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REVIEW

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Ochratoxin A in food and feed: occurrence, legislation and mode of action

Ochratoxin A in Lebens- und Futtermitteln: Vorkommen, gesetzliche Regelung und Wirkungsmechanismen

Summary Ochratoxins, of which ochratoxin A (OA) is the most prevalent, are secondary fungal metabolites of some toxigenic species of *Aspergillus* and *Penicillium*. OA has been shown to be nephrotoxic, hepatotoxic, teratogenic, carcinogenic and immunosuppressive. The natural occurrence of OA in food and feed stuffs is widespread, especially in temperate areas such as Canada, Denmark, Germany, Sweden and the United Kingdom, and detectable amounts were even found in randomly collected human milk samples in Germany, Sweden and Italy. Of greatest concern in humans is its implicated role in an irreversible and fatal kidney disease referred to as Balkan Endemic Nephropathy. The mean dietary intake for humans in the European Union was found to be in the range of 1 to 2 ng/kg bw/day. Compared with the Provisional Tolerable Daily Intake (PTDI) proposed by the WHO of 16 ng OA/kg bw/day for humans, the average OA intake in Europe seems to be rather low. The main contributor to the OA intake in humans are cereals and cereal products, other possible contributors are coffee, beer, pork, products containing pig blood/plasma, pulses and spices. Only very few countries have regulations for OA in food and feed products. Based on the current literature, the mechanisms involved in the toxicity of OA indicate three major effects: (1) inhibition of mitochondrial respiration correlated with a depletion of ATP; (2) inhibition of tRNA-synthetase accompanied by a reduced protein synthesis; and (3) enhanced lipid peroxidation. Generation of free radicals and lipid peroxidation as an important mode of action of OA *in vitro* and *in vivo* is discussed in detail, as well as counteracting effects of dietary antioxidants.

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Zusammenfassung Ochratoxine sind Metabolite des Sekundärstoffwechsels von verschiedenen Pilzarten der Gattungen *Aspergillus* und *Penicillium*, wobei Ochratoxin A (OA) am bedeutensten ist. OA weist nephrotoxische, teratogene, carcinogene und immunsuppressive Eigenschaften auf. Insbesondere in gemäßigten Klimazonen, wie in Kanada, Dänemark, Deutschland, Schweden und dem Vereinigten Königreich kommen OA-Kontaminationen weit verbreitet in Lebens- und Futtermitteln vor. In Deutschland, Schweden und Italien konnte sogar in Muttermilch OA nachgewiesen werden. Beim Menschen ist OA möglicherweise an der Genese der endemischen Balkan-Nephropathie, einer nierenschädigenden Erkrankung, beteiligt. Die mittlere OA-Aufnahme der Bevölkerung in den Staaten der Europäischen Union wird auf 1-2 ng/kg Körpergewicht und Tag geschätzt. Der Wert liegt weit unter der von der WHO empfohlenen maximal tolerierbaren OA-Aufnahme in Höhe von 16 ng/kg Körpergewicht und Tag.
OA wird beim Menschen vor allem über Getreide und Getreideprodukte aufgenommen, daneben kommen als weitere OA-Quellen Kaffee, Bier, Schweinefleisch, Schweinefleischerzeugnisse, Hülsenfrüchte und Gewürze in Frage. Eine gesetzliche Regelung für Höchstgehalte an OA in Lebens- und Futtermitteln existiert zur Zeit nur in sehr wenigen Ländern. In der aktuellen Literatur werden drei verschiedene Toxizitätsmechanismen von OA beschrieben: (1) eine Hemmung der mitochondrialen Atmung im Zusam-

menhang mit einer ATP-Depletion, (2) eine Hemmung der tRNA-Synthetase und damit eine reduzierte Proteinbiosynthese, (3) eine gesteigerte Lipidperoxidation. Die Erzeugung freier Radikale und eine gesteigerte Lipidperoxidation als ein wichtiger Toxizitätsmechanismus von OA *in vitro* und *in vivo* sowie eine mögliche Gegensteuerung bzw. Schutzwirkung nutritiver Antioxidantien wird ausführlich diskutiert.

