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Title: TOXICITY AND METABOLISM OF OCHRATOXINS IN RAINBOW TROUT (SALMO GAIRDNERI)

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Seven structurally related ochratoxin compounds were prepared and their acute intraperitoneal toxicities determined using rainbow trout (Salmo gairdneri) as a test animal. The compounds tested were: ochratoxins A, B, C, B ethyl ester, a ethyl ester, and the alanine and leucine analogues of ochratoxin A. The ten-day $LD_{50}$ values of ochratoxins A, B ethyl ester and C in six-month-old rainbow trout were 5.53, 13.0 and 3.0 mg/kg body weight, respectively. Ochratoxin B administered at 66.7 mg/kg body weight, was non-lethal. The two amino acid analogues of ochratoxin A as well as ochratoxin a ethyl ester, were non-lethal at the molar $LD_{50}$ level of ochratoxin A. Histopathological damage caused by these toxins is described.

Ochratoxin A-$^{14}$C was biologically prepared using sodium acetate-1-$^{14}$C as a precursor added to Aspergillus ochraceus liquid cultures. The specific activity of the purified toxin was found to be

93.3 μc/mmole. Ochratoxin B-$^{14}$C was prepared by catalytic dehalogenation of the purified ochratoxin A-$^{14}$C.

The 24-hour excretion and tissue distribution of $^{14}$C from ochratoxin A-$^{14}$C and B-$^{14}$C administered to trout was determined in an effort to explain the difference in toxicity of these two toxins. The $^{14}$C from ochratoxin B-$^{14}$C was found to be excreted in the urine more rapidly than that from ochratoxin A-$^{14}$C. This low urinary excretion of $^{14}$C from ochratoxin A-$^{14}$C was directly paralleled by a greater biliary excretion.

The metabolite pattern in chloroform-soluble extracts of urine, bile, intestinal contents, liver and kidney from trout dosed with ochratoxin A-$^{14}$C and B-$^{14}$C was determined. Ochratoxin B is metabolized to its isocoumarin acid hydrolysis product and a hydroxy derivative to a greater extent than ochratoxin A is metabolized to its corresponding analogues of these products.

The greater toxicity of ochratoxin A can be attributed, at least partially, to the fact that it is more resistant to metabolism and excretion in the urine and may persist in the body for a longer time than ochratoxin B. Possible reabsorption from the gut could result in greater exposure of the liver to unaltered ochratoxin A than ochratoxin B.

# Toxicity and Metabolism of Ochratoxins in Rainbow Trout (*Salmo gairdneri*)

by

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# TOXICITY AND METABOLISM OF OCHRATOXINS 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 studied mycotoxins, the aflatoxins, and by suggestions that these toxins offer a reasonable explanation for the high incidence of human liver cancer in those parts of the world where large quantities of moldy foods are consumed. Other mold metabolites besides aflatoxin cause significant pathological changes in experimental animals and may be involved in the etiology of certain livestock and human disorders.

Metabolites of the Aspergilli molds have 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 a potential hazard to animal and human health.

Studies of the toxic effect of ochratoxins have shown that the

chlorine and phenylalanine-containing molecules are by far the most toxic. Ochratoxin compounds lacking one or both of these moieties are substantially less toxic.

The metabolism of the ochratoxins in animals has been limited because of their extreme lethality, the unavailability of radiolabeled toxins and lack of sensitive analytical methods for detection of fluorescent and non-fluorescent biologically formed derivatives.

This study had three main objectives. The first was by acute toxicity trials to determine the possible relationship of chemical structure to biological activity. Seven ochratoxin compounds were administered intraperitoneally to rainbow trout and mortalities and histopathological damage were noted. The second objective was to prepare radiolabeled ochratoxins A and B with sufficient specific activities to permit metabolism studies in the extremely sensitive rainbow trout. This requires substantially greater specific activities than those attained by other workers. The third objective was to determine the excretory and tissue distribution pattern of $^{14}C$ from ochratoxins A-$^{14}C$ and B-$^{14}C$ in rainbow trout. Characterization of excreted $^{14}C$-labeled products from ochratoxins A-$^{14}C$ and B-$^{14}C$ was performed.

# LITERATURE REVIEW

## Ochratoxin

### Mycological Aspects

*Aspergillus ochraceus* has been isolated from a variety of agricultural commodities (13, 14, 15, 16, 83, 107). Symptoms of chronic and acute toxicity have been observed in experimental animals when *A. ochraceus* was grown in pure culture on corn, wheat, rye, sorghum, rice, buckwheat, soybeans or peanuts and the molded substrates fed to test animals (14, 15, 16, 33, 34, 107). The toxic agent produced by the mold was first isolated and named ochratoxin by van der Merwe *et al.* (106, 107).

In 1969, Shotwell *et al.* (89) found ochratoxin A as a naturally occurring contaminant in sample grade corn at levels up to 150 parts per billion (ppb). Scott *et al.* (87) 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 (58) found that corn moldy with *P. viridicatum* poisoned horses and pigs in Argentina, and Krogh and Hassellager (53) showed that barley inoculated with this fungus contained a toxic principle which caused kidney damage in rats and pigs.

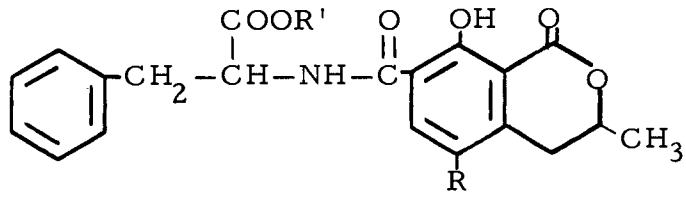
Ochratoxin A has since been found to be produced by P. viridicatum as well as A. melleus and A. sulphureus (54, 55, 109). The Penicillium species which produced ochratoxin A was isolated from a surface growth of packaged ham (108). Van Walbeek et al. (109) state that it is possible that ochratoxin A detected by Shotwell et al. (89) 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 a part of the disease syndrome caused experimentally by P. viridicatum in animals (10, 53). Toxicity, pathology and metabolism of ochratoxins are reviewed in a later section.

## Structure and Chemical Characteristics

Ochratoxin is a collective term used to describe several related compounds of the isocoumarin type. The chemical structure of the principal toxic compound, ochratoxin A, was determined (95, 106) and verified by direct synthesis (79, 96). 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.

Other ochratoxins which have been isolated from mold cultures and identified are the methyl esters of ochratoxins A and B and the

ethyl ester of ochratoxin B. These structures are shown in Figure 1.

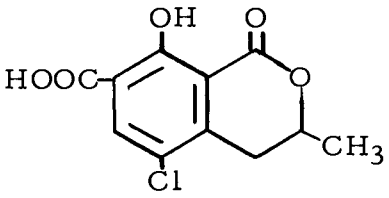


Ochratoxin A; R=Cl, R'=H
Ochratoxin B; R=H, R'=H
Ochratoxin C; R=Cl, R'=$C_2H_5$
Ochratoxin A methyl ester; R=Cl, R'=$CH_3$
Ochratoxin B ethyl ester; R=H, R'=$C_2H_5$
Ochratoxin B methyl ester; R=H, R'=$CH_3$

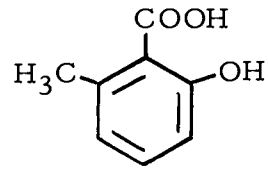
Figure 1. Structure of the ochratoxins.

Other secondary metabolites which have been isolated from A. ochraceus cultures are L-prolyl-L-valine anhydride and L-prolyl-L-leucine anhydride (51), mellein (114), ochratoxin a (103), hydroxyaspergillic acid (95), pencillic acid (95, 64), 3-hydroxymellein (80), 4-hydroxymellein (25, 80), secalonic acid A (117), 6-methylsalicylic acid (80) and the cyclotripeptide, aspochracin (62, 63). The structures of these compounds are shown in Figure 2.

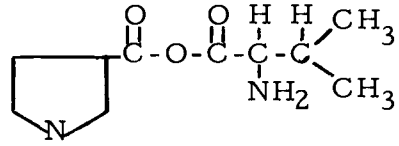
Besides the obvious fact that the presence of these compounds in A. ochraceus culture extracts makes purification of the ochratoxins more difficult, it is significant that they also contribute to the toxicity of ochratoxin in fungal extracts or cultures. For example, the minimum lethal dose (MLD) of L-prolyl-L-leucine anhydride to silk worm larva was 100 μg/g and 200 μg/g for L-prolyl-L-valine anhydride (51).



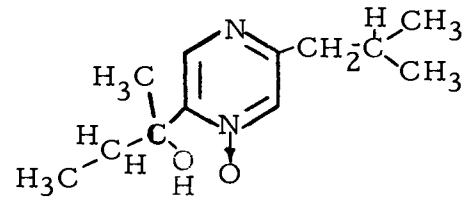
Ochratoxin a



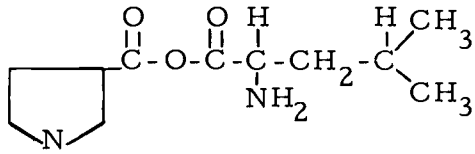
6-methyl salicylic acid



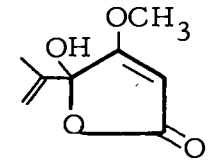
L-prolyl-L-valine anhydride



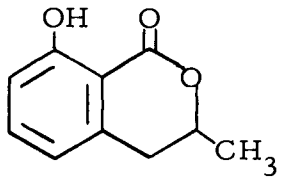
Hydroxyaspergillic acid



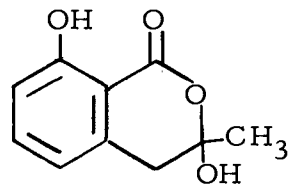
L-prolyl-L-leucine anhydride



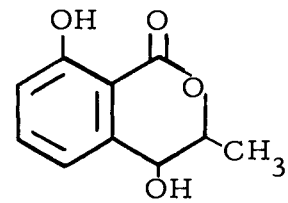
Penicillic acid



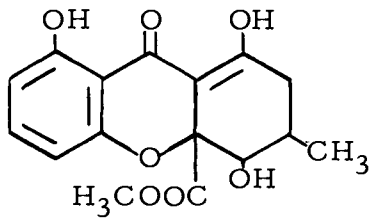
Mellein



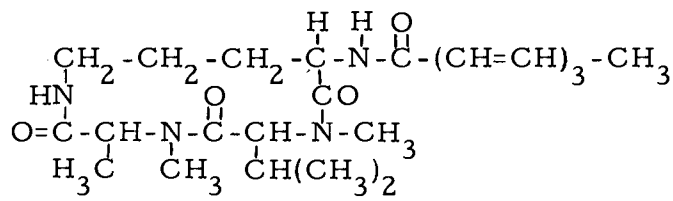
3-hydroxymellein



4-hydroxymellein



Secalonic acid A



Aspochracin

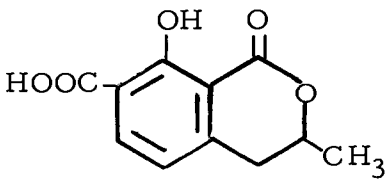
Figure 2. Secondary metabolites isolated from Aspergillus ochraceus cultures.

Sasaki et al. (80) found the intraperitoneal (IP) $LD_{50}$ of 3- and 4-hydroxymellein in mice was 262 mg/kg and 1000-1500 mg/kg body weight, respectively. These authors also reported the IP $LD_{50}$ of mellein in mice as 250-500 mg/kg body weight. Secalonic acid A killed all rats when injected IP at 100 mg/kg (117). Myokei et al. (63) reported a MLD of aspochracin in silkworm larvae of 17 μg/g and 170 μg/g in fall webworm larvae. Low toxicity was noted in microbial and mammalian organisms, however.

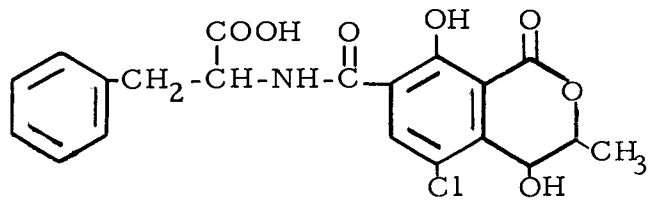
Another mold producing large amounts of ochratoxin A, Penicillium viridicatum, has also been shown to produce a number of secondary metabolites, namely, ochratoxin b, the acid hydrolysis product of ochratoxin B (49), 4-hydroxyochratoxin A (49), citrinin (40) and oxalic acid (40). Four-hydroxyochratoxin A was non-toxic to rats when injected at the $LD_{100}$ ochratoxin A level of 40 mg/kg (49) but citrinin and oxalic acid are nephrotoxic agents which may well contribute to the toxicity of ochratoxins produced by P. viridicatum cultures or culture extracts. The structures of these compounds are shown in Figure 3.

## Physical Characteristics

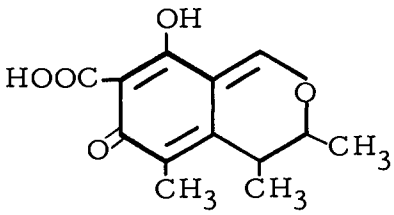
Ochratoxins A and B are colorless crystalline compounds that fluoresce green and blue, respectively, under ultraviolet radiation at 365 nm. Ochratoxin C is amorphous and appears dull green on



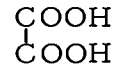
Ochratoxin b



4-hydroxyochratoxin A



Citrinin



Oxalic acid

Figure 3. Secondary metabolites isolated from Penicillium viridicatum cultures.

developed chromatograms as does the methyl ester of ochratoxin A. The methyl and ethyl esters of ochratoxin B both appear light blue under ultraviolet radiation (95). Ochratoxins a and b, the acid hydrolysis products of A and B appear dark blue on developed chromatograms under long-wave ultraviolet radiation. On holding thin-layer chromatograms over ammonia fumes, ochratoxins A, B and C exhibit a bright blue fluorescence. Trenk and Chu (102) used this fact to develop an improved spectrofluorometric analysis of ochratoxin which involved measurement of the fluorescence spectra of ammoniated ochratoxin. They were able to measure ochratoxin A in the range of 0.25-0.5 ng/spot, a two-fold improvement over the previous method using non-ammoniated ochratoxin.

The ultraviolet absorption spectra data of the ochratoxins and their derivatives are shown in Table 1. An inspection of these data shows that most workers are in close agreement on the wavelengths of maximum absorption of the ochratoxins but discrepancies are apparent in the molar extinction coefficients for a given compound in the same solvent system. This is an important problem to resolve since toxin solutions are routinely quantitated spectrophotometrically. The discrepancies are likely attributable to the fact that the absorption spectra of the ochratoxins are pH dependent (72), e. g., absorption maxima for ochratoxin A are at 333 and 380 nm at pH 7. 1 but a single maxima is noted at 333 and 380 nm at pH 6. 8 and 7. 4, respectively (67). Ochratoxin B shows absorption maxima at 318 and 366 nm at pH 7. 5 but a single maxima at 318 and 366 nm at pH 6. 5 and 8. 5, respectively. Therefore, molar extinction coefficients determined with toxins dissolved in ethanol without reference to the pH are of dubious value.

Besides ultraviolet spectral analysis, the ochratoxins have also been characterized by infrared, nuclear magnetic resonance, optical rotatory dispersion and mass spectrometry (67, 96, 106).

## Analytical Methodology

Preparation of Ochratoxins A and B. Natural ochratoxins A and B have been produced in highest concentrations by A. ochraceus

Table 1. Ultraviolet spectra data of ochratoxins.

| Compound | $\lambda$ max (nm) | $\epsilon$ | Solvent[a] | Reference |
|---|---|---|---|---|
| Ochratoxin A | 333 | 5550 | A | 66 |
| | 215, 333 | 34000, 2400 | EtOH | 106 |
| | 213, 333 | 35400, 6100 | EtOH | 67 |
| | 213, 332 | 36800, 6400 | EtOH | 96 |
| | 211, 334, 379 | 34650, 4000, 3300 | MeOH | 79 |
| | 217, 331 | 37000, 6325 | EtOH | 84 |
| Ochratoxin B | 318 | 6000 | B | 66 |
| | 218, 318 | 37200, 6900 | EtOH | 106 |
| | 218, 318 | 36200, 6700 | EtOH | 67 |
| | 218, 318 | 34300, 6750 | EtOH | 96 |
| Ochratoxin a | 217, 336 | 39090, 6440 | EtOH | 67 |
| | 212, 338 | 30000, 5600 | EtOH | 106 |
| | 212, 338 | 26000, 5200 | EtOH | 97 |
| | 214, 336 | 28000, 5400 | EtOH | 96 |
| Ochratoxin b | 219, 332 | 33800, 6800 | EtOH | 96 |
| Ochratoxin C | 333 | 6200 | B | 66 |
| | 214, 333 | 30000, 7000 | EtOH | 106 |
| | 213, 331, 378 | 32700, 4100, 2050 | NG | 95 |
| Ochratoxin B ethyl ester | 318 | 6700 | $CHCl_3$ | 67 |
| | 218, 318, 364 | 32000, 5200, 1250 | NG | 95 |
| Ochratoxin A methyl ester | 333 | 6500 | MeOH | 67 |
| Ochratoxin B methyl ester | 318 | 6500 | $CHCl_3$ | 67 |

[a]Solvent A = benzene:glacial acetic acid 99:1 v/v.
Solvent B = benzene:glacial acetic acid:acetonitrile 97:1:2 by volume.
NG = not given.

on wet corn meal (95, 98), rice (116) and shredded wheat breakfast cereal (68, 81). Schindler and Nesheim (81) studied various parameters such as moisture level, incubation time and temperature on toxin yields and obtained 2.39 mg ochratoxin A per gram of shredded wheat at water levels between 40 and 70 ml/100 g substrate for an incubation period of 17-25 days.

Although large amounts of ochratoxin are required for toxicological studies, synthetic and semisynthetic media are preferred for the isolation of mycotoxins and for the study of ochratoxin biosynthesis (93). Several workers have developed liquid media suitable for ochratoxin production. Van Walbeek et al. (108) reported that A. ochraceus produced 1.2 mg ochratoxin A per kg mycological broth enriched with 0.5% yeast extract (MYE) and 9.0 mg ochratoxin A per kg of 15% sucrose-2% yeast extract (YES) media. Davis et al. (27) obtained a yield of 29 mg ochratoxin A per 100 ml of a semi-synthetic medium of 4% sucrose and 2% yeast extract. Ochratoxin A was the sole metabolite in chloroform extracts of the medium if the sucrose content was kept below 8%. Sucrose concentrations above this level resulted in ochratoxin B production as well.

Ferreira (36, 37) obtained yields of 10 mg ochratoxin A per 100 ml synthetic medium in shaken flasks and 10-liter fermentors. The preferred carbon source was sucrose over glucose and fructose, and a concentration of 3% sucrose was optimal. The preferred nitrogen

source was glutamic acid, but proline was also satisfactory. Ferreira speculated that glutamic acid and proline might have an indirect effect on formation of ochratoxin A. The induction of ochratoxin A production by proline and glutamic acid was inhibited by 22 other amino acids and their derivatives as well as analogues and antagonists of proline and glutamic acid. This inhibition was decreased by increasing the concentration of either glutamic acid or proline or by adding lactic acid to the medium. The fermentation was found to proceed in three stages characterized by a 2-3 day lag phase in which there was slow substrate utilization, slow increase in mycelial weight and pH and no ochratoxin A production. This was followed by a logarithmic phase characterized by rapid substrate utilization, pH and mycelial weight increase, and ochratoxin A production which was completed in 12-24 hours. This is in contrast to production of secondary metabolites by other molds in which these metabolites are usually produced after the log phase. In the third phase everything remained constant except mycelial weight which at first remained constant and then declined.

Lai et al. (54, 55) described conditions for the production of ochratoxin A by A. ochraceus and two members of the A. ochraceus group, A. sulphureus and A. melleus in a semisynthetic medium similar to that described by Ayde and Mateles (1). Ochratoxin A biosynthesis started on the third or fourth day and ended by the eighth

day. The A. sulphureus (NRRL 4077) produced the most ochratoxin A (ca. 16 mg/100 ml) at a pH of 6.0-6.3. Of the carbon sources studied, glucose and sucrose were best utilized while lactose was not utilized. The effect of various trace elements on ochratoxin A production was studied and it was found that A. ochraceus (NRRL 3174) and A. melleus (NRRL 3519) required the addition of copper, boron, iron, manganese, molybdenum and zinc in some combination with one another for good mycelial growth and ochratoxin A production. These elements were unnecessary for ochratoxin A production by A. sulphureus (NRRL 4077).

Yamazaki et al. (116) examined the effect of different nitrogen sources on ochratoxin A production by A. ochraceus (MR 31-1) when added to a basal medium similar to that described by Ferreira (36). They found that 2% polypeptone has as great an effect on toxin production as 2% yeast extract. However, malt extract produced no effect. The addition of 1% L-phenylalanine and 2% yeast extract resulted in 2.5 times more ochratoxin A (60 mg/100 ml) than yeast extract alone (24 mg/100 ml).

Extraction and Purification. Several procedures have been proposed for extraction and purification of ochratoxins (27, 65, 81, 85, 95, 98, 106). In addition, screening methods for detecting the presence of several mycotoxins together including ochratoxin have been devised (35, 94, 111).

The methods that are being used to obtain ochratoxins from mold cultures involve extraction of the toxins with a suitable organic solvent (chloroform, chloroform:methanol 1:1 v/v or ethyl acetate), extraction of this extract with aqueous 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 the chloroform extract and ochratoxin standards on silica gel thin-layer plates which are developed in one of several acidic solvent systems (35, 85, 94, 98). Spots on the developed chromatograms are visualized by exposure to ultraviolet illumination of 365 nm wavelength. Investigators have estimated the ochratoxin content of the cultures or culture extracts by comparing the size of the observed spot with that of a standard reference of ochratoxin either by visual examination (27, 36, 54, 81, 85, 98, 109) or fluorodensitometric analysis (18, 21, 102, 116).

Extracted ochratoxins A and B have been purified by column or preparative thin-layer chromatography using one of several adsorbents (6, 67, 84, 95, 106). 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 is the procedure described by Nesheim (67) in which large amounts of ochratoxin A and B mixtures were separated from each other by gradient silica gel chromatography using a benzene:glacial acetic acid

eluant. Scott et al. (84) were encouraged by the excellent TLC separation of ochratoxins A and B achieved by Steyn (94) on oxalic acid impregnated silica gel after development with a neutral solvent system and consequently developed an improved column chromatography procedure using oxalic acid treated silica gel.

## Ochratoxin A Biosynthesis

The biosynthesis of ochratoxin A has been investigated using radiolabeled precursors by Searcy et al. (88), Ferreira and Pitout (38), Steyn et al. (97) and Yamazaki et al. (115). In general, these studies have revealed that the chlorolactone acid (ochratoxin a) is derived by head-to-tail condensation of five acetate (or malonate) units. Steyn et al. (97), using methionine-methyl-$^{14}C$, showed the carboxyl group at position seven of the chlorolactone acid was derived from the $C_1$-pool. This was further substantiated by results of Yamazaki et al. (115) which indicated ethionine inhibited ochratoxin A production. The $C_1$ unit is subsequently oxidized to give rise to the carboxyl group. Little is known concerning the mechanism of the reaction in which the chlorine is incorporated into ochratoxin A. Figure 4 shows the expected labeling pattern of ochratoxin A using acetate-1-$^{14}C$ as a precursor assuming no randomization of the label.

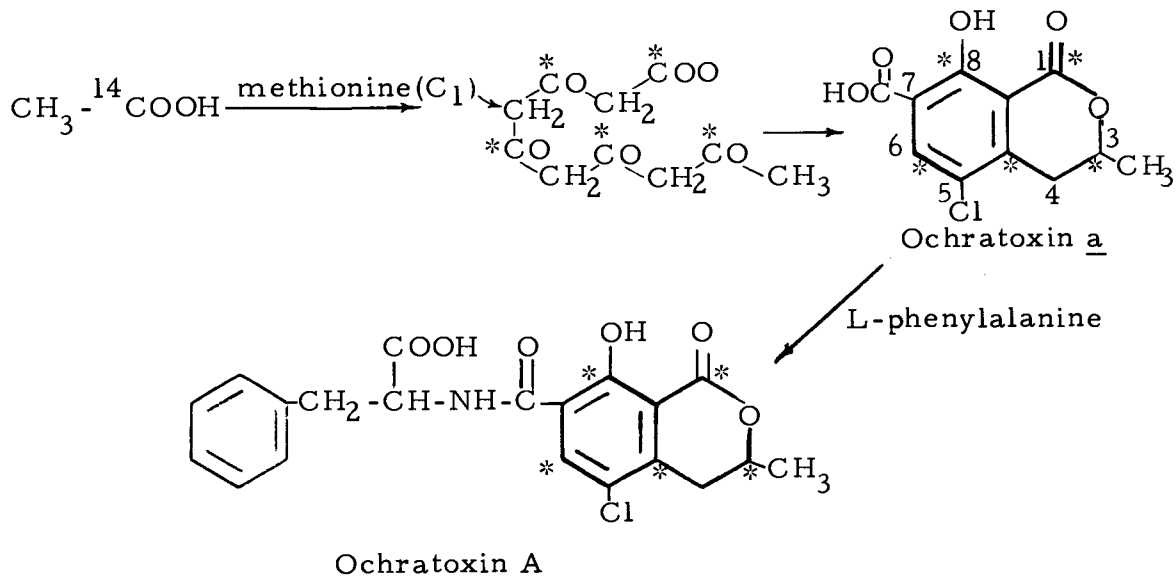



Figure 4. Biosynthesis of ochratoxin A from acetic acid-1-$^{14}$C.

