needed to inject into each fish was calculated from the following formula:

$$\mu l \text{ dose} = \text{weight of fish, g}/0.15$$

Therefore, a 30 g fish dosed with 200 µl of the 1.2 mg/ml solution of ochratoxin A received a dose level of 8 mg/kg body weight. Table 2 lists the dose levels injected of the four ochratoxin compounds.

Immediately before intraperitoneal dosage, each fish was anaesthetized with tricaine methanesulfonate, weighed, and marked by a differential fin clip. Fish were injected at a point just anterior and dorsal to the right pelvic fin, then placed in a large revival tub before returning to 150 gallon fiberglass tanks. Trout were not fed at any time after dosage. Mortality during the ten-day experimental period was recorded and the LD_{50} was determined by the method described by Litchfield and Wilcoxon (44).

The surviving trout were killed on day ten with tricaine methanesulfonate and autopsied. Gross abnormalities were recorded and the livers and kidneys were excised and preserved in Bouin's fixative. These tissues were microtome sectioned at 4 μ and stained with hematoxylin and eosin (38, p. 130) for light microscope examination.

An unsuccessful attempt to dose trout orally with ochratoxin A was made. Weighed, unanaesthetized trout averaging 30 g were orally dosed with #2 gelatin capsules[9] containing a bicarbonate solution of ochratoxin A and a red food coloring. The capsules were forced into the stomach with a glass rod. As many as 40% of all fish in a group were found to regurgitate capsules within the first 24 hours. Some trout dosed with capsules containing bicarbonate only were also seen to regurgitate their capsules. Thus, it appears likely that the toxin is not the only factor initiating the regurgitation response. It was concluded that oral dosage conducted in this manner is not practical with rainbow trout. It may be possible to incorporate the toxin in a food pellet which may be retained with greater probability than a gelatin capsule.

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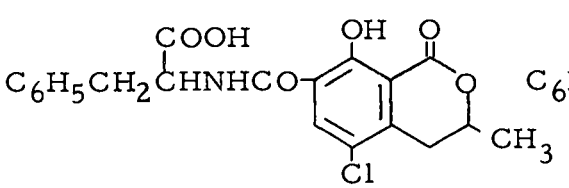
[9] Eli Lilly and Co., Indianapolis, Indiana.

RESULTS AND DISCUSSION

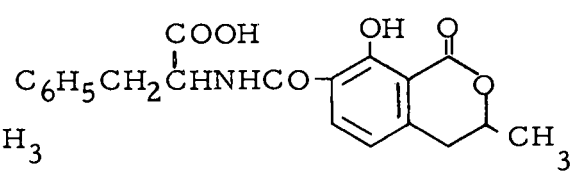
Acute Toxicity of Ochratoxins

General Observations

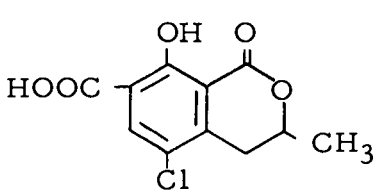
Of the four compounds dosed (see Figure 4 for structures) only ochratoxin A was found to be toxic (Table 2). Single intraperitoneal doses of ochratoxin A at 3, 4, 5, and 8 mg/kg body weight produced ten-day mortalities of 0/10, 3/10, 6/10, 8/10, and 10/10 respectively. Mortality did not occur until five days after treatment. Generally, the trout exhibited inappetance and weakness before death. No noticeable change in color was noted as previously found in aflatoxin B_1 poisoning of rainbow trout (4).



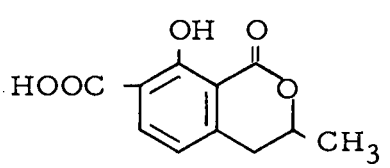
Ochratoxin A



Ochratoxin B



Ochratoxin a



Ochratoxin b

Figure 4. Structures of ochratoxin compounds studied.

Table 2. Mortality in rainbow trout ten days after intraperitoneal administration of ochratoxins.

Total dose (mg/kg body weight)	Mortality	Percent
0.1 N bicarbonate control	0/10	0
3.0 ochratoxin A	0/10	0
4.0 ochratoxin A	3/10	30
5.0 ochratoxin A	6/10	60
6.0 ochratoxin A	8/10	80
8.0 ochratoxin A	10/10	100
66.7 ochratoxin B	0/10	0
28.0 ochratoxin a	0/10	0
26.7 ochratoxin b	0/10	0

Autopsied trout dosed with ochratoxin A showed edema and multiple hemorrhages in adipose fat and the pyloric caeca area. This was particularly noticeable in those trout dosed with the highest levels of ochratoxin A. Livers were light brown to bright orange in color instead of their normal light to dark reddish brown (Figures 5 and 6). Kidneys appeared pale instead of their normal deep red color. Excised livers were swollen and tended to float briefly when placed in Bouin's fixative, indicating a liver of lower density than normal. This was especially noticeable with very orange livers produced by the highest levels of ochratoxin A.

The intraperitoneal LD_{50} of ochratoxin A was found to be 4.67 mg/kg body weight with 95% confidence limits of 4.03-5.42 mg/kg body

Figure 5. Normal macroscopic appearance of internal organs of a rainbow trout dosed with 0.1 N sodium bicarbonate. Note characteristic dark reddish brown liver.

Figure 6. Trout dosed with 4.0 mg/kg ochratoxin A showing characteristic orange liver seen in most fish dosed with this toxin.

weight. The slope function was 1.34 with 95% confidence limits of 1.21-1.486 mg/kg body weight.

Only the highest levels of ochratoxins B, a, and b that were dosed are shown in Table 3. The amounts of ochratoxins a and b shown correspond to ochratoxin A and B dose levels of 44 mg/kg body weight when calculated on an equimolar basis with the latter two toxins. No macroscopic abnormalities were found in autopsied trout dosed with ochratoxins B, a, and b, or the 0.1 N sodium bicarbonate solution.

Histology

Normal Trout Liver. The histology of a normal trout liver is adequately described in the following excerpts from Simon et al. (70):

> The most commonly encountered cell plates are 2 cells in thickness, bounded by a distinct endothelium and separated from other plates by sinusoid spaces. Weak polarization of nuclei is often observed with nuclei tending to be displaced toward the sinusoids. In younger fish (age 2-6 months) the sinusoids appear to be distended. This feature is consistent and apparently normal in young trout (p. 21).
>
> Bile canaliculi appear to be arrayed in branching, web-like fashion between contiguous parenchymal cells. The width of canaliculi is approximately 1 μ (p. 21).
>
> Trout fed with standard levels of dry diets typically display moderate cytoplasmic basophilia in hepatic cells. Not infrequently the parenchymal cells are highly vacuolated when examined in H and E paraffin sections. Fresh frozen sections with the same liver, when stained by the PAS method (with complementary diastase controls) and with oil red-O, indicate that glycogen is responsible for the great bulk of this vacuolation. At high magnifications a general dispersion of fine fat droplets (1 μ or less) is demonstrated by oil red-O staining. It has further been demonstrated that glycogen is

> absent and no vacuoles are present in liver cells of hatchery trout following withdrawal of food for 2 weeks (p. 22).
>
> Parenchymal nuclei are round or spherical with nuclear membranes sharply defined by basic stains. A single, prominent nucleolus is most frequent and often approximates a central position. Fine strands or clumps of chromatin constitute a typical feature. The nuclear space otherwise appears vacant following routine fixations and staining (p. 22).

Figure 7 shows a section of normal liver tissue taken from a trout dosed with 0.1 N sodium bicarbonate.

Livers from Ochratoxin Dosed Trout. Livers from trout dosed with the highest levels of ochratoxin A (4.0 mg/kg and above) had a normal architecture but many parenchymal cells were necrotic. Nuclei showed swelling, irregularities in shape, and chromatin margination. The most striking effect of ochratoxin A on the liver was the large number of cytoplasmic and nuclear vacuoles (Figure 8). Livers from trout dosed with the highest levels of ochratoxin A were beyond their normal functioning capacity.

Livers from trout dosed with less than 4.0 mg/kg ochratoxin A showed some disturbances but liver function was presumably little altered. Many nuclei were slightly enlarged, irregular, and lacking polarity. Isolated areas of vacuolation and parenchymal cell degradation were noted.

Livers from trout dosed with 28.0 mg/kg ochratoxin a, 26.7 mg/kg ochratoxin b and 0.1 N sodium bicarbonate were all relatively normal. Livers from trout dosed with 66.7 mg/kg ochratoxin B did

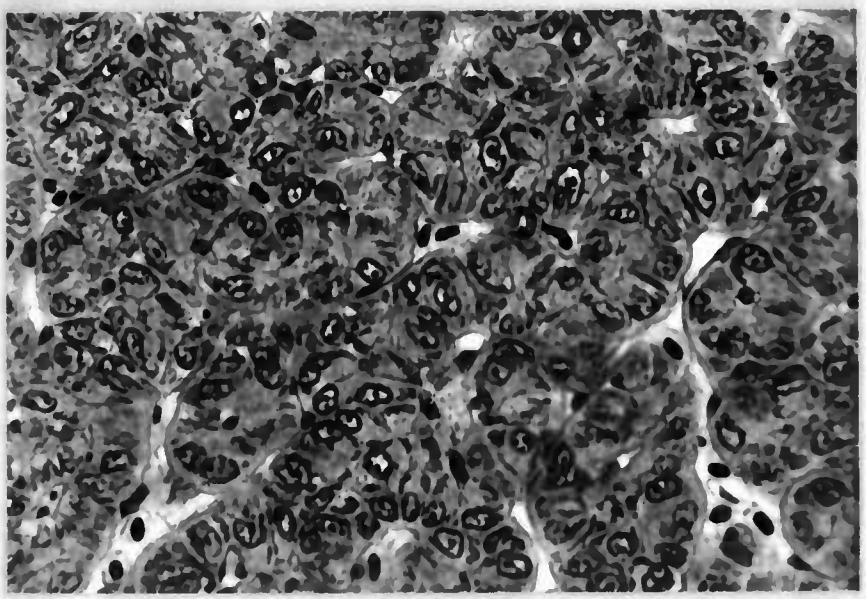


Figure 7. Normal trout liver. Note the prominent sinusoids and muralia or cords characteristically two cells thick. Parenchymal nuclei vary only slightly in size and shape and have a uniform chromatin distribution. Hematoxylin and eosin. X320.

