THE INCIDENCE OF OCHRATOXIN A IN SWINE AND HUMAN SERUM AND PREDICTIONS OF ITS CONCENTRATION IN TISSUE

BY

KIMBERLY HEATHER OMINSKI

A thesis submitted to the Faculty

of Graduate Studies in Partial Fulfillment

of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

University of Manitoba

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THE INCIDENCE OF OCHRATOXIN A IN SWINE AND HUMAN SERUM AND PREDICTIONS OF ITS CONCENTRATION IN TISSUE

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KIMBERLY HEATHER OMINSKI

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

Ominski, Kimberly Heather. Ph.D., The University of Manitoba, July, 1994. The Incidence of Ochratoxin A in Swine and Human Serum and Predictions of Its Concentration in Tissue. Major Professor: R.R. Marquardt.

Ochratoxin A (OA), a nephrotoxic secondary metabolite produced by several species of *Aspergillus* and *Penicillium* fungi, has been shown to cause adverse effects in animals. It has been identified in a variety of feed ingredients, as well as human blood and human breast milk in several counties. The reported presence of this toxin in feed and feed ingredients in western Canada suggests that this toxin may be transmitted up the food chain in Canada, as has been observed elsewhere. These investigations were undertaken to examine the occurrence of OA in swine serum and human serum over time, and to evaluate, on a preliminary basis, the variability of this occurrence.

A survey of swine destined for slaughter in Manitoba indicated that 36% of the serum samples from 1600 pigs contained detectable levels of OA. The incidence and concentration of OA varied with season, as well as geographic location from which the samples were collected ($p < 0.05$). Analysis of the incidence of OA within a herd suggested that the OA status of the herd can be estimated by sampling a few animals in a given herd.

A survey of renal and nonrenal patients indicated and confirmed that Manitobans are exposed to low levels of OA. High performance liquid

chromatographic analysis of serum samples indicated that 40 and 38% of the samples analyzed in 1990 and 1991, respectively contained detectable levels of OA. There was no relationship between the presence or concentration of OA in serum and the occurrence of renal disease ($p > 0.05$). The variability in the incidence of OA observed in the swine survey was also apparent in the individuals sampled in this two-year survey.

The presence of OA in swine serum indicates that it is a possible source of OA contamination in the food chain, and a means of predicting its presence in tissue is desirable. Therefore, feed studies were undertaken to systematically establish the relationship between blood and tissue concentrations of OA at equilibrium in swine fed diets containing different concentrations of the toxin. The concentration of OA in serum and tissues were found to be highly correlated (R^2 = 0.88-0.97). This relationship between blood and tissue was not affected by dietary concentration of the toxin or by the sex of the animal. Regression equations were developed to predict the concentrations of OA in tissue based on that present in the serum or other tissues. A high correlation was also observed between plasma, serum and whole blood fractions (R^2 = 0.97-0.99), indicating that the OA concentration in the plasma or whole blood can be converted to a serum equivalent, and be used to predict the OA concentration in tissue.

The presence of OA in swine and human serum indicate thats OA is transmitted through the food chain. Further studies are required to establish the significance of this toxin in Canadian food products.

ACKNOWLEDGEMENTS

I would like to extend my gratitude to my supervisor, Dr. R.R. Marquardt. His extensive knowledge of science and sincere interest in research were highly valued and greatly appreciated. Thanks and appreciation are also extended to Dr. Andrew Frohlich, for his advice and assistance with laboratory procedures throughout my program. I am grateful to the other members of my graduate committee, Dr. David Abramson, Dr. Alma Kennedy, Dr. Trieste Vitti for their guidance and advice. I would also like to thank the external examiner, Dr. Peter Scott, for reviewing this thesis.

Thanks to Dr. Norman Stanger for his surgical expertise, enthusiasm for sharing knowledge, as well as his encouragement and friendship. I am also indebted to John Baptist, Bob Stuski and Rheal Parent, who were always willing to go above and beyond the call of duty to ensure that the animals were well cared for, and to Dr. Gary Crow for the precise and patient statistical advice.

I would like to extend a sincere and heartfelt thank you to all the staff in the Department of Animal Science. Their assistance, advice, and kind words were greatly appreciated. Special thanks to Dr. Bob Parker, Dr. Laurie Connor, Karen Carrette, Helena Stelsovsky, Margaret Ann Baker, Janice Haines, and Terri Garner.

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DEDICATION

This thesis is dedicated to my husband and friend, Jim Signatovich, for his love and support. I am especially grateful for his encouragement and understanding during the pursuit of this degree, particularly during seemingly grim moments when he was always able to make me smile. His assistance during those unforgetable 24-hour blood and urine collections when the temperature dipped below -35°C was greatly appreciated.

FOREWORD

This thesis is written in manuscript style. The first manuscript has been submitted to the "Natural Toxins", and the second manuscript has been submitted to "Food Additives and Contaminants". The third manuscript will be submitted to "Food and Chemical Toxicology".

The authors of the manuscripts are:

I. K.H. Ominski, R.R. Marquardt, A. Frohlich, F. Madrid, T. Kuiper-Goodman, J. Manfreda, J. McKenzie and A. Fine

II. K.H. Ominski, A.A. Frohlich, R.R. Marquardt, G.H. Crow and D. Abramson

III. K.H. Ominski, R.R. Marquardt, A.A. Frohlich, K. Carrette, G.H. Crow, and N.E. Stanger

Two additional manuscripts which have not been included in this thesis will also be submitted for publication.

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GENERAL INTRODUCTION

Storage of cereal grains, oilseeds, and their products is practiced throughout the world to ensure an even food supply throughout the year, to facilitate transport of surplus food to a deficit area, and to use as seeds for the next growing season (Salunkhe et al. 1985). These commodities which are an important dietary component for livestock and humans, may become infected with fungi during the storage period. Although *Aspergillus* and *Penicillum* species are the predominant storage fungi (Sinha et al. 1986), *Penicillium* species are more abundant in temperate climates, such as that found in Canada (Krogh 1987). Given the appropriate growing conditions, some species of fungi may produce toxic secondary metabolites called mycotoxins. Ochratoxin A (OA) is one of the main toxins produced by *Penicillium* species on stored cereals in western Canada (Scott et al. 1972).

Since its discovery in 1965, considerable research has been devoted to the acute and chronic effects of OA, as well as the mechanisms of its toxicity. This toxin has been shown to cause nephropathy in swine, and has also been implicated as the causal agent of Balkan Endemic Nephropathy (BEN), a fatal kidney disease in humans. The recently reported carcinogenic potential of this toxin can, in part, be attributed to its long half-life in several animals species, ability to accumulate in animal tissue, and structural stability at high temperatures (i.e. cooking). These factors have stimulated several countries to examine the extent to which it has been

transported through the food chain. Ochratoxin A has been identified as a natural contaminant in plant products (Scott et al. 1972, Mills and Abramson 1982, Abramson et al. 1983a) and swine blood (Marquardt et al. 1988) in Canada. In addition, it has also been detected in human body fluids in several countries (Bauer and Gareis 1987; Breitholtz et al. 1991; Creppy et al. 1991; Breitholtz-Emanuelsson et al. 1993a). The health risks of Canadians due to the presence of OA in food products was examined by the Health Protection Branch of Health Canada (Kuiper-Goodman and Scott 1989). It was recommended that further monitoring programs to assess the presence of OA in agricultural commodities and human blood were necessary to evaluate dietary exposure, as well as the need for regulation. Currently, there are no regulations regarding OA in agricultural products. The implementation of such regulations would therefore require edible tissues to be monitored for OA or at least devise an accurate method of predicting their concentrations.

The objectives of the research presented in this thesis were to:

1) Monitor the presence of OA in swine serum, and assess the effect of season and geographic location on its incidence.

2) Determine if serum concentrations of OA in animals from the same herd were similar, and thus indicative of the OA status of the entire herd.

3) Monitor the presence of OA in human serum samples over a two-year period, and determine if a relationship exists between renal disorders and the presence of OA.

4) Systematically establish the relationship between blood and tissue concentrations of OA at equilibrium in swine fed diets containing different dietary concentrations of the toxin.

LITERATURE REVIEW

1. Fungal Contamination

Fungi are a major cause of spoilage in stored grains and seeds, and rank only second to insects as a cause of deterioration and loss (CAST 1989). The presence of storage fungi in agricultural commodities in Canada has been well documented (Wallace and Sinha 1962; Scott et al. 1972, Prior 1976, Abramson et al. 1983a). Fungal infection may result in a decrease in quality, grade and market value of cereal grains and their products. Furthermore, some species of fungi have the potential to produce toxic secondary metabolites, called mycotoxins, and thus have implications for human and animal health. The occurrence of fungi and/or mycotoxins is of utmost importance to grain exporting countries like Canada, which are concerned with ensuring high standards of quality in their produce.

1.1 Ochratoxin-Producing Fungal Species

Aspergillus and *Penicillium* species are the most important filamentous fungi invading cereals in stored agricultural commodities in many countries (Frisvad and Samson 1991), including Canada (Sinha et al. 1986). Many *Aspergillus* and *Penicillium* species are capable of producing toxic secondary metabolites or mycotoxins, such as ochratoxin A (OA). This nephrotoxic mycotoxin was initially isolated from *Aspergillus ochraceus* Wilhelm (van der Merwe et al. 1965a). Since this time several other

Aspergillus species, capable of producing OA, have been identified and include *A. ostianus*, *A. quercinus* (*A. mellus*), *A. sulphureus* (*A. fresenii*) (Frisvad and Samson 1991). Ochratoxin A production by *Eurotium herbariorum*, a member of the *Aspergillus glaucus* group, has also been reported (Chelkowski et al. 1987). Frisvad and Samson (1991) have suggested that *Eurotium repens* may also produce OA. *Eurotium* species are very common in cereals and possible production of mycotoxins would be of considerable concern.

Although numerous species of *Penicillium* have been reported to produce OA, *Penicillium verrucosum* Dierckx is the only species to date, for which OA production has been confirmed (Pitt 1987; Frisvad and Filtenborg 1989). The apparent production of OA by other *Penicillium* species is attributed to misidentification of species, the use of different systems of nomenclature and synonymy of fungal names (El-Banna et al. 1987; Frisvad 1989).

In recent years, new techniques for fungal identification including chemotaxonomy, electrophoresis, genetic and ultrastructure studies, as well as immunological techniques, have been used in conjunction with traditional morphology-based taxonomy to clarify the taxonomy of fungal species. In the literature cited below, the species are cited as they appear in the original reference.

1.2 Factors Influencing Fungal Growth and Toxin Production

Factors which are likely to affect fungal growth and OA formation include water activity, temperature, time, damage to the seed, oxygen and carbon dioxide

levels, composition of the substrate, fungal abundance, prevalence of toxigenic strains, spore load, microbial interactions and invertebrate vectors (Hesseltine 1974). Spoilage, fungal growth and mycotoxin formation may result from the complex interaction of these factors. Thus, the species of ochratoxin-producing fungi which predominate in a given area are dependent upon climatic conditions, particularly temperature. *Aspergillus* species tend to predominate in warmer climates such as Yugoslavia, while *Penicillium* species tend to predominate in more temperate climates such as Sweden, Denmark and Canada (Krogh 1987).

The minimum water activity (a_w) for most species colonizing stored cereal grains is about 0.70 (Lacey 1989). Adequate water for fungal growth in grain may result from inadequate drying before storage, penetration of rain or wind driven snow into storage structures or moisture migration because of temperature gradients within a bin (Christensen and Kaufmann 1974; Sauer 1978; Lacey 1989). Temperature has considerable influence on water requirements as the minimum requirements for growth are different at different temperatures and on different substrates. The minimum a_w for growth can be lowest at the optimum temperature and highest near minimum and maximum growth temperatures (Bullerman et al. 1984). The water and temperature requirements for several ochratoxin-producing species of fungi have been determined by Northolt et al. (1979). The minimum a_w for ochratoxin formation were 0.83-0.87, 0.87-0.90 and 0.83-0.86 for *A. ochraceus*, *Penicillium cyclopium* Westling and *Penicillium viridicatum* Westling, respectively. At 24°C, optimum a_w was 0.99 for *A. ochraceus* and 0.95-0.99 for *P. cyclopium* and *P.*

viridicatum. At optimum a_w, the temperature range for OA production was 12-37°C for *A. ochraceus* and 4-31°C for *P. cyclopium* and *P. viridicatum*.

1.3 Natural Occurrence of Ochratoxin-Producing Fungal Species

Aspergillus and *Penicillium* species are widespread in nature. *Aspergillus ochraceus* have been isolated from a variety of agricultural commodities including stored feed wheat, oats and barley (Abramson et al. 1983a), poultry feed (Bacon et al. 1973), rice (Yamazaki et al. 1970)), brewery hops (van Walbeek et al. 1968), black and red pepper (Christensen et al. 1967), peanuts (Doupnik and Peckman 1970), brazil nuts (van Walbeek et al. 1968), pecans (Doupnik and Bell 1971), bread (Visconti and Bottalico 1983), cheese (Sinha and Ranjan 1991), and cured ham (Escher et al. 1973).

Ochratoxigenic *Penicillium* species have been isolated from rapeseed (Mills and Abramson 1982), wheat (Scott et al. 1970,1972; Shotwell et al. 1976), rye, oats, mixed feed, dried white beans, peanuts (Scott et al. 1972), corn (Misilevic and Tuite 1970), bread and flour (Osborne 1980; Goliński et al. 1991), cheese (Bullerman 1976) and smoked pork shoulder (Bueno et al. 1982).

2. Ochratoxin A and Related Compounds

2.1 Structures and Relatives Toxicities

Ochratoxin A is comprised of a 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin linked through the 7-carboxyl group to L-ß-phenylalanine by an

amide bond (van der Merwe et al. 1965a, 1965b). The ochratoxins are a group of structurally-related compounds (Figure 1) with ochratoxin A, B, C, α, (van der Merwe et al. 1965b) and hydroxylated-ochratoxin A (Hutchinson and Steyn 1971) being the most common. The ochratoxins are biosynthesized via the formation of an unbranched polyketide from acetyl and malonyl CoA, which undergoes cyclization and aromatization to an isocoumarin, and oxidation to the carboxy derivative which is chlorinated before active acylation for reaction with an ester of phenylalanine which arises via the shikimic acid pathway (Steyn 1984). Ochratoxin A is one of the most toxic compounds of the ochratoxins (Steyn 1984), and therefore is of greatest concern in terms of human and animal health.

Ochratoxin A can be hydrolyzed by inorganic acid (van der Merwe et al. 1965a, 1965b), or by proteolytic enzyme action, to a non-toxic 7-carboxyisocoumarin (ochratoxin α) and phenylalanine (Pitout 1969; Creppy et al. 1983a). Ochratoxin B, the dechloro-derivative of OA, is considerably less toxic than OA (Steyn 1971; Cole and Cox 1981), while ochratoxin C, the ethyl ester of OA, is comparable in toxicity to OA (Steyn 1984). Størmer et al. (1983) has identified several hydroxylated forms of OA, including (4R)-OH-OA, (4S)-OH-OA, and 10-OH-OA. It has been shown that the (4R)-OH-OA metabolite is almost as toxic as OA (Creppy et al. 1983a, 1983c). Analogues of OA, in which phenylalanine was substituted with other amino acids, have been prepared (Creppy et al. 1983b). The tyrosine, valine, serine, and alanine analogs were the most toxic, the methionine, tryptophan, and glutamic acid analogs had intermediate toxicity, while the glutamate and proline analogs had the

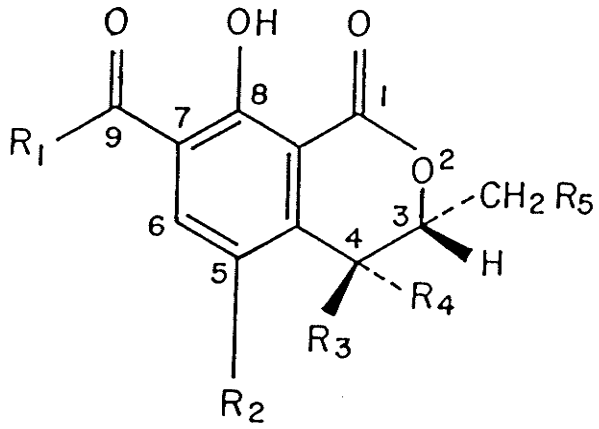

Figure 1: Structure of the ochratoxins. An explanation of the R groups can be found in Table 1.

Table 1. Naturally occurring forms of the ochratoxins[1]

Common name	Abbreviation	R_1[1]	R_2	R_3	R_4	R_5
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 ß	Oß	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

[1] R^1 through R^5 refers to that group bound to carbons 9, 5, 4, 4 and the number 3 methyl carbon, respectively, of ochratoxin.

lowest toxicity when tested on yeast aminoacyl-tRNA synthetases as well as on the growth and protein synthesis of hepatoma cells. All analogues were, however, less toxic than OA. A natural occurrence of the serine, hydroxyproline, and lysine analogues (Hadidane et al. 1991) as well as formation of tyrosine-ochratoxin A in vivo (Creppy et al. 1990) have been reported.

2.2 Physicochemical Properties

Ochratoxin A is a colorless, crystalline compound with a melting point of 90°C when crystallized from benzene (Steyn 1971). It has a molecular weight of 403 and a molecular formula of $C_{20}H_{18}ClNO_6$ (van der Merwe et al. 1965a,1965b). Ochratoxin A is poorly soluble in water, although, the sodium and potassium salts are soluble in polar solvents (Röschenthaler et al. 1984). The ultraviolet absorption spectrum of OA varies with pH and polarity of the solvent. It has maxima in aqueous solutions of 333 nm at pH 1.5 and 380 nm at pH 8.5 (Röschenthaler et al. 1984). The infrared spectrum peaks at 1690, 1665, 1535, 3420, 1745 (van der Merwe et al. 1965a). Major peaks in the electron mass impact spectrum are at *m/z* 239/241 and 255/257 with a low-intensity parent ion at *m/z* 403 (Harwig et al. 1983).

Ochratoxin A has two ionizable groups under physiological conditions, the carboxyl group from phenylalanine and the 8-hydroxyl group. The dissociation constant (pKa) of the 8-hydroxyl group is influenced by the structure; the pKa values are 7.05 to 7.10 for OA and OC, 8.0 for OB, and 11.0 for Oα (Marquardt and Frohlich 1992). Chu et al. (1972) have demonstrated that the toxicity of ochratoxins

is closely related to the pKa of the hydroxyl group, as the 8-hydroxyl group must be in the dissociated form for ochratoxin intoxication. Ochratoxin A and OC, which have comparable molar toxicities, have the same pKa values. Ochratoxin B, which is approximately ten times less toxic than OA, has an acid dissociation constant that is ten times smaller than that of OA. Ochratoxin α is nontoxic and does not dissociate under physiological conditions (Chu et al. 1972).

3. Occurrence of Ochratoxin A in Agricultural Commodities

3.1 Natural Occurrence in Plant Products

The first natural occurrence of OA in plant products was reported on corn (Shotwell et al. 1969) and wheat (Scott et al. 1970). In addition to the commodities listed above which have been found to support the growth of ochratoxigenic species of fungi, OA has also been detected in sorghum and groundnuts (Betina 1989), cowpea (Kane et al. 1991), dried peas and beans (Williams 1985; Fuchs et al., 1991), green coffee beans (Levi et al. 1974) and roast coffee (Tsubouchi et al. 1988). Krogh (1987) summarized survey data on OA from 30 countries. Although the mean level of OA in foodstuffs surveyed to 1979 was 1035 μg/kg, 83% of the samples contained less than 200 μg/kg. In most cases, the frequency of contamination and concentration of OA were generally higher in feeds than in foods.

3.2 Natural Occurrence in Animal Products

Although direct fungal contamination has been observed in several animal

products including cheese (Bullerman 1976) and ham (Escher et al. 1973), the presence of OA in animal products resulting from exposure to OA-contaminated feedstuffs is a greater concern (Kuiper-Goodman and Scott 1989). A natural occurrence of OA has been observed in several species, including swine and poultry, and is of considerable concern due to its demonstrated stability in pork products (Josefsson and Møller 1980).

Ochratoxin A has been detected in the serum and kidneys of swine in several countries, including Sweden (Rutqvist et al. 1977; Hult et al. 1984; Holmberg et al. 1990a,b; 1991), Denmark (Krogh 1977; Buchmann and Hald 1985; Hald 1991b), Poland (Goliński et al. 1984,1985), Germany (Frank et al. 1991), Japan (Ueno et al. 1991), Czechoslovakia (Fukal 1992), and Canada (Marquardt et al. 1988; Kuiper-Goodman et al. 1993). Recent data on the presence of OA in pork products, particularly serum and kidneys, from 15 countries has been summarized by Kuiper-Goodman and Scott (1989). For most countries, the mean OA level in serum containing OA varied between 4.4 and 13.8 ng/ml, but was as high as 85 ng/ml in Yugoslavia. A number of surveys have been carried out in Sweden to monitor the presence of OA in swine. Based on observations of the natural occurrence of OA in swine herds, Swedish researchers have identified several parameters which are associated with ochratoxin development during storage. Holmberg et al. (1990a) observed a positive relationship between the incidence of OA-positive swine blood samples and average moisture content in barley over a six-year period. They concluded that OA production and subsequent contamination of swine blood, and

feed, are influenced by the moisture content of the feed at harvest.

Holmberg et al. (1990b) noted that length of storage, origin of the grain and the drying procedure which was used, influenced the incidence of OA. Grain stored for a long period of time (approximately 12 months) resulted in a higher incidence of OA-positive herds compared to that which was stored for a shorter period of time (six months). A larger number of herds fed grain dried with forced ambient air had detectable levels of OA compared to those fed grain dried with heated forced air. Furthermore, herds fed cereal grain produced on-farm had a higher incidence of OA-positive blood samples than those fed commercial feed in the beginning of the storage period. At the end of the storage period, however, this situation was reversed. At higher contamination levels (5 ng/mL), no difference was observed between the two types of grain after long-term storage. Annual, seasonal, and geographic variations with regards to the presence of OA were also noted.

Mycological analysis of feed samples from OA-positive herds (>2 ng/ml blood) and OA-negative herds (<2.0 ng/ml) demonstrated that there was no difference in mold content, as detected by colony forming units (Holmberg et al. 1991). There were, however, differences in the microflora as the incidence of storage fungi (*Penicillium* and *Aspergillus*), particularly *P. verrucosum*, was higher in feed from OA-positive herds.

In addition to its presence in swine, OA has also been detected in the muscle of chickens destined for slaughter at concentrations as high as 29 μg/kg (Elling et al. 1975). Residues of OA are generally not found in ruminants as OA is cleaved to Oα

by bacteria and protozoa in the rumen (Hult et al. 1976; Patterson et al. 1981; Xiao 1991a,b).

3.3 Experimental Transmission into Animal Tissue

Numerous studies have been carried out to determine the transmission of OA in several species including poultry, cattle, and swine, and have been reviewed by Kuiper-Goodman and Scott (1989).