Key words Ochratoxin A – mycotoxins – occurrence – legislation – mode of action

Schlüsselwörter Ochratoxin A – Mykotoxine – Vorkommen – gesetzliche Regelung – Wirkungsmechanismen

Introduction

Mycotoxins are secondary fungal metabolites and remain an area of major concern throughout the world. Where present, they usually occur as trace contaminants in agricultural products, in concentrations ranging from nanogram to microgram quantities per gram. Intensive research on mycotoxins has been carried out since 35 years. The first group of mycotoxins which was isolated and described in 1961 were the aflatoxins as the result of several acute animal disease outbreaks in 1960 (Goldblatt, 1969). The ochratoxins comprised the first major group of mycotoxins identified after the discovery of the aflatoxins (van der Merwe *et al.*, 1965 a, b). It was typical for mycotoxicoses in general, that a seasonal peak in toxin occurrence drew attention to the agents through production of an acute clinical disease. These acute intoxications can be dramatic and economically devastating, but they constitute only a small fraction of the biological and economic consequences of the more usual chronic intoxications caused by lower levels of toxin (Pier, 1981, 1987). These conditions are of particular interest since the Food and Agriculture Organization (FAO) of the United Nations estimated in 1985, that 25% of the world's grain supply is contaminated with mycotoxins. Hesseltine (1986) ranked mycotoxins in the following order of relative importance: aflatoxins (hepatotoxin), ochratoxins (nephrotoxin), trichothecenes (dermatotoxin), zearalenone (estrogen), deoxynivalenol (dermatotoxin), citrinin (nephrotoxin).

Ochratoxins, of which ochratoxin A (OA) is the most prevalent, are secondary fungal metabolites of some toxigenic species of *Aspergillus* and *Penicillium*. OA occurrence in food and feed is widespread (Krogh and Nesheim, 1982), and it has been shown to be hepatotoxic, nephrotoxic, teratogenic and carcinogenic to single-stomached animals (Kuiper-Goodman and Scott, 1989). Of greatest concern in humans is its implicated role in an irreversible and fatal kidney disease referred to as Balkan Endemic Nephropathy (BEN) (Krogh et al., 1977, Pavlovic et al., 1979). The increase of renal disease is accompanied with a high risk for urinary tract tumours. In addition to OA, a very low selenium status of the population could be a risk factor for the BEN and urinary tract tumors, too (Maksimovic et al., 1991). OA contains 7-carboxy-5-chloro-8-hydroxy-4,4-dihydro-3R-methylisocoumarin (Oα) that is linked through the 7-carboxy group to L-ß-phenylalanine by an amide bond. Several forms of ochratoxin occur (see Table 1, Figure 1; Marquardt and Frohlich, 1992; Hoehler et al., 1996 b), which will be discussed subsequently. This paper reviews current information on the occurrence, legislation and mode of action of ochratoxins. Within the latter part, free radical generation and lipid peroxidation as one important mechanism of action of ochratoxins is discussed in more detail.

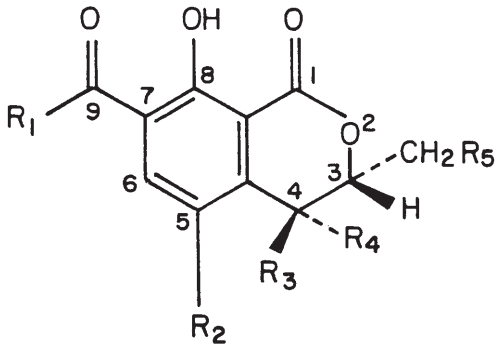

Figure 1 General structure of ochratoxin. See Table 1 for structure of the different R groups.

Occurrence

The OA producing fungi have been referred to as storage fungi. In tropical and subtropical areas OA is mainly produced by *Aspergillus* species *(A. ochraceus, A. ostianus, A. melleus* and others; *A. ochraceus* is now referred to as *A. alutaceus*), whereas in temperate regions *Penicillium* species are of greater importance, especially *P. viridicatum* (Krogh, 1978). *P. viridicatum* can produce OA at a minimum temperature of 4 °C and a minimum moisture content of wheat of 18.5 % (Table 2). Fungal growth without toxin production can also occur below this moisture content.

Table 1 Naturally occurring and synthetic forms of ochratoxins (Marquardt and Frohlich, 1992; Hoehler et al., 1996 b; 1997 a)

Name	Abbreviation	R1	R2	R3	R4	R5
Naturally occurring ochratoxins						
Ochratoxin A	OA	Phenylalanyl	Cl	H	H	H
Ochratoxin B	OB	Phenylalanyl	H	H	H	H
Ochratoxin C	OC	Phenylalanyl, ethyl ester	Cl	H	H	H
Ochratoxin A methyl ester		Phenylalanyl, methyl ester	Cl	H	H	H
Ochratoxin B methyl ester		Phenylalanyl, methyl ester	H	H	H	H
Ochratoxin B ethyl ester		Phenylalanyl, ethyl ester	H	H	H	H
Ochratoxin α	Oα	OH	Cl	H	H	H
Ochratoxin β	Oβ	OH	H	H	H	H
4R-Hydroxyochratoxin A	OH-OA	Phenylalanyl	Cl	H	OH	H
4S-Hydroxyochratoxin A	OH-OA	Phenylalanyl	Cl	OH	H	H
10-Hydroxyochratoxin A	OH-OA	Phenylalanyl	Cl	H	H	OH
Ochratoxin A, tyrosine analog		Tyrosine	Cl	H	H	H
Ochratoxin A, serine analog		Serine	Cl	H	H	H
Ochratoxin A, hydroxyproline analog		Hydroxyproline	Cl	H	H	H
Ochratoxin A, lysine analog		Lysine	Cl	H	H	H
Synthetic ochratoxins						
d-Ochratoxin A	d-OA	d-Phenylalanyl	Cl	H	H	H
Ochratoxine A, ethyl amide	OE	Phenylalanyl, ethyl amide	Cl	H	H	H
O-methylated Ochratoxin A	OM-OA	Phenylalanyl, OCH_3 at C-8	Cl	H	H	H
Lactone-opened Ochratoxin A	OP-OA	Phenylalanyl	Cl	H	H	-

