THE UNIVERSITY OF MANITOBA

EFFECT OF A HAY A GRAIN DIET ON THE HYDROLYSIS AND BIOAVAILABILITY OF OCHRATOXIN A IN SHEEP

BY

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ABSTRACT

Factors affecting the hydrolysis of ochratoxin A (OA) by rumen fluid was studied in sheep fed diets that contained high concentrations of hay or grain. The pH of rumen fluid was much lower in sheep fed grain (pH 5.5±0.1) than in those fed hay (pH 7.0±0.1), ($P<0.001$). The rate of hydrolysis of OA in vitro by the particulate fraction of the rumen fluid, as indicated by the formation of its hydrolyzed product, ochratoxin α (Oα), was eight times greater for rumen obtained from hay-fed than from grain-fed sheep ($P<0.001$). No Oα was detected when OA was incubated with the supernatant fraction of rumen fluid collected from sheep fed either diet. The rate of hydrolysis of OA by rumen fluid was influenced by time of feeding ($P<0.03$), and the changes in rate paralleled changes in rumen pH, especially for sheep fed the grain diet. The rate of OA hydrolysis, however, was not affected by the pH (5.5 or 7.0) of the incubation media ($P=0.67$). An in vivo study was carried out involving the direct administration of OA (0.5 mg/kg body weight) into the rumen. OA disappeared from the rumen 2 to 5.5 times faster in sheep fed hay than in sheep fed grain ($P<0.05$). The results suggest that while a change in rumen pH may be associated with a change in species and population of rumen microflora, it is the change in microflora but not pH which affects the rate of OA hydrolysis.

In another in vivo experiment, OA was injected intravenously (0.2 mg/kg body weight) into four sheep (two fed, two fasted). The serum concentration of OA declined immediately after injection following a bi-phasic pattern with the initial disappearance half-life ($T_{\frac{1}{2}}$) being 1.5±0.1 h and the final elimination half-life ($T_{\frac{1}{2}}$) being 17.3±1.4 h. The cumulative excretion data revealed that 90-97% of OA was excreted into the urine, only small amounts of OA (1.5-5.0%) was excreted as Oα into urine and feces; no OA was found in the feces and no Oα was detected in the serum. Another in vivo experiment involving the administration of OA (0.5 mg/kg body weight) orally to four sheep (two fed hay, two fed grain). 90-99% of OA was converted into Oα with 68-89% of OA being excreted as Oα in urine and only 0.5-3% of OA being found in the urine. Of the OA that was excreted, a much greater amount was excreted by sheep fed-grain as compared to those fed-hay. The results suggest that OA is nearly quantitatively hydrolyzed to Oα in both grain-fed and hay-fed sheep, but the rate of hydrolysis is much faster in the hay-fed sheep. In a final study, eight sheep (four fed hay, four fed grain) were given a single oral dose of OA (0.5 mg/kg body weight). The disappearance of OA from the rumen was much faster for sheep fed hay than for sheep fed grain with the half-life ($T_{\frac{1}{2}}$) being 0.63 and 2.7 h, respectively. The corresponding rate of formation of Oα in the rumen was much greater for sheep fed hay compared to those fed grain with the formation half-life

($T_{\frac{1}{2}}$) being 0.9 and 1.9 h, respectively. The maximum concentration of O in the rumen was obtained 1.4 h after dosing in sheep fed hay (C_{max}=220 μg/ml), whereas it was obtained at 7 h in sheep fed grain (C_{max}=88 μg/ml). The apparent relative bioavailability of OA for sheep fed hay was only 20% of that for sheep fed grain. The results of this experiment demonstrate that the hydrolysis of OA in the rumen of sheep reduces the amount of OA absorbed into the blood. Overall these results would suggest that the rumen of sheep plays a very important role in detoxification of OA and also suggest that the type of diet influences the metabolism of OA in the rumen and therefore its bioavailability. Sheep fed grain hydrolyze OA at a much lower rate than those fed hay.

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LIST OF ABBREVIATIONS

aw	water activity
cAMP	cyclic adenosine-3′,5′-monophosphate
DNA	deoxyribonucleic acid
h	hours
kg	kilogram
l	litre
LD50	median lethal dose
mg	milligram
min	minute
ml	millilitre
mRNA	messenger-ribonucleic acid
ADP+	oxidized form of nicotinamide adenine dinucleotide phosphate
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
ng	nanogram
NMR	nuclear magnetic resonance
OA	ochratoxin A
Oα	ochratoxin α
pK_a	dissociation rate constant
$T_{1/2}$	biological half life
tRNA	transport ribonucleic acid
µg	microgram

1. INTRODUCTION.

Ochratoxin A (OA) is a mycotoxin produced by fungi of Aspergillus and Penicillium (Van der Merwe et al, 1965a, b, Van Walbeek et al, 1969a, b). Structurally (Figure 1), it is a substituted 7-carboxy-5-chloro-8-hydroxy-3-4-dihydro-methylisocoumarin (Cole and Cox, 1981). The toxin has been isolated from variety of agricultural commodities in various geographical regions of the world (Chu, 1974 and Krogh, 1987) and has been shown to be a contaminant in many foods and feedstuff (Dwivedi and Burns, 1986a, b, and Roschenthaler, 1984). In animals, OA is absorbed into the systemic circulation across the intestinal mucosa and then distributed to various tissues and organs of the body (Krogh, 1983). Residues of OA has been found in the blood and many tissues of animals and man (Hult et al, 1980, Krogh et al, 1977, and Marquardt et al, 1988). Exposure to OA causes nephropathy in swine (Krogh, 1977) and liver damage in chicken (Warren and Hamilton, 1981). Numerous studies have demonstrated that OA affects many tissues in many different ways. It is primarily known as a nephrotoxin, a hepatotoxin, a carcinogen and an immunosuppressive agent (Chu, 1974, Steyn, 1984, Dwivedi and Burns, 1986a, b). In most species the principle target organs for OA toxicity appear to be the kidney and liver (Krogh, 1983).

OA is hydrolyzed in vitro and in vivo to an isocoumarin

compound, ochratoxin α (Oα), by the action of some proteolytic enzymes, especially carboxypeptidase A (Nel and Purchase, 1968, Pitout, 1969). Hydrolysis of OA to Oα has been suggested as one mechanism by which OA is detoxified since Oα has been shown to be non-toxic to the animal (Chu et al, 1972). Studies have shown that OA is hydrolyzed to Oα by the enzymes in the small intestine (Doster and Sinnhuber, 1972) and by the rumen microflora (Hult et al, 1976, Kiessling et al, 1984). Sreemannarayana et al (1988) reported that the relative bioavailability of OA for ruminant calves was only 26 to 37% of that for preruminant calves in which the rumen microflora had not yet developed. It has also been shown that rumen protozoa are able to hydrolyze OA at a much faster rate than other rumen microflora (Kiessling et al, 1984). It is well known that diet high in grain depresses the population of protozoa (Eadie et al, 1970, Jouany et al, 1988), It may be hypothesized that the rate of hydrolysis of OA would be faster in the rumen of hay-fed as compared to grain fed sheep. The present studies were designed to evaluate the effects of diet (hay or grain) on the hydrolysis of OA in the rumen of sheep and therefore possibly affect the bioavailability of OA in sheep, and to test the hypothesis that the rate of hydrolysis of OA in the rumen of sheep fed hay is faster than those fed grain.

2. THE REVIEW OF THE LITERATURE

2.1. Ochratoxins.

Ochratoxins are a group of mycotoxins produced by toxigenic species of Aspergillus and Penicillium (Chu, 1974). The family of ochratoxins has been described by Cole and Cox (1981) and Applegate (1973).

2.1.1. Ochratoxin A (OA) (Figure 1). OA was first isolated from the culture of Aspergillus ochraceus by extraction with aqueous sodium hydrogen carbonate and purified by chromatography on acidic silica gel (Van der Merwe et al, 1965a,b.). Chemically ochratoxin is 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin-7-L-ß-phenylalanine with a molecular weight of 403 (Cole and Cox, 1981). OA is a colourless, heat stable crystalline compound, and is highly soluble in polar organic solvents and slightly soluble in water. It also dissolves in aqueous sodium bicarbonate (Steyn, 1984). In aqueous medium it exists in both the non- and ionized forms because of dissociation of the phenolic group (pk_a=7.04) in the dihydroisocoumarin ring (Chu, 1974). These chemical and physical properties of ochratoxin A are responsible for its biological activity. Among the ochratoxins, OA is the most toxic to animals (Cole and Cox, 1981). OA can be hydrolyzed by inorganic acid and by the enzyme carboxypeptidase A into phenylalanine and 7-

carboxylisocoumarin (ochratoxin α) which is non-toxic to the animals (Steyn, 1984).

2.1.2. **Ochratoxin B (OB)** (Figure 1). Ochratoxin B, which is the dechloro form of OA, is a colourless crystalline compound closely related to ochratoxin A (Steyn, 1984). Ochratoxin B is considerably less toxic than OA. It was reported to be hydrolyzed into the beta form of OA by bovine carboxypeptidase or various rat tissue extracts much faster than OA (Doster and Sinnhuber, 1972). The LD50 in day-old chicks was 54 mg/kg while the corresponding value for OA was 3.3-3.9 mg/kg (Cole and Cox, 1981).

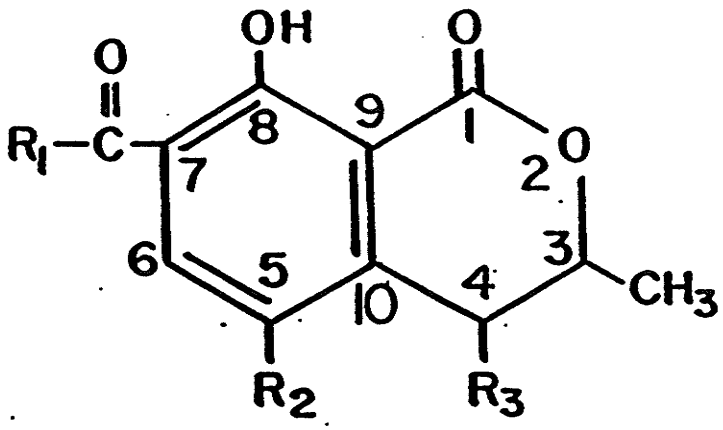
2.1.3. **Ochratoxin C (OC)** (Figure 1). Ochratoxin C simply is the ethyl ester of OA. Ochratoxin C is relatively less toxic than OA but more toxic than ochratoxin B (Cole and Cox, 1981). Ochratoxin C can be converted into OA in vivo (Fuchs et al, 1984).

2.1.4. **Hydroxylated-ochratoxin A (OH-OA)** (Figure 1). Hydroxylated-ochratoxin A includes 4- and 10-hydroxy OA. 4-Hydroxy-ochratoxin A has two epimeric forms (4R) and (4S). Hydroxylated-ochratoxin A is found in both the culture of A.ochraceus and urine of the animals (Cole and Cox, 1981). The 4-hydroxy-ochratoxin A's are relatively non-toxic to animals, possibly being the detoxification forms in animals

dosed with OA (Cole and Cox, 1981).

Figure 1. Ochratoxins

OA	Ochratoxin A
OB	Ochratoxin B
OC	Ochratoxin C
OA-OH	Hydroxylated ochratoxin A



	R_1	R_2	R_3
OA	$-CH_2CH(COOH)NH-$	–Cl	–H
OB	$-CH_2CH(COOH)NH-$	–H	–H
OC	$-CH_2CH(COO\overset{*}{E}t)NH-$	–Cl	–H
OAOH	$-CH_2CH(COOH)NH-$	–Cl	–OH

* methyl or ethyl ester

2.1.5. Biosynthesis of Ochratoxin A.

OA has been chemically synthesized (Steyn et al,1969, Roberts and Woollven, 1970, Rousseau et al, 1984). Acid hydrolysis reveals that OA is composed of L-ß-phenylalanine and 7-carboxylisocoumarin which can be synthesized by both microbes and higher plants. An OA synthetase in fungi forms the amide bound between the 7-carboxyl group of the dihydroisocoumarin and the amino group of phenylalanine
(Ferrira and Pitout, 1969). The mode by which the chlorine atom is introduced into the molecule of OA is not yet clear though incorporation of radio-labelled chlorine into OA has been readily observed in a culture of A.ochraceus (Wei et al, 1971). From a theoretical consideration of the chemical structure of OA, the aromatic ring of phenylalanine may be formed directly from the pure aliphatic precursor, phosphor-enol-pyruvate via the shikimic acid pathway as described by Lehninger (1979). The labelling pattern of acetate-2-^{14}C strongly supported the hypothesis that the 7-carboxylisocoumarin moiety of OA was synthesized via the acetate-malonate pathway (Searcy et al, 1969). Incorporation of labelled acetate has been used for the production of radio-labelled OA (Lillehoj et al, 1978). Based on the experimental results, a suggestion was made that biosynthesis of OA involved both the shikimic acid and the acetate-malonate pathway (Roschenthaler, 1984).

2.2. Occurrence of Ochratoxin A.

2.2.1. Ochratoxin Producing Fungi.

Fungi mold are one of the most ubiquitous types of microbes in the nature. Only a few toxigenic fungi produce toxic metabolites which are harmful to animals and humans. Ochratoxin producing fungi are found in only some species of Aspergillus and Penicillium (Krogh, 1987).

Aspergillus ochraceus was the first fungus found to be an ochratoxin producer (Van der Merwe et al, 1965a, b). Since then other species of Aspergillus and Penicillium have been isolated and identified as ochratoxin producers (Semeniuk et al, 1971 and Van Walbeek et al, 1969). More and more studies have indicated that production of ochratoxins is common to many fungi in the Aspergillus group (Lai et al, 1968, 1970, Lillehoj et al, 1978).

Production of ochratoxins by different species of the A.ochraceus family was investigated by Hesseltine et al (1972); 44 strains of nine species in the group were selected for the culture. The results indicated that production of ochratoxins occurred through out the A.ochraceus group: A.ochraceus, A.sclerotiorum, A.alliaceus, A.melleus, A.sulphureus, A.ostianus, and A.petrakii with the first four species having the highest capacity to produce the toxin. The

highest yielding species were identified as being two strains of A.ochraceus NRRL 3174 and NRRL 3519. The same result was observed by Lillehoj (1978).

In addition to the fungi in the A.ochraceus group, different species of Penicillium have been isolated and shown to be producers of the ochratoxins (Van Walbeek et al, 1969, Scott, 1965, Scott et al, 1970). Included in this group are P.variabile, P.commune, P.cyclopium, P.purpurescens and P.palitans (Scott et al, 1972, Ciegler, 1972, Ciegler et al, 1972). In summary, the species known to produce OA are A.ochraceus, A.ostianus, A.melleus, A.petrakii, A.sclerotiorum, A.sulphureus, A.alliaceus, and P.viridicatum, P.cyclopium, P.commune, P.palitans, P.purpurescens, and P.variabile (Kurata, 1978, Krogh, 1987).