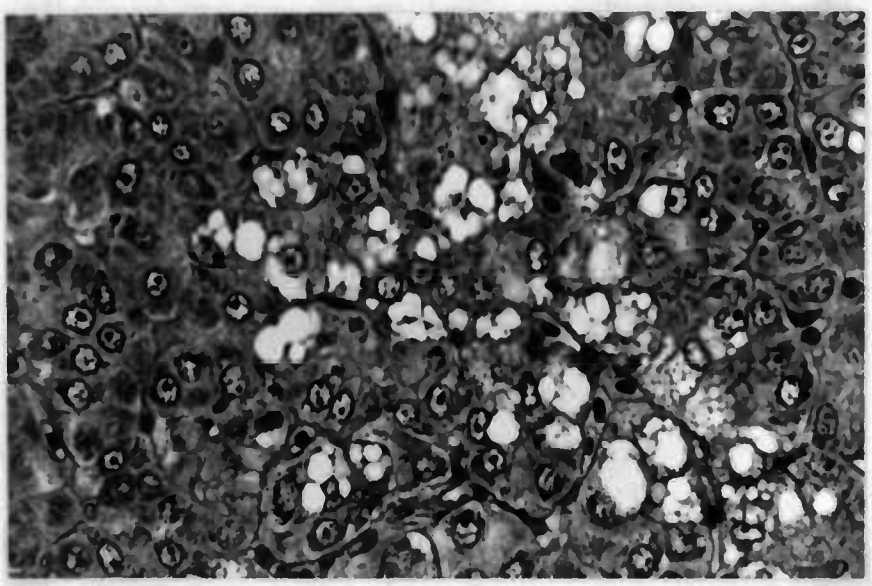


Figure 8. Liver from trout dosed with 2.0 mg/kg ochratoxin A showing nuclear and cytoplasmic vacuolation. Several nuclei have a relatively clear nucleoplasm and prominent nuclear membranes. Hematoxylin and eosin. X320.

contain swollen and irregularly shaped nuclei. Damage, however, was not nearly as severe as that caused by even the lowest levels of ochratoxin A.

Normal Trout Kidney. The normal structure of the euryhaline glomerular kidney of the rainbow trout has been clearly defined (14, 37). Hickman and Trump (37) describe the typical freshwater toleost nephron as being composed of the following regions: 1) renal corpuscle with well-vascularized glomerulus; 2) ciliated neck region; 3) first proximal segment with prominent brush border and numerous lysosomes; 4) second proximal segment with a less developed brush border; 5) narrow ciliated intermediate segment; 6) distal segment with relatively clear cells; and 7) collecting duct system. Figure 9 shows a representation of the various morphological regions of the rainbow trout nephron. Some of these regions are also shown in Figure 10.

The functional significance of homologous regions of the fish nephron have been adequately described by Hickman and Trump (37). The glomerulus functions basically as a filter to selectively pass materials from the blood into the tubular lumen. These materials then pass into the neck region of the nephron. The ciliary activity of this region is assumed to be important in movement of materials from Bowman's capsule which surrounds the glomerulus.

The first proximal segment, with its prominent lysosomes, has

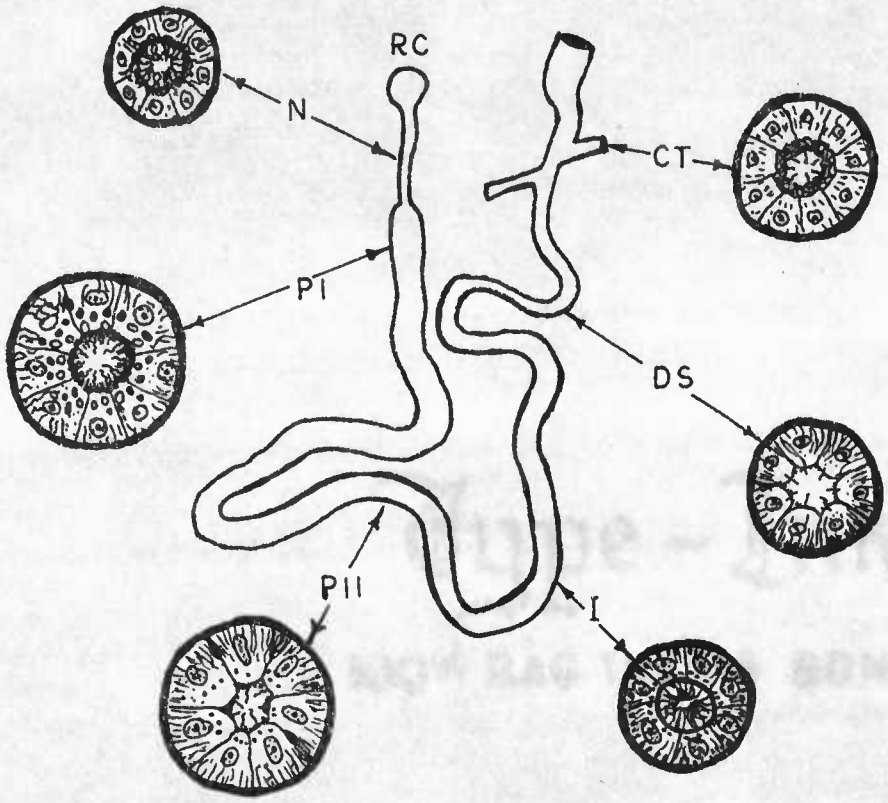


Figure 9. Schematic representation of the nephron segments in the rainbow trout. Relative sizes are approximately as shown. The morphological regions are renal corpuscle (RC), neck (N), first proximal segment (PI), second proximal segment (PII), distal segment (DS), and collecting tubule (CT).

been shown to be involved in pinocytic uptake of particles and probably many macromolecules (5). Other functions include reabsorption of monovalent ions such as sodium and chloride.

The second proximal segment constitutes the longest portion of the nephron. Its poorly developed lysosome and pinocytic vesicle systems suggest that its macromolecular uptake is minimal. This segment is probably responsible for the bulk of the divalent ion secretion.

The narrow intermediate segment, with its highly developed ciliary activity is thought to act as a pump to aid in the movement of fluids passing along the nephron.

The distal segment participates in active sodium resorption. Since this resorption is not isosmotic, it appears that the distal segment may be important in monovalent ion retention.

The collecting tubules and ducts appear to facilitate the formation of dilute urine by reabsorbing monovalent ions from the glomerular filtrate.

Besides the structures of the kidney associated with the common kidney functions of excretion and resorption, the kidney also contains a considerable amount of interstitial hematopoietic tissue. Contrary to mammals where red blood cells are produced in the bone marrow, the hematopoietic tissue of the kidney serves as the primary producer of red blood cells in the fish.

Kidneys from Ochratoxin Dosed Trout. All elements of kidneys including tubules, glomeruli, and hematopoietic tissue were necrotic in trout dosed with the highest levels of ochratoxin A. Disruption of plasma membranes along the lumen surface of the tubular epithelium with sloughing of cellular components into the lumens was a striking alteration (Figures 11 and 13). This was particularly noticeable in tubules of the first and second proximal segments. The cellular fragments appeared to be membrane limited and contained a variable number of organelles and occasionally a nucleus. In many cases it was difficult to determine the section of the nephron from which a tubule came because of the necrotic changes. Many tubule nuclei were pycnotic, especially in those tubules containing casts. Many symmetrically round vacuoles suggestive of lipoidal material were also seen in tubules of the first and second proximal segments (Figures 11 and 13). All other regions of the nephron showed few morphological changes other than nuclear and cytoplasmic swelling.

Some kidneys from trout dosed with lower levels of ochratoxin A showed lesions similar to those already described. Lesions were much less extensive, however, and function was presumably not seriously impaired.

Damage done to kidneys by the highest levels of ochratoxins B, a and b shown in Table 3 was quite variable. Kidneys from trout dosed with ochratoxins a and b showed relatively minor abnormalities such

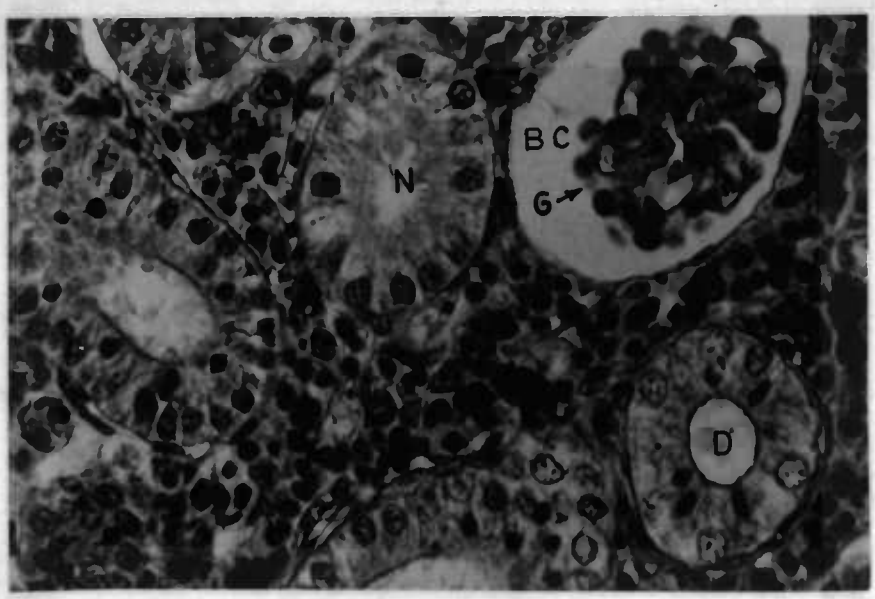


Figure 10. Normal kidney from trout dosed with 0.1 N sodium bicarbonate showing Bowman's capsule (BC), glomerulus (G), tubule from neck region (N), and distal tubule (D). Hematoxylin and eosin. X320.

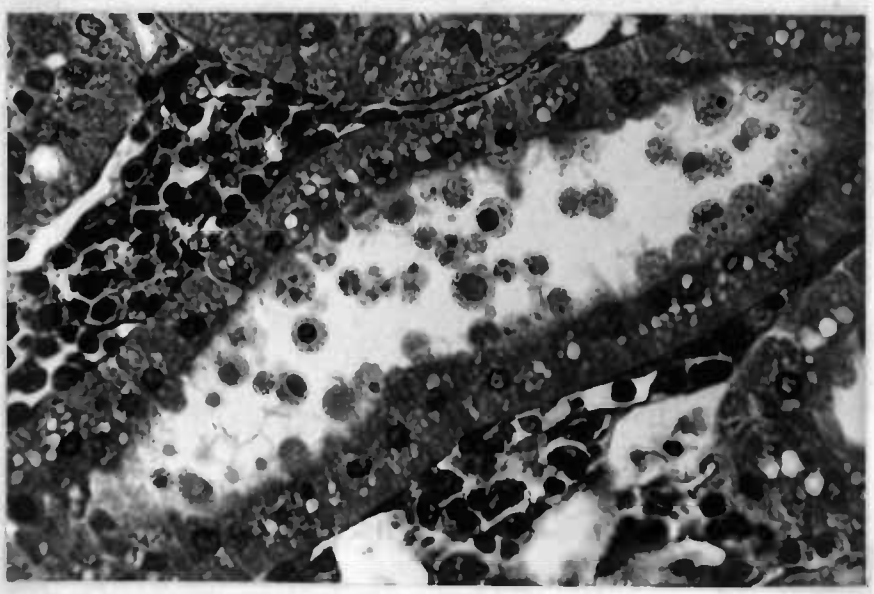


Figure 11. Necrotic proximal tubule from trout dosed with 4.0 mg/kg ochratoxin A. Note pycnotic nuclei, cytoplasmic vacuolation, and cast formation. Hematoxylin and eosin. X320.

