Pure & Appl. Chem., Vol. 64, No. 7, pp. 1029–1046, 1992.
Printed in Great Britain.

INTERNATIONAL UNION OF PURE
AND APPLIED CHEMISTRY

APPLIED CHEMISTRY DIVISION
COMMISSION ON FOOD CHEMISTRY*

OCHRATOXIN A: A REVIEW

(Technical Report)

Prepared for publication by

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Ochratoxin A: A review (Technical Report)

Abstract - Ochratoxin A is a mycotoxin of current interest from a public health point of view; consequently, a considerable effort has been and is being expended to document human and animal exposure to this mycotoxin and the consequences of such exposure. This paper reviews the current status of knowledge with respect to ochratoxin A.

INTRODUCTION

In recent years the attention of most mycotoxinologists has been focused to a great extent on the continuing quest for better ways to control exposure to aflatoxins, resolution of the questions relating to aflatoxin's acute and chronic toxicity (i.e., carcinogenicity) for humans, and an ever-expanding study of the myriad metabolites of the Fusaria (especially trichothecenes). Study of the ochratoxins, except in some localized areas of the world (e.g., Denmark, Sweden), has generally been of much lower priority. This situation seems to be changing, as evidenced by the recent efforts of public health officials in various countries (Germany, Canada, United States) to develop estimates of human risk from exposure to ochratoxin A. The main difficulty in developing such risk estimates seems to be the paucity of data on human exposure to this mycotoxin and comparative toxicology data for translating observations in animals to humans. It has always been somewhat of an enigma that human exposure to ochratoxin seems to be localized in some countries of northern Europe and the Balkans and may possibly be responsible for a serious human nephropathy, but is not generally found in other areas of the world. Nevertheless, the International Program on Chemical Safety/World Health Organization, in its latest review of environmental contaminants, has recently selected ochratoxin as one of the mycotoxins for which sufficient new information indicates a need for further study and evaluation with respect to human health potential. Furthermore, the Commission on Food Chemistry, IUPAC, has embarked on a worldwide effort to document the natural occurrence of ochratoxin. It is the purpose of this paper to summarize recent information on the occurrence, toxicology and public health significance of human exposure to ochratoxin A.

CHEMISTRY AND METHODOLOGY

Biosynthetically the ochratoxins are pentaketides, consisting of a dihydro-isocoumarin coupled to ß-phenylalanine (ref. 1), with the following properties:

White, odorless, crystalline solid
MP 168-173°C (90°C, benzene)
α_D^{21} -46.8° (2650 µmol/L, $CHCl_3$)
Mol. Wt. 403.8

λ_{max}^{MeOH} 214 nm (ϵ_{max} 37,200),
282 nm (ϵ_{max} 890),
332 nm (ϵ_{max} 63,300)

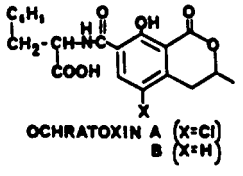

Fig. 1.
Structure of ochratoxin A.

Only ochratoxin A, and very rarely its dechloro analog, ochratoxin B, have been found in foods and feeds, whereas in culture filtrates of the generating molds, the methyl and/or ethyl esters as well as the 4-hydroxy-derivative and the isocoumarin nucleus of ochratoxin B (ochratoxin α) have been observed (ref. 2). These compounds are produced by a variety of fungi, with the more frigophilic Penicillia (e.g., Penicillium viridicatum, P. verrucosum) being the primary producers in the cooler areas of the world (ref. 3).

ANALYTICAL METHODOLOGY

Several excellent reviews of analytical methods for determining ochratoxin A in foods and feeds (refs. 4, 5) are available. In general the major steps in all of the methods are the same: lot sampling, test sample preparation, extraction, cleanup, determination, confirmation of identity. Because the ochratoxin in most commodities is heterogeneously distributed, a relatively large random primary sample must be taken from the lot. In the case of grain, a 5-10 lb. sample is usually taken. This primary sample is then ground fine enough to pass a U.S. No. 20 sieve and blended thoroughly, and a 50-g test portion is withdrawn. It has been found that ochratoxin A in grains tends to degrade after grinding as a result of enzyme activity; consequently the test portion must be analyzed (extracted) immediately after grinding. The cleanup step most frequently used involves extraction into a basic solution ($NaHCO_3$), followed by acidification and partition into chloroform.

The method that has received the widest acceptance has been collaboratively studied by the Association of Official Analytical Chemists (AOAC) and subsequently adopted by that organization as an official method (ref. 6). The IUPAC also selected it as a recommended method. In one of the earliest multimycotoxin methods, and one that has been extensively used (refs. 7, 8), the chloroform extract is put directly onto a silica gel column and aflatoxin and zearlenone are eluted; finally ochratoxin is eluted with acetic acid-benzene and determined as the free acid by thin-layer chromatography (TLC) and visual estimation or by TLC-densitometry.

In all methods using fluorescence, sensitivity can be greatly improved by exposure of the developed TLC plate to NH_3, which converts ochratoxin A to its ammonium salt and in so doing shifts the absorption maximum from 330 to 370 nm and increases the fluorescence emission at 460 nm. An added advantage of this technique is that the salt (anion form) of ochratoxin A appears to be more stable on a TLC plate than the free acid.

An excellent high-performance liquid chromatographic (HPLC) method (ref. 9) applicable to the parts-per-billion level has been developed. The method includes the silica gel column cleanup described above preceded by an acid-base partition. A minicolumn screening method (ref. 10) has also been developed.

Most of the older methods are time-consuming and use considerable amounts of solvent. To avoid these disadvantages, a method has been developed and validated which traps the ochratoxin on a 0.5-g C_{18} column impregnated with $NaHCO_3$. This method has been evaluated in a joint AOAC-IUPAC collaborative study (ref. 11), and on the basis of the results, was recommended to the AOAC for official first action status. The method consists of extraction of the test portion (50 g) by blending 3 min with 25 mL of 0.1 M H_3PO_4 + 250 mL of $CHCl_3$, filtering, and extracting the filtrate with 3% $NaHCO_3$. The ochratoxin is trapped on a methanol-$NaHCO_3$-C_{18} column and recovered from the column by elution with EtOAc-MeOH-HOAc. Ochratoxin A in the extract is determined by HPLC.