Table 2 Minimum and optimum conditions for the production of ochratoxin A (moisture content and temperature) of *Penicillium viridicatum* when grown on wheat or corn (Schuh and Schweighardt, 1981)

Moisture content	Wheat	Barley
Optimum:		
Moisture content, %	21.8	27.1
Temperature, °C	24	24
Minimum:		
Moisture content, %	18.5	21.6
Temperature, °C	24	24
Temperature	**Wheat**	**Barley**
Minimum:		
Temperature, °C	4	4
Moisture content, %	28.6	40.5

The natural occurrence of ochratoxin A (OA) in food and feed stuffs is widespread (Dwivedi and Burns, 1986), especially in temperate areas such as Canada, Denmark, Germany, Sweden and the United Kingdom (Jorgensen et al., 1996). Detectable amounts of OA (0.017 to 0.030 ng/ml) were even found in randomly collected human milk samples in Germany, with an 11 % incidence of positive samples (Gareis et al., 1988). A comparable study performed in Sweden reported similar concentrations of OA in human milk, but an incidence of positive samples of 58 % (Breitholtz-Emanuelsson et al., 1993). Miraglia et al. (1993) concluded that human milk is likely to be a matrix where OA accumulates in women. In a further study Miraglia et al. (1995) evaluated OA contamination levels over one week in human milk samples and in selected foodstuffs consumed by the milk donors in Italy. Values of daily OA intakes between 9.6 and 64.8 ng/kg bw/day were reached, which the authors considered to be alarming since the amount of OA ingested daily by the babies was almost always above the estimated tolerable daily intake (TDI), i.e. 0.2 ng/kg bw and 4.2 ng/kg bw (Kuiper-Goodman, 1991), or 16 ng/kg bw (WHO, 1991). The authors also state that babies represent a particularly sensitive population group for which a specific TDI should be evaluated, especially in consideration of the unfavourable dose/body-weight ratio.

During the last few months, there came out several reports in the daily press on the occurrence of OA in different foodstuffs, such as wine, beer and coffee. Some of these reports dramatically overestimated the possible risks of OA for human health. Nevertheless, OA occurrence is widespread, as shown by the OA content of red wines from various origins (Table 3). Although the number of samples analysed was not very high, the amount of positive samples as well as a maximum value of 7 µg OA/l of one sample from Italy are quite significant. It was also stated in this report, that white wines in general contain much less OA than red wines, which can be explained by the different processing methods.

Table 3 Occurrence of ochratoxin A in red wine ("Rheinpfalz", 1997)

Country	Number of wines analyzed	Positive	Median µg/l	Maximum µg/l
Germany	11	3	0.03	0.24
Switzerland	1	1	0.07	0.07
France	20	6	0.13	0.78
Italy	10	7	0.30	7.00
Spain	6	3	0.13	0.19
Greece	2	1	-	0.11
Portugal	2	2	0.32	0.34
Mazedonia	6	4	0.43	0.89
Tunisia	5	5	1.63	1.85
South Africa	2	1	0.05	0.05
USA	2	1	0.08	0.08
Chile	9	2	-	0.21
Australia	5	1	-	0.22

White grapes are directly pressed out, whereas red grapes are left mashed for a certain time period, which obviously permits fungal growth and production of the toxin.