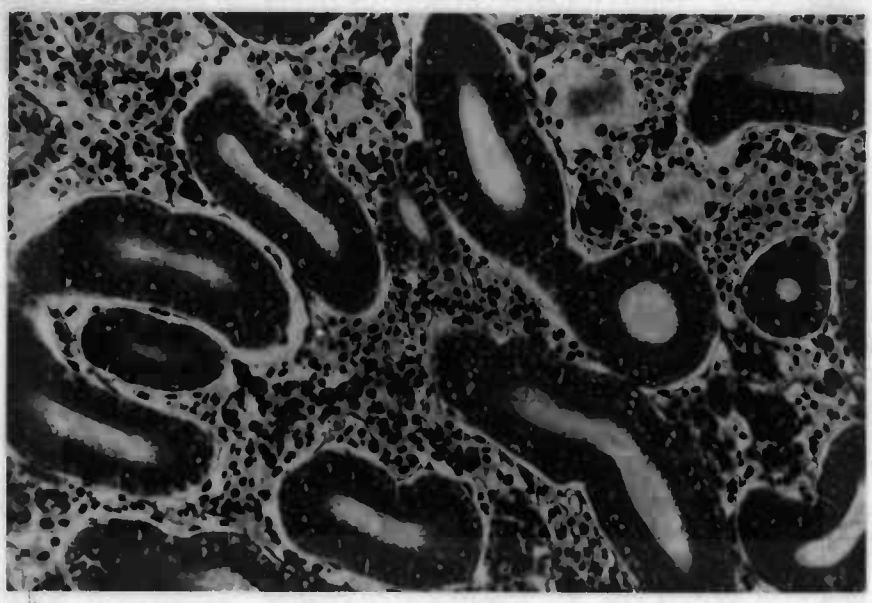


Figure 12. Normal kidney from trout dosed with 0.1 N sodium bicarbonate. Note characteristic patent lumens of tubules and the absence of vacuoles. Hematoxylin and eosin. X128.

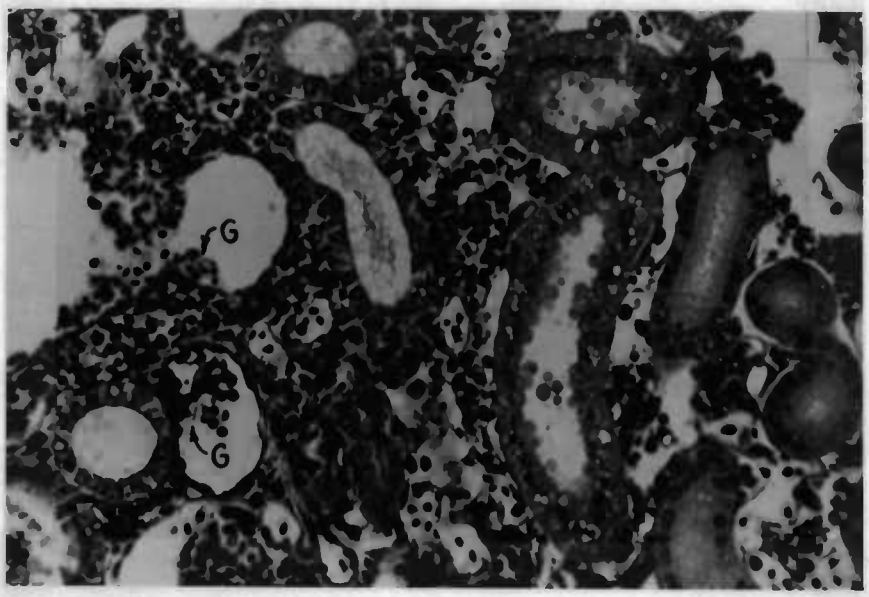


Figure 13. Kidney from trout dosed with 4.0 mg/kg ochratoxin A showing necrotic renal tubules containing pycnotic nuclei and vacuoles. Note also the necrotic glomeruli (G). Hematoxylin and eosin. X128.

as swollen nuclei and vacuolation of the proximal tubules. The vacuoles were much smaller and fewer in number than those seen in kidneys from trout dosed with ochratoxin A. Kidney function in trout dosed with ochratoxins a and b is presumably little impaired.

Kidneys from trout dosed with 66.7 mg/kg ochratoxin B definitely showed injury (Figure 15). Cast formation, vacuolation, and swollen nuclei were seen in proximal tubules. No pycnotic nuclei were observed. Damage done to these kidneys was comparable to that done by the lowest levels of ochratoxin A that were dosed.

The pathological changes noted in kidneys from trout dosed particularly with ochratoxin A are quite common changes that are associated with focal cellular necrosis induced by toxins and infectious diseases (7, 14, 23, 33, 84). The changes are also similar to those produced by ochratoxin A administration to rats and ducklings (59, 71).

Thus, the dechloro ochratoxin compounds are relatively non-toxic to rainbow trout at quite high levels. Also, the phenylalanine moiety must be attached to the chlorine-containing dihydroisocoumarin (ochratoxin a) for the toxic properties to prevail. It seems likely that an animal metabolizing ochratoxin A to ochratoxin a is performing a detoxification mechanism. Parke and Williams (53) consider the metabolism of toxic substances to be a two phase process in which a toxin is usually converted into more polar products than the parent compound in phase I reactions. Phase II products are classified as

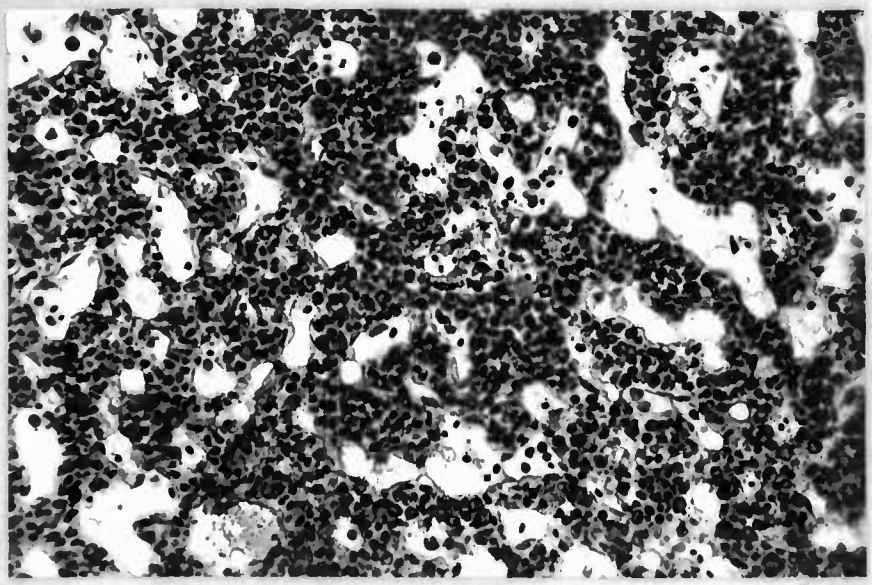


Figure 14. Necrotic hematopoietic tissue in kidney from trout dosed with 4.0 mg/kg ochratoxin A. Hematoxylin and eosin. X128.

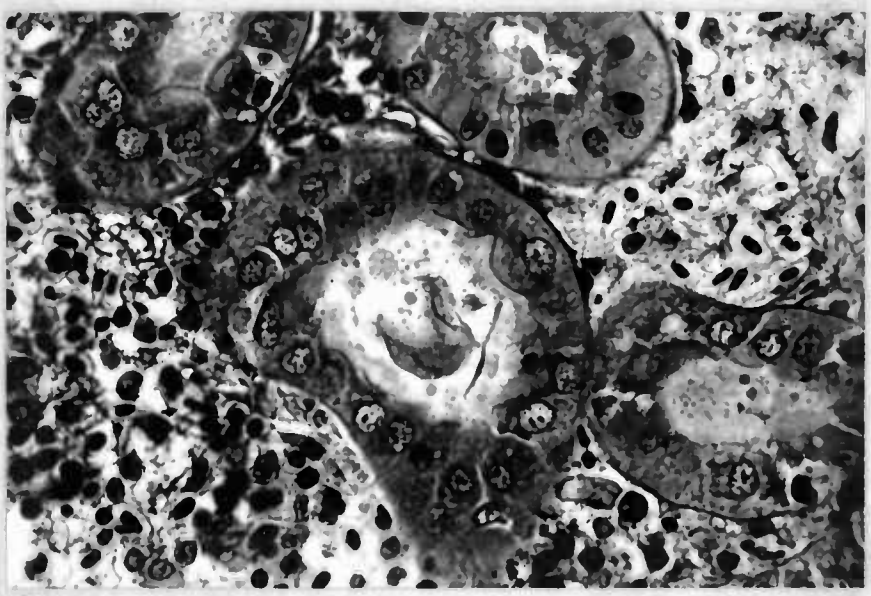


Figure 15. Kidney from trout dosed with 66.7 mg/kg ochratoxin B. Note center proximal tubule with extremely large nuclei. Lumen contains a portion of the ciliated epithelium in addition to several membrane limited casts. Hematoxylin and eosin. X320.

syntheses usually resulting in the production of acidic compounds. The parent compound is thus converted into water-soluble polar products which are readily excreted. Since the dihydroisocoumarin derivatives are more polar than their parent toxins (Table 1), it seems likely that these derivatives may represent phase I products.

It is interesting to note, as previously stated, that Moore and Truelove (47) found ochratoxin a to be more inhibitory to ATP-stimulated mitochondrial respiration than ochratoxin A. The significance of this in terms of the present study cannot be stated from the available information. It can, however, be stated that if inhibition of mitochondrial respiration occurs in the livers of trout dosed with ochratoxin a, it has virtually no toxic effect on the animal.

Histochemical Study of Trout Liver and Kidney Vacuolation

Procedure

Since the vacuoles seen in the hematoxylin and eosin stained livers and kidneys from ochratoxin A dosed trout were perfectly round in shape it was felt that they probably contained lipid material. In order to investigate this possibility five 30 g trout were intraperitoneally dosed at a level of 4.0 mg/kg body weight with ochratoxin A dissolved in 0.1 N sodium bicarbonate. After a ten-day incubation period the trout were killed with tricaine methanesulfonate and the

livers and kidneys were excised and fixed in neutral formalin. Tissues were frozen and cryostat sectioned at 15 μ then stained with Sudan IV (38, p. 255) and examined by light microscopy.

Livers

Hepatic parenchymal cells were found to contain a large number of fat droplets stained red by the Sudan IV (Figures 17 and 18). Particularly interesting was the presence of intranuclear lipoid inclusions in liver nuclei. Liver sections were examined to determine the distribution of lipid in the nucleus. In some cases lipoidal materials appeared to be in the interior of the nucleolus, while in others, globular lipoidal droplets appeared in other areas of the nucleus not directly related to the nucleolar structure. As many as five distinctly separate lipid droplets were seen particularly in swollen nuclei in areas of the liver where the surrounding cytoplasm was similarly infiltrated with lipid droplets (Figure 18).