Krogh et al. (1974) demonstrated that when swine were fed dietary levels of OA of 200, 1000 and 4000 μg/kg feed for 3-4 months, OA was found in increasing concentrations in the muscle, adipose, liver and kidney. Interestingly, the OA concentration in the four tissues was considerably higher after 68 days of feeding, than after nine days and 3-4 months. Furthermore, pigs fed 4000 μg/kg accumulated equal quantities of OA in the liver and fat. A second study by Krogh et al. (1976a) demonstrated that pigs slaughtered at 30-35 kg after one month on feed containing 1000 μg/kg crystalline OA had a slightly different pattern of OA accumulation in the tissues, with the greatest concentration found in the kidney (25.7 μg/kg), followed by the liver (17.8 μg/kg), muscle (11.5 μg/kg), and fat (6.0 μg/kg). Ochratoxin A disappeared from the muscle and fat two weeks after OA was withdrawn. Corresponding disappearance times from the liver and kidney were three and four weeks respectively. Hult et al. (1979) reported a slower rate of disappearance from the blood than from the kidney, liver, muscle and fat and did not observe pathological lesions during the two month exposure period. Elling (1983) observed

that the characteristic renal lesions which are associated with animals exposed to OA, developed after six weeks of exposure, with young animals demonstrating greater susceptibility than older animals. Furthermore, the renal lesions induced early in the growth period persisted even when animals were given a toxin-free diet. Krogh (1976b,1979) fed crystalline OA for a three-month and two year period, and observed a pattern of OA accumulation in the tissues similar to that of Krogh et al. (1976a). In the later study, OA residues in the kidney, liver, and fat were not significantly different for the two exposure periods, but were significantly different in the muscle. Madsen et al. (1982a) fed 310 pigs varying concentration of OA for different weight ranges and observed the highest concentrations of OA in the kidney; muscle and liver contained approximately one-half of that in the kidney; and fat was the least. A similar pattern of accumulation in each of the four tissues was observed when pigs were fed a diet containing either crystalline OA or naturally contaminated barley (Madsen et al. 1982b). The residues of OA in the tissues were, however, higher in the pigs fed naturally contaminated barley than in those fed crystalline OA. Mortensen et al. (1983a) developed the following regression equations of OA in kidney, lean, liver and fat on OA in serum at slaughter:

μg OA/kg kidney = 0.0651 x μg OA/l serum: R^2 = 0.79

μg OA/kg lean = 0.0346 x μg OA/l serum: R^2 = 0.78

μg OA/kg liver = 0.0259 x μg OA/l serum: R^2 = 0.79

μg OA/kg fat = 0.0181 x μg OA/l serum: R^2 = 0.71

Hult et al. (1979) demonstrated that OA in swine blood may be used to predict OA in feed, and also suggested that one sample from a single pig in a herd could be used to identify herds contaminated with OA (correlation coefficient = 0.80) (Hult et al. 1980). Thus, it may be possible to predict OA levels in the tissues of pigs consuming an OA-contaminated diet based on that which is present in the serum.

Although OA has been shown to accumulate in the tissues, Mortensen et al. (1983b) demonstrated that OA residues were not detected in the piglets or the sow's milk when sows were fed naturally contaminated OA at doses of 700 and 1400 μg/kg of feed, for two consecutive periods of gestation and lactation. Breitholz-Emanuelsson et al. (1993b) observed a dose-dependent transfer of OA into the milk of lactating rats given a single oral dose of OA (ranging from 10-250 μg OA/kg body weight). A linear relationship between the concentration of OA in the dam's milk and the blood and kidney of the pups was observed, indicating that the OA concentration in the milk can be used as indicator of the dose to the suckling.

In addition to the observed accumulation of OA in the carcass, performance of the animal was also effected in the above studies but varied with dose and duration of exposure (Krogh et al. 1974,1976b,1979; Madsen et al. 1982a,1982b; Mortensen et al. 1983a). Pig health, in general, was not affected in these studies; however, reduced feed intake, decreased feed efficiency, decreased growth rates, increased water consumption and polyuria were related to increasing levels of OA. Growth rate appeared to be restored once the OA-contaminated diet was removed (Madsen et al. 1982a)

3.4 Occurrence of Ochratoxin A in Human Body Fluids

The presence of OA in human blood was initially investigated in countries such as Yugoslavia where a high incidence of urinary tract tumors and/or endemic nephropathy was apparent (Hult et al. 1982). Since this time, numerous other countries have reported the presence of OA in human blood including Poland (Goliński and Grabarkiewicz-Szczęsna 1986), Germany (Bauer and Gareis 1987), Sweden (Breitholtz et al. 1990, Breitholtz-Emanuelsson et al. 1993a), Bulgaria (Petkova-Bocharova et al. 1988), France (Creppy et al. 1991), Denmark (Hald 1991a), and Czechoslovakia (Fukal and Reisnerova 1990). Ochratoxin A has also been detected in human kidneys (Bauer and Gareis 1987) and human breast milk (Bauer and Gareis 1987; Gareis et al. 1988; Micco et al. 1991; Breitholtz-Emanuelsson et al. 1993a).

4. Toxicity of Ochratoxin A

4.1 Acute Toxicity

Ochratoxin A is acutely toxic to many species of animals including chicks, hens, ducklings, quail, dogs, rats, mice, guinea pig, sheep (see reviews by Chu 1974 and Kuiper-Goodman and Scott 1989). There is considerable variability between these species with regards to their susceptibility to OA, as oral LD_{50} values range from 0.2 mg/kg body weight for the dog to 58.3 mg/kg body weight for the mouse. The dog and the pig appear to be the most sensitive, and rats and mice the least sensitive (Kuiper-Goodman and Scott 1989).

Ochratoxin A has been demonstrated to cause nephropathy, characterized by polyuria, glucosuria, proteinuria, decreased osmolality of the urine, changes in renal function, as well as histopathological changes in the proximal convoluted tubules (Berndt et al. 1980), in numerous mammalian species. Although the kidney is the target organ, changes in the liver and lymph tissue have also been noted (Szczech et al. 1973a,b,c; Krogh 1987) and will be discussed subsequently.

The main clinical features resulting from ingestion of OA (0.2 - 3.0 mg OA/kg body weight) in beagle dogs were anorexia, weight loss, emesis, and retching followed by tenemus, elevated rectal temperature, bilateral purulent conjunctivitis, tonsillitis, polydipsia, polyuria, passage of clots of blood stained mucous from the rectum, dehydration, prostration and death (Szczech et al. 1973a,b). Manifestation of the symptoms occurred earlier when higher doses of OA were given. Gross pathological lesions included mucohemorrhagic enteritis of the cecum, colon and rectum and enlargement of the lymph nodes. The main features of the toxicoses were renal damage, as well as necrosis of the lymphoid tissue. Renal damage was characterized by necrosis and desquamation of the epithelial cells in the convoluted tubules as well as by elevated concentrations of protein, glucose, lactic dehydrogenase, isocitric dehydrogenase, glutamic-pyruvic transaminase, glutamic-oxalacetic transaminase, and alkaline phosphatase in the urine.

Oral administration of 1.0-2.0 mg/kg/body weight OA to pigs resulted in a mycotoxicosis characterized by reduced feed consumption and decreased body weight followed by diarrhea, polyuria, and dehydration (Szczech et al. 1973c). Fatty changes

and discoloration were apparent in the liver and packed cell volume, hemoglobin, total plasma protein and blood urea nitrogen were increased. Lactic dehydrogenase, isocitric dehydrogenase, and glutamic-oxalacetic transaminase concentrations in the urine were also increased.

4.2 Chronic toxicity

Several studies have been carried out to assess the chronic toxicity of OA in pigs (Krogh et al. 1974, 1976a,b, 1979). Doses of 200, 1000 and 4000 μg of OA per kg of feed administered to pigs, resulted in changes which appeared to be localized in the proximal tubule of the kidney (Krogh et al. 1974). Renal function was characterized by impairment of proximal tubular function, indicated by a decrease in the transport maximum for *p*-aminohippuric acid ($Tm\text{-}_{PAH}$) and $Tm\text{-}_{PAH}$/inulin clearance, as well as a decreased ability to concentrate urine. An increase in the urinary concentration of glucose, leucine aminopeptidase and protein was also observed. Protein excretion in the urine was dose-dependent, consisting of albumin and other proteins with a large molecular weight. These changes in renal function correspond to changes in renal structure which include degeneration of proximal tubules and interstitial formation of connective tissue, as well as sclerotized glomerular tufts in the group receiving the highest dose of OA. Krogh et al. (1979) subsequently observed that doses of 1 mg/kg feed, resulted in a decrease in the ratio of Tm_{PAH}/C_{in}, increased glucose excretion, and a decreased ability to concentrate urine within a few weeks of exposure to OA. No further changes in Tm_{PAH} were

noted after two years of exposure to OA, although the resorptive capacity for glucose and the ability to produce a hyperosmolar urine were more pronounced. The morphological renal changes were aggravated after two years of exposure, however, there was no apparent decrease in glomerular filtration, and a state of terminal renal failure was never reached. Gekle and associates observed a reduction in glomerular filtration rate and of para-aminohippuric acid clearance, increased Na, K, and Cl excretion (Gekle et al. 1992). They concluded that reductions in glomerular filtration rate, are due, in part, by a change in renal hemodynamics mediated by angiotensin II and not tubulo-glomerular feedback. Ochratoxin A treatment led to a reduction in renal plasma flow and in an increase in total renal vascular resistance (Gekle and Silbernagl 1993). Furthermore, they suggested that OA acts acutely on "post proximal" parts of the nephron, possibly in the collecting duct, where it exhibits an inhibitory action on plasma membrane anion conductance (Gekle et al. 1993a) and disrupts pH and chloride homeostasis, leading to functional and morphological alterations (Gekle et al. 1994).

The target organ for ochratoxicoses in poultry is the kidney, although other systems such as the liver, gastrointestinal tract, lymphoid organs, skeletal system, hematopoietic tissues and reproductive organs may also be affected (Burns and Dwivedi 1986). Huff et al. (1988) have demonstrated that chickens fed 0.0, 1.0, 2.0, and 4.0 μg OA/g feed experienced growth depression, as well as increased relative weights of liver, kidney, spleen, pancreas and gizzard. Anemia characterized by a decrease in packed-cell volume and hemoglobin was evident. Nephrotoxicity was

characterized by an increase in serum uric acid and creatinine levels. A reduction in serum levels of total protein, albumin, globulin, cholesterol, triglycerides and blood urea nitrogen and an increase in serum activities of gamma glutamyl transpeptidase and cholinesterase were indicative of hepatotoxicity.

Administration of OA has also been shown to reduce the levels of membrane bound enzymes (ATPase, Na^+ K^+ dependent ATPase and alkaline phosphatase) and macromolecules in the intestine of rats, resulting in decreased in vivo absorption of ^{14}C-glycine and ^{14}C-glucose (Subramanian et al. 1991). Impairment of spermatogenesis in rats given 2.0 ppm of OA per day for a period of eight weeks has also been observed (Gharbi et al. 1993).

Ochratoxicoses has been assessed in several species by measuring the activity of select urinary and renal enzymes (Elling 1979, Kane et al. 1986a,b; Krogh et al. 1988). Gluconeogenesis from pyruvate was decreased by 26% and renal phosphoenolpyruvate carboxykinase activity (PEPCK) was decreased by 55% in kidney-cortex slices from rats fed 2.0 mg OA/kg orally for two days (Meisner and Selanik 1979). It was concluded that OA is an in vivo inhibitor of renal PEPCK and thus accounts for the block in renal gluconeogenesis. A subsequent study by Meisner and Meisner (1981) demonstrated that rats fed OA orally for 2 days show a dose-dependent decrease in renal PEPCK activity. At a dose of 1 mg OA/kg per day, PEPCK activity was reduced by 50%. Other enzymes located in the proximal convoluted tubules including phosphate-dependent glutaminase, gamma glutamyltranspeptidase, pyruvate carboxylase and Na, K-ATPase were not affected.

Elling (1979) demonstrated a reduction in NADH-tetrazolium reductase and succinate dehydrogenase in the proximal tubule of all nephrons after five days of OA exposure, which remained reduced after three months and two years of OA exposure. Krogh et al. (1988) demonstrated that PEPCK activity was decreased by 40% in pigs fed 1 ppm of OA after 1 week, and remained inhibited until the termination of the experiment at 5 weeks. Gamma glutamyl transpeptidase activity was reduced to a similar degree. The decrease in activity of these two enzymes was dose dependent. Hexokinase, a cytosolic enzyme located in the nephron, and phosphate dependent glutaminase, an enzyme located in the distal tubule, were not affected. Kane et al. (1986b) demonstrated that the activity of gamma glutamyl transferase and leucine aminopeptidase (located in the brush border) and alkaline phosphatase (located primarily in the lumenal plasma membrane, with low amounts in the endoplasmic reticulum) in the urine and tubules were well correlated to the nephrotoxic effects of low doses of OA administered orally.

4.3 Carcinogenicity

Two studies conducted recently in mice (Bendele et al. 1985a) and rats (NTP 1989), have provided strong evidence that OA is a potent renal carcinogen. Male and female (C57BL/6J X C3H)F_1 (B6C3F_1) mice were fed 1 ppm OA and 40 ppm OA for 24 months (Bendele et al. 1985a). Renal neoplasms, both carcinomas and adenomas, were found in 26 of the 49 male mice fed the 40 ppm diet. The incidence of hepatocellular neoplasms was slightly increased in both males and females fed OA.

It was concluded that OA is a renal carcinogen in male mice.

The National Toxicology Program evaluated the carcinogenic potential of OA in male and female Fisher 344/N rats (NTP 1989). Ochratoxin A was administered in corn oil by gavage at 0, 1, 4, or 16 mg/kg five days per week for a two year period. The incidence of renal tubular adenomas and carcinomas observed in male rats was much higher than that observed in females. Furthermore, the incidence in males was the highest seen in any study of the National Cancer Institute/National Toxicology Program studies to date. The neoplasms were often multiple and bilateral at the highest dose level. In female rats, an increased incidence of mammary gland fibroadenomas was also observed.

4.4 Immunotoxicity

In addition to its carcinogenic potential, OA has been shown to be cause immunomodulation (Hong et al. 1988) and depletion of lymphoid cells in the thymus, bursa of Fabricus, spleen and pyers patches (Szczech et al. 1973a,b; Dwivedi and Burns 1984a, 1985, 1986).

At the cellular level, administration of OA to turkeys caused severe lymphoid depletion of the thymus with a concurrent reduction in delayed hypersensitivity responses, indicating suppression of cell mediated immunity (Dwivedi and Burns 1985, 1986). Growing gilts treated with 2.5 mg OA per kg of feed for 35 days also showed signs of a reduction in delayed hypersensitivity response (Harvey et al. 1992). Singh et al (1990) have also observed significant reductions in cell-mediated immunity

in broiler chicks, as measured by diminished skin sensitivity, graft versus host reactions and T-lymphocyte counts. Furthermore, OA has been shown to specifically inhibit natural killer cell activity and increase the growth of transplantable tumor cells without altering T-cell or macrophage-mediated antitumor activity in mice ingesting OA at levels of 6.7-13.4 mg/kg body weight (Luster et al. 1987). Natural killer cell activity appeared to be suppressed by inhibition of basal interferon by OA. Lea et al. (1989), however, have reported that OA has inhibitory effects on both T and B lymphocytes. Holmberg et al. (1988) have demonstrated a dose-dependent inhibition of cell-mediated immune response by OA in porcine blood lymphocytes. A 10% inhibition in lymphocyte response occurred at a dose of 0.06 mg OA/l, a level which has been detected in the blood of slaughter pigs.

At the humoral level OA has been shown to reduce serum IgA, IgG and IgM concentrations in broiler chicks (Dwivedi and Burns 1984b) but had no effect on serum immunoglobulin levels in cows (Patterson et al. 1981). The complement system was not affected by oral administration of OA (0.45 or 10.45 mg/day) to guinea pigs (Richard et al. 1975).

4.5 Genotoxicity

The genotoxicity of OA has been reviewed by Bendele et al. (1985b) and Kuiper-Goodman and Scott (1989). The genotoxic potential of OA was evaluated in several in vitro and in assays (Bendele et al. 1985b). Ochratoxin A was not mutagenic to *Salmonella typhimurium*, nor was there any evidence of unscheduled

DNA syntheses in primary rat hepatocytes exposed to concentrations of OA ranging from 0.000025 to 500 μg/mL. In addition, exposure of L5178Y tk+/- mouse lymphocytes to OA did not increase the number of mutants. DNA damage (single strand breaks), however, was observed in vivo in mouse spleen, kidney and liver cells (Creppy et al. 1985), as well as in rat liver and kidney (Kane et al. 1986c). DNA adducts were also observed in kidney, liver, and spleen of mice after oral treatment with OA (Pfohl-Leszkowicz et al. 1991) and in tumerous kidney and bladder tissue from Bulgarian patients undergoing treatment for cancer (Pfohl-Leszkowicz et al. 1993). Ochratoxin A was also found to induce aberrations on X chromosomes, including X-trisomy, in cultured human lymphocytes (Manolova et al. 1990). Therefore, although OA is not mutagenic, it is weakly genotoxic to mammalian cells.

4.6 Teratogenicity

Species differences exist in terms of the teratogenic effects of OA, as OA has been shown to be a potent teratogen in chick embyros (Vesely and Vesela 1992), mice (Hayes et al. 1974; Szczech and Hood 1981) rats (Brown et al. 1976), and hamsters (Hood et al. 1976) but not in pigs (Shreeve et al. 1977). Ochratoxin A has been shown to cause prenatal mortality, reduced fetal growth and a wide variety of anomalies (Hayes et al. 1974; Hood et al. 1976). Observed differences in toxicity have been attributed to the species differences in placental transfer of OA, as well as the time and route of administration (Fukui et al. 1987; Arora et al. 1981). Ochratoxin A caused the greatest effect when given intraperitoneally to mice, eight

days post conception (Hayes et al. 1974), whereas oral administration resulted in maximum effect nine days post conception (Arora et al. 1981). A single i.p. dose of OA on gestation day seven or eight, resulted in excessive cell death in mice (Wei and Sulik 1993). Cranial malformations and body wall defects were observed subsequently on day 14 and 18 of gestation. Shirai et al. (1984) observed a dose-related teratogenic response when OA was given intraperitoneally on day seven of pregnancy. Arora and Fölén (1981) have demonstrated that the stages of embryogenesis prior to day seven or after day nine are not very sensitive to oral administration and subsequent teratogenic effects of OA. Fukui et al. (1987), however, have demonstrated that OA crossed the placental barrier on days 11 and 13 of gestation in mice. Administration of OA to pigs from day 26 to 42 of pregnancy has no effect on the development of the fetuses (Bauer and Gedek 1992). In teratogenicity studies with OA, the central nervous system, the eye and the axial skeleton were the most susceptible targets at early organogenesis (Kuiper-Goodman and Scott 1989).

5. Biochemical Mechanisms of Ochratoxicoses

5.1 Effect on Carbohydrate Metabolism

Administration of OA has been shown to result in accumulation of glycogen in the cytoplasm of liver cells in rats (Pitout 1968; Purchase and Theron 1968) and chickens (Huff et al. 1979). Warren and Hamilton (1980) have demonstrated that this accumulation can be attributed to an inhibition in the activation of glycogenolysis

via cAMP-dependent protein kinase. However, Munro et al. (1983) and Subramanian et al. (1989) observed reduced glycogen levels in rats fed OA. Subramanian et al. (1989) monitored key enzymes of carbohydrate metabolism in the liver and observed decreases in the activity of glycolytic enzymes such as hexokinase, aldolase and lactate dehydrogenase and increases in gluconeogenic enzymes such as glucose-6-phosphatase and fructose-1,6-diphosphatase. Glycogen synthetase activity was decreased while glycogen phosphorylase activity was increased. As discussed previously, OA has also been found to inhibit PEPCK, a key gluconeogenic enzyme in the kidney (Meisner and Selanik 1979; Meisner and Meisner 1981; Krogh et al. 1988).

5.2 Effect on Protein Synthesis

Numerous studies have demonstrated that OA affects RNA and protein synthesis (Konrad and Röschenthaler 1977; Bunge et al. 1978; Meisner and Selanik 1979; Meisner and Meisner 1981; Creppy et al. 1983a,1983b,1984; Elling et al. 1985, Meisner and Polsinelli 1986). Ochratoxin A has been shown to reduce protein synthesis in *Bacillus subtilis* (Bunge et al. 1978), *B. stearothermophilus* (Konrad and Röschenthaler 1977), and yeast (Creppy et al. 1979a), by competing with phenylalanine in the reaction catalyzed by phenylalanine-tRNA synthetase. Creppy et al. (1984) demonstrated that a 1 mg/kg body weight dose of OA given i.p., resulted in an inhibition of protein synthesis by 26, 68 and 75% in the liver, kidney and spleen respectively. Inhibition can be prevented or reversed with administration of

phenylalanine-tRNA or phenylalanine (Bunge et al. 1978; Creppy et al. 1984; 1979a). Braunberg et al. (1992) have demonstrated that protein and DNA synthesis are particularly sensitive to OA, at 0.01 and 0.001 nM respectively, while RNA synthesis was somewhat less sensitive with 60% inhibition at 1 mM.

Ochratoxin B, unlike OA, does not inhibit aminoacylation of phenylalanine-tRNA synthetase, nor does it act as an antagonist and prevent OA-inhibition of protein synthesis (Roth et al. 1989).

As indicated above, OA has been shown to inhibit the activity of renal PEPCK (Meisner and Selanik 1979; Meisner and Meisner 1981). A subsequent study by the same researchers demonstrated that OA lowers total renal mRNA concentration, and certain species, such as PEPCK mRNA, are reduced to a greater extent (Meisner et al. 1983). Meisner and Posinelli (1986) have demonstrated that the rate of transcription of the genes coding for OA sensitive RNA's is not affected. They concluded that OA affects the concentration of certain mRNA's by a post-transcriptional mechanism such as RNA processing (poly A addition, splicing or capping), translational efficiency, or degradation rate.

Incubation of OA with rat hepatocytes results in inhibition of both phenylalanine hydroxylation to tyrosine, which is catalyzed by phenylalanine hydrolase, as well as metabolism of tyrosine (Creppy et al. 1990). Furthermore, the in vivo formation of tyrosine-OA was also observed. This is important toxicologically, as synthetic tyrosine-OA has been shown to be as toxic as OA in hepatoma cell cultures (Creppy et al. 1983b). These researchers have suggested that blockage of

phenylalanine hydroxylase could not only reduce the flux of phenylalanine to tyrosine and related metabolites but also produce "phenylketonuria-like" effects, including an enhanced production of phenylpyruvate and phenylacetate.

4.3 Effect on Lipid Peroxidation

Another mechanism which has been proposed for OA toxicity is lipid peroxidation. The addition of OA to rat liver and kidney microsomes resulted in an enhanced rate of NADPH or ascorbate-dependent lipid peroxidation as measured by malondialdehyde formation, indicating that OA toxicity may be attributed to lipid peroxidation (Rahimtula et al. 1988). The authors provided further evidence for this hypothesis as not only were the toxicities of OA, OB, OC and Oα, well correlated with their ability to enhance lipid peroxidation, but administration of OA to rats resulted in enhanced lipid peroxidation in vivo as measured by an increase in exhaled ethane. Subsequent studies indicated that OA stimulates lipid peroxidation by complexing Fe^{3+}, thereby facilitating its reduction (Omar et al. 1990), and that cytochrome P-450 could substantially enhance the rate of OA-induced lipid peroxidation in phospholipid vesicles (Omar et al. 1991a). The Fe^{3+} complex of OA has previously been shown to produce hydroxyl radicals in the presence of the NADPH-cytochrome P-450 reductase system (Hasinoff et al. 1990). Khan et al. (1989) reported enhanced lipid peroxidation in the presence of OA, as well as inhibition of ATP-dependent calcium uptake in liver microsomes. Furthermore, they showed that various agents that were able to inhibit OA-enhanced lipid peroxidation

could block this destruction of calcium uptake activity. It was concluded that OA causes enhanced lipid peroxidation, thereby impairing the endoplasmic reticulum membrane, and disrupting microsomal calcium homeostasis. Chong and Rahimtula (1992) observed an increase in the renal cortex endoplasmic reticulum calcium pump activity in rats given a single high dose or multiple low doses of OA. Furthermore, there was no evidence of lipid peroxidation, as measured by malondialdehyde levels and a variety of antioxidant enzymes. It was therefore concluded that OA does not have a significant effect on renal cortex calcium uptake nor does it induce lipid peroxidation in the renal cortex. Subsequent studies by Omar and Rahimtula (1993) have demonstrated that lipid peroxidation is induced by an active Fe^{2+}-oxygen complex, formed by NADPH-cytochrome P450 reductase and cytochrome P450-dependent reduction of free Fe^{3+}, followed by oxygen binding.