Besides the mentioned publications, there exist numerous reports on the occurrence of OA as well as on the occurrence of other mycotoxins in different commodities. Most of these investigations were not performed systematically, and therefore it is almost impossible to derive conclusions for a general risk for the population resulting from OA intake. In 1994 the European commission set up a Scientific Co-Operation on Questions Relating to Food (SCOOP, 1996) task to provide the Scientific Committee for Food with information on European dietary exposure to OA. Thirteen countries participated in this large scale task and delivered data on occurrence of OA in food products, data on consumption of these food products and data on occurrence of OA in human blood plasma and human milk. The objective was to provide the scientific basis for the evaluation and management of risks to public health arising from dietary exposure to OA. The exposure was estimated by 2 different methods (Table 4). First, the exposure was estimated from the average concentration of OA in blood plasma samples, and second, the exposure was estimated from the average level of OA in food products combined with consumption data. The estimation of 1.34 x C_p = Estimated daily intake of OA had been made from data as described by Breitholtz et al. (1991). Already Mortensen et al. (1983), based on studies with pigs, reported that blood concentrations of OA could be used as a good index for predicting OA intake and tissue concentrations of OA. The high binding affinity of OA to plasma albumin retards its elimination by limiting the transfer of OA from the bloodstream to the hepatic and renal cells and therefore consequently contributes to a prolonged half-life of OA in the body. OA can be cleaved into phenylalanine and the chlorinated dihydroisocoumarinic moiety Oα, which was found not to be toxic, but retaining some genotoxicity (Föllmann et al., 1995). In addition to being converted to Oα, a small percentage of absorbed OA is converted to hydroxyochratoxin A by liver microsomes. Recently, Omar et al. (1996) have shown that OA is metabolized

Table 4 Comparison of estimates of mean dietary intake for an average adult person calculated from occurrence and consumption data and from mean human blood plasma levels (SCOOP, 1996)

Country	Average concentration in blood plasma (C_p) (ng/ml)	Estimated daily intake calculated from blood plasma concentration (1.34 x C_p) (ng/kg bw)	Estimated daily intake based on occurrence data and consumption data (ng/kg bw)
Denmark	1.8	2.4	2.0
France	0.4	0.5	1.5
Germany	0.45	0.6	0.9
Italy	0.53	0.7	4.6
Netherlands			2.0
Spain			0.7
Sweden	0.18	0.24	1.5
United Kingdom			1.4

by the main cytochrome P450 isoforms IA1/IA2, IIB1, and IIIA1/IIIA2 of rats mainly to 4*R*- and to a smaller extent to 4*S*-hydroxyochratoxin A, leading to its detoxication. In humans blood analyses have been used to investigate the possible role of OA in the mentioned disease Balkan endemic nephropathy and in urinary tract tumours on the basis of its nephrotoxicity. The hypothesis is to find an increased incidence and higher concentrations of the toxin in patients suffering from the disease (Hult, 1995). A significant difference was also found, when subjects attending dialysis were compared with healthy subjects (Breitholtz-Emanuelsson et al., 1994). As a result of the SCOOP investigation (1996), the mean dietary intake for humans based on occurrence and consumption data is 1.8 ng/kg bw/day, the mean daily intake from blood data is 0.9 ng/kg bw/day, showing that the values are in the same order of magnitude (Table 4). Compared with the Provisional Tolerable Daily Intake (PTDI) proposed by the WHO (1991) of 16 ng OA/kg bw/day for humans, the average OA intake in Europe seems to be rather low. It should be considered that the OA intake can be considerably higher for certain populations depending on the harvest year or the geographical region. The above mentioned PTDI was based on the lowest adverse affect level for kidney damage in pigs and a safety factor of 500. In the SCOOP investigation (1996) it is also pointed out that young children will probably have a higher intake per kg bw than adults and that the main contributor to the OA intake seems to be cereals and cereal products, other possible contributors are coffee, beer, pork, products containing pig blood/plasma, pulses and spices. Also, Jorgensen et al. (1996) state that cereals are probably the main source of OA intake in Denmark. A recent large scale survey, where 1431 samples of wheat, barley, rye, oats and bran were analysed for OA in Denmark, showed that 40 % of the samples were contaminated with OA. Among the commodities analysed, bran products showed a higher OA contamination than cereal kernels, e.g. OA was detected in wheat in 62 % of the bran samples and in only 30 % of the samples of kernels. The survey covers the period from 1986 to 1992 and the samples were taken from products intended for human consumption. Surveillance of OA in cereals has been part of the Danish monitoring system since 1986. In this study, especially in years with wet weather during harvest, the daily intake of OA for some individuals in the Danish population could reach levels above the TDI of 5 ng/kg bw suggested by The Nordic Working Group on Food Toxicology and Risk Evaluation (1991). The authors also state that the OA production in cereals takes place mainly in the first period just after harvest before the water activity has decreased by drying. The most important way to protect consumers from the toxic effects of OA therefore remains to ensure good agricultural practice to prevent fungi start growing and producing OA during the production processes. The importance of management practices in this context was also demonstrated in the study of Jorgensen et al. (1996), where higher contents of OA in rye kernels from ecological farms than from conventional farms were found. The authors conclude that this might be due to the generally smaller and less efficient drying facilities available in the ecological farms compared with conventional farms.