Several possibilities exist which might account for the presence of intranuclear lipid droplets. Luse et al. (45) suggested that nuclear pores and/or pinocytosis by the nuclear membrane are involved in nuclear penetration of colloidal materials. Since most nuclei containing intranuclear fat were quite swollen, it seems likely that a disruption of the nuclear membrane has occurred, thus facilitating the transfer of lipoidal material into the nucleus where it coalesces to form a

droplet. This possibility could be investigated using electron microscopy.

Several mechanisms have been implicated in the pathogenesis of hepatic steatosis (39, 43). Most of the information concerning this problem comes from studies with rats and other warm-blooded animals. It should be emphasized, therefore, that proposed mechanisms of fatty liver production might not be applicable to rainbow trout. Figure 16 indicates a composite of the mechanisms for production of fatty liver proposed by Lieber (43) and Judah (39). Free fatty acids move from peripheral fat deposits to the liver. There they are either oxidized to CO_2 (β-oxidation scheme) or formed into triglycerides and phospholipids. The triglycerides and phospholipids are conjugated with a globulin to form lipoproteins which are then secreted.

The points at which the fat cycle is interrupted resulting in a fatty liver are shown at points a through m in Figure 16. Previous research indicates that various chemical compounds induce fatty livers by acting at one or more of these points. For example, carbon tetrachloride acts by blocking synthesis of the carrier globulin (mechanism l) resulting in a block in the synthesis of lipoprotein causing lipid to accumulate (43). At k is the proposed site of ethionine acting by blocking lipoprotein transport (39). Ethanol has been shown to act at point c resulting in a fatty liver due to increased hepatic fatty acid synthesis (43).

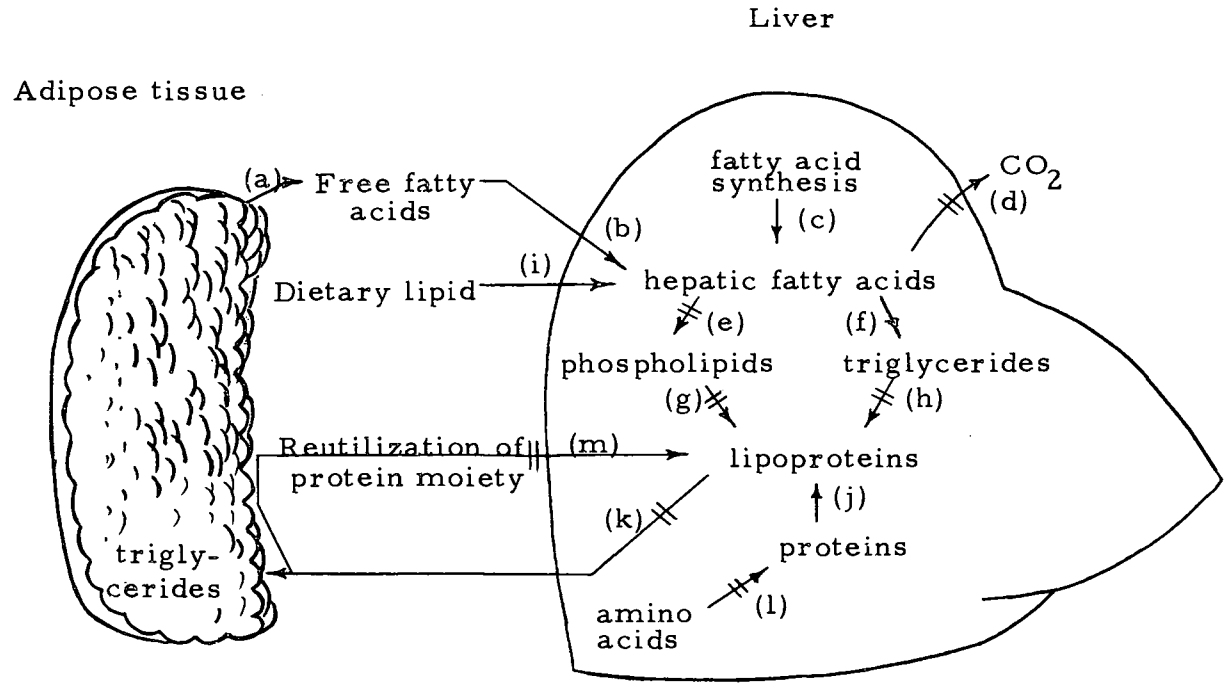


Figure 16. The fat cycle, showing mechanisms for fatty liver production, either through increase(⟶) or decrease(⟶‖) in metabolic pathways.

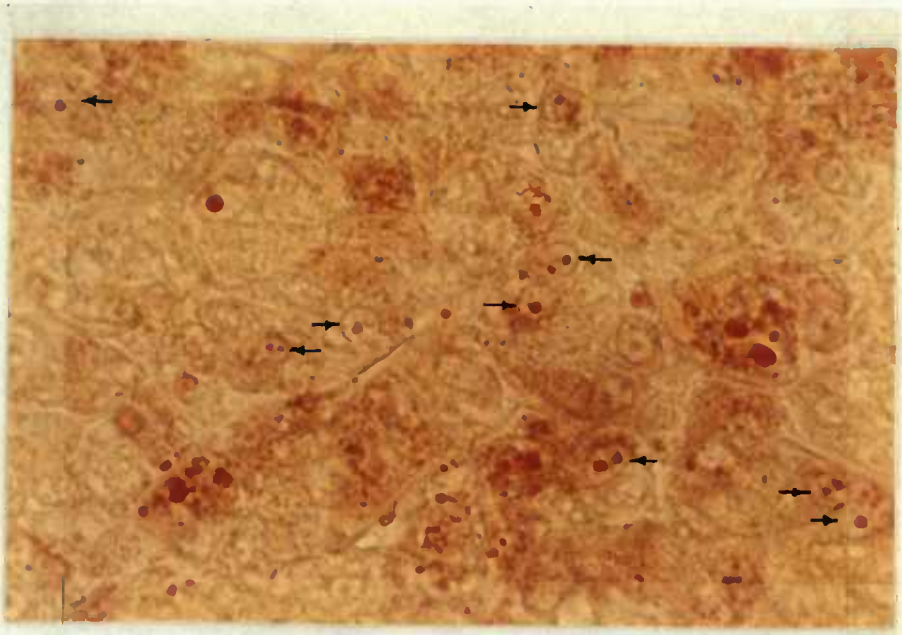


Figure 17. Liver from trout dosed with 4.0 mg/kg ochratoxin A showing lipid droplets in the cytoplasm and nuclei (arrows). Lipid droplets in nuclei were most commonly seen in areas of the cytoplasm which were heavily infiltrated with lipid. Sudan IV. X320.

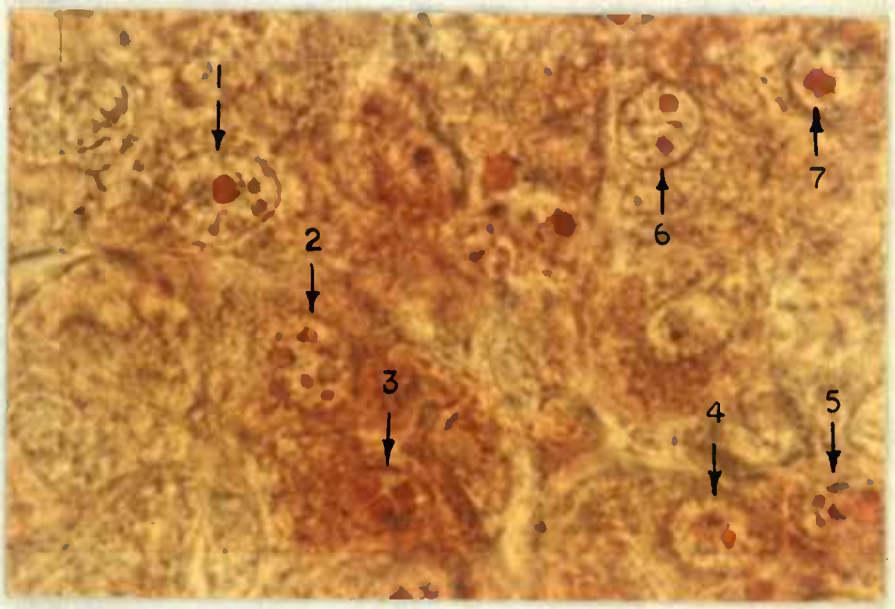


Figure 18. Liver from trout dosed with 4.0 mg/kg ochratoxin A showing at least seven nuclei containing lipid vacuoles. Nucleus #7 contains lipoidal material in the interior of the nucleolus while nucleus #1 also contains several small lipid droplets in the nucleoplasm surrounding the central lipid-containing nucleolus. Sudan IV. X800.

It seems likely that analogous mechanisms may be involved in hepatic steatosis in rainbow trout. While the exact nature of the mechanism in trout is unknown, it is conceivable that lipid accumulation in circumstances of liver poisoning by ochratoxin A may, in general, be due to lipid mobilization from peripheral fat (adipose tissue) to the liver and/or a diminished capacity of injured hepatic cells to degrade fatty acids.

The problem of fatty liver development is summed up very aptly in the following statement by Lieber (43):

> Thus, a variety of intoxications and deficiency states lead to development of hepatic steatosis. The problem of pathogenesis, however, is complicated by the fact that for the various individual types of fatty livers, multiple possible pathogenic mechanisms have been demonstrated. Generally it has not been possible to determine which of these mechanisms play a primary role and which are consequences of other defects or simply result from unusual experimental conditions (p. 762).

Kidneys

Renal tubules of the first and second proximal segments were found to contain a large number of lipid droplets (Figure 19). In all cases the droplets were extranuclear. The cytoplasm of many tubules appeared to be completely infiltrated with these droplets.

The presence of lipid droplets in renal tubules has been observed in a variety of pathological states that result in acute tubular injury.

Figure 19. Fat laden proximal tubules in kidney of trout dosed with 4.0 mg/kg ochratoxin A. Note distal tubules (D) completely devoid of lipid. Sudan IV. X128.

Lipid droplets have been reported in experimental pathology (7, 14, 35, 63) and in man in cases of chronic nephropathy (33). The lipid droplets most likely originate either from decreased cellular utilization of lipid normally brought to the cell or from some abnormality of lipid transport (14, 33). Chatelanat and Simon (14) present evidence indicating that "it is not likely that they can be due directly to fatty degeneration or lipophanerosis of cellular structures" (p. 465). Thus, the mechanism may be similar to that believed to occur in acutely injured hepatic parenchymal cells.

If the lipid is arising from an abnormality of lipid transport that brings about increased mobilization of fat, uptake of these colloidal materials by way of pinocytosis is a likely mechanism for transport

across the surface membranes in different cell types of the kidney (30). The fact that the first and second proximal segments of the nephron possess pinocytic vescicle systems tends to support this view. This also explains why vacuoles were not seen in distal tubules since the distal segment participates primarily in monovalent ion resorption (37).

Ochratoxin A Feeding Trial

From the results shown in Table 3 it can be seen that all levels of ochratoxin A fed to trout failed to induce hepatoma in the 12 month study period. Also, no other gross abnormalities were observed in any of the trout.