5.4 Effect on Mitochondrial ATP

Several studies have indicated that another mechanism of OA toxicity may be directed towards mitochondrial ATP. Administration of OA in rats altered mitochondrial morphology (Suzuki et al. 1975) and caused liver mitochondrial degeneration (Purchase and Theron 1968). Ochratoxin A, which is taken up by rat liver mitochondria in an energy-dependent manner (Meisner 1976), inhibits respiration and oxidative phosphorylation in rat liver mitochondria (Meisner and Chan 1974; Wei et al. 1975) as it acts as a competitive inhibitor of liver mitochondrial transport carrier proteins and phosphate transport and depletes mitochondrial ATP

(Meisner and Chan 1974; Meisner 1976; Wei et al. 1985). Dose-dependent depletion of mitochondrial ATP (Jung and Endou 1989) and reduced respiration (Aleo et al. 1991) has also been observed in rat renal proximal tubules. In the latter study it was suggested that OA toxicity in the kidney may be attributed to an inhibition of mitochondrial function as measured by lactate dehydrogenase release and oxygen consumption rather than lipid peroxidation as measured by malondialdehyde.

5.5 Effect on Platelet Aggregation

In addition to the above mechanisms of OA toxicity, several studies have suggested that OA may also interfere with blood clotting mechanisms. Ochratoxin A has been shown to increase clotting times in mice (Gupta et al. 1979) and decrease plasma fibrinogen and factors II, VII, and X in rats (Galtier et al. 1979b). However, these inhibitory effects on platelet aggregation have recently been attributed to a disruption of the platelet plasma membrane rather than to a specific OA-induced effect (Omar et al. 1991b).

6. Ochratoxin A as a Causal Agent of Specific Diseases

6.1 Porcine Nephropathy

A nephropathy in Danish pigs was initially described by Larsen in 1928 (Krogh 1987). This disease, characterized by tubular atrophy and interstitial fibrosis, was associated with feeding batches of moldy grain. Animals suffering from this disease had kidneys which were enlarged and greyish-brown in color. The changes in the

kidney consisted of progressive interstitial fibrosis and regressive tubular changes with thickening of the basement membrane and hyalinization of many glomeruli. In the later stages, atrophy of the neurons and dilation of the tubules was observed (Elling and Møller 1973). A relationship between OA and the observed renal lesions was suggested by Elling and Møller (1973) and Krogh et al. (1973).

Feeding studies utilizing crystalline OA provided additional evidence that OA was the causal agent of porcine nephropathy (Krogh et al. 1974). The reproduced nephropathy was similar to the naturally-occurring nephropathy. The resulting changes were confined to the kidneys and proximal tubules. Early signs of the induced nephropathy included polydipsia and polyuria. The changes in renal function were characterized by impairment of the proximal tubule function. The changes of renal structure were characterized by degeneration of the proximal tubules resulting in interstitial atrophy and interstitial fibrosis. Thus, it was concluded that OA was a major causal determinant of this nephropathy called porcine nephropathy. Citrinin, another nephrotoxic mycotoxin, is frequently a co-contaminant with OA, and may act synergistically with OA in this disease (Krogh 1978; Hald 1991b). Cases of porcine nephropathy have also been observed in Sweden, Germany, Hungary, and Poland (Rutqvist et al. 1978; see Krogh 1987 and Hald 1991b for a review).

6.2 Balkan Endemic Nephropathy

Balkan endemic nephropathy (BEN) is a primary chronic bilateral kidney disease encountered in the Balkan countries - Yugoslavia, Bulgaria and Romania -

and has been described in detail (Plestina 1992; Vukelić et al. 1992; Radonić and Radošević 1992). Histopathologically, the disease is characterized by degenerative and regenerative processes in the proximal tubules, diffuse cortical fibrosis, and either complete or partial hyalinization of the glomeruli, as well as degenerative and necrotic changes in the epithelium (Vukelić et al. 1992).

The main epidemiological features of this disease are as follows: focal occurrence in rural areas, residence in an endemic area for over ten years, unnoticeable onset without an acute episode, lack of an inheritance pattern of other known genetic diseases (Pleština 1992). In most areas, nearly twice as many women as men are affected by the disease, while the highest morbidity occurs in individuals between 35 and 55 years of age (Austwick 1975). Furthermore, all ethnic groups and religions are affected (Pleština 1992). The morbidity or prevalence of BEN lies somewhere between 2-10%. Specific mortality for the Slavonski Brod area averaged 1.54 per thousand per year during the period 1957-1987 (Čeović et al. 1992).

Many studies have investigated the main etiology of BEN. Nephrotoxins have been suggested as causal agents include lead, natural or synthetic drugs, silica, cadmium, uranium, aluminum, arsenic and selenium (Pleština 1992). Barnes et al. (1977) suggested fungal metabolites as a possible nephrotoxic agent. Based on a comparison of the morphological and functional kidney impairment between OA-induced porcine nephropathy and Balkan endemic nephropathy, (Krogh 1974 cited in Krogh 1992), OA has been suggested as a disease determinant of BEN. Further to this, OA was observed in an endemic area of Yugoslavia in human food and

animal feed (Krogh et al. 1977; Pavlović et al. 1979), as well as in human blood (Hult et al. 1982). In addition, a study by Castegnaro and Chernozemsky (1987) observed that one-third of the patients suffering from BEN had papillomas and/or carcinomas of the renal ureter and bladder, with individuals in endemic areas having a 90-fold greater risk of developing urinary tract tumors compared to patients from other nonendemic areas.

7. Pharmacokinetics of Ochratoxin A

7.1 Absorption

The two ionizable groups in OA - the phenolic hydroxy in the dihydroisocoumarin ring and the carboxylic group in phenylalanine - may exist in either ionized or nonionized forms in aqueous media, depending of the pH of the media (Chu et al. 1972). The main mechanism of gastrointestinal transfer for many weak electrolytes is diffusion of the nonionized form across the lipid membrane (Gibaldi 1984). Absorption of OA probably occurs by the same mechanism, as in vitro uptake of OA increased when the pH of the medium was decreased, as a result of an increase in the proportion of the nonionized form (Kumagai 1988). The content of OA in the stomach of rats given OA orally was high one hour after administration, decreased rapidly at two hours, and then increased to 65% of the maximum level at four hours. Higher concentrations of OA were found in the duodenum as compared to the ileum and colon. Therefore, it was concluded that OA was absorbed mainly from the stomach and small intestine (Suzuki et al. 1977).

Kumagai and Aibara (1982) reported maximum absorption of OA in the proximal jejunum, as portal blood was found to contain the largest concentration of OA when the toxin was placed in this region of the gastrointestinal tract. Absorption was intermediate in the duodenum and mid jejunum to mid ileum, and lowest in the distal ileum. The latter occurring probably as a result of the low ileal concentration of OA (Galtier 1991). Immunohistochemical staining for OA in mice was demonstrated in the esophagus, stomach and small intestine (Lee et al. 1984), confirming previous findings that OA absorption occurs in the upper portion of the gastrointestinal tract. Intravenous administration of OA indicated that jejunal absorption of OA occurred even when the jejunal lumen has a lower concentration than plasma (Kumagai 1988). This was attributed to the low mucosal pH that is present in the jejunum.

7.2 Distribution

Ochratoxin A has a high binding affinity to plasma constituents (Chu 1971; Chang and Chu 1977; Galtier 1979a; Stojković et al. 1984; Kumugai 1985). The interaction of OA with bovine serum albumin has been demonstrated by several researchers (Chang and Chu 1977; Galtier 1979a; Kumagai 1985) with approximately 1.81-2.47 moles of OA able to bind to one mole of BSA (Chu 1971). Kumagai (1985) has demonstrated that the rate of OA excretion in albumin-deficient rats was 20-70 fold faster than that in normal rats. Therefore, it was concluded that the effect of the interaction between OA and albumin is to restrict the entry of OA into hepatic and renal cells thereby retarding elimination. The binding of OA to serum in vitro may

be competitively inhibited by several acidic drugs - phenylbutazone, ethylbiscoumacetate and sulfamethazone - indicating that these drugs and the toxin probably bind at the same site on the albumin molecule (Galtier et al. 1980).

The in vitro binding properties of OA to other plasma constituents has been investigated by Stojković et al. (1984). OA was shown to bind to plasma constituents (MW 20,000) other than albumin with higher affinity than that observed for albumin. They suggested that the binding of OA to these low molecular weight compounds may account for the nephrotoxic effect of OA, as these small molecules could pass through the glomerular membrane and accumulate in the kidney.

The plasma binding properties of OA have been investigated in several species including quail, rat, monkey, pig and humans. In all species, 99-100% of the toxin was bound to plasma proteins except for the fish in which only 78% of the toxin was bound (Hagelberg et al. 1989). The half-life of OA after intravenous injection varied considerably between species and ranged from 12 hours for the quail to 840 hours for the monkey.

The interaction between OA and plasma proteins facilitates the passive absorption of OA from the digestive tract even when the overall concentration is higher in the blood (Kumagai 1988) and also delays elimination and prolongs the its half-life (Kumagai 1985).

The profile of animals administered OA orally indicate a rapid phase of absorption, followed by a slow elimination (Galtier 1991). Several pharmacokinetic parameters such as peak concentration in plasma and its corresponding time,

biological half-life and bioavailability have been determined for several species (see Galtier 1991 for a review). Not only are these parameters species-dependent, but may also be dose-dependent. Half-life appears to be relatively long in monogastric species such as the monkey, pig and rat, and comparatively shorter in avian and fish species. Bioavailability is relatively uniform for all species (approximately 60%) except for the chicken and fish, which are somewhat lower (40 and 16% respectively) (Galtier 1991).

A pharmacokinetic study in rats utilizing radiolabelled OA (^{14}C-ochratoxin A) indicated that OA is distributed in two kinetically distinct compartments (Galtier et al. 1979a). The central compartment corresponded to well perfused organs including kidneys, liver, spleen and brain, and the peripheral compartment included muscle, fat, eyes and glands.

The use of autoradiography has provided considerable information about the distribution of OA in a variety of species including the rainbow trout (Fuchs et al. 1986), mouse (Fuchs et al. 1988a, Breitholtz-Emanuelsson et al. 1992), quail (Fuchs et al. 1988b) and pregnant mouse (Applegren and Arora, 1983). Breitholtz-Emanuelsson et al. (1992) have also used autoradiography to study the distribution and metabolism of OB in the mouse. Although the distribution patterns were similar, OB was excreted more rapidly than OA.

The transmission of OA into animal tissue and the resulting residues have been discussed in a previous section and will not be discussed in this section.

Biliary recycling, as indicated by secondary distribution peaks of OA has been

demonstrated in rats (Fuchs et al. 1988c), mice (Roth et al. 1988) and calves (Sreemannarayana et al. 1988). Reabsorption of OA from the gastrointestinal tract back into the circulation, as a result of biliary recycling, leads to redistribution and re-exposure of OA (Marquardt and Frohlich 1992). Roth et al. (1988) has suggested that a considerable portion of the OA may be conjugated in the liver and excreted as glucuronide and sulfate conjugates which are hydrolyzed by intestinal microflora to OA and Oα.

Renal reabsorption in the proximal tubule, as depicted in Figure 2, is another mechanism by which OA persists in the body and may account for the gross changes associated with ochratoxicoses (Stein et al. 1985, Gekle et al. 1993b).

7.3 Metabolism of Ochratoxin A

Ochratoxin A can be metabolized in the body by several mechanisms. Pitout (1969) concluded that OA could be hydrolyzed by carboxypeptidase A and possibly trypsin, α-chymotrypsin and cathepsin C. Subsequent incubation of OA with rat tissue homogenates indicated that the conversion to Oα occurred in the pancreas and small intestine, and not in the liver and kidney (Doster and Sinnhuber 1972; Suzuki et al. 1977). Studies with rats treated with OA indicated that Oα was primarily located in the large intestine and incubation of OA with rat caecal contents revealed that hydrolysis resulted from the bacterial flora (Galtier et al. 1974 cited by Galtier 1991). Hydrolytic detoxification by microflora also occurs in the rumen fluid (Hult et al. 1976; Ribelin et al. 1978; Xiao 1991a,b). This mechanism has been further

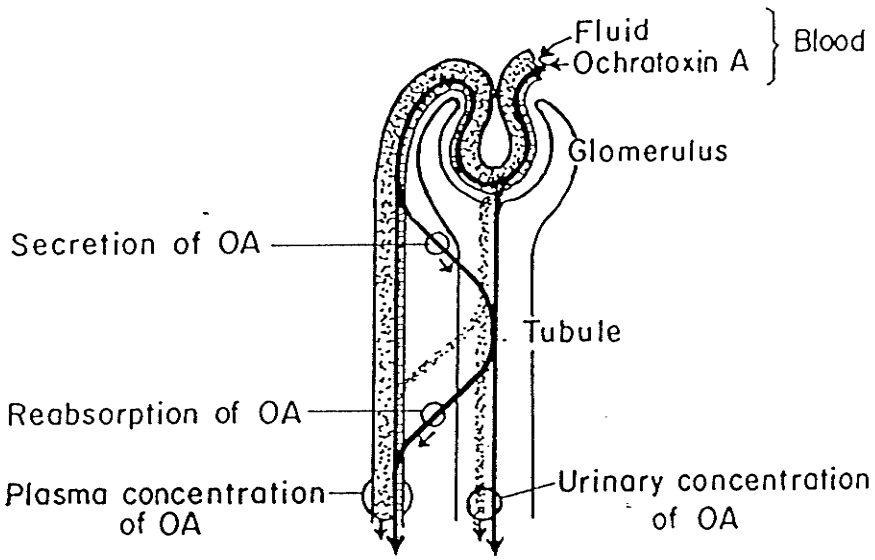

Figure 2: Illustration of renal tubular excretion of OA (taken from Stein et al. 1985).

confirmed by Madhyasta et al. (1992a) who demonstrated that the presence of neomycin, a broad spectrum antibiotic, decreased the amount of OA excreted in the feces. Thus, hydrolysis of OA occurs by enzymes produced in vivo and by intestinal microorganisms, but does not appear to occur via kidney and liver homogenates.

Another mechanism by which OA is metabolized is via hydroxylation. Several studies have demonstrated that liver microsomes of several species are capable of metabolizing OA in the presence of NADPH to (4R)- and (4S)-hydroxyochratoxin A (Størmer and Pederson 1980; Størmer et al. 1981; Støren et al. 1982). The (4R)-4-hydroxyochratoxin A formation is formed by mixed-function oxidation, while (4S)-4-hydroxyochratoxin A is formed, in part, by an active Fe^{2+}-oxygen complex, formed via NADPH-cytochrome P450 reductase and cytochrome P450-dependent reduction of free Fe^{3+}, followed by oxygen binding (Omar and Rahimtula 1993). The toxicity of the (4R)-hydroxyochratoxin appears to be comparable to that of OA (Creppy et al. 1983a,b,c). Approximately 1.5% of an interperitoneal or per os dose of OA was excreted as (4R)-4-hydroxyochratoxin A, while none of the (4S)-4-hydroxyochratoxin A was detected (Støren et al. 1982). Incubation of OA with rabbit liver microsomes resulted in the formation (4R)- and (4S)-hydroxyochratoxin A, as well as another metabolite identified as 10-hydroxyochratoxin A (Størmer et al. 1983). This compound, however, has not been described in vivo (Galtier 1991). Compounds such as phenobarbital and 3-methylcholanthrene, which are inducers of drug metabolizing enzymes, have been shown to decrease the toxicity of ochratoxin A (Størmer et al. 1983; Chakor et al. 1988). Comparison of two different strains of rats have

revealed differences in production of 4-hydroxyochratoxin A which was attributed to genetic polymorphism (Hietanen et al. 1986), suggesting a co-segregation of genes that regulate OA and debrisoquine 4-hydroxylation (Castegnaro et al. 1989).

7.4 Elimination

Ochratoxin A is eliminated from the body primarily as the native toxin OA and as the metabolite, Oα (Galtier 1991). Stein et al. (1985) have demonstrated that administration of probenecid, an inhibitor of anion secretory processes in the proximal tubules, prior to administration of OA significantly decreased the clearance of OA indicating that secretion of OA via organic anion transport is an important mechanism in OA excretion. Subsequently, excretion of OA via this mechanism has been confirmed by several other researchers (Friis et al. 1988; Sokol et al. 1988). Probenecid has also been shown to diminish OA-induced ATP inhibition in the mitochondria located in the middle and terminal portions of the proximal tubule, suggesting that OA enters the plasma membrane in these regions via the organic anion transport pathway and inhibits oxidative phosphorylation (Jung and Endou 1989). Reabsorption of OA by the kidney has been discussed previously.

8. Management Practices to Reduce Intake or Toxicity of Ochratoxin A

8.1 Prevention and/or Control of Fungal Growth

The factors which influence fungal growth include moisture, temperature, time, damage to the seed coat, O_2 and CO_2 concentrations, microbial interactions,

invertebrate vectors, composition of the substrate, fungal abundance and prevalence of toxigenic strains, as discussed previously (Hesseltine 1974). From a practical standpoint, the producer has limited control over these variables, with the exception of moisture. Abramson and his colleagues have carried out numerous experiments in a variety of agricultural commodities to assess the fungal growth and subsequent toxin production in stored grain ecosystems in which initial moisture was controlled (Abramson et al. 1980, 1983b, 1985, 1987, 1990a,b). From these studies it may be concluded that OA production in grain may be controlled by storage at less than 15% moisture.

Grain dryers are often utilized to ensure that grain is sufficiently dry to store. Holmberg et al. (1990b) has demonstrated that forced heated air dryers are more efficient at preventing OA production than are ambient air dryers.

Several other techniques directed at variables other than the moisture content of the grain have been employed to prevent or control fungal growth. Paster et al. (1983) observed that toxin production on a synthetic medium was completely inhibited at 30% CO_2, regardless of the level of O_2. Fungal growth, however, was not inhibited until CO_2 levels reached 60%. Ochratoxin A production resumed when the high CO_2 environment was terminated suggesting that this technique has limited practical application.

Radiation is another technique for food preservation currently used in several countries (Paster and Bullerman 1988). Chelack et al. (1991a,b) reported that spores of *A. alutaceus* were destroyed with either gamma or electron beam irradiation.

Borsa et al. (1992) reported that OA production was enhanced relative to untreated controls if the toxigenic fungus was inoculated into the grain following irradiation. Ochratoxin A production was diminished if the toxigenic fungus was inoculated prior to treatment.

Antimicrobial agents such as sodium propionate, ethyl paraben or potassium sorbate, have also been used as an effective means of controlling fungal growth and subsequent production of OA (Tong and Draughon 1985). The presence of competing microorganisms tends to restrict fungal growth and mycotoxin production and thus inoculation of grain with competing microorganisms such as lactic acid bacteria, may be an alternative to the above techniques (Bullerman et al. 1984).

Utilizing sound management techniques such as ensuring that grain is sufficiently dry to store, minimizing grain damage due to insects or harvest equipment, repairing leaky bins, and cleaning transport and storage equipment from the previous years debris, are highly effective means of preventing fungal growth.

8.2 Reduction in Intake

Reducing the intake of OA by animals has been attempted through destruction of the toxin. Several techniques, such as treatment of OA-contaminated barley with NH_3, NaOH and autoclaving (Madsen et al. 1983), and inoculation with *Lactobacillus* species prior to ensiling (Rotter et al. 1990) have been investigated. Although these techniques were somewhat effective in reducing the concentration of OA, they had little or no effect on toxicity as measured by residues in the tissue or feeding studies.

More recently, Trivedi et al. (1992) investigated the destruction of OA upon heating under acidic and alkaline conditions. Heating under dry conditions did not destroy the OA nor did it affect cytotoxicity as measured by the proliferation of HeLa cells, whereas heating under moist conditions resulted in small changes in the molecule, but had no effect on cytotoxicity. Ochratoxin A was decomposed when heated with HCl, but once again, cytotoxicity was not changed. Heating with NaOH, however, resulted in decomposition and detoxification of OA.

8.3 Reduction in Uptake of Ochratoxin A

8.3.1 Maximize Hydrolysis

Microorganisms located in the gastrointestinal tract of monogastric as well as ruminants are capable of hydrolyzing OA (Hult et al. 1976; Ribelin et al. 1978; Xiao et al. 1991a,b), as discussed previously. Xiao et al. (1991a,b) have demonstrated that the extent of microbial hydrolysis and therefore bioavailability may be influenced by the type of diet fed. Rumen fluid from hay-fed sheep was able to hydrolyze OA as much as five times more quickly than that of grain-fed sheep.

8.3.2 Addition of Dietary Adsorbents

The uptake of OA may also be modified through the use of dietary adsorbents such as sodium calcium aluminosilicate, bentonite, and charcoal. Bauer et al. (1990) demonstrated that dietary addition of hydrated sodium calcium aluminosilicate (1%) and bentonite (1 and 10%) to swine diets had no effect on OA levels in serum,

tissues and bile. However, activated charcoal, at 1%, resulted in a slight decrease in serum OA, while when added at 10%, resulted in a 50-80% decrease in the OA concentration in serum, tissues and bile. Grunkemeier (1992) also demonstrated a reduction in the OA content in the blood and tissues of pigs fed a diet containing 1 mg OA per kg of feed and 10% activated charcoal. The charcoal was ineffective at a dietary concentration of 2%. Other less effective adsorbents tested included acid-, neutral-, and alkaline bentonites, BT-yeast, cholestyramine, kaolinite, silica gel, diatomaceous earth, lignin, hydrated sodium calcium aluminosilicate, Nutrimin, perlite, and vermiculite. In contrast, Rotter et al. (1989a) and Huff et al. (1992) concluded that addition of charcoal and hydrated sodium calcium aluminosilicate, respectively, to OA-contaminated diets were ineffective methods for reducing the toxic effects of OA to growing chicks. Fischer (1992) demonstrated that pigs fed an OA-contaminated diet supplemented with 1% charcoal, bentonite or sodium calcium aluminum silicate showed no reduction in renal excretion of the toxin.

Madhyastha et al. (1992b) observed that dietary inclusion of cholestyramine, an anion exchange resin used to reduce hypercholesteremia in humans by sequestering bile salts, reduced blood OA levels by 50% and reduced urinary excretion of OA while increasing cumulative fecal excretion.

8.4 Reduction in Toxicity

8.4.1 Addition of Antioxidants

Lipid peroxidation appears to be one mechanism of OA toxicity (Rahimtula

et al. 1988). Thus, the addition of antioxidants, such as vitamin C and vitamin E, to the diet may reduce the toxicity of OA. Haazele et al. (1992) observed that ascorbic acid supplementation to a laying hen diet reduced OA toxicity as measured by improvement in production characteristics such as egg production and egg mass as well as increased in blood sodium and a decrease in blood calcium as compared to birds receiving OA only.