In a survey where 514 grain samples were analysed for OA in the south of Germany, between 1 and 54 % of the samples were positive for OA (Richter and Schuster, 1995, Table 5). In this study, grain samples from 1991 to 1993, which were stored for different periods of time, were analysed. The maximum value of these randomly selected samples was 60 ng OA/g (µg/kg) in a sample from 1991. Overall, a total of 2 % of the samples had an OA content of more than 3 ng/g.

Besides cereals and other vegetable products the contribution to OA intake from animal products should also be taken into consideration. Because of the above mentioned strong binding of OA especially to plasma albumin, a relatively high amount of OA in animal feed is transmitted to animal tissues (Kuiper-Goodman and Scott, 1989). As low to moderate levels of OA are rapidly

Table 5 Occurrence of ochratoxin A in grain samples in Bavaria 1991-1993 (Richter und Schuster, 1995)

Year	Storage (Months)	Number of samples	Positive samples				Maximum (ng/g)
			n	%	> 3 ng/g		
					n	%	
1991	4	172	2	1.2	2	1.2	60.3
1991	6	195	106	54	1	0.5	3.5
1991	10	121	63	52	2	1.7	5.5
1992	6	87	18	21	1	1.1	21.5
1992	9	179	27	15	5	2.8	19.7
1993	10	163	82	50	7	4.3	23.9

Table 6 Present regulation of ochratoxin A in the EU member states (SCOOP, 1996)

Country	Commodity	Max. limit (µg/kg)	Legal basis	Comments
Austria	wheat, rye, durum	5	guideline	
Denmark	pig kidneys	25	official	whole carcass condemned
	pig kidneys	10	official	viscera condemned
	cereals and cereal products	5	official	since 1. July 1995
France	cereals	5	guideline	
Greece	raw coffee	20	draft	

degraded by the microorganisms in the forestomachs of ruminants (Marquardt and Frohlich, 1992), pork and poultry meat are regarded as the main animal sources of OA intake by humans, although, according to Kuiper-Goodman and Scott (1989) the OA levels in these products are rather insignificant compared with the levels in vegetable food products. In a recent survey in Northern Germany (Heer et al., 1995) the occurrence of OA was monitored in blood serum pools of pigs of a total of 1801 pig farms. An incidence of positive samples of 62 and 54 % was found for fattening and breeding herds, respectively. The mean OA concentration in the positive samples was 0.88 ng/ml serum, the maximum value was 148 ng/ml serum, and the amount of samples containing more than 5 ng OA/ml of serum was 4 %. Based on data of Kühn (1993), the OA concentration under steady state conditions in kidney, liver, muscle and adipose tissue of pigs amounts to 7.2, 6.9, 4.7 and 3.8 % of the serum concentration, respectively. That would result in an OA concentration of the edible tissues of the pigs, which were OA positive in the study of Heer et al. (1995), on average of 50 ng/kg, the maximum value (based on 148 ng/ml of serum) would be about 8 µg OA/kg of pork. Considering the Provisional Tolerable Daily Intake (PTDI) proposed by the WHO (1991) of 16 ng OA/kg bw/day for humans, the average OA concentration of pork in Northern Germany would be below the PTDI, whereas the maximum value of 8 µg OA/kg of pork would result in an OA intake (assumed: 300 g of pork/day, adult 70 kg bw) of 34 ng/kg bw, which would be above the proposed safety limit. This calculation demonstrates that food derived from animals fed on OA contaminated feed (mainly cereals) can be of significant importance for the total dietary intake of OA in humans.

Legislation

Based on the carcinogenicity of OA, the WHO (1991) proposed a maximum limit for OA of 5 µg/kg in cereals. So far, no barriers to trade have been reported. According to van Egmond (1991), a total of 60 countries have enacted or proposed regulations for levels of mycotoxins in food and feed. Although most of the existing regulations refer to aflatoxins, the number of other mycotoxins for which regulations are being developed is growing. Within the countries of the European Union, only Austria, Denmark, France and Greece have regulations for ochratoxin A in food products (Table 6). Solely in Denmark there exists an official legal basis for different commodities, whereas the legal status in the remaining 3 countries is either a guideline level or a draft. It can be expected that a maximum level of 5 µg OA/kg for cereals and cereal products, which was also proposed by the WHO, will be established in more countries especially in Europe in the future.

Mode of Action

Most of the mycotoxins can exhibit various toxicities, e.g. OA has been shown to be nephrotoxic, hepatotoxic, teratogenic, carcinogenic and immunosuppressive. Although mycotoxins can exert such a great variety of toxic effects, knowledge about the mode of action of mycotoxins is rather limited. According to Ueno (1991), the interaction of OA with the enzyme phenyl alanyl-tRNA synthetase is the only mode of action for which there is direct evidence in the biochemical sense.