Table 3. Hepatoma incidence in rainbow trout fed experimental diets.

Diet number	Description	4 Month sample	8 Month sample	12 Month sample
1-1970	control	0/20	0/24	0/80
68-1970	16 ppb ochratoxin A	0/20	0/36	0/80
69-1970	32 ppb ochratoxin A	0/20	0/33	0/80
70-1970	64 ppb ochratoxin A	0/20	0/41	0/80

Histological examination revealed a considerable amount of damage in livers from trout fed diets containing ochratoxin A, particularly at 8 and 12 months. Livers had a normal architecture but in many cases showed foci of swollen or hypertrophic parenchyma

cells with heavily vacuolated cytoplasm. The vacuoles lacked the characteristic symmetrical shape of lipid vacuoles and were therefore assumed to contain glycogen. Similarly, large irregularly shaped nuclei were seen in some cases scattered around the periphery of the muralia (Figure 20). Figure 21 shows a normal liver for comparison.

Some livers also showed a considerable number of lymphocytes. In most cases the lymphocytes were scattered in small foci, while in others, quite large foci were seen presumably attacking parenchymal cells (Figure 22). The large focus shown in Figure 22 appeared at first to be attacking seemingly more basophilic parenchymal cells but upon closer examination, the cells appeared no more basophilic than those outside the lymphocyte focus.

Other more discrete abnormalities seen in livers from trout fed ochratoxin A include an occasional focus of bile ductule proliferation (Figure 23) and a mild arterial hyperplasia (Figure 24). The proliferations appear diffuse and poorly differentiated but definitive in many cases. The hyperplasia of the arterial wall was not a significant characteristic of livers from trout fed diets containing ochratoxin A and only a few mild cases were seen.

Most of the prominent pathological changes noted in livers from trout fed diets containing ochratoxin A have been associated with various types of damage which precede hepatomagenesis in trout (1, 81). Since ochratoxin A appears to be a relatively mild toxin compared

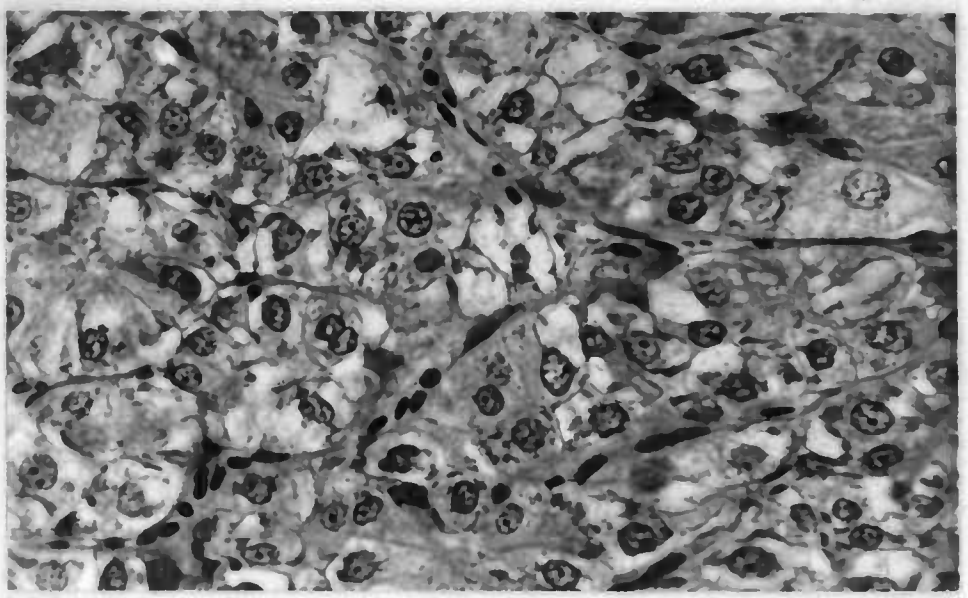


Figure 20. Liver from trout fed 64 ppb ochratoxin A for eight months. Note swollen nuclei in some cases scattered around the periphery of the muralia. The large masses of cytoplasmic deposits characteristic of glycogen appear to displace the nuclei to the periphery of the muralia. Hematoxylin and eosin. X320.

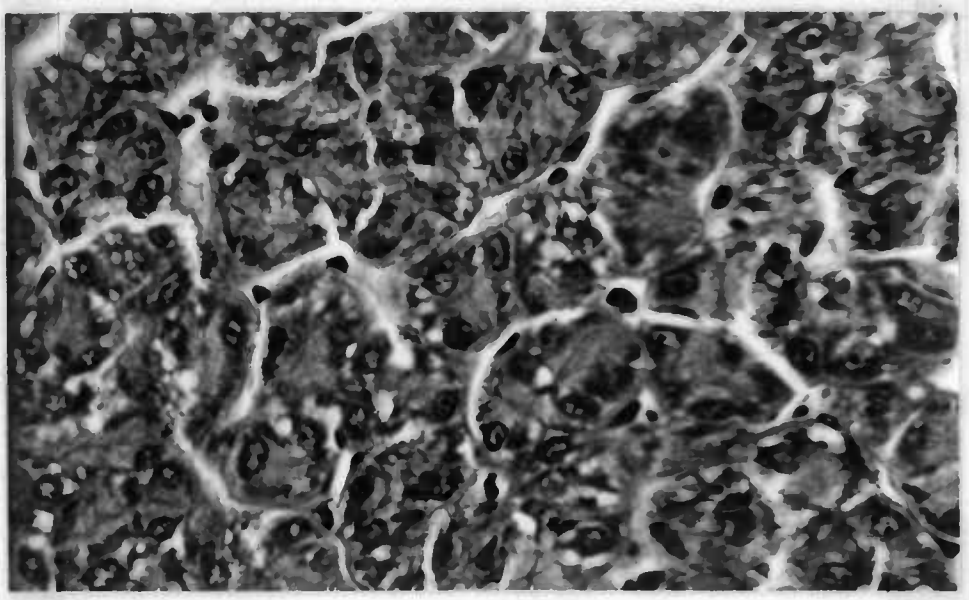


Figure 21. Normal liver from trout fed Diet 1 for eight months. Note characteristic small oval nuclei varying only slightly in size and shape. This liver shows typical amount of deposits found in trout that have not been fasted. Hematoxylin and eosin. X320.

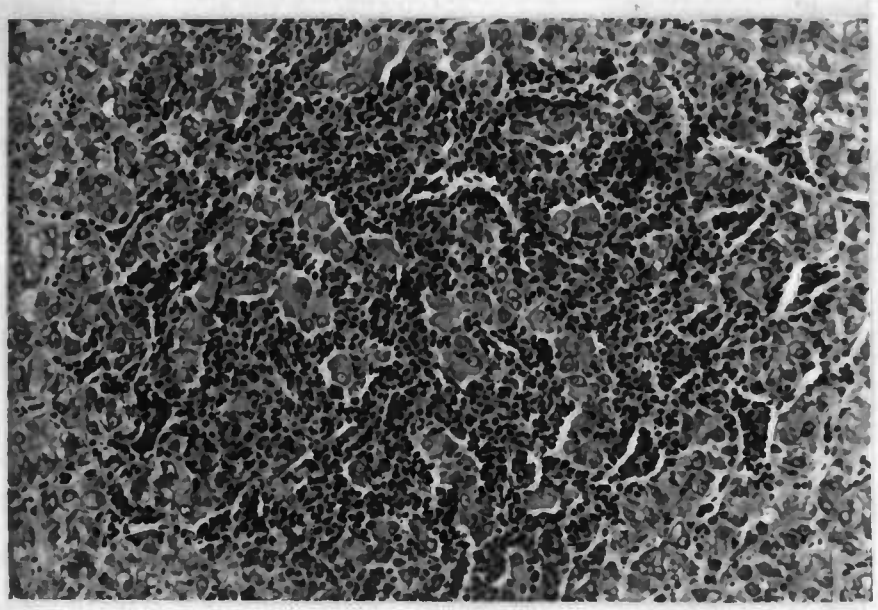


Figure 22. Large focus of lymphocytes in liver from trout fed 64 ppb ochratoxin A for 12 months. Hematoxylin and eosin. X128.

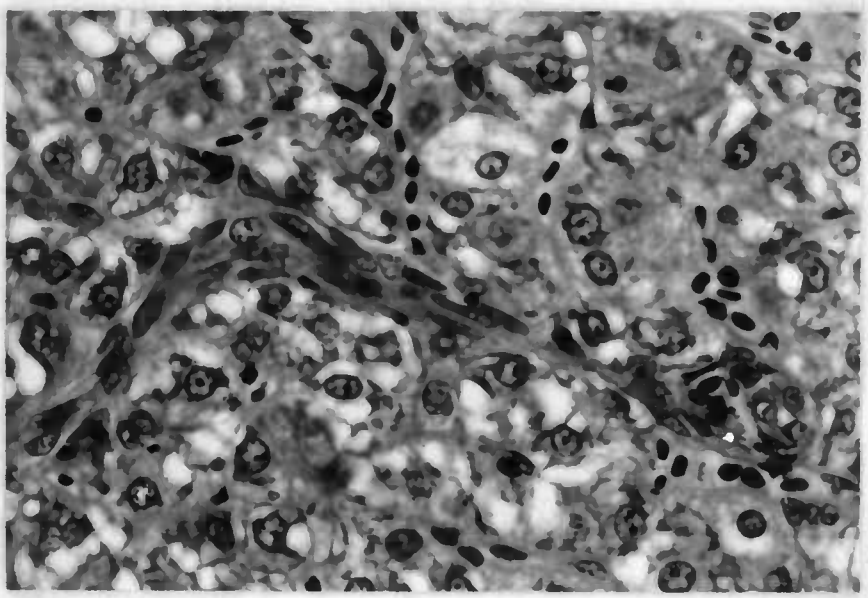


Figure 23. Focus of bile ductule proliferation in liver from trout fed 64 ppb ochratoxin A for eight months. Hematoxylin and eosin. X320.

to aflatoxin B_1, it would seem that it might also be a very mild carcinogen. In contrast to this, it is also possible that ochratoxin A might in fact be a very potent toxic and carcinogenic agent but because of its extreme water solubility, it is excreted much more readily than aflatoxin B_1. Therefore, the levels used here could have been too low or the feeding trial not conducted long enough to induce hepatomagenesis. For these reasons the feeding trial experiments are currently being repeated at ochratoxin A levels ranging from 128 to 512 ppb.