Aspergillus ochraceus has been regarded as the predominant ochratoxin producer and has been frequently used for lab production of OA (Chu, 1974).

2.2.2. Natural Occurrence of Ochratoxin Producing Fungi.

Ochratoxin producing fungi are widespread in nature and have been characterized as storage fungi (Chu, 1974). Ochratoxigenic species of Aspergilli and Penicilli have been associated with the natural occurrence of OA (Van walbeek et al, 1969a,b, Scott et al, 1972, Mills and Abramson, 1982).

Aspergillus ochraceus was isolated from stored grains (Wallace and Sinha, 1962, Yamazaki et al, 1970). Toxigenic A. ochraceus has been frequently isolated from a variety of food and feedstuffs (Doupnik and Peckham, 1970, Christensen et al, 1968), cereal and sorghum (Scott, 1965, 1977), black and red peppers (Christensen and Kaufman, 1969, Christensen et al, 1967), hay (Still, 1973), corn (Richard et al, 1969), poultry feed (Bacon, 1973), rice (Udagawa et al, 1970, Yamazaki et al, 1970), dried fish (Udagawa et al, 1970), soyameal and poultry feed (Connole, 1973), coffee beans (Levi et al, 1974), wheat (Manning and Wyatt, 1984), bread and flour (Osborne, 1980, Visconti and Bottalico, 1983), and country cured ham (Escher et al, 1973). A.sulphureus and A.melleus has been isolated from Brazil nut and dried fish (Van Walbeek et al, 1969a, b, Hesseltine et al, 1972).

Toxigenic stains of P.viridicatum have been isolated from ham (Escher et al, 1973), and corn (Shotwell et al, 1969). Penicillium ochraceus was isolated from corn (Carlton et al, 1972). P.viridicatum, P.palitans have been isolated from heated grains and animal feeds (Scott et al, 1972, Scott, 1977). P.verrucosum, P.cyclopium, P.chrysogenum and P.janthinellum have been isolated from rapeseed and cereal (Mills and Abramson, 1982).

P.viridicatum frequently produces both OA and citrinin (Scott et al, 1972, Krogh, 1973, and Krogh et al, 1973). Conversion

of mycotoxin citrinin into OA by P.viridicatum has been reported (Patterson and Damoglou, 1987). However a report indicated that P. viridicatum was not a producer of both the ochratoxins and citrinin. The discrepancy was due to the misidentification of the species and the association of P. viridicatum with the ochratoxin producing species (Pitt, 1987). P. viridicatum produces other nephrotoxins including xanthomegin and viomellein (Hald et al, 1983, Pitt, 1987).

2.2.3. Occurrence of Ochratoxin A

Influence of Environmental Conditions. The occurrence of OA is influenced by environmental conditions (Welling, 1974) as the toxin producing fungi require suitable conditions for growth. Moisture is a critical component in the determination of the species of fungi that occur on an organic substrate (Lillehoj and Elling, 1983). In terms of water activity (aw), Harwig and Chen (1974) observed that an aw of 0.90-0.93 provided optimum conditions for the growth and production of OA by Penicillium viridicatum.

Water activity is expressed as the ratio of water vapour pressure of a substrate to the vapour pressure of pure water at equal temperature and pressure (Northolt et al, 1979).

Studies on Aspergillus ochraceus, the maximum OA production was found at an aw of 0.95 in poultry feeds with no toxin

being detected below 0.90 (Bacon et al. 1973). A water content of 30-31% provided the largest yield (2,000-4,000 ug/g) of OA when A. ochraceus was grown in barley and wheat (Lindenfelser and Ciegler, 1975). The optimum aw for P.cyclopium was 0.95-0.99 (Northolt et al, 1979).

In conjunction with moisture, temperature plays the same important role for growth and production of OA (Lillehoj and Elling, 1983). The range of temperature for production of OA by A.ochraceus was found to be between 12 and 37°C with optimal yield between 31 and 37°C. In the case of P.viridicatum, the maximum production of the toxin was found at temperatures between 16 and 24°C (Northolt et al, 1979). When A.ochraceus NRRL 3174 grew on wheat, corn and rice, the optimal temperature for OA production was observed to be 28°C; no toxin was detectable at temperatures lower than 4°C (Trenk et al, 1971). Production of OA by A.ochraceus in barley was completely inhibited when the temperature was lower than 10°C, while P.viridicatum still produced OA even at a temperature 4°C (Haggblom, 1982 and Damoglou et al, 1984). Natural occurrence of OA was also shown to be influenced by the climatic conditions in western Canada (Sinha et al, 1986).

Substrate such as plant products provide nutrients for fungi to grow and produce toxins, which can also affect the production of OA (Lillehoj and Elling, 1983). Concentrations of OA in the medium is not proportional to the growth

(mycelial) of the fungus but is related to nutritional factors (Lai et al, 1968). No OA was detectable if trace elements were absent in the culture of A.ochraceus (Lai et al, 1970, Steele et al, 1973). Sucrose is an important factor for production of OA in liquid medium with 4% of sucrose providing maximum production of OA by A.ochraceus (Yamazaki et al, 1970). Production of OA by A.ochraceus in barley increased with an increase in the concentration of protein and the amino acids, glutamic acid and proline (Haggblom and Ghosh, 1985). However, methionine and some antimicrobial additives were found to inhibit the production of OA by A.ochraceus and P.viridicatum (Lisker et al, 1983, Tong et al, 1985). Exposure to radioactive ^{60}Co seems to stimulate the production of OA by A.ochraceus (Applegate and Chipley, 1976, Paster et al, 1985).

Natural Occurrence of Ochratoxin A. Natural occurrence of OA on a number of grain substrate and other agricultural commodities has been demonstrated (Chu, 1974, Krogh, 1987, Dwivedi and Burns, 1986a, b).

The first report of the natural occurrence of OA in low grade samples of corn was noted in United States (Shotwell, 1969). The report revealed that Penicilli were isolated and OA was detected at 110-150 ug/kg from corn sample which had a musty odour and contained 18% moisture. Then natural occurrence of OA has been observed in barley and wheat (Shotwell et al,

1976), coffee (Levi et al, 1974, Stack et al, 1982), cereal products (Chu and Butz, 1970) in the United States.

Investigations in western Canada indicated that the natural occurrence of OA could be observed in tough, damp stored wheat, barley (Sinha et al, 1986), and field grain (Mills et al, 1982, Abramson et al, 1983). OA as a natural contaminant was detected up to 27,000 ug/kg in heated grain, and in animal feedstuffs (Scott et al, 1970, 1972, Prior, 1976, 1981).

In Denmark, Krogh (1973) reported that OA occurred at 28-27,500 ug/kg of barley. Natural occurrence of OA was also observed in country cured ham (Escher, 1973), bread and flour (Osborne, 1980). The highest level of OA as a natural contaminant of poultry feeds was reported in Australia (Connole et al, 1981).

Using contaminated grain for brewing, OA could be recovered in beer (Nip et al, 1975, Krogh et al, 1974a, b). The carry-over of OA from natural contaminated feed to tissues and milk of dairy cows was reported by Shreeve et al (1979).

OA has been of particular concern in Bulgaria and Yugoslavia as this region is affected by an endemic nephropathy referred to as Balkan endemic nephropathy which has been suggested to be related to OA (Petkova-Bocharova and Castegnaro, 1985). Natural contamination with OA was detected at 45-5,125 ug/kg

in corn, barley, and cereal in Yugoslavia (Muntanola-Cvetkovic and Klemenc, 1983).

When contaminated food or feed was consumed by humans or animals, residues of OA were found in the blood and tissues of the body (Dwivedi and Burns, 1986). Residues of OA were found in broiler and laying hen dosed long term with low concentrations of OA (Micco et al, 1987). Natural occurrence of OA residues was detected in the blood of slaughter pigs in Canada with the serum concentration of ochratoxin A in positive samples exceeding 20 ng/ml (Marquardt et al, 1988). Feeding experiments with bacon pigs ingesting OA contaminated barley revealed residues of OA were in kidney, liver, lean and fat (Madsen et al, 1982). Residues of OA were detected in the kidneys (16-77 ug/kg), liver (0-21/kg), and blood (36-77 ug/kg) of swine from nephropathic areas in Yugoslavia (Pepeljnjak and Cvetnic, 1985). Spontaneous occurrence of OA in the kidneys of fattening pigs was noted in Sweden (Rutqvist et al, 1977, 1978) and in porcine kidney, and serum samples in Poland (Golinski et al, 1984, 1985). Krogh (1977) and Krogh et al (1977) reported the occurrence of OA residues in tissues from slaughter pigs with nephropathy and reported a correlation between OA concentration and swine nephropathy. Disappearance of OA from the body is a time dependent process (Krogh et al, 1976). It has been suggested that the concentration of OA in the blood of animal can be used to estimate the concentration of the toxin in the feed (Hult et

al, 1979, 1980). Natural occurrence of OA residues has been reported in human blood (Hult et al, 1982).

2.3. Toxicological and Pathological Effects of Ochratoxin A.

2.3.1. The LD50 of Ochratoxin A.

The acute toxicity of OA has been reported for different species. The LD50 (median lethal dose) of OA given orally to one-week-old New Hampshire Leghorn cross chicks was 166 ug (Chu and Chang, 1971). In broiler chicks the LD50 for day-old chicks was 2.14 mg/kg body weight, and for 3 week-old birds was 3.6 mg/kg body weight (Peckham et al, 1971). Initial studies on day-old ducklings indicated that the oral LD50 of OA was 25 ug (Van der Merwe et al, 1965a). But later work by Steyn and Holslapfel (1969) estimated that the oral LD50 of OA in day-old ducklings ranged between 135-170 ug. Chang et al (1981) found the oral LD50 of OA for day-old and 3 week-old turkey poults to be 4.63 and 7.84 mg/kg body weight, respectively, while the corresponding intraperitoneal values were 0.16 and 0.34 mg/kg body weight, respectively. The LD50 of OA for beagle dogs was 0.2-0.3 mg/kg daily estimated within 14 days (Chu, 1974). Studies on the rat indicated that the oral-stomach tube LD50 of OA was 20-22 mg/kg body weight (Purchase and Theron, 1968, Purchase). The oral LD50 in 24-h-old infant rat was determined to be 3.90 mg/kg (Hayes et al, 1977). The LD50 of OA in sheep infused intravenously with OA

was estimated for 24 h to be 1 mg/kg (Munro et al, 1973). The LD50 in swine was found to be 1-2 mg/kg when fed daily over a 5-6 day period (Chu, 1974). Acute toxicity of OA to 60-day-old rainbow trout indicated that the LD50 value for intraperitoneal injection was 4.65 mg/kg body weight (Doster et al, 1972). The acute intraperitoneal toxicity of OA over a seven day period for adult female Swiss mice was found to be 48 mg/kg and to be 10 mg/kg daily dose when administered for 10 days (Prior and Sisodia, 1982).

2.3.2. The General Toxicity of Ochratoxin A.

OA is toxic to many test animals and bacteria. The minimum dose showing an inhibiting effect on Bacillus megaterium was 4 ug/ml (Clements, 1968). The growth of B. subtilis is inhibited by 50% with 6 ug/ml of OA (Roschenthaler et al, 1984). Induction of autolysis of Bacillus subtilis by OA was observed in a medium with 10-20 ug/ml of OA (Singer and Roschenthaler, 1978). A cytotoxic effect on mammalian cell cultures was first reported by Natori et al (1970). Hepatoma tissue culture cells showed growth inhibition at an OA concentration of 18 ug/ml with cytotoxic effects being observed at concentrations of 36 ug/ml of OA in the culture medium (Creppy et al, 1979b). Inhibition of growth by OA has been found in young chicks, the minimum growth inhibitory dietary level being 2 ppm. Enlargement of kidney,liver, crop, proventriculus and gizzard was observed (Huff et al, 1975).

Dwivedi and Burns (1984a) found a dose-dependent depression of growth and an enlargement of kidney and liver together with regression of the thymus, bursa of Fabricius and spleen. Kubena et al. (1984, 1985, 1986) reported that administration 2.5 mg/kg dietary OA significantly increased the relative weight of kidney, liver and gizzard and significantly decreased the relative weight of bursa of Fabricius. The report also indicated significant changes in haematological parameters. Histological changes induced by 4 ppm dietary OA was reported by Duff et al (1987) which induced skeletal osteopenia with disturbed endochondral and intramebranous formation. The diameter of bone was decreased by 4 ppm OA in the diet (Huff and Doerr, 1981). Warren and Hamilton (1980b) reported that OA caused a decease in collagen concentration and consequently increased intestinal fragility. An examination of production characteristics of laying hens on a weekly basis indicated that ochratoxin A significantly depressed egg weight, fertility and hatchability at a dietary concentration of 4 ppm and 16 ppm (Prior et al, 1978a, b).

Histopathologic and electron microscopic studies on rats treated with an oral dose of 22 mg OA/kg indicated that in many of the rats, which were moribund or began dying within 12 to 24 h after dosing, fibrin deposits were found in spleen, brain choroid plexus, liver, kidney and heart (Albassam et al, 1987). Renal tubular nephrosis, hepatic and lymphoid necrosis and necrotic enteritis with villous atrophy were also

observed. A dose response decease in weight gain occurred in neonatal rats given 1 to 5 mg/kg of OA 24 h after birth (Hayes et al, 1977). Administration of OA to pregnant rats increased fetal resorption and decreased fetal weight. The highest number of resorption and greatest depression of fetal weight occurred when the toxin was injected on days 5, 6 or 7 of pregnancy (Mayura et al, 1982). Suppression of marrow granulocyte macrophage progenitors was observed in mice treated with 20 mg/kg OA (Hong et al, 1988).

Natural occurrence of ochratoxicosis in cattle was observed by Lloyd and Stahr (1979, 1980). Nephrosis with hyaline casts, dilated tubules and fibrosis in the kidney, and fatty change in the liver occurred in cattle suffering from ochratoxicosis. Although degradation of OA by the microorganisms of the rumen has been reported, toxicity of OA to the ruminants has been reported by several workers. Ribelin et al. (1978) reported that a cow given up to 1.66 mg/kg for 4-5 days still remained normal while a cow given 13.3 mg/kg OA had only transit illness with OA being transferred to the milk. The lethal dose for goats was found to be 3 mg/kg OA. Lloyd (1980) reported that renal uraemia, hepatoses, gastrointestinal ulcers and pneumonia in cattle were related to the ochratoxicosis that occurred naturally. OA has also caused abortion and fetal death in cattle (Macklin et al, 1971).