Based on the current literature, the mechanisms involved in the toxicity of OA indicate three major effects:

(1) inhibition of mitochondrial respiration correlated with a depletion of ATP, (2) inhibition of tRNA-synthetase accompanied by a reduced protein synthesis, and (3) enhanced lipid peroxidation (Röschenthaler et al., 1984, Marquardt and Frohlich, 1992, Fink-Gremmels et al., 1995).

According to Creppy et al. (1995), inhibition of protein synthesis and implication of oxidative pathways due to production of free radicals play the key role in the mechanism of action of OA, which therefore will be discussed in more detail in the following. Rahimtula et al. (1988) showed that OA, when added to rat liver microsomes, enhanced the rate of NADPH or ascorbate-dependent lipid peroxidation as measured by malondialdehyde formation. *In vivo* administration of OA to rats also resulted in enhanced lipid peroxidation. Studies by Hasinoff et al. (1990) and Omar et al. (1990, 1991) suggested that OA chelated iron and that this chelate in the presence of the NADPH-cytochrome P-450 reductase system produced reactive oxygen species which in turn reacted with lipids to form lipid peroxides. Malaveille et al. (1994) reported that the antioxidant Trolox C, a water soluble form of vitamin E, completely quenched the genotoxic effects of OA in *E. coli*. A similar protective effect of vitamin C against OA genotoxicity in mice was demonstrated by Bose and Sinha (1994). Stormer and Hoiby (1996) recently stated that an additional important main function of OA might be an implication in the iron metabolism. One function of OA in nature could be to affect the iron uptake of competing microorganisms. As iron redox cycles and generates free radicals, the promotion of lipid peroxidation might be connected to this mechanism. The ability of OA to generate free radicals and to enhance lipid peroxidation has been linked to the genotoxicity expressed by DNA adduct formation and to the disturbance of calcium homeostasis due to an impairment of the endoplasmic reticulum membrane (Pfohl-Leszkowicz et al., 1993). Zanic-Grubisic et al. (1995) found that treatment of rats with low doses of OA resulted in a reduction of the activities of the membrane bound enzymes in rat pancreas, as a result of the impairment of the functional integrity of cell membranes. It is known that enhanced peroxidation of polyunsaturated fatty acids from membrane lipids might seriously impair cell membrane integrity, and produce structural and functional changes leading to cellular necrosis. Additionally, perturbation of the liver microsomal membranes has been attributed to ochratoxin A induced increased NADPH-dependent lipid peroxidation (Siraj et al., 1981). Omar et al. (1996) showed that OA is metabolized in the rat mainly by cytochrome P450 isoforms IA1/IA2, IIB1 and IIIA1/IIIA2. These authors also found that OA-induced lipid peroxidation lead to cytochrome P450 destruction, whereas a decreased lipid peroxidation and consequently lesser destruction of cytochrome P450 lead to increased OA metabolism. More evidence for the involvement of oxidative metabolism in the toxicity of OA is given by the finding that the genotoxicity of OA involves a glutathione conjugation reaction in bacteria suggesting the formation of a cytotoxic thiol-containing derivative (Malaveille et al., 1994). However, the importance of lipid peroxidation as a direct cause of the resulting toxicity of OA and the nature of the radicals that are formed remained to be proven.

Tissue damage due to lipid peroxidation directly results of free radical-mediated toxicity. Free radicals are atoms or molecules with unpaired electrons that have escaped the solvent cage or enzyme-active site in which they were produced (Bonorden and Pariza, 1994). The targets of oxidative damage are usually critical biomolecules such as nucleic acids, proteins and lipids (Gutteridge and Halliwell 1990, Imlay and Linn, 1988, Stadtman, 1990). The role of oxidation in the ageing processes (Stadtman, 1992), carcinogenesis (Sun, 1990) and many metabolic diseases (Halliwell and Gutteridge, 1989) has gained increasing attention. As tumorigenicity and depression of immunological response also occur in the toxicity of OA and some other mycotoxins, the promotion of free radicals might be partly responsible for some of these toxic effects, especially if the organism is exposed to the toxin for a prolonged period of time.