8.4.2 Addition of Phenylalanine

Addition of phenylalanine to cell cultures containing OA (Creppy et al. 1979b) or simultaneous administration of OA and phenylalanine prevented the inhibition of protein synthesis (Creppy et al. 1984), reduced the acute toxicity of OA by increasing the LD_{50} (Creppy et al. 1980; Moroi et al. 1985) and reduced renal tubular injury in the rat as measured by the change of enzyme activity in urine and tubules (Kane et al. 1986a,b). Furthermore, the addition of phenylalanine to OA-contaminated diets resulted in reduced mortality and reduced serum uric acid and creatinine in broiler chicks, suggesting that the kidneys may have been protected from OA (Bailey et al. 1990). However, other production parameters such as body weight gain and feed consumption were not significantly improved (Gibson et al. 1989; Rotter et al. 1989b).

9. Risk Assessment and Current Regulatory Guidelines for Ochratoxin A

9.1 Risk Assessment

An extensive risk assessment for OA has recently been completed by Kuiper-

Goodman and Scott (1989). A tolerable daily intake of 0.2-4.2 mg/kg body weight was established by applying a safety factor of 5000 to the experimentally observed no effect limit of 21 μg/kg body weight/day (NTP 1989) for upper limit of 4.2 and using the lower 95% confidence level for the virtually safe dose for males for a risk of $1:10^6$ for the lower limit of 0.2. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) suggested that the tolerable intake of OA be equal to 16 ng/kg body weight per day. This recommendation is based on a 500-fold margin of safety applied to the lowest observed effect level of 0.008 mg/kg body weight/day (Hermann 1991).

9.2 Rationale for Current Regulations Regarding Ochratoxin A

Currently there are is no regulations in Canada regarding allowable limits for OA in food or feed. Eleven countries have either proposed or set official limits for OA, with acceptable limits ranging from 1-50 μg/kg for food and from 100-1000 μg/kg for feed (van Egmond 1991). Denmark is one of the countries that has legislated safety levels for the presence of OA in pork products. All animals which have macroscopically changed kidneys are analyzed for the presence of OA, although there are limitations associated with this screening technique (Büchmann and Hald 1985). If the OA content of the kidney is greater than 25 μg/kg, then the entire carcass is condemned; if it is 10-25 μg/kg then the kidney, liver, and other visceral organs are condemned; if it is less than 10 μg/kg only the kidneys are condemned. An OA content of 25 ng/g in the kidney corresponds to 125 ng/mL in the blood or 219 ng/mL

in the serum (Hult et al., 1980; Marquardt et al., 1988). Van Egmond (1991) has identified the following factors which influence the limits which have been set by the eleven countries: availability of data on dietary exposure and toxicology, distribution of the toxin in agricultural commodities, availability of analytical methodology, legislation in countries with which trading occurs, availabilities of methods of analysis, and a sufficient food supply. It was concluded that the scientific basis for the established regulations is weak except in Canada where risk assessments have been compiled for several mycotoxins, including ochratoxin A (van Egmond 1991, 1993; Stoloff 1991).

10. Summary and Conclusions

The presence of OA-producing fungal species in western Canada has been well documented. The potential for OA production under the appropriate conditions is confirmed by its presence in feed ingredients and swine blood. Ochratoxin A exerts its toxicological effects through several biochemical mechanisms, including promotion of lipid peroxidation, inhibition of the enzymes associated with phenylalanine metabolism, and inhibition of ATP production. The concentration of OA in contaminated feed may be reduced or prevented by a variety of techniques, however, a corresponding reduction in toxicity is not always associated with this apparent reduction in concentration.

Several countries have proposed or set official limits for OA in food and feed. Although the occurrence of OA in agricultural commodities in Canada appears to be

sporadic, its presence in isolated cases of animal toxicoses and as detected by a limited number of provincial surveys, confirms the need for continued surveillance of agricultural commodities to monitor the level and extent of exposure and to identify increases in incidence if they arise. Furthermore, rapid and accurate means of predicting OA concentration in plants and animal products are required for use on-farm and at the point of processing.

The Incidence and Distribution of Ochratoxin A in Western Canadian Swine

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Abstract

A survey of swine destined for slaughter in Manitoba was conducted to examine the incidence of ochratoxin A (OA) in swine herds from different regions of Manitoba throughout the year. Thirty-six percent of the blood samples from 1600 pigs contained detectable levels of OA. The identity of this toxin was confirmed using liquid chromatography-mass spectrometry and enzymatic hydrolysis. There was a significant effect of the region from which the herds originated, as well as the season in which the samples were collected, on both the incidence ($p < 0.001$) and concentration of OA ($p < 0.001$). In July, 65% of the samples contained detectable levels of OA, compared to 38, 21 and 16%, in April, October and January respectively. Furthermore, 24% of the samples collected in July contained greater than 15 ng/mL of OA, while only 2, 9, and 1% of the samples collected in April, October and January respectively, contained greater than 15 ng/mL of OA. Based on the six samples collected from each herd, it appears that the presence and concentration of OA within a herd may be estimated by sampling a limited number of animals per herd.

Introduction

Ochratoxin A (OA) is a toxic secondary metabolite mainly produced by two species of fungi, *Aspergillus ochraceus* Wilhelm and *Penicillium verrucosum* Dierckx (Frisvad and Samson 1991). The species which predominates in a given area is dependant upon climate: *Penicillium* species predominate in temperate climates such as Canada, Norway, Denmark and Sweden; *Aspergillus* species predominate in tropical and subtropical climates such as Yugoslavia (Krogh 1987). In the past two decades OA and its metabolites have received considerable attention. In the early 1970's, OA was implicated as a causal agent of mycotoxic porcine nephropathy (Krogh et al. 1973). This disease is characterized by impairment of proximal renal function, glucosuria, proteinuria and decreased maximal tubular clearance of *para*-aminohippurate, clearance of inulin, and ability to concentrate urine, as well as growth depression (Hald 1991a). The renal changes are characterized by proximal tubular degeneration and atrophy, interstitial fibrosis in the renal cortex, and in the most advanced cases, hyalinization of some glomeruli (Elling and Møller 1973; Krogh 1978). Since this time, OA has been found to nephrotoxic in many other species (Harwig et al. 1983). Furthermore, this ubiquitous toxin has been found to occur in plant products, as well as in animal sera and tissue, in many countries (see Kuiper-Goodman and Scott 1989 for a review).

The presence of OA in animal products raises a number of concerns. Ochratoxin A has been shown to be carcinogenic in mice (Bendele et al. 1985a) and rats (NTP 1989), and has been implicated as a causal agent of Balkan Endemic

Nephropathy (BEN) (Hult et al. 1982; Krogh 1987) and/or the renal tumors associated with this disease (Castegnaro and Chenozemsky 1987). The presence of OA in human sera in several countries including Germany (Bauer and Gareis 1987), Sweden (Breitholtz et al. 1991, Breitholtz-Emanuelsson et al. 1993a), Bulgaria (Petkova-Bocharova et al. 1988), Yugoslavia (Hult et al. 1982), Czechoslovakia (Fukal and Reisnerova 1990), Poland (Goliński and Grabarkiewicz-Szczęsna 1986), and Denmark (Hald 1991a) indicates that OA is transmitted through the human food chain, and thus poses a human health concern. This transmission is further confirmed by the detection of this toxin in human breast milk (Gareis et al. 1988; Micco et al. 1991, Breitholtz-Emanuelsson et al. 1993a). The presence of OA in human body fluids can be attributed to its ability to withstand the relatively high temperatures which are reached during cooking of food products (Josefsson and Møller 1980), as well as its long half-life in humans (Hagelberg et al. 1989).

The presence of OA in animal tissue also raises some concern for the livestock industry. Pigs consuming OA have decreased growth rates (Madsen et al. 1982a; Mortensen et al. 1983b; Elling 1983) and may be more susceptible to secondary infections (Pestka and Bondy 1990) as a result of the immunosuppressive effects of this toxin (Dwivedi and Burns 1984b, 1985; Holmberg et al. 1988). These decreases in productivity may result in a substantial losses to the livestock industry.

The potential for OA production and its presence in cereal products in western Canada have been well documented (Scott et al. 1972; Prior 1976; Abramson et al. 1980, 1983a, 1983b; Mills 1990; Frohlich et al. 1991). A study conducted by

Marquardt et al. (1988) demonstrated that OA is present in swine blood in Canada, and indicated that season influenced both the incidence and concentration of OA. Several surveys conducted elsewhere in the world have indicated that the incidence and concentration of OA in swine sera and tissues are also influenced by geographic location (Goliński et al. 1984, 1985; Hult et al. 1984), moisture content of the feed at harvest, origin of the feed, length of storage, and the drying procedure used (Holmberg et al. 1990a,b; Hult 1991).

This study was undertaken (1) to monitor the presence of OA in swine serum in Canada, (2) to assess the effect of season and geographical location on the incidence and concentration of OA, and (3) to determine if the blood concentration of OA in animals from the same herd were similar and thus indicative of the OA status of the herd.

Materials and Methods

Ochratoxin standards

Ochratoxin A standard was purchased from Sigma Chemical Co. (St. Louis, MO). The alpha form of ochratoxin was prepared by acid hydrolysis (van der Merwe et al. 1965b).

Sample Collection and Extraction

Blood samples were obtained from two slaughterhouses in Manitoba; one located in Winnipeg and the other in Neepawa. The two slaughterhouses received

animals from five regions of Manitoba, as well as a limited number of animals from Saskatchewan (Figure 3). Herds were randomly selected, and blood from six animals from each herd was collected. Blood samples were collected in April (n=369), July (n=429), October (n=389) of 1989 and January (n=401) of 1990. Information regarding origin of the herd was provided by Manitoba Pork est. In some cases, information regarding origin of the herd was not available.

Immediately following evisceration, blood was removed from the heart, and placed in a nylon centrifuge tube. Blood samples remained at room temperature for 12 hours to allow clotting to occur, and were then spun at 3400*g* for 15 min. Serum was decanted and stored at -20°C until analyzed. Samples were extracted according to the method of Hult et al. (1979), prior to high performance liquid chromatographic (HPLC) analysis. Using this technique, recovery of OA from saline was 96%, while recovery from serum was 87%.

HPLC System

The HPLC system consisted of an LKB 2150 HPLC pump and LKB 2152 HPLC controller (LKB, Pharmacia Canada Inc, Baie d'Urfé, Quebec), and a Waters 712 WISP automatic injector (Millipore, Waters Chromatography Division, Mississauga, Ontario). Analysis for the presence of OA was performed with an HP 1046A fluorescence detector and HP 3392A integrator (Hewlett-Packard (Canada) Ltd., Mississauga, Ontario). Confirmation of OA was performed using a Shimadzu RF-535 fluorescence HPLC monitor and a Shimadzu CR501 Chromatopac HPLC

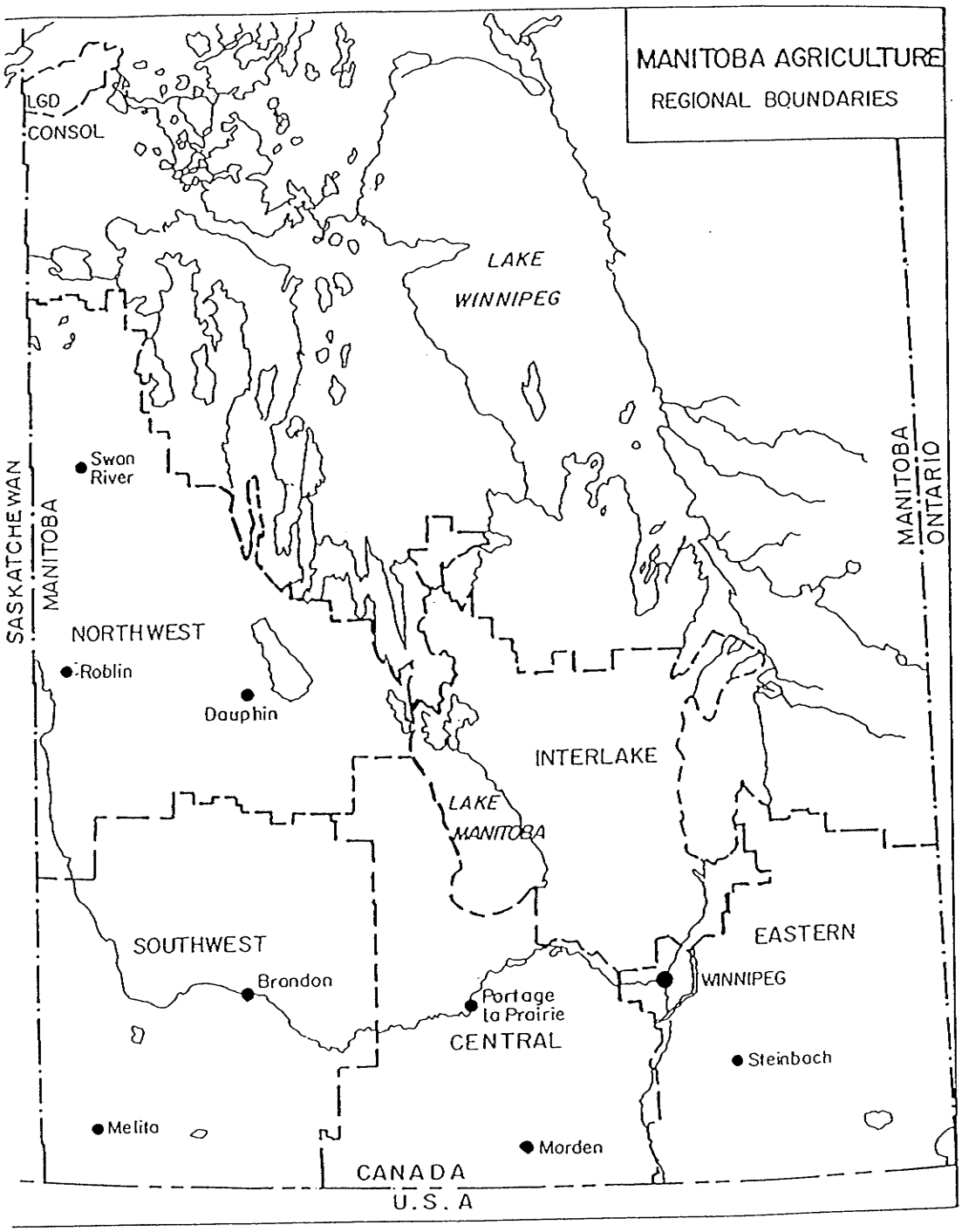

Figure 3: Herds from which the blood samples were collected originated from the five agricultural regions of Manitoba. A limited number of the herds also originated from Saskatchewan.

data processor (Shimadzu Corporation, Kyoto, Japan). The excitation and emission wavelengths used in both systems were 333 nm and 450 nm, respectively.

Ochratoxin A Analysis via HPLC

Residues of the serum extract were reconstituted in 500 μL of HPLC-grade methanol (Fisher Scientific Ltd., Ottawa, Ontario) and aliquots of 50 μL were injected into a 4.6 mm x 25 cm column maintained at 40°C, containing 5 μm diameter C-18 bonded adsorbent (Ultrasphere ODS, Beckman Canada Ltd., Mississauga, Ontario). The mobile phase consisted of methanol and isopropanol (90:10) and de-ionized, double distilled water adjusted to pH 2.1 with phosphoric acid, at a flow rate of 1.4 mL/min. The gradient profile used for the serum samples and the retention time of the OA standard and OA in swine serum are given in Figure 4. Reference standards were injected between every 12th sample, followed by a methanol wash. There was no carry over of OA between samples. The peak area of the reference standard was consistent throughout the analysis. The lower limit of detection was 0.3 ng/mL. All values below this were considered to be negative.

Ochratoxin A Confirmation via Enzymatic Hydrolysis

The presence of OA in a minimum of five serum samples collected from each saampling period was confirmed using enzymatic hydrolysis as outlined by Hult and Gatenbeck (1976), with slight modifications. Serum samples were extracted as indicated above. Residues were reconstituted with 1.2 mL 0.04 M TRIS buffer, (pH

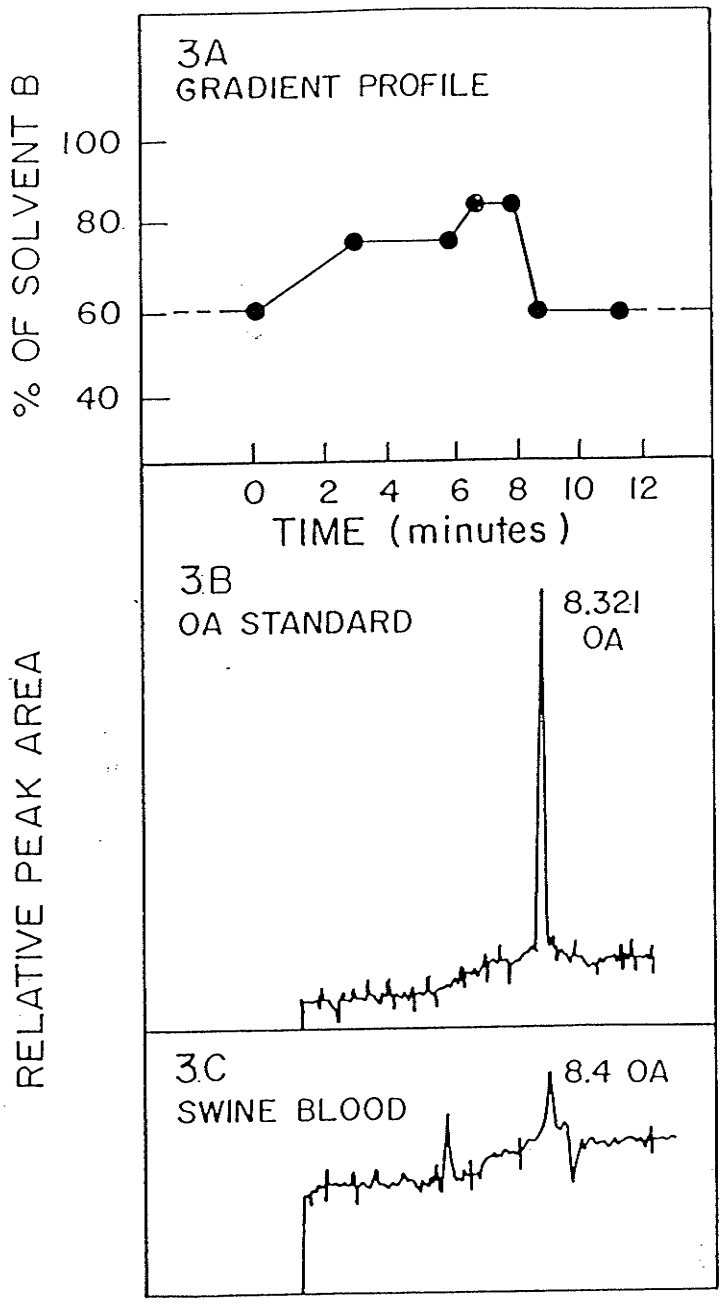

Figure 4. Serum samples were analyzed by HPLC. A: Gradient profile used (solvent A: acidified H_2O; solvent B: methanol:isopropanol (90:10)). B: Retention time of the OA standard. C: Swine serum sample.

7.6) and divided into two equal portions. Carboxypeptidase A (Sigma Chemical Co., St. Louis, MO) was diluted with 1 N NaCl, 0.04 M TRIS, pH 7.6 to obtain a concentration of 2 mg protein/mL. Two hundred microlitres of the diluted enzyme was added to one portion and 200μL of 0.04 M TRIS buffer to the other. Samples were incubated at 37°C for 180 minutes, immersed in boiling water to terminate enzyme activity and centrifuged at 12,500*g*. Two hundred microlitres of the supernatant was diluted with an equal portion of methanol, and aliquots of 50 μL were injected into the HPLC system described above, with a flow rate of 1.3 mL/min. The gradient profile used for enzymatic confirmation and the retention times of the OA and the ochratoxin α (Oα) standards are given in Figure 5.

Ochratoxin A Confirmation by LC/MS

In addition to enzymatic hydrolysis, high-performance liquid chromatography-mass spectrometry (LC/MS) was used to confirm the presence of OA in one individual and three pooled samples. Serum extracts were chromatographed on a C-18 bonded-phase column (Spherisorb S3 ODS2, PhaseSep, Deeside Industrial Estate, Queensferry, Clwyd, U.K.) and the effluent passed through a direct liquid introduction interface into a quadrupole mass spectrometer equipped for negative-ion chemical ionization (Model 5985B, Hewlett-Packard Canada Ltd., Edmonton, Alberta). Ochratoxin A standards were chromatographed and scanned from masses 150-450 to determine retention time and ion mass. Sample extracts were analyzed by monitoring the ion of mass 403.1 for maximum sensitivity (Abramson, 1987), as seen in Figure 6.

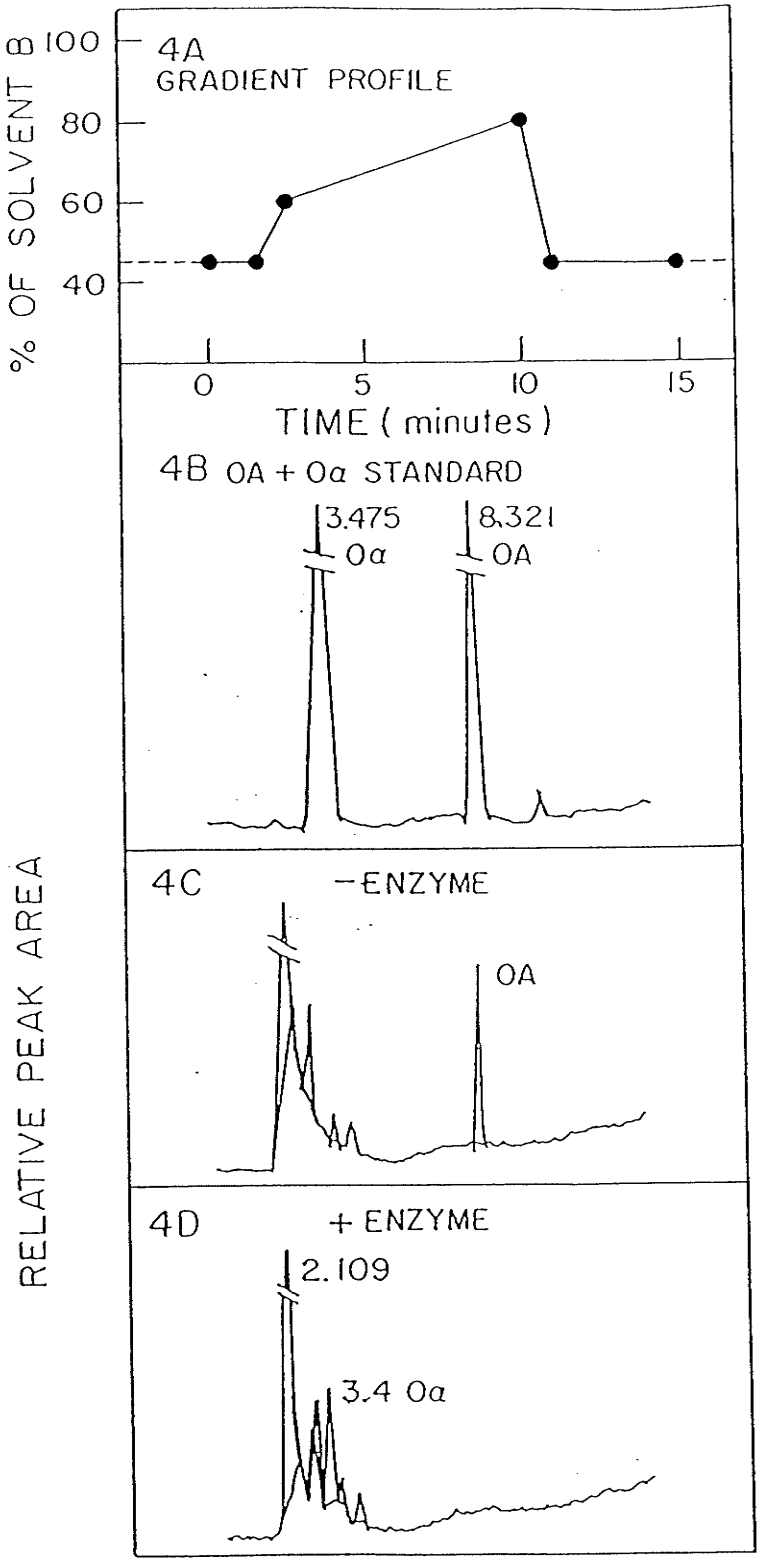

Figure 5. HPLC chromatogram confirming the presence of OA in swine serum by enzymatic hydrolysis. A: Gradient profile used (solvent A: acidified H_2O; solvent B: methanol:isopropanol (90:10)). B: Retention times of ochratoxin A and ochratoxin α standards. C: Swine serum sample plus TRIS buffer. D: Swine serum sample plus carboxypeptidase A.