2.3.3. Ochratoxin A as a Causal Agent of Nephropathy.

The toxic effects of OA include acute nephritis, hepatic degeneration and enteritis (Steyn, 1984), and renal failure (Stein et al, 1984). Experimental porcine nephropathy has been induced in pigs fed diets that contained from 0.2 to 4 ppm OA (Krogh et al, 1974b). Kidney lesions developed which were identical to the naturally occurring porcine nephropathy. High concentrations of OA were detected in nephropathic kidneys from slaughter pigs (Krogh, 1977 and Rutqvist et al, 1978, Peterson and Ciegler, 1978). Spontaneous occurrence of OA in the kidney and blood of nephropathic swine was observed in Poland (Golinski et al, 1984). The toxin has been suggested to be a causal agent of swine nephropathy (Krogh, 1987). Though OA altered the transport functions of the rat kidney, gross evidence of nephropathy was not observed (Berndt, 1980). Nephropathy in poultry caused by OA was also observed by Elling et al (1975). Balkan nephropathy is an endemic chronic and fatal disease of unknown etiology found in Bulgaria, Yugoslavia and Romanian and is produced by a nephrotoxin from P. vrucosum (Austwick et al, 1979). OA has been suspected to be one of the causal agents of Balkan (endemic) nephropathy (Hult et al, 1982). Comparisons of the mycotoxicolgical contamination of food by OA in a nephropathic and non-nephropathic area in Yugoslavia indicated a high incidence of OA in blood, kidney, liver of swine and higher average concentrations of OA in stored cereals in the nephropathic

area (Pepeljinjak and Cvetnic, 1985). OA levels occurred in 16.7% (25-27ug/kg) of the bean samples from the endemic area and 7.1% (25-50 ug/kg) in those from the control area; in maize samples, the concentrations were 27.3% (25-35ug/kg) and 9.0% (10-25ug/kg), respectively (Petkova-Bocharova and Castegnaro, 1985). These results suggest that OA may be a causal agent of Balkan (endemic) nephropathy.

2.3.4. Ochratoxin A as a Carcinogen and Mutagen.

The mycotoxin, aflatoxin B1 has been suggested by Itakura, 1983 to be a carcinogen for human primary hepatocellular carcinoma. Since then, induction of renal carcinoma by OA was observed by Kanesawa and Suzuki, (1978), Kanesawa (1984) and Bendele (1983). The observation was confirmed by Bendele et al (1985b) who found that both renal carcinoma and adenomas were present only in male mice treated with OA above 33 mg/kg and that the incidence of hepatocellular neoplasms was slightly increased in male and female mice fed with diets containing OA. The results indicate that OA is a renal carcinogen in male mice and a hepatic carcinogen in both male and female mice.

Genotoxicity of ochratoxin A was investigated in a battery of *in vitro* and in vivo assays with mammalian cells. The toxin was found not to be mutagenic to *Salmonella typhimurium*, either with or without metabolic activation and no induction

of unscheduled DNA synthesis was evident in primary cultures of rat hepatocytes exposed to 0.000025 to 500 ug/ml of OA (Bendele et al, 1985a). OA caused fetal malformation in rats injected with the toxin above 1.75 mg/kg (Mayura et al. 1982). A single subcutaneous teratogenic dose of OA (1.75 mg/kg) on day 7 of gestation resulted in significantly increased fetal resorption, fetal malformation in both impaired renal function rats and sham-operated rats and impairment of renal function increased the sensitivity to the teratogenic effect of OA (Brown, 1973, Mayura et al, 1984a, b). Teratogenesis induced by OA in rats was partially protected by phenylalanine (Mayura et al, 1984b).

2.3.5. Immunosuppressive Effect of Ochratoxin A.

The direct effect of OA on the immune system was investigated in broiler chicks fed graded dietary level of the toxin up to 4 ppm for 20 days from hatch. The results revealed that OA caused a significant depression in the numbers of immunoglobulin containing cells in all lymphoid organs studied and total serum immunoglobulin levels were reduced in serum from birds fed 2 and 4 ppm dietary concentration of OA (Dwivedi et al, 1984, Dwivedi and Burns, 1984a, b, c). OA caused a regression of the thymus and bursa of Fabricius and lymphoid depletion in lymphoid organs in turkeys fed 4 ppm of dietary ochratoxin A for 10 weeks (Dwivedi and Burns, 1984c). These experiment also found that cell-mediated immune response

was strongly affected by OA in turkey. However the concentration of antibody was not altered in broiler chickens treated with 2 ppm of OA; only depression of lymphocyte and heterophils was observed (Campbell et al, 1983). Chang and Hamilton (1980) reported that the phagocytic activity in chickens was deceased with increase in dosage of OA.

A dose related decrease in thymic mass was observed in mice treated with graded intraperitoneal injections of OA (Boorman et al, 1984). Dose related suppression of the immune response to sheep red blood cells was observed by Haubeck et al.(1981); OA caused a significant depression in the antibody response of Swiss mice against Brucella abortus (Prior and Sisodia, 1982); the mice were treated with 5 ppm OA daily for 50 days prior to being challenged with the antigens. Decrease in serum protein and antibody against Brucella abortus was also noticed in guinea pig treated with OA (Richard et al, 1975). The effect of OA on immune mechanisms and associated tumour resistance has also been examined (Luster et al, 1987). OA seems to suppress natural killing cell activity by inhibiting production of basal interferon. The mechanism for immunosuppression induced by OA seems to be inhibition of protein or enzyme synthesis by competitively inhibiting phenylalanine tRNA-synthetase. The immunosuppressive effect of OA can be inhibited by phenylalanine (Haubeck et al, 1981).

2.4. Pharmacokinetics of Ochratoxin A.

Pharmacokinetics is the study of the time-course of changes in the concentration of drug or chemical and its metabolites in the body (Shargel and Yu, 1985). Pharmacokinetics of OA therefore involves the study of the rate processes involved in the absorption, distribution, metabolism and excretion of this compound and its metabolites. When animals are fed ochratoxin contaminated feedstuffs, it is absorbed from the alimentary tract, mainly the small intestine. On passing through the intestinal mucosa, part of the toxin is cleaved into the alpha form of ochratoxin and phenylalanine due to the activity of carboxypeptidase A and chymotrypsinogen. It is transported in blood, tightly bound to serum albumin, to various tissues and organs of the animal. The toxin is excreted into bile, urine, and feces (Krogh, 1983).

2.4.1. Absorption of Ochratoxin A.

Natural occurrence of ochratoxicosis mainly results from the consumption of feed contaminated with OA which is absorbed from the alimentary tract into the circulatory system. The absorption of the toxin across the gastro-intestinal tract is fast and efficient. Peak plasma concentrations (T_{max}) of the toxin is obtained in 1 h after treatment. A rapid absorption phase (absorption half-life, $T_{1/2}$=18 min) follows the oral administration of OA in the rat (Galtier et al, 1979). Studies

on cattle indicate that the maximum plasma concentration of OA (0.44 ug/ml) is obtained 2 to 4 h after a single oral dose of 1 mg OA/kg body weight (Sreemannarayana et al, 1988). The time to reach the maximum concentration is independent of dose, but is dependent on rate of absorption. Other studies on pigs, rabbits, and chickens also show the rapid absorption of OA from the gastrointestinal tract (Galtier et al, 1981, Lee et al, 1984).

Studies on the absorption site indicate that absorption of OA may occur along the whole alimentary tract, starting from the stomach and continuing into the small intestine (Kumagai and Aibara, 1982, Lee et al, 1984). When OA was introduced into the lumen at various sites of the gastrointestinal tract, the highest concentration of the toxin in portal blood was found when the toxin was injected into the lumen at the proximal jejunum (Kumagai and Aibara, 1982). The results indicated that absorption of OA occurred mainly in the proximal jejunum. However, results obtained by the use of immunohistochemical stains suggested that absorption of OA took place maximally in the esophagus, duodenum, and to a lesser degree in the jejunum (Lee et al, 1984). The primary absorption route of OA was suggested to be the portal vein although the contribution of the lymphatic route could not be excluded when a low dose of OA was given (Kumagai and Aibara, 1982). The absorption site of OA in the ruminant is not clear as the three pre-stomachs in the ruminant are very unique, differing from the

gastrointestinal tract of monogastro animals. No report has revealed the role of the rumen in absorption of OA.

OA exists in both ionized and non-ionized forms in aqueous medium because of the partial dissociation of the phenolic hydroxy group in the dihydroisocoumarin ring (Chu, 1974). Regarding the absorption of OA, facilitated diffusion or mediated transport has been assumed to be the main mechanism of intestinal transfer of this weak electrolyte. Although secretion and absorption of OA was observed to occur together, these can be attributed to a transfer of OA across the intestinal mucosa into the blood stream against a concentration gradient (Kumagai and Aibara, 1982, Kumagai, 1985).). The absorption of OA in the jejunal segment was linearly related to the concentration of OA in blood plasma. OA was absorbed even when its level was higher in plasma than in the jejunal lumen. The jejunal uptake of OA increased with a decrease in medium pH, with the increase in uptake coinciding with an increase in the proportion of OA present in the non-ionized form (Kumagai, 1988). OA uptake by the intestinal mucosa was found to be Na+ dependent, but afflux of OA from mucosa layer was found to be Na+ independent (Kumagai, 1988). The results suggest that diffusional movement of organic anion plays a major role in OA absorption. A concentration gradient of the non-ionized form from the jejunum lumen to the blood and lymph may occur even though the concentration of OA is higher in the blood plasma than that

in the lumen since the pH value is different between plasma (pH=7.4) and gastric juice (pH=1.25).

Binding of OA to plasma albumin also contributes to the maintenance of a concentration gradient of OA between plasma and body tissues (Kumagai and Aiara, 1982).

2.4.2. Distribution of Ochratoxin A.

OA is rapidly absorbed across the intestinal mucosa into the bloodstream and distributed to various tissues (Nel and Purchase, 1968). Experimental data suggest that a two compartment open model may account for the pharmacokinetics of OA (Galtier et al, 1979). According to blood concentration profile, disappearance of OA follows a bi-exponential decline. The distribution half-life ($T_{1/2}$ distribution) is 1.8 hour in cattle (Sreemanarayana et al, 1988), and 2.1 hours in rat (Galtier et al, 1979). In terms of the distribution rate constant, its value is 0.087/h in pigs, 0.36/h in rabbits, 1.52/h in chickens following oral administration of OA (Galtier et al, 1981).

In blood, OA strongly binds to serum albumin. It is then distributed in the free and bound forms to liver, kidney, spleen and other tissues and organs of the animals (Nel and Purchase, 1968, Lee et al, 1984). The distribution of (^{3}H)-labelled OA varied with time elapsed after administration; at

5 h the highest specific label was found in a decreasing order in: the intestinal content, lung, liver, kidney, heart, fat, intestine, testes, with the lowest specific activity occurring in muscles, spleen and the brain (Kane et al, 1986a). Studies on the distribution of ^{14}C-OA in Japanese quail indicated that after dosing with radio-labelled OA, it was detected in almost every organ and tissue: blood, myocardium, liver, proventriculus, gizzard intestinal mucosa, pancreas, kidney, ovary, eggyolk, brain, red and white muscle, lung, and the uropygial gland (Fuchs et al, 1988). When pregnant hamsters were given an intravenous dose of OA, diminished fetal growth and malformation occurred (Hood et al, 1976). OA was detected in maternal serum and reached its maximum concentration within 2 h in pregnant mice given an intraperitoneal injection of 5 mg OA/kg on day 11 or 13 of pregnancy (Fukui et al, 1987). Distribution of OA was detected in the liver, kidney, blood, salivary gland, brown fat, myocardium, uterus and lymphatic tissues of pregnant mice receiving intravenously administrated ^{14}C-labelled OA. The toxin was shown to cross the placental barrier on day 9 of pregnancy (Appelgren and Arora, 1983). OA was detected in kidney, liver, muscle, placenta and fetus in pregnant pigs dosed with OA (Patterson et al, 1976). The distribution of labelled OA in serum, muscle, skin, kidney, liver, heart, lung, uterus, placenta, and fetus was also observed in the pregnant rat (Ballinger et al, 1986). The results indicated that the principal target organs were the kidney and liver, having the highest concentrations of OA in

the body and that OA can cross the placental barrier into the maternal circulation thereby affecting the fetus.

2.4.3. Metabolism of ochratoxin A.

Conversion of Ochratoxin A to Ochratoxin α. Studies on the fate of OA in rats showed that OA was cleaved into dihydroisocoumarin (Oα) and phenylalanine (Nel and Purchase, 1968). Oα is considered to be non-toxic to animals (Chu et al, 1972). Oα has been found as a metabolite of OA in the feces and urine of animals which have received OA either intravenously or orally (Patterson et al, 1976, Galtier et al, 1979, Storen et al, 1982, and Sreemannarayana et al, 1988). Some proteolytic enzymes may be able to hydrolyze the peptide bond connecting 7-carboxylisocoumarin (Oα) and phenylalanine. Studies have indicated that OA is hydrolyzed by carboxypeptidase A and α-chymotrypsin, with a greater affinity between the carboxypeptidase A and OA (Pitout, 1969). Both carboxypeptidase A and α-chymotrypsin are produced by the pancreas of animals (Pitout, 1969). Carboxypeptidase A requires a free carboxyl group and has a specificity for peptide. OA competitively inhibited the activity of carboxypeptidase A with other peptides (Pitout and Nel, 1969). Hydrolysis of OA by extracts of various tissues of rats has been reported (Doster and Sinnhuber, 1972). The results indicated that extracts from both small and large intestine

were able to degrade OA to ochratoxin α with the hydrolysis by extract from the small intestine being two times greater than that from the large intestine, suggesting the involvement in the small intestine of proteolytic enzymes from the pancreas. Liver extracts were only able to hydrolyze trace amounts of OA.

The Role of Rumen in Metabolism of Ochratoxin A. Hydrolysis of OA in the gastrointestinal tract of the ruminant greatly reduce the amount of OA entering the systemic circulation (Sreemannarayana et al, 1988). The enzymes produced by the microorganisms and possibly those from the pancreas were able to hydrolyze the toxin to Oα. The pharmacokinetic study revealed that up to 90% of the administered toxin was converted to Oα. The microorganisms in the rumen played an important role in this respect as the bioavailability of OA in ruminant cattle was only 26-36% of that in pre-ruminant cattle (Sreemannarayana et al, 1988). Degradation of OA by rumen microorganisms has been shown to be very efficient as 40% of OA incubated with rumen fluid is converted to Oα within 1-4 h (Hult et al, 1976). The rate of hydrolysis of OA by the rumen is dependent on the species of the rumen microflora. The majority of activity against the toxin was found to be attributed to rumen protozoa (Kiessling et al, 1984). The pattern of hydrolysis of OA was the same in the reticulum and omasum.

The faunal composition of protozoa in the rumen can vary with dietary composition. The densities or the number of protozoa in the rumen of animals fed hay plus concentrates is generally greater than in animals fed hay only (Nakamura and Kanegasaki, 1969). The ecological condition of the rumen influences the composition of the microflora. Low pH values in the rumen does not favour the growth of protozoa, and a reduction in the total numbers of protozoa results in an increased number of bacteria (Jouany et al, 1988). The total disappearance of protozoa from the rumen can be obtained by feeding ground barley to ruminants, which creates conditions favouring rapid fermentation and low pH values (Eadie and Mann, 1970). The ability to degrade OA fell by about 20% in sheep after being fed a diet high in grain concentrates (Kiessling et al, 1984).