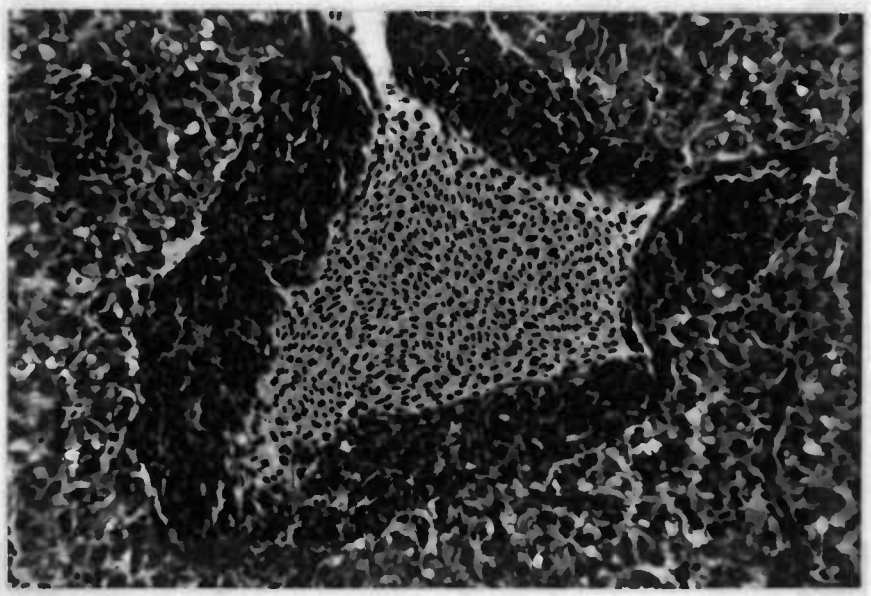


Figure 24. Mild arterial hyperplasia in liver from trout fed 64 ppb ochratoxin A for eight months. Hematoxylin and eosin. X128.

SUMMARY AND CONCLUSIONS

Ochratoxin A was the only one of four structurally related compounds found to be lethal to rainbow trout. Lack of either the chloride group or phenylalanine moiety of ochratoxin A renders the compound completely non-toxic at levels approximately ten times greater than the original toxic levels of ochratoxin A. This probably accounts for at least one detoxification mechanism whereby the phenylalanine moiety of ochratoxin A is hydrolytically cleaved from the original structure by proteolytic enzymes such as carboxypeptidase in the small intestine of the animal which has ingested the toxin.

In summary, the primary lesions seen in ochratoxin A poisoned trout were the following:

1. Hepatic parenchymal cell degeneration including nuclear swelling and cytoplasmic and nuclear lipid vacuolation.
2. Necrosis in proximal tubules, hematopoietic tissue, and glomeruli of kidneys.
3. Pycnotic nuclei, cast formation, and lipid vacuolation particularly in the first and second proximal segment of the renal tubules.

Ochratoxin A was not found to be carcinogenic to trout when fed at levels up to 64 ppb in a semi-synthetic diet for 12 months. Prominent pathological changes in the livers such as nuclear swelling,

parenchymal cell degeneration, and bile ductule proliferation which characteristically precede hepatomagenesis were seen. For this reason it was felt that ochratoxin A may indeed be a mild carcinogen. This possibility is currently being investigated using higher levels of ochratoxin A in experimental trout diets.

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APPENDICES

APPENDIX I

Preparation of Mold Spores for Inoculation

1. Fill a one liter Roux culture bottle with 100 ml Czapek's solution agar:

   35 g Czapek Dox broth
   20 g Bacto mycological agar
   One liter distilled water

   Plug with cotton and sterilize at 121°C, 15 psig for 15 minutes. Cool to room temperature.

2. Inoculate Aspergillus ochraceus spores onto the agar face in 5 ml distilled water and distribute evenly.

3. Incubate at 25°C for four days in normal position then incubate upside down for an additional six days.

4. Add 100 ml of sterile soap solution (0.02% sodium dodecyl sulfate in distilled water) to the bottle and rinse spores from agar surface and place in a sterile 200 ml centrifuge bottle. Centrifuge at 2000 x g for five minutes, then wash spores with two 150 ml portions of sterile distilled water by centrifugation.

5. Suspend spores in 150 ml of sterile distilled water and store in 50 ml bottles at 2°C.

6. Viable spores are counted by pour plates with Czapek's agar on serial dilutions of the spores made in sterile distilled water (four-100 fold). Triplicate plates containing three-30 spores are compared after a four-day incubation period at 25°C and the number of viable spores per ml is calculated.

APPENDIX II

Growth of *Aspergillus ochraceus* on Shredded Wheat

1. Add 100 g crumbled shredded wheat and 55 ml distilled water to a 2.8 l Fernbach flask. Plug the flask with gauze-wrapped cotton and autoclave 20 minutes at 121°C. Allow to cool to room temperature.
2. Each flask is evenly inoculated with at least 10^6 spores in 2 ml sterile distilled water with a syringe.
3. Flasks are placed in an incubator at 25°C for 19 days.

Moisture accumulates as condensation on the shredded wheat and sides of flask on the fourth day of incubation. Toxin yields from the shredded wheat using ATCC 18642 are about 270 mg ochratoxin A and 180 mg ochratoxin B per 100 g shredded wheat.

Reference: Schindler and Nesheim (64).

APPENDIX III

Extraction of Ochratoxin from Shredded Wheat

1. During extraction of toxins, the worker should wear a mask to protect against inhalation of mold spores and gloves to protect the hands from toxins. Gloves should be worn during all other procedures involving the handling of toxins.
2. Add 500 ml chloroform to each Fernbach flask using a funnel to bypass the cotton plug. The chloroform is rinsed around the flask by swirling, the plug is then removed, and the mycelial mat broken up with a wooden dowel. The solvent-shredded wheat mixture is transferred to a 5000 ml reaction flask. Rinse each flask with 100 ml chloroform and transfer chloroform to the reaction flask.
3. The reaction flask is made up of a spherical lower section provided with a stopcock for drainage of chloroform extracts and a top section with three ℑ 24/40 outer necks which accommodate a condensor and ground glass stirrer assembly. The reaction flask is heated with a hemispherical heating mantle with a bottom outlet to permit passage of the stopcock.
4. The first extraction consists of gently refluxing the mixture for one hour while stirring the flask contents. The chloroform extract is drained from the flask and force filtered through a Buchner funnel to remove spores.
5. Two additional extractions are performed exactly as before using 500 ml portions of chloroform per 100 g culture added to the reaction flask. All filtrates are combined for sodium bicarbonate fractionation.

APPENDIX IV

Bicarbonate Fractionation of Extracted Ochratoxins

1. A 1000 ml portion of the combined chloroform extract is placed in a 2000 ml separatory funnel. To this is added a 500 ml portion of 0.5 N sodium bicarbonate. Ochratoxins A and B are partitioned into the aqueous bicarbonate phase with shaking. If a heavy emulsion forms, it can be broken by adding a small amount of 95% ethanol while swirling the separatory funnel.
2. After separation of the two phases, the lower chloroform layer is drawn into a flask and saved for further bicarbonate extractions. After draining the bicarbonate layer into another flask, the chloroform layer is returned to the separatory funnel and extracted two additional times with 500 ml portions of 0.5 N sodium bicarbonate.
3. The combined bicarbonate extracts containing the ochratoxins are acidified to pH 1-2 with 2 N HCl to form the free ochratoxin acids. Two volumes of the acidified aqueous phase are then extracted with one volume of chloroform. Repeat this extraction three times.
4. The combined chloroform solution is dried over anhydrous sodium sulfate, filtered, evaporated to dryness with a vacuum rotary evaporator, then dissolved in minimal chloroform.

The original chloroform extract from the shredded wheat cultures is a dark reddish color. The sodium bicarbonate fractionation procedure effectively removes most of this color in addition to neutral or less acidic fluorescent contaminants such as ochratoxin esters. The weight of crude ochratoxins obtained by bicarbonate fractionation of the initial chloroform extract is about 1 g per 100 g shredded wheat culture extracted.

APPENDIX V

Separation of Ochratoxins A and B by Column Chromatography

1. Water-jacketed columns[1] with standard "O"-ring connectors at each end to permit connection to adapters or extenders are used for all column chromatography of ochratoxins. At the bottom of each column is connected an "O"-ring adapter[2] with a tapered Teflon stopcock. The stopcocks are plugged with cotton or a medium porosity fritted disc which will not allow passage of the silica gel adsorbent. Another "O"-ring adapter[3] with a Luer joint on the delivery tip is connected to the top of the column. Adapters are sealed with the column by means of Viton "O"-rings and pinch clamps.
2. A slurry of 454 g dried silica gel[4] in benzene is packed into a 1000 x 40 (id) mm water-jacketed column. A 500 x 40 mm glass extender[5] is attached to the top of the column so that all the silica gel can be added at once.
3. Some of the benzene is drained to aid in settling of the silica gel. After the silica gel has settled, a 2 cm layer of anhydrous sodium sulfate is carefully layered on top. If the silica gel is disturbed, poor separation of toxins will result due to streaking and overlapping of eluting bands. Finally, the benzene is drained just to the top of the sodium sulfate layer. The column height

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[1] Jacketed Chromaflex extender, K-42230, Kontes Glass Co., New Jersey.

[2] Chromaflex adapter, K-422390, Kontes Glass Co., New Jersey.

[3] Chromaflex adapter, K-422370, Kontes Glass Co., New Jersey.

[4] Mallinckrodt SilicAR CC-7, 100-200 mesh, dried two hours at 110°C.

[5] Chromaflex extender, K-422440, Kontes Glass Co., New Jersey.

when packed is 800 mm.

4. A 3 g sample of crude ochratoxins dissolved in 24 ml chloroform is added to the column and drained just to the top of the sodium sulfate layer. Four 5 ml portions of benzene are successively added and drained to the top of the sodium sulfate layer to aid in completely transferring the sample to the silica gel adsorbent. The headspace in the column is then completely filled with benzene.

5. The column is connected to a gradient elution apparatus which provides a linear increase in eluting force (6). In the first of the two reservoirs, 3 l benzene is added and in the second, 3 l benzene:glacial acetic acid 85:15 v/v. The two reservoirs are joined by a glass siphon tube. The reservoir originally containing 100% benzene is linked to the delivery tip at the top of the column by a 0.044" (id) Teflon tube. The Teflon tube is attached to the delivery tip by means of a female Luer Kel-F hub. Thus the column is completely sealed at the top so it is continuously fed solvent as the elution is carried out. The solvent in the reservoir originally containing 100% benzene is stirred with a stirring bar and magnetic stirrer. Elution is carried out with the stopcock completely opened thus allowing a flow rate of approximately 240 ml per hour.

6. During elution the various bands are visualized briefly with a long wave ultraviolet light.[6] The first major fluorescent band to elute is the green ochratoxin A. The blue ochratoxin B follows immediately afterwards but well separated from the ochratoxin A. One minor fluorescent band will elute just before the ochratoxin A. This should be collected separately and discarded.

7. The ochratoxin A fraction is collected <u>in toto</u> then placed in a tared 500 ml ℸ 24/40 round bottom flask and evaporated to

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[6] Blak-Ray UVL-22, San Gabriel, California.

dryness with a rotary vacuum evaporator. A water bath at 37°C is used to aid the evaporation. It will be found that solvent mixtures containing greater than 2% glacial acetic acid in benzene will not evaporate completely to dryness under the conditions described. This is because benzene and glacial acetic acid form an azeotropic solution which will distill forming a vapor consisting of 2% glacial acetic acid in benzene (82). To remove any remaining glacial acetic acid, estimate its volume and add a portion of benzene that will give a final solution containing at least 98% benzene, then evaporate to dryness. This may need to be repeated if the estimation was incorrect and glacial acetic acid remains in the flask after evaporation.