## Biological Preparation of Radiolabeled Ochratoxin

As previously stated, radiolabeled ochratoxin A has been prepared using sodium acetate-$^{14}$C (38, 88, 97), phenylalanine-$^{14}$C (38, 88, 97), methionine-methyl-$^{14}$C (38, 97) and formate-$^{14}$C (38) as precursors supplied to A. ochraceus in liquid culture media. In addition, Wei et al. (112) supplied $^{36}$Cl to an A. ochraceus culture for incorporation into ochratoxin A. Since all but the latter of these studies primarily emphasized the biogenesis of ochratoxin A, little has been done on the preparation of radiolabeled ochratoxins. Production factors of importance would include: 1) cultural conditions favoring label incorporation; 2) suitable purification methods;

3) verification of radiopurity of purified products; and 4) development of suitable methods and procedures to be used in counting radiolabeled ochratoxins. Results of the studies in which labeled ochratoxin A has been prepared are summarized in Table 2. Several facts are apparent from this table:

1) Studies utilizing acetate-$^{14}C$ as an ochratoxin precursor result in very low incorporation percentages. This is likely due to the fact that under the cultural conditions used, competition for this common biochemical intermediate is intense. Ferreira and Pitout (38) reported 50% of the acetate-$^{14}C$ is incorporated into the lipid fraction of their cultures and 25% was oxidized to $CO_2$.

2) Acetate-$^{14}C$ precursors are incorporated exclusively into the isocoumarin moiety of ochratoxin A. Searcy et al. (88) found phenylalanine contained 16.5% of the activity of acetate-2-$^{14}C$ but this was likely due to the long incubation time employed and the resulting randomization of the label.

3) Label incorporation percentages resulting from phenylalanine-$^{14}C$ precursors are considerably higher primarily because of less competition for this compound than acetate and because it is probably incorporated intact into ochratoxin A (38).

4) Label incorporation percentages resulting from the use of acetate-1-$^{14}C$ (97) and 2-$^{14}C$ (88) precursors are nearly the same, 0.139 and 0.132%, respectively. However, a direct

Table 2. Summary of ochratoxin A labeling experiments.

| Labeled precursor | Culture[a] type | Incubation time (days) | Ochratoxin A yield (mg) | % Activity in ochratoxin a | % Activity in phenylalanine | Ochratoxin A specific activity (μc/mmole) | % Incorporation | Ref. |
|---|---|---|---|---|---|---|---|---|
| Phenylalanine-1-$^{14}$C, 100 μc | A | 8 | - | 0.95 | 91.0 | - | 0.2 | 88 |
| Phenylalanine-1-$^{14}$C, 50 μc | B | 2 | 20 | 0 | 97.8 | 26.5 | 1.0 | 97 |
| Phenylalanine-1-$^{14}$C, 50 μc | B | 2 | - | 0 | 100.0[b] | - | 1.0 | 38 |
| Sodium acetate-2-$^{14}$C, 250 μc | A | 8 | - | 62.0 | 16.5 | - | 0.139[c] | 88 |
| Sodium acetate-1-$^{14}$C, 500 μc | B | 2 | 34 | 100.0[b] | 0 | 7.8 | 0.132 | 97 |
| Sodium acetate-1-$^{14}$C, 5 μc | B | 2 | - | 100.0[b] | 0 | - | 0.29[d] | 38 |
| Methionine-Me-$^{14}$C, 150 μc | B | 2 | 7.6 | 100.0[b] | 0 | 11.1 | 0.37 | 97 |

(Continued on next page)

Table 2. (Continued)

| Labeled precursor | Culture type | Incubation time (days) | Ochratoxin A yield (mg) | % Activity in ochratoxin a | % Activity in phenylalanine | Ochratoxin A specific activity (μc/mmole) | % Incorporation | Ref. |
|---|---|---|---|---|---|---|---|---|
| Formic acid-$^{14}$C, 500 μc | B | 2 | - | - | - | - | 0.08 | 38 |
| Na$^{36}$Cl, 10 μc | C | 6 | 11.2 | 100.0 | 0 | 27.0 | 7.5 | 112 |

[a]Culture types: A = 100 ml 4% sucrose, 2% yeast extract incubated stationarily; B = resting culture of A. ochraceus cultured on 1 liter resuspension medium on rotary shaker, labeled precursor added 18-24 hours subsequent to resuspension; C = 100 ml modified Ferreira medium (36) rotary shaken, $^{36}$Cl added 2 days after inoculation.

[b]Percentage values not given; however, statement was made that all activity was present in this moiety.

[c]Reported as 1.39% by Searcy et al. (88) but incorporation actually was 0.139% (N. D. Davis, personal communication).

[d]Highest incorporation achieved, usually less than 0.1% though.

comparison may not be valid since cultural conditions and precursors were not the same in these two studies. From the standpoint of simplicity, however, the method used by Searcy et al. (88) is by far more easily performed than that used by Steyn et al. (97).

As previously stated, Ferreira (36) found that ochratoxin A is produced during the log phase of mold growth. Wei et al. (112) substantiated this finding by observing that addition of $^{36}Cl$ two or three days after inoculation of the cultures resulted in the highest ochratoxin A specific activities.

None of these studies have mentioned the production of radio-labeled ochratoxins other than ochratoxin A. This is probably because of low yields of other ochratoxins in the liquid media used.

## Biological Activity of Ochratoxins

### Acute and Long-term Toxicity Experiments

Ochratoxin A was first shown to be toxic by van der Merwe et al. (107) when dosed orally in aqueous bicarbonate solution to day-old ducklings. The $LD_{50}$ value obtained in this initial study was 25 μg/50 g (0.5 mg/kg) body weight. Van der Merwe et al. (106) reported that ochratoxin B and C were non-toxic to ducklings at a thousand-fold higher dose level than the toxic ochratoxin A level. Further studies by

Steyn and Holzapfel (95) showed that the $LD_{50}$ values of ochratoxin A and its methyl and ethyl esters were similar, namely, 135-170 μg/duckling. Purchase and Nel (78) reported a similar ochratoxin A $LD_{50}$ value of 150 μg/duckling. For comparison, Carnaghan et al. (11) reported the $LD_{50}$ of the most potent mycotoxin known, aflatoxin $B_1$, at 18.2 μg/duckling (0.364 mg/kg). Steyn and Holzapfel (95) confirmed the originally reported non-toxic nature of ochratoxin B and its derivatives.

Steyn (99) reported that O-methylochratoxin A methyl ester and ochratoxin a were essentially non-toxic (see Figure 5 for structure of the former compound). Chu and Chang (19) reported oral $LD_{50}$ values of ochratoxins A and C of 166 and 216 μg/day-old chick, respectively. No toxic effect was demonstrated when chicks were fed up to 500 μg/chick of ochratoxin a.

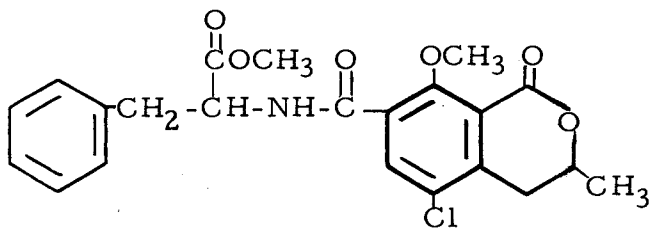



Figure 5. Structure of O-methylochratoxin A methyl ester.

Yamazaki et al. (118) studied the toxicity of ochratoxins A and a in the chicken embryo and found the 72-hour $LD_{50}$ of ochratoxin A injected into egg yolk sacs was 16.96 μg/egg. Ochratoxin a injected at a level of 100 μg/egg killed only three of ten chicks 96 hours after injection. It was concluded that ochratoxin a showed no definite toxicity to the chicken embryo. In a study supporting results of Steyn (99), Chu et al. (20) found that ochratoxin a and O-methylochratoxin A ethyl ester were non-toxic to day-old chicks when orally dosed at levels of 1000 and 500 μg/chick, respectively. In the first report of ochratoxin B toxicity, Peckham et al. (70) found the seven-day oral median lethal dose of ochratoxin A in day-old Babcock B-300 cockerels to be 116-135 μg/chick (3.3-3.9 mg/kg) and 1890 μg/chick (54 mg/kg) for ochratoxin B.

Purchase and Theron (76) found the oral $LD_{50}$ values of ochratoxin A to be 22 and 20 mg/kg for male and female rats, respectively. This is about twice 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 (8).

In a study of considerable practical significance, Still et al. (100) provided evidence that feed contaminated with ochratoxin may be the cause of previously undiagnosed bovine abortion. These investigators force-fed 10-day pregnant rats with bicarbonate solutions of ochratoxins A and a. Ochratoxin A at doses of 6.25-25 mg/kg induced

fetal death and resorption while ochratoxin a at a dose level of 18 mg/kg had no effect. In contrast, however, Butler and Wigglesworth (9) failed to produce fetal resorption in 180 g Wistar rats which were orally dosed with 1 mg aflatoxin $B_1$.

In studies investigating the possible carcinogenic activity of ochratoxin A, Purchase and van der Watt (77) subcutaneously injected ochratoxin A at a level of 2.5 mg/kg twice weekly into Wistar rats until 35 doses were given. Survivors were killed and examined 87 weeks after beginning these experiments. Another group of rats were dosed orally with 100 or 300 μg ochratoxin A, 5 days/week for 50 weeks. Survivors were killed on week 110. Results of both of these studies indicated that ochratoxin A is not carcinogenic in rats.

Doster et al. (32) studied the toxicity of various ochratoxins in six-month-old rainbow trout and found the 10-day intraperitoneal $LD_{50}$ of ochratoxin A to be 4.67 mg/kg body weight. Ochratoxin B produced no mortalities at levels up to 66.7 mg/kg but produced histological abnormalities in livers and kidneys. Ochratoxins a and b were non-toxic at levels up to 28.0 and 26.7 mg/kg, respectively.

In summarizing the toxicity studies it would be useful to rank various ochratoxins according to their expected relative toxicities. The ochratoxins could be ranked in order of decreasing toxicity as follows: ochratoxin A $\geq$ ochratoxin C = ochratoxin A methyl ester $>>$ ochratoxin B $>$ ochratoxin a (non-toxic) = ochratoxin b (non-toxic) =

O-methylochratoxin A methyl ester (non-toxic) = O-methylochratoxin A ethyl ester (non-toxic).

## Histopathological Effects of Ochratoxin in Experimental Animals

Histopathological studies of ochratoxin toxicity have revealed that ochratoxin A primarily affects the liver and kidneys of ducklings (101, 107), rats (76, 78, 101, 110), chicks (34, 70) and rainbow trout (30, 32).

Van der Merwe (107) fed 50 g Peking ducklings single doses of 20, 50 and 75 μg ochratoxin A and noted acute fatty infiltration occurred in liver parenchyma cells. Fat was present throughout liver lobules from portal tracts to the central veins. Some cells contained nuclei displaced to the periphery of the cell by fat droplets. Occasionally intranuclear fatty vacuolation was noted. The portal tract appeared normal.

Theron et al. (101) 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. The structural changes became more severe

with time until the matrix of the mitochondria became coarsely granular with compression of the cristae against the external mitochondrial membranes. There was also an increase in the number of free ribosomes in the cytoplasm.

Theron et al. (101) found that 60 g weanling rats given 100 μg ochratoxin A develop hyaline degeneration and focal necrosis of the liver. Hypertrophy of the smooth endoplasmic reticulum (ER) forming "fingerprints" which appeared as hyaline degeneration under the light microscope 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 ochratoxin since many of the enzymes that play a role in the metabolism of hepatotoxic drugs are localized in the ER (39). No changes were observed in other organelles and it was concluded that the primary site of ochratoxin A action is on the ER and other cellular lesions develop from initial alterations in this subcellular structure.

The main pathological changes seen in ochratoxin A dosed rats by Purchase and Theron (76) 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. These necroses resulted in obstruction of the collecting tubules in the medulla with resulting dilation of other tubules in the nephron. No

abnormal glomeruli were reported in kidneys. Vacuolation due to glycogen accumulation was also seen in periportal cells of the liver.

Van Walbeek et al. (110) similarly concluded that ochratoxin A acts primarily as a nephrotoxin in rats. They noted cloudy swelling of proximal convoluted tubules of rats after feeding 500 μg ochratoxin A per day for 3 days. Later changes included foci of epithelial vacuolation and cytolysis in proximal tubules and desquamation across the mid-cortex.

Doupnik and Peckham (34) cultured several A. ochraceus strains on cracked corn and fed these cultures in a diet to 1-day-old Babcock B-300 cockerels. Corn infected with strains NRRL 3174 and P-657 were the most toxic and the toxicity correlated with the amount of ochratoxin A present. Gross postmortem findings included dehydration and emaciation, dry firm mucosal linings in the gizzard and small hemorrhages in the mucosa of the proventriculi. Histopathological examination of various tissues revealed hepatic lesions which varied from mild scattered foci of fatty changes to severe diffuse foci of necrosis. Occurring consistently in chicks fed NRRL 3174 and P-657 infected corn was suppression of hematopoietic activity in the bone marrow and depletion of lymphoid elements from the spleen and bursa of Fabricius.

In acute toxicity experiments in which purified ochratoxins A and B were orally dosed to 1-day-old Babcock B-300 cockerels,

Peckham et al. (70) noted principally visceral gout with white flake-like deposits (uric acid crystals) in the kidneys and ureters as well as on the heart, pericardium, liver and spleen of affected birds. Also noted was emaciation, dehydration and firm mucosal linings in the gizzards. Histopathological findings included acute nephrosis in chicks dying from ochratoxin A or B intoxication. Proteinaceous casts, urates, scattered heterophiles and localized necrosis occurred in renal tubules. Hepatic lesions occurred less frequently than renal lesions and varied in severity from mild diffuse vacuolation of hepatocytes to necrotic foci. Only one chick given ochratoxin B had liver lesions and these differed markedly from the lesions seen in chicks given ochratoxin A. The necrotic foci seen in the chick given ochratoxin B were scattered and irregularly shaped as opposed to the regularly shaped necrotic foci in chicks given ochratoxin A. Other histological findings in birds from this study included suppression of hematopoiesis in the bone marrow and depletion of lymphoid elements from the spleen and bursa of Fabricius. Catarrhal enteritis with heterophils in dilated intestinal glands were seen in a few birds from both ochratoxin groups. These authors suggested that the toxic effects of ochratoxins A and B were similar but felt additional pathological studies of ochratoxin B toxicity would be needed for conclusive data.

Choudhury et al. (12) fed A. ochraceus wheat cultures to White Leghorn pullets from 14 weeks to one year of age. The cultures were

assayed for ochratoxin A and added to the standard grower and layer diets to bring the level of ochratoxin A to 1, 2 and 4 mg per kg of diet. The lowest levels of ochratoxin delayed sexual maturity and reduced egg production. Increasing levels of ochratoxin reduced production further and pullets became very emaciated. With the 4 ppm level, most of the pullets that survived had not laid eggs through one year of age. The morbidity and mortality were severe for the 2 and 4 mg/kg treated groups during the first six weeks of the experiment but subsided toward the end of the experiment as birds apparently gained resistance to the toxin. Histopathological examination of livers and kidneys from treated birds revealed large numbers of heterophiles in the interstitial areas of the liver. No changes were noted in any of the other tissues examined.

Doster (30) and Doster et al. (32) dosed 6-month-old Mt. Shasta strain rainbow trout intraperitoneally with aqueous bicarbonate solutions of purified ochratoxins A, B, a and b and found pathological changes in livers and kidneys of trout dosed with ochratoxins A and B but not ochratoxins a and b. Ochratoxin A produced degenerative changes in hepatic parenchymal cells including nuclear swelling and cytoplasmic and nuclear lipid vacuolation. Necrosis in proximal segments of nephrons, the hematopoietic tissue and glomeruli as well as pycnotic nuclei, cast formation and lipid vacuolation were seen in renal tubules. Ochratoxin B administered at levels up to approximately

14 times the toxic level of ochratoxin A produced no deaths but induced pathological changes in the liver and kidney similar to those produced by relatively low levels of ochratoxin A.

Doster (30) fed ochratoxin A at levels up to 64 ppb in a semi-synthetic diet to rainbow trout for one year and failed to note any carcinogenic effect. Periodic histological examination of these trout revealed liver damage including nuclear swelling, parenchymal cell degeneration and bile duct proliferation.

## Metabolism of Ochratoxin A

Nel and Purchase (65) studied the metabolic fate of intraperitoneally dosed ochratoxin A in rats. They detected ochratoxin A in the blood, liver and kidneys 30 minutes after dosage. Maximum excretion occurred 6 to 18 hours after injection and decreased thereafter until only traces were detectable at 78 hours. Another more polar product ($R_f$=0.5) with blue fluorescence, characterized as ochratoxin *a*, was excreted in the urine and feces 6 hours after dosage and increased until maximum excretion was noted between 30 and 42 hours. In addition, a green fluorescent spot having an $R_f$ value less than ochratoxin *a* was found consistently in all the extracts of urine and feces. The amount of this compound excreted diminished slowly until at 78 hours only a trace was visible. Hutchison *et al.* (49) reported that this green fluorescent compound had an $R_f$ value identical

to 4-hydroxyochratoxin A (Figure 3) in five different $SiO_2$-TLC systems. It is noteworthy that the latter authors also reported that ochratoxin A caused 100% mortality of male Wistar rats after 6 days at a dose level of 40 mg/kg, but 4-hydroxyochratoxin A had no effect at this level.

Van Walbeek et al. (110) intubated rats daily with 500 μg of ochratoxin A and noted an average of 10% of the amount intubated in the urine and feces daily. They noted up to 27 μg ochratoxin a/rat/day in the urine and 10 μg/rat/day in the feces. There was little accumulation of ochratoxin A and no ochratoxin a in the liver or kidneys.

## In vitro Ochratoxin Studies

Since Purchase and Theron (76) found that administration of ochratoxin A to rats at a dose of 10 mg/kg body weight caused accumulation of glycogen in the liver, Pitout (72) speculated this probably results from ochratoxin interacting with nucleic acids and/or certain enzymes involved in carbohydrate metabolism. Pitout (72) subsequently found that ochratoxin A inhibits the hepatic phosphorylase enzyme system. At a concentration of 0.108 mg/ml reaction mixture, the inhibition was 70%. Ochratoxin A had no effect on the enzymes glucomutase, glucose-6-phosphate dehydrogenase, phosphorylase a or hexokinase. Pitout suggested that the inhibitory effect of ochratoxin A on the hepatic phosphorylase enzyme system might be at the level

of the phosphorylase b kinase reaction. Phosphorylase b kinase catalyzes the activation of phosphorylase b to phosphorylase a which in turn catalyzes the phosphorylytic cleavage of $\alpha$-glucosidic-1, 4-linkages of glycogen to $\alpha$-glucose-1-phosphate. Additionally, analysis of spectra and difference spectra in the visible region, column chromatography on Sephadex G-25 and thermal denaturation studies revealed no interaction of ochratoxin A with nucleic acids and their derivatives.

After Nel and Purchase (65) reported ochratoxin A to be metabolized by rats to ochratoxin a, Pitout (73) studied the hydrolysis of ochratoxin A by some proteolytic enzymes. He found that ochratoxin A was hydrolyzed by carboxypeptidase and $\alpha$-chymotrypsin, while trypsin had no effect. The apparent $K_m$ value, first order reaction constant and apparent proteolytic coefficient for carboxypeptidase A at 25°C were $1.5 \times 10^{-4}$ M, $1.1 \times 10^{-2}$ $min^{-1}$ and 4.4, respectively. It was suggested that ochratoxin A may also be hydrolyzed by cathepsin C from lysosomes. In view of this, Pitout (73) stated from information contained in a personal communication with Dr. I. F. H. Purchase, that ochratoxin A is hydrolyzed in the liver to ochratoxin a which is excreted in the bile. This information was obtained using isolated liver perfusion experiments.

Yamazaki et al. (118) incubated ochratoxin A labeled with L-$\beta$-phenylalanine-U-$^{14}$C in rat liver homogenates and microsomal

preparations and noted no effective hydrolytic activity in any of the systems used. Only a trace of the fluorescent spot of ochratoxin a was detected when ochratoxin A was incubated with the homogenate or microsomal fraction in 0.1 M NaCl-0.02 M Tris buffer at pH 7.5 at 37°C for one hour.

In a study attempting to explain at least a portion of the difference in toxicity of ochratoxins A and B, Doster and Sinnhuber (31) showed that ochratoxin B is hydrolyzed to a greater extent than ochratoxin A by bovine carboxypeptidase A and by enzymes in tissue extracts of rat liver, small intestine and large intestine. The $V_{max}$ values for hydrolysis of ochratoxins A and B by carboxypeptidase A were $5.15 \times 10^{-7}$ and $4.35 \times 10^{-5}$ moles/liter/min, respectively. Incubation of rat tissue extracts with equimolar amounts of ochratoxin A or B for 6 hours at 37°C indicated that ochratoxin B was hydrolyzed 6-7 times faster than ochratoxin A by enzymes in the three tissue extracts.

Moore and Truelove (61) found that ochratoxins A and a inhibited ADP-stimulated respiration when applied at low concentration to rat liver mitochondria. One hundred percent inhibition was noted at concentrations of $1 \times 10^{-5}$ and $4.2 \times 10^{-4}$ M for ochratoxins a and A, respectively, indicating ochratoxin a was more inhibitory than the parent ochratoxin A. The significance of these results in view of the marked difference in toxicity of ochratoxins A and a is not readily apparent.

Chu (17) demonstrated the interaction of ochratoxin A with bovine serum albumin (BSA) by spectrophotometric, spectrofluorometric, equilibrium dialysis and Sephadex gel filtration analyses. These results indicated that 1 mole of BSA binds 1.87, 2.23 and 2.47 moles of ochratoxin A with binding constants of 0.317, 1.86 and 3.17 x $10^6$ $M^{-1}$ at 25°, 16° and 6°C, respectively. Chu speculated the ochratoxin A-BSA binding may be significant *in vivo* and albumin may serve as a transport agent by providing a constant liberation of the toxin from the complex to the target tissue. Also, the ochratoxin A-BSA complex may inhibit the hydrolysis of ochratoxin A by proteolytic enzymes.

Chu *et al.* (20) presented evidence that the dissociation of the phenolic hydroxyl group of ochratoxin is necessary for ochratoxin intoxication. The apparent dissociation constant of the phenolic hydroxyl group in ochratoxins B, C and *a* were found to be 7.95, 7.14 and 11.0, respectively. It was noted that the toxicities of these compounds correlated well with the dissociation constants, e.g., the acid dissociation constant of ochratoxin B was ten times smaller than ochratoxin A, while the toxicity in day-old chicks was about ten times less. Additionally, O-methylochratoxin A ethyl ester which has the phenolic hydroxyl group blocked by methylation was non-toxic to day-old chicks. It was postulated that the toxic effect of ochratoxin arises from the interaction of the phenolic group in the toxin with proteins and enzymes *in vivo*.

# EXPERIMENTAL

## Ochratoxin Toxicity Experiments

### Preparation of Ochratoxin Compounds

Ochratoxins A, B and Ethyl Esters of A, B and a. For the toxicity experiments, various ochratoxins were prepared and administered to rainbow trout in an attempt to define a structure-activity relationship. Seven ochratoxin compounds were prepared for these studies, namely, ochratoxins A, B, A and B ethyl esters, a ethyl ester, and the alanine and leucine analogues of ochratoxin A. The structures of these compounds are shown in Figure 15 (p. 69).

Ochratoxins A and B were prepared and purified as previously described (30, 32). Ochratoxin a was prepared from ochratoxin A by the acid hydrolysis method of van der Merwe et al. (106), then purified (30). Approximately 10 mg of the ethyl esters of ochratoxins A, B and a were prepared from pure samples of ochratoxins A, B and a, respectively, as described in Appendix I. These were then purified by column chromatographic procedures similar to those described for purification of radiolabeled ochratoxin A (Appendix IX, 7) except the column was eluted with carbon tetrachloride:glacial acetic acid 98:2 v/v. [1]

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

Alanine and Leucine Analogues of Ochratoxin A. Of particular interest in this study was the question of whether substitution of other amino acids in place of phenylalanine in ochratoxin A would have an influence on toxicity. To test this possibility, the alanine and leucine analogues of ochratoxin A were prepared for use in toxicity studies with rainbow trout. Synthesis was achieved by cleavage of the phenylalanine from ochratoxin A, then replacement of this amino acid with each of the other two amino acids by amide formation. The reactions used in this synthesis are summarized in Figure 6. Details of the procedures used are described in Appendix II.

Ochratoxin Purity. The purity of the compounds prepared for the toxicity studies was checked by spotting 8-10 μg on a silica gel chromatoplate (Appendix X), developing the plate in carbon tetrachloride: glacial acetic acid 9:1 v/v, examining under long-wave ultraviolet radiation (365 nm), and establishing that only one fluorescent spot was present on the plate. Developed plates were also placed in an iodine chamber for 30 minutes to detect possible non-fluorescent impurities.