2.4.2. Formation of Hydroxylated Ochratoxin A

Formation of hydroxyochratoxin A is one of the features of OA metabolism. OA in the presence of the reducing agent, NADPH, was converted by a culture of rat liver microsome into two metabolites. The metabolites were identified as (4R)- and (4S)-4-hydroxyochratoxin A by NMR spectroscopy (Stormer and Pedersen, 1980). (4R) and (4S)-4-Hydroxyochratoxin A were found in the culture of liver microsome with NADPH from various species: humans, pigs, and rats. The result suggested that microsomal hydroxylation occurs in the cytochrome P-450 system (Stormer et al, 1981 and Hansen et al, 1982). In vivo

metabolism of OA by rats showed that 1-1.5% of the toxin orally administered (6.6 mg/kg) was excreted as (4R)-4-hydroxyochratoxin A and 25-27% as Oα in the urine (Storen et al, 1982). Beside the (4R) and (4S)-4-hydroxyochratoxin A, a 10-hydroxyochratoxin A was found in a culture of rabbit liver microsome when incubated in the presence of NADPH (Stormer et al, 1983). The formation of hydroxylated metabolites was inhibited by carbon monoxide and methyrapone, and was stimulated when the animals were pretreated with phenobarbital (Stormer et al, 1983). Among the three hydroxylated ochratoxin A's, only (4R)-4- and 10-hydroxyochratoxin A were oxidized by alcohol dehydrogenase in the presence of NAD (Syvertsen and Stormer, 1983).

Metabolism of OA has been considered to be a means by which it is detoxified. OA metabolism in phenobarbital treated mice increases and as a result its toxicity decreased (Stormer et al, 1983, Moroi et al, 1985).

Interaction with other chemicals. In vitro and in vivo studies showed that phenylbutazone, ethyl biscounmacetate and sulphamethoxypyridazine each competitively inhibited the binding of OA to porcine serum albumin. Phenylbutazone and ethyl biscoumacetate increased the toxicity of OA as expressed in terms of LD50 and histological examination. In contrast, sulphamethoxypyridazine decreased the toxicity of OA (Galtier et al, 1980). When OA and OB were given in combination, the

animals were not clinically affected and histologically there was only slight damage of the renal proximal tubules. These observations indicate that OB considerably reduces the toxic effects of OA (Stormer et al, 1985). Ochratoxin C, which is less toxic than OA, can be converted into OA in the body of the animals (Fuchs et al, 1984).

2.4.4. Excretion of ochratoxin A.

Elimination of OA involves both biotransformation and excretion. Elimination of OA can be expressed in terms of either the elimination rate constant (Ke) or the elimination half-life ($T_{1/2}$). The elimination half-life of OA is 77.3 h in young cattle (Sreemannarayana et al, 1988), 84.5 h in pigs, 10.8 h in rabbits, 3.0 h in chickens (Galtier et al, 1981), and 55.88 h in pregnant mice (Ballinger et al, 1986). The elimination rate constant of OA in the rat when administered orally is 0.028/h for oral dose and 0.039/h when administered by an intravenous injection (Galtier et al, 1979). Studies on young cattle injected intravenously with OA demonstrated that about 7% of the OA was excreted unchanged in urine and 11.1% in feces indicating that ochratoxin A is excreted into the bile (Sreemannarayana et al, 1988).

Biliary recycling is very significant in terms of the bioavailability of OA. The secondary peak in the pharmacokinetic profile in ruminant calves given an oral dose

of OA suggests that the toxin is excreted into the intestinal tract via the bile and is reabsorbed from the intestine with reabsorption acting as a second small dose of OA (Sreemannarayana et al, 1988). Cholestyramine, which blocks enterohepatic cycling, eliminates the secondary peak of OA, thereby changing the elimination profile of OA. These findings further support the proposal that biliary recycling of OA occurs (Roth et al, 1988). Secretion and reabsorption of OA by the renal proximal tubule has been investigated in rats. Blocking the renal secretion decreased the renal clearance of OA and increased the nephrotoxicity of the toxin (Stein et al, 1985). The results also indicated that secretion of OA via organic anion transport is an important mechanism by which the kidney handles the toxin, and renal tubular secretion and reabsorption may facilitate the residual persistency of OA in kidney. The mechanism of OA transport in the kidney has been confirmed to be mediated via the renal organic anion transport system (Sokol et al, 1988).

OA is strongly bound to serum albumin (Chu, 1971, Krogh, 1983). Binding of OA seems to retard its elimination and thereby prolong its half-life. Plasma concentration of OA drops to 0.5 ug/ml 10 min after injection (2.2 mg/kg) in the albumin-deficient rat, while it remains at 50.0 ug/ml after 90 min in the normal rat (Kumagai, 1985). The concentration of OA in plasma is higher than that in the bile and urine in normal rats, while the opposite result is obtained in the

albumin-deficient rat (Kumagai and Aibara, 1982, Kumagai, 1985). The bioavailability of OA in the calf fed with milk immediately after dosing is 3-7 times higher than that in non-fed calf, suggesting that binding of OA to milk protein will increase the toxicity of the toxin (Sreemannarayana et al, 1988).

2.5. Biochemical Effects of Ochratoxin A.

2.5.1. Effect of Ochratoxin A on Carbohydrate Metabolism.

Excessive accumulation of glycogen in the liver is a notable effect of ochratoxicosis in rats (Purchase and Theron, 1968, Suzuki and Satoh, 1973, Munro and Theron, 1973), and in the broiler chicken (Warren and Hamilton, 1981). Hyperglycogenation associated with ochratoxicosis in broiler chickens has been shown to be type X glycogen storage disease (Warren and Hamilton, 1981). The accumulation of glycogen in liver of the broiler chicken is due to a decrease in the mobilization of glycogen during ochratoxicosis (Huff et al, 1979). Further research showed that phosphorylase α and phosphorylase kinase in the glycogen synthesis and degradation system were not inhibited by OA, but that protein kinase was inhibited in a non-competitive manner by OA in the presence or absence of cAMP (Warren and Hamilton, 1980a). The results indicate that OA inhibits glycogeneolysis by inhibiting the

cAMP-dependent protein kinase in a non-competitive fashion, which agrees with the observation that the glycogen accumulation during ochratoxicosis is not mobilized by glucagon. Studies on bacteria have shown that the binding of cAMP to cAMP-binding protein is inhibited by ochratoxin A (Heller and Roschenthaler, 1978). Decreases in the amount of glycogen in kidney of rats was reported to be the inhibitory effect of OA on renal gluconeogensis of rats (Meisner and Selanik, 1979). The report indicates that gluconeogensis from pyruvate, lactate, malate, and glutamine is significantly decreased and renal phosphor-enol-pyruvate carboxykinase activity is decreased by about 55% in rats fed 2.0 mg/day of OA. The decrease in activity of phosphor-enol-pyruvate carboxykinase may be due to the inhibitory effect of OA which greatly reduces the level of translatable mRNA for phosphor-enol-pyruvate carboxykinase (Meisner et al, 1983).

In the respiration system, the effect of OA on metabolite anion uptake by mitochondria reveal a competitive type of inhibition with respect to the binding of dicarboxylic acids (succinate and malonate), adenine nucleotides (ADP and ATP), and inorganic phosphate. OA, therefore, acts as a competitive inhibitor of mitochondrial transport carrier protein (Meisner and Chan, 1974). OA exerts its effect on mitochondrial respiration and oxidative phosphorylation through impairment of the mitochondrial membrane and inhibition of the succinate-supported electron transfer activities of the respiratory

chain in rat liver mitochondria. Inhibition kinetic studies revealed that OA is an uncompetitive inhibitor of both succinate-cytochrome c reductase and succinate dehydrogenase (Wei et al, 1985).

2.5.2. Action of Ochratoxin A on DNA, RNA, and Protein Synthesis.

Inhibition of protein synthesis by OA has been reported in mice (Creppy et al, 1984), and bacteria (Konrad and Roschenthaler, 1977, Bunge et al, 1978, Heller and Roschenthaler, 1978). The degree of inhibition of protein synthesis is greatest in the spleen and kidney, and least in liver of mice dosed with 15 mg OA/kg intraperitonally. The inhibitory effect of OA is prevented by injection of phenylalanine (Creppy et al, 1984). Studies on the action of OA on protein synthesis demonstrated that OA inhibited the binding of cAMP to cAMP-binding protein and the activity of cAMP-dependent protein in a non-competitive fashion (Heller and Roschenthaler, 1978). Inhibition of phenylalanine-tRNA synthetase has been reported to act in a competitive manner (Creppy et al, 1979a, Konrad and Roschenthaler, 1977). OA has the highest affinity for its aminoacyl-tRNA sythetase and acts as a competitive inhibitor of amino acids (Creppy et al, 1983). This inhibitory effect is structurally unique as OB, the dechloro form of OA, has no effect on cellular protein synthesis and on the formation of phenylalanyl-tRNA (Roth et

al, 1989). There is a report indicating that OA has no effect on the translation system, but OA significantly reduces the amount of translatable mRNA (Meisner et al, 1983). Transcription of RNA is not affected by OA, but OA changes the abundance of mRNA at the post transcription level (Meisner and Polsinnelli, 1986). Synthesis of protein, RNA, and DNA is inhibited by OA in kidney cells (Creppy et al, 1986).

OA has been shown to induce liver and kidney tumours in experimental animals (Kanisawa and Suzuki, 1978, Creppy et al 1985a, b). They reported that OA induced in vitro as well as in vivo DNA damage in the liver, spleen and kidney with the degree of damage being dose-dependent. Evidence for DNA single-strand breaks in liver and kidney caused by OA was reported by Kane et al (1986a). Induction of DNA single-strand breaks and inhibition of DNA synthesis by OA was obtained in Chinese hamster ovary cells and transformed fibroblasts (Stetina and Votava, 1985). The results as discussed subsequently indicate that OA is a mutagen and a carcinogen.

2.5.3. Effect of Ochratoxin A on Activity of Some Enzymes.

The first report of an inhibitory effect of OA on an enzyme noted that OA was a competitive inhibitor of bovine carboxypeptidase A which also hydrolyzed OA to ochratoxin α (Pitout and Nel, 1969). Studies with OA-induced porcine nephropathy showed that the reduction of protein synthesis

and enzymatic activity occurred mostly in samples from kidney tissue with only limited changes occurring in liver samples (Elling et al, 1985). In the case of ochratoxicosis, decreases in cytochrome P-450 level, aminopyrine demethylase and aniline hydroxylase activities were observed in a drug biotransformation system of rats (Galtier et al, 1984, Gilberte et al, 1985). The effect of OA on the activity of five enzymes in the urine and renal tubules was studied (Kane et al, 1986b), and it was found that the activities of gamma-glutamyl transferase, alkaline phosphorytase, leucine aminopeptidase, lactate dehydrogenase, and N-acetyl-B-D-glucosaminidase in the tubules were inhibited by OA. The inhibitory effect of OA on other enzymes such as those involved in carbohydrate metabolism and synthesis of protein, RNA, and DNA, was previously discussed.

2.5.4. Effect of Ochratoxin A on Lipid Peroxidation.

Rahimtula et al, (1988) proposed that the mechanism of OA toxicity involved an increase in the degree of lipid peroxidation. It was found that OA greatly enhanced the rate of NADPH or ascorbate-dependent lipid peroxidation as measured by malondialdehyde formation. NADPH-dependent lipid peroxidation in kidney microsome was similarly enhanced by OA. The enhancement of lipid peroxidation by an ochratoxin analog was related to the presence and reactivity of the phenolic group. Administration of OA to rats also resulted in

an enhanced rate of lipid peroxidation in vivo as evidenced by a seven fold increase in the rate of ethane exhalation. The results also indicated that the P-450 cytochrome system was not involved in the enzymatic and chemically induced lipid peroxidation by OA. In the ochratoxin family, only ochratoxin A, C and B but not ochratoxin and hydroxylated-ochratoxin A induced lipid peroxidation, the degree of lipid peroxidation being correlated with their toxicity to chicks.

The peroxidation of polyunsaturated fatty acid present in membrane lipids has been proposed as a mechanism by which a number of foreign compounds produce structural injury to tissues. Damage of mitochondrial membrane by OA has been noted in rat liver mitochondria (Wei et al, 1985). OA also impaired the endoplasmic reticulum membrane probably via enhanced lipid peroxidation and consequently disturbed microsomal calcium homeostasis (Khan et al, 1989). The inhibition of endoplasmic reticulum calcium uptake is a common pathway in toxic cell death induced by various agents. Enhanced lipid peroxidation was proposed as the basis for the cytotoxicity of OA.

2.6. Summary and Conclusion.

Ochratoxin A (OA), a secondary metabolite of fungus, is commonly found as a natural contaminant of food and feed stuffs. The toxin exerts its effects by inhibiting the activity of enzymes, limiting the availability of proteins and energy of cells, and finally causing death of the exposed animal. OA is a nephrotoxin. The toxin causes human nephropathy and kidney damage in animals. OA alters the functions of liver and immune organs with necrosis and severe immunosuppression. OA is a carcinogen, which induces liver and kidney carcinoma in mice. Enhancement of the rate of lipid peroxidation of unsaturated fatty acid by OA may account for its cytotoxicity and the impairment of membrane of the cells. OA, as an antibiotic, inhibits the growth of gram-positive bacteria like B.subtilis

3. STUDY ONE: EFFECT OF A HAY AND A GRAIN DIET ON THE RATE OF HYDROLYSIS OF OCHRATOXIN A IN THE RUMEN OF SHEEP.

Abstract

The degree of hydrolysis of Ochratoxin A (OA) and the corresponding formation of its hydrolyzed product, ochratoxin α (Oα), by the rumen digesta and in the rumen of hay-fed and grain-fed sheep were compared. The rumen contents from sheep fed hay or grain were able to hydrolyze OA in vitro with the majority of the activity against OA being associated with the particulate fraction of the rumen fluid as the rate of hydrolysis of OA was much greater in the precipitate fraction than in the supernatant fraction ($P<0.009$). Also in contrast to the precipitate, Oα was not detected in the supernatant fraction of the fluid. The rate of hydrolysis of OA by rumen fluid adjusted to different pH's, was not greatly influenced ($P>0.6$) by the pH of the samples (pH range was from 5.5 to 7.0). In contrast, rumen fluid obtained from hay fed animals (pH 7.0) was able to hydrolyze OA in vitro at a much greater rate (5 fold) than rumen fluid obtained from grain fed animals (pH 5.5), ($P<0.01$). In vivo, the disappearance of OA from the rumen was compared in hay-fed and grain-fed sheep which were given OA orally at a rate of 0.5 mg/kg body weight. The half-lives ($T_{\frac{1}{2}}$) for disappearance of OA from the rumen of sheep fed grain (normal feed intake, rumen pH 5.7), fed grain at a

reduced intake level (30% feed intake, pH 6.5), and fed hay (pH 7.1) were 3.6, 1.3 and 0.6 h, respectively. The results suggest that OA is hydrolyzed much faster in the rumen of sheep fed hay as compared to sheep fed grain, and that the decrease in rumen pH due to rapid fermentation of starch from the grain may affect the species or population of rumen microflora, which in turn influences the rate of hydrolysis of OA.