8. The dry ochratoxin A fraction is dissolved in 50 ml chloroform. This fraction is then analyzed by spotting a 2 µl portion onto a silica gel thin layer plate and developing the plate with a suitable solvent. The system which was used in this study unless otherwise stated was a 250 micron layer of MN-silica gel G-HR, developed in an unlined chamber with benzene:glacial acetic acid 9:1 v/v. The ochratoxin A fraction generally contains trace amounts of other fluorescent contaminants and the concentrated toxin solution has a straw color.

9. The ochratoxin B fraction is also collected in toto and evaporated to dryness using the procedure outlined for the ochratoxin A fraction. It is taken up in 100 ml chloroform and analyzed by TLC. It generally contains trace amounts of ochratoxin A and other fluorescent contaminants. The concentrated ochratoxin B fraction at this point is quite yellow.

APPENDIX VI

Column Chromatography of Ochratoxin A

1. The ochratoxin A fraction obtained from the first large preparative column contains small amounts of a high (0.7) and low (0.3) R_f fluorescent impurity. Also the concentrated toxin solution has a straw color. These impurities are effectively removed by column chromatography followed by recrystallization.
2. A slurry of 75 g dried silica gel[7] in benzene is packed into a 500 x 25 (id) mm water-jacketed column. A 500 x 25 mm glass extender is attached to the top of the column so that all the slurry can be added at once. The column is equilibrated and a 2 cm layer of anhydrous sodium sulfate is layered on top of the silica gel. The benzene is drained to the top of the sodium sulfate layer. The column height when equilibrated is 35 cm.
3. The ochratoxin A fraction obtained from the first large preparative column is evaporated to dryness, weighed, and dissolved in minimal chloroform. A 75 mg sample is then added to the column. The level of the chloroform solution is lowered to the top of the sodium sulfate layer, then four 5 ml portions of benzene are similarly added to aid in completely transferring the sample to the adsorbent.
4. The headspace in the column is filled with benzene then an adapter with a 0.044" (id) Teflon tubing connected as described in Appendix V is clamped to the top of the column. The free end of the Teflon tube is placed into a reservoir containing 2500 ml benzene:glacial acetic acid 98:2 v/v. Elution is carried out with this solvent until all the ochratoxin A has eluted. A yellow

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[7] Mallinckrodt SilicAR CC-7, loc. cit.

pigment and high R_f fluorescent impurity will elute before the ochratoxin A while the low R_f fluorescent impurity will remain at the top of the column. The high R_f impurity is collected and discarded, then the ochratoxin A fraction is collected *in toto* and evaporated to dryness with a rotary vacuum evaporator.

5. The sample is dissolved in 50 ml 95% ethanol and a 2 µl portion is analyzed by TLC to determine the degree of purity. At this point the sample is generally free from other fluorescent and pigmented impurities. The sample can now be crystallized to attain absolute purity necessary for toxicity and carcinogenicity studies.

APPENDIX VII

Recrystallization of Ochratoxin A

1. The ochratoxin A sample is evaporated to dryness and dissolved in minimal hot benzene. The solution is transferred to a 40 ml conical bottom centrifuge tube and the solvent is evaporated in a steam bath until crystals just begin to form.
2. The solution is covered and then cooled overnight at 10°C. The crystals that form are collected by centrifugation. The mother liquor is removed and saved for a second crop.
3. The crystallization is repeated three times. The final product, a white crystalline substance, contains one molecule benzene of crystallization (79). The benzene is removed by transferring the sample to a suitable round bottom flask then successively evaporating three 100 ml portions of 95% ethanol from the flask using a rotary vacuum evaporator. This azeotropic technique effectively removes all traces of benzene from the sample. The final product is dissolved in 100 ml 95% ethanol and stored at -15°C.

APPENDIX VIII

Recrystallization of Ochratoxin B

1. The ochratoxin B fraction obtained from the first preparative column (Appendix V) is very yellow and contains trace amounts of ochratoxin A and other fluorescent impurities. The yellow pigment material cannot be removed by column chromatography but can easily be completely removed by crystallization in methanol. The fluorescent impurities are then removed by column chromatography.
2. Transfer the ochratoxin B sample dissolved in minimal hot methanol to a 40 ml conical bottom centrifuge tube. Evaporate the methanol from the tube with gentle heating over a steam bath while constantly stirring until the solution begins to cloud when cooled to room temperature.
3. Cover the tube and place it in a freezer overnight at -15°C. Collect the crystals by centrifugation and remove the yellow mother liquor and save for second crops.
4. Repeat the recrystallization three times. The crystallized toxin now appears pure white and will contain trace amounts of ochratoxin A and other fluorescent impurities which are not completely removed by crystallization in methanol.

APPENDIX IX

Column Chromatography of Ochratoxin B

1. A slurry of 75 g dried silica gel[8] in benzene is packed into a 500 x 25 (id) mm water-jacketed column. The column is prepared and equilibrated as in Appendix VI, 2. The benzene is drained just to the top of the sodium sulfate layer.
2. A 50 mg sample of ochratoxin B partially purified as in Appendix VIII is dissolved in 5 ml chloroform and added to the column. This solution is drained to the top of the sodium sulfate layer. Three additional 5 ml portions of chloroform are used to transfer the sample to the silica gel adsorbent.
3. The column is connected to a gradient elution apparatus identical to the one described in Appendix V, 5. In the two reservoirs are placed 750 ml benzene and 750 ml benzene:glacial acetic acid 8:2 v/v.
4. Develop the column and begin collecting 20 ml fractions once the ochratoxin B starts to elute. The progress of elution is followed by briefly holding an ultraviolet light next to the column to determine when to start collecting fractions.
5. Two µl portions of each fraction are analyzed by TLC as in Appendix V, 8. Fractions containing only ochratoxin B are combined and evaporated to dryness as described in Appendix V, 6.
6. Dissolve the sample in minimal hot methanol and recrystallize three times as described in Appendix VIII. This yields a white crystalline toxin completely free of all impurities. The final product is dissolved in 100 ml 95% ethanol and stored at -15°C.

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[8] Mallinckrodt SilicAR CC-7, loc. cit.

APPENDIX X

Preparation of Ochratoxin a

The phenylalanine moiety of ochratoxin A is removed by a modification of the acid hydrolysis method of van der Merwe (79).

1. Place a 65 mg sample of pure ochratoxin A dissolved in 95% ethanol into a 250 ml round bottom flask having three ℈ 14/20 outer necks. Stopper the two outer holes and place the flask on a rotary vacuum evaporator and take to dryness.
2. Suspend the sample in 75 ml 6 N HCl and reflux for 30 hours at 100°C. A small condenser is placed in the center hole of the flask; nitrogen is bubbled through a gas tube inserted in one of the outer holes; and the third hole is stoppered.
3. Cool the mixture, transfer to a 250 ml separatory funnel, and extract three times with 100 ml portions of chloroform. Dry the combined chloroform extract with anhydrous sodium sulfate, filter, and evaporate to dryness with a rotary vacuum evaporator.

Yield from this procedure is about 39 mg ochratoxin a or 95% of the calculated yield. TLC reveals that the sample contains some unreacted ochratoxin A in addition to traces of low R_f fluorescent impurities which can be removed by column chromatography.

Butanol extraction of the aqueous layer (step 3, above) adjusted to pH 5.85 with sodium hydroxide, gave L-β-phenylalanine identified by chromatography on Whatman #1 chromatography paper using butanol: glacial acetic acid:water 4:1:5 v/v/v as the mobile phase. Developed chromatograms were dried, sprayed with ninhydrin spray reagent, and heated ten minutes at 110°C to confirm the presence of phenylalanine. An external standard of L-β-phenylalanine had the same R_f (0.58) as the amino acid in the butanol extract.

APPENDIX XI

Column Chromatography of Ochratoxin a

1. Slurry 60 g silica gel[9] in benzene and pack into a 500 x 25 mm water-jacketed column. The column is prepared and equilibrated as in Appendix VI, 2. Column height when packed is 29 cm.
2. Drain the benzene in the column just to the top of the sodium sulfate layer, then apply a 39 mg ochratoxin a sample dissolved in 5 ml chloroform. Three additional 5 ml portions of benzene are successively applied to aid in transferring the sample to the adsorbent.
3. Fill the headspace in the column with benzene and connect the column to a gradient elution apparatus identical to the one described in Appendix V, 5. In the two reservoirs are placed 600 ml benzene and 600 ml benzene:glacial acetic acid 97:3 v/v. A thin band of unreacted ochratoxin A will elute first and is collected and saved for future use. The ochratoxin a will then elute and is collected *in toto* and evaporated to dryness with a rotary vacuum evaporator. Bands are visualized briefly with an ultraviolet lamp to follow the elution progress.
4. The ochratoxin a fraction is dissolved in 10 ml 95% ethanol and analyzed by TLC as in Appendix V, 8 to determine the degree of purity.

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[9] Mallinckrodt SilicAR CC-7, *loc. cit.*

APPENDIX XII

Recrystallization of Ochratoxin a

The ochratoxin a sample purified in Appendix IX generally contains a trace amount of ochratoxin A that can be completely removed by crystallization in ethanol.

1. Transfer the ochratoxin a sample in minimal hot 95% ethanol to a 40 ml conical bottom centrifuge tube. Evaporate the ethanol from the tube with gentle heating over a steam bath while constantly stirring until the solution begins to cloud when cooled to room temperature.
2. Cover the tube and place it in a freezer overnight at -15°C then collect the crystals by centrifugation. Remove the mother liquor and save for second crops.
3. Repeat the recrystallization three times. The crystallized toxin appears white and is completely free of all impurities. Dissolve the final crystals in 100 ml 95% ethanol and store at -15°C.

APPENDIX XIII

Preparation of Ochratoxin b

The phenylalanine moiety of ochratoxin B is removed by a modification of the acid hydrolysis method of van der Merwe (79).

1. Place a 50 mg sample of pure ochratoxin B dissolved in methanol into a three-holed 100 ml round bottom flask having three ℸ 14/20 outer necks. Stopper the two outer holes and place the flask on a rotary vacuum evaporator and take to dryness.
2. Suspend the sample in 50 ml 6 N HCl and carry out remainder of the hydrolysis and extraction procedure exactly as described in Appendix X, 2 and 3.

Yield from this procedure is 29 mg or about 97% of the calculated yield. The unreacted ochratoxin B is removed by column chromatography. The presence of L-β-phenylalanine in the extracted aqueous layer was again confirmed using the method described in Appendix X.