Hoehler et al. (1996 b) investigated the generation of free radicals by OA and seven of its analogs. OA; its three natural analogs, OB, OC and Oα; and four synthetic analogs, *d*-OA, the ethyl amide of OA (OE-OA), O-methylated OA (OM-OA), and the lactone-opened OA (OP-OA) were used to study free radical generation in bacteria with *Bacillus brevis* as a model system (see Table 1, Figure 1). In addition to that, the uptake of OA and its analogs by the microorganism was evaluated. The uptake of the different ochratoxins by *B. brevis* varied substantially depending on the molecular structures. The absence from OA of either the chlorine atom (OB) or the phenylalanine side chain (Oα) completely prevented the uptake of these analogs by the bacteria compared with OA and most of the other analogs. Electron paramagnetic resonance spectroscopy (EPR) using 4-POBN as a spin trapping agent to directly detect generated free radicals was also applied to this model system and showed an enhanced free radical generation (increase of the amplitude of the EPR spectra) due to the addition of OA and most of the analogs. Also, the above mentioned analogs generated only either a weak (OB), or no detectable EPR signal, as in the case of Oα. The *in vivo* toxicities reported more than two decades ago for OA, OB and Oα fit with the current results on the mode of action in bacteria. Chu (1974) found LD_{50} values for the young chick as follows: OA, 150 µg (3-4 mg/kg of body weight); OB, 1900 µg; and Oα was not toxic at 1000 µg. These comparative findings on *in vivo* toxicities (LD_{50} values in chicks) and *in vitro* mechanisms of action (uptake and free radical generation of different OA ana-

logs in bacteria) further support the results on free radical-mediated toxicity of ochratoxins as well as the suitability of the employed model organism (Hoehler et al., 1996 a).

The EPR signals could be further enhanced by the addition of Ca^{2+}, a calcium ionophore and an ATPase uncoupler, whereas the EPR signals were eliminated by incubating growing cells with the antioxidant vitamin E. The spin adduct hyperfine splitting constants indicate the presence of α-hydroxy ethyl radicals resulting from generated extremely damaging hydroxyl radicals, which are trapped by 4-POBN. The results further suggest that OA induces free radical production in this model system by enhancing the permeability of the cellular membrane to Ca^{2+}. It has been hypothesized by Chu et al. (1972) that the toxicity of OA is directly related to the degree that the C-8 phenolic hydroxyl group is ionized (see Figure 1). Rahimtula et al. (1988) hypothesized that an Fe complex of the C-8 phenolate ion and not the non-dissociated form of OA facilitated the reversible oxidation-reduction of iron and that this complex was involved in enhanced lipid peroxidation. However, structure activity studies of our group with different analogs of OA on *B. brevis*, HeLa cells and the mouse indicated that there was not a clear relationship between the degree of ionization of the C-8 phenolate group of the different analogs of OA at physiological pH values and their corresponding toxic effects in different biological systems (Xiao et al., 1996). The results of Hoehler et al. (1996 b) support these conclusions as OM-OA, an analog in which the phenolate is blocked, is able to generate a relative strong EPR signal. These results suggest that there does not appear to be a close relationship between degree of ionization of the C-8 phenolate group and the production of the hydroxyl radical in *B. brevis* or its overall toxicity to different organisms.

A further study (Hoehler et al., 1997 a), where free radical generation due to OA and its analogs was evaluated in hepatocytes, mitochondria and microsomes from rats, strongly supports these observations. EPR spectroscopy using 4-POBN as a spin trapping agent showed an enhanced free radical generation due to the addition of NADPH to the microsomes. Addition of OM-OA together with NADPH and Fe^{3+} to the microsomes resulted in a strong EPR signal compared with the other analogs, whereas the signal could be quenched by the addition of catalase. OM-OA does not have a dissociable phenolate group and does not chelate Fe^{3+}. OM-OA even greatly increased the EPR signal compared with that obtained with OA. Taken together, the results further suggest that some other groups within the OA molecule must be involved in the toxicity of OA. Studies by Xiao et al. (1996) have suggested that the toxicity of OA is associated with its isocoumarin moiety and that its lactone carbonyl group appears to be involved.

Antioxidants are substances that are capable of inhibiting oxidation. According to Bonorden and Pariza (1994) antioxidants are classified into two different categories: the preventive inhibitors, which retard the inition phase of oxidation and free radical chain-braking antioxidants, which effectively remove free radicals from a given system. Preventive inhibitors include ß-carotene, α-tocopherol, several enzymes and chelating agents, whereas ascorbic acid represents a free radical chain-breaking antioxidant. In regards to the pro-oxidative effects of OA, the effects of the physiological antioxidants α-tocopherol and ascorbic acid on lipid peroxidation and generation of free radicals were evaluated in *in vivo* studies involving rats and chicks (Hoehler and Marquardt, 1996, Hoehler et al., 1997 b). A quenching effect of the antioxidants on the effects of the mycotoxins would not only be an additional hint towards the mode of action of the mycotoxin, but antioxidants could also be of interest as possible protective agents against the toxic effects of mycotoxins.