3.1. Introduction.

Ochratoxin A (OA) is a secondary metabolite of the fungal genera Aspergillus and Penicillium (Chu, 1974). It has been found as a natural contaminant in many food and feedstuffs (Shotwell et al, 1969, Krogh, 1987). Exposure to OA causes a variety of pathological responses in different animal species, with nephropathy commonly occurring in swine (Krogh, 1973). OA is hydrolyzed by enzymes such as carboxypeptidase A and microbes to ochratoxin α (Oα) which is believed to be non-toxic to animals (Chu, 1972). Rumen microflora are able to hydrolyze OA (Hult et al, 1976). It has been suggested that rumen protozoa are responsible for OA hydrolysis in the rumen (Kiessling et al, 1984). It has been well established that the protozoa numbers and the pH of rumen digesta decrease dramatically when starch is rapidly fermented, which occurs more extensively in sheep fed grain than in those fed hay (Eadie and Mann, 1970, Jouany et al, 1988). It may therefore be hypothesized that rate at which OA is hydrolyzed to Oα and phenylalanine in the rumen of sheep is influenced by the type of diet and that the rate would be faster for hay-fed as compared to grain-fed sheep. The objectives of this study were to determine the rate of hydrolysis of OA and corresponding formation of Oα by rumen digesta from sheep fed diets containing a high concentration of grain or hay. Both in vitro and in vivo studies were to be used.

3.2. Methods and Materials.

Ochratoxin A. OA that was used for most of the experiments was produced by surface liquid fermentation with Aspergillus ochraceus NRRL 3174 grown on PDA-NaCl slants (Yamazaki et al, 1970, Davis et al, 1969, 1972). The fermentation was carried out for 10 to 12 days at 28°C. After removal of mycelia mats, the liquid medium was concentrated in a cyclone steam evaporator at reduced pressure and dried by freeze-drying. The final concentration of the toxin in different batches of the OA freeze-dried medium (OA-FDM) varied between 500 and 2,500 mg/kg. Several batches of OA-FDM were pooled into a uniform preparation containing 1,300 mg OA/kg, as determined according to the methods of Josefsson and Moller (1979) and AOAC (1980). Multitoxin analysis (Wilson et al, 1976, Josefsson and Moller, 1977) confirmed the presence of OA alone.

Pure crystalline OA was used as reference standard and was purchased from Sigma Chemical Co., St. Louis, MO. Oα used as a reference standard was prepared by acid hydrolysis of OA (Van der Merwe et al, 1965).

Preparation of OA in buffers. Phosphate buffers (0.1M) were prepared as described by Colowick and Kaplan (1955). Crystalline OA (2 mg) was dissolved in 20 ml of chloroform and quantitatively transferred to ten 10ml-vials. Each vial

containing 200 ug OA was dried with nitrogen. The OA when used for the incubation studies was first dissolved in 0.5 ml of absolute alcohol with the aid of an ultrasonic cleaner (Mettler Electronics, Anaheim, CA, USA), and then diluted with phosphate buffers (pH 5.5 or pH 7.0) to a concentration of 25 or 50 ug OA/ml depending on the experiment. The OA which was introduced into the rumen (0.5 mg/kg body weight) for the *in vivo* test (Experiment Three), was dissolved in buffers (pH 5.5 or pH 7) to a concentration of 1 mg/ml.

Animals and dietary treatment for *in vitro* study. Four male sheep which were approximately six month of age and weighed from 50 to 70 kg were obtained from the University farm. A rumen canula was surgically implanted into the rumen (the first stomach) of each sheep to facilitate the introduction of the toxin directly into the rumen and the collection of rumen fluid (Komarek, 1981). The canula was made of soft plastic (F.H. & SONS MANUFACTURING LTD, CONCORD, ONTARIO) and had a diameter of 20 to 30 mm depending on the age of the sheep (Komarek, 1981). During recovery from surgery, the animals were enclosed in separate pens and fed hay for at least 10 days before being placed on the dietary treatments.

The hay diet from the University farm was mainly alfalfa (1-00-063). The grain diet which was prepared in the University feed mill contained 45% barley (4-00-549), 45% wheat (1-05-268) and a 10% supplement of minerals and vitamin to meet

the NRC requirement (NRC, 1985). Two of the four sheep were placed in a separate pen and gradually adapted to the grain diet by increasing the amount of grain in the diet by 0.2 kg grain/sheep/two days for 10 days until it totally replaced the hay. The other two sheep continued to receive hay. All sheep were fed once a day from 9:30 am to 4:00 pm at libitum. The feed was removed at 4:00 pm.

Preparation of rumen samples. Rumen fluid was collected via a rumen cannula into a 25 ml pipette which was connected to a 50 ml plastic syringe. The rumen fluid was transferred into an insulated container (thermos) and stored anaerobically at a temperature 37-39°C. The rumen fluid was filtered through cheese cloth before use.

Most of the experiments used fresh intact rumen fluid. When fractions of supernatant and particulate of rumen fluid were needed, rumen fluid was centrifuged at 150 xg for 10 min. The particulate fraction was resuspended with an equal volume of the phosphate buffers having the same pH as the intact rumen fluid. The pH of the rumen samples and buffers was measured directly by use of a PHM82 standard pH meter (Copenhagen, Bach-Simpson Limited, London, Ont, Canada).

Microflora in the rumen sample were fixed with methylgreen-formalin-saline (MFS) solution for nuclear staining and for identification of ciliates (Ogimoto and Imai, 1981). Protozoa

in the fixed sample was identified under the light microscope (Fisher Scientific) at a magnification of 400 using the procedure of Ogimoto and Imai (1981).

Incubation of rumen samples. Fresh rumen fluid (10 ml) was mixed with 0.5 ml of a solution of OA (25 to 50 g/ml). Anaerobic conditions were maintained by bubbling through the incubation medium a gas mixture (5:95) of CO_2 and N_2. The incubation was stopped by adding 1.5 ml of 1 M phosphoric acid. The control incubations were made by adding the same amount of OA to the same volume of rumen fluid in which the microorganisms were killed by addition of 1.5 ml of 1 M phosphoric acid before incubation. The percent of OA degraded during incubation was based on the recovery of OA from the control since the recovery of OA from spiked samples would not be quantitive due to irreversible binding of some of the OA to the particular matter in the sample.

Analytical methods. The acid-inactivated samples were extracted for 20 min with 20 ml of chloroform with constant shaking. The samples were centrifuged at 1200 xg for 10 min at 2°C in 50 ml polyethylene tubes to separate the chloroform phase, containing OA and its metabolite (Oα) extracted from the aqueous phase. An aliquot of the chloroform fraction (10ml) was transferred to a 15 ml centrifuge tube containing 2 ml of distilled water and the mixture was shaken by hand to assure thorough mixing, followed by centrifugation at 1200 xg

for 10 min at 2°C. Finally, 5 ml of the chloroform fraction was transferred to a 10 ml vial and dried under a stream of nitrogen for TLC and HPLC analysis.

The OA and Oα in the dried samples were reconstituted with 2 ml of chloroform and mixed by use of a ultrasonic cleaner. OA was applied to RPTLC plates using the procedure of Frohlich et al (1988). In brief the procedure involves triple applications of 10 or 20 ul of the reconstituted chloroform solution to a RPTLC plate type KC 18F (Whatman, Inc., Clifton, NJ.) along with separate applications of standard OA and Oα. The RPTLC plate was developed first in hexane for 20 min, allowed to dry, and then in a solvent of methanol-water (70/30) for 20 min at 20C. OA and Oα were located on the plates by use of UVGL-25 ultraviolet lamp (UVP, Inc, San Cabriel, CA.). The spots containing OA and Oα were removed using the procedure described by Frohlich et al (1988). The concentration of OA and Oα were determined by using the HPLC procedures of Josefsson and Moller (1979).

Experiment One. The objective of Trial 1 of the experiment was to establish the pattern of pH change over different time periods in the rumen of sheep that were being adapted to the grain diet, and to compare the pattern with that obtained from sheep fed hay. Hay-fed sheep continued to receive hay while grain-fed sheep were fed the grain diet gradually for

adaptation. Rumen samples were collected 5 h after feeding from each of two sheep per treatment on days 3, 6, 9, 15, and 30 of the experiment.

A second trial established the pattern of pH change in the rumen over a 10 h period in adapted sheep which had been fed the two diets for 45 days. Rumen samples were collected from two sheep in each treatment group immediately (0) before feeding and at 2, 4, 6, 8, and 10 h after feeding on two sussesive days. The sample was prepared for pH measurement as described above.

Experiment Two. The objective of this experiment was to determine some of the factors that influence the rate of hydrolysis of OA *in vitro*.

Trial 1 was carried out to determine whether the particulate or soluble fraction of rumen digesta was predominately responsible for the hydrolysis of OA. Rumen fluid that was collected 3 h after feeding from both grain and hay-fed sheep (two for each diet) was centrifuged. The two fractions (particulate and supernatant) were incubated separately with OA using the procedure previously described. OA (12.5 ug) was incubated with 10 ml of supernatant or resuspended precipitate for 3 h at 38-39°C. The reaction was stopped and the sample was assayed for OA and Oα as previously described.

The objective of Trial 2 was to determine if the rate of hydrolysis of OA was influenced by the site at which the rumen sample was collected. Fluid was collected 5 h after feeding from the upper, middle, and bottom portion of the rumen from each of two sheep in each dietary treatment. The samples were prepared as described above and were incubated at 38-39°C with OA (25 ug/10 ml rumen fluid) for 3 h before being assayed according to procedures outlined above.

The objective of Trial 3 was to determine whether any difference in rate of hydrolysis of OA between hay-fed and grain-fed sheep was attributable to a difference in the pH of the rumen fluid. Rumen fluid was collected from two sheep on each dietary treatment 6 h after feeding. The samples were centrifuged at 150 xg for 10 min and the particulate fraction was diluted with 0.1 M pH 5.5 and pH 7.0 buffers to the original volume of the intact rumen fluid. Intact rumen fluid and the reconstituted particulate were each incubated with OA (25 ug/10ml sample) for 3 h at 38-39°C, followed by analysis as previously described.

The objective of Trial 4 was to determine whether the rate of hydrolysis of OA was affected by collection time after feeding, which was reported to influence the population of the microflora (Michalowski and Muszyynski, 1978). Rumen fluid was collected from two sheep on each dietary treatment before (0 h) and after 3, 6 h feeding. Incubation of rumen fluid with

OA and their analysis were carried out as previously described.

Experiment Three. The objective of this study was to follow the rate of hydrolysis of OA in the rumen of sheep.Four female sheep weighing from 50 to 80 kg that had been fitted with a rumen cannula were given a single oral dose of 0.5 mg OA/kg body weight. The toxin was introduced directly into the rumen via the rumen cannula 1 h after feeding. Two of four sheep received grain and the other two received hay daily at 9:00 am to 4:00 pm ad libitum. The experiment was repeated after two months with the same sheep under the same conditions. Feed consumption in one of the grain-fed sheep decreased to 30% of normal and as a result the pH of the rumen did not decrease. The experiment therefore consisted of three treatments: treatment one, two hay-fed sheep, repeated twice; treatment two, one grain-fed sheep (30% of normal intake), repeated twice; treatment three, one grain-fed sheep (normal intake), repeated twice. Rumen fluid was collected at times 2, 4, 6 and 10 h after dosing. Samples were analyzed for pH, OA and Oα as described previously.

In all experiments, each sample was assayed in duplicate.

Calculation of rate of hydrolysis of OA and Oα/OA ratio.

Rate of hydrolysis of OA=((OA μg in control)-(OA μg in sample))/((sample volume (l) x period of incubation (h)).

The ratio of Oα to OA upon completion of incubation was determined by dividing the molar concentration of Oα by the molar concentration of OA:

Oα/OA=(Molar concentration of Oα)/(Molar concentration of OA).

Experimental design and statistic analysis. The experiments included two dietary treatments with two sheep in each treatment. Statistical analysis of the difference between dietary treatments was performed using GLM in SAS system (SAS INSTITUTE INC. SAS CIRCLE PO BOX 8000 CARY, N.C. 27511-8000). For Experiment Three, the rate constant which was used to calculate the half-life of OA disappearance from the rumen was determined by a non-linear regression technique as programmed in NONLIN (Ralston and Jennrich, 1978).

3.3. Results of the Experiments.

Experiment One. Results (Trial 1) presented in Figure 1 demonstrate that the pH in the rumen of the hay fed sheep ranged from 6.5 to 7.2 with the mean value being 6.9. The pH in the rumen of sheep being adapted to the grain diet decreased dramatically over a 15 day period to pH 5.2, after which it did not change much over the next 15 day-period.

In the second trial (Figure 2), sheep that had been adapted to the diets for 45 days were fed only once daily for a 7 h period. In hay-fed sheep, the rumen pH decreased to a near minimal value of pH 6.9 within 2 h of feeding whereas the pH decreased over a 6 h period in grain-fed sheep to a low level of 5.5, after which the values remained stable up to 10 h after feeding, then the pH returned to the beginning value next day before feeding. The magnitude of pH fluctuation was much greater in grain-fed (0.99 pH units) than in hay-fed sheep (0.33 pH units). The difference in pH between hay- and grain-fed sheep, 6 h after feeding, was 1.45 pH units ($p<0.004$). Athough the difference in population of protozoa was not established in this study, an increased growth of small size of ciliate was observed in grain-fed as compared to hay-fed sheep.

Figure 2. Change in pH in the rumen of sheep during adaptation to a grain diet and in sheep that had only received a hay containing diet, (Experiment One, Trial 1). Values represent mean SE of two sheep for each diet. The grain fed sheep were changed from a hay diet to a grain diet at day zero. The coefficients of regression are -0.094 ($P<0.003$), for sheep fed grain and -0.021 ($P>0.12$), for sheep fed hay for the 3 to 15 day time period.