APPENDIX XIV

Column Chromatography of Ochratoxin b

1. Slurry 60 g silica gel[10] in benzene and pour into a 500 x 25 (id) mm water-jacketed column. The column is prepared and equilibrated as in Appendix VI, 2. Drain the benzene just to the top of the sodium sulfate layer.
2. A 15 mg sample of ochratoxin b obtained as in Appendix XIII is dissolved in 5 ml chloroform and added to the column. This solution is drained to the top of the sodium sulfate layer and three additional 5 ml portions of benzene are successively added to aid in transferring the sample to the adsorbent.
3. After filling the headspace of the column with benzene, an "O"-ring adapter with a 0.044" (id) Teflon tubing connected is clamped to the top of the column as described in Appendix V, 5. The free end of the Teflon tube is connected to a metering pump[11] which is used to pump solvent through the column from a gradient elution apparatus identical to the one described in Appendix V, 5. The metering pump is necessary to attain a sufficient flow rate to prevent diffusion of the eluting toxin. In the two reservoirs of the gradient elution apparatus are placed 500 ml benzene and 500 ml benzene:glacial acetic acid 97:3 v/v. All of this solvent is pumped through the column at a rate of 120 ml per hour, then elution is completed by pumping an additional 450 ml benzene: glacial acetic acid 97:3 v/v through the column.

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[10] Mallinckrodt SilicAR CC-7, 200-325 mesh, dried two hours at 110°C.

[11] Cheminert metering pump, Model CMP-1, Chromatronix, Inc., Berkeley, California. The use of this pump was first described in Wiley, M. and A. C. Waiss, Jr. An improved separation of aflatoxins. Journal of the American Oil Chemists' Society 45:870-871. 1968.

4. The first band to elute is the unreacted ochratoxin B and is collected and saved for future use. The bright blue-fluorescing ochratoxin b elutes completely free from all impurities. It is collected *in toto* and evaporated to dryness with a rotary vacuum evaporator. All traces of glacial acetic acid are removed as described in Appendix V, 7. Dissolve the dried sample in 5 ml 95% ethanol.

APPENDIX XV

Recrystallization of Ochratoxin b

1. Transfer the final ochratoxin b sample prepared in Appendix XIII to a 40 ml conical bottom centrifuge tube and remove the ethanol under a stream of nitrogen. Completely dissolve the sample in minimal hot benzene:hexane 8:2 v/v then evaporate this solvent from the tube with gentle heating over a steam bath until the solution begins to cloud when cooled to room temperature.
2. Cover the tube and place it in a refrigerator at 10°C overnight then collect the crystals by centrifugation. Remove the mother liquor and save for second crops. Repeat the crystallization three times then remove all traces of benzene from the sample by the procedure outlined in Appendix VII, 3. Dissolve the final white crystalline sample in 50 ml of 95% ethanol and store at -15°C.

The silica gel adsorbents used in column chromatography of ochratoxins in the preceding appendices were found to be quite satisfactory. Two other adsorbents which were tried were silica gel H[12] and florisil[13]. Silica gel H was completely unsatisfactory because of streaking of eluting toxins when developing columns with benzene: glacial acetic acid mixtures. Florisil was found to be quite suitable but no more so than the adsorbents used in this study.

Carbon tetrachloride was found to be a satisfactory solvent for column chromatography of ochratoxins but again was no better than

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[12] Merck Silica Gel H acc. to Stahl, Brinkman Instruments, Inc., Westbury, New York.

[13] Florisil, 200 mesh, Floridin Co., Tallahassee, Florida.

benzene which was used in this study. The toxic and carcinogenic properties of carbon tetrachloride were significant factors eliminating it as a possible developing solvent.

It should be pointed out that the SilicAR CC-7, 100-200 mesh silica gel, which is suitable for purification of ochratoxins A, B, and a, is not suitable for purification of ochratoxin b. It will not separate traces of ochratoxin B from ochratoxin b to the degree that the SilicAR CC-7, 200-325 mesh silica gel will.

APPENDIX XVI

Quantitation of Ochratoxins

The following data are used in calculating concentration of ochratoxin compounds:

Toxin	Molecular weight	λ_{max} (nm)	ϵ	Solvent system[a]
Ochratoxin A	403	333	5550	A
Ochratoxin B	369	318	6000	B
Ochratoxin a	256	336	5600	C
Ochratoxin b	222	322	6800	C

[a]Solvent system A - benzene:glacial acetic acid 99:1 v/v

Solvent system B - benzene:acetonitrile:glacial acetic acid 97:3:1 v/v/v

Solvent system C - 95% ethanol

1. The purified sample is dissolved in the proper solvent system and its absorbance recorded with a Beckman DK-1 recording spectrophotometer in the region 280 to 360 nm.
2. The amount of toxin in the sample in micrograms is calculated using the equation

$$\mu g = \frac{(A)(MW)(1000)(CF)(V)}{\epsilon}$$

where A is the absorbance at the wavelength of maximum absorption; MW is the molecular weight of the toxin; CF is the correction factor for the spectrophotometer[14]; V is the volume in ml; and ϵ is the molar absorptivity of the toxin in its designated solvent system.

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[14]Calculated by the method used by Rodricks and Stoloff (62).

3. A portion of the pure stock ochratoxin A or B sample in 95% ethanol is measured into a small pear-shaped flask and evaporated to dryness using a rotary vacuum evaporator. It is then dissolved in the designated solvent system for quantitation.

Several different dilutions of each toxin will probably have to be tried until a measurable absorbance is obtained so the concentration of the stock solution can be determined. Ochratoxins A and B will give a measurable absorbance at a concentration less than 50 μg/ml while ochratoxins a and b can be measured at concentrations less than 25 μg/ml.

APPENDIX XVII

Preparation of Ochratoxin A and B Ethyl Esters

This procedure is a modification of that used by Morin et al. (48) for esterification of fatty acids. It was used because it is quite suitable for preparation of small amounts of ochratoxin esters.

1. Transfer approximately 150 μg of purified ochratoxin A or B in 95% ethanol to a small pear-shaped flask and take to dryness with a rotary vacuum evaporator. Dissolve the sample in 5 ml of an ethanolysis mixture consisting of 123 ml benzene, 61.5 ml 95% ethanol, and 1.0 ml concentrated sulfuric acid.
2. Transfer the sample to a 15 ml culture tube that has a screw cap with a Teflon lining. Screw the cap tightly onto the tube and reflux for eight hours in an oven at 72-76°C. Check the tube for leakage after it has been in the oven for 30 minutes.
3. Remove culture tube from the oven and cool for 15 minutes before removing cap. The ochratoxin esters are analyzed by directly spotting 10 μl of the ethanolysis mixture and external standards of authentic ochratoxin A and B ethyl esters on a silica gel thin layer plate. Develop the plate in benzene:glacial acetic acid 9:1 v/v and compare the R_f's of the spots with the external standards while holding the developed chromatogram under both long and short wave ultraviolet radiation.

APPENDIX XVIII

Composition of Semi-synthetic Trout Diet

Ingredient	Percent[15]
Casein	49.5
Gelatin	8.7
Dextrin	15.6
Salmon oil	10.0
Mineral mix[a]	4.0
Carboxymethylcellulose	1.3
Cellulose (Alphacel)[b]	7.7
Vitamin mix[c]	2.0
Choline chloride (70%)	1.0
Vitamin E conc. (α-tocopherol 330 I.U./g	0.2

[a]Modified Barnhart-Tomarelli (3) salt mix (0.002% NaF and 0.02% $CoCl_2$ added).

[b]Nutritional Biochemicals Corporation, Cleveland, Ohio.

[c]Vitamins supplied at the following levels (mg/kg): thiamin (HCl), 64; riboflavin, 144; niacinamide, 512; biotin, 1.6; Ca D-pantothenate, 288; pyridoxine (HCl), 48; folic acid, 19.2; menadione, 16; cobalamine (B_{12}), 0.159; i-inositol (meso), 2500; ascorbic acid, 1200; para-amino-benzoic acid, 400; Vitamin A concentrate (250,000 I.U./g), 200; Vitamin D_2 (500,000 I.U./g), 8.

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[15]Modification of that reported by Lee et al. (42).

APPENDIX XIX

Mass Spectral Analysis of Ochratoxin Ethyl Esters

1. Ochratoxin ethyl esters were prepared by incubating approximately 500 mg of each purified toxin (A, B, a, and b) in 5 ml of the ethanolysis mixture described in Appendix XVII at 78°C for 48 hours. The toxins were purified by preparative TLC on a 250 μ layer of silica gel developed in benzene:glacial acetic acid 9:1 v/v. The toxin band was scraped from the developed chromatogram into a small beaker containing about 25 ml chloroform:methanol 1:1 v/v. The silica gel was removed by filtration through a 1.5 cm cotton plugged glass column containing about 10 cm of anhydrous sodium sulfate. The filtered sample was evaporated to dryness then dissolved in about 1/2 ml of 95% ethanol.

2. Mass spectra for each sample were obtained on a Varian MAT direct inlet CH-7 mass spectrometer using the following experimental conditions:

   ion source temperature: 265°C
   vacuum: 10^{-5} - 10^{-6} Torr
   ionization voltage: 70 ev
   acclerating voltage: 3000 v
   scan range: 18
   recorder: 32 ips
   internal standard: perfluorodecalin (via liquid inlet)
   anode current: 60 μa

3. Twenty μl of each sample was inserted into the mass spectrometer following evaporation of the ethanol. After insertion into the mass spectrometer the direct probe was programmed upward at 5-10°C per minute and spectra for ochratoxin a and b ethyl

esters were recorded at 50-60°C and ochratoxin A and B ethyl esters were recorded at about 150°C.

4. The significant peaks and their suggested possible assignments are as follows:

	m/e	
Ochratoxin A ethyl ester	431	molecular ion
	386	loss of OEt
	358	loss of · COOEt
	340	loss of · COOEt and water
	255	loss of C_6H_5-CH=CH-COOEt
	239	base peak, loss of C_6H_5-CH_2-CH-COOEt-NH
	221	loss of C_6H_5-CH_2-CH-COOEt-NH-CO
Ochratoxin B ethyl ester	397	molecular ion
	352	loss of OEt
	324	loss of · COOEt
	306	loss of · COOEt and water
	221	unknown
	205	base peak, loss of C_6H_5-CH_2-CH-COOEt-NH
	187	loss of C_6H_5-CH_2-CH-COOEt-NH-CO
Ochratoxin a ethyl ester	284	molecular ion
	239	loss of OEt
	212	base peak, loss of · COOEt
	194	loss of · COOEt and water
Ochratoxin b ethyl ester	250	molecular ion
	205	loss of OEt
	178	loss of · COOEt
	160	base peak, loss of · COOEt and water

In addition, the mass spectrum for ochratoxin A ethyl ester showed a molecular ion peak at m/e 431 and an isotope peak at m/e 433 with relative intensities of about 3:1 indicating one chlorine atom. Similarly, the cholrine-containing ochratoxin a ethyl ester showed a molecular ion peak at m/e 284 and an isotope peak at m/e 286 also with relative intensities of about 3:1.

It should be emphasized that the interpretation of the above fragmentation patterns is only tentative as the patterns were not confirmed by mass spectra of deuterium-labelled analogues. The fact that the proper molecular ion and other expected ions were obtained for each toxin presents good evidence of proper assignment of structures to the toxins.