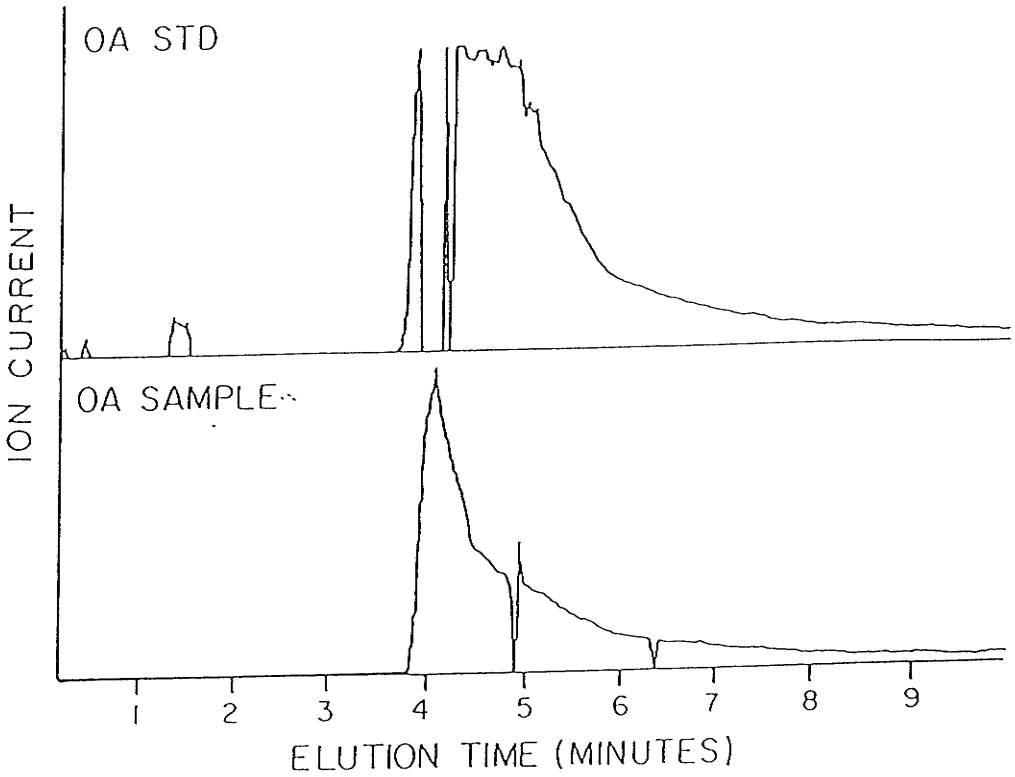

Figure 6. Confirmation of OA in swine serum by liquid chromatography-mass spectrometry. Negative ions generated by chemical ionization were monitored at a mass of 403.1 (M^-, ^{35}Cl isotope).

Statistical Analysis

These data were not completely suitable for a typical analysis of variance and thus were analyzed in two ways. The frequency of samples with detectable levels of OA was analyzed using chi square (Statistical Analysis Systems Institute, Inc. 1989) to determine the effect of season and geographic region. In order to determine if animals within herds tended to be similar for OA incidence, herds were classified according to the number of animals with detectable levels of OA (0 of 6, 1 of 6, etc.). The number of herds falling in each category should follow a binomial distribution if the incidence of OA in herds was a uniform and random occurrence. Hypotheses were tested using an α of 0.05.

An analysis of variance of all non-zero OA values was conducted to determine the quantitative effects of month, region, and herd (SAS, 1989) as well as to determine variation in OA concentration among animals within herds. The model was:

$$Y_{ijkl} = \mu + m_i + r_j + h_{k(j)} + e_{ijkl}$$

where Y_{ijkl} is the concentration of OA in the serum of the l'th animal in the k'th herd within the j'th region and in the i'th month. Region effects were tested using the mean square for herds while all other effects were tested using the error mean square.

Results

The incidence and mean concentration of OA in swine serum are summarized

in Table 2. HPLC analysis of the samples (figure 4) indicated that 36% of the 1588 samples analyzed contained detectable levels of OA. The range of positives was from 0.3 to 211 ng/mL.

This presence of OA was confirmed in selected samples using enzymatic hydrolysis and LC/MS. Addition of carboxypeptidase A, which hydrolyzes OA to Oα, resulted in the formation of a new peak at 3.4 min which corresponds to the elution time of Oα, as seen in figure 5. Disappearance of OA and appearance of Oα confirmed the presence of OA in the serum and confirmed that there were no compounds present which co-eluted with OA. Ion chromatograms (figure 6) generated by LC/MS illustrate that the presumptive OA peaks elute at the same retention time as the OA standard, and give the same ions.

There was a significant effect of month of sampling on the incidence ($p < 0.001$) and the concentration of OA ($p < 0.001$). In July, 65% of the samples contained detectable levels of OA, compared to 38, 21 and 16% in April, October, and January respectively. In addition, 24% of the samples collected in July contained greater than 15 ng/mL of OA while only 2, 9, and 1% of the samples collected in April, October, and January respectively, contained greater than 15 ng/mL of OA.

There was also a significant effect of the region from which the herds originated on the incidence ($p < 0.001$) and concentration of OA ($p < 0.001$). The incidence and concentration of OA within a region were not consistent across sampling periods. A significant relationship between the slaughterhouse in which the samples were collected and the presence of OA ($p < 0.001$) and the concentration

Table 2: Incidence of OA in Swine Blood in Western Canada

	Number of samples collected	Percent of samples above the detection limit	Mean of positives (ng/mL)± S.E.	Overall Mean (ng/mL)±S.E.
Month**				
April	369	38	8.3 ± 0.3	3.2 ± 0.2
July	429	65	17.6 ± 1.3	11.5 ± 0.9
October	389	21	19.4 ± 1.9	4.1 ± 0.6
January	401	17	5.4 ± 0.6	0.9 ± 0.2
Total	1588	36	14.1 ± 0.7	5.1 ± 0.3
Region**				
Interlake	138	26	8.9 ± 1.0	2.3 ± 0.4
Northwest	24	54	42.3 ± 11.5	22.9 ± 7.5
Southwest	180	49	15.9 ± 2.8	7.8 ± 1.5
Central	597	24	14.3 ± 0.9	3.5 ± 0.3
Eastern	519	41	11.8 ± 0.9	4.9 ± 0.5
Saskatchewan	12	33	17.6 ± 6.8	5.9 ± 3.2
Slaughterhouse**				
#1	1445	32	12.6 ± 0.6	4.1 ± 0.2
#2	143	72	21.0 ± 2.9	15.1 ± 2.3

**Significant effect on the incidence and concentration of OA ($p < 0.001$).

of OA ($p < 0.001$) was also evident. This difference may be attributed to a regional effect as the two slaughterhouses did not represent the same regions.

The proportion of herds with a given number of animals with detectable levels of OA within a herd was not a random occurrence and therefore did not follow a binomial distribution, but rather followed a U-shaped curve (Figure 7). This indicates that a large portion of the herds tended to have most animals with detectable levels of OA or no animals with detectable levels of OA. These analyses suggest that a few animals sampled in a herd may be used to indicate the presence or absence of OA in other animals in the same herd. This is further supported by the observation that as the number of animals in a herd with detectable levels of OA increased, the mean OA concentration of the herds tended to increase. However, as the number of animals in a herd with detectable levels of OA increased, the observed variability among animals in the herd increased concurrently. This would make the problem of sampling to accurately assess the herd average of OA more difficult.

Discussion

Results from this study confirm the presence of OA in swine serum in Manitoba, as demonstrated in a less extensive survey by Marquardt et al. (1988). The number of samples which contained detectable levels of OA (35%) was lower than that determined previously (65%), demonstrating that there may be annual variation in the incidence of OA for a given area of the province. The concentration of OA in both studies was similar, with 11.3% and 16.8% of the samples containing greater

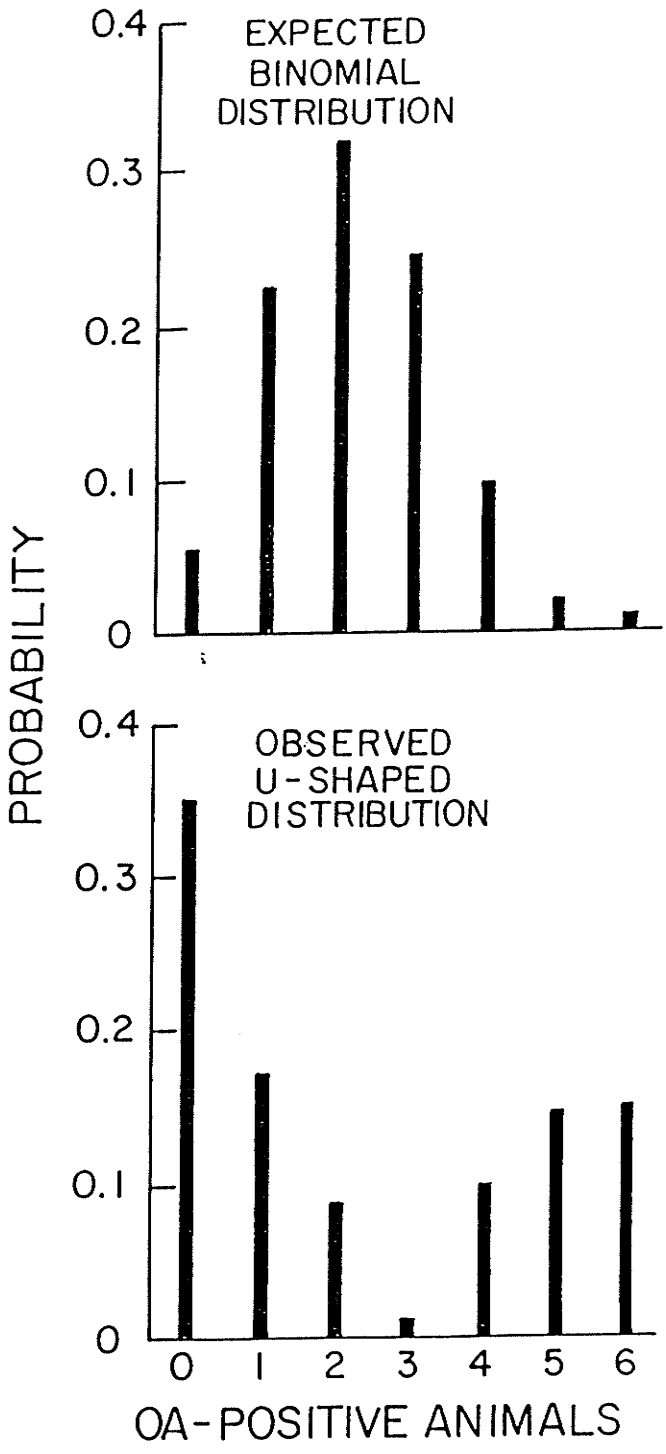

Figure 7. The proportion of herds with a given number of animals with detectable levels of OA within a herd followed a U-shaped curve, rather than a binomial distribution. The distribution is expected to be binomial if OA occurred randomly in the population of animals. The observed distribution is significantly different ($p < 0.01$) from the expected distribution.

than 10 ng/ml in 1986 and 1989/90, respectively.

The increased incidence of OA in the samples collected in April and July, suggest that the risk of fungal growth and subsequent toxin production is greater in the spring and summer. This may be attributed to a lengthy storage period, combined with increasing temperatures and possibly higher moisture levels than those which occur in autumn and winter. This temporal effect has been demonstrated elsewhere (Goliński et al. 1984,1985; Marquardt et al. 1988, Holmberg et al. 1990b)). Although the number of samples that contained detectable levels of OA was greatest in July, the mean OA concentration was slightly higher in October (19.4 ng/mL). The lower incidence and slightly higher concentration of OA in October may be attributed to a few producers utilizing feed which has been stored for a prolonged period of time.

The geographical differences for both the incidence and concentration of OA that are observed in this study have been reported elsewhere (Hult 1991), and may occur as a result of regional differences in moisture content at storage, resulting from differences in rainfall at harvest (Holmberg et al. 1990a) or higher relative humidities (Trenholm et al. 1985). However, as indicated by Hult (1991), the OA concentration within a region may vary on a monthly, as well as yearly basis. It is, therefore, difficult to draw any conclusions about geographic trends without several successive years of data.

Hult et al. (1979) demonstrated that OA in swine blood may be used to predict OA in feed and also suggested that one sample from a single pig in a herd

may be used to identify herds contaminated with OA (correlation coefficient = 0.80) (Hult et al. 1980). This latter observation was based on herds which were represented by two pigs. Based on data presented here, the following expression shows the 95% confidence limits within which the average OA concentration of a herd would be expected to fall based on sampling blood from one animal (Y_1) in a herd:

$$Yi \pm t_{263,0.05} \times s = Y_i \pm 35 \text{ ng/mL OA}$$

where $t_{263,0.05}$ x s = 35 ng OA/mL is the "within herd" standard deviation in OA concentration. Thus, at most, the OA concentration in the serum of an animal sampled in a herd may vary by ± 35 ng/mL from the herd average. Thus, one animal in a herd is not only an indicator of the presence or absence of OA in a herd, but also provides an estimate of the OA concentration of other animals in a herd. This information may then be used to determine the need for further testing. Hult (1991) has suggested that for small herds, one sample may be adequate but in larger herds it may be beneficial to take several samples.

Holmberg et al. (1990b) has demonstrated that grain dried with forced ambient air was more susceptible to fungal growth and subsequent OA production than grain dried with forced heated air. However, regardless of the method of preservation, 9% of the samples contained OA at the beginning of the storage period. This indicates that a low level of toxin production may be unavoidable as storage fungi may be present prior to harvest. In the same study, differences were noted in the occurrence of OA from feed which was produced on-farm compared to that

purchased from a feed mill. Information regarding grain handling procedures and origin of the feed was not gathered in the present survey.

Currently there is no legislation in Canada regarding allowable limits of OA in food or feed. Eleven countries have either proposed or set official limits for OA, with acceptable limits ranging from 1-50 μg/kg for food and from 100-1000 μg/kg for animal feed (van Egmond 1991). Van Egmond (1991) has identified the following factors which influence these limits: availability of data on dietary exposure and toxicology; distribution of the toxin in agricultural commodities; availability of analytical methodology; legislation in countries with which trading occurs; and a sufficient food supply. He concludes that the scientific basis for the established regulations is weak.

Denmark has legislated safety levels for the presence of OA in pork products. All animals which have macroscopically changed kidneys are analyzed for the presence of OA, although there are limitations associated with this screening technique (Büchmann and Hald 1985). If the OA content of the kidney is greater than 25 μg/kg, then the entire carcass is condemned; if it is 10-25 μg/kg then the kidney, liver, and other visceral organs are condemned; if it is less than 10 μg/kg only the kidneys are condemned. An OA content of 25 μg/kg in the kidney corresponds to 125 ng/mL in the blood or 219 ng/mL in the serum (Hult et al. 1980; Marquardt et al. 1988). None of the animals sampled in this study exceeded this latter value.

Based on the results of this study, OA must be monitored on a continuous basis throughout the regions to accurately assess the extent of OA contamination, as

annual differences may lead to an inaccurate assessment of the problem. This issue has been addressed by the Health Protection Branch of Health Canada as it has initiated a nation-wide survey of agricultural commodities, including breakfast cereal food products, pork sera and pork kidney, over several successive years (Kuiper-Goodman et al. 1993).

Although the OA content in swine sera in Canada observed in the present study, and elsewhere (Marquardt et al. 1988; Kuiper-Goodman et al. 1993) are low when compared to European countries, particularly Denmark (Hald 1991b), it is difficult to assess the long term implications with regard to human health, as well as the resulting financial loss to the livestock industry. Therefore, it is important to minimize the incidence of this toxin in agricultural commodities. This may be achieved through identification and implementation of the appropriate production practices at the farm level.

Ochratoxin A in Human Serum in Western Canada

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Abstract

Ochratoxin A (OA), a nephrotoxic secondary metabolite produced by several species of *Aspergillus* and *Penicillium*, has been detected in a variety of agricultural commodities in many countries, including Canada. This study was undertaken to monitor the exposure of Manitobans to this toxin and to determine if renal patients exhibited any of the epidemiological characteristics associated with Balkan Endemic Nephropathy. HPLC analysis of the samples indicated that 40 and 38% of the individuals sampled in 1990 and 1991 respectively, contained detectable levels of OA. There was no relationship between: renal and nonrenal patients ($p > 0.05$); males and females ($p > 0.05$); age ($p > 0.05$) or serum creatinine ($p > 0.05$) for either the presence or the concentration of OA. These data can be used to calculate an estimate of average probable daily intake of 1.7 ng/kg bw per day in 1991, which is below the upper limits of the tolerable daily intake of 0.2 - 4.2 ng/kg bw per day proposed by Kuiper-Goodman and Scott (1989)and below the limit of 16 ng/kg bw per day proposed by the Joint FAO/WHO Expert Committee on Food Additives (WHO, 1991). The presence of OA in the blood of Manitobans indicates the need for ongoing monitoring of foodstuffs in Canada to determine the frequency and concentration of this mycotoxin in the human food chain.

Introduction

The mycotoxin ochratoxin A (OA) was initially discovered in the 1960's, (van der Merwe et al. 1965a,1965b). However, it was not until 1969 that this nephrotoxic secondary metabolite was observed as a natural contaminant of maize (Shotwell et al. 1969). Since that time, OA has been detected in food and feed of plant origin, in animal tissue, and more recently, in human body fluids, in many areas of the world (Krogh 1987; Jelinek et al. 1989; Kuiper-Goodman and Scott 1989).

Ochratoxin A is produced by two genera of fungi, *Penicillium* and *Aspergillus*. *Penicillium verrucosum* Dierckx is the only known and confirmed *Penicillium* species to produce OA (Pitt 1987,1988; Frisvad and Filtenborg 1989; Frisvad and Samson 1991), and tends to predominate in temperate climatic regions such as Canada, Norway, Denmark, and Sweden (Krogh 1987). In tropical and subtropical climates, *Aspergillus ochraceus* Wilhelm is the most important cereal-borne producer of OA (De Scott 1965; Krogh 1987; Frisvad and Samson 1991).

Numerous forms of ochratoxin exist, each with varying toxicity. The most common naturally occurring compound is OA, a dihydroisocoumarin ring linked through its 7-carboxyl group by an amide bond to L-ß-phenylalanine.

Ochratoxin A is nephrotoxic in all species tested to date (Harwig et al. 1983). Renal changes are characterized by degeneration and atrophy of the proximal tubules, interstitial fibrosis in the renal cortex, and in the most severe cases, hyalinization of some glomeruli (Elling and Møller 1973; Krogh 1978). This toxin is the major causal agent of the disease mycotoxic porcine nephropathy, which has been

identified in Denmark, Sweden and other European countries (Krogh 1978,1992). Citrinin, another nephrotoxic mycotoxin, is a frequent co-contaminant with OA, and may act synergistically with OA in this disease (Krogh et al. 1973; Hald 1991a). In addition to its nephrotoxic effects, OA has also been shown to be teratogenic, genotoxic and immunotoxic (see Kuiper-Goodman and Scott 1989, for a review). Ochratoxin A is also a potent renal carcinogen in rats, as shown by the National Toxicology Program (NTP 1989).

The natural occurrence of OA in plant products, animal tissue, and animal sera in numerous countries has been summarized by Kuiper-Goodman and Scott (1989). Ochratoxin A has been identified in a variety of agricultural commodities in Canada, including barley, wheat, oats, legumes, and forages. Furthermore, it has also been detected in the blood of swine in western Canada (Marquardt et al. 1988; Manuscript I). Detectable levels of OA have been found in breakfast cereal products, pork sera and pork kidney from locations across Canada in a recent survey carried out by the Health Protection Branch of Health Canada (Kuiper-Goodman et al. 1993).

The presence of this toxin in animal products not only indicates a production loss for the livestock industry (Madsen et al. 1982a; Elling 1983; Mortensen et al. 1983b), but more importantly, that OA is penetrating the human food chain. As such, OA has been detected in human blood in Bulgaria (Petkova-Bocharova et al. 1988), Yugoslavia (Hult et al. 1982), Germany (Bauer and Gareis 1987), Sweden (Breitholtz et al. 1991, Brietholtz-Emanuelsson et al. 1993a), Denmark (Hald 1991b),

Czechoslovakia (Fukal and Reisnerova 1990), and Poland (Goliński and Grabarkiewicz-Szczęsna 1986). Furthermore, OA has also been detected in human breast milk in Germany (Gareis et al. 1988), Italy (Micco et al. 1991) and Sweden (Breitholtz-Emanuelsson et al. 1993a).

Ochratoxin A may be a possible causal agent of Balkan Endemic Nephropathy (BEN), a fatal chronic kidney disease that occurs primarily among rural populations along the Balkan Peninsula (Hult et al. 1982; Castegnaro and Chernozemsky 1987; Krogh 1987). In Yugoslavia, the morbidity or prevalence rate of this disease is between 2 and 10%, however, when suspected cases are also included, the prevalence rate rises to 20%. Specific mortality for the Posavlje region around Slavonski Brod from 1957 to 1984 averaged 1.54 per thousand (Čeović et al. 1992). In most areas, nearly twice as many women as men are affected by the disease, while the highest morbidity occurs in individuals between 35 and 55 years of age (Austwick 1975).

In this study, human sera samples were collected from renal and nonrenal patients from two hospitals in Manitoba to examine the exposure of a select population of Manitobans to this toxin, and to determine if a relationship existed between renal disorders and the presence of OA.

Materials and Methods

Ochratoxin standards

Ochratoxin A standard was purchased from Sigma Chemical Co. (St. Louis, MO). The alpha form of ochratoxin was prepared by acid hydrolysis (van der Merwe

et al. 1965b).