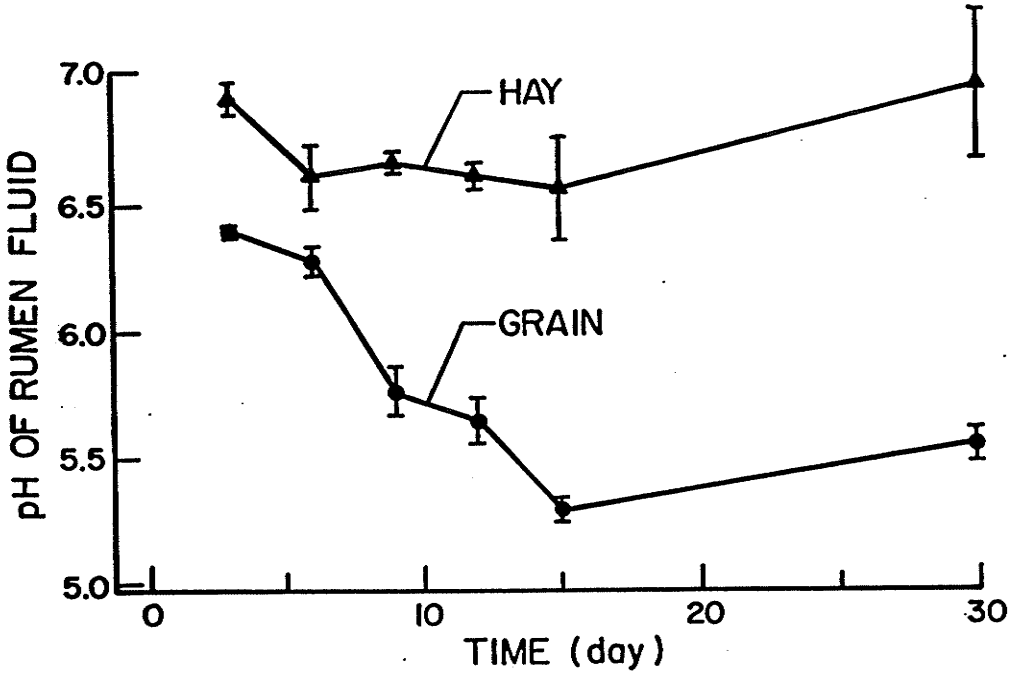
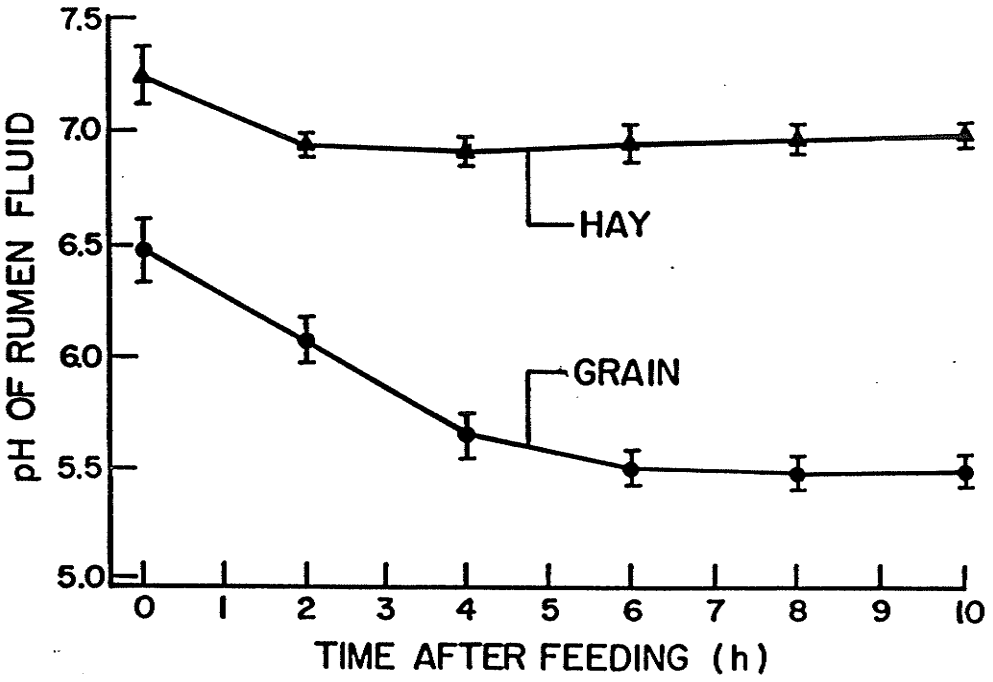

7.0
6.5
6.0
5.5
5.0
pH OF RUMEN FLUID
HAY
GRAIN
0
10
20
30
TIME (day)

Figure 3. The change in pH over a 10 h period in the rumen of sheep fed hay or grain once a day. (Experiment One, Trial 2). Values represent mean SE of two sheep fed each diet on two sussesive days. The coefficients of regression are 0.097 ($P<0.02$) for sheep fed grain and 0.01 ($P>0.32$) for sheep fed hay.


7.5
7.0
6.5
6.0
5.5
5.0
pH OF RUMEN FLUID
HAY
GRAIN
0
1
2
3
4
5
6
7
8
9
10
TIME AFTER FEEDING (h)

Experiment Two. Results of Trial 1 (Table 1) indicated that the rate of OA hydrolysis was relatively low in all fractions except for the particulate fraction obtained from hay-fed sheep, which was 4 times greater than the particulate fraction obtained from grain-fed sheep ($P<0.009$). The total amount of Oα formed as estimated from the Oα/OA ratio, followed a similar pattern except the results would indicate that the supernatant fraction from both treatment group did not produce any Oα whereas the particulate fractions from both treatment groups were able to form Oα, further suggesting that the hydrolysis of OA occurred in the particulate fraction of rumen fluid. The rate of formation of Oα in this fraction from the grain-fed sheep, however, was only 13% of that obtained with the fraction from hay-fed sheep ($P<0.001$).

Results from Trial 2 (Table 2), demonstrated that the site in the rumen from which rumen fluid was obtained did not affect ($P>0.53$) the rate of hydrolysis of OA whereas diet had a pronounced effect ($P<0.01$).

Results from Trial 3 (Table 3) demonstrate that the pH of the incubation medium did not affect ($P>0.67$) the rate of hydrolysis of OA by microflora from either treatment group. There was, however, a significant difference ($P<0.01$) in rate of hydrolysis of OA by hay-fed compared to grain-fed sheep regardless of the buffer type or pH of the incubation mixture.

Table 1. Effect of different fractions of rumen fluid on the degradation of ochratoxin A (Experiment Two, Trial 1).

	Grain		Hay	
	Rate of OA hydrolysis (µg/h/l)	Ratio (Oα/OA)	Rate of OA hydrolysis (µg/h/l)	Ratio (Oα/OA)
Supernatant	52.8	0	17.4	0
Particulate	49.0	0.12	200.7	0.92
SE	24	0.01	8	0.00

SE: Standard error of difference in means.

Table 2. In vitro hydrolysis of OA by rumen fluid from different sections of the rumen. (Experiment Two, Trial 2).

	Grain	Hay
Section of the rumen	Rate of OA hydrolysis (µg/h/l)	Rate of OA hydrolysis (µg/h/l)
Upper	10	358
Middle	91	321
Lower	50	328
SE	18	16

SE: Standard error of difference in means.

Table 3. Hydrolysis of OA by the content of the rumen when incubated at different pH values. (Experiment Two, Trial 3).

	Hay	Grain	SE
Intact rumen fluid			
pH	6.7	5.3	0.1
Rate of OA hydrolysis			
(μg/h/l)	345	73	19
Ratio of Oα to OA	0.24	0.04	0.02
Particulate of rumen fluid			
In pH 7.0 buffer			
pH	7.0	6.8	0.1
Rate of OA hydrolysis			
(μg/h/l)	291	165	20
Ratio of Oα to OA	0.22	0.10	0.01
In pH 5.5 buffer			
pH	5.6	5.5	0.02
Rate of OA hydrolysis			
(μg/h/l)	334	132	13
Ratio of Oα to OA	0.25	0.04	0.01

SE: Standard error of difference in means.

The results of Trial 4 (Table 4) demonstrate that both diet ($P<0.04$, $P<0.01$) and collection time ($P<0.03$, $P<0.01$) affected the rate of hydrolysis of OA and the Oα/OA ratio, respectively, and that in both cases there was an interaction that was significant ($p<0.01$) or approached significance ($p<0.09$). The interaction may be attributed to a greater relative decrease in rate of hydrolysis of OA between 0 time (approximately 22 h after feeding) and the 3 and 6 h values for the grain fed animals as compared to the hay fed animal. As observed in the previous studies, both the rate of OA hydrolysis and the ratio of Oα/OA was much greater in hay-fed as compared to the grain-fed sheep.

Table 4. Hydrolysis of OA by the rumen fluid collected at various time after feeding, (Experiment Two, Trial 4).

	Time after feeding (h)					
	0		3		6	
	pH	Rate (μg/h/l)	Ratio Oα/OA	pH	Rate (μg/h/l)	Ratio Oα/OA	pH	Rate (μg/h/l)	Ratio Oα/OA
Hay	7.1	316	0.32	7.0	248	0.25	6.6	346	0.24
Grain	6.3	204	0.12	5.6	117	0.09	5.3	73	0.07
SE	0.1	13	0.02	0.2	8	0.02	0.1	19	0.01

SE: Standard error of difference in means.

Experiment Three. A single dose of OA (0.5 mg/kg body weight) was administered directly into the rumen of four sheep (two fed hay and two fed grain). The feed intake for one sheep on grain in both the first and second trial was 30% of normal and as a result the pH of the rumen fluid did not decrease to the same degree as for the sheep that consumed a normal amount of feed (Table 5). The average feed consumption for sheep in treatment group hay, grain-30% and grain-100 were approximately 2.0 kg of hay, and 0.5 and 1.5 kg of grain/day/sheep, respectively. The concentration of OA in the rumen of sheep fed hay fell rapidly with the total disappearance of OA from the rumen occurring within almost 6 h of dosing. On the other hand, the disappearance of OA in the rumen of sheep fed grain was considerably more gradual, still being detectable in the rumen 10 h after dosing (Figure 4). As shown in Table 5, the corresponding half-lives ($T_{\frac{1}{2}}$) for disappearance of OA from the rumen were 0.65, 1.30 and 3.38 h for hay-fed, grain-fed (30% of normal intake) and grain-fed (normal intake) sheep, respectively, indicating that the rate of OA hydrolysis in the rumen was greater in sheep fed hay as compared to those fed grain. The pH of rumen digesta appeared to be closely associated with the rate of hydrolysis of OA in the rumen. The results indicate that when the rumen pH decreases the rate of hydrolysis of OA also decreases. Apparently the low pH of the rumen which is caused by the rapid fermentation of the starch rich grain diet may have attributed to the decreased rate of hydrolysis of OA.

Figure 4. Concentrations of OA in the rumen of sheep after receiving a single oral dose of OA (0.5 mg/kg body weight). HAY: hay fed sheep (two sheep and two time periods); GRAIN-100%: grain fed sheep (normal feed intake for one sheep and two time periods); GRAIN-30%: grain fed sheep (feed intake 30% of normal for one sheep and two time periods). (Study One, Experiment three).

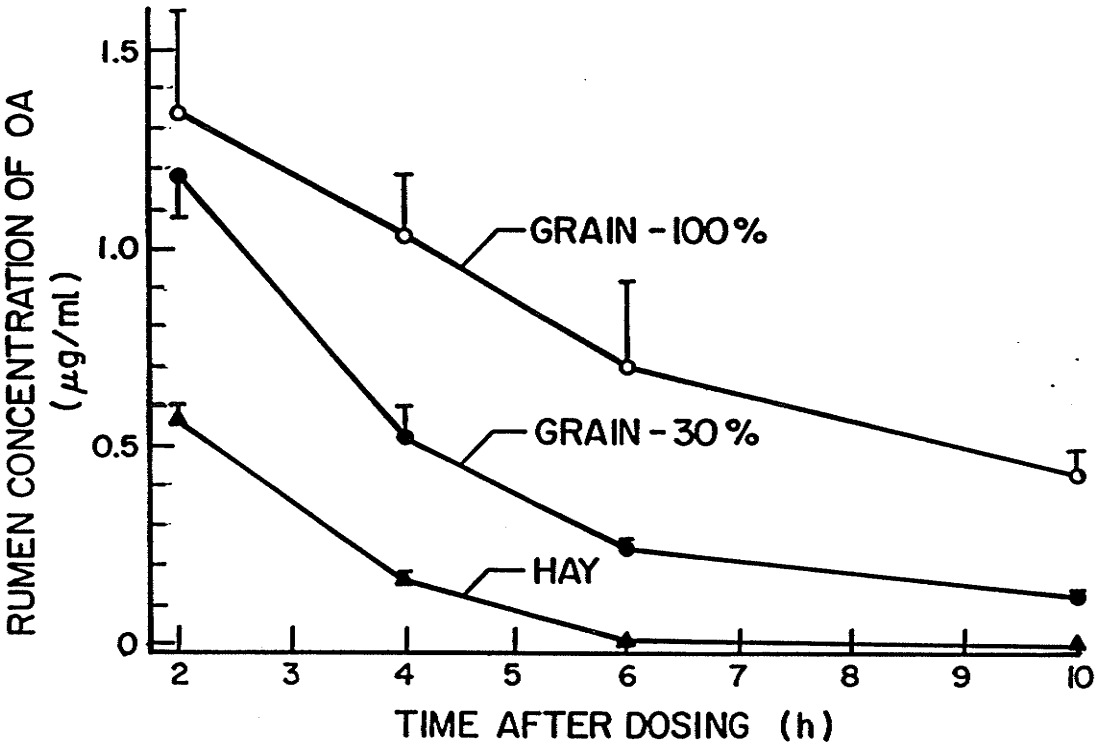

RUMEN CONCENTRATION OF OA
(μg/ml)
1.5
1.0
0.5
0
GRAIN - 100%
GRAIN - 30%
HAY
2
3
4
5
6
7
8
9
10
TIME AFTER DOSING (h)

Table 5. Time-course of the concentration of OA (μg/ml) in the rumen of sheep after receiving a single oral dose of OA (0.5 mg/kg body weight).

	Time after dosing (h)								
	2		4		6		10		
Treatment[a]	pH	OA	pH	OA	pH	OA	pH	OA	$T_{\frac{1}{2}}$[b] (h)
Hay	7.1	0.58	6.9	0.16	7.0	0.10	7.1	0.0	0.65
Grain-30%	6.2	1.18	6.5	0.52	6.9	0.24	6.9	0.12	1.33
Grain-100%	5.7	1.34	5.8	1.03	5.7	0.70	6.0	0.43	3.58
SE	0.33		0.16		0.09		0.10		0.60

a. Hay, sheep fed hay: grain-30%, sheep fed grain with 30% of normal feed intake: grain-100%, sheep fed grain with normal feed intake.

b. $T_{\frac{1}{2}}$: the half-life for disappearance of OA from the rumen.

SE: Standard error of difference in means.