Quantitation. The ochratoxin test compounds were quantitated spectrophotometrically using the molar absorptivity values given in Appendix XIII. The molar absorptivity value for ochratoxin a ethyl ester was determined experimentally while that of ochratoxin A was used for quantitation of the alanine and leucine analogues of ochratoxin

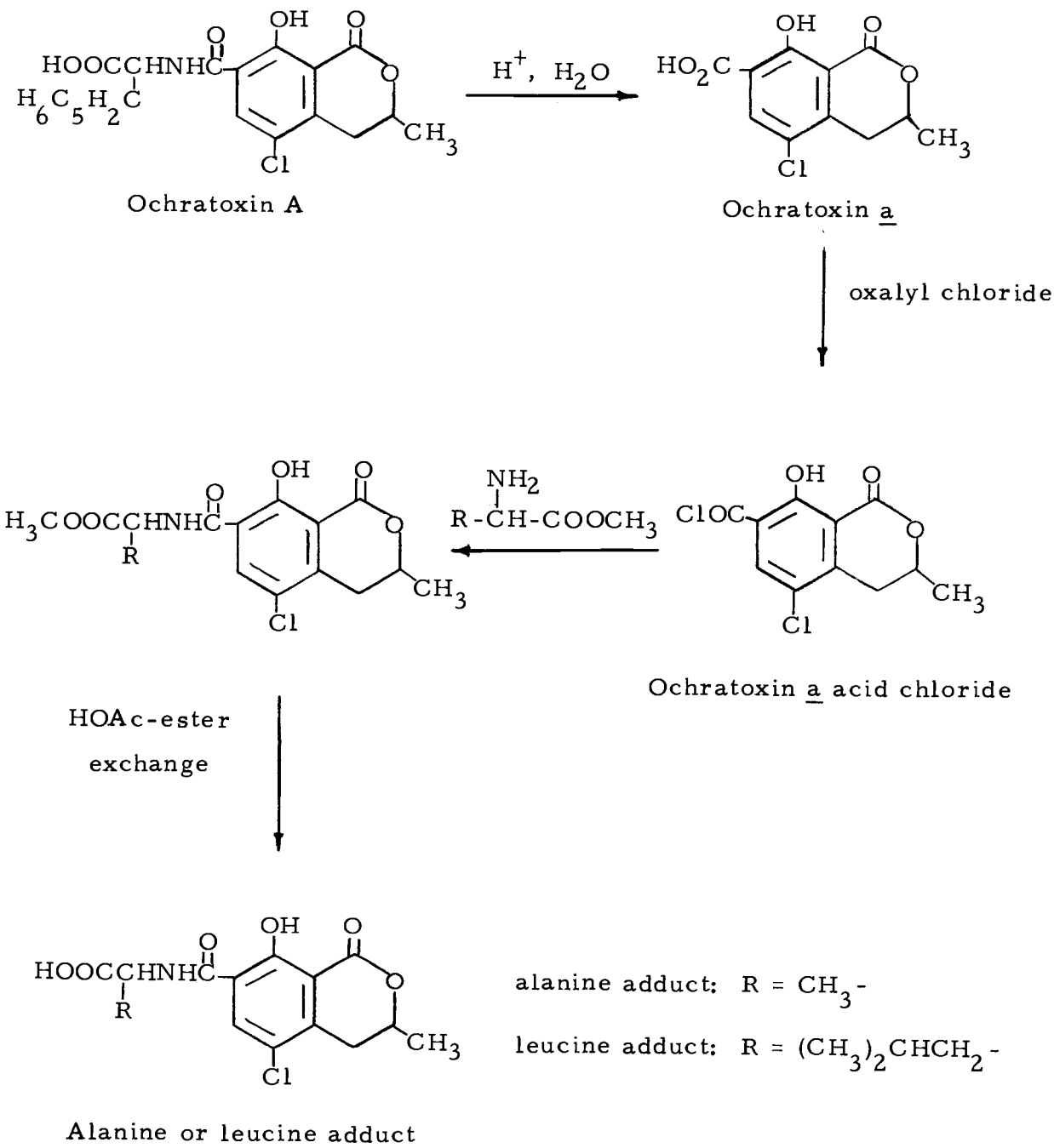


Figure 6. Formation of alanine and leucine analogues of ochratoxin A.

A since insufficient quantities of the latter two compounds were prepared for accurate weighing. The molar absorptivity values of the other ochratoxins were taken from the literature (see Table 1).

Great care was taken to assure complete removal of carbon tetrachloride and other harmful organic solvents from all ochratoxin compounds used in toxicity studies because of the hepatotoxic effects of these solvents. This was done by repeatedly dissolving the toxin solutions in 95% ethanol and evaporating to dryness in vacuo to aid in driving off these solvents. Final quantitated ochratoxin solutions were dissolved in 95% ethanol and stored at -10°C until initiation of the toxicity trials.

## Toxicity Trials

Rainbow trout (Salmo gairdneri) eggs of the Mt. Shasta strain were spawned and hatched at our laboratory facility[2] and held on a control diet (30) for 6 months before initiation of the toxicity experiments. Trout were reared in 150 gallon fiberglass tanks with a water flow rate of four gallons per minute at a temperature of 12°C with an oxygen content of 8.5 to 9.5 ppm.

Six-month-old trout weighing an average of 35 g were fasted 48 hours then given a single dose of ochratoxin by intraperitoneal (IP)

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

injection. This route was used because of the strong regurgitation response of trout to orally dosed toxins (3, 30). Quantitated ethanol solutions of ochratoxins A and B and the alanine and leucine analogues of ochratoxin A were evaporated to dryness and dissolved in 0.1 N sodium bicarbonate. Ethanol solutions of ochratoxins C, a ethyl ester and B ethyl ester were added to aliquots of corn oil and the ethanol removed in vacuo. The final concentrations of the ochratoxin compounds in their respective carriers which were administered to trout are shown in Table 3. Control trout were dosed with 0.1 N sodium bicarbonate or corn oil. The amount of each solution injected into each trout was calculated from the following formula:

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

Therefore, a 30 g trout 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 3 also lists the dose levels of the seven ochratoxin compounds injected.

Immediately before IP dosage, each fish was anaesthetized with MS-222 (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. Mortalities during the ten-day experimental period were recorded and the $LD_{50}$ values for ochratoxins causing mortalities

Table 3. Concentrations and dose levels of ochratoxins administered to rainbow trout.

| Ochratoxin | Concentrations (mg/ml) | Toxin carrier | Dose levels (mg/kg body weight) |
|---|---|---|---|
| A | 0.6, 0.9, 1.2 | 0.1 N $NaHCO_3$ | 4.0, 6.0, 8.0 |
| B | 10.0 | 0.1 N $NaHCO_3$ | 66.7 |
| A ethyl ester | 0.3, 0.45, 0.6 | corn oil | 2.0, 3.0, 4.0 |
| B ethyl ester | 1.5, 1.88, 2.25 | corn oil | 10.0, 12.5, 15.0 |
| a ethyl ester | 0.585 | corn oil | 3.9 |
| alanine analog of A | 0.673 | 0.1 N $NaHCO_3$ | 4.49 |
| leucine analog of A | 0.763 | 0.1 N $NaHCO_3$ | 5.06 |

were determined by the method described by Litchfield and Wilcoxon (56).

Surviving trout were sacrificed on day ten and immediately autopsied. Gross abnormalities were recorded and the livers and kidneys excised and preserved in Bouin's fixature. These tissues were microtome sectioned at 4 μ and stained with hematoxylin and eosin (46, p. 130) for light microscope examination.

## Preparation of Radiolabeled Ochratoxins

### Choice of Radiolabeled Precursor

The most suitable precursor for use in preparation of radiolabeled ochratoxins A and B was acetate-$^{14}C$. The reasons are

threefold: 1) labeling with phenylalanine-$^{14}C$ is of no value since this would not allow tracing possible hydrolysis products of ochratoxin, e. g., ochratoxin a; 2) labeling with $^{36}Cl$ is of no value because this would not permit tracing ochratoxin B since it lacks chlorine in the molecule; and 3) labeling with acetate-$^{14}C$ would make it possible to prepare ochratoxins A and B having identical molar specific activities if labeled ochratoxin A could be converted to ochratoxin B by a dehalogenation reaction. Also, since much lower levels of ochratoxin B than A are generally produced in mold cultures, it was felt that the ochratoxin B would most efficiently be obtained by dehalogenation of ochratoxin A obtained from the cultures.

Preparation of Sodium Acetate-1-$^{14}C$

Due to the low incorporation percentages of acetate-$^{14}C$ into ochratoxin A in previous studies, it was felt that large amounts of acetate would be needed for preparation of labeled ochratoxin A having a sufficiently high specific activity for conduction of the desired metabolism studies. Therefore, in the interest of economy, it was decided to prepare carboxy-labeled acetate rather than purchase it from a commercial source. This was done using a modification of the semi-micro procedure described by Van Bruggen et al. (105), involving carbonation of a methyl Grignard reagent. The apparatus described by these authors was modified by the addition of a 70 cm capillary

mercury manometer to monitor the pressure inside the reaction vessel. The manometer was connected to a short tube branching off the delivery tube between the distillation flask and $CO_2$ generator. Also, the generator vessel was modified to permit insertion of a 11 x 40 mm vial containing the $Ba^{14}CO_3$ which was purchased from a commercial source.[3] This obviated the necessity of transferring the $Ba^{14}CO_3$ from the vial to the $CO_2$ generator, a hazardous procedure, even under ideal circumstances (24). The side arm reservoir on the $CO_2$ generator was replaced with a short serum-capped tube through which 40% perchloric acid was slowly dripped for generation of the $CO_2$. All other parts of this apparatus as well as the distillation assembly were identical to that described by Van Bruggen et al. (105). A schematic of the apparatus as modified is shown in Figure 7.

The synthesis was performed several times with non-isotopic $BaCO_3$ and yields of 80-90% were obtained using 0.5-1.0 mmole amounts of $BaCO_3$. Yields were determined by titration to phenolphthalein end point with 0.25 N NaOH. Reaction conditions used were identical to those described by Van Bruggen et al. (105). The methyl Grignard reagent used in the reaction was prepared as described in Appendix IV. A four-fold excess of this reagent was used in the acetic acid synthesis.

Sodium acetate-1-$^{14}C$ was prepared in two reactions using 25 mc

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[3] Amersham/Searle, Arlington Heights, Illinois.

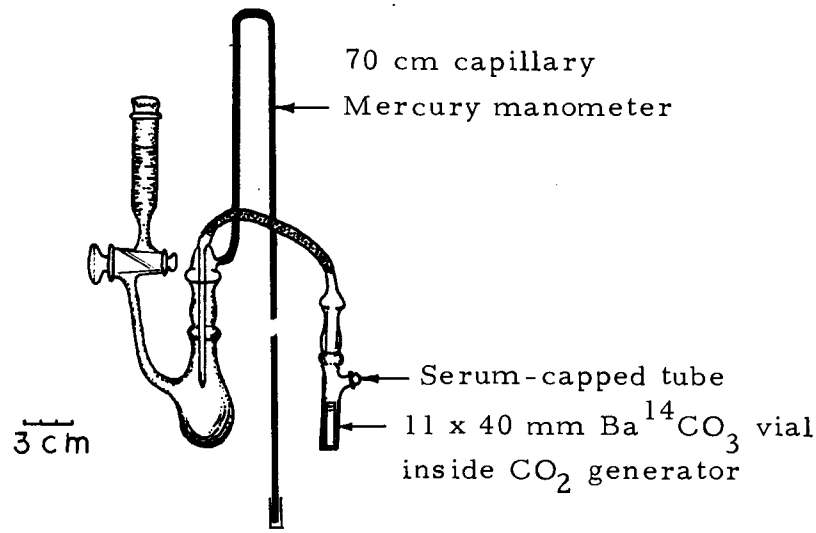


Figure 7. Modified carbonation apparatus for production of acetic acid-1-$^{14}$C.

$Ba^{14}CO_3$. The average yield from these two reactions was 83.2%. The product was frozen and dried by lyophilization, then dissolved in sterile mineral mix (Appendix VI) at a concentration of 5 mc/ml. The experimentally determined specific activity of the sodium acetate-1-$^{14}$C product was 54.6 mc/mmole. This is within 93% of the theoretical specific activity assuming the $Ba^{14}CO_3$ had a specific activity of 58.6 mc/mmole as given by the manufacturer. The product was counted by liquid scintillation spectrometry in dioxane fluor (Appendix XIV).

The radiopurity of the prepared sodium acetate-1-$^{14}$C was checked by spotting 0.037 μc onto a 5-cm wide Whatman #1 paper strip and developing in acetone:t-butanol:n-butanol:ammonium hydroxide

2:1:1:1 by volume. This strip was scanned with a Packard Model 7201 Radiochromatogram scanner. The radiopurity was found to be greater than 99%. The $R_f$ of the sodium acetate in this system was about 0.35.

## Preparation of Radiolabeled Ochratoxin A

Radiolabeled ochratoxin A was prepared by inoculating 40 10-ml sucrose yeast extract liquid cultures with Aspergillus ochraceus (NRRL 3174)[4] and growing the cultures for two days at 25°C. One mc of the synthesized sodium acetate-1-$^{14}$C was added and the cultures were incubated an additional five days at 25°C. The crude ochratoxin was obtained by chloroform extraction of the liquid portions of the cultures. The mycelial mat was homogenized and extracted with chloroform and saturated sodium bicarbonate. The sodium bicarbonate was acidified and back extracted with chloroform. All chloroform extracts were combined for purification by column chromatography, bicarbonate fractionation and recrystallization. Details of these procedures are reported in Appendices V through XII.

Because of the instability of ochratoxin in daylight (74) all procedures performed in this study with ochratoxin were conducted in subdued light and toxin solutions were stored in darkness at -10°C. By taking these precautions it was found that purified toxin solutions

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[4]Obtained from Dr. C. W. Hesseltine at Northern Regional Research Laboratories, Peoria, Illinois.

retained their chemical and radiopurity throughout the course of this study.

Before the final preparation of radiolabeled ochratoxin A using acetate-1-$^{14}$C, it was first established that addition of non-isotopic acetate at chemical levels up to 40 mg/10 ml culture had no effect on ochratoxin A production. The maximum amount of acetate-1-$^{14}$C used in the 10 ml cultures was about 1.52 mg. Also, it was established that the amount of phenolphthalein added to the acetic acid before titration (ca. 0.5 μg) had no effect on ochratoxin A production.

## Determination of Chemical and Radioactive Purity of Ochratoxin A-$^{14}$C

After recrystallization (Appendix XII) the final purified ochratoxin A-$^{14}$C was dissolved in its appropriate quantitation solvent (Appendix XIII) and its absorbance recorded in the 280 to 360 nm region. The wavelength of maximum absorption of ochratoxin A in this solvent system is at 333 nm and the absorbance values at 360 and 290 nm are nearly the same for a chemically pure ochratoxin A sample. Some samples which are chemically impure produce a spectra in which the absorbance at 290 is greater than that at 360 nm (> 0.05 O. D. unit difference). However, ochratoxin A samples in which the absorbance at 290 nm is not 0.05 O. D. unit greater than that at 360 nm are not necessarily pure, but those samples in which the difference in

absorbance at these two wavelengths is greater than 0. 05 O. D. unit are always impure. See Figure 8 for an example of this.

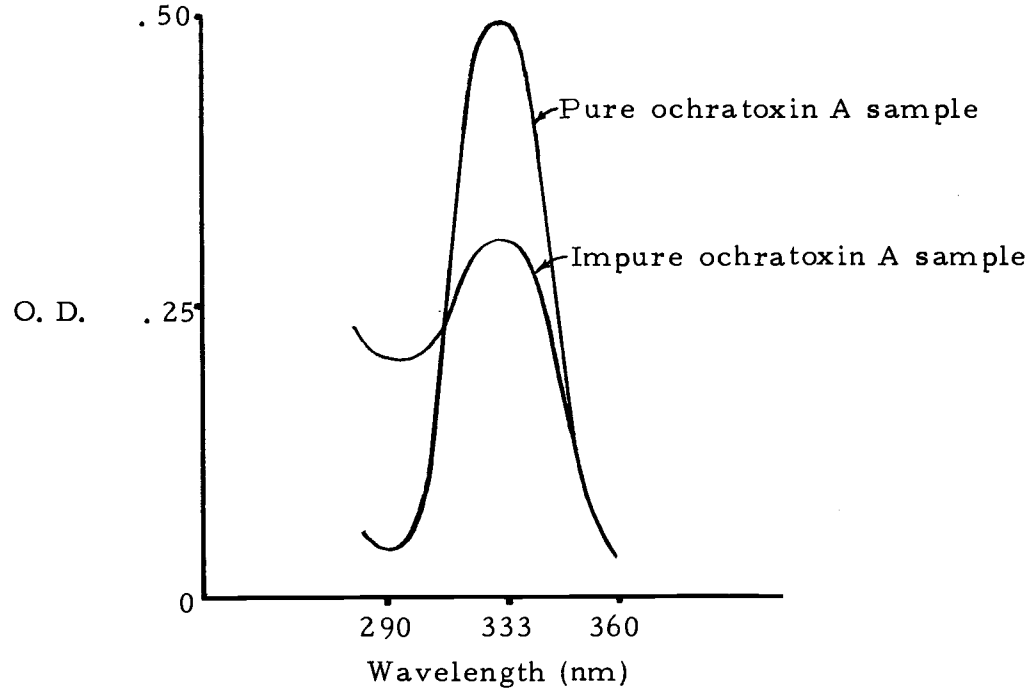


Figure 8. Ultraviolet absorption spectra of pure and impure ochratoxin A samples. Solvent: benzene:glacial acetic acid 99:1 v/v.

This criteria of purity is not always reliable but it is often more reliable than visualizing developed chromatogram plates and identifying fluorescent spots other than ochratoxin A. This is so because non-fluorescent (often yellow) impurities are present in partially purified ochratoxin A samples which are not easily detectable on thin-layer chromatograms. This method is useful in rejecting a suspected

sample as being pure before other more time-consuming procedures are used to check the chemical purity.

The purified ochratoxin A-$^{14}$C sample which passed the above purity criteria was dissolved in a small volume of benzene and 0.005 μc applied to a silica gel plate (Appendix X) and the plate developed in benzene:glacial acetic acid 9:1 v/v. This level of activity was considerably greater than that chromatographed in any portion of the ochratoxin metabolism studies. This was repeated using two other solvent systems, namely, carbon tetrachloride:glacial acetic acid 9:1 v/v and toluene:ethyl acetate:glacial acetic acid:90% formic acid, by volume. These plates were scanned with a Varian Model 6000 Radio Scanner. After scanning each plate, the ochratoxin A spot was scraped into a scintillation vial and counted in dioxane fluor (Appendix XIV) to 1% standard error. Also, the silica gel above and below the ochratoxin A spot was counted to detect the presence of possible radioactive impurities.

After establishing that the ochratoxin A sample was pure, the specific activity was determined by quantitation as described in Appendix XIII and counting an aliquot of the quantitated sample in triplicate by liquid scintillation spectrometry (Appendix XIV) to 1% standard error.

Dehalogenation of Ochratoxin A

Catalytic dehalogenation is a convenient and usually efficient method of removing halogen under mild conditions. During the synthesis of ochratoxins A and B, Steyn and Holzapfel (96) used Raney nickel to catalyze the dehalogenation of 5-chloro-8-methoxy-3-methylisochroman to produce 8-methoxy-3-methylisochroman as shown in Figure 9.

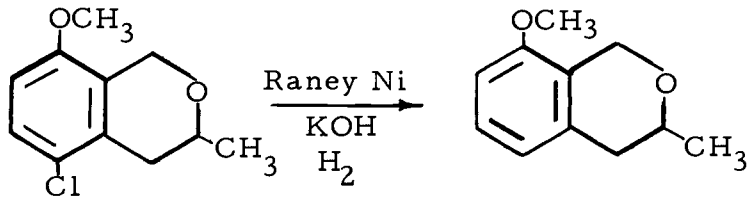


Figure 9. Dehalogenation of 5-chloro-8-methoxy-3-methylisochroman.

Neither these nor any other authors have reported the dehalogenation of ochratoxin A, however. The procedure described in the above dehalogenation was used in an attempt to dechlorinate ochratoxin A but was found to be unsatisfactory as no detectable levels of ochratoxin B were found after the reaction was carried out for 24 hours.

Huffman (45) prepared 5-methoxy-1-tetralone from

8-chloro-5-methoxy-1-tetralone by preferential hydrogenolysis of the halogen atom (Figure 10). The hydrogenolysis was carried out over 10% palladium-on-carbon in ethanol containing an equivalent of triethylamine. The reaction proceeded smoothly and virtually ceased after absorption of one mole of hydrogen. The dechlorinated product was obtained in 53% yield after recrystallization.

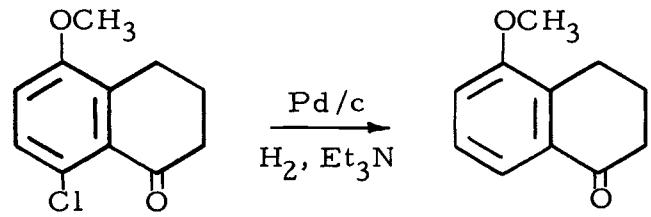


Figure 10. Dehalogenation of 8-chloro-5-methoxy-1-tetralone.

A modification of this procedure was used to convert ochratoxin A to ochratoxin B. The optimum conditions to be used in the reaction were first determined with non-isotopic ochratoxin A, then 20 mg radiolabeled ochratoxin A having a specific activity of 93.3 μc/mmole was subjected to the dehalogenation reaction. Details of the procedure used are described in Appendix XV. The ochratoxin B formed was purified by silica gel column chromatography and bicarbonate fractionation as described in Appendices XVI and XI, respectively.

## Determination of Chemical and Radioactive Purity of Ochratoxin B-$^{14}$C

The radiolabeled ochratoxin B was much more easily purified than ochratoxin A since it was made from ochratoxin A already having a high degree of purity. The purified ochratoxin B sample was dissolved in a small volume of benzene and 0.005 μc applied at the bottom of a 5-cm wide silica gel (Appendix X) plate and the plate developed with benzene:glacial acetic acid 4:1 v/v. This was repeated using two other solvent systems, namely, carbon tetrachloride: glacial acetic acid 4:1 v/v and toluene:ethyl acetate:90% formic acid 5:4:1 by volume. These plates were scanned with a Varian Model 6000 Radio Scanner. After scanning, the ochratoxin B spot was scraped into a scintillation vial and counted in 10 ml dioxane fluor to 1% standard error (Appendix XIV). As with the ochratoxin A plates, the silica gel above and below the ochratoxin B spot was also counted. After establishing that the ochratoxin B sample was pure, it was quantitated spectrophotometrically (Appendix XIII) and the specific activity determined in triplicate.

## Excretion and Tissue Distribution of $^{14}$C from Ochratoxins A-$^{14}$C and B-$^{14}$C

If one assumes that the relative toxicological response of ochratoxins A and B is a function of their internal cell concentration, then

not only are their rates of transport important in explaining their toxicity difference but also their rates of metabolism. These two factors are interrelated since conversion to more water-soluble metabolites often results in a faster excretion rate. Therefore, the development of possible reasons for the difference in toxicity of ochratoxins A and B necessitates determining the distribution of the unaltered toxins as well as their metabolic products in various excretions and tissues of an experimental animal.

## Experimental Animal

For these studies rainbow trout of the Mt. Shasta strain were held in fiberglass tanks and fed a control ration (30) until they were 9-12 months old. Fasted trout were catheterized, placed in metabolism chambers, then dosed IP with radiolabeled ochratoxin A or B dissolved in 0.1 N sodium bicarbonate. The injection was made with a 500 µl syringe with a 27 gauge needle just anterior and dorsal to the left pelvic fin. Ayres (2) determined that IP dose leakage in trout was less than 1%. As before, great care was taken to assure complete removal of benzene as well as other toxic solvents from the radiolabeled ochratoxin solutions used in the metabolism studies.

## Trout Metabolism Chambers

Proper conduction of metabolism experiments with drugs or

toxic agents necessitates collection of the major metabolic excretions of the experimental animal and analysis of these excretions for metabolites. Trout excrete metabolic wastes primarily through four possible pathways: the urine, the bile, carbon dioxide and direct excretion through the gills. Biliary excretions which are not reabsorbed from the gastrointestinal tract are excreted in the feces.

Because the "gill does not seem to provide a very efficient method of eliminating many of the more readily diffusible foreign substances tested to date" (43, p. 326), efforts were concentrated on collection of urinary and fecal excretions. Procedures have been devised for continuous collection of urine, free of fecal matter from trout (44, 47, 75, 91). All of these methods utilize some type of plexiglass chamber in which a catheterized trout is held. The methods used by Post et al. (75) and Smith (91) permit collection of both fecal and urinary excretions through the use of a rubber dam between the front and rear parts of the chamber through which the trout is placed. The chambers used by Holmes and Stainer (44) and Hunn et al. (47) do not permit collection of fecal excretions, as aerated water enters at one end, flows through the entire length of the chamber, and exits at the opposite end. The chamber used by Smith (91) has a 12-liter reservoir around the head end of the chamber as an added feature to permit collection of gill excretions.

In the preliminary stages of this study a chamber similar to the

one used by Smith (91) was built and used for collection of urinary and fecal excretions from trout. After using this chamber briefly it was felt that a different design was needed which permitted a number of necessary operations to be performed which were not easily accomplished using the chamber of Smith's design.

The final design of the chamber used in these studies is shown in Figure 11. The chamber, constructed with plexiglass was divided into a head and tail section between which was bolted a sheet of dental rubber dam material.[5] A vertical slit about 2" long was cut in the rubber dam material through which the trout was placed. This effectively prevented exchange of water between the two chamber sections. The tail of the trout was held relatively stationary by means of a V-shaped restrainer attached to the chamber lid with two brass bolts. This reduced movement of the tail of the fish to prevent working loose the urinary catheter and consequent leakage of urine. The lid was slotted to allow longitudinal adjustment of the bolted tail restrainer to fit various sizes of trout.

Experimental trout were fasted four days prior to anaesthesia with 50 ppm MS-222, weighing and catheterization. PE 90 catheter tubing[6] was prepared for insertion by cutting obliquely at one end, then

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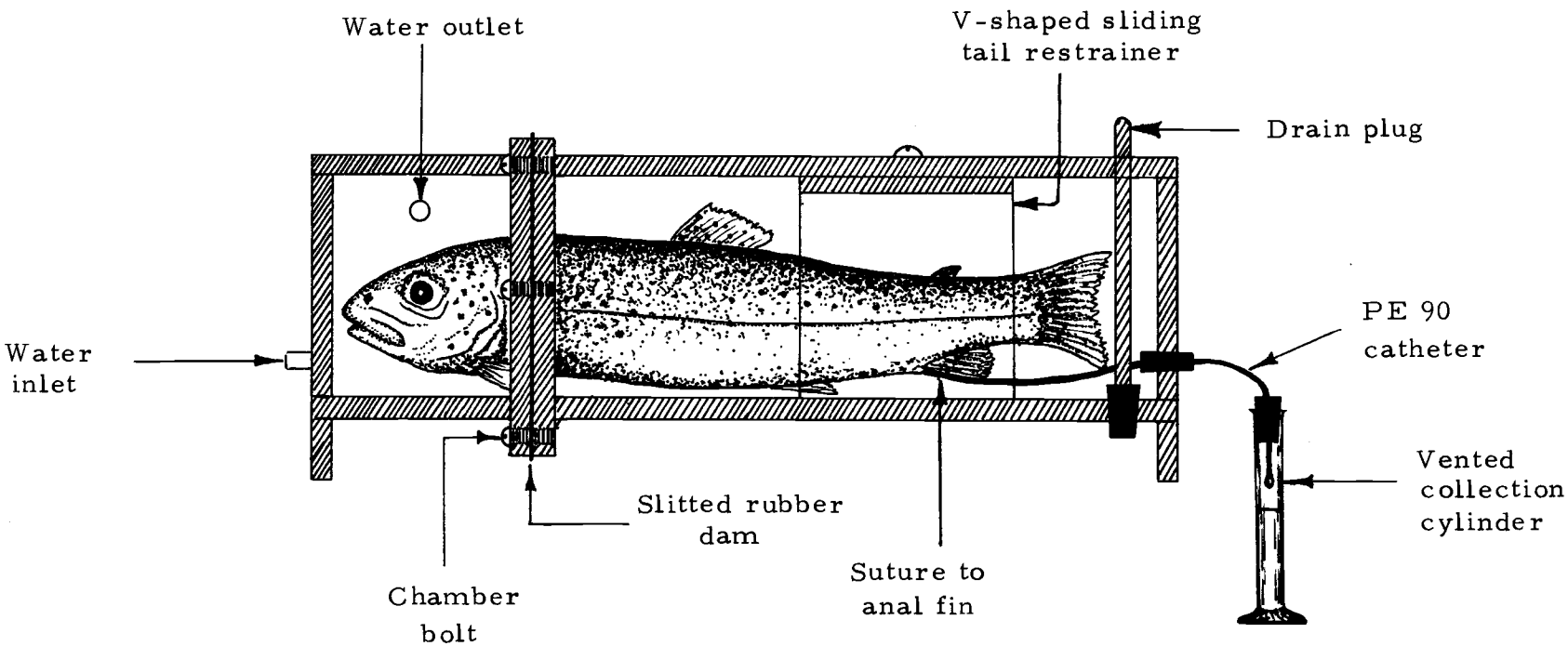
[5] Dental dam, Scientific Products, Redmond, Washington.

[6] Ibid.




Scale: 1/2" = 1"

Figure 11. Longitudinal diagram of complete metabolism chamber.

a 1/2" wide piece of masking tape was wrapped around the tubing 1/2" from the end about four times. A short piece of 5-0 silk thread was tied around the tape. The catheter was primed with distilled water and inserted into the urino-genital papilla and sutured to the base of the anal fin with the silk thread tied around it. Figure 12 shows this in detail. With practice this entire operation can be performed in about 5 minutes.

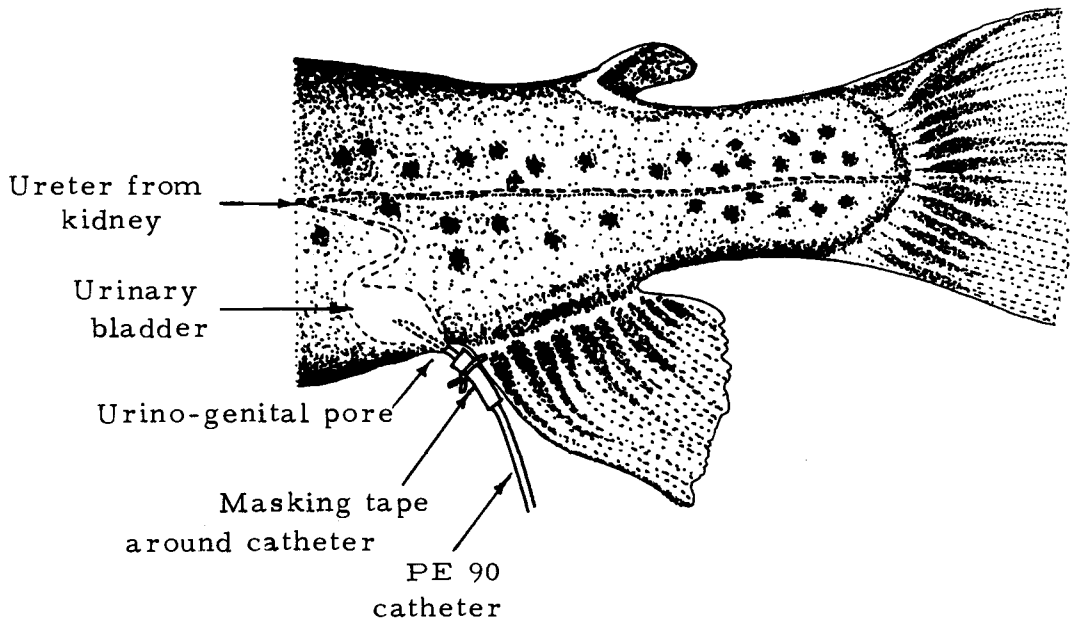



Figure 12. Detail of catheterized trout.

After catheterization, the trout was placed into the metabolism chamber and the catheter passed through a hole in the rubber stopper at the back of the tail section of the chamber and the tip allowed to hang 2" below the lower level of the fish. This provided sufficient negative pressure to aid urine flow but was not great enough to collapse

the bladder on the end of the catheter. The lid and tail restrainer were placed on the chamber and held down with a heavy lead weight. Water with a temperature of 12°C and oxygen content of 10 ppm was flowed through the head section of the chamber at a rate of 300 ml/minute. A lid was placed on the head chamber and held down with a lead weight. This was found to be necessary to prevent the trout from escaping from the chamber. All metabolism experiments were conducted in a cold room adjusted to 10°C (± 3°).

Urine Collection and Toxin Injection

Trout placed in the chambers were held 24 hours before injection with radiolabeled ochratoxins. This time was used to allow urine output to stabilize and also to establish that urine was flowing properly through the catheter. Trout in which the urine flow was lower than normal (indicating probable catheter leakage) were not used in the metabolism studies. Twenty-four hour urine output in trout of the size used in these experiments (average weight, 115 g) is 7-10 ml. Generally improperly catheterized trout will show urine flows markedly less than this. Urine output was found to reach its highest level about 2-3 hours after anaesthetization and catheterization and tended to stabilize at a constant level after about 12 hours. Hunn and Willford (48) noted virtually the same results in trout weighing 305 to 660 g.

At the end of the 24-hour preliminary period, the water entering the head section of the chamber was stopped and trout were anaesthetized with MS-222 (three drops of a 10% solution into the head section) and turned upside down to facilitate injection. Trout were injected with 6.86 μmoles/kg radiolabeled ochratoxins A or B dissolved in 0.1 N sodium bicarbonate at a concentration of 2.06 μmoles/ml. The water flow to the head section was turned back on, 150 ml distilled water added to the tail section of the chamber and urine collection begun. Twenty μl urine samples were taken at 1, 2, 3, 5 and 7 hours and 40 μl samples at 12 and 24 hours and counted by liquid scintillation spectrometry to 1% standard error in 10 ml dioxane fluor (Appendix XIV). Cumulative recoveries were calculated based on the dpm of radiolabeled ochratoxin dosed. Three ml aliquots of the water in the tail section of the chamber were taken at 5, 12 and 24 hours and counted in 15 ml dioxane fluor to 3% standard error. This water was changed after 12 hours to reduce bacterial and fungal growth.

The collected urine was removed from the collection cylinder after the time intervals 0-5 hours, 5-12 hours and 12-24 hours, then frozen at -10°C until further use. The collecting cylinder was rinsed with distilled water and the rinsings added to the urine collected during these three time intervals.

It should be pointed out that two separate metabolism chambers were maintained in these studies, one for metabolism studies with

ochratoxin A-$^{14}$C, the other for ochratoxin B-$^{14}$C. In this manner, the possibility of cross-contamination of ochratoxin A and B was eliminated.

All liquid scintillation counting in these and other portions of the metabolism studies yet to be described were done with duplicate samples. Results were calculated from the average of these duplicates.

## Distribution of $^{14}$C in Trout Tissues at 24 Hours

At the end of the 24-hour urine collection period, trout were sacrificed and immediately dissected. The liver, gall bladder, pyloric ceca, intestine, kidney and a blood sample taken by decapitation were weighed into screw-capped bottles and frozen at -10°C until further use. Before freezing, the intestine was cut longitudinally and washed with 0.5 N sodium bicarbonate. The washings were acidified to approximately pH 7.0 and frozen at -10°C also.

The frozen tissue samples were thawed and digested with alkali as indicated in Appendix XVII and 1 ml aliquots of the digests counted in tissue dioxane gel (Appendix XIV). Before counting, the liver and kidney tissues were thawed, cut into small pieces with a razor blade, and a representative portion approximating one-half the total weight taken for digestion and counting. The other half of these tissues was

extracted by methods to be described shortly. Bile samples were thawed and aliquots counted by liquid scintillation spectrometry in 10 ml dioxane fluor (Appendix XIV) to 1% standard error. Recoveries in tissue digests and bile were calculated by dividing the dpm found in the digests and bile by the dpm of the ochratoxin-$^{14}C$ dosed.

Metabolite Pattern in Tissues and Excretions

All handling of tissues and excretions extracted in these studies was done in a 0$^{o}$C room after initial thawing of samples.

The levels of unaltered ochratoxin A or B and their metabolites in chloroform extracts of urine collected during the three time intervals previously mentioned were determined. Urine samples collected from five trout for a given time interval were thawed, divided into two equal volumes, acidified to pH 2.0 with 1 N HCl and successively extracted six times in a separatory funnel with two volumes of chloroform. The chloroform extracts of urine collected during a given time interval were pooled, evaporated to dryness and dissolved in a small volume of chloroform. Aliquots of the chloroform extracts and the aqueous phases after chloroform extraction were counted by liquid scintillation spectrometry in dioxane fluor to 1% and 3% standard error, respectively.

Aliquots of the chloroform extracts as well as ochratoxin

standards were applied to silica gel chromatoplates (Appendix X) and developed in benzene:glacial acetic acid 9:1 v/v to resolve individual metabolites. Developed chromatograms were examined under long-wave ultraviolet light and fluorescent spots not seen in chloroform extracts of untreated trout urine marked by outlining with a needle. The $R_f$ values of these spots were recorded, then the plates scanned with a Varian Model 6000 Radio Scanner. Radioactive fluorescent spots as well as other selected areas of the plates were scraped into vials and counted by liquid scintillation spectrometry in 10 ml dioxane fluor to 3% standard error.

The bile as well as the intestinal wash samples from five trout dosed with either radiolabeled ochratoxin A or B were thawed, pooled and chloroform extracted exactly like the urine samples. The chloroform extracts were evaporated to dryness in vacuo and dissolved in a small volume of chloroform for liquid scintillation counting and determination of metabolite pattern by thin-layer chromatographic analysis as with chloroform extracts of the urine samples. The aqueous phases remaining after chloroform extraction were also counted. Samples were counted in dioxane fluor to the standard errors shown in Table 4.

The other halves of the liver and kidney tissues from five trout dosed with radiolabeled ochratoxin A or B which were left after removal of a representative portion for digestion and counting, were

Table 4. Standard errors to which bile and tissue samples were counted by liquid scintillation spectrometry.

| Sample | Portion | Standard error (%) |
|---|---|---|
| Bile | chloroform soluble | 1 |
| | chloroform insoluble | 1 |
| | chloroform TLC[a] | 3 |
| Intestinal contents | chloroform soluble | 3 |
| | chloroform insoluble | 5 |
| | chloroform TLC[a] | 5 |
| Liver and kidney | chloroform soluble | 3 |
| | chloroform insoluble | 5 |
| | chloroform TLC[a] | 5 |

[a]Selected areas of thin-layer plates on which chloroform solubles were chromatographed.

pooled and homogenized in ten volumes of cold (0 °C) saturated aqueous sodium bicarbonate in a Virtis tissue homogenizer for one minute at full speed. The mixture was centrifuged in a clinical centrifuge at top speed for 15 minutes, the supernatant drawn off and the pellet then extracted in this manner two more times. The bicarbonate extracts were combined in a separatory funnel, acidified to pH 2.0, then successively extracted three times with three volumes of chloroform. The chloroform extracts were evaporated to dryness in vacuo and dissolved in a small volume of chloroform for liquid scintillation counting and thin-layer chromatographic analysis. Aqueous phases remaining after extraction were also counted. The metabolite

pattern in the chloroform extracts were determined as before with other chloroform extracts. All samples were counted in dioxane fluor to the standard errors shown in Table 4.

All of the thin-layer chromatographic analyses were expressed as the percentage of $^{14}C$ in the chloroform extracts accounted for by the $^{14}C$ levels found at various portions of the plate which were scraped and counted.

Flow diagrams of the methods used in extraction and counting excretions and tissue samples are shown in Figures 13 and 14.

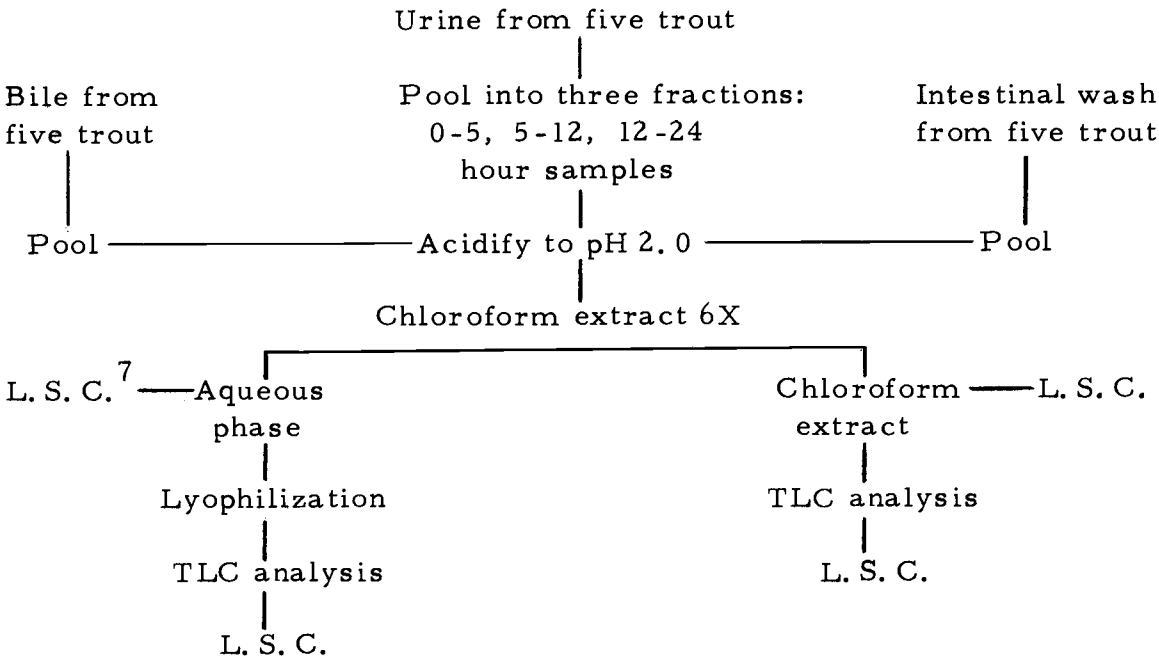



Figure 13. Flow diagram of procedure used in extraction of trout urine, bile and intestinal wash samples.

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[7] Liquid scintillation counting.

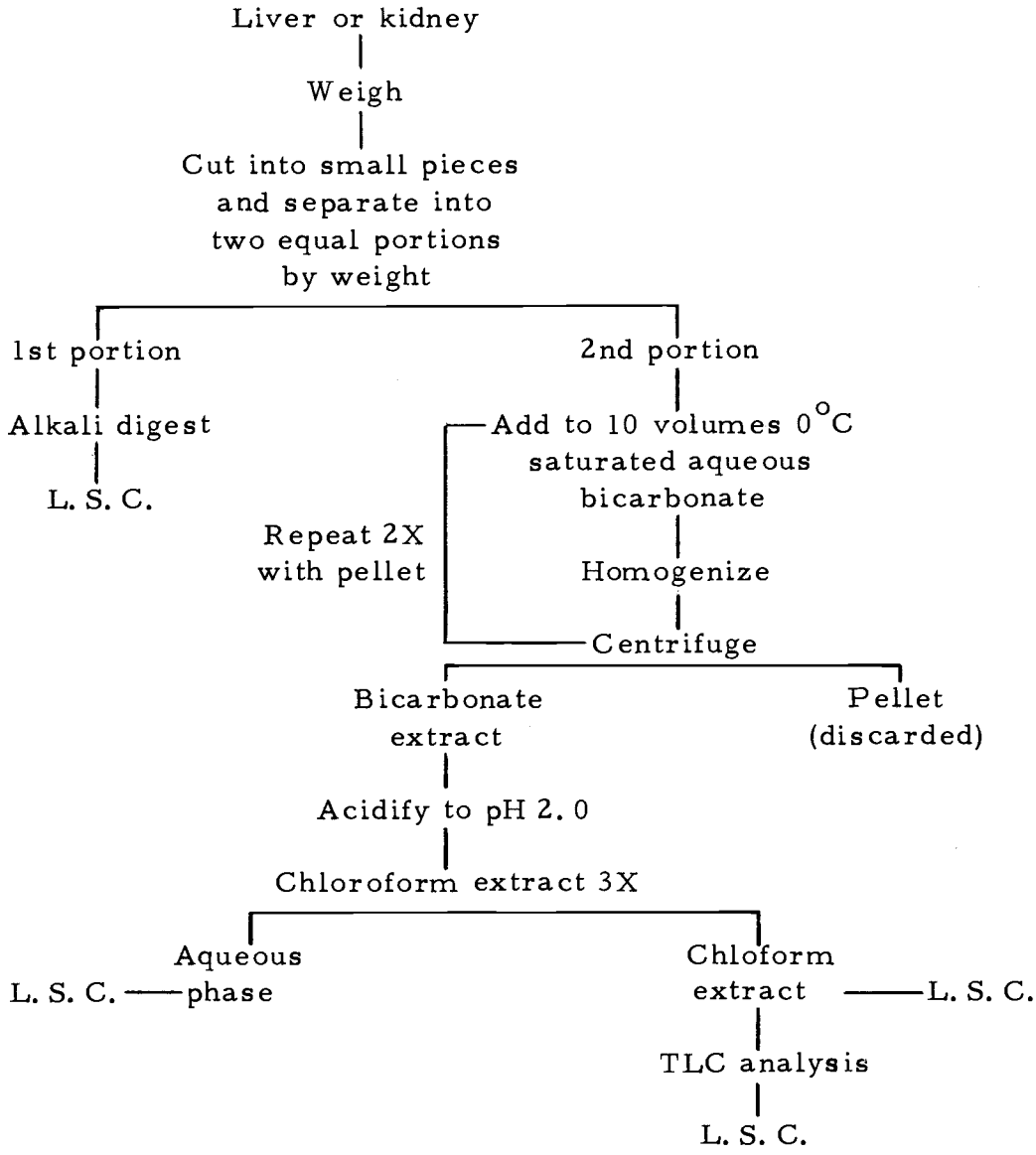



Figure 14. Flow diagram of procedures used in extraction of trout livers and kidneys.

## Analysis of Chloroform-insoluble $^{14}C$

The aqueous phases of the urine, bile and intestinal wash from five trout dosed with radiolabeled ochratoxin A and B were found to contain significant levels of radioactivity after chloroform extraction. It was felt that this radioactivity may either be due to 1) incomplete chloroform extraction of these phases or 2) the presence of water-soluble ochratoxin metabolites and/or possible oxidative degradation artifacts. In order to investigate the first possibility, the aqueous phases were frozen and lyophilized to a small volume. Portions of these concentrates were spotted on silica gel chromatoplates (Appendix X) and developed in benzene:glacial acetic acid 9:1 v/v. The developed plates were examined under long-wave ultraviolet light, then fluorescent as well as other areas of the plates were scraped into vials and counted in dioxane fluor.

## Evaluation of Extraction Procedure

Chloroform extracts of various excretions and bicarbonate tissue extracts from trout dosed with radiolabeled ochratoxins A and B were found to contain suspected ochratoxin metabolites. The possibility that these could have been formed artifactually was considered. To test this possibility, 0.04 μc radiolabeled ochratoxin A and B were separately mixed with 8 ml freshly collected trout urine

and these mixtures frozen and stored at -10 °C for three weeks. The mixtures were then thawed and processed in accordance with the isolation procedures previously described. The $^{14}C$ in the chloroform extracts and aqueous phases remaining after extraction was counted in dioxane fluor. The chloroform extracts were also subjected to thin-layer chromatographic analysis followed by radiochromatogram scanning. After scanning, various areas of the plate were scraped into vials and counted in dioxane fluor.

# RESULTS AND DISCUSSION

## Ochratoxin Toxicity Experiments

### Preparation of Ochratoxins and Derivatives

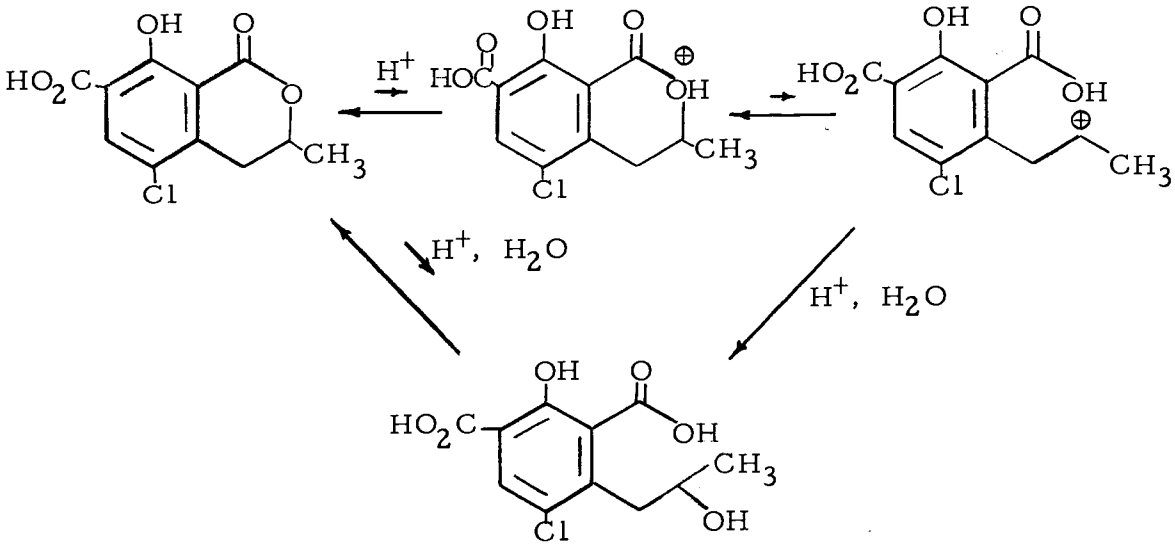
No problems were encountered in the preparation of natural ochratoxins A and B and the ethyl esters of A, B and a as these compounds have been prepared before in other studies (30, 32).

The leucine and alanine analogues of ochratoxin A were prepared and purified with yields of 38 and 3%, respectively, by methods described in Appendix II. The mass spectra of the ethyl esters of these analogues were consistent with their structures (Appendix III). There remained the question of possible racemization of the final products as a result of findings reported in previous ochratoxin synthesis studies (79, 96). Since too small amounts of the analogues were prepared for measurement of optical activity, a sufficient quantity of ochratoxin A was prepared from ochratoxin a and L-phenylalanine by the same procedures described for the preparation of the ochratoxin A analogues. The specific rotation of this product was then measured. As stated in Appendix II, the racemization of the prepared ochratoxin A was about 11%.

When the acid chloride method is used in peptide synthesis, racemization can occur at the amino-carbon alpha to the acyl chloride

group (52). In the coupling of an amino ester with ochratoxin a, the acyl carbon formed on the ochratoxin a molecule would have no alpha carbon, and would, therefore, be safe from racemization during the coupling.

Other than the attached amino acid, ochratoxin A has only one asymmetric carbon giving rise to optical activity, the number three carbon in the lactone ring. The functional group at this position is a secondary alcohol bonded to the carboxyl group through an ester (lactone) linkage. In aqueous acid solution, the equilibrium between a six-membered lactone and its hydroxylated ring-opened form lies far in the direction of the lactone. Albeit, racemization could still occur in solutions of strong acid by two possible routes:



The isocoumarin moiety of ochratoxins is exposed to acid twice during the interchange of amino acids. First when the original amino acid is hydrolyzed off, and again during the formation of the acyl chloride. Van der Merwe et al. (106) report the resulting isocoumarin, ochratoxin a, has very high activity following acid hydrolysis of ochratoxin A. This suggests that under the conditions used for hydrolysis, racemization is slow. Therefore, from experimental results, as well as theoretical considerations discussed above, it seems logical to assume that racemization of the alanine and leucine analogues would also be minimal.