Collection of Samples

In 1990, 159 renal and nonrenal patients from the Renal and Hypertension Clinics of the Nephrology Section at the Health Sciences Centre and St. Boniface Hospital in Winnipeg, Manitoba, Canada were asked to volunteer blood samples (7 ml) which were drawn after full explanation of the study and informed consent. Sixty-eight of the individuals from whom the samples were taken, suffered from some form of renal disease or renal impairment including glomerulonephritis, pyelonephritis, interstitial nephritis and nephrosclerosis (hypertensive renal disease). The remaining 91 samples were collected from individuals who had neither renal disease nor renal impairment. Age, sex, and serum creatinine were recorded. However, in some cases, serum creatinine was not available. Sixty of the individuals (34 renal, 26 nonrenal) donated blood again in 1991. These patients had not received any information regarding the presence or absence of OA in their blood from the previous year.

Sample Extraction

Following collection, blood samples were allowed to clot and centrifuged at 3500*g*. The serum portion was decanted and stored at -70°C prior to analysis. Samples were extracted according to the method of Hult et al. (1979), prior to high-performance liquid chromatography (HPLC), with slight modifications in volume.

Two millilitres of serum and 0.5 ml of 0.145 M sodium chloride were placed in a nylon centrifuge tube and 10 ml of 0.05 M HCl and 0.1 M $MgCl_2$ was added. The mixture was shaken in a horizontal shaker (Eberbach Corporation, Ann Arbor, Michigan) with 6.0 ml of chloroform for 10 min. The samples were then placed on ice for 10 min, centrifuged at 13,000*g* for 15 min, and 4.0 ml of the chloroform phase was transferred to a glass tube and washed with 1.5 ml of water. After centrifugation at 900*g*, 3.0 ml of the chloroform fraction was transferred to a glass vial and dried under a stream of nitrogen. The samples were stored at -20 °C prior to HPLC analysis.

HPLC System

The HPLC system consisted of an LKB 2150 HPLC pump and LKB 2152 HPLC controller (LKB, Pharmacia Canada Inc, Baie d'Urfé, Quebec), a Waters 712 WISP automatic injector (Millipore, Waters Chromatography Division, Mississauga, Ontario), a Shimadzu RF-535 fluorescence HPLC monitor (Shimadzu Corporation, Kyoto, Japan), and a Shimadzu CR501 Chromatopac HPLC data processor (Shimadzu Corporation, Kyoto, Japan). The excitation wavelength was 333 nm and emission wavelength was 450 nm.

Ochratoxin A Analysis via HPLC

Residues were reconstituted in 500 μL of HPLC-grade methanol (Fisher Scientific Ltd., Pittsburg, Pennsylvania) and aliquots of 50 μL were injected into a 4.6

mm x 25 cm column maintained at 40°C, containing 5 μm diameter C-18 bonded adsorbent (Ultrasphere ODS, Beckman Canada Ltd., Mississauga, Ontario). The mobile phase consisted of methanol containing 10% isopropanol (90:10) and de-ionized, double distilled water adjusted to pH 2.1 with phosphoric acid, at a flow rate of 1.4 mL/min. The gradient profile used for the serum samples, the retention time of the OA standard, and OA in human serum are given in Figure 8. The minimum detectable limit for OA was 0.1 ng/mL in 1990 and 0.5 ng/mL in 1991. This increase in the detection limit was attributed to a slight deterioration in the performance of the fluorescence monitor lamp.

Ochratoxin A Confirmation via Enzymatic Hydrolysis

The presence of OA in the serum samples was confirmed using enzymatic hydrolysis as outlined by Hult and Gatenbeck (1976), with slight modifications. Serum samples were extracted as indicated above. Residues were reconstituted with 1.2 mL 0.04 M TRIS buffer, (pH 7.6) and divided into two equal portions. Carboxypeptidase A (Sigma Chemical Co., St. Louis, MO) was diluted with 1 N NaCl, 0.04 M TRIS, pH 7.6 to obtain a concentration of 2 mg protein/mL. Two hundred microlitres of the diluted enzyme was added to one portion and 200μL of 0.04 M TRIS buffer to the other. Samples were incubated at 37°C for a period of 180 minutes, immersed in boiling water to terminate enzyme activity and centrifuged at 12,500*g*. Aliquots of 150 μL were injected into the HPLC system described above,

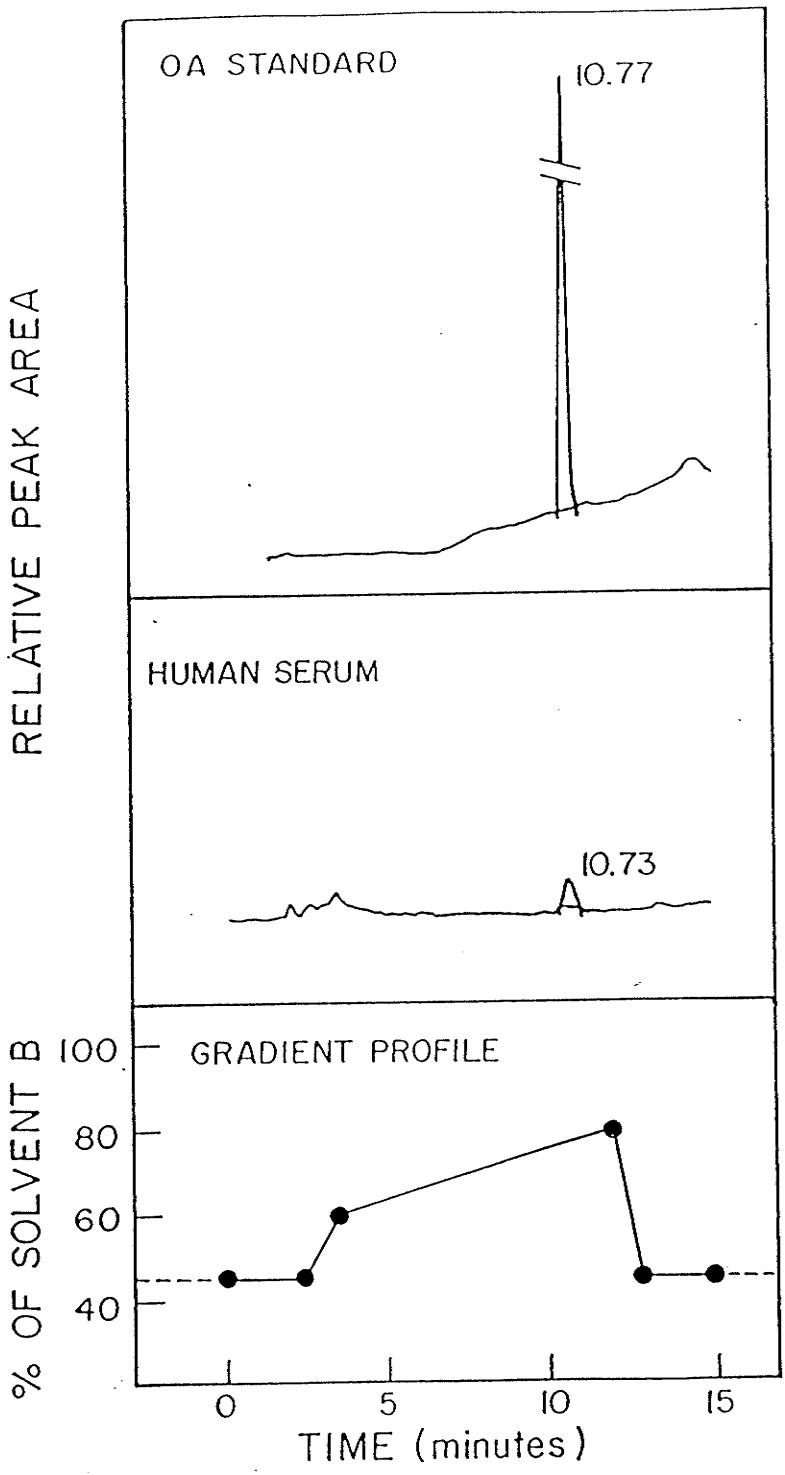

Figure 8: HPLC analysis of OA in human serum (solvent A: acidified H_20, solvent B: methanol:isopropanol (90:10)). The sample contained 0.8 ng/mL OA.

with flow rate modified to 1.3 mL/min. The gradient profile used for enzymatic confirmation and the retention times of the OA and the ochratoxin α (Oα) standards are given in Figure 9. Prior to enzymatic hydrolysis, an unidentified peak was present at 4.7 min. Addition of carboxypeptidase A, which hydrolyzes OA to Oα, resulted in the co-elution of the two peaks at 4.7 minutes. The complete disappearance of OA in all samples provided further evidence that OA is present in human serum and that there were no compounds which co-eluted with OA. Due to a limited sample volume, a total of ten positive samples were confirmed using this technique.

Statistical Analysis

The data were analyzed using chi square (χ^2) analysis. The non-parametric method described below was used to estimate overall mean. The empirical cumulative distribution function (ECDF) of the non-censored data was calculated and a fifth-degree polynomial fit to $-\log_e(1\text{-ECDF})$. The polynomial was fit without an intercept and with the value $-\log_e(1-(nc+½)/n)$ at the detection limit (DL), where nc is the number of censored observations. The probability density function and the expected value of the censored observations were estimated from the polynomial. The overall estimated mean was calculated as a weighted average of the estimated expected value of the censored observations and the mean of the non-censored observations. A paired t-test was used to compare the OA values for the individuals sampled in both 1990 and 1991.

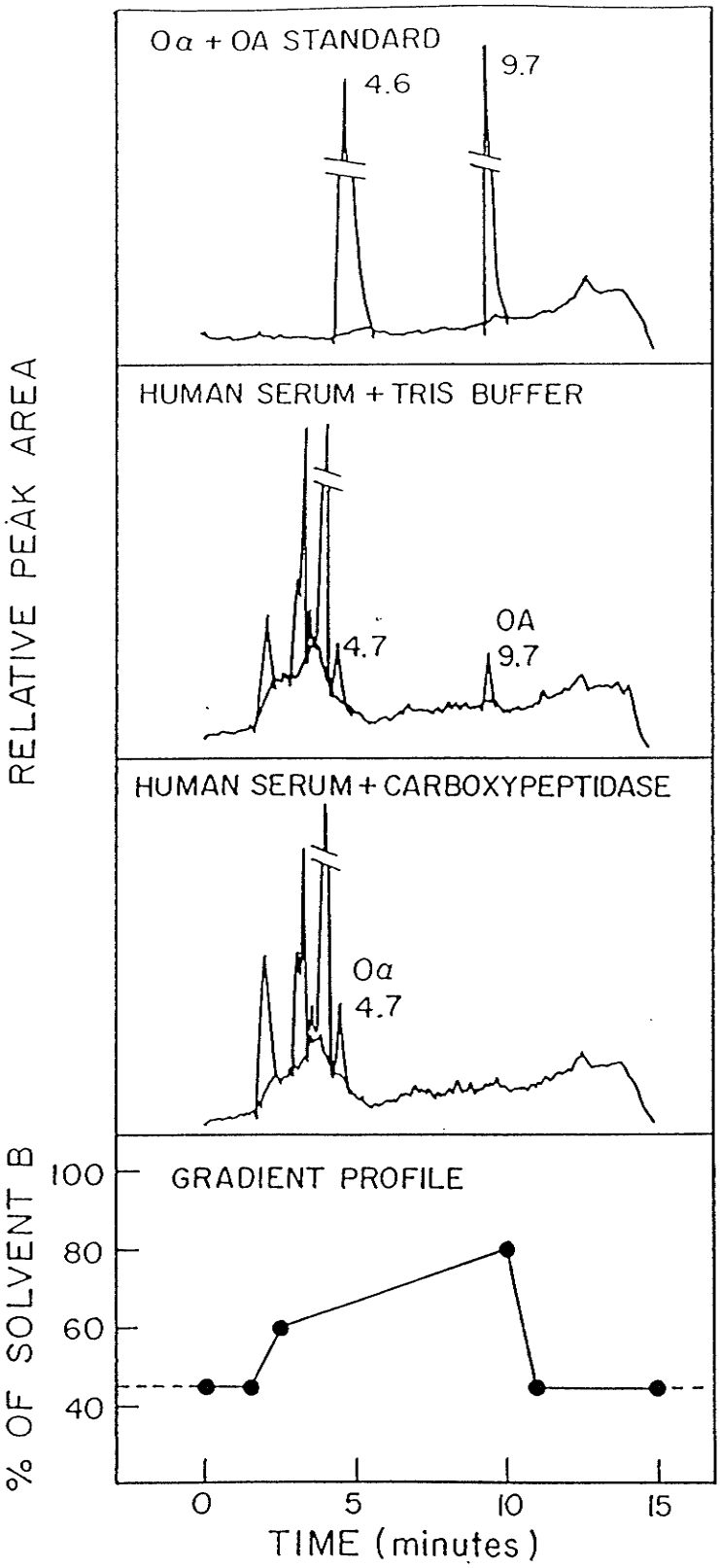

Figure 9: HPLC chromatogram confirming the presence of OA in human serum by enzymatic hydrolysis (solvent A: acidified H_20, solvent B: methanol:isopropanol (90:10)).

Results

Results from the study are summarized in Table 3. HPLC analysis of the samples indicated that 63/159 of the individuals sampled in 1990, and 23/60 of the individuals sampled in 1991, contained detectable levels of OA. The values ranged from 0.16 to 35.3 ng/mL and from 0.8 to 8.96 ng/mL in 1990 and 1991 respectively. Enzyme treatment of selected positive samples resulted in complete disappearance of the OA peak in all cases and the co-elution of a peak at the same retention time as Oα.

There was no relationship between renal and nonrenal patients nor between males and females for the presence or concentration of OA in 1990 and 1991, respectively. Nor was there a relationship between serum creatinine or age of patients for the presence and concentration of OA in 1990 and 1991, respectively. In 1990, one sample was found to contain 35 ng/mL of OA. Ochratoxin A was not detected in the blood of this patient in 1991, and although this value was considered to be true, it was deleted from the data set. With this outlier deleted the mean OA values for the 59 individuals sampled in 1990 and 1991 are significantly different ($p < 0.05$). This difference was also evident when the detection limit in 1990 was increased from 0.10 ng/mL to the 1991 detection limit of 0.50 ng/mL (by setting values less than 0.5 ng/mL equal to zero). There was a significant relationship for the presence or absence of OA among the same individuals sampled in the two year period ($p < 0.05$). The number of individuals with detectable levels of OA (detection limit set to 0.5 ng/mL in 1990) in both years was as predicted from a binomial

Table 3. Ochratoxin A in Human Serum Collected from Renal and Nonrenal Patients in Manitoba

Year	n	DL[a] (ng/mL)	Number of samples > DL	Mean of positives (ng/mL)	Estimated mean[b] (ng/mL)	Calculated mean (ng/mL)	Median of positives
1990	159	0.1	63	1.13	0.47	0.45	0.30
1990	158[c]	0.1	62	0.58	0.25	0.23	0.30
1990	159	0.5	18	3.28		0.37	0.89
1990	158[c]	0.5	17	1.39		0.15	0.77
1990	59[c]	0.5	8	1.26		0.76	
1991	60	0.5	23	3.10	1.29	1.19	
1991	59[c]	0.5	23	3.10		1.21	3.14

[a]DL, detection limit.
[b]Estimated using a non-parametric approach.
[c]The individual with a serum value of 35.3 ng/mL OA was deleted from the data set.

expansion. Similarly, the number of individuals with nondetectable levels of OA in both years was also as expected. However, the number of individuals with detectable levels in 1990 and nondetectable levels in 1991, was lower than expected, while the number of individuals with nondetectable levels of OA in 1990 and detectable levels in 1991 was higher than expected (Figure 10).

Discussion

Although OA has been detected in human blood in several European countries its presence in human blood has not been reported in Canada, to date. Nor, to the authors' knowledge, have the same individuals been studied over time. Although data presented here indicate that OA is present in the blood of Manitobans, there was no relationship between OA and renal disease. These results are not unexpected due to the low levels of OA found in the serum. Further, many factors other than OA cause renal disease. However, they do not negate the hypothesis that OA is the causal agent of BEN, nor does it preclude the development of this disease in Manitoba, as the expression and incidence of the disease may be different between the two locations. Also, the disease may be present in individuals long after the toxin has been cleared from the body.

Ochratoxin A has recently been monitored in a variety of agricultural commodities by the Health Protection Branch of Health Canada (Kuiper-Goodman et al. 1993). Detectable levels of OA were found in 46% of pork sera samples and 0% of organ meats in 1990, 25% and 6% of pork kidneys in 1991 and 1992

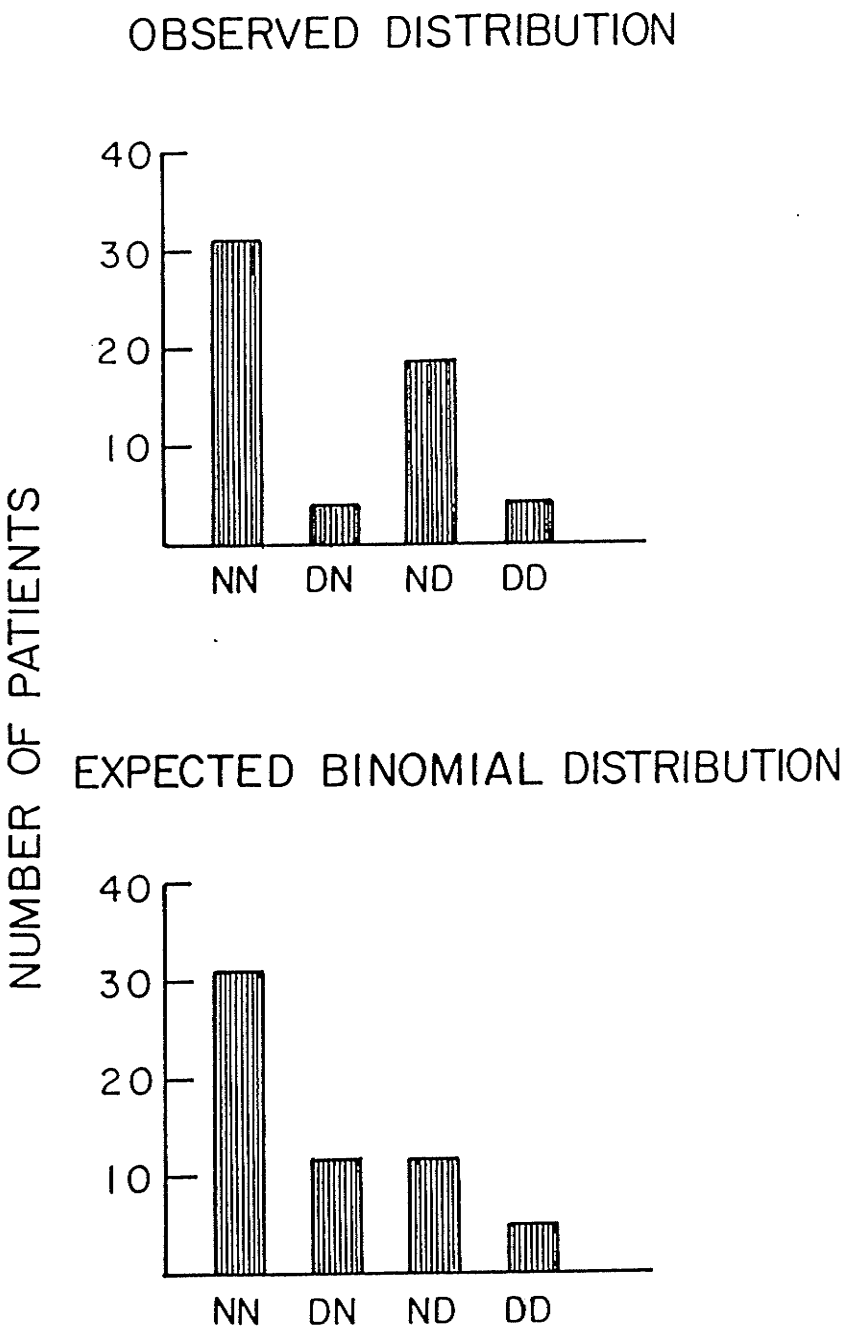

Figure 10: Comparison of expected versus observed distributions of individuals sampled in 1990 and 1991 for the presence of OA. NN = not detected in 1990, not detected in 1991; ND = not detected in 1990, detected in 1991; DN = detected in 1990, not detected in 1991; DD = detected in 1990, detected in 1991.

respectively, and 8 and 9% of cereal breakfast products in 1991 and 1992 respectively. Marquardt et al. (1988) and Ominski et al. (Manuscript I) have also observed detectable levels of OA in swine sera in western Canada. These results indicate that both cereal products, as well as pork products are possible points of entry for OA into the human food chain. Furthermore, the incidence of contamination was quite variable from one year to the next, for both pork sera and pork kidneys, and somewhat more stable for cereal products in the two year period. This annual variability in agricultural commodities was reflected in the human sera, as only four of the 59 individuals sampled in both years had detectable levels of OA (detection limit set to 0.5ng/mL in 1990) in 1990 and 1991. Twenty-three patients, however, had detectable levels of OA in one year and not the other. The remaining patients were negative in both years. The incidence of OA in the sample population was, nevertheless, similar in 1990 and 1991, although the mean OA serum concentrations differed. This suggests that a portion of the population is exposed to variable levels of OA, depending on the OA content of the commodities consumed, and that the exposure to OA is not consistent among the same individuals over time. Regional differences were also observed in the swine blood surveyed by Ominski et al. (Manuscript I) and the agricultural commodities surveyed by Kuiper-Goodman et al. (1993). These differences indicate that exposure to OA is variable from one region to another, and suggest that the incidence and concentration of OA in human blood sera may also vary between regions. Therefore, serum should be monitored at a regular interval to accurately assess the extent of OA contamination in human

blood.

An estimation of average intake of OA can be calculated using the following equation given by Breitholtz et al. (1991)

$$k_o = Cl_p \cdot C_p/A \text{ (Klaassen 1986)}$$

where continuous intake (k_o, ng/kg body weight per day) is related to clearance (Cl_p, 0.67 ml/kg body weight per day (Hagelberg et al. 1989)), the plasma concentration (C_p, ng/mL) and the bioavailability (A, 0.50 (Hagelberg et al. 1989)). The estimated average intake of OA from all food sources, based on a mean serum level of 1.29 ng/mL, is 1.73 ng/kg body weight per day in 1991. This value corresponds to the OA intake estimated from consumption of contaminated food products in Canada proposed by Kuiper-Goodman et al. (1993). Tolerable daily intakes (TDI) of 0.2 - 4.2 ng/kg body weight per day and 16 ng/kg/bw per day have been proposed by Kuiper-Goodman and Scott (1989) and the Joint FAO/WHO Expert Committee on Food Additives (WHO 1991), respectively. Thus, the estimated average intake of 1.73 ng/mL is below the upper limit of these values. As indicated by Breitholtz et al. (1991), this equation has some limitations. Firstly, it assumes that most of the OA is cleared by renal filtration, as the value used for plasma clearance only involves glomerular filtration, and does not account for other routes of elimination such as hepatic clearance via metabolism and thus may be an underestimate of intake. Conversely, it may be an overestimate of intake if factors such as enterohepatic recycling and renal reabsorption (Marquardt and Frohlich 1992) are significant in humans. Nevertheless, without additional information regarding plasma clearance in

humans, this equation may provide a reasonable estimate of intake.