3.4. Discussion.

Results from the in vitro studies demonstrated that the pH of the rumen of grain-fed sheep was lower than that of hay-fed sheep and that it was influenced to some extent by the time of feeding. Similar results have been reported by other researchers (Jouany et al, 1988). The rate of hydrolysis of OA to Oα was also influenced by diet as digesta from hay-fed sheep hydrolyzed OA in vitro at a rate 2 to 5 times greater than that from grain-fed sheep. In addition, both the rumen pH and the rate of hydrolysis of OA appeared to depend on the time after feeding, particularly for the grain fed sheep. The effect on rate of hydrolysis of OA may be attributed to differences in population of microflora and therefore amount of active enzyme but not to a direct effect of pH on the activity of the enzyme as the rate of hydrolysis of OA in vitro by digesta from the two groups of sheep was not influenced by pH of the incubation mixture. It has been reported that the population of the microflora decrease in the rumen after feeding (Michalowski and Muszynski, 1978). Also the OA hydrolyzing ability of the microflora appeared to be evenly distributed throughout the rumen and was primarily associated with the particulate fraction rather than the soluble fraction. This would indicate that intact microflora were required for hydrolysis of OA and that the soluble extracelluar enzymes were of less importance. The

microorganisms tend to be associated with the feedstuffs (Ogimoto and Imai, 1981) whereas the soluble enzymes would be located in the supernatant fraction.

The in vivo study demonstrates that the half-life ($T_{1/2}$) for disappearance of OA from the rumen was 2 (30% of normal feed) to 5 (normal feed) times greater in grain-fed as compared to the hay-fed sheep, indicating the faster hydrolysis of OA in sheep fed hay than in sheep fed grain. The results also indicated that rate of hydrolysis of OA in the rumen of sheep fed grain (30% normal) decreased since its half-life was 2 times greater than for sheep fed hay. It, nevertheless, was 2.6 times less than for sheep that consumed a greater quantity of grain. In general, the rate of hydrolysis of OA deceased with decreasing pH in the rumen. The results suggest that the pH of the rumen influenced the species and population of rumen microflora which in turn affected the rate of hydrolysis of OA.

The enzymes responsible for the hydrolysis of OA in the rumen were not identified in the present study but other researchers have shown that carboxypeptidase A and related enzymes are able to cleave OA in vitro (Pitout, 1969). Presumably several different proteolytic enzymes in the rumen are capable of cleaving the peptide bound of OA between the isocourmarin moiety and phenylalanine. Of considerable interest was the

fact that the rate of OA conversion to its non-toxic hydrolytic product (Oα), was much lower in grain as compared to hay-fed sheep. This presumably could be attributed, as indicated above, to a differences in the type of microflora that inhabits the rumen in the presence of different feedstuffs (Jouany et al, 1988). It is particularly well known that the population of protozoa dramatically decrease in grain fed ruminants especially at rumen pH values below 5.0 (Eadie et al, 1970). In the current study, complete defaunation may not have occurred as the pH values of the rumen decreased from 6.9 to 5.2-5.5 but never below 5.0. The species of microorganism responsible for the hydrolysis of OA were not identified in the current study. Kiessling et al (1984), however, in a study with chemically defaunated sheep concluded that the major activity against OA was protozoa and not bacteria. They did not examine rumen digesta for active protozoa or bacteria. Conceivably, the bacterial population may have also been affected. The results of the present study are consistent with their conclusion since the rate of hydrolysis of OA is lower in grain- as compared with the hay-fed sheep and since it is well known that protozoa are abundant in the rumen of hay-fed but not grain-fed animals.

The rate of OA hydrolysis may have also been affected by the peptide content of the rumen fluid as it is well known that peptides will compete with OA for carboxypeptidase A (Pitout

and Nel, 1969) and thereby influence subsequent rate of hydrolysis of OA. It is possible that the peptide concentration in the rumen of the two treatment groups was different and therefore the activity of the hydrolytic enzymes may have been affected.

In summary, the results of this study clearly demonstrate that the rate of hydrolysis of OA to Oα *in vitro* and *in vivo* is much less in grain-fed as compared to hay-fed sheep which is most likely due to a difference in rumen pH and/or associated microflora. Presumably if the pH of the rumen had decreased even further than obtained in the current study, which can be readily obtained by a more rapid fermentation rate, there would have been a greater change in the species of rumen microflora and therefore the rate of OA hydrolysis. These observations have practical significance in that OA is generally found in grain and since the consumption of grain reduces the rate of OA hydrolysis it may be expected that the consumption of an OA contaminated feedstuff will reduce the ability of ruminants to counteract its toxic effect. OA contaminated grain presumably would be most effectively detoxified if fed to ruminants that consume in addition considerable quantities of hay.

Further research is required to identify the species of microorganism that are responsible for the hydrolysis of OA

and to develop feeding schemes that will promote the development of a microbial population that will enhance rather than reduce the ability of the rumen to hydrolyze OA.

4. STUDY TWO: EFFECT OF A HAY AND A GRAIN DIET ON THE BIOAVAILABILITY OF OCHRATOXIN A IN SHEEP

Abstract

The role of the rumen in the detoxification of OA and the effect of the type of diet on the metabolism of OA in the rumen were studied. Four sheep (two fasted, two fed) were given an intravenous injection of OA (0.2 mg/kg body weight). Of the administered dose, 90-97% was excreted into the urine, only a small amount was excreted as Oα into urine and feces (0.5-2.5%), and no OA was found in the feces. A second experiment involved the oral administration of OA (0.5 mg OA/kg body weight) to four sheep (two fed hay, two fed grain). Nearly all of the OA (90-99%) was converted into Oα with 68-89% being excreted in urine and 15-20% in feces. Only 0.5-3% of the intact OA was found in the urine with the total amount being much higher in the urine of grain-fed as compared to hay-fed sheep. In a final study, eight sheep (four on hay, four on grain) were given a single oral dose of OA (0.5 mg/kg body weight). The disappearance of OA from the rumen was much faster for sheep fed hay than for sheep fed grain with the half-lives ($T_{\frac{1}{2}}$) being 0.63 h and 2.7 h respectively. The corresponding formation of Oα in the rumen was much greater for sheep fed hay compared to sheep fed grain with the formation half-lives ($T_{\frac{1}{2}}$) being 0.9 h and 1.9 h respectively. The maximum concentration of Oα in the rumen of sheep given OA orally was obtained at 1.4 h in sheep fed hay (C_{max}=220 μg/ml), whereas it was obtained at 7 h in sheep fed grain

(C_{max}=88 μg/ml). The relative bioavailability of OA for sheep fed hay was only 20% of that for sheep fed grain. The results demonstrate that the rumen of sheep plays a very important role in the detoxification of OA and that the type of diet influences the metabolism of OA in the rumen, which together affect the absolute bioavailability of OA and probably its toxicity to the animal.

4.1. Introduction

Degradation of ochratoxin A (OA) to its alpha metabolite, ochratoxin α (Oα), is well known to be the principle mechanism for the detoxification of OA (Chu et al, 1972). Pitout (1969) demonstrated that OA was hydrolyzed to Oα by enzymes such as carboxypeptidase A which was secreted from the pancreas of the animal. Hult et al (1976) and Kiessling et al (1984) reported that OA was degraded to Oα by rumen microbes particularly by rumen protozoa. Hydrolysis of OA in the small intestine may also contribute to the detoxification of OA as reported by Doster and Sinnhuber (1972). Little, however, is known of the importance of the rumen in the detoxification of OA until Sreemannarayana et al (1988) reported that the bioavailability of OA in ruminant calf was only 27% to 36% of that in the preruminant calf, indicating that a functional rumen may reduce the toxicity of OA to the calf. The faunal composition of microflora in the rumen of sheep is also known to be influenced by the composition of the diet (Nakamura and Kanegasaki, 1969, Eadie and Mann, 1970). Until recently, there has been no report about the influence of diet and microbes on the bioavailability of OA in sheep. Recent studies in our laboratory (study one) have, however, demonstrated that OA was hydrolyzed <u>in vitro</u> more rapidly by rumen digesta from sheep fed hay compared to those fed grain. The results suggested that the hydrolysis of OA by the microflora in the rumen

should reduce the total amount of OA that is absorbed into the blood and should therefore reduced its toxicity to the animal and that this effect should be greater in sheep fed hay as compared to those fed grain. In this study, the influence of diet on the hydrolysis of OA in the rumen of sheep and its subsequent bioavailability was evaluated. Experiments involving intravenous and oral (intra-rumen) administration of OA were conducted, in which the concentration of OA and Oα in the rumen, serum, urine and feces were monitored.

4.2. Methods and materials

Source of toxins and animals, and dietary treatments. OA was prepared as previously describe for Study One. Pure crystalline OA that was used as a reference standard was purchased from Sigma Chemical Co., St. Louis, MO. OX used as a reference standard was prepared by acid hydrolysis of OA (Van der Merwe et al, 1965). The source of hay and grain diet was the same as reported in Study One.

Experiment One. Four female sheep which weighted approximately 50 kg were injected intravenous with 0.2 mg OA/kg body weight. Two of the four sheep were fasted for 24 h before the injection, and fed 4 h after injection, the other two were injected 1 h after feeding. All sheep were kept in individual metabolic cages and were fed hay once a day ad libitum for a 7 h period.

Blood was collected via catheters that had been surgically implanted into the jugular vein one day before administration of the toxin. Two catheters were used in order to avoid contamination of OA, one for injection of OA and the other for collection of blood samples.

Urinary catheters were surgically inserted into the bladder one day before administration of the toxin for the collection of urine samples. Fecal bags were attached to all sheep for

the collection of feces (Frohlich et al, 1987).

Blood samples were collected at the following times after OA adminstration: 0, 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 24 h and then every 12 h up to 144 h. Urine and feces were collected every 4 h up to 12 h then every 12 h up to 144 h after adminstration of OA. Blood samples were allowed to clot, and following centrifugation, sera were separated and frozen at -20°C until analyzed for both OA and Oα. Urine and feces samples were also stored at -20°C prior to being analyzed.

Experiment Two. This experiment was in part described in Study One (Experiment Three). Two of the four sheep received the grain diet and the other two received hay. Blood samples were collected as described in Experiment One of this study except only a single catheter was implanted in the jugular vein. Rumen fluid was collected at time 0, hourly up to 4 h, and then at 6, 12, 24, 48, and 72 h after dosing. After the determination of pH, the samples were filtered through cheese cloth and stored at -20°C until extracted for OA and Oα determination.

Experiment Three. Eight female sheep weighing approximately 20 kg each were each fitted with a rumen canula (Komarek, 1981). Fecal bags and blood and urinary catheters were not fitted to the sheep. Compared to Experiment Two, theses

changes resulted in easier management of the sheep with greatly reduced stress on the animals. Four of the sheep received hay and the other four received grain daily ad libitum from 9:00 am to 4:00 pm after which the feed was removed. OA (0.5 mg/kg body weight) was administered as a single dose into the rumen via the cannula 1 h after feeding. Blood samples were collected from the jugular vein directly into a vacuum tube at 0, 6, 12, 24, 48 and 72 h after dosing. Serum was prepared from blood samples as described above. Rumen samples were collected into a 25 ml pipette connected to a 50 ml syringe at 0, 1, 2, 3, 4, 6, 12, 24, 48, and 72 h after dosing and were placed in ice. Immediately after the measurement of pH, the rumen samples were filtered through cheese cloth and stored at -20°C until assayed for both OA and Oα.

Chemical analysis. The same extraction (Hult et., 1979) and HPLC assay was used to measure the concentration of both OA and Oα in the same samples of serum, urine and feces. The extracts of urine, feces and rumen fluid required a final separation and clean-up of the OA and Oα by reverse-phase TLC prior to HPLC quantitation.

Prior to extraction, diluted serum samples were acidified with a $HCl/MgCl_2$ solution, urine samples with 85% H_3PO_4 to pH 2.5, and an aliquot of feces homogenate, prepared in $HCl/MgCl_2$,

with 8 N HCl to pH a value of 2.0. Both OA and Oα were quantitively extracted into chloroform. The chloroform extracts of serum, urine and rumen fluid were separated from the aqueous phase by centrifugation. The extracts of the fecal samples were separated from the aqueous slurry by filtration through filter paper. Each chloroform extract was washed once with water to remove dissolved acid and dried under a stream of nitrogen gas. The dried residue containing OA and Oα was stored at -20°C until further processed.

The residues obtained from the extracts of urine, feces and rumen fluid were reconstituted in chloroform and purified via reverse-phase TLC plates, type KC 18F (Whatman, Inc., Clifton, NJ). The plates were developed with hexane, dried and redeveloped with methanol: water (70:30), yielding Oα that was well separated from the OA. Each compound was recovered in methanol which, prior to HPLC analysis, was filtered through a 3-mm membrane nylon filter (Micron Separation, Inc., Honeoye Falls NY), having a pore size of 0.45 m.

Each serum, urine, feces and rumen sample was dissolved in HPLC-grade methanol and was injected onto a 250 mm x 4.6 mm i.d. column containing 5-μm diameter C-18 bonded phase adsorbent, Altex Ultrasphere ODS (Beckmen Canada, Ltd., Mississauga, Ontario) and a 50 mm x 4.6 mm pre-column packed with CO:PELL C-18 ODS groups chemically boned to 30- to 38- m

glass beads (Whatman, Inc., Clifton, NJ). The main column was maintained at 50°C. The mobile phase was methanol:water (70:30) acidified with 1 M phosphoric acid to pH 2.1. Its flow rate was adjusted to 1.5 ml/min except for the blood and urine samples. For the latter samples, to facilitate the separation of OA and Oα from other interfering compounds, a two-solvent gradient system was used. Solvent A contained water adjusted to pH 2.1 with 1 M phosphoric acid, while solvent B consisted of methanol and isopropanol in ratio of 90:10 (V/V). The percent of solvent B was initially changed linearly from 60 to 70 over 3 min, then remained isocratic for the next 3 min. This was followed by a two linear gradients in which solvent B changed from 75 to 85 over 2 min and from 85 to 60 over 1 min. During the last 2 min of the run and the 5 min equilibration period, the percent of solvent B was 60. Fluorometric detection and measurement were achieved by means of a fluorometer (Hewlett-Packard, Ltd., Canada) with excitation at 333 nm and emission at 418 nm. All samples and standards were analyzed in duplicate. The retention times for OA and Oα were 4.4 and 8.9 min respectively. The minimum detectable level of both OA and Oα was 0.05 ng/μl and recoveries were 94%.

Statistical and Pharmacokinetic Analysis. The concentration-time profile of OA in the serum was subjected to pharmacokinetic analysis. The data was fitted to a linear sum

of exponential terms by use of an iterative, nonlinear, least squares regression technique as programmed in NONLIN (Ralston and Jennrich, 1978). The rate constants and coefficients from the computer fitted function were used to calculate absorption and elimination (or disappearance) half-lives of OA and Oα and the rate of formation of Oα. The area under the curve (AUC), which was used to estimate the apparent relative bioavailability of both OA and Oα, was calculated according to the trapezoidal rule. The difference between dietary treatments was compared using the GLM procedure as programmed in SAS (SAS INSTITUTE INC. SAS CIRCLE PO BOX 8000 CARY, N.C 27511-8000).

4.3. The Experimental Results.

Experiment One. Four sheep (two fed and two fasted) were given an intravenous injection of OA (0.2 mg/kg body weight). They appeared normal except urine output appeared to increase after administration of OA, suggesting that the toxin may have affected the kidney.

The serum OA concentration-time profiles were almost identical in both fed and fasted sheep after intravenous administration of OA. The combined profiles are presented as a single curve in figure 5. Serum OA concentration fell rapidly with no toxin being detectable 120 h after injection. No Oα or other

metabolites of OA was found. The disappearance of OA from the serum of sheep followed a bi-exponential decline (Figure 5). There appeared to be no differences in the elimination of OA between the fed and fasted sheep. The combined corresponding half-life ($T_{1/2}$) values were:

$T_{1/2}$(initial disappearance)±SE=1.5±0.1 h

$T_{1/2}$(final elimination)±SE=17.3±1.4 h

The cumulative excretion data (Table 6) showed that most of the injected OA (90-97%) was excreted as unchanged OA into the urine of both fed and fasted sheep with 45 to 56% being excreted in first 12 h period after injection. About 5% of the injected OA was excreted as Oα in urine in two sheep (one fed and one fasting). No OA was detected in the feces but trace amounts of OA was excreted as Oα in the feces. The formation of Oα in the feces may be due to the proteolytic enzymes or microflora in the intestinal tract.

Figure 5. Overall mean serum concentration of OA in four sheep (two fed, two fasted) after intravenous injection of OA (0.2 mg/kg body weight), (Experiment One). Each point represents mean ± SE.

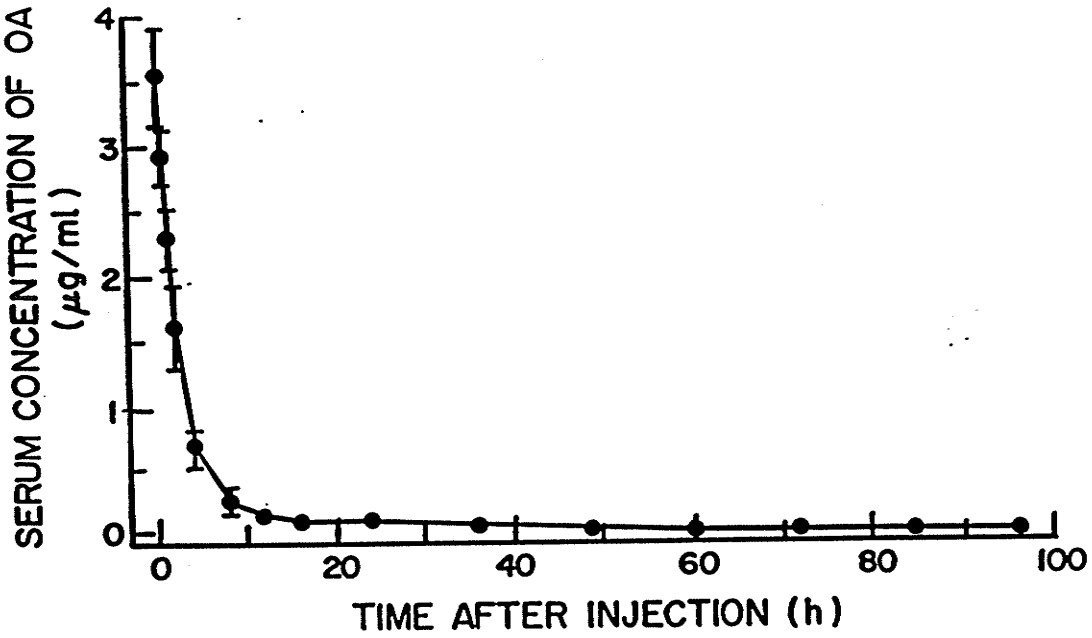

SERUM CONCENTRATION OF OA
(μg/ml)
4
3
2
1
0
0
20
40
60
80
100
TIME AFTER INJECTION (h)

Table 6. Cumulative excretion of OA and its metabolite (Oα) by sheep (two fed and two fasted) after intravenous injection of 0.2 mg OA/kg body weight (Experiment One).

Diet treatment	Weight (kg)	Cumulative excretion (mg)				
		OA		Oα[a]		Percent
		Urine	Feces	Urine	Feces	of dose (%)
Fasted	42-52	5.13	0	0.17	0.12	56.46
Fed	49-56	5.70	0	0.20	0.41	60.67
SE		1.69		0.16	0.29	

a. Cumulative excretion of Oα expressed in terms of OA equivalents according to: molecular weight of OA/molecular weight of Oα (403/256) x mg of Oα.

SE: Standard error of difference in means.

Experiment Two. In this experiment, four sheep (two on hay , two on grain) were given a single oral dose of OA (0.5 mg/kg body weight). No gross illness due to OA treatment was observed. One of the sheep on grain was stressed somewhat because of discomfort experienced at inserting the urine and blood catheters and possibly as a result of its reduction in feed consumption to 30% of normal. This caused an increase in pH of the rumen by 0.5 to 1 unit as compared to the normal sheep on grain. The pH values in the rumen of hay fed, grain fed and grain fed at 30% of normal intake were 6.9, 5.5 and 6.3, respectively. Urine output tended to increase in all sheep after dosing. Feces and urine collection was more difficult in sheep fed grain than those fed hay.

The average serum concentrations of OA at different times following OA dosing for sheep fed grain (normal intake), a reduced level of grain (30% of normal intake) and hay from both trials of the experiment are outlined in Figure 6. The rationale for separating the grain-fed sheep into two groups is discussed subsequently. The results suggest that the disappearance of OA in serum in this experiment, as in Experiment One, followed a biphasic decline. A small secondary peak was found after the absorption phase as part of the elimination phase suggesting that biliary recycling may have occurred. The absorption and elimination of OA for all treatment groups followed the same pattern which was not influenced by the dietary treatment. The pharmacokinetic

parameters for overall absorption and elimination (or disappearance) of OA were:

$T_{\frac{1}{2}}$(absorption)±SE=2.6±0.1 h

$T_{\frac{1}{2}}$(initial disappearance)±SE=3.1±0.1 h

$T_{\frac{1}{2}}$(final elimination)±SE=33.8±1.2 h

Although the peak serum concentration of OA (C_{max}) varied between dietary treatments (grain or hay), the time to reach the peak concentrations (T_{max}) was not different ($P>0.05$) (overall T_{max}=4.2±0.3 h). The apparent relative bioavailability measured in terms of area under the curve (AUC) was 3 times greater in sheep fed grain (100% normal intake) as compared to that in sheep fed hay and those fed reduced amount of grain (30% of normal intake), ($p<0.05$). The corresponding AUC (0-120 h)±SE for these three groups were 7232±443, 1853±154 and 2730±403 ng x h/ml, respectively. Table 7 gives the cumulative excretion of OA and of Oα in the urine and feces of sheep. Most of the administered OA (90-99%) was converted to Oα, which was mostly excreted in urine, and only 0.5 to 3% of OA was excreted unchanged in urine with 50 to 60% being found in the first 12 h period after dosing. The total amount of OA excreted in the urine was also substantially greater in grain fed sheep (0.70 mg for normal intake, 0.25 mg for 30% of normal intake) than in hay fed sheep (0.09 mg), ($P<0.05$).

Figure 6. Mean serum concentrations of OA in sheep after given a single oral dose of OA (0.5 mg/kg body weight), (Experiment Two). HAY: hay fed sheep (two sheep and two time periods); GRAIN-100%: grain fed sheep (normal feed intake for one sheep and two time periods); GRAIN-30%: grain fed sheep (feed intake 30% of normal for one sheep and two time periods). Each point represents mean ± SE.

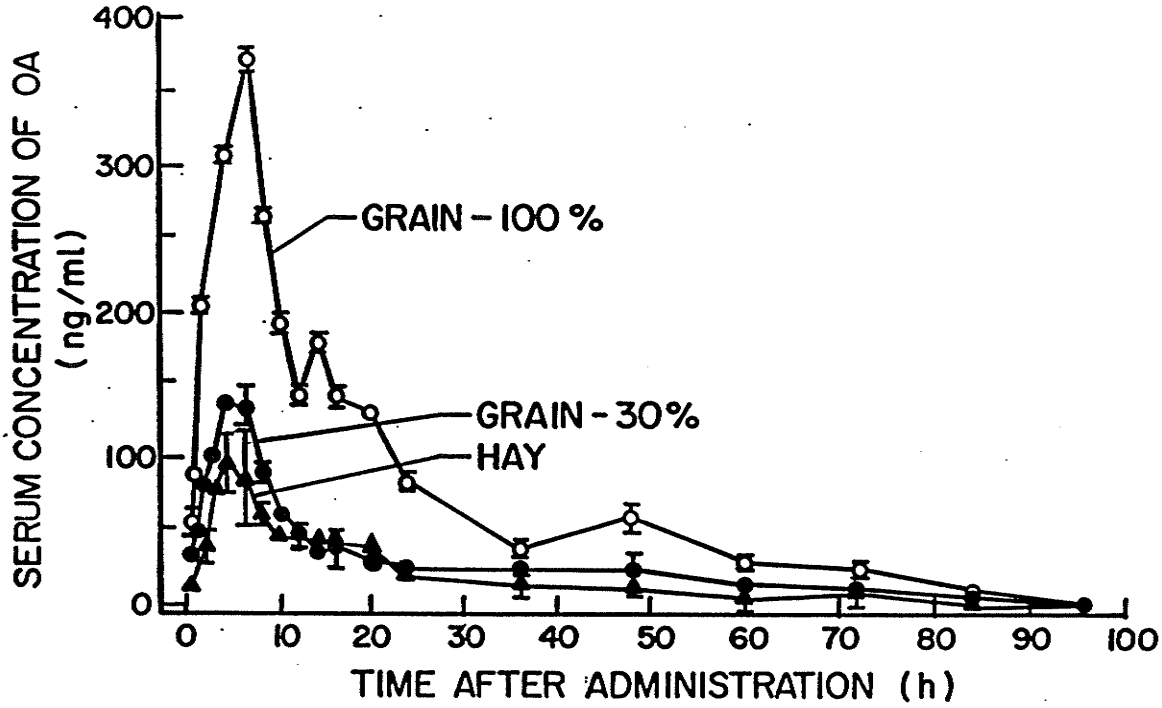

SERUM CONCENTRATION OF OA (ng/ml)
400
300
200
100
0
GRAIN - 100 %
GRAIN - 30%
HAY
0
10
20
30
40
50
60
70
80
90
100
TIME AFTER ADMINISTRATION (h)

Table 7. Cumulative excretion of OA and its metabolite (Oα) by sheep given a single oral dose of OA (0.5 mg/kg body weight), (Experiment Two).

Dietary treatment	Weight (kg)	Cumulative excretion (mg)				Percent of dose (%)
		OA		Oα[a]		
		Urine	Feces	Urine	Feces	
Grain-30%[b]	73-78	0.25	0.06	16.63	3.72	64.37
Grain-100%[c]	70-72	0.70	0.07	22.28	1.89	70.25
Hay	47-65	0.09	0.15	12.02	3.86	57.57
SE		0.10	0.09	1.76	1.55	

a. Cumulative excretion of Oα expressed in terms of OA equivalents according to: molecular weight of OA/molecular weight of Oα (403/256) x mg of Oα.

b. Feed intake was normal.

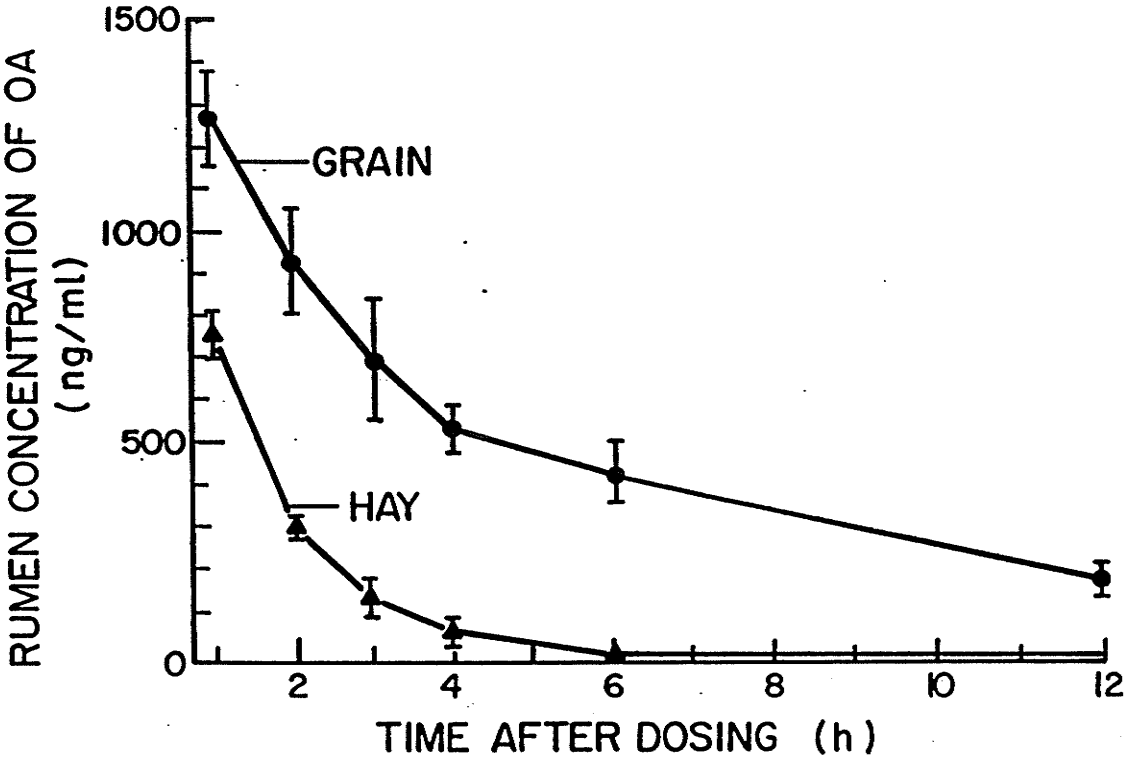
c. Feed intake was only 30% of normal.

SE:Standard error of difference in means.

Experiment Three. This experiment was conducted to confirm the results from Experiment Two. A substantial portion of the variation within the grain treatment group was attributed to the stress experienced by the sheep, which probably reduced appetite and therefore the type of microflora in the rumen. All sheep survived without gross illness or abnormal rumen pH. The pH±SE of the rumen of sheep fed hay or grain were 6.9±0.1 and 5.6±0.1, respectively.

Figure 7 shows the mean rumen concentration of OA in sheep fed hay or grain after being dosed with oral OA. The disappearance of OA from the rumen followed a mono-exponential decline which was more rapid for sheep fed hay than for sheep fed grain. As shown in