Over the last 15 years a number of immunochemical methods have been developed. These are very sensitive, specific and rapid. Some of these methods have been incorporated into test kits in the last few years and are commercially available.

As is the case with all methodologies for trace contaminants, a source of standard material is required. Commercial sources of standard materials are listed in Table 1. The standard material as purchased, and solutions prepared from it, must be characterized for concentration by UV absorption measurements and for chromatographic purity by HPLC or TLC. Solution concentrations of about 30 μg/mL are required for adequate UV response, and about 100 μg/spot is needed for a TLC purity check.

The identity of ochratoxin A in "positive" test extracts is usually confirmed by formation of the methyl or ethyl ester derivatives (ref. 6), followed by rechromatography (TLC, HPLC) or gas chromatography-mass spectrometry.

Table 1 Commercial Sources of Ochratoxin A and Approximate Cost

Makor Chemical Co.	$9.00/mg
Aldrich Chemical Co.	12.25/mg
Sigma Chemical Co.	22.25/mg
CSIR, S. Africa	7.00/mg
Chemical Dynamics Corp.	13.20/mg

Table 2 Incidence of Ochratoxin A in U.S. Grains

Commodity	Incidence No. (% Positive)	Level (ng/g)	Reference
Barley	164 (14)	<10-29	13
Barley	127 (14.2)	10-40	3
Barley malt	138 (0)	---	13
Corn	283 (0.3)	130	12
Corn	293 (1)	83-166	14
Corn	300 (0)	---	13
Feed	102 (1)	27	13
Feed	395 (2)	t-191	13
Oats	28 (2)	52, 110	13
Sorghum	32 (0)	---	13
Wheat	848 (1.3)	15-115	15

NATURAL OCCURRENCE

Since the first report by Shotwell, Hesseltine and Goulden of ochratoxin A as a natural contaminant of corn (ref. 12), many attempts have been made to document the occurrence of this mycotoxin in foods and feeds. In a vigorous, continuing survey effort in the United States (Table 2), less than 2% of test all samples analyzed contained ochratoxin. In these surveys, ochratoxin was found most frequently in barley, but at the highest level in corn.

The worldwide occurrence of ochratoxin A contamination of raw agricultural products (Table 3) has been amply documented (refs. 3, 16-18).

Comparatively high levels have been reported in some types of feed. In the United Kingdom, where an effort was made to measure the occurrence of ochratoxin A in agricultural products (although the actual numbers of test samples analyzed were quite low), the incidence of test samples contaminated (16-40%) and levels found (50-100 μg/kg) were much higher than those encountered in the United States (ref. 3). Similar high frequencies of contamination have been observed in other Balkan countries where nephropathy is endemic; e.g., in areas of Bulgaria with endemic nephropathy the incidence of contamination of corn was reported to be 27.3% (25-35 μg/kg) (ref. 19). In northern European countries, high ochratoxin A contamination of certain agricultural commodities has also been documented; e.g., in the Federal Republic of Germany, in a 5-year survey, 12.9% of 984 feed test samples were found to contain ochratoxin at levels up to 206 μg/kg (ref. 20).

It is not surprising, given the significant occurrence of ochratoxin A in raw agricultural products, that fluids and tissues of animals exposed to

Table 4 Transmission of Ochratoxin A from Feed to Tissues

Species	Level in Feed (ng/g)	Tissue	(Contamination Level) (ng/g)	Reference
Broiler chickens	50-2000	Kidney Liver Muscle Blood	0.8 11-59 3.0-8.5 1.2-4.6	18
Laying hens	50-5200	Kidney Liver Muscle Blood Eggs	3.8-8.0 1.5-18 ≤0.8-2.7 4.0-14 1.6-4	18
Cow	1125	Kidney	5	21
Pigs	25-1400	Kidney Liver Muscle Fat Blood	1.8-67 2-30 ND-37 ND-11 665	18

Table 5 Natural Occurrence of Ochratoxin A in Pig Tissues/Fluids (18)

Tissue/Fluid	Incidence No. (% Positive)	Level (ng/g, ng/mL)
Blood	767 (11)	2-270
Blood serum	2989 (39)	0.1-520
Kidney	10407 (39)	0.1-200
Liver	78 (6)	20-100

Table 3 Worldwide Occurrence of Ochratoxin A in Raw Agricultural Products

Country	Commodity	Incidence (%)	Max. Level (ng/g)	Reference
Australia	Feeds	<1	70,000	18
Austria	Feeds	5-10	1,000	18
Canada	Feeds	<1	6000	16,17
	Heated grains*	50-60	27000	17
	Wheat, barley	<1	51	18
	Dried peas, beans	1-5	21	18
Czechoslovakia	Feeds	100	17	18
Denmark	Feeds*	50-60	27500	3,17
	Barley malt	6	189	3
	Wheat, rye	1	50	3
Finland	Feeds	30-40	100	18
France	Corn	1-5	200	16,17
Germany (FRG)	Barley	10-20	206	18
	Corn	5-10	82	18
	Feeds	10-15	13	18
	Oats	10-15	59	18
	Wheat	5-10	137	18
(GDR)	Corn	1-5	22	17
Hungary	Feeds	?	?	18
India	Grains	5-10	2000	16
	Sorghum	5-10	?	18
Indonesia	Feed	?	500	18
Italy	Corn	40-50	1	18
Poland	Feed	1-5	200	18
	Grains	5	200	16
	Grains	?	3000	17
	Rye	15-20	200	18
	Wheat	10-15	100	18
Sweden	Barley/Oats	5-10	410	16
Taiwan	Chicken feed	40-50	?	18
United Kingdom	Corn	30-40	500	18
	Feeds	1-5	250	18
	Oats	5-10	80	18
	Wheat/Barley	10-15	5000	18
Yugoslavia	Corn	25-30	5125	3
	Grains	60-70	68900	18

*Visibly moldy.

ochratoxin A in the feed would be found contaminated. This transmission of ochratoxin A to animal fluids and tissues has been documented (Table 4).

In pigs it has been observed that the kidney is generally the most heavily contaminated tissue, and that the levels in the blood are about five times as high (ref. 22) as in the kidney. As a practical illustration, Krogh et al. (ref. 23) have calculated that if the level of ochratoxin A in swine kidney is 12.1 ng/g (resulting from about 1000 ng/g in the feed), the other levels would be expected to be 7.8 ng/g in the liver, 4.2 ng/g in the muscle and 2.8 ng/g in the adipose tissue. Ochratoxin A in ruminants is usually hydrolyzed in the forestomach by protozoans and bacterial enzymes, and consequently little ochratoxin is found in the tissues (ref. 21).

The natural occurrence of ochratoxin A in animal tissues and fluids has been well documented, particularly with respect to occurrence in pig tissues/fluids (Table 5).

Table 6 Worldwide Occurrence of Ochratoxin A in Human Foodstuffs

Country	Commodity	Incidence (%)	Max. Level (ng/g)	Reference
Brazil	Cassava flour	<1	65	28
	Dried beans	<1	160	28
	Dried white corn	<1	32	28
Bulgaria*	Beans	17	27	18
**	Beans	7	50	18
*	Corn	27	35	18
**	Corn	9	25	18
Czechoslovakia	Cereals	5-10	17.5	18
France	Beer	10	110	18
Germany (GDR)	Coffee beans*	50	90	17
(FRG)	Sausage	17.8	3.4	18
India	Cocoa pdts.	<1	50	18
Italy	Bread*	?	80000	18
	Green coffee	20-30	23	18
Japan	Rice*	?	430	3
	Green Coffee	1-5	46	18
	Roasted coffee	5-10	17	18
Morocco	Olives/oil	1-5	80	18
Norway	Cereals	1-5	180	18
Poland	Cereals	5-10	1200	18
	Flours	20-30	100	18
	Gruels	5-10	20	18
Sweden	Beans	5-10	442	3
	Peas	1-5	10	3
	Barley	1-5	11	17
Switzerland	Figs	10	160	18
	Flour (brown)	90	1.9	18
	Sausages	8	0.8	18
	Wheat pdts.	90	3.5	18
Tunisia	Couscous			18
United	Bread*	40-50	80	18
Kingdom	Cereals	10-15	108	18
	Cocoa beans, raw	18	500	3
	Cocoa beans, roasted	15.8	100	3
	Coffee, green	20-30	200	18
	Corn flour	30-40	200	3
	Corn oil	30	50	18
	Flour*	20-30	6250	17
	Kidney (pork)	15.5	44	18
	Nuts	40	1	18
	Soya bean	36	500	3
	Soya flour	19	500	3
	Wheat flour	28	2900	29
United States	Coffee beans (green)	5	360	30,31
Yugoslavia	Corn/Beans	40	5000	18
	Beans	5-10	53	18
	Bread*	19	?	32

*Moldy.

In northern Europe and Scandinavia, it is common practice to examine pig kidneys for signs of ochratoxin pathology; typically about 5% of the kidneys examined contain ochratoxin A (refs. 24-26). The vast majority of kidney specimens analyzed (from swine with nephrotic kidneys) contained <10 ng of ochratoxin A/g; the highest level reported, from the United States, was 200 ng/g. The contamination level appears to vary considerably with geographic location; for example, according to a recent report (ref. 27), 52% of 85 specimens of swine blood collected in Germany contained ochratoxin A at levels up to 17.6 ng/g (median level, 0.85 ng/g). What is somewhat surprising is the high incidence of positive specimens in areas where the documentation of ochratoxin A in feeds is lacking.

The natural occurrence of ochratoxin A in human foods has been observed (Table 6).

Clearly these data are insufficient to estimate human exposure. The published data do indicate higher exposure levels in areas of the Balkans with endemic nephropathy vs. non-endemic areas. The highest reported levels of ochratoxin A (2900 ng/g) were observed in moldy wheat flour in the United Kingdom and a moldy bread test sample (80,000 ng/g) in Italy. Although transmission to eggs in hens fed ochratoxin A has been demonstrated (ref. 18), no natural occurrence of ochratoxin A in commercial eggs has been reported, nor have there been any reports of contamination of commercial chicken meat with ochratoxin. Given the frequent occurrence of ochratoxin A in pig blood and tissues in Scandinavian countries and the finding of ochratoxin A in human blood in some countries of northern Europe, it is somewhat surprising that so little documentation of contamination of pork sausage from these areas is available; in a small survey of pork-containing sausage in the United States (ref. 33), no ochratoxin A was found.

The fate of ochratoxin A after processing and cooking has been investigated (Table 7) with mixed results.

Table 7 Ochratoxin A: Fate

Commodity	Process	% Reduction	Reference
Barley (4 ng/g)	Ensiling	68	34
Cheddar cheese	Storage (48 h, 25°C)	41	18
Coffee beans	Roasting	97-100	35
Coffee beans	200°C, 10-20 min	0-12	18
Coffee, green	198-240°C	80-88	24
Coffee, ground	Brewing	100	35
Corn	Wet milling	4% in germ 51% in bits	18
Corn	Ammoniation (96 h, 70°C, 5% NH_3)	95	18
Faba beans	Cooking	16-20	36
Flour, bread	Baking (220°C, 25 min	0	24
Flour, biscuits	Baking (180°C, 5½ min)	62	24
Pig kidneys	Storage (-18°C, 7½ years)	0	18
Polished wheat	Cooking	6	36

The experimental results reflect the general thermal/hydrolytic stability of ochratoxin A, although it should be remembered that one of the problems encountered in developing analytical methods for ochratoxin A in grains was the instability (presumably enzymatic) of this compound after grinding of the grain during test sample preparation. In the processing of flours contaminated with ochratoxin A, it appears that some loss of toxin occurs, depending on the end product. The preparation of blood sausages and puddings results in slight losses. Roasting of coffee beans results in anywhere from 12 to 100% destruction of ochratoxin A depending on the conditions used; brewing of coffee results in 100% loss of ochratoxin A. As was the case with aflatoxin, ammoniation is effective in removing ochratoxin A.

Given the lack of evidence for significant human exposure to date, it is surprising to note reports on the occurrence of ochratoxin A in human blood and milk (Table 8).

Table 8 Natural Occurrence of Ochratoxin A in Human Specimens

Tissue/Fluid	Incidence No. (% Positive)		Level ng/g(mL)	Country	Reference
Blood serum*		(26)	20(mean)	Bulgaria	37
Blood serum**		(7.7)	10(mean)	Bulgaria	37
Blood serum*	143	(24.5)	To 1.26	Czechoslovakia	38
Blood serum	306	(56.6)	0.1-14.4	Germany(FRG)	21
Blood serum	216	(4)	1.3-4.8	Poland	18
Blood serum*	420	(6)	1-40	Yugoslavia	18
Blood serum**	219	(8)	1-10	Yugoslavia	18
Kidney	46	(6.5)	0.1-0.3	Germany(FRG)	18
Milk	36	(11)	0.017-0.03	Germany(FRG)	18

*Collected from areas with endemic nephropathy.
**Collected from non-endemic areas.

BIOLOGICAL EFFECTS OF OCHRATOXIN A

Ochratoxin A has been shown to be a potent nephrotoxin in all animal species tested, with the exception of mature ruminants (ref. 39). Based on field studies carried out in Denmark, Hungary, Scandinavia and Poland, this toxin is thought to constitute an important etiologic agent in the disease called porcine nephropathy (ref. 3). The disease, characterized by degeneration of the proximal tubules, atrophy of the tubular epithelium, interstitial fibrosis in the renal cortex and hyalinized glomeruli, has been reproduced in animals under laboratory conditions by the acute or chronic administration of ochratoxin A (refs. 40-42). Renal lesions in poultry have also been associated with ingestion of this toxin (ref. 43).

A. Acute and subacute effects

Species as well as sex and route of administration affect the toxicity of ochratoxin A. LD_{50} values range from 3.4 mg/kg(bw) for white leghorn chickens to 30.3 for male rats. Female rats are more sensitive. The intraperitoneal route of administration is more effective than the oral route (ref. 3). The kidney is a target organ, as evidenced by functional and morphological changes, but other effects have been observed as well. For example, in the rat abnormalities were noted in liver and heart morphology and in blood clotting factors (refs. 40, 44, 45). In mice administered 40 mg of ochratoxin A/kg(bw) ip and chickens fed 2-4 mg of the toxin/kg diet, evidence of myelotoxicity was seen (refs. 46, 47). Ochratoxin A was seen in cows during milk production (refs. 3, 48). Intestinal fragility was one consequence of feeding an ochratoxin A-containing diet (2.8 mg/kg(bw)) to broiler chickens for 3 weeks (ref. 49), and feeding 1.0 mg/kg of toxin in the diet of white leghorn chickens reduced egg production by about 14% (ref. 50). An early study revealed major gastrointestinal as well as renal and lymphoid tissue lesions in male and female guinea pigs treated daily with 5.6-20 mg/kg(bw) of ochratoxin A for 14 days (ref. 51). Ultrastructural examination of kidneys from pigs treated with ochratoxin A (800 μg/kg(bw)/daily for 5 days) indicated a process in which cellular materials were condensed and membranes disappeared in the proximal convoluted tubules (ref. 41). Probably contributing to the toxicity of ochratoxin A was the finding of a high degree of bioavailability (refs. 52, 53), low plasma clearance rate (refs. 52, 54) and long tissue half life (refs. 23, 55) of the toxin after its ingestion by the mammals studied.

B. Chronic effects (tumorigenesis)

In the first report (1971) of the possible carcinogenicity of ochratoxin A by Purchase and Van der Watt (ref. 56), one rat developed "hamortoma" of the kidney in a group of Wistar-derived rats, each of which received 300 mg of ochratoxin A 5 days a week for 50 weeks. Kanisawa and Suzuki (ref. 57) reported renal and hepatic tumors in male ddY mice after they were fed a diet containing 40 mg/kg for 45 weeks and sacrificed at 50 weeks. In a study conducted by Bendele et al. (ref. 58), 24 of 49 B6C 3F1 mice given a diet containing 40 mg of ochratoxin A/kg for 20 months developed renal carcinoma. Like the males, the treated female mice had nephropathy but no renal tumors. Both male and female mice fed diets containing ochratoxin A had a slightly increased incidence of hepatocellular neoplasms (ref. 58).

In a study recently carried out for the National Toxicology Program (NTP), using male and female F344/N rats, the carcinogenic activity of ochratoxin A was confirmed, although again the female animals were relatively resistant; 72% of the males and 16% of the females developed renal tubule neoplasms (adenomas and carcinomas) after 2 years at an ochratoxin A dose of 210 mg/kg(bw); the incidence was 39% and 4%, respectively, for males and females treated with the next lowest dose of ochratoxin A (70 mg/kg(bw)). At these two dose levels virtually all the rats, male and female, had renal tubule karyomegaly and renal tubule degeneration. In female rats an increased incidence of mammary gland fibroadenomas was also observed (ref. 59).

Thus, the evidence is compelling for ochratoxin A to be considered as a renal carcinogen, at least in the species and strains of animals studied during long-term feeding regimens. Whether the tumors are secondary to chronic injury of the kidney cells is a question that must await further studies.

C. Mutagenicity and genotoxicity

Use of several standard systems designed to measure the mutagenicity of test compounds yielded negative results for ochratoxin A. These tests included Ames-type assays in which several strains of Salmonella typhimurium are used to measure 8-azaguanine resistance in C3H mouse carcinoma cells or changes at the ade 2 locus of Saccharomyces cerevisiae (ref. 3). Ochratoxin A produced single-strand DNA breaks in CHO and AWRF cell lines, but only at the very high concentration of 200 μg/mL (ref. 60). However, the apparent genotoxicity of the toxin has been demonstrated in in vivo experiments in which DNA single-strand breaks were observed in the livers and kidneys of rats (ref. 55) and livers, kidneys and spleens of mice (ref. 61) by alkaline elution methods. The rats were administered a dose of 288.8 μg/kg(bw) by intubation each 48 h for 12 weeks; mice were given a single ip dose of 2.5 mg/kg(bw). DNA damage occurred within 24 h after treatment. These workers also reported single-strand breaks in PHA-stimulated spleen cells in culture exposed to ochratoxin A at 2.48×10^{-5} M. Furthermore, the results of a hepatocyte primary culture/DNA repair test were positive for ochratoxin A (although weakly positive relative to some other mycotoxins tested) (ref. 62), suggesting that this compound may indeed be a genotoxic agent.

In another study, Meisner and Cimbala (ref. 63) reported that ochratoxin A affected gene expression in rat kidneys but apparently did so by a post-transcriptional mechanism. Very recently Manolova et al. (ref. 64) demonstrated chromosomal aberrations, particularly on X chromosomes, produced in human lymphocytes in culture by ochratoxin A at a concentration of 15 nM, possibly a type found in lymphocytes of patients suffering from endemic nephropathy. In any case, such studies provide limited evidence that, at least in some cases, ochratoxin A may exert its carcinogenic action directly through a genetic mechanism.

D. Reproductive effects

Abnormalities involving limbs, neck, viscera and body size have been noted in chick embryos after the injection of ochratoxin A into the air sacs of fertile eggs at doses as low as 0.0005 mg/egg (ref. 65). Teratogenic as well as other developmental effects have resulted from treatment of pregnant mice, rats and hamsters with ochratoxin. A rather broad range of gestational periods was studied, as the following examples show. A 5 mg/kg(bw) interperitoneal dose of ochratoxin A given to pregnant mice on one of gestation days 7-12 produced a spectrum of effects ranging from fetal malformations to prenatal mortality. Exencephaly and anomalies of the eyes, digits and tail were the most common defects (ref. 66). Treatment of pregnant mice fed diets containing different levels (2 and 3 mg/kg) of ochratoxin A by gavage on gestation day 8 reduced prenatal survival and inhibited fetal growth in all groups at the higher dose. The extent of gross malformations associated with ochratoxin A were dependent on the dietary protein level; eye, limb and tail malformations increased in the lowest dietary protein group, whereas skull and facial defects were noted in all groups (ref. 67).

In mice treated ip with ochratoxin A at 5 mg/kg(bw) on day 11 or 13 of pregnancy, pyknotic cells began to increase in the telencephalon of the fetal brain 12 h after injection and peaked between 36 and 48 h, coinciding with

the time of peak concentration of the toxin found in the embryo (ref. 68). More severe effects, e.g., cerebral necrosis in the fetuses, resulted from the oral and ip administration of ochratoxin A (3-5 mg/kg(bw)) to two strains of mice on days 15 through 17 of gestation (ref. 69). In rats administered ochratoxin A (1.75 mg/kg(bw)) as a single subcutaneous injection, the highest number of resorptions, and largest number of malformations (external hydrocephaly, omphalocele, anophthalmia, internal hydrocephaly and abnormalities of the sternebrae, vertebrae and ribs) occurred when the toxin was injected on day 5, 6 or 7 of gestation (ref. 70). In another study, the incidence of gross malformations in rat pups was greater when pregnant rats treated with ochratoxin on day 7 were partially nephrectomized to impair kidney function (ref. 71).

E. Immunotoxicity

There is now ample evidence, resulting from studies involving several species of animals, that under certain conditions of treatment, ochratoxin A can produce defects in the structure and/or function of elements comprising the immune system. The leucocyte count was dramatically decreased in guinea pigs administered ochratoxin A at 5.6 mg/kg(bw) (the lowest dose) by gastric intubation for 14 days; necrosis and edema were also observed in the lymph nodes, spleen and thymus (ref. 52). Lymphocytopenia and a regressed thymus were observed in turkey poults given ochratoxin A (4 and 8 mg/kg feed) from hatching until 3 weeks of age (ref. 72). Ochratoxin A suppressed the immune response to sheep erythrocytes in BALB/c mice at single ip doses as low as 0.005 mg/kg(bw). This effect was prevented by simultaneous treatment with phenylalanine at approximately twice the dose of the ochratoxin A (ref. 73). The weight of the spleen and splenic cell count, total bone marrow cells and precursors of erythrocytes, leucocytes and megakaryocytes in the femoral bone marrow were decreased in mice after 6 weekly ip injections of ochratoxin A at 5 mg/kg(bw) per week (ref. 74). Similar results were found in another study in which mice were administered 20-80 mg/kg(bw) of ochratoxin A ip on alternate days over an 8-day period. Besides evidence of nephrotoxicity and myelotoxicity (bone marrow hypocellularity), thymic mass was decreased to 33% of the controls. Interestingly, however, phagocytic capacity and capacity to inhibit tumor cell growth were increased (ref. 46).

In guinea pigs (475-525 g) administered 0.45 mg of ochratoxin per day for 4 weeks, no treatment-related effect was seen on complement activity or antibody response to B abortus antigen, but the level of serum ß-globulin was lowered (ref. 75). More recently, however, ochratoxin A, 3.4-13.4 mg/kg(bw), administered orally or subcutaneously daily over a 12-day period, was shown to specifically inhibit natural killer cell activity, apparently by inhibiting production of basal interferon. The growth of transplantable tumor cells was increased (ref. 76).

A study designed to test the reversibility of ochratoxin A-induced myelotoxicity revealed that in female mice treated ip with ochratoxin (total dose: 20 mg/kg(bw) over a week), suppression of marrow granulocyte macrophage progenitors (CFU-C) returned to normal by 2 weeks. Reduction in CFU-C by whole body radiation was more pronounced in ochratoxin-treated mice, and recovery from radiation-induced lowering of CFU-C and peripheral white blood cell count was delayed by ochratoxin A in a dose-related fashion (ref. 77). Contradictory to the immunologic effects of ochratoxin A in mammalian systems was the finding that, although injection of 2.5 mg of ochratoxin A into 13-day-old chick embryos via the chorioallantoic membrane slightly suppressed growth of immunoglobulin-bearing cells of the bursa, chicks hatched from these treated embryos responded as well as controls to an antigen (*Escherichia coli* bacterin) and resisted infection when challenged with B-hemolytic *E. coli* (ref. 78).

F. Metabolic effects

One of the first observed and most dramatic effects of ochratoxin A on metabolic systems was its *in vitro* and *in vivo* inhibition of protein synthesis. An ip dose of 1 mg/kg(bw) given to mice produced a 50% inhibition of protein synthesis in kidney, whereas 13 mg/kg(bw) was necessary to achieve this degree of inhibition of protein synthesis in the liver. This inhibition was prevented by administering phenylalanine concurrently with the toxin, which was evidence that the inhibition resulted from competition of

ochratoxin with phenylalanine in the reaction catalyzed by phenylalanyl-t-RNA synthetase (ref. 79). Ochratoxin A and a metabolite of ochratoxin A, (4R)-4-hydroxyochratoxin A, were subsequently demonstrated to directly inhibit the yeast t-RNA aminoacylation reaction (ref. 80). The extreme sensitivity of these reactions and of protein synthesis in general to ochratoxin A prompts speculation that other effects of the toxin - e.g., immunosuppression - may be secondary to or at least related to these particular biochemical effects.

The addition of phenylalanine to the culture media of hepatoma tissue cells prevented the inhibition of cell multiplication by ochratoxin A. RNA synthesis (after a lag) but not DNA synthesis by these cells was also inhibited by ochratoxin A. This inhibition apparently was also reversed by phenylalanine (ref. 81). DNA synthesis was mildly inhibited in CHO and AWRF cells by ochratoxin A at concentrations of 1.2×10^{-4} M (ref. 62). Ochratoxin A concentrations greater than 5×10^{-6} M inhibited RNA, DNA and protein synthesis at about the same degree in mouse L cells (ref. 82). Braunberg et al. (ref. 83), using minipig cortical explants as a test system, found that 10^{-6} M ochratoxin A inhibited RNA, DNA and protein synthesis. At 25 μM, ochratoxin A inhibited the activity of several renal tubular enzymes (e.g., gamma-glutamyl transferase, leucine aminopeptidase and N-acetyl-ß-D-glucosaminidase), and also inhibited macromolecular synthesis in cultures of Madin Derby Canine kidney cells (ref. 84). The activity of renal tubule enzymes as measured *in situ* and in urine was also decreased after administration of ochratoxin A to Wistar rats by intubation at a daily dose of 145 μg/kg(bw) for 8-12 weeks (refs. 85, 86).

Renal gluconeogenesis and the activity of cytosolic phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme in this pathway, were reduced by daily administration of 0.1 mg/kg(bw) in rats (ref. 87) and 0.008 mg/kg(bw) in swine (ref. 88). This effect appears to be selective, since hepatic PEPCK activity as well as the activity of some other renal enzymes are unaffected by similar treatment (ref. 87). The level of mRNA for this enzyme was also decreased but not its synthesis, indicating that the effect of the toxin was to change the mRNA abundance at the post-transcriptional level (ref. 89). A corresponding dose-dependent decrease in renal cytosolic PEPCK and gamma-glutamyl transpeptidase activities and decrease in renal function was found by Krogh et al. (ref. 90) in pigs fed ochratoxin A at 0-1 mg/kg, suggesting that these enzymes are selective indicators of ochratoxin A-induced porcine nephropathy, and furthermore that they may have mechanistic implications for the pathogenesis of ochratoxin A-induced renal damage (ref. 91).

Another enzyme involved in carbohydrate metabolism, protein kinase, was studied in chickens in an effort to explain the accumulation of glycogen in the livers of some animals treated with ochratoxin A (refs. 92, 93). The activity of this enzyme, which initiates the cascading glycogen phosphorylase system, was inhibited by the lowest level of ochratoxin fed, namely, 0.5 mg/kg of diet (ref. 94). Other investigators (ref. 95), however, reported a depletion of hepatic glycogen by ochratoxin A, albeit at higher dosages or differing dosing regimens. Activities of hepatic mixed function oxidase enzymes, including aminopyrene demethylase and aniline hydroxylase, and levels of cytochrome P-450 were decreased in rats administered ochratoxin A by intubation at a daily dose of 1.5 mg/kg(bw) for 15 days. Hepatic phase II biotransformation enzyme activities were unaffected (ref. 96).

Several studies (including some already referred to) have been carried out by using *in vitro* systems to explore possible mechanisms responsible for the nephrotoxic action of ochratoxin A. A number of studies have shown that ochratoxin A interferes with both the organic anion (e.g., para-aminohippurate or PAH) and the organic cation (e.g., tetraethylammonium acetate) renal transport systems (refs. 97-100). Examples of this phenomenon are inhibition of PAH uptake by pig renal cortical slices (ref. 98) at an ochratoxin A concentration of 10^{-4} M and inhibition of PAH uptake by renal brush borders and basolateral membrane vesicle of canine kidney cortices with IC_{50} values of 2.0 and 3.2×10^{-5} M ochratoxin A, respectively (ref. 99). Furthermore, evidence was presented indicating that ochratoxin A transport in the kidney is mediated by the same renal organic anion transport system (refs. 98, 99). Since this is an active transport system requiring adenosine triphosphate (ATP) as the energy source, it is of interest that ochratoxin has also been demonstrated to reduce the cellular ATP contents of nephron segments *in vitro* at concentrations as low as 10^{-8} M.

In addition, ATP synthesis in mitochondria isolated from the renal cortex was significantly inhibited by 10^{-6} M ochratoxin A (ref. 101). These findings suggest that both this action and competition by organic anions and ochratoxin A for a common carrier may be responsible for the inhibition of organic ion transport by the toxin. With liver mitochondria also, uncoupling of respiration was noted with concentrations of ochratoxin starting at 1.2×10^{-6} M (ref. 102). Possibly a reflection of this phenomenon was the finding that respiration was stimulated in renal cortical explants at ochratoxin A concentrations of 10^{-5} M and higher (ref. 82).

Enhanced calcium uptake by the cells is frequently proposed as an event leading to disruption of cell morphological integrity by specific toxic chemicals. Berndt et al. (ref. 103) reported that calcium accumulation was stimulated by the addition of 10^{-5} M ochratoxin A to rat renal cortical slices. However, other investigators (ref. 104), using hepatic microsomes, found that addition of ochratoxin A (2.5-100 μM) caused a concentration-dependent inhibition of calcium uptake. Finally, at a concentration of 125 μM, ochratoxin A greatly enhanced the rate of lipid peroxidation of rat liver microsomes as measured by malondialdehyde formation. Similarly, oral administration of ochratoxin A to rats (6 mg/kg) resulted in enhanced lipid peroxidation *in vivo*, as evidenced by an increase in the rate of ethane exhalation (ref. 105).

A more recent study involving an ultrastructural investigation of kidney in ochratoxin-treated pigs revealed a loss of membrane integrity of peroxisomes in the tubular epithelial cells along with enhanced levels of peroxisomal ß-oxidation in the soluble kidney fraction (ref. 41), strengthening the possibility that oxidation-induced membrane damage is a factor in the pathogenesis of ochratoxin A toxicity. Investigating the mechanism of ochratoxin A-induced lipid peroxidation with a reconstituted system of phospholipid vesicles, flavoprotein and iron ions, Omar et al. (ref. 106) reported evidence that the toxin stimulates the production of an iron-oxygen complex which initiates the peroxidation reaction. However, another very recent report of a preliminary nature tends to cast some doubt on the significance of this iron-mediated lipid peroxidation (ref. 107).

G. Interaction of ochratoxin A with other mycotoxins

Because cultures of *P. viridicatum* have been shown to produce both ochratoxin A and citrinin, and because these two toxins have been found as natural contaminants of barley and various cereals used as feed for pigs (ref. 108), most of the studies investigating possible interactions between ochratoxin A and other mycotoxins involved examination of the effects of these two toxins, acting separately and together, on biological systems. As shown in Table 9, the results depended on the biological system used, and ranged from antagonism to additive effects to synergism or enhanced effects.

Of interest is the finding that ochratoxin B greatly reduced the toxic effect of ochratoxin A on renal proximal tubules in the rat (ref. 109). On the other hand, apparently neither the *in vivo* metabolism of ochratoxin A (ref. 110) nor the ability of ochratoxin A to inhibit phenylalanyl-tRNA formation or protein synthesis was affected by ochratoxin B (ref. 110). Penicillic acid, in contrast, apparently enhanced the toxic effect in mice (refs. 120, 127), which may have been due to the demonstrated ability of penicillic acid to inhibit carboxypeptidase A and the *in vivo* conversion of ochratoxin A to the nontoxic metabolite, ochratoxin α (ref. 128). A very interesting finding, which may help to reveal the mechanism of the teratogenic effect of ochratoxin A, was that zearalenone and diethylstilbestrol, both estrogenic substances, greatly reduced the effect of ochratoxin A in mice when either was administered together with this toxin (ref. 126).

Also to be noted is the finding that whether or not an interaction is observed in a particular system is sometimes dependent on the particular parameter of measurement applied in that system. For example, in the case of hepatorenal carcinogenesis associated with ochratoxin treatment of mice, synergism with citrinin was observed only for kidney and not liver carcinogenesis (ref. 114). Even sexual status appears to be a determinant of this phenomenon, as seen in the case of citrinin and ochratoxin A interacting synergistically to produce enhanced lethality in female but not male guinea pigs (ref. 121). Another example is the *in vivo* inhibition of renal ATPase by ochratoxin. Synergism with citrinin was found for Na^{+}-K^{+}-dependent ATPase but not for the Mg^{+2}-dependent enzyme (ref. 119).

Finally, other compounds besides mycotoxins are also reported to interact with ochratoxin A. Caffeine was reported to enhance the disturbance of fetus development induced by ochratoxin A (ref. 129). Biscoumacetate or phenylbutazone treatment of rats increased the toxicity of ochratoxin A when administered together with the toxin, possibly because of displacement of ochratoxin A from binding sites on plasma proteins, which was determined *in vitro* (ref. 130).

SIGNIFICANCE AND CONCLUSIONS

Although the fungi capable of producing ochratoxins are frequently encountered on foods and feeds, surveys have shown that contamination of raw agricultural products is region-dependent. Analytical methods capable of determining ochratoxin A down to 10 μg/kg in a wide variety of substrates have been used. In the United States, a contamination rate of selected raw agricultural products of approximately 3% has been observed, with contamination levels usually <100 μg/kg. In other areas of the world, especially northern Europe and the Balkans, higher contamination incidences and levels have been observed. As might be expected, occurrence rates and levels of ochratoxin A have been far lower in human foods than in raw agricultural products.

In animal studies, ochratoxin A has clearly been shown to be a potent nephrotoxin, immune suppressant, teratogen and carcinogen. A developing body of information indicates that ochratoxin A may be a factor in the etiology of Balkan endemic nephropathy, a disease in which there appears to be a highly significant relationship with tumors of the urinary tract (particularly tumors of the renal pelvis and ureters) (ref. 20). This evidence includes (1) surveys of foodstuffs in Yugoslavia and Bulgaria which indicate a higher level of ochratoxin A contamination in endemic areas than in non-endemic areas; (2) a striking similarity between renal pathology associated with Balkan endemic nephropathy and renal pathology in field cases of porcine nephropathy induced by ochratoxin A; and (3) the finding of higher levels of ochratoxin A in blood of patients with urinary system tumors than in blood of healthy persons from the same area (Bulgaria). However, data on human blood levels of ochratoxin A should not be interpreted as proof of the causality of human cancer by ochratoxin A, given the normally accepted long latency period between exposure and appearance of cancer in humans.

There have been several recent reports of a high incidence (up to 54%) of ochratoxin A in human blood specimens in some northern European countries, although the source(s) of ochratoxin A has not been satisfactorily established. In one of these countries (Germany), a risk evaluation has been made for humans (124) with a calculated virtual safe daily dose of 0.1 ng/kg(bw) and an acceptable daily intake of 0.007 μg (based on carcinogenicity in mice and a safety factor of 1 in a million). The basis for these calculations (especially human exposure) has not been satisfactorily described.

The Health Protection Branch of Health and Welfare Canada very recently published (ref. 18) a risk assessment dealing with ochratoxin A. Along with the usual caveats in the development of such documents (ref. 131), e.g., the uncertainties in the extrapolation of data from laboratory animals to humans and from high doses used in laboratory studies to the intake levels resulting from the consumption of ochratoxin A-contaminated foods, two approaches were used with regard to carcinogenicity. In one a safety factor of 5,000 was applied to the experimentally observed NOEL (no effect level) derived from the NTP study to arrive at an estimated tolerable daily intake for humans of 4.2 ng/kg(bw). The other approach was to use the lower 95% confidence level for the VSD (virtually safe dose) for a risk of $1:10^6$, arriving at 200 ng/kg(bw) as the estimate for the tolerable VSD in humans. Thus the estimated tolerable daily intake in humans ranged from 200 to 4.2 ng/kg(bw), depending on the method of extrapolation used. This publication further states that, based on estimated human intake, these doses were exceeded on occasion. The difficulty in risk assessment has been in accurately documenting human exposure. The Commission on Food Chemistry, IUPAC, has taken steps to document the worldwide incidence and levels of ochratoxin in foods and feeds.

Table 9 Interaction of Ochratoxin A with Other Mycotoxins

Mycotoxin	Test System/Measurement	Effect	Reference
Ochratoxin B	Rat renal tubular morphology	Greatly reduced (antagonistic)	109
Ochratoxin B	Mouse liver - protein synthesis	No effect or additive	110
Citrinin	Hepatoma cells - RNA, DNA, protein synthesis	Synergistic	111
Citrinin	Renal cortical cells - organic ion transport	Additive or slightly synergistic	97
Citrinin	White leghorn pullets - kidney function	Reduced citrinin effect	112
Citrinin	Layer chick - renal ultrastructure	Additive	113
Citrinin	Mouse - hepatorenal carcinogenesis	Synergistic (only for renal effect)	114
Citrinin	Chick embryo - morphology	Additive	115
Citrinin	Broiler chicks - growth depression, water consumption increase	Reduced (antagonistic)	116, 117
Citrinin	Fetal rat - malformation	Synergistic	118
Citrinin	Rat - renal Na^+-K^+ and Mg ATPase	Synergistic (only Na^+-K^+ATPase)	119
Citrinin	Mouse - lethal effect	Synergistic	120
Citrinin	Guinea pig - lethal effect	Synergistic (female) Additive (male)	121
T-2 toxin	Broiler chickens - body weight, serum protein LDH triglyceride, GGt and Ca++	Antagonistic for Ca++ and GGt; synergistic for triglyceride	122
Aflatoxin	Poultry - fatty livers and growth weight	Reduced aflatoxin effect for fatty liver; synergistic for growth weight and nephropathy	112 123
Aflatoxin B_1	Swine - renal interstitial fibrosis	Mild antagonism	124
Deoxynivalenol	Broiler chick - general toxicity	Reduced (antagonistic)	125
Zearalenone	Fetal mouse - teratogenesis	Reduced (antagonistic)	126
Penicillic acid	Mouse - renal tubular morphology	Synergistic	127
Penicillic acid	Mouse - lethal effect and RNA synthesis	Synergistic	120

Table 10 Regulatory Limits - Ochratoxin A

Country	Commodity	Regulatory Limit (μg/kg)
Brazil	Rice, barley, beans, corn	50
Czechoslovakia	Most foods	20
	Infant foods	1
	Children's foods	5
Denmark	Pork kidney	<10
Hungary	All foods	20
Israel	Grain for feed	300
Romania	All foods, feeds	5

In spite of the paucity of data on which to base a human risk assessment, six countries have taken steps to limit human exposure to ochratoxin A (Table 10) (ref. 132).

In Denmark, before 1978, the entire carcass of the pig was discarded as unfit for human consumption if the concentration of ochratoxin A exceeded 10 μg/kg. Only kidneys showing nephropathy (pale kidneys, enlarged kidney, mottled renal surface, cysts, cortical fibrosis) were analyzed. In 1980 this limit was raised to 25 μg/kg. Experience in Denmark shows that 10-29% of all pig kidneys that demonstrate evidence of abnormal pathology contain ochratoxin A (>25 μg/kg) (ref. 13). Sweden (ref. 10) and Poland (ref. 12), using the same system, have similar experiences.

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