This study demonstrates that although OA is present in the blood of Canadians, there was no relationship between this toxin and renal disease. In addition, although the average estimated daily intake of OA is within the TDI proposed by Kuiper-Goodman and Scott (1989), 1.8% of the individuals in 1990 and 18.3% of the individuals in 1991, had estimated daily intakes that exceeded the upper limit of this TDI. None of the individuals exceeded the TDI proposed by the Joint FAO/WHO Expert Committee on Food Additives (WHO, 1991). These results indicate that some individuals intermittently have high serum levels of OA presumably from periodic exposure to high OA levels in food. This study reinforces the need for ongoing observations of agricultural products for the presence of OA, and the provision of information to the farming community regarding the prevention and control of this toxin in agricultural commodities.

The Effect of Different Dietary Concentrations of Ochratoxin A on its Accumulation in Swine Blood and Tissue

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Abstract

Feeding studies were conducted to determine if the OA concentrations in swine serum and tissue were correlated in pigs fed dietary concentrations of OA ranging from 200-5000 μg/kg of feed (0.2-5.0 ppm). The concentration of OA in the serum was found to be highly correlated with that of the liver ($R^2 = 0.94$), kidney ($R^2 = 0.88$), muscle ($R^2 = 0.97$), and fat ($R^2 = 0.97$) at the equilibrium phase of distribution within the animal. The relationship between blood and tissue was not affected by the dietary concentration of OA ($p > 0.05$) or the sex of the animal ($p > 0.05$). Furthermore, the concentration of OA among tissues was also highly correlated, with R^2 values ranging from 0.91 - 0.97. Regression equations were developed to predict the concentrations of OA in tissue based on that present in the serum, or other tissues. A high correlation was also observed between plasma, serum and whole blood fractions ($R^2 = 0.97 - 0.99$), indicating that the concentration of OA in plasma or whole blood can be converted to a serum equivalent and used to predict OA concentration in tissue. Further studies are under way to confirm that the regression equations established in this study are applicable at the pre- and post-equilibrium phases of distribution. Such evidence would indicate that it may be possible to reduce the presence of OA in the food chain by quantitating the concentration of OA in swine blood at the slaughterhouse or preferably, on-farm, and using regression equations, determine if the OA concentration in the tissue exceeds acceptable concentrations.

Introduction

Ochratoxin A (OA), a toxic secondary metabolite produced primarily by fungi belonging to the species, *Penicillium* and *Aspergillus*, is a potent nephrotoxin in many mammalian species (Harwig et al. 1983). The nephropathy is characterized by polyuria, glucosuria, proteinuria, decreased osmolality of the urine, changes in renal function, as well as histopathological changes in the proximal tubules such as tubular degeneration and atrophy (Berndt et al. 1980; Krogh 1978). The carcinogenic (Bendele et al. 1985a; NTP 1989), immunomodulatory and immunosuppressive (Hong et al. 1988; Dwivedi and Burns 1984b,1985; Szczech et al. 1973a,b,c), genotoxic (Creppy et al. 1985, Kane et al. 1986c) and teratogenic (Hayes et al. 1974; Szczech and Hood 1981; Brown et al. 1976, Hood et al., 1976) effects of OA have been reported. Furthermore, OA has been implicated as a causal agent of several diseases - porcine and avian nephropathy (Elling and Moller, 1973; Elling et al., 1975; Krogh et al., 1973) and Balkan endemic nephropathy (Krogh 1974 cited in Krogh 1992). A nephropathy of pigs was initially described by Larsen in 1928 (Krogh 1987). This disease was associated with feeding batches of moldy grain. The similarity between this disease and that induced by OA, led Elling and Moller (1973) and Krogh et al. (1973) to suggest that OA may be a causal agent. Feeding studies utilizing OA provided additional evidence to support this hypothesis (Krogh et al. 1974).

Balkan endemic nephropathy is a fatal chronic kidney disease encountered in the Balkan countries of Romania, Bulgaria, and Yugoslavia. The similarity in both morphological and functional impairment between OA-induced porcine nephropathy

and Balkan endemic nephropathy (Krogh 1974 cited by Krogh, 1992), as well as the presence of this toxin in human food and animal feed (Krogh et al. 1977; Pavlović et al. 1979) and human blood (Hult et al. 1982) in endemic areas of Yugoslavia, also suggest that OA may be a causal agent of this disease.

In addition to its presence in the Balkans, OA has been detected in food and feed (see Krogh 1987 for a review) as well as in human blood (see Kuiper-Goodman and Scott 1989 for a review, Manuscript II) and human breast milk (Bauer and Gareis 1987; Gareis et al. 1988; Micco et al. 1991; Breitholtz-Emanuelsson et al. 1993) in several countries. This transmission of toxin through the food chain raises some concern for human and livestock health. Kuiper-Goodman et al. (1993) have identified cereal products and swine blood as two possible sources of OA in the food chain. Although chicken livers were also sampled in the above mentioned survey, none of the samples collected contained detectable levels of OA.

To date, there have been no regulations established in Canada, regarding the presence of OA in pork products. Denmark, however, has legislated safety levels for the presence of OA in pork products. All slaughtered animals which have macroscopically changed kidneys are tested for OA (Hald 1991b). If the OA content of the kidney is found to be greater than 25 μg/kg, then the entire carcass is condemned; if it is 10-25 μg/kg, then the kidney, liver, and other visceral organs are condemned; if it is less than 10 μg/kg, only the kidneys are condemned (Hald 1991b; van Egmond 1991). The shortcomings of this program have been outlined by Büchmann and Hald (1985). The kidneys from animals which had recently been

exposed to OA would appear normal at slaughter based on gross observation, even though the carcass may contain a high level of OA, as the gross changes in the kidney may take several weeks to develop (Elling 1983). Moreover, lesions which are induced early in the growth period, when animals are more susceptible to OA, do not disappear when pigs are fed an OA-free diet (Elling 1983), and are therefore analyzed at the expense of the farmer (Büchmann and Hald 1985).

In the last decade, new analytical techniques such as immunoassays have made it possible to screen a considerable number of samples for the presence of mycotoxins such as OA, both quickly and accurately (Chu 1990). The innovation of commercial test kits for OA enables these analyses to be performed at the slaughterhouse, as well as on-farm, and thus herds which contain detectable levels of OA are identified and prevented from entering the human food chain. A more reliable and simple procedure than that described above would be to monitor OA in the blood and use these values to predict tissue concentrations of OA. This not only permits direct on-farm monitoring of OA, but also addresses the aforementioned concerns. Furthermore, serum values of OA tend to be higher than that of the tissues (Mortensen et al. 1983a), and do not require extensive sample preparation.

Several studies have been carried out to assess the transmission of OA into swine blood and tissue. Slightly different patterns of OA accumulation in the tissue have been observed. An increasing concentration of OA was observed in the muscle, fat, liver and kidney in swine fed 200, 1000, and 4000 μg/kg of OA (Krogh et al. 1974). Pigs fed the 4000 μg/kg diet, accumulated equal quantities of OA in the liver

and the fat. Krogh et al. (1976a), however, in a subsequent study, found the highest concentration of OA in the kidney, followed by the liver, muscle and fat in pigs fed 1000 μg/kg OA for one month. This same pattern of accumulation was also observed by Krogh et al. in other studies (1976b, 1979). Madsen et al. (1982a) observed the highest concentration of OA in the kidney, approximately equal quantities in the muscle and liver, and lowest in the fat. Using this data, Mortensen et al. (1983a) developed regression equations of OA in the kidney, liver, muscle and fat, based on that present in the blood.

The objective of this study was to systematically establish the relationship between blood and tissue concentrations of OA at steady state in swine fed diets containing different concentrations of the toxin. The four experiments studied (1) the time at which OA reached equilibrium in the blood after daily consumption of 1000 μg/kg of OA; (2) the relationship between blood and tissue concentrations of OA in pigs fed 0, 200, 500, 1000, and 5000 μg/kg OA; and (3) the accumulation of OA in serum, plasma, whole blood and red blood cells in animals fed 200 and 1000 μg/kg OA and the relationship between these fractions. These experiments should provide a basis for not only predicting OA concentration in tissues from that in the blood, but also for predicting OA concentrations in tissue from OA values in the various blood fractions. This data should facilitate the routine testing of OA in the intact animal prior to slaughter and for predicting OA in tissues after slaughter.

Materials and Methods

Management of Animals. Castrated male and female Managra pigs weighing 80-100 kg body weight were obtained from the University of Manitoba swine herd. The animals were fed a standard finisher swine diet consisting of 89% barley, 8.5% soybean meal and 2.5% vitamin and mineral premix as described by the National Research Council (NRC). Modifications of the diet are described below. Blood was collected during experiments 1 and 3, via indwelling jugular vein catheters. The jugular vein was surgically exposed, and approximately 45 cm of a one meter length of catheter tubing (1.00 mm inner diameter; 1.50 mm outer diameter, Dural Plastics, Australia) was inserted into the jugular vein. The remaining 55 cm was externalized at the back of the neck. Animals were cared for according to the guidelines established by the Canadian Council of Animal Care.

Preparation of OA-contaminated diets. Inoculum was prepared as outlined by Madhyastha et al. (1990) with slight modifications. *Aspergillus alutaceus* var. *alutaceus* Berkeley et Curtis (Kosakiewicz, 1989), formerly *Aspergillus ochraceus* Wilhelm, was grown on potato dextrose agar (PDA) slants with 3% NaCl at 28°C for seven days. The spores were harvested following the addition of sterilized 0.05% Tween in water and were aseptically dislodged with a sterile inoculating loop.

Sixty gram allotments of soybeans (*Glycine max* L.) were dispersed into 500 ml Erlenmeyer flasks and autoclaved for 20 min at 121°C. The moisture content of the flasks was adjusted to 24-25% by the addition of the appropriate volume of distilled water. Moisture content was determined by the use of a moisture determination balance (Ohaus Corp, Florham Park, NJ). Following a 24 h

equilibration period, each flask was inoculated with 2 mL of the spore suspension under aseptic conditions and incubated for a period of 28 days. Soybeans from each flask were pooled, dried at room temperature for 48 hours, and ground using a Cyclotec 1093 Sample Mill (Tecator, Sweden) grinder with a 1-mm screen. Ground soybeans were remixed to ensure a uniform preparation and stored at -20°C prior to extraction. Subsamples of the soybeans were extracted with $CHCl_3$-0.1 M H_3PO_4 (20:1 v/v) and subjected to reverse-phase thin-layer chromatography for cleanup. Ochratoxin A was eluted with methanol according to the procedure of Frohlich et al. (1988) and quantified using high performance liquid chromatography with an HP 1046A fluorescence detector (Hewlett-Packard Ltd.) according to the procedure of Josefsson and Møller (1979). Aliquots of 20 μL were injected into a 250 X 4.6 mm column, maintained at 50°C, containing 5-μm diameter C-18 bonded-phase adsorbent (Beckman Inc., Altex Division). The mobile phase consisted of 65% methanol containing 10% isopropanol and 35% double distilled water at a pH of 2.7, with a flow rate of 1.5 mL/min. Ochratoxin A reference standard was obtained from Sigma Chemical Co. (St. Louis, MO). The OA-contaminated soybeans (148 μg OA/kg), were diluted with clean soybeans to obtain the desired dietary concentration of OA.

Experiment 1. This experiment was designed to determine the relationship between blood and tissue concentrations of OA after blood concentrations reached equilibrium. Three gilts and three barrows, weighing 80-100 kg body weight were fed a standard finisher mash diet containing 1000 μg OA per kg of feed, one hour each day for a period of 14 days. Blood samples were taken at two hour intervals for the

first 24 h and then twice per day (0800 and 1500 hours) for the remainder of the experiment. Blood and tissue (kidney, liver, muscle and fat) samples were collected at slaughter and stored at -20 °C.

Experiment 2. This experiment was designed to study the effects of different dietary concentrations of OA on its accumulation in blood and tissues. Twenty-four pigs, 80-100 kg body weight, were randomly divided into four groups and fed standard finisher mash diets, on a restricted basis, containing 0, 500, 1000 and 5000 μg OA per kg of feed for a period of 21 days. Blood and tissue (kidney, liver, muscle and fat) samples were collected at the end of the experimental period.

Experiment 3. This experiment was designed to further explore the effects of different dietary concentrations of OA on its accumulation in swine blood and tissues. Six pigs weighing 80-100 kg were fed, on a restricted basis, a standard finisher diet containing 200 μg OA per kg of feed for one hour each day for a period of 21 days. Blood samples were collected once per day for 28 days. On day 21, three pigs were given clean feed, while the remaining three pigs continued to receive OA.

Experiment 4. This experiment was designed to examine the distribution of OA in whole blood, serum, plasma and red blood cells of pigs fed OA, as well as to determine the relationship among these fractions. Eight pigs (four males, four females) weighing 40-60 kg body weight were randomly divided into two groups and fed a standard finisher diet containing 200 and 1000 μg of OA per kg of feed for a period of 16 days. Blood samples were collected via vena puncture from pigs on day 0,4,8,12 and 16 of feeding. Samples for whole blood and serum analysis were

collected in untreated tubes. Samples for serum analysis were allowed to clot, centrifuged at 850*g* and the serum fraction was decanted. Whole blood was extracted immediately following collection according to the procedure described below. Samples for plasma and red blood cell analysis were collected in heparinized tubes, centrifuged at 1600*g*, and the plasma fraction was decanted. The remaining red blood cells were washed twice with saline and reconstituted to the original volume with saline.

Analytical methods. Ochratoxin A was extracted from serum according to the method of Hult et al. (1979). Kidney, liver, and muscle were extracted as follows. One gram of tissue was placed in a nylon centrifuge tube, and homogenized in a Polytron PT 10 OD (Brinkman, Rexdale, ON) with 10 mL of 0.1 M $NaHCO_3$. The samples were extracted, following acidification to pH 2.1 with 85% phosphoric acid, with 12 mL of $CHCl_3$ in a horizontal shaker (Eberbach Corporation, Ann Arbor, Michigan) and then centrifuged at 1200*g* for 10 min. The lower layer of chlorofrom was withdrawn and dried under a stream of nitrogen. Using this extraction procedure, recovery of OA from kidney, liver and muscle was 78%, 79%, and 84%, respectively. The HPLC assay for OA was as described by Sreemannarayana et al. (1988). Fat was extracted as follows. Twenty-five grams of fat were placed in a 250 ml plastic bottle, and homogenized as described above, with 100 mL of methanol and sodium bicarbonate (3:7). Samples were then centrifuged at 4400*g* for 20 min at 2°C, and the supernatant was transferred into a separatory funnel and extracted with hexane. Following acidification with 85% phosphoric acid, the aqueous methanol

solutions were extracted with $CHCl_3$ as described above, centrifuged at 1200*g* and dried under a stream of nitrogen. Extracts were then reconstituted with 500 μL methanol prior to HPLC analysis. Using this procedure, recovery of OA from fat was 67%.

Statistical Analysis. Data were analyzed using Analysis of Variance (ANOVA) and the General Linear Model (GLM) procedures of the Statistical Analysis Systems Institute, Inc. (1989). For experiments 1, 2 and 3, regression equations were derived to relate OA concentration in certain target tissues to OA concentration in serum and other tissues. Polynomial regressions were used, with non-significant ($p > 0.05$) terms removed in a stepwise fashion. No intercept was assumed. Patterns of accumulation in serum over time were also described with polynomial regressions with non-significant terms ($p > 0.05$) removed.

In experiment 4 the relationship between OA in serum and OA in whole blood and plasma was examined over the course of the trial; the model included polynomial terms for each fraction, as well as interaction of these with day of trail and dietary concentration of OA. No intercept was assumed.

Results

Experiment 1. The average concentration of OA in the blood of pigs over a 24-hour period following the oral administration of OA are depicted in Figure 11. Ochratoxin A was detected in the blood within two hours of feeding, and continued to rise until about 18 hours after feeding, attaining a concentration of approximately

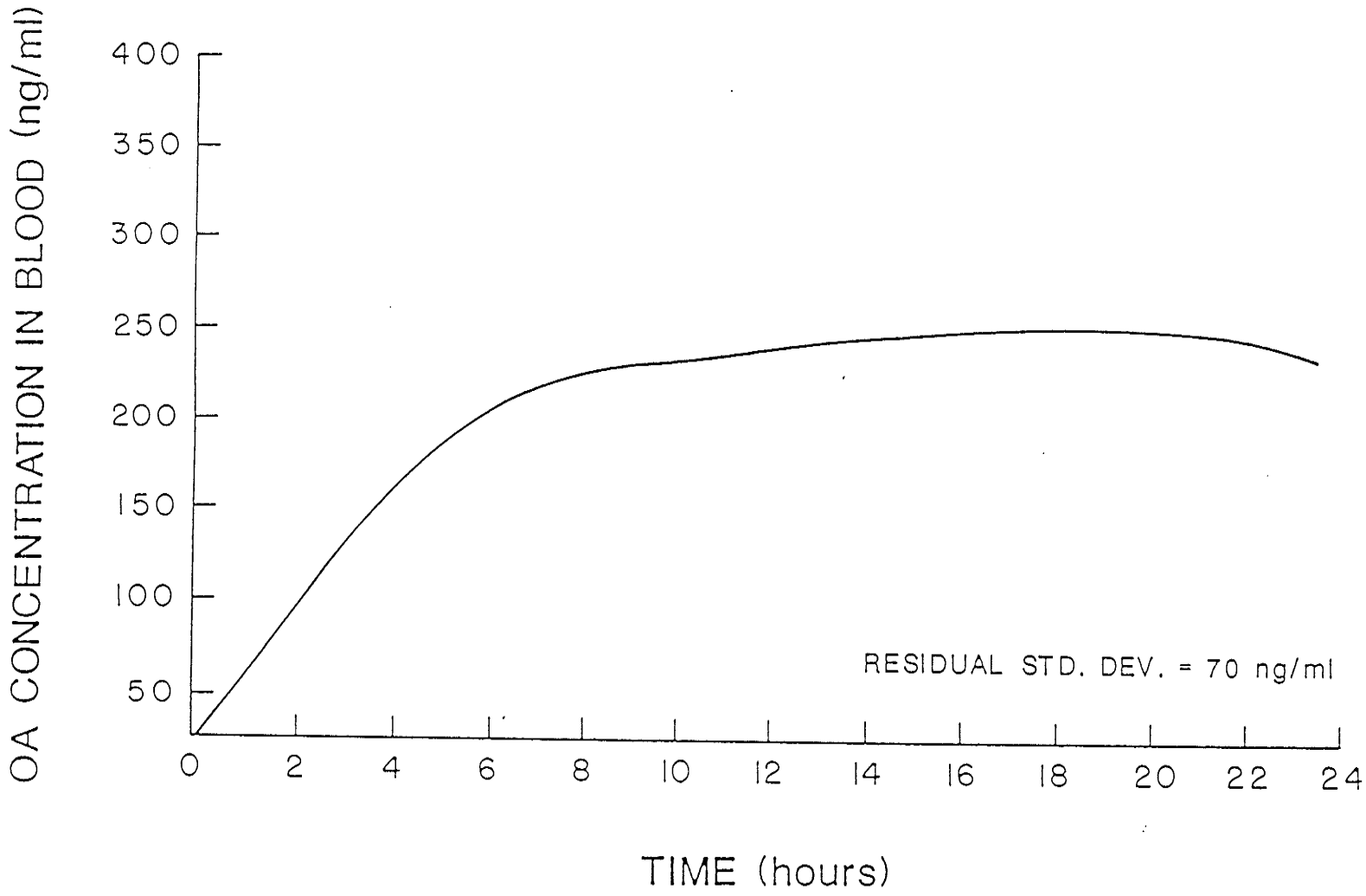

Figure 11: Ochratoxin A in the blood of swine (n=6) fed a diet containing 1.0 ppm OA for a one-hour period. Blood samples were collected every two hours for 24 hours. Figures were obtained using the General Linear Model (GLM) procedures of the Statistical Analysis Systems Institute, Inc. (1989).

250 ng/mL. At this point it appears that an equilibrium was reached. The concentration of OA in the blood started to decline by the 23rd hour, indicating that OA was being metabolized and excreted. One-half maximal blood concentrations were reached within three hours, which indicates that uptake is rapid and probably occurs in the upper portion of the gastrointestinal tract.

The concentration of OA in the serum continued to rise as feeding of OA continued over the 14 day trial, as expected (Figure 12). An equilibrium appears to be reached at approximately the 290th hour or day 12. The one-half maximal blood concentration was reached within 70 hours, suggesting that it takes several days to saturate the blood with OA. The average OA concentration ± S.E. in the serum at the end of 14 days was 1281 ± 81 ng/mL. There was no difference between males and females ($p > 0.05$) in serum OA values. The average concentration of OA in the kidney, liver, muscle and fat at slaughter was 150 ± 3, 189 ± 4, 37 ± 0.2, and 50 ± 0.8 ng/g, respectively.

Experiment 2. The concentration of OA attained in each of the tissues and the blood after 14 days of feeding OA was dependent upon the concentration in the diet, as the OA concentration attained in the serum, kidney, liver, muscle and fat were significantly different for each treatment group ($p < 0.05$). However, residues of OA, regardless of the dietary concentration fed, were highest in the serum, followed by the liver and kidney, and lowest in the muscle and fat (Figure 13). The diet containing 5000 μg OA/kg yielded the highest tissue concentrations of OA, the 1000 μg/kg diet intermediate levels and the 500 μg/kg diet the lowest levels.

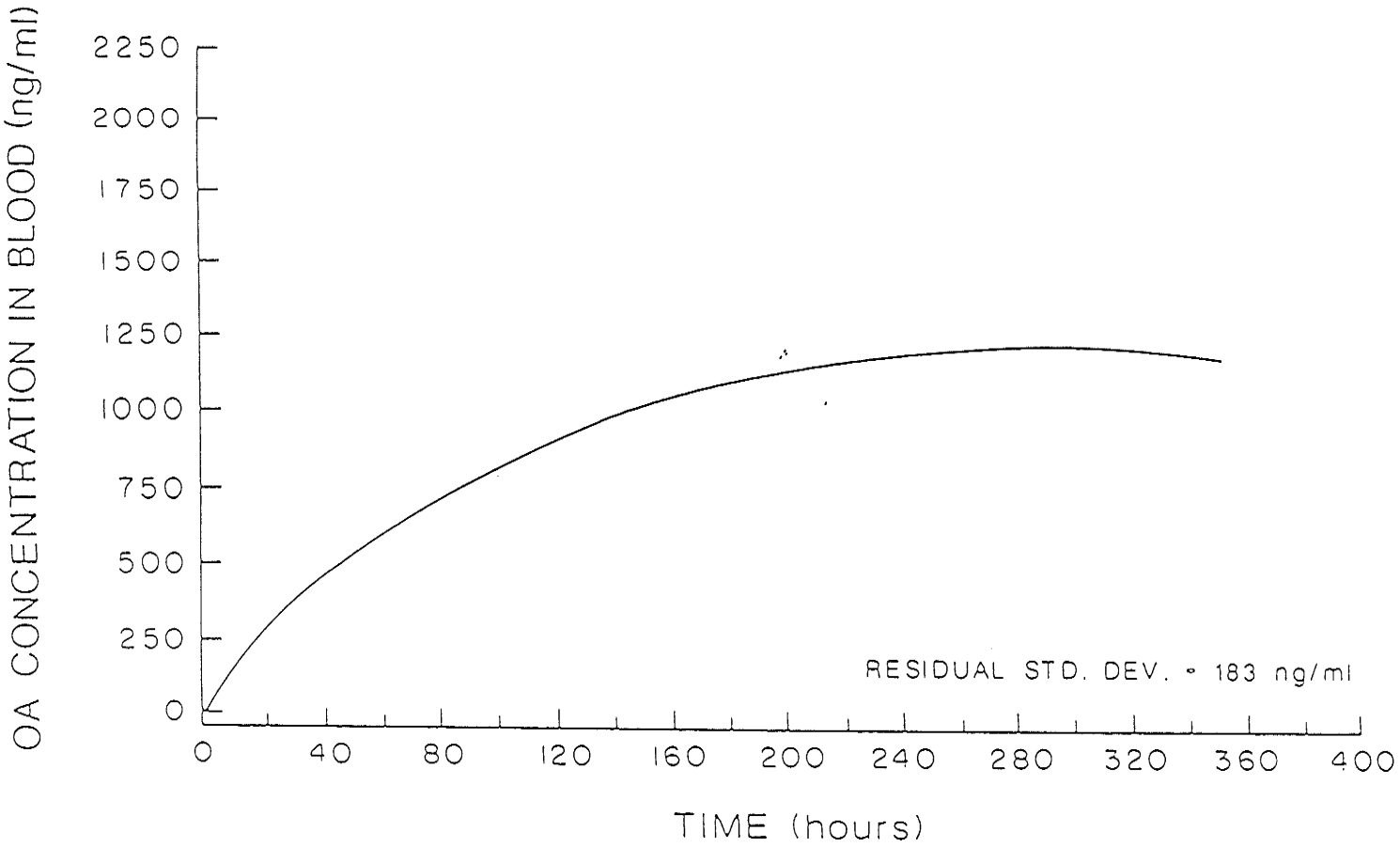

Figure 12: Ochratoxin A in the blood of swine (n=6) fed a diet containing 1.0 ppm of OA for a one-hour period each day for 14 days. Blood samples were collected every two hours for the first 24 hours (see Figure 1), and then twice per day for the remainder of the experiment. Figures were obtained using the General Linear Model procedures of the Statistical Analysis Institute, Inc. (1989).