General Observations on Toxicity Studies

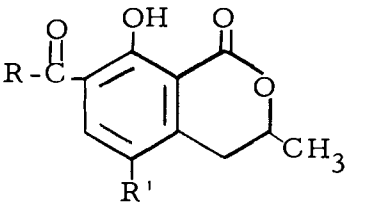
Of the seven ochratoxins dosed (Figure 15) only ochratoxins A, A ethyl ester (C) and B ethyl ester were found to be toxic. Single IP doses of ochratoxin A at 4, 6 and 8 mg/kg body weight produced mortalities of 1/10, 7/10 and 9/10, respectively. Doses of ochratoxin A ethyl ester in corn oil at 2, 3 and 4 mg/kg body weight produced mortalities of 1/10, 5/10 and 9/10, respectively, and doses of ochratoxin B ethyl ester in corn oil at 10, 12.5 and 15 mg/kg produced mortalities of 1/10, 4/10 and 8/10, respectively.

Autopsy of ochratoxin A dosed trout showed the same symptoms as previously described (30, 32), while trout dosed with ochratoxin A ethyl ester showed symptoms virtually identical to these. Trout dosed

with ochratoxin B ethyl ester showed pale kidneys and livers like those seen in trout dosed with low levels of ochratoxin A. However, liver damage appeared less severe as no orange livers were seen which are always prevalent in trout dosed with toxic levels of ochratoxin A and its ethyl ester.

The $LD_{50}$ of ochratoxin A was found to be 5.53 mg/kg (13.72 μmoles/kg) body weight with 95% confidence limits of 4.45-6.86 mg/kg body weight. The slope function was 1.28 with 95% confidence limits of 0.94-1.73 mg/kg body weight. This is comparable to the ochratoxin A $LD_{50}$ previously found for rainbow trout (30, 32). The $LD_{50}$ of ochratoxin A ethyl ester was 3.0 mg/kg (6.96 μmoles/kg) body weight with 95% confidence limits of 2.37-3.80 mg/kg body weight. The slope function was 1.31 with 95% confidence limits of 0.97-1.77 mg/kg body weight. The $LD_{50}$ of ochratoxin B ethyl ester was 13.0 mg/kg (32.75 μmoles/kg) with 95% confidence limits of 10.30-16.41 mg/kg body weight. The slope function was 1.21 with 95% confidence limits of 0.89-1.63 mg/kg body weight.

Ochratoxin B, previously shown to be non-lethal to trout at levels up to 66.7 mg/kg (30, 32), was again non-lethal in this study. The alanine and leucine analogues of ochratoxin A produced no mortalities in ten trout dosed at levels of 4.49 and 5.06 mg/kg body weight, respectively. Similarly ochratoxin a ethyl ester dosed at a level of 3.9 mg/kg body weight produced no mortalities in ten trout.



Ochratoxin A: R=$C_6H_5$-$CH_2$-CH(COOH)-NH-, R'=Cl

Ochratoxin C: R=$C_6H_5$-$CH_2$-CH(COOEt)-NH-, R'=Cl

Ochratoxin B: R=$C_6H_5$-$CH_2$-CH(COOH)-NH-, R'=H

Ochratoxin B ethyl ester: R=$C_6H_5$-$CH_2$-CH(COOEt)-NH-, R'=H

Ochratoxin a ethyl ester: R=EtO-, R'=Cl

Alanine analogue: R=$CH_3$-CH(COOH)-NH-, R'=Cl

Leucine analogue: R=$(CH_3)_2$-CH(COOH)-$CH_2$-CH-NH-, R'=Cl

Figure 15. Structures of ochratoxins dosed in toxicity trials.

The dose levels of the latter three compounds are equivalent to the ochratoxin A $LD_{50}$ level of 13.72 μmoles/kg when expressed on a molar basis. No macroscopic abnormalities were seen in any of these trout or trout dosed with 0.1 N sodium bicarbonate or corn oil.

Histological Changes

Livers and kidneys from trout dosed with ochratoxin A or its

ethyl ester showed necrotic changes identical to those previously described in trout dosed with ochratoxin A (30, 32). Figures 16 through 19 show some of these changes.

Livers from trout dosed with ochratoxin B ethyl ester showed definite injury but this damage was not as severe as that seen in trout dosed with toxic levels of ochratoxin A or its ethyl ester. In general, these livers had a normal architecture, but in many cases showed areas of swollen or hypertrophic parenchyma cells. Some livers contained large nuclei scattered around the periphery of the muralia (Figure 20). Livers were also seen to contain an occasional focus of bile duct proliferation (Figure 21). These proliferations appeared diffuse and poorly differentiated but definitive in many cases.

Livers from trout dosed with 13.72 μmoles/kg of the leucine analogue of ochratoxin A or 66.7 mg/kg ochratoxin B definitely showed minor signs of injury. This was determined by the presence of occasional foci of bile duct proliferation (Figure 22) as well as slightly swollen nuclei. The injury seen in livers from trout dosed with the high level of ochratoxin B is consistent with results of previous studies (30, 32).

Livers from trout dosed with 13.72 μmoles/kg of the alanine analogue of ochratoxin A, 13.72 μmoles/kg ochratoxin a, 0.1 N sodium bicarbonate or corn oil showed no histological abnormalities.

Kidneys from trout dosed with ochratoxin B ethyl ester were

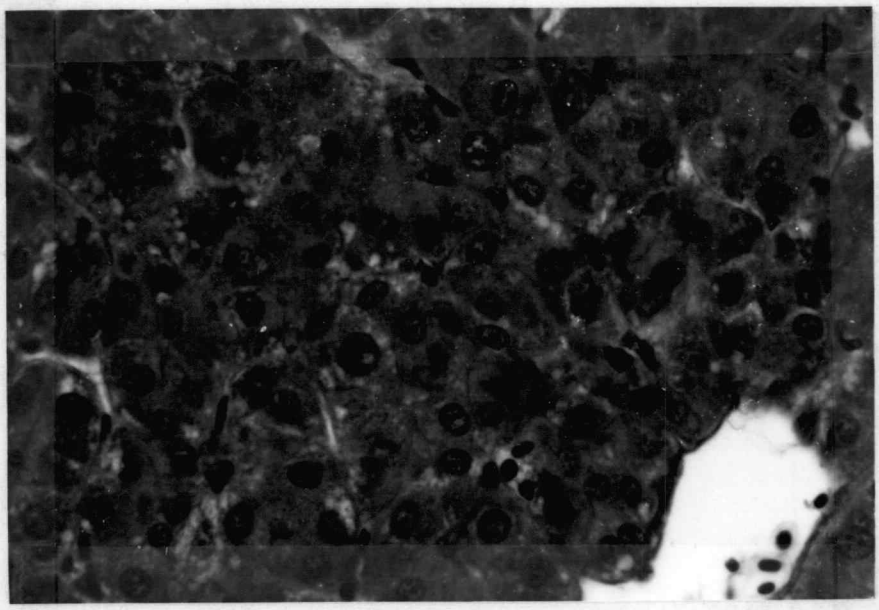


Figure 16. Liver from trout dosed with 2.0 mg/kg ochratoxin A ethyl ester. Note several abnormally large nuclei, a characteristic feature seen in livers of trout dosed with this toxin. Hematoxylin and eosin. X320.

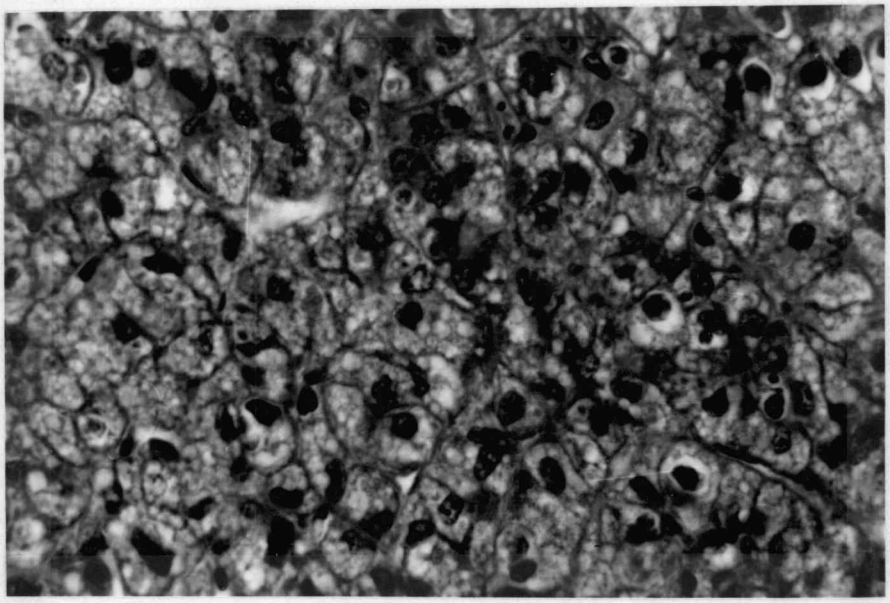


Figure 17. Liver from trout dosed with 4.0 mg/kg ochratoxin A showing advanced stages of necrosis characterized by the raisin-like pycnotic nuclei and lipid vacuolation. Hematoxylin and eosin. X320.

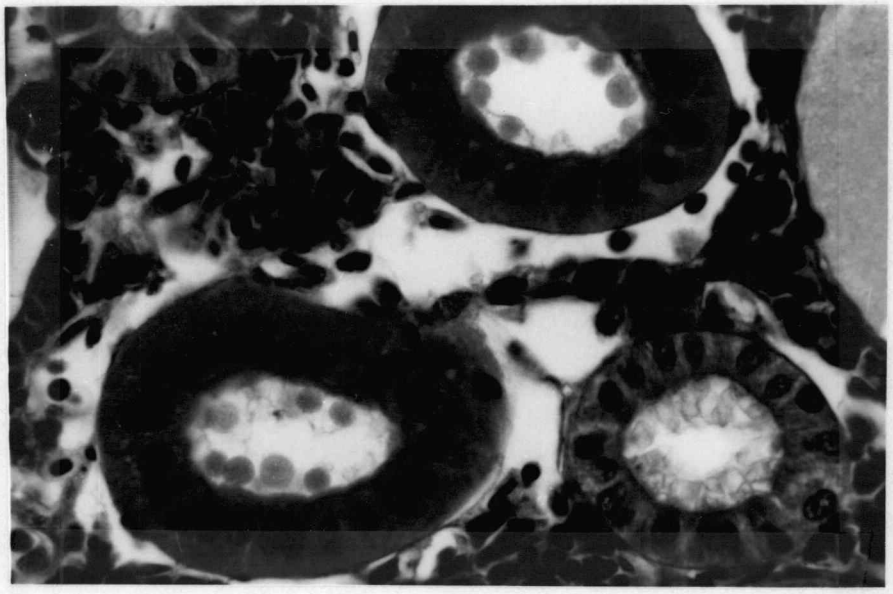


Figure 18. Necrotic proximal tubules from trout dosed with 4.0 mg/kg ochratoxin A. Note presence of membrane limited casts in lumen of tubules. Hematoxylin and eosin. X320.

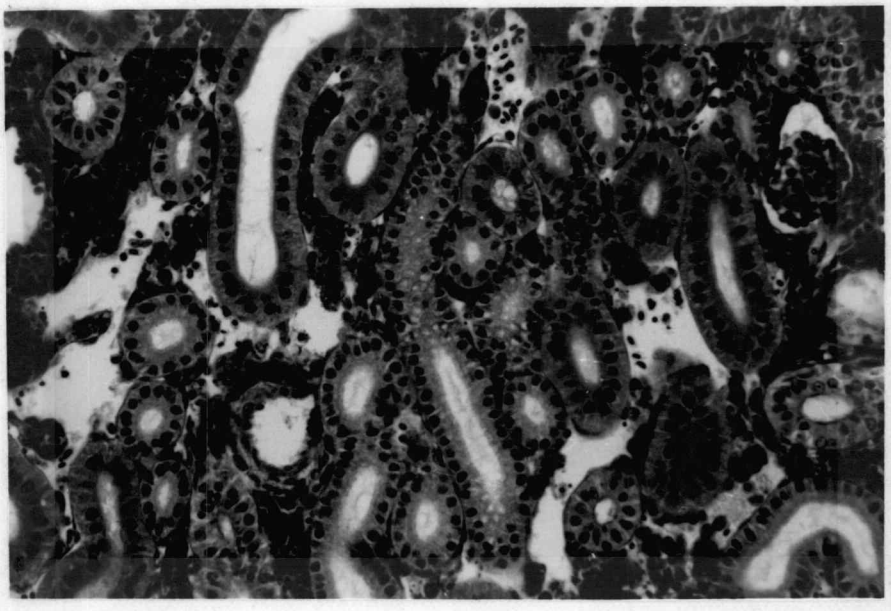


Figure 19. Pycnotic nuclei in proximal tubules of kidney from trout dosed with 2.0 mg/kg ochratoxin A ethyl ester. Hematoxylin and eosin. X128.

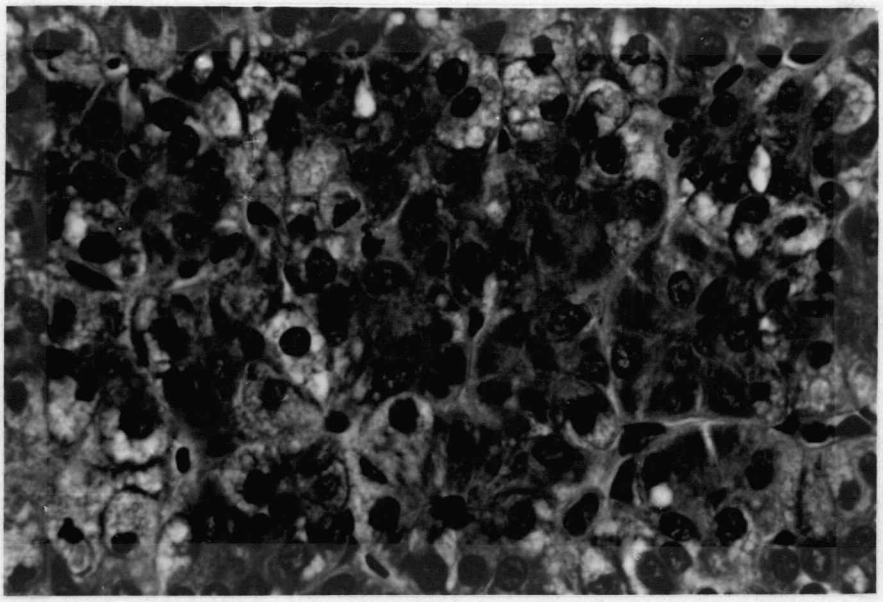


Figure 20. Liver from trout dosed with 10 mg/kg ochratoxin B ethyl ester showing several large swollen nuclei. Hematoxylin and eosin. X320.

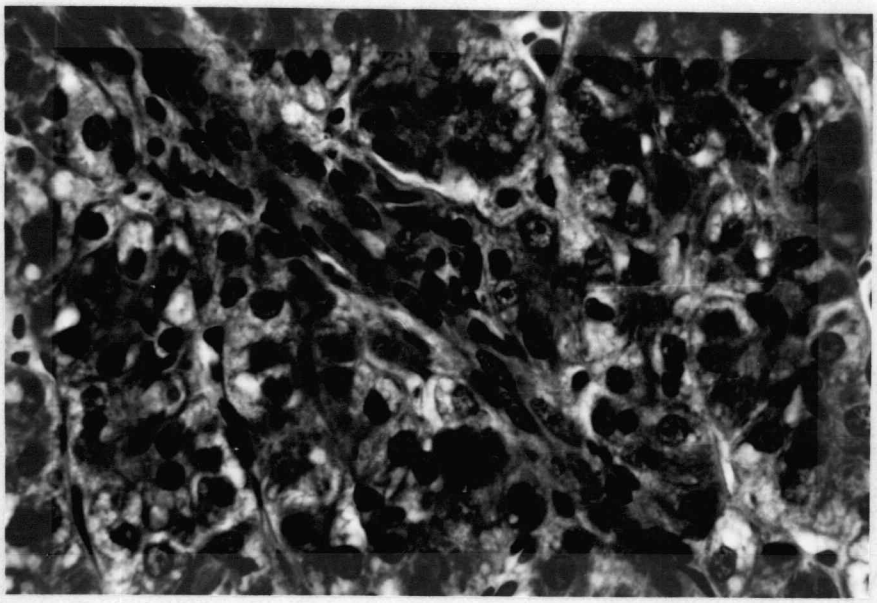


Figure 21. Bile duct proliferation in liver of trout dosed with 10 mg/kg ochratoxin B ethyl ester. Hematoxylin and eosin. X320.

found to contain necrotic hematopoietic tissue and swollen or pycnotic tubule nuclei. Also seen was a general loss of tubular patency and casts in renal tubules (Figure 23). The latter were seen especially in kidneys from trout dosed with the higher levels of ochratoxin B ethyl ester.

Kidneys from trout dosed with 13.72 μmoles/kg of the leucine analogue of ochratoxin A and 66.7 mg/kg ochratoxin B showed damage similar to that caused by non-lethal levels of ochratoxin A. The most noticeable abnormality was a general necrosis of the hematopoietic tissue of these kidneys (Figure 24). In fact, if damage was seen in kidneys of trout dosed with any ochratoxin, it usually was visible first in the hematopoietic tissue. Other abnormalities in kidneys from trout dosed with the leucine analogue of ochratoxin A and ochratoxin B included swollen tubule nuclei, loss of tubular patency and cast formation.

Necrotic hematopoietic tissue as well as some swollen tubule nuclei were also seen in kidneys of trout dosed with 13.72 μmoles/kg of the alanine analogue of ochratoxin A. These abnormalities were not nearly as noticeable as those seen in kidneys from trout dosed with the leucine analogue of ochratoxin A or the high level of ochratoxin B. Also, no casts were evident in the lumen of tubules, indicating damage was much less severe in these kidneys.

Kidneys from trout dosed with 13.72 μmoles/kg ochratoxin a

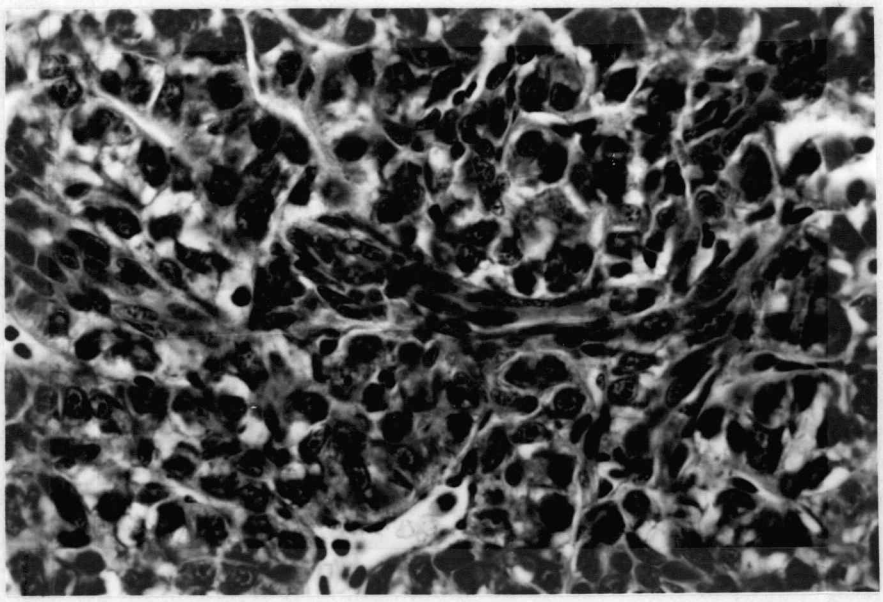


Figure 22. Bile duct proliferation in liver of trout dosed with 13. 72 μmoles/kg of the leucine analogue of ochratoxin A. Hematoxylin and eosin. X320.

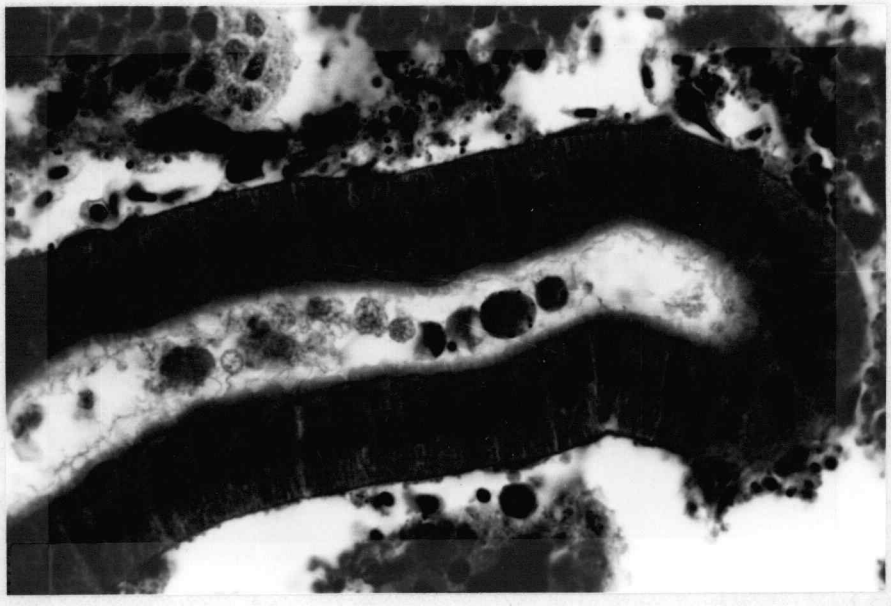


Figure 23. Casts in proximal segment of renal nephron from trout dosed with 15 mg/kg ochratoxin B ethyl ester. Hematoxylin and eosin. X320.

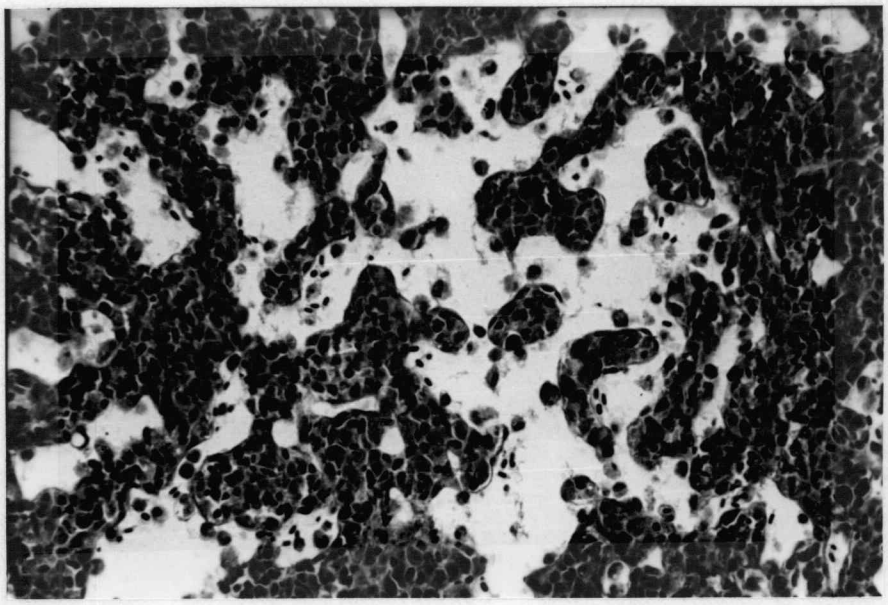


Figure 24. Necrotic hematopoietic tissue in kidney from trout dosed with 13.72 μmoles/kg of the leucine analogue of ochratoxin A. Hematoxylin and eosin. X128.

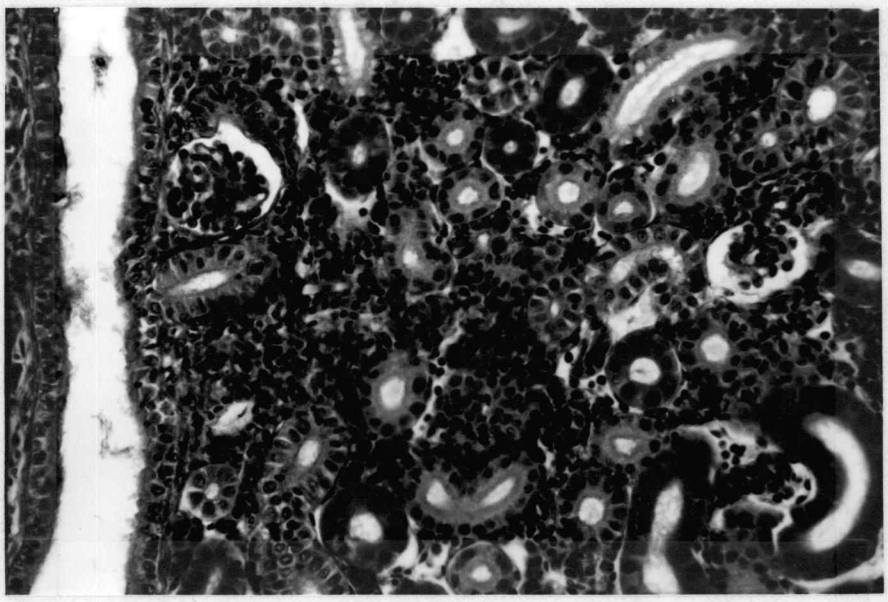


Figure 25. Section of kidney from trout dosed with 13.72 μmoles/kg ochratoxin a ethyl ester showing completely normal histological features. Hematoxylin and eosin. X128.

ethyl ester (Figure 25) as well as those from control trout showed no histological abnormalities.

In summarizing results of the toxicity studies, the ochratoxin compounds tested can be ranked in order of decreasing toxicity in the following manner: ochratoxin C > ochratoxin A > ochratoxin B ethyl ester > leucine analogue of ochratoxin A $\geq$ ochratoxin B > alanine analogue of ochratoxin A > ochratoxin a ethyl ester (non-toxic).

From these results it appears as though changes in the basic structure of the non-chlorinated dihydroisocoumarin moiety of ochratoxin which increases its molecular weight to near 400 but still maintains the anionic nature of the molecule, increases its toxicity. It is proposed that these changes may alter the distribution of the molecule in trout to bring about a greater exposure to target sites in various tissues where the lethal action is apparent. This hypothesis is based upon the thesis that changes which alter the distribution of a molecule to increase its interaction with a cellular component (often called a receptor) leads to an expression of toxicological activity. This assumes that the toxic agent merely initiates the chain of events for expression of toxicological activity, but before it can do this, it obviously must reach the receptor to initiate the response.

The influence of molecular weight is borne out by the observation that the relatively non-toxic ochratoxin B molecule (mol. wt. 369) is made much more toxic when esterified to produce ochratoxin B ethyl

ester (mol. wt. 397). Also, the toxicity of ochratoxin A (mol. wt. 403) is about one-half that of its ethyl ester (mol. wt. 431). It is realized that the different carriers used for administration of these compounds may have had some influence on this toxicity. This was necessary as the esters are not soluble in bicarbonate and the free acids have poor solubility in corn oil. It would be expected that the esters dosed in corn oil would normally be absorbed more rapidly than the free acids in aqueous bicarbonate (82) and this may influence their comparative toxicities to some extent.

It is noteworthy that Chu and Chang (19) did not find ochratoxin A ethyl and methyl esters to be more toxic than ochratoxin A to day-old chicks. Also, Steyn and Holzapfel (95) found ochratoxin B esters to be non-lethal to day-old ducklings. In the latter study, no details of the experiment such as dose levels, toxin carrier, route of administration, etc., were given, thus, making comparisons with this study impossible. In the former study, however, chicks were dosed orally with bicarbonate solutions of ochratoxin A and corn oil solutions of ochratoxin A esters. It seems possible that since all of the toxins were dosed orally, the ochratoxin esters were hydrolyzed to free acids in the lower gut of the chicks before absorption took place. In the present study the IP dosed ochratoxin esters would not likely be subjected to conditions which would bring about significant ester hydrolysis until after absorption and excretion into the bile.

Kitamikado and Tachino (50) reported a strong esterase activity in the liver, spleen and bile of rainbow trout. It was also detected but much weaker, in the intestine, pyloric ceca and stomach.

Possibly of greater value in exemplifying the effect of molecular weight on possible distribution differences and consequent toxicity are results obtained for the alanine (mol. wt. 327) and leucine (mol. wt. 369) analogues of ochratoxin A. Both these compounds caused no mortalities in trout when dosed at the molar ochratoxin A $LD_{50}$ level. The leucine analogue did, however, induce some histological abnormalities in the liver and kidney of trout.

An explanation of possible reasons for the difference in toxicity of ochratoxins A and B necessitates consideration of the influence of ionic property differences as well as molecular size and structure differences. As previously mentioned in the Literature Review, Chu et al. (20) found that the apparent dissociation constant of the phenolic hydroxyl group in ochratoxins B and A ethyl ester were 7.95 and 7.14, respectively. Therefore, at a physiological pH of 7.2-7.4, proportionally more ochratoxin A is present in ionized form than ochratoxin B. These authors reasoned that this difference in ionic properties may cause ochratoxin A to bind more readily to cellular components than ochratoxin B. Further support for this hypothesis was presented in the finding that O-methylochratoxin A ethyl ester is non-toxic to day-old chicks (20). It is conceivable that this difference

in ionic properties of ochratoxin A and B could also play a role in altering the distribution of the molecules to hasten or hinder their transport to receptor sites within cells of tissues where the lethal action is apparent. This hypothesis, attempting to relate ochratoxin toxicity to potential differences in biological distribution, does not take into account possible differences in ochratoxin transformation to metabolites which are more readily excreted than the parent toxins. As mentioned previously, Doster and Sinnhuber (31) noted ochratoxin B is more readily hydrolyzed in vitro to its non-toxic isocoumarin moiety than ochratoxin A. These differences may also alter the biological distribution of the ochratoxins and affect their ultimate toxicity.

It is important to note that the two factors which appear to influence ochratoxin toxicity, namely, molecular size and polarity, also play an important role in affecting biliary excretion of organic anions (60, 90). The possible significance of increased biliary excretion of ochratoxins in relation to their toxicity will become apparent after results of the ochratoxin A and B comparative metabolism studies are presented.

## Ochratoxin Metabolism Studies

### Preparation of Radiolabeled Ochratoxins A and B

Chloroform extracts from the liquid cultures used in the

biological preparation of ochratoxin A were found to contain mainly ochratoxin A and little or no ochratoxin B or other fluorescent metabolites. About 6% of the added $^{14}C$ activity was found in the chloroform extract of the culture contents. Ochratoxin A accounted for only about 0.4% of the activity in the chloroform extract. The average yield of ochratoxin A/10 ml culture was just slightly less than 1 mg. The specific activity of the radiolabeled ochratoxin A purified as described in Appendices IX to XII was 514 dpm/μg or 93.3 μc/mmole. Further recrystallizations did not result in a lowering of this specific activity. The overall incorporation of $^{14}C$ activity into ochratoxin A was about 0.023%. This incorporation percentage is considerably less than the values reported by other authors (88, 97). This is possibly because the radiolabeled ochratoxin A produced in this study was subjected to more rigorous purification procedures. The ochratoxin A produced in these other studies was purified by preparative thin-layer chromatography only, a procedure which, in the author's hands, does not yield ochratoxin A which has radiopurity. This point should be apparent in view of the fact that only 0.4% of the $^{14}C$ in the chloroform culture extract was accounted for by ochratoxin A-$^{14}C$.

It should be pointed out that the conditions used in this study for preparation of radiolabeled ochratoxin A may be suboptimal. A study conducted to determine the optimum conditions for incorporation of

$^{14}C$ into ochratoxin A would involve examining a wide range of variables which affect toxin production and incorporation of acetate into ochratoxin A such as incubation time, level of energy source, effect of various nitrogen sources, time after inoculation at which acetate-$^{14}C$ is added, etc. Some of these parameters were studied briefly in arriving at the conditions used in this study but not to the extent they would have been if the object of this study had been to optimize $^{14}C$ incorporation into ochratoxin A. The significant point is that ochratoxin A having a sufficiently high specific activity for conduction of the desired metabolism studies was obtained using the conditions described.

The radiolabeled ochratoxin A was found to be at least 98% pure by the procedures described. Less than 2% of the $^{14}C$ activity applied to the thin-layer plates was found in the remainder of the plate scrapings counted separately from the ochratoxin A scrapings. The majority of this activity probably results from a fluorescent trace which can be seen to trail behind the ochratoxin A spot on the chromatoplate especially when large quantities are spotted on the plate.

The dehalogenation reaction carried out under the conditions described in Appendix XV resulted in virtually complete conversion of ochratoxin A to ochratoxin B in about 3 hours. The specific activity of the purified ochratoxin B-$^{14}C$ was found to be 573 dpm/μg or 95.2 μc/mmole. This is within 2% of the specific activity of the

ochratoxin A from which the ochratoxin B was prepared. The purified ochratoxin B-$^{14}$C was found to be at least 99% pure. An average of less than 1% of the activity applied to the thin-layer plates was found in the remainder of the plate scrapings counted separately from the ochratoxin B scrapings.

## Excretion and Tissue Distribution of $^{14}$C from Ochratoxins A-$^{14}$C and B-$^{14}$C

Urinary Excretion. The cumulative excretion of $^{14}$C from five trout dosed with ochratoxin A-$^{14}$C or B-$^{14}$C is shown in Figure 26. It can be seen from this graph that the average 24-hour cumulative excretion of $^{14}$C from ochratoxin B-$^{14}$C is 44.2%, while the excretion of $^{14}$C from ochratoxin A-$^{14}$C is 35.8%, a difference of 19%. In both cases, it is apparent that urinary excretion of $^{14}$C is quite rapid, and little additional $^{14}$C from these toxins is excreted after about 7 hours after dosage.

Biliary Excretion and Tissue Distribution. Table 5 shows the averages and ranges for the 24-hour biliary excretion and tissue distribution of $^{14}$C from five trout dosed with ochratoxin A-$^{14}$C or B-$^{14}$C. Mean values were compared statistically according to the Student 't' test (59). Only differences between the urinary $^{14}$C excretion and blood content of $^{14}$C in trout dosed with radiolabeled ochratoxins A and B were statistically significant. Other differences

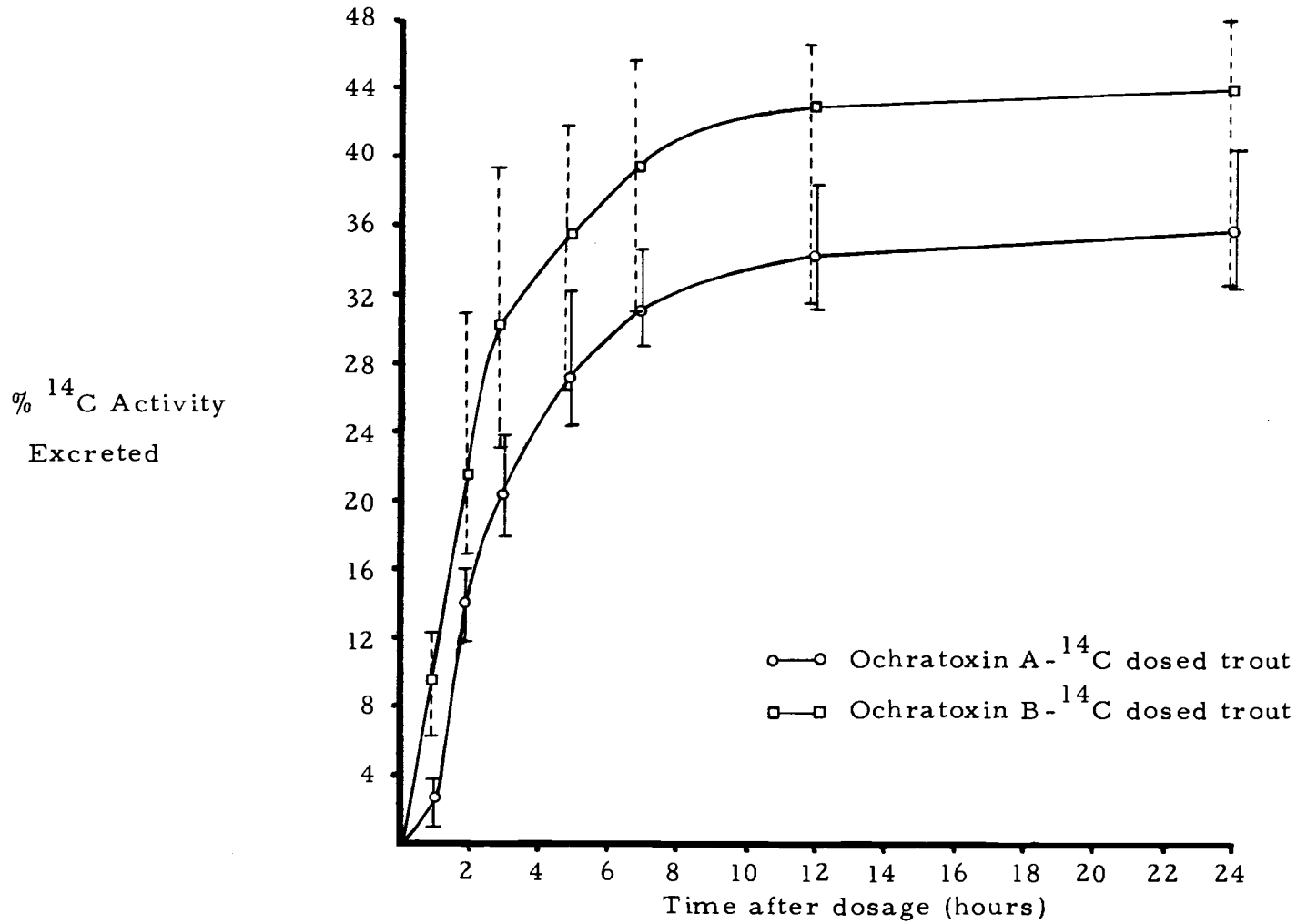



Figure 26. Recovery of $^{14}$C in urine from trout dosed with ochratoxins A-$^{14}$C and B-$^{14}$C.

Table 5. Twenty-four hour excretion and tissue distribution of $^{14}C$ from five trout dosed with radiolabeled ochratoxin A or B.

| Excretion or tissue | $^{14}C$ recovered, % of dose[a] | | Difference between mean values (%) |
|---|---|---|---|
| | Ochratoxin A-$^{14}C$ dosed trout | Ochratoxin B-$^{14}C$ dosed trout | |
| Urine | 35.8 (33.4-40.4) | 44.2 (33.6-48.3)[c] | 19.0 |
| Bile | 57.1 (49.9-64.4) | 48.2 (38.3-64.5) | 15.6 |
| Blood[b] | 0.51 (0.43-0.57) | 0.41 (0.35-0.45)[d] | 19.6 |
| Intestinal contents | 0.55 (0.11-1.34) | 0.96 (0.09-2.27) | 42.7 |
| Intestine | 1.79 (0.93-4.43) | 2.87 (0.34-5.73) | 37.6 |
| Pyloric ceca | 6.1 (1.7-9.6) | 6.4 (1.6-14.8) | 4.7 |
| Liver | 1.54 (1.10-2.03) | 1.74 (0.56-3.03) | 11.5 |
| Kidney | 0.49 (0.15-1.68) | 0.28 (0.10-0.52) | 42.9 |

[a]Mean and range.

[b]Based on blood = 5% body weight (92).

[c]Difference between means significant at the alpha = 0.025 level.

[d]Difference between means significant at the alpha = 0.05 level.

were noted but because the variation between individual trout was so great, these differences were not statistically significant.

It became apparent upon examining the combined 24-hour excretion of $^{14}C$ in urine and bile from individual trout dosed with radiolabeled ochratoxin A or B that a lower level of urinary excretion was paralleled by an increase in biliary excretion in all cases. Parke (69) points out that biliary excretion is dependent on molecular size and increases as the molecular weight of a compound increases. Using excretion of substituted fluorescein dyes as an example, he showed that an increase in biliary excretion which occurs with an increase in molecular weight is paralleled by a decrease in excretion in the urine. This is precisely the situation noted in the ochratoxin excretion studies.

The levels of $^{14}C$ were quite low 24 hours after dosage of radiolabeled ochratoxins A and B in all tissues except the pyloric ceca. This is likely due to the fact that bile in trout is excreted first into the pyloric ceca; therefore, because of the high levels of $^{14}C$ in the bile, the pyloric ceca would naturally be expected to contain substantial levels of $^{14}C$ also.

Low levels of $^{14}C$ were found in the water in the tail section of the metabolism chamber, indicating fecal excretion of $^{14}C$ from radiolabeled ochratoxin A and B is negligible in the first 24 hours after dosage. The average cumulative levels of $^{14}C$ recovered in the tail sections of the metabolism chambers after 24 hours were 0.58% and

1.8% of the dose of ochratoxin A-$^{14}$C and B-$^{14}$C injected, respectively. Occasionally, a trout was found to excrete a lower than normal amount of urine and consequently, showed lower than normal cumulative $^{14}$C excretion in the urine after injection with radiolabeled ochratoxins. Counting the water in the tail section of the metabolism chamber revealed considerable levels of $^{14}$C. This was interpreted as being due to leakage of urine around the urinary catheter; therefore, data from these trout were not used. The low fecal excretion of $^{14}$C is also indicated by the low levels of $^{14}$C found in the intestine of trout dosed with both ochratoxins (Table 5).

The higher blood levels of $^{14}$C in trout dosed with ochratoxin A-$^{14}$C could simply reflect the greater percentage of $^{14}$C still present in these trout after 24 hours. It may also reflect a greater plasma binding of ochratoxin A. As previously mentioned, Chu (17) found ochratoxin A to bind to bovine serum albumin. He also noted that ochratoxin B binds to this protein but to a lesser extent (personal communication). This may have important toxicological significance, but further studies on this possible interaction are suggested before any relevance can be assigned to it.

The slightly higher levels of $^{14}$C in the pyloric ceca, intestinal contents and intestinal digest from trout dosed with ochratoxin B-$^{14}$C possibly reflect a more rapid excretion of this toxin in the bile. In view of the large amount of variation in results obtained in individual

trout, conclusions from these data must be made with caution. The apparent more rapid excretion of $^{14}C$ from ochratoxin B may possibly be due to a faster rate of metabolism of ochratoxin B by enzyme systems within the liver as will be seen when results of the ochratoxin metabolite pattern in the bile are presented in the next section.

The slightly higher average $^{14}C$ level in livers from trout dosed with ochratoxin B-$^{14}C$ is due largely to the observation that one liver from an ochratoxin B-$^{14}C$ dosed trout was found which contained 3.03% of the $^{14}C$ dose, thus increasing the average $^{14}C$ level to the higher value. Most $^{14}C$ levels were much lower than this; therefore, no special significance can be assigned from these results to the difference in $^{14}C$ levels in livers from trout dosed with the two toxins.

The higher $^{14}C$ levels in kidneys from trout dosed with ochratoxin A may simply reflect the fact that proportionally more $^{14}C$ is probably still being excreted in the urine of trout dosed with ochratoxin A-$^{14}C$ at 24 hours than in urine of trout dosed with ochratoxin B-$^{14}C$. Between 12 and 24 hours, trout dosed with ochratoxin B-$^{14}C$ excreted an average of 1.2% of the $^{14}C$ dosed. During this same time interval, trout dosed with ochratoxin A-$^{14}C$ excreted an average of 1.6% of the $^{14}C$ dosed. This alone may account for at least 50% of the difference between mean $^{14}C$ levels in kidneys of trout dosed with ochratoxins A-$^{14}C$ and B-$^{14}C$. Also important in this regard are the higher levels of $^{14}C$ in the blood of trout dosed with ochratoxin A-$^{14}C$.

## Ochratoxin Metabolite Pattern in Chloroform Extracts of Excretions and Tissues

Urinary Metabolites. As previously described, the urine was separated into collections obtained during three time intervals after dosage, namely, 0-5 hours, 5-12 hours and 12-24 hours. The metabolite pattern in chloroform extracts of each of the urine samples collected during these time intervals is shown in Table 6. There appeared to be a shift away from chloroform-soluble toward chloroform-insoluble compounds. This was more noticeable with urine extracts from ochratoxin A-$^{14}$C dosed trout.

Table 6. Distribution of $^{14}$C excretion in the urine of ochratoxin A-$^{14}$C and B-$^{14}$C dosed trout.

| Ochratoxin dosed | Time interval (hrs) | % Chloroform soluble | % Chloroform insoluble |
|---|---|---|---|
| A-$^{14}$C | 0-5 | 95.0 | 5.0 |
| | 5-12 | 88.3 | 11.7 |
| | 12-24 | 77.0 | 23.0 |
| B-$^{14}$C | 0-5 | 98.4 | 1.6 |
| | 5-12 | 93.4 | 6.6 |
| | 12-24 | 95.4 | 4.6 |

The TLC pattern of the chloroform extracts shown in Table 7 indicated the presence of two radioactive metabolites of ochratoxin A. One of these has the same fluorescent properties and $R_f$ value as ochratoxin a ($R_f$=0.34), while the other, a green fluorescent compound

Table 7. Chromatographic distribution of $^{14}C$ in chloroform extracts of urine from trout dosed with radiolabeled ochratoxin A or B.

| Ochratoxin dosed | Spot on thin-layer chromatoplate | | | Extract I (%) | Extract II (%) | Extract III (%) |
|---|---|---|---|---|---|---|
| | | Color of fluorescence | $R_f$ [a] | | | |
| A-$^{14}C$ | Origin | variable | 0 | 0.5 | 1.2 | 1.3 |
| | 2nd | green | 0.13 | 0.8 | 1.0 | 3.5 |
| | 3rd | dark blue | 0.34 | 2.5 | 4.4 | 5.0 |
| | Ochratoxin A | green | 0.50 | 95.6 | 92.6 | 88.6 |
| | Remainder of plate | variable | - | 0.6 | 0.8 | 1.6 |
| B-$^{14}C$ | Origin | variable | 0 | 0.4 | 0.9 | 1.9 |
| | 2nd | blue | 0.06 | 3.5 | 2.1 | 5.4 |
| | 3rd | dark blue | 0.24 | 0.9 | 2.2 | 3.5 |
| | Ochratoxin B | blue | 0.34 | 94.5 | 93.4 | 87.2 |
| | Remainder of plate | variable | - | 0.7 | 1.4 | 2.0 |

[a] $R_f$ values in this and other tables determined from chromatoplates developed in benzene:glacial acetic acid 9:1 v/v.

[b] Extract I = 0-5 hours, Extract II = 5-12 hours, Extract III = 12-24 hours.

has an $R_f$ value of 0.13. The $^{14}C$ activity detected in thin-layer plate scrapings of both of these spots was found to increase with time. The percentage of $^{14}C$ in the chloroform extract accounted for by ochratoxin A-$^{14}C$ showed a consequent decrease with time.

The TLC pattern of the chloroform extracts shown in Table 7 also indicated the presence of two radioactive metabolites of ochratoxin B. Of the chloroform-soluble compounds, these two metabolites accounted for nearly the same percentage of the $^{14}C$ as the two metabolites found in the chloroform extracts of urine from trout dosed with ochratoxin A-$^{14}C$. One of these metabolites had the same fluorescent properties and $R_f$ value as ochratoxin b ($R_f$=0.24), while the other, a blue fluorescent compound, has an $R_f$ value of 0.06. As was the case with the ochratoxin A metabolites, these metabolites accounted for an increasing percentage of the $^{14}C$ with time.

Biliary Metabolites. The proportion of $^{14}C$ activity in bile from trout dosed with radiolabeled ochratoxins A and B which was chloroform-soluble was 96.2 and 96.4%, respectively.

The TLC patterns of the bile chloroform extracts shown in Table 8 indicate primarily the presence of unaltered ochratoxins A and B as well as the two fluorescent compounds previously seen in urine extracts which had $R_f$ values less than ochratoxins a and b, respectively. The $^{14}C$ activity detected at the level of the low $R_f$ blue fluorescent metabolite of ochratoxin B was over ten times that detected

Table 8. Chromatographic distribution of $^{14}C$ in chloroform extracts of bile from trout dosed with radiolabeled ochratoxin A or B.

| Ochratoxin dosed | Spot on thin-layer chromatoplate | | | % of Chloroform soluble $^{14}C$ |
|---|---|---|---|---|
| | | Color of fluorescence | $R_f$ | |
| A-$^{14}C$ | Origin | variable | 0 | 3.9 |
| | 2nd | green | 0.13 | 0.6 |
| | Ochratoxin A | green | 0.50 | 93.5 |
| | Remainder of plate | variable | - | 2.0 |
| B-$^{14}C$ | Origin | variable | 0 | 1.9 |
| | 2nd | blue | 0.06 | 6.7 |
| | Ochratoxin B | blue | 0.34 | 90.7 |
| | Remainder of plate | variable | - | 0.7 |

at the level of the low $R_f$ green fluorescent metabolite of ochratoxin A. No detectable levels of $^{14}C$ activity were found at positions on the thin-layer plates corresponding to ochratoxins a and b in chloroform extracts of bile samples from trout dosed with radiolabeled ochratoxins A and B, respectively.

Intestinal Content Metabolites. The proportion of $^{14}C$ activity in the chloroform extract of the bicarbonate-soluble portion of the intestinal contents from trout dosed with radiolabeled ochratoxins A and B was 91.1 and 94.1%, respectively.

The TLC patterns of these chloroform extracts shown in Table 9 indicate the presence of considerably more $^{14}C$ at the $R_f$ of ochratoxin b than ochratoxin a (38.5% vs. 1.0%, respectively). None of the low

Table 9. Chromatographic distribution of $^{14}C$ in chloroform extracts of intestinal bicarbonate extract from trout dosed with radiolabeled ochratoxin A or B.

| Ochratoxin dosed | Spot on thin-layer chromatoplate | | | % of Chloroform soluble $^{14}C$ |
|---|---|---|---|---|
| | | Color of fluorescence | $R_f$ | |
| A-$^{14}C$ | Origin | variable | 0 | 4.0 |
| | 2nd | dark blue | 0.34 | 1.0 |
| | Ochratoxin A | green | 0.50 | 91.2 |
| | Remainder of plate | variable | - | 3.8 |
| B-$^{14}C$ | Origin | variable | 0 | 2.6 |
| | 2nd | blue | 0.06 | 12.6 |
| | 3rd | dark blue | 0.24 | 38.5 |
| | Ochratoxin B | blue | 0.34 | 43.7 |
| | Remainder of plate | variable | - | 2.6 |

$R_f$ (0.13) green fluorescent metabolite was visible in the chloroform extract of the intestinal bicarbonate wash from trout dosed with ochratoxin A-$^{14}C$. In contrast, 12.6% of the $^{14}C$ in the chloroform extract of the intestinal bicarbonate wash from trout dosed with ochratoxin B-$^{14}C$ was present in the low $R_f$ (0.06) blue fluorescent metabolite.

Liver Metabolites. The proportion of $^{14}C$ in the bicarbonate extract of livers from trout dosed with radiolabeled ochratoxin A and B which was chloroform-soluble was 94.3 and 93.9%, respectively.

As seen in Table 10 the same general metabolite pattern seen in bile was also seen in the chloroform-soluble extracts of the liver. No

Table 10. Chromatographic distribution of $^{14}C$ in chloroform extracts of livers from trout dosed with radiolabeled ochratoxin A or B.

| Ochratoxin dosed | Spot on thin-layer chromatoplate | | | % of Chloroform soluble $^{14}C$ |
|---|---|---|---|---|
| | | Color of fluorescence | $R_f$ | |
| A-$^{14}C$ | Origin | variable | 0 | 1.4 |
| | 2nd | green | 0.13 | 1.4 |
| | Ochratoxin A | green | 0.50 | 94.4 |
| | Remainder of plate | variable | - | 2.8 |
| B-$^{14}C$ | Origin | variable | 0 | 1.7 |
| | 2nd | blue | 0.06 | 6.7 |
| | Ochratoxin B | blue | 0.34 | 86.5 |
| | Remainder of plate | variable | - | 5.1 |

fluorescence was seen at the $R_f$ values of ochratoxins a and b in extracts of livers from trout dosed with ochratoxin A-$^{14}C$ and B-$^{14}C$, respectively. However, $^{14}C$ activity was detected at the level of the two lowest $R_f$ metabolites of ochratoxins A and B. The percentage of $^{14}C$ in the chloroform extract detected at the level of the low $R_f$ blue fluorescent metabolite of ochratoxin B was nearly five times greater than that detected at the level of the low $R_f$ green fluorescent metabolite of ochratoxin A.

Kidney Metabolites. The proportion of $^{14}C$ in the bicarbonate extract of kidneys from trout dosed with radiolabeled ochratoxin A and B which was chloroform-soluble was 95.9 and 96.8, respectively.

Table 11. Chromatographic distribution of $^{14}C$ in chloroform extracts of kidneys from trout dosed with radiolabeled ochratoxin A or B.

| Ochratoxin dosed | Spot on thin-layer chromatoplate | | | % of Chloroform soluble $^{14}C$ |
|---|---|---|---|---|
| | | Color of fluorescence | $R_f$ | |
| A-$^{14}C$ | Origin | variable | 0 | 2.1 |
| | 2nd | green | 0.13 | 26.8 |
| | Ochratoxin A | green | 0.50 | 59.4 |
| | Remainder of plate | variable | - | 11.7 |
| B-$^{14}C$ | Origin | variable | 0 | 2.3 |
| | 2nd | blue | 0.06 | 29.2 |
| | Ochratoxin B | blue | 0.34 | 60.4 |
| | Remainder of plate | variable | - | 8.1 |

Table 11 shows the distribution of $^{14}C$ in chloroform extracts of kidneys from trout dosed with radiolabeled ochratoxins A and B. No detectable fluorescence was noted in these extracts at the $R_f$ values of ochratoxins a and b. However, considerable percentage of the activity in these extracts was accounted for by $^{14}C$ at the levels of the two lowest $R_f$ metabolites of ochratoxins A and B. The percentage of the total chloroform-soluble $^{14}C$ accounted for at these $R_f$ levels was nearly the same in chloroform extracts of kidneys from trout dosed with radiolabeled ochratoxins A and B. It should be pointed out that a large proportion of the chloroform extracts had to be applied to the silica gel plates in order to obtain sufficient levels of $^{14}C$ on the plate for counting. This, therefore, hindered good resolution of

various labeled compounds on the plates as a result of interference of natural chloroform-soluble materials in the kidneys.

Characterization of Chloroform-soluble Metabolites

From results of the metabolite distribution studies, it is apparent that ochratoxin A is transformed in significant quantities to at least two metabolites which are chloroform-soluble. One of these metabolites was found to have the same $R_f$ and fluorescent properties as ochratoxin a when chromatographed on silica gel. The other was found to have an $R_f$ value less than ochratoxin a and had a green fluorescence much like ochratoxin A.

In order to further confirm the identity of the ochratoxin a metabolite and obtain presumptive evidence of the chemical identity of the low $R_f$ green fluorescent metabolite, five trout (ave. weight, 125 g) were fasted four days, catheterized and IP injected with 13.72 μmoles/kg non-isotopic ochratoxin A by methods previously described. Urine was collected for 24 hours, the trout were sacrificed and the bile removed from the gall bladder. The urine and bile were acidified and chloroform extracted as previously described. The chloroform extract was evaporated to dryness in vacuo then dissolved in a small volume of chloroform. This extract was then streaked onto a preparative silica gel chromatoplate (Appendix X) and the plate developed in carbon tetrachloride:glacial acetic acid 4:1 v/v. Also applied at one

side of the plate before development was an ochratoxin a standard and the original chloroform extract of urine from trout dosed with ochratoxin A-$^{14}$C. After developing the plate, the band corresponding to ochratoxin a as well as the low $R_f$ green fluorescent metabolite were scraped from the plate into 95% ethanol and the silica gel removed by filtration. The resulting ethanol solutions were evaporated to dryness, dissolved in minimal ethanol, and again purified by preparative TLC as described above.

The purified metabolite having the same $R_f$ as ochratoxin a was chromatographed with an internal and external ochratoxin a standard in three other solvent systems, namely, benzene:glacial acetic acid 4:1 v/v and solvent systems C and D in Appendix X. Results of these studies revealed that this metabolite had the same $R_f$ values and fluorescent properties as authentic ochratoxin a on thin-layer chromatoplates developed in all solvent systems tested.

Finally, the ethyl ester of the suspected ochratoxin a metabolite was prepared (Appendix I) and chromatographed on silica gel with an internal and external standard of authentic ochratoxin a ethyl ester using carbon tetrachloride:glacial acetic acid 9:1 v/v, benzene:glacial acetic acid 9:1 v/v and toluene:ethyl acetate:90% formic acid 5:4:1 v/v/v solvent systems. $R_f$ values and fluorescent properties of the ethyl ester of the metabolite and ochratoxin a ethyl ester were identical on chromatoplates developed in these solvent systems.

The purified low $R_f$ green fluorescent metabolite of ochratoxin A was dissolved in a small volume of 95% ethanol and chromatographed with internal and external standards of authentic 4-hydroxyochratoxin A[8] (Figure 3) in carbon tetrachloride:glacial acetic acid 4:1 v/v, benzene:glacial acetic acid 4:1 v/v and toluene:ethyl acetate:90% formic acid 5:4:1, by volume. Results of these studies revealed that this metabolite had the same $R_f$ value and fluorescent properties as 4-hydroxyochratoxin A on chromatoplates developed in the three solvent systems.

Ochratoxin B was similarly found to be metabolized in significant quantities to at least two other compounds found primarily in chloroform extracts of urine and bile of trout dosed with ochratoxin B-$^{14}$C. Analogous to the situation with ochratoxin A, one of the compounds had the same $R_f$ value as ochratoxin b on silica gel chromatoplates developed in benzene:glacial acetic acid 9:1 v/v. Also, the other compound had an $R_f$ value less than ochratoxin b and exhibited a blue fluorescence identical to ochratoxin B.

Sufficient quantities of these two metabolites were obtained for characterization by injecting four catheterized trout (ave. weight, 120 g) with 13.72 μmoles/kg non-isotopic ochratoxin B and collecting

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[8]Obtained from Dr. P. M. Scott, Food Research Laboratories, Health Protection Branch, Department of National Health and Welfare, Ottawa, Ontario, K1A OL2 Canada.

urine and bile from these trout as before with the trout injected with non-isotopic ochratoxin A. The urine and bile were chloroform extracted in the usual manner and the two metabolites purified two times by preparative TLC.

The purified metabolite corresponding to ochratoxin b was chromatographed with internal and external standards of authentic ochratoxin b in the same solvent systems used to chromatograph the ochratoxin A metabolite corresponding to ochratoxin a. The ethyl ester of this metabolite was prepared and the product chromatographed on silica gel with internal and external standards of authentic ochratoxin b ethyl ester in the three solvent systems used to chromatograph the ethyl ester of the ochratoxin A metabolite. The ochratoxin B metabolite and its ethyl ester had the same chromatographic and fluorescent properties as ochratoxin b and its ethyl ester, respectively.

The purified low $R_f$ blue fluorescent metabolite of ochratoxin B was dissolved in 95% ethanol and the ultraviolet spectra recorded between 280 and 360 nm. The spectrum was virtually identical to that of ochratoxin B as its wavelength of maximum absorption was at 318 nm; thus, ochratoxin B and this metabolite contain the same chromophore.

Since a considerable amount of this metabolite was obtained in purified form, its ethyl ester was prepared as described in Appendix I then purified by preparative TLC using carbon tetrachloride:glacial

acetic acid 4:1 v/v as an eluant. The mass spectra of the purified ester was determined using conditions specified in Appendix III except the probe temperature used was 200°C. It was felt that this compound might also be a hydroxy derivative of ochratoxin B, which would require a molecular ion at m/e 413 in the mass spectrum. A weak signal was detected at this mass but was not strong enough to be conclusive. However, the fragmentation pattern (Table 12) was consistent with the expected pattern for a hydroxy derivative of ochratoxin B.

Table 12. Mass spectral fragmentation pattern of low $R_f$ blue fluorescent metabolite of ochratoxin B.

| m/e | Suggested assignment |
|---|---|
| 413 (weak) | molecular ion |
| 368 | loss of · OEt |
| 339 | loss of $HCOCH_3$ and $COH_2$ from lactone ring |
| 313 | unknown |
| 237 | loss of $C_6H_5CH{=}CHCOOEt$ |
| 221 | loss of $C_6H_5CH(COOEt)NH$ |
| 177 | loss of $C_6H_5CH(COOEt)NH$ and $HCOCH_3$ from lactone ring |

Further evidence in support of the identity of the proposed hydroxy derivatives of ochratoxin A and B was obtained by performing a catalytic dehalogenation with the ochratoxin A metabolite. This

reaction was carried out with about half the proposed hydroxy derivative of ochratoxin A obtained from five trout. The sample was dissolved in 1 ml 0.25 M methanolic KOH and then hydrogenated for two hours as described in Appendix XV. About 10 ml water was added to the product, then the mixture was acidified to pH 2.0 and extracted with chloroform. The chloroform extract was evaporated to a small volume in vacuo. This extract was chromatographed with an internal and external sample of the proposed hydroxy derivative of ochratoxin B on silica gel using benzene:glacial acetic acid 4:1 v/v and carbon tetrachloride:glacial acetic acid 4:1 v/v as eluants. Results revealed that the product of hydrogenation of the ochratoxin A metabolite had the same chromatographic and fluorescent properties as the proposed hydroxy derivative of ochratoxin B in both solvent systems. A portion of the standard 4-hydroxyochratoxin A sample was also hydrogenated, and the product of the reaction had the same chromatographic and fluorescent properties as the proposed hydroxy derivative of ochratoxin B in the two solvent systems described above.

These results do not conclusively establish the identity of the proposed hydroxy derivatives of ochratoxins A and B. They do, however, present evidence that the two derivatives are analogues and they may be hydroxy derivatives. As to the position of the hydroxyl group on the molecule, further evidence is necessary, viz., determination of

nuclear magnetic resonance spectra. This would require a larger quantity of the compounds than was isolated in these studies.

Support for the formation of the 4-hydroxy derivatives is presented by Parke (69) who states that with "compounds containing both alicylic and aromatic rings, the saturated ring is more readily hydroxylated" (p. 40). He states that the major metabolites of tetralin (1, 2, 3, 4-tetrahydronaphthalene) in the rabbit are conjugates of 1- and 2-tetralol, and only small amounts of the phenolic product are formed.

It is likely that the proposed hydroxy derivatives are formed in the liver of trout. Several investigators have shown that trout are capable of hydroxylating a number of xenobiotic agents including aniline (7), biphenyl (26) and 2-acetylaminofluorene (57). Buhler (7) was able to induce NADPH-dependent hepatic hydroxylation of aniline by pretreating trout with DDT or phenylbutazone.

Of further significance to the present study, Dewaide (29) detected aniline p-hydroxylation activity in microsomal preparations from trout livers as well as other organs including the kidney. The activity in the kidney was less than that of the liver but was still substantial. As shown in Table 11, a considerable portion of the $^{14}C$ in chloroform extracts of kidneys from trout dosed with ochratoxins A-$^{14}C$ and B-$^{14}C$ was present at the $R_f$ values corresponding to the proposed ochratoxin hydroxy derivatives. There may be some question

as to the accuracy of the amount of $^{14}C$ detected at these $R_f$ values because of interference of other materials which caused poor separation of radiolabeled compounds in the extracts. However, this does not preclude the possibility that a portion of these metabolites were produced by the kidney. Further studies on this possibility seem warranted.

Chloroform-insoluble $^{14}C$ in Urine, Bile and Intestinal Contents

Significant levels of chloroform-insoluble $^{14}C$ were found in urine, bile and intestinal contents from trout dosed with radiolabeled ochratoxins A and B. These samples were frozen and lyophilized to a small volume and portions of these concentrates chromatographed on silica gel to determine if the $^{14}C$ resulted from incomplete chloroform extraction of these samples. Results of these studies indicated, without exception, that the radioactivity in the concentrates was accounted for by $^{14}C$ at the origin of silica gel chromatoplates developed in benzene:glacial acetic acid 9:1 v/v. Therefore, the activity is not likely due to incomplete extraction of the chloroform-soluble $^{14}C$. It seems likely that the $^{14}C$ in these samples is due to formation of ochratoxin conjugates and/or polar decomposition or oxidation products formed artifactually during sample storage or extraction.

As previously described, radiolabeled ochratoxins A and B were mixed with trout urine, stored and extracted in accordance with the isolation procedures used to extract urine from ochratoxin $^{14}C$-dosed trout. Results of this study indicated that 3.5 and 4.3% of the $^{14}C$ from the added ochratoxin A-$^{14}C$ and B-$^{14}C$, respectively, was not extractable from the urine with chloroform. Thin-layer chromatography of the chloroform extracts revealed $^{14}C$ at the $R_f$ of ochratoxins A and B as well as the origin of the plates. Specifically, the percentages of the $^{14}C$ in the chloroform extracts accounted for by activity at the origin were 3.6 and 2.9% for extracts of urine mixed with radiolabeled ochratoxins A and B, respectively. No other fluorescent or non-fluorescent areas were seen in these chloroform extracts, clearly demonstrating that the two metabolites of ochratoxins A and B previously described are not artifacts formed during the isolation procedure.

In summary, these results indicate that a large proportion of the chloroform-insoluble $^{14}C$ detected in urine, bile and tissue extracts may be accounted for by artifactually formed products of ochratoxin. Also, $^{14}C$ detected at the origin of thin-layer plates on which the various chloroform extracts were chromatographed may also (at least partially) represent extraction artifacts. These results do not, however, preclude the possibility that some of the chloroform-insoluble $^{14}C$ in the urine and bile is not accountable for by the presence of

ochratoxin conjugates. This possibility is supported by the observation that the percentage of chloroform-insoluble $^{14}C$ in the urine from trout dosed with ochratoxins A-$^{14}C$ and B-$^{14}C$ was found to increase with time (Table 6). However, the possibility of ochratoxin conjugate formation was not further pursued because the chloroform-insoluble $^{14}C$ levels in the urine, bile and tissues not accounted for by possible artifacts would be quite low, making purification and characterization extremely difficult.

## Significance of Differences in Ochratoxin A and B Excretion and Metabolism

From results obtained in the ochratoxin excretion and metabolism studies, certain facts are apparent: 1) IP dosed ochratoxins A and B are primarily excreted in both the urine and bile of trout; 2) trout excrete ochratoxin B and its metabolites more efficiently than ochratoxin A; and 3) ochratoxin B is converted to other metabolites to a greater extent than ochratoxin A.

Smith (90) states that excretion of foreign compounds in the bile and urine are often complimentary to each other. The relative amounts of a compound excreted by a given species in the bile and urine will depend upon the molecular size and ionic properties of the compound. In general, these compounds contain a polar anionic group and have a molecular size intermediate between those compounds excreted largely

in the urine and those excreted primarily in the bile. In rats the molecular weight of foreign compounds and/or their metabolites which appear in the bile in quantity is 300-400 or more (60, 90). Certain anionic compounds having relatively low molecular weights are more efficiently secreted by the renal tubules. Based on molecular weight differences alone, one would correctly predict that ochratoxin B would be excreted in the urine to a greater extent than ochratoxin A. It would certainly be of greater scientific value to be able to predict or explain this outcome based on knowledge of the biological processes involved in excretion of foreign substances in the urine and bile. However, these biological processes are quite complex and are affected by a large number of variables, some of which are poorly characterized. Consequently, it is difficult to propose mechanisms which might explain why proportionally more of a given compound is excreted in the urine or bile.

The processes involved in excretion of a substance by the mammalian kidney include glomerular filtration, active tubular secretion and active and passive tubular reabsorption (113). It can be assumed partly from evidence obtained directly with fish and partly from studies which indicate the similarity of various portions of the fish and mammalian renal nephron (43), that these same basic processes are involved in urinary excretion of foreign substances by trout. Specific factors of importance in these processes are included

in the following excerpts from Weiner (113);

> The rate of delivery of a drug to the kidney is determined by the flow of plasma to the kidney and the volume of distribution of the drug. The degree of protein binding determines the fraction of drug that is filtered. . . . The size of the molecule will determine its ability to pass through aqueous channels in tubular membranes, its polarity will determine the extent of diffusion through non-aqueous areas of membranes, its pKa will determine the fraction of nonionized drug at a particular tubular pH and this will modify the extent and possibly the net direction of non-ionic diffusion. The rate of urinary flow will determine the extent of drug concentration in tubular fluid and consequently will be a major influence on all passive processes. Finally, the extent and direction of active transport of a particular drug can greatly influence its excretory route (p. 331-332).

Factors generally recognized as being involved in the excretion of substances in the bile include hepatic uptake and storage, filtration and active secretion. Millburn (60) states that a low liver concentration of small organic anions can be explained in two ways: the liver cell membranes may be permeable to such compounds and/or these compounds do not enter hepatic cells but are rapidly returned to the plasma by active secretion. Smith (90) explains this same observation on the basis of possible reabsorption from the bile. He states that low biliary excretion of small organic anions may be due to their rapid reabsorption from the primary bile while high biliary excretion of large anions may be due to their passage into the bile and failure to be reabsorbed.

Defining a mechanism explaining excretion of a foreign compound in the bile is further complicated by the fact that drug metabolizing

enzymes of the liver may alter the compound to bring about a change in its intracellular distribution leading to a greater excretion in the bile (90). In addition, metabolism by gut flora and an enterohepatic circulation may influence the route of excretion of a compound and hence, its ultimate persistance in the body (60, 90).

If one assumes that excretion of a compound in the bile implies that the hepatobiliary system is exposed to higher levels of the compound and its metabolites, it would follow that ochratoxin A should be more strongly hepatotoxic than ochratoxin B. Results of toxicity trials conducted in this and other studies (30, 32) show this to be the case. Ochratoxin B causes minor liver damage but extensive renal necrosis, while ochratoxin A causes extensive liver and kidney damage. It would follow from this that tissues of the pyloric ceca and intestine of trout dosed with ochratoxin A are exposed to high levels of this compound. It is not surprising, therefore, that hemorrhagic lesions are also seen in these tissues in trout dosed with ochratoxin A but not in trout dosed with ochratoxin B.

These differences probably also reflect the fact that ochratoxin B is more extensively metabolized than ochratoxin A to its proposed hydroxy derivative by microsomal enzymes in the liver. Also of importance in this regard is the greater extent of hydrolysis of ochratoxin B than ochratoxin A.

Two important consequences of toxicological significance may

arise as a result of excretion of ochratoxins A and B and their metabolites in the bile: 1) they may pass into an enterohepatic circulation and 2) they may be metabolized by the gut flora (90). The influence of the latter has already been mentioned in that the ratio of ochratoxin b to ochratoxin a in the intestinal contents of trout dosed with ochratoxins A and B, respectively, was about 38:1. This indicates that flora and/or hydrolases in the gut are better able to hydrolyze ochratoxin B than A.

Enterohepatic circulation is defined as the reabsorption of a substance from the intestine into the portal blood after its excretion in the bile, followed by further recycling (90). In general, compounds which are strongly polar will not undergo extensive reabsorption while the more lipophilic compounds will (90). In the present study, therefore, one would predict that the ochratoxin hydrolysis products as well as the proposed hydroxy derivatives would not be reabsorbed as rapidly as the unaltered parent ochratoxins because of their greater polarity. In view of the fact that the hydrolysis products of ochratoxins A and B were found only in the intestinal contents and urine (and not in the bile or liver), it seems likely that they are produced predominately in the gut after excretion of the parent ochratoxins in the bile. These compounds must, therefore, be reabsorbed from the gut to be excreted in the urine. If this conclusion is correct, one would expect the parent ochratoxins A and B to also be reabsorbed. Further

support for this reabsorption is provided from the fact that the intestine, less its contents, from trout dosed with ochratoxin A-$^{14}$C and B-$^{14}$C contained significant levels of $^{14}$C (Table 5).

The reabsorbed portions of ochratoxins A and B and their metabolites could then cycle back through the liver and be metabolized and excreted in the usual manner. The ultimate result of this would be for ochratoxin B to be transformed into more polar metabolites and be excreted relatively rapidly. Ochratoxin A, being more resistant to metabolism as well as excretion in the urine, could possibly persist in the body a very long time.

This brings up an important question regarding the nature of the toxicological response of trout to ochratoxin. Does the toxic response result from repeated exposure of target sites in various tissues to the toxin by its recycling through the body, or is the initial cycle after injection sufficient to initiate the response? The answer to this question could likely be obtained by injecting an $LD_{50}$ dose of ochratoxin A into trout, than after about 12 hours, orally dosing these trout with vegetable oil to stimulate release of the bile and induce faster recycling of the toxin. If recycling is important, a greater toxicity for a given dose of toxin might be noted. It would, of course, first have to be established for certain that recycling of ochratoxin does occur by carrying out this same experiment for a longer time than that used in this study with catheterized trout in metabolism chambers and

determining the amount of ochratoxin excreted in the feces. This may be more easily demonstrated by injecting ochratoxin into the gut via the anal pore then determining the presence of ochratoxin in the bile and/or urine.

## Areas for Future Study

1. Carry out ochratoxin metabolism studies for longer periods of time than that used in this study to allow tracing the remaining toxin still in the trout after 24 hours. Because of the problems involved in keeping trout catheterized for long periods of time without urine leakage, it might be necessary to inject the trout with the radiolabeled toxin then wait 24 hours before catheterization.
2. Conduct a time-course subcellular distribution of $^{14}C$ in the liver and kidney of trout dosed with radiolabeled ochratoxins. At the same time, a gross distribution in other tissues could be carried out. This study might give clues as to why ochratoxin B is excreted to a greater extent in the urine than ochratoxin A, and conversely, why ochratoxin A is excreted to a greater extent in the bile.
3. Study the *in vivo* interaction of ochratoxins A and B with various cellular components such as proteins, nucleic acids, carbohydrates and lipids.

4. Study the effect of inducers and/or inhibitors of microsomal enzymes on metabolism, excretion and toxicity of ochratoxins A and B.

5. Study the comparative metabolism and excretion of ochratoxin A and B ethyl esters as well as other amino acid analogues.

6. Determine the toxicity and/or excretion rate and metabolism of ochratoxin A and B metabolites.

# SUMMARY AND CONCLUSIONS

The ten-day $LD_{50}$ values of ochratoxins A, C and B ethyl ester in six-month-old rainbow trout were 5.53, 3.0 and 13.0 mg/kg body weight, respectively. Ochratoxin B administered at 66.7 mg/kg was non-lethal but caused histopathological abnormalities in trout livers and kidneys. Ochratoxin a ethyl ester and the alanine and leucine analogues of ochratoxin A administered at the molar $LD_{50}$ level of ochratoxin A were non-lethal. The alanine and leucine analogues of ochratoxin A caused minor kidney damage primarily. These results indicate that structural changes in the non-chlorinated isocoumarin moiety of ochratoxin which increase its molecular weight to near 400, but still maintain the presence of the phenolic hydroxyl anionic group, increase its toxicity in trout.

Ochratoxins A and B are metabolized in significant quantities by rainbow trout to two compounds: the isocoumarin acid hydrolysis product and a proposed hydroxy derivative of the parent toxins. The acid hydrolysis products appear to be formed in the gastrointestinal tract after excretion in the bile, while the hydroxy derivatives are probably formed by microsomal enzymes in the liver.

The difference in toxicity of ochratoxins A and B in trout is probably at least partially due to a greater excretion of ochratoxin B in the urine as well as a greater rate of conversion to its isocoumarin

and proposed hydroxy derivatives. The low rate of conversion of ochratoxin A to its isocoumarin and hydroxy derivatives as well as its resistance to excretion in the urine may cause it to persist in the body a relatively long time compared to ochratoxin B.

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# APPENDICES

# APPENDIX I

## PREPARATION OF OCHRATOXIN ETHYL ESTERS

1. Dry 95% ethanol by passage through a short column of anhydrous sodium sulfate. Weigh portion into glass-stoppered Erlenmeyer flask, stopper and cool on ice bath.
2. Bubble $BF_3$[1] into the ethanol until concentration by weight reaches 16%.
3. Transfer ochratoxin sample into small pear-shaped flask and evaporate to dryness in vacuo. Dissolve in 5-30 ml 16% $BF_3$ in ethanol depending upon amount present. Stopper flask and place in 75°C oven for about 30 minutes or until esterification is complete.
4. Remove sample from oven and determine progress of esterification by spotting a few µl of the mixture on a silica gel thin-layer plate alongside the appropriate ochratoxin ester standard. The plate can be developed in benzene:glacial acetic acid 9:1 v/v or carbon tetrachloride:glacial acetic acid 9:1 v/v for examination of all esters made in this study. Visualize by holding the developed chromatograms under long-wave ultraviolet (365 nm) radiation.[2, 3]

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[1] Matheson Co., Inc., Newark, California.

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

[3] Chromato-vue cabinet, U. V. Model C-5, Ultraviolet Products, Inc., San Gabriel, California.

# APPENDIX II

## PREPARATION OF ALANINE AND LEUCINE ANALOGUES OF OCHRATOXIN A

### Amino Acid Esterification

1. Three g of the levo forms of phenylalanine, alanine or leucine are dissolved in 50 ml of dry methanol containing 2 ml concentrated sulfuric acid. Equilibrate at room temperature for several days or one day at 50°C. Dilute ten-fold with water and repeatedly extract with ether.
2. Dry phenylalanine methyl ester by adding benzene and distill off a benzene water azeotrope *in vacuo*. Because of the volatility of the esters of alanine and leucine, dry over anhydrous sodium sulfate, then molecular sieves.
3. Infra-red spectra of these esters showed significant absorption at 3460, 3395, 1730 and 1200 $cm^{-1}$.

### Ochratoxin A Hydrolysis

1. Ochratoxin A is hydrolyzed by the acid hydrolysis method of van der Merwe *et al.* (106).
2. Recovery of ochratoxin *a* is achieved by chloroform extraction of the hydrolysate. Thin-layer chromatography (Appendix X) using benzene:glacial acetic acid 9:1 v/v as an eluant will reveal the presence of unhydrolyzed ochratoxin A which is removed by column chromatography as previously described (30).

### Preparation of Ochratoxin *a*, Leucine Amide

1. Add 1 ml oxalyl chloride to 13 mg purified ochratoxin *a* in 30 ml

dry benzene. After 1 hour, remove benzene and excess oxalyl chloride in vacuo then immediately add 30 ml dry benzene.

2. While stirring rapidly, slowly add one equivalent of L-leucine methyl ester. After 10 minutes, add a five-fold excess of leucine methyl ester. After 12 hours, wash the benzene solution with 0.01 N HCl, and dry.
3. Thin-layer chromatography revealed the presence of a green fluorescent spot at $R_f$ 0.70 which was purified by preparative TLC (Appendix X) using benzene:glacial acetic acid 9:1 v/v as an eluant. This purified compound was added to 100 ml acetic acid containing 5 ml 12 N HCl and the progress of the ester exchange followed by TLC. After 24 hours at 35°C TLC revealed only one green fluorescent spot at $R_f$ 0.55.
4. Add several volumes water and extract with chloroform. Evaporate to dryness and purify by preparative TLC as described above. Overall yield from this reaction was 6 mg or 36% determined spectrophotometrically (Appendix XIII) using a molar extinction coefficient of 5550 at 333 nm, the same as ochratoxin A.
5. The ethyl ester of a portion of this compound was prepared (Appendix I) and its mass spectrum determined (Appendix III).

Preparation of Ochratoxin a, Alanine Amide

1. Prepare the alanine amide by methods similar to those employed in the preparation of the leucine amide. Rapid stirring during and after the addition of the amino-ester is important due to the low solubility of L-alanine methyl ester in benzene. This low solubility plus the hygroscopic nature of the alanine ester probably account for the low yield.
2. After work-up of the reaction mixture, TLC using benzene: glacial acetic acid as an eluant showed the appearance of a green

fluorescent spot ($R_f$ 0.51). This was separated from the unreacted ochratoxin a by preparative TLC using benzene:glacial acetic acid 9:1 v/v as an eluant. The esterifying methyl was exchanged to acetic acid and the free acid product extracted and purified by preparative TLC using carbon tetrachloride:glacial acetic acid 9:1 v/v as an eluant. Overall yield was 3% determined spectrophotometrically (Appendix XIII).

3. As with the leucine adduct, the ethyl ester of a portion of this compound was prepared (Appendix I) and its mass spectrum determined (Appendix III).

Preparation of Ochratoxin A

The possibility of racemization in the synthesized ochratoxin A analogues was considered. Since insufficient amounts of these compounds were prepared for determination of their optical activity, ochratoxin A was prepared using methods identical to those described for preparation of the two amino acid analogues. This method of checking the expected extent of racemization is also facilitated by the fact that the published specific rotation of natural ochratoxin A and (-, ±) ochratoxin A are available for comparison (79).

1. The product of the reaction of ochratoxin a acid chloride and the methyl ester of L-phenylalanine had an $R_f$ value of 0.70 on silica gel plates developed in benzene:glacial acetic acid 9:1 v/v. The unreacted ochratoxin a was separated from the product by repeated extraction of the benzene solution with 5% aqueous sodium bicarbonate.
2. The esterifying methyl group was removed again by interchange with acetic acid. TLC of the product using benzene:glacial acetic acid 9:1 v/v revealed a green fluorescent spot having an $R_f$ value identical to authentic natural ochratoxin A.

3. The synthetic ochratoxin A product (yield, 40%) was recrystallized once from benzene, then repeatedly from glacial acetic acid:tetrahydrofuran 3:1 v/v. The ultraviolet spectra was identical to natural ochratoxin A. The synthetic ochratoxin A had a specific rotation (sodium D-line) of $-79^{\circ}$ (determined in glacial acetic acid:chloroform 1:1 v/v). Roberts and Woollven (79) report the specific rotation of (-, ±) ochratoxin A as $+38^{\circ}$ (the L-phenylalanine is active, but the isocoumarin moiety is racemic), and $-93^{\circ}$ for natural ochratoxin A. Thus, the ochratoxin A prepared as described here is 11% racemized.

# APPENDIX III

## MASS SPECTRAL ANALYSIS OF ETHYL ESTERS OF ALANINE AND LEUCINE ANALOGUES OF OCHRATOXIN A

1. Samples are dissolved in 95% ethanol at a concentration of 0.5 mg/ml.
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

   acceleration voltage: 3000 v

   scan range: 18

   recorder: 8 ips

   internal standard: perfluorodecalin (via liquid inlet)

   source current: 100 μa
3. Ten μl of the sample was inserted into the mass spectrometer following evaporation of the ethanol. Spectra were recorded at a probe temperature of 100°C.
4. The significant peaks and their suggested possible assignments are as follows:

| | m/e | |
|---|---|---|
| Ethyl ester of alanine analogue | 355 | molecular ion |
| | 282 | loss of · COOEt |
| | 239 | loss of $CH_3CH(COOEt)NH$ |
| | 221 | loss of $CH_3CH(COOEt)NH$ and water |
| Ethyl ester of leucine analogue | 397 | molecular ion |
| | 324 | loss of · COOEt |
| | 239 | loss of $(CH_3)_2CHCH_2CH$-(COOEt)NH |
| | 221 | loss of $(CH_3)_2CHCH_2CH$-(COOEt)NH and water |

# APPENDIX IV

## PREPARATION OF METHYLMAGNESIUM IODIDE REAGENT FOR SODIUM ACETATE-1-$^{14}$C SYNTHESIS

1. Reaction apparatus:

   The reaction is carried out in a 50-ml three-neck (14/20 ⧖) round bottom flask. In one of the outer necks is inserted a 25 ml addition separatory funnel with pressure equalizer,[4] in the other, a short ⧖ gas inlet tube.[5] In the center neck is inserted a ⧖ condenser with a 14/20 ⧖ drying tube charged with anhydrone inserted at the top of it. A magnetic stirring bar is inserted into the round bottom flask and magnetic stirrer placed under it.
2. Flame dry the reaction apparatus then pass dry nitrogen through the gas tube until the apparatus is cool.
3. Weigh out 0.8 g Mg turnings and add to round bottom flask. Continue flushing with dry nitrogen.
4. Weigh 4.315 g methyl iodide into a 25 ml Erlenmeyer flask with ground glass stopper. Stopper and cool in an ice bath.
5. Stop nitrogen flow and add 12.5 ml dry ether[6] to the addition funnel. Add 8 ml dry ether to methyl iodide and transfer to addition funnel. Wash weighing flask with two 3-ml portions of dry ether and transfer to addition funnel. Make all transfers rapidly to avoid losing anhydrous conditions.
6. Start stirring bar and add methyl iodide solution over a 45 minute period. Wash last traces from addition funnel with two

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[4] Separatory funnel, K-299200, Kontes Glass Co., New Jersey.

[5] Inlet adapter, K-275400, Kontes Glass Co., New Jersey.

[6] Dry over freshly cut sodium, then distill, maintaining anhydrous conditions.

3-ml portions of dry ether. Bring total volume to approximately 30 ml and stir an additional 5 minutes.

7. Stopper drying tube at top of condenser and let reaction mixture stand 1 hour to allow settling of suspended material. Quickly transfer solution to dry 50 ml volumetric flask. Wash round bottom flask with two 10-ml portions of dry ether and transfer to volumetric flask after allowing suspended material to again settle. Bring final volume of methylmagnesium iodide solution to exactly 50 ml.
8. In determining the amount of the Grignard reagent required for preparation of acetic acid, it was assumed that the yield from the above reaction described was 85%. Therefore, the total amount of Grignard reagent produced was assumed to be 25.8 mmoles (85% of 30.4 mmoles or 4.315 g methyl iodide).

Procedure adapted from Gilman and Meyers (41)

# APPENDIX V

## PREPARATION OF MOLD SPORES FOR INOCULATION

1. Add 100 g of garden soil (free of fertilizer and pesticides) to a 250 ml Erlenmeyer flask and plug with cotton. Sterilize at 121°C, 15 psig for 30 minutes. After cooling, inoculate soil with Aspergilus ochraceus NRRL 3174 spore suspension which was obtained by washing spores from potato dextrose agar slant with sterile distilled water. Incubate at 28°C for 10 days.
2. Fill a 1-liter Roux culture bottle with 100 ml Czapek's solution agar:

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

   Plug with cotton and sterilize at 121°C, 15 psig for 15 minutes. Cool to room temperature.
3. Inoculate Aspergillus ochraceus spores on agar face of Roux bottle by streaking repeatedly with sterile loopfuls of spores from the soil culture. Add 5 ml sterile water and distribute evenly.
4. Incubate at 25°C for four days in normal position then incubate upside down for an additional six days.
5. 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 5 minutes, then wash spores with two 150-ml portions of sterile distilled water by centrifugation.
6. Suspend spores in 150 ml of sterile distilled water and store in 50 ml bottles at 2°C.
7. 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 3-30 spores are compared after a four-day incubation period at 25 $^{o}$C and the number of viable spores per ml is calculated.

# APPENDIX VI

## MINERAL MIX

Mineral mix for use in sucrose-yeast extract A. ochraceus cultures:

1.0 g $KH_2PO_4$

0.5 g KCl

0.5 g $MgSO_4 \cdot 7H_2O$

24.4 mg $FeCl_3 \cdot 6H_2O$

21.99 mg $ZnSO_4 \cdot 7H_2O$

10.98 mg $MnSO_4 \cdot 5H_2O$

3.93 mg $CuSO_4 \cdot 5H_2O$

2.52 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$

in 1 liter distilled water.

Reference: Yamazaki et al. (116)

# APPENDIX VII

## BIOLOGICAL PREPARATION OF RADIOLABELED OCHRATOXIN

1. Pipette 9.4 ml mineral mix described in Appendix VI into 25 ml Erlenmeyer flask. Add 0.4 g sucrose and 0.2 g yeast extract. Plug with foam rubber and autoclave 20 minutes at 121°C. Allow to cool to room temperature.
2. Inoculate each flask with at least $10^4$ spores in 0.1 ml sterile distilled water with a syringe. Place flask in an incubator at 25°C for 2 days.
3. After initial 2-day incubation period, add to each 10 ml culture 1 mc of sodium acetate-1-$^{14}$C in 0.2 ml sterile mineral mix. Mix thoroughly by swirling then incubate an additional 5 days in a water bath at 25°C in a well vented hood.

# APPENDIX VIII

## EXTRACTION OF OCHRATOXIN A FROM LIQUID CULTURES

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 10 ml chloroform to each culture flask using a funnel to bypass the foam plug. The chloroform is rinsed around the flask by swirling, the plug is removed and the mycelial mat pushed back to allow decanting of the liquid portions of the cultures.
3. The liquid portions of the cultures are decanted into a 1-liter separatory funnel and the mycelial mat is then worked out of the flask with a glass rod and set aside for further extraction. The flask is rinsed with two 5-ml portions of distilled water and these washings transferred to the separatory funnel.
4. The liquid portion of the cultures in the separatory funnel is acidified to pH 2.0 with 1 N HCl and then extracted four times with two volumes of chloroform. The chloroform extract is drained from the flask and filtered through Whatman #1 phase separating paper.
5. The mycelial mats which were removed from the culture flasks are ground with a Virtis tissue homogenizer for 1 minute at full speed in 25 ml chloroform/mycelial mat. This is force-filtered through Whatman #1 paper. The filtrate is added to a separatory funnel, the chloroform phase drawn off and filtered through Whatman #1 phase separating paper. This chloroform extraction procedure is repeated two more times with the ground mycelial mat which was left after the initial filtration.
6. The ground mycelial mat contains additional ochratoxin which is

difficult to remove by further chloroform extractions. However, the majority of it can be removed using saturated sodium bicarbonate. The ground mycelial mat is placed in a Virtis homogenizer jar containing about 25 ml saturated sodium bicarbonate solution/culture mycelial mat and the mixture homogenized for 1 minute at full speed. This is force-filtered through Whatman #1 paper and the filtrate added to a separatory funnel, acidified to pH 2.0 with 1 N HCl and extracted three times with chloroform. The filtered mycelial mat is extracted two more times by the bicarbonate extraction procedure.

7. All chloroform solutions are combined, evaporated to dryness with a vacuum rotary evaporator, then dissolved in minimal benzene and stored at -10 $^{o}$C until further use.

# APPENDIX IX

## PURIFICATION OF OCHRATOXIN A BY COLUMN CHROMATOGRAPHY

1. Glass columns[7] with standard "O"-ring connectors at each end to permit connection to adapters are used for all chromatography of ochratoxins. At the bottom of each column is connected an "O"-ring adapter[8] with a tapered Teflon stopcock. The stopcock is plugged with cotton to prevent passage of the silica gel adsorbent. Another "O"-ring adapter[9] 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 75 g dried silica gel[10] in benzene is poured into a 500 x 25 (id) mm water-jacketed column. 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. Finally, the benzene is drained just to the top of the sodium sulfate layer.
3. The crude chloroform extract from twenty 10-ml liquid cultures dissolved in minimal benzene is applied 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

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[7] Jacketed Chromaflex extender, K-422430, Kontes Glass Co., New Jersey.

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

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

[10] Mallinckrodt SilicAR CC-7 100-200 mesh, dried 2 hours at 110°C.

sodium sulfate layer to aid in completely transferring the sample to the silica gel adsorbent. The headspace in the column is completely filled with benzene.

4. The column is connected to a gradient elution apparatus which provides a linear increase in eluting force (4). In the first of the two reservoirs, 1 liter benzene is added and in the second, 1 liter benzene:glacial acetic acid 93:7 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.

5. During elution the various bands are visualized briefly with a long-wave ultraviolet light.[11] The bright green ochratoxin A band is the first major fluorescent band to elute after elution of a thin green fluorescent band ahead of it. The ochratoxin A fraction is collected in toto then placed in a Ʇ 24/40 round bottom flask and evaporated to dryness with a vacuum rotary evaporator. 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. To remove any remaining glacial acetic acid, estimate its volume and add a portion of benzene that will give a final

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[11] Blak-Ray UVL-22, loc. cit.

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.

6. The dry ochratoxin A fraction is dissolved in 25 ml chloroform. This fraction is analyzed by spotting a 2 µl portion onto a silica gel thin-layer plate (Appendix X) and developing the plate with benzene:glacial acetic acid 9:1 v/v. The sample generally 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 partition column chromatography followed by bicarbonate fractionation and recrystallization.

7. A slurry of 30 g 1% water-treated silica gel[12] in carbon tetrachloride is packed into a 250 x 20 (id) mm unjacketed column. The column is equilibrated and a 1 cm layer of anhydrous sodium sulfate is layered on top of the silica gel. The carbon tetrachloride is drained to the top of the sodium sulfate layer.

8. The ochratoxin A fraction from the first larger preparative column is evaporated to dryness and one-half the amount present (ca. 10 mg ochratoxin A) applied to the column in minimal carbon tetrachloride. Three 5-ml portions of carbon tetrachloride are added to aid in transferring the sample to the silica gel.

9. The headspace of the column is filled with carbon tetrachloride and the column eluted with about 1.5 liters carbon tetrachloride: glacial acetic acid 92:8 v/v. The ochratoxin A band is collected in 50 ml fractions. The fractions are analyzed by thin-layer

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[12] Brinkman Silica gel 60 dried overnight at 110°C, cooled and 1% distilled water added and thoroughly mixed by vigorous shaking.

chromatography as described in section 6 of this Appendix, and those containing only ochratoxin A are combined and evaporated to a small volume in vacuo. The combined concentrated sample generally contains no other fluorescent impurities but will retain a slight straw-colored impurity which can be removed by bicarbonate fractionation and recrystallization.

# APPENDIX X

## PREPARATIVE AND ANALYTICAL THIN-LAYER CHROMATOGRAPHIC SYSTEMS

1. Preparation of thin-layer plates:
   Shake one part MN-silica gel G-HR[13] with two parts water for 1 minute. Spread plates 250 μ thick for analytical and 375 μ thick for preparative TLC. Useful plate sizes include 5 x 20 and 10 x 20 cm for analytical chromatography and 20 x 20 cm for preparative chromatography. After spreading plates, air dry 1/2 hour, then activate 2 hours at 110°C. Cool and store in desiccator.
2. Apply sample with micropipette about 1/2" from lower edge of chromatoplate then develop in solvent system of choice in an unlined chamber protected from the light. Useful solvent systems for separation of various ochratoxins and derivatives include:

   Solvent A: benzene:glacial acetic acid 9:1 v/v or 4:1 v/v.
   Solvent B: carbon tetrachloride:glacial acetic acid 9:1 v/v or 4:1 v/v.
   Solvent C: toluene:ethyl acetate:90% formic acid 5:4:1 by vol.
   Solvent D: benzene:methanol:glacial acetic acid 24:2:1 by vol.

   Plates are developed until solvent has moved 15 cm from the point of sample application.
3. After air drying, plates are examined with a 365 nm ultraviolet lamp.[14]
4. For preparative work, bands are scraped from developed plates into 95% ethanol and the silica gel removed by either filtration or centrifugation.

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[13] Brinkman Instruments, Inc., Westbury, New York.

[14] Blak-Ray UVL-22, loc. cit.

# APPENDIX XI

## BICARBONATE FRACTIONATION OF OCHRATOXINS A AND B

1. The ochratoxin A purified as described in Appendix IX or the ochratoxin B purified as described in Appendix XVI, is dissolved in about 200 ml chloroform and placed in a 500 ml separatory funnel. To this is added a 200 ml portion of 0.5 N sodium bicarbonate. The toxin is partitioned into the aqueous bicarbonate phase by shaking.
2. After separation of the two phases, the lower chloroform layer is drawn off 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 200 ml portions of 0.5 N sodium bicarbonate.
3. The combined bicarbonate extracts containing the ochratoxins are acidified to pH 2.0 with 2 N HCl and back extracted three times with an equal volume of chloroform.
4. The combined chloroform solution is dried by passing through a short column of anhydrous sodium sulfate. It is evaporated to dryness <u>in vacuo</u> and the ochratoxin dissolved in 25 ml benzene and held at $-10^{\circ}C$ until further use.

# APPENDIX XII

## RECRYSTALLIZATION OF OCHRATOXIN A

1. The ochratoxin A sample purified as described in Appendix XI is evaporated to dryness in vacuo and dissolved in minimal (ca. 5 ml) hot benzene. The solution is transferred to a 40 ml conical bottom centrifuge tube and hot hexane added dropwise until the solution just begins to get cloudy. This mixture is heated briefly to completely dissolve the ochratoxin, then is covered and placed in a refrigerator overnight at about 5°C. The crystals that form are collected by centrifugation. The mother liquor is removed and saved for second crops.
2. The crystallization is repeated three additional times. The final product, a white crystalline substance, contains one molecule benzene of crystallization (106). 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 vacuum rotary 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 -10°C.

# APPENDIX XIII

## QUANTITATION OF OCHRATOXINS AND DERIVATIVES

The following data are used in calculating the concentration of ochratoxins and their derivatives:

| Toxin | Molecular weight | $\lambda$max (nm) | $\epsilon$ | Solvent system[a] |
|---|---|---|---|---|
| Ochratoxin A | 403 | 333 | 5550 | A |
| Ochratoxin B | 369 | 318 | 6000 | B |
| Ochratoxin C (A ethyl ester) | 431 | 333 | 6200 | B |
| Ochratoxin B ethyl ester | 397 | 318 | 6700 | C |
| Ochratoxin a ethyl ester | 284 | 336 | 6250 | D |
| Ochratoxin A Alanine analog | 327 | 333 | 5550 | A |
| Ochratoxin A Leucine analog | 369 | 333 | 5550 | A |

[a]Solvent system A: benzene:glacial acetic acid 99:1 v/v.
Solvent system B: benzene:acetonitrile:glacial acetic acid 97:2:1 by vol.
Solvent system C: chloroform
Solvent system D: 95% ethanol

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

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

where A is the absorbance at the wavelength of maximum

absorption; MW is the molecular weight of the toxin; V is the volume in ml; and $\epsilon$ is the molar absorptivity of the toxin in its designated solvent system.

## APPENDIX XIV

## LIQUID SCINTILLATION COUNTING FLUOR SOLUTIONS

| | Fluor solution[a] components | Solution volume (ml) | Sample | | Counting[b] | |
|---|---|---|---|---|---|---|
| | | | Volume (ml) | Type | Efficiency[c] (%) | Background (cpm) |
| Dioxane | 440 ml dioxane, 2 g PPO, 100 mg POPOP, 30 g naphthalene, 50 ml methanol, 20 ml ethylene glycol | 10 | <. 1 | aqueous | 82 | 24 |
| | | 15 | . 1-3 | aqueous | 65-82 | 24 |
| | | 10 | 0-1 | TLC scrapings | 82 | 24 |
| Tissue dioxane gel | 1 L dioxane, 50 ml toluene, 100 g naphthalene, 16 g PPO, 1 g POPOP, 50 g cab-o-sil | 20 | 1 | Tissue OH[e] digest | 80 | 30 |

[a] PPO: 2, 5-diphenyloxazole (Scintillation grade); POPOP: 1, 4-bis[2-(5-phenyloxazolyl)] (Scintillation grade); Naphthalene, dioxane, toluene, methanol and ethylene glycol (reagent grade); Cab-o-sil: Godfrey L. Cabot, Inc.

[b] Nuclear Chicago Liquid Scintillation Spectrometer.

[c] Efficiency determined by optimum settings of unquenched samples. Channels ratio and internal standard used to determine efficiency of samples.

References: Bray (5) and Tye and Engel (104).

# APPENDIX XV

## DEHALOGENATION OF OCHRATOXIN A

1. Dissolve 10 mg pure ochratoxin A in 5 ml 0.25 M methanolic KOH and place in a 100 ml Ⓢ 24/40 round bottom flask. Then 10 mg catalyst[15] is added, magnetic stirrer supplied and the flask placed on a Brown hydrogenation apparatus.[16]
2. The flask is purged twice with hydrogen and hydrogenation begun. Hydrogenation is usually complete after about 3 hours. The course of the reaction can be followed by removing the flask from the hydrogenation apparatus and spotting an aliquot of the methanol mixture on a silica gel chromatoplate alongside ochratoxin A and B standards. Develop the plate in benzene: glacial acetic acid 9:1 v/v.
3. When the ochratoxin A is no longer present or is present only in trace quantities, the reaction is stopped and the contents of the round bottom flask transferred to a separatory funnel. Wash the sides of the flask with distilled water and transfer to the separatory funnel.
4. Acidify the solution to pH 2.0 with 1 N HCl and extract three times with an equal volume of chloroform. Evaporate the chloroform extract to dryness in vacuo. Dissolve in 25 ml benzene and store at -10°C until further use.

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[15] Ten percent palladium-on-carbon, Engelhard Industries, Inc., Newark, New Jersey.

[16] Delmar Scientific, Maywood, Illinois.

# APPENDIX XVI

## COLUMN CHROMATOGRAPHY OF OCHRATOXIN B

1. The ochratoxin B produced in Appendix XV will likely contain a trace of ochratoxin A. Also, the concentrated solution will have a slight straw color which must be removed before the sample can be used in biological studies.
2. Slurry 30 g 1% water-treated silica gel 60[17] in carbon tetrachloride and pour into a 250 x 20 (id) mm unjacketed column and equilibrate as previously described in Appendix IX. Pour a 1 cm layer of anhydrous sodium sulfate on top of the silica gel and lower the solvent level to the top of this layer.
3. Evaporate the ochratoxin B fraction produced by dehalogenation of 10 mg ochratoxin A to dryness and dissolve in minimal carbon tetrachloride and apply to the silica gel. Three 5-ml portions of carbon tetrachloride are added to aid in transferring the sample to the silica gel.
4. The headspace of the column is filled with carbon tetrachloride and the column eluted with about 2 liters carbon tetrachloride: glacial acetic acid 85:15 v/v. The eluting blue ochratoxin B band is visualized briefly with an ultraviolet lamp.[18] It is collected in 50 ml fractions. The fractions are analyzed by TLC along with an ochratoxin B standard to determine the degree of purity. Fractions containing only ochratoxin B are combined, evaporated to dryness in vacuo, then dissolved in chloroform and stored at -10°C until further use. The combined concentrated sample should be free of other fluorescent impurities but

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[17] Brinkman Silica gel 60, loc. cit.

[18] Blak-Ray UVL-22, loc. cit.

will retain the straw color which can be removed by bicarbonate fractionation as described in Appendix XI.

# APPENDIX XVII

## TISSUE DIGESTION FOR SCINTILLATION COUNTING

1. Weighed tissues are placed in screw-capped vials or test tubes and 0.5 N NaOH added at a concentration of 4 ml/g tissue.
2. The tissues are digested in a 60 $^{o}$C oven with occasional mixing until a homogenous mixture is obtained. One ml of this mixture is transferred to a counting vial and if highly colored, 3 drops of hydrogen peroxide added to bleach the sample. Twenty ml tissue dioxane gel (Appendix XIV) is added and the sample mixed and counted in a liquid scintillation spectrometer to 3% standard error. The total volume of the digest is measured and recoveries calculated by dividing the dpm found in each whole organ by the amount of dpm of the dosed ochratoxin-$^{14}$C. Recoveries in blood are based on blood representing 5% of the body weight of trout (92). Counting efficiencies are determined by internal standardization with $^{14}$C-toluene.

Reference: Tye and Engel (104)