Recently, there have been a few reports on possible beneficial effects of antioxidant vitamins on mycotoxicoses. Haazele *et al.* (1993) demonstrated that ascorbic acid supplementation (300 mg/kg) of laying hen diets that contained 3.0 mg/kg of OA partially ameliorated OA toxicity, including its negative effects on egg mass production, rigidity of eggshells, plasma Ca, Na, and Cl concentrations and the size of the thyroid glands. More recently Malaveille *et al.* (1994) reported that the antioxidant Trolox C, a water-soluble form of vitamin E, completely quenched the genotoxic effects of OA in *Escherichia coli*. A similar protective effect of the antioxidant vitamin C against OA genotoxicity in mice was demonstrated by Bose and Sinha (1994).

In the study of Hoehler and Marquardt (1996) the presence of OA in the diet decreased the concentration of α-tocopherol in the livers of chicks. Consistent with these findings, increased values of malondialdehyde (MDA, the commonest parameter to assess lipid peroxidation) in the livers due to OA were observed. Vitamin E supplementation of the diets (10 x and 100 x the requirement) partially ameliorated the pro-oxidative effects of OA by decreasing the concentrations of MDA. In the study of Hoehler et al. (1997 b), involving growing rats and chicks, an increase in lipid peroxidation could be obtained due to a higher dietary level of unsaturated fatty acids. In the chick experiment there were marked effects of the dietary treatment on MDA concentrations of different tissues, as both a higher supply with unsaturated fatty acids and OA increased most of the MDA values significantly. Collectively, these data also show that lipid peroxides are formed *in vivo* by OA.

The major biochemical function of α-tocopherol in membranes and lipoproteins is believed to be the scavenging of oxygen-centered free radicals to provide antioxidant protection, however, a clearly defined metabolic

function has not been established. The mode of action of α-tocopherol has been reviewed by Kappus (1985). α-tocopherol reacts with lipid radicals, thereby breaking the radical chain reaction. The resulting tocopherol semiquinone radical is relatively stable and may be reduced by cellular components such as ascorbic acid. Interestingly, the formation of free radicals following the addition of OA and Ca^{2+} to bacteria (Hoehler et al., 1996 b) could only be quenched if vitamin E was previously incorporated into the membranes of the microorganism. Higher dosages of each of vitamin E or Trolox C, a water soluble form of vitamin E, when added just prior to the EPR analysis did not have any effects on the formation of free radical adducts. This indicates that different forms of vitamin E, even in relatively high concentrations, failed to prevent cells from free radical-mediated toxicity when they are present in the aqueous phase and not in the membranes of the cells.

The significance and the practical impacts of the findings in the literature together with our results can be discussed in terms of reduction of risks to animals and humans, and in long term effects in humans. It is conceivable that the long-term uptake of small amounts of mycotoxins collectively, particularly in individuals with a poor antioxidant status, would enhance incidence of cancer and the suppression of the immune system. The effects of mycotoxins on the immune system have been reviewed by Pestka and Bondy (1994) and by Pier (1991). Suppression of the immune response or of the native defense mechanisms have been demonstrated for the aflatoxins, OA, T-2 toxin, DON and other mycotoxins. The major effect of OA on the immunologic response appears directly related to antibody formation. According to Creppy *et al.* (1995), it seems impossible to prevent toxinogenic fungi from growing and producing mycotoxins and OA in particular, therefore the emphasis on detoxification and detoxication of OA is an important task from both medical and ethical points of view.

Currently no economically feasible procedure has been developed for detoxification of OA in food and feed supply (Marquardt and Frohlich, 1992). Therefore, studies on the mode of action of certain mycotoxins are not only of academic interest, they also provide the basis to counteract or neutralize the toxic effects of mycotoxins. The possible benefits of antioxidants in this respect have been discussed previously, but other promising approaches have also been reported. Creppy *et al.* (1995) have recently demonstrated that Aspartame®, a structural analog of OA and phenylalanine, when given to rats combined with OA, prevented nephrotoxicity and genotoxicity for several weeks.

The present review shows that some of the many different toxic effects of the mycotoxin OA can be partially controlled by increasing the dietary supply with antioxidants, particularly vitamin E and possibly other agents such as carotenoids and flavonoids. Of particular importance might be the ability of these compounds to counteract the immunosuppressive effects of mycotoxins and possibly their carcinogenic effects. The findings that vitamin E reduced the toxicity, the lipid peroxidation and free radical generation due to OA *in vitro*, as well as the counteracting effects of vitamin E *in vivo*, are additional hints on the role that free radicals have on the etiology of ochratoxicosis. The findings on free radical generation due to OA therefore explain some of the previously reported deleterious effects of OA, for example the perturbation of microsomal calcium homeostasis (Khan *et al.*, 1989) and the genotoxicity (Malaveille *et al.*, 1994). Finally, this mechanism might be involved in the described general liver and kidney damage (Harwig, 1974; Krogh, 1978), as well as in the occurrence of renal and hepatic tumors (Bendele *et al.*, 1985).

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