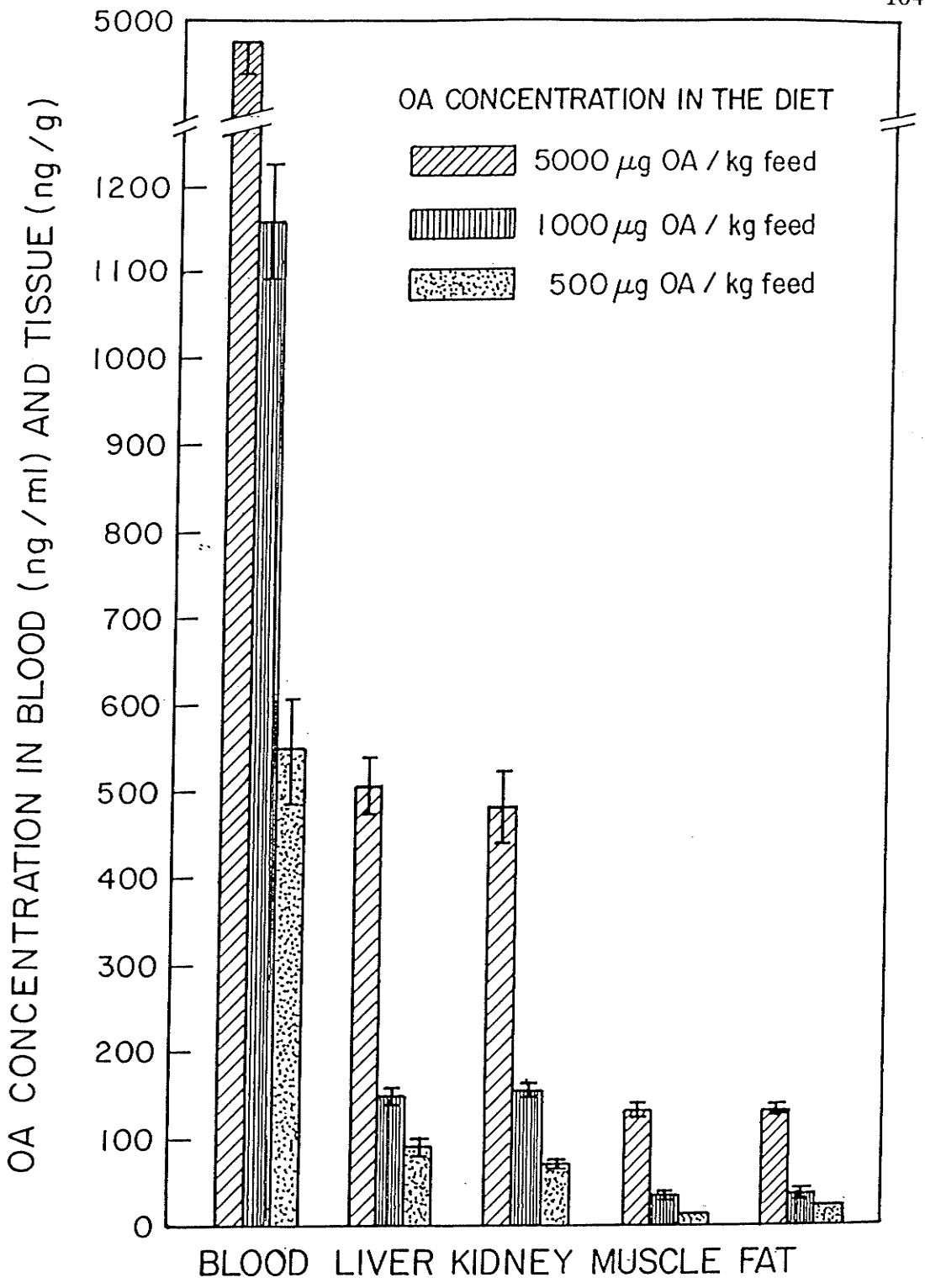

Figure 13: Residues of OA in tissues and blood from swine fed different dietary concentrations of OA. Each bar represents the mean ± S.E.M. for 6 pigs.

Experiment 3. The blood profile of OA in animals fed OA at 200 μg OA per kg of diet followed the same trend as that observed in Experiment 1. The concentration of OA in the blood and the examined tissues on day 28 of the experiment was 100.5 ± 17 and 33 ± 1, 21 ± 0.4, 16 ± 0.7 and 10 ± 0.4 ng/g, in the kidney, liver, muscle and fat, respectively.

Experiment 4. The concentration of OA in the serum, plasma and whole blood increased with time, as was expected. The red blood cells, however, did not contain detectable levels of OA during any of the sampling periods, while the serum and plasma concentrations were similar. Whole blood has values intermediate to those of plasma and serum, and the red blood cells. The best fit regression data demonstrated that R^2 values were slightly higher when the data was corrected for the small but significant ($p < 0.05$) polynomial terms, as well as interaction of these with day and diet, as indicated in Table 4. However, the R^2 for the simple linear regression were high (greater than 0.97); therefore, a simple linear regression was used to predict the concentration of OA in one fraction from that of another. Simple regression equations for the various fractions are given in Table 5.

Experiments 1, 2 and 3. The relationship between blood and tissue data from experiments 1, 2 and 3 was not affected by the dietary concentration of OA ($p > 0.05$) or the sex of the animal ($p > 0.05$), although the latter approached significance ($p < 0.10$). Therefore, the data was pooled and regression equations were developed to predict the OA concentration in tissue based on that in the serum, as well as in other tissues. The simple linear and best fit regression data demonstrated that R^2

Table 4: Comparison of Simple and Best Fit Regression Data for Predicting the Concentration Of OA in Different Blood Fractions

	Simple Linear Regression	Best Fit Regression	
Predicted Fractions	R^2	Significant Factors	R^2
Plasma from serum	0.98	serum, serumXserum, serumXdiet, serumXserumXday	0.98
Whole blood from serum	0.97	serum, serumXserum, serumXday, serumXdiet, serumXserumXday, serumXdietXday	0.99
Serum from whole blood	0.97	whole blood, whole bloodXday	0.96
Plasma from whole blood	0.99	whole blood, whole bloodXwhole blood whole bloodXdiet, whole bloodXday	0.99
Whole blood from plasma	0.99	plasma, plasmaXplasma, plasmaXdiet	0.99
Serum from plasma	0.98	plasma, plasmaXday, plasmaXplasmaXday, plamsaXdietXday	0.98

Table 5: Regression Equations to Predict the Concentration of OA in Different Blood Fractions

Regression Equations	R^2
OA_{plasma} (ng/mL) = 1.01 x OA_{serum} (ng/mL)	0.98
$OA_{whole\ blood}$ (ng/mL) = 0.57 x OA_{serum} (ng/mL)	0.97
OA_{serum} (ng/mL) = 1.70 x $OA_{whole\ blood}$ (ng/mL)	0.97
OA_{plasma} (ng/mL) = 1.76 x $OA_{whole\ blood}$ (ng/mL)	0.99
$OA_{whole\ blood}$ (ng/mL) = 0.55 x OA_{plasma} (ng/mL)	0.99
OA_{serum} (ng/mL) = 0.97 x OA_{plasma} (ng/ml)	0.98

values were slightly higher when the data was corrected for small but significant ($p > 0.05$) polynomial terms as indicated in Table 6. Simple linear regressions were, however, used to predict the OA concentration in tissues from that of blood and other tissues, as the associated R^2 values were comparable to those of the best fit regressions (Table 6). Simple linear regression equations are given in Table 7.

Discussion

The data indicate that the concentration of OA in serum and tissues (liver, kidney, muscle and fat) from pigs fed different dietary concentrations of OA (200-5000 μg OA/kg diet) are highly correlated. Therefore, a serum sample may be used to predict the concentration of OA in the liver, kidney, muscle and fat of the same animal. Serum, as indicated in Table 7, appears to be a better predictor of the concentration of OA in liver, muscle and fat than kidney, as the R^2 associated with the regression equation for kidney was lower ($R^2 = 0.88$) than that of the other tissues.

In addition to predicting OA concentration in tissue from serum values, it is also possible to predict the tissue concentration of OA from other tissues (Table 7). However, as stated above, serum is a better predictor of the OA concentration in the liver, muscle and fat than is the kidney in predicting these same tissues, as demonstrated by the associated R^2 values. This observation, combined with the difficulty of collecting tissue samples and the extensive sample preparation required for tissue analysis, suggests that it is simplier and more precise to predict tissue OA

Table 6: Comparison of Simple and Best Fit Regression Data for Predicting the Concentration of OA in Tissue from Serum and Other Tissues

	Simple Linear Regression	Best Fit Regression	
Predicted Fraction	R^2	Significant Factors	R^2
Kidney from serum	0.88	serum, serumXserum	0.91
Liver from serum	0.94	serum, serumXserum	0.96
Muscle from serum	0.97	serum, serumXserumXserum	0.94
Fat from serum	0.97	serum, serumXserum	0.98
Liver from kidney	0.92	kidneyXkidney	0.95
Muscle from kidney	0.91	kidney, kidneyXkidney, kidneyXkidneyXkidney	0.97
Fat from kidney	0.89	kidney, kidneyXkidney, kidneyXkidneyXkidney	0.97
Kidney from liver	0.92	liver	0.92
Muscle from liver	0.96	liver	0.96
Fat from liver	0.95	liver	0.95
Kidney from muscle	0.91	muscle	0.91
Liver from muscle	0.96	muscle	0.96
Fat from muscle	0.95	muscle, muscleXmuscle	0.96
Kidney from fat	0.89	fatXfat, fatXfatXfat	0.92
Liver from fat	0.95	fat	0.95
Muscle from fat	0.95	fat, fatXfat	0.97

Table 7. Regression Equations to Predict the Concentration of OA in Tissue from the OA Concentration in Serum and Other Tissues

Regression Equations	R^2
OA_{liver} (ng/g) = 0.11 x OA_{serum} (ng/mL)	0.94
OA_{kidney} (ng/g) = 0.10 x OA_{serum} (ng/mL)	0.88
OA_{muscle} (ng/g) = 0.03 x OA_{serum} (ng/mL)	0.97
OA_{fat} (ng/g) = 0.03 x OA_{serum} (ng/mL)	0.97
OA_{liver} (ng/g) = 1.00 OA_{kidney} (ng/g)	0.92
OA_{muscle} (ng/g) = 0.26 OA_{kidney} (ng/g)	0.91
OA_{fat} (ng/g) = 0.25 OA_{kidney} (ng/g)	0.89
OA_{kidney} (ng/g) = 0.92 OA_{liver} (ng/g)	0.92
OA_{muscle} (ng/g) = 0.25 OA_{liver} (ng/g)	0.96
OA_{fat} (ng/g) = 0.25 OA_{liver} (ng/g)	0.95
OA_{kidney} (ng/g) = 3.54 OA_{muscle} (ng/g)	0.91
OA_{liver} (ng/g) = 3.78 OA_{muscle} (ng/g)	0.96
OA_{fat} (ng/g) = 0.97 OA_{muscle} (ng/g)	0.95
OA_{kidney} (ng/g) = 3.54 OA_{fat} (ng/g)	0.89
OA_{liver} (ng/g) = 3.81 OA_{fat} (ng/g)	0.95
OA_{muscle} (ng/g) = 0.99 OA_{fat} (ng/g)	0.95

concentrations from serum rather than other tissue. The high correlation between the concentration of OA in plasma, serum, and whole blood fractions demonstrate that plasma or whole blood may be used to estimate OA, or be converted to a serum equivalent (Table 5), and then used to predict OA in tissue using the predictive equations provided in Table 7. Red blood cells did not contain detectable levels of OA.

The predictive equations obtained by Mortensen et al. (1983a) are somewhat different than those obtained here. Ochratoxin A concentrations in that study were highest in the kidney, followed by the lean, liver, and fat, with R^2 values ranging from 0.71-0.79. In the present study, OA concentrations were highest in the kidney followed by the liver, muscle and fat, with R^2 values ranging from 0.88-0.97. It is not unexpected that OA concentrations in the liver and kidney would be greater than those in the muscle and fat for several reasons. Firstly, these organs have a high cardiac output and are therefore, well-perfused with blood. Utilizing radiolabelled OA (^{14}C-ochratoxin A) Galtier et al. (1979) demonstrated that OA is distributed in two kinetically distinct compartments. The central compartment corresponds to well-perfused organs including the liver and kidney, while the peripheral compartment includes the muscle and fat. Secondly, liver and kidney have a high capacity to bind chemical toxins (Klaassen 1976). Finally, the demonstrated metabolism of OA in the liver (Størmer et al. 1981), suggests that the concentration of OA leaving the liver and reaching other tissues might be less, depending on the extent of metabolism of the toxin.

Hult et al. (1980) has demonstrated that animals within a herd tend to have similar concentrations of OA in the serum (R^2 = 0.80) and has suggested that one animal in a herd may be used to predict the concentration of the entire herd. In larger herds, however, several samples may be required (Hult 1991). This relationship was also demonstrated in Manuscript II (Ominski et al). Therefore, it may be possible to reduce the presence of OA in the human food chain by quantitating the concentration of OA prior to slaughter using modern immunological techniques and by using predictive equations such as those provided by this study, estimate the OA concentration in the respective tissues. Any animals with serum samples exceeding an acceptable limit could then be fed until acceptable concentrations of OA were reached. The shortcomings of the Danish program, as desribed above, would thereby be eliminated. Further studies are required to determine if the regression equations presented here apply at the pre- and post-equilibrium phase of OA intake. Such studies are currently underway.

GENERAL DISCUSSION

The potential for OA production in stored products in western Canada (Abramson et al. 1980,1985,1987), its presence in a variety of feed ingredients (Scott et al. 1972; Abramson et al. 1983a), and more recently, in swine blood (Marquardt et al. 1988, Manuscript II) suggests that although the incidence of OA in agricultural commodities is sporadic and variable, it may have implications for human and livestock health. Furthermore, it may cause considerable financial loss to the livestock industry during outbreaks of acute toxicoses, or more frequently when at levels which render the animal susceptible to secondary infections (Pier et al. 1980).

Risk assessments for several mycotoxins, including OA, prepared by Health Canada (Kuiper-Goodman and Scott 1989), indicate the need for continued monitoring of animal-derived food products and human blood, and thus directed the research undertaken.

Although conducted on a limited basis, the surveys of human and swine serum indicate and confirm that Manitobans experience low level exposure to OA. This exposure is inconsistent, as indicated by its presence in swine serum which showed both seasonal and geographic variation. The inconsistent nature of this exposure, also apparent in human serum, creates considerable difficulty with regards to regulations for OA, as well as other mycotoxins. An accurate assessment of the incidence of OA in agricultural commodities requires frequent nationwide monitoring

to identify and minimize sources of OA in the human food chain. The presence of OA in swine serum suggests that it is a possible source of OA and a means of predicting its presence in tissue is highly desirable to minimize exposure. The data provided suggest that the overall OA status of the herd may be predicted by sampling a few animals in the herd. Regression equations, such as those provided, indicate that a strong relationship exists between serum and tissue concentrations of OA, and may be used to predict the OA concentration in the respective tissues. If the OA concentration in the tissue exceeded acceptable limits, the appropriate feeding procedure could be employed to reduce its concentration to an acceptable value. A collaborative effort between regulatory agents and commodity groups to reduce this source of contamination would be mutually beneficial.

CONCLUSIONS

Based on the research conducted, it can be concluded that:

1) Ochratoxin A is present, at low levels, in the serum of swine in Manitoba. Geographic and seasonal differences were observed for both the incidence and concentration of OA. Analysis of the incidence of OA within a herd suggests that the OA status can be estimated by sampling a few animals in a given herd.

2) Ochratoxin A is present, at low levels, in a select population of Manitobans. There was no relationship between the presence of OA in human serum and the occurrence of renal disease.

3) There is a high correlation, over a range of dietary concentrations of OA, between the concentration of OA in the serum and that in several tissues (kidney, liver, muscle and fat). Regression equations were developed which can be used to predict the concentration of OA in the tissues from its concentration in the serumat steady-state.

Further research:

1) Additional research is underway to confirm that the regression equations developed at steady state are applicable at pre- and post-equilibrium phases

of distribution.

2) Management practices used by swine producers in Manitoba should be evaluated to identify those which facilitate or enhance fungal growth and subsequent toxin production. Viable alternatives to such practices should be sought.

3) Practical and cost-effective techniques to reduce the concentration and toxicity of OA in contaminated feeds should be explored.

4) It is my experience that swine producers are unaware of the implications of feeding mycotoxin-contaminated feed, including potential financial losses due to decreased production. Although the mycotoxin issue was given considerable media attention in Manitoba in the fall of 1993 with the outbreak of Fusarium headblight in wheat and barley and the corresponding contamination of these grains with deoxynivalenol, a basic understanding of mycotoxin production and its transmission into livestock is lacking. Researchers and provincial extension personnel should work closely to provide relevant information to livestock producers, thereby reducing this source of OA contamination in the food chain.

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Winnipeg Research Center
195 Dafoe Rd
Winnipeg MB R3T 2M9

6 October 1994

Helen Agar
Faculty of Graduate Studies
Room 500, University Center
University of Manitoba
Winnipeg MB R3T 2N2

Ph.D. Thesis, Kim Ominsky, Dept of Animal Science

Kim Ominsky contacted me today regarding a request for copyright permission. I am a co-author on one of the manuscripts included in her thesis.

To the extent that I can claim copyright for any portion of the material in question, I give the University of Manitoba permission to reproduce or transmit the said material, in whole or in part.

Sincerely,

D. Abramson, Ph.D.,
Research Scientist

THE UNIVERSITY OF MANITOBA

FACULTY OF AGRICULTURAL AND FOOD SCIENCES
Department of Animal Science

Winnipeg, Manitoba
Canada R3T 2N2

Tel.: (204) 474-9383
FAX: (204) 275-0402

September 27, 1994

Helen Agar
Thesis Officer
Faculty of Graduate Studies
500I University Centre
University of Manitoba

Dear Helen:

Dr. Kim Ominski has asked that I send a letter of permission concerning her thesis to you. As co-author of some of the material in Dr. Ominski's thesis, I give permission to the Faculty of Graduate Studies and the University of Manitoba Library to distribute the material as they see fit.

Sincerely

Gary H. Crow, Ph.D.
Associate Professor

GHC:lmf

Mrs. Helen Agar
500I University Centre
Faculty of Graduate Studies
University of Manitoba
Winnipeg, MB
R3T 2N2

Dear Mrs. Agar:

Please accept this letter as verification of my consent to be listed as a co-author of the paper entitled "Incidence and Distribution of Ochratoxin A in Western Canadian Swine" which is included in Kim Ominski's Ph.D. thesis.

Thank you.

Sincerely,

Mrs. Karen Carrette

JUL 17 1995

Mrs. Helen Agar
500I University Centre
Faculty of Graduate Studies
University of Manitoba
Winnipeg, MB
R3T 2N2

Dear Mrs. Agar:

Please accept this letter as verification of my consent to be listed as a co-author of the paper entitled "Ochratoxin A in Human Serum in Western Canada" which is included in Kim Ominski's Ph.D. thesis.

Thank you.

Sincerely,

Dr. J. McKenzie

JAN 24 [illegible]

Mrs. Helen Agar
500I University Centre
Faculty of Graduate Studies
University of Manitoba
Winnipeg, MB
R3T 2N2

Dear Mrs. Agar:

Please accept this letter as verification of my consent to be listed as a co-author of the papers entitled "Ochratoxin A in Human Serum in Western Canada" and "Incidence and Distribution of Ochratoxin A in Western Canadian Swine" which are included in Kim Ominski's Ph.D. thesis.

Thank you.

Sincerely,

Dr. R.R. Marquard

Mrs. Helen Agar
500I University Centre
Faculty of Graduate Studies
University of Manitoba
Winnipeg, MB
R3T 2N2

Dear Mrs. Agar:

Please accept this letter as verification of my consent to be listed as a co-author of the paper entitled "Ochratoxin A in Human Serum in Western Canada" which is included in Kim Ominski's Ph.D. thesis.

Thank you.

Sincerely,

Dr. Adrian Fine

17/1/95

Mrs. Helen Agar
5001 University Centre
Faculty of Graduate Studies
University of Manitoba
Winnipeg, MB
R3T 2N2

Dear Mrs. Agar:

Please accept this letter as verification of my consent to be listed as a co-author of the paper entitled "Ochratoxin A in Human Serum in Western Canada" which is included in Kim Ominski's Ph.D. thesis.

Thank you.

Sincerely,

Dr. Jure Manfreda

Mrs. Helen Agar
500I University Centre
Faculty of Graduate Studies
University of Manitoba
Winnipeg, MB
R3T 2N2

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Thank you.

Sincerely,

Dr. A. Frohlich

Mrs. Helen Agar
500E University Centre
Faculty of Graduate Studies
University of Manitoba
Winnipeg, MB
R3T 2N2

Dear Mrs. Agar:

Please accept this letter as verification of my consent to be listed as a co-author of the paper entitled "Ochratoxin A in Human Serum in Western Canada" which is included in Kim Ominski's Ph.D. thesis.

Thank you.

Sincerely,

Dr. Tina Kniper-Goodman

Winnipeg, Man. R3T 2N2
December 1, 1994.

Mrs. H. Agar,
Graduate Studies,
University of Manitoba

Please accept this letter as verification of my consent to ~~keep~~ have the paper entitled "Incidence and Distribution of Ostracoda in Western Canadian Lakes", included in Miss Kim Swigchild's Ph.D. Thesis

Yours very truly,

DEC 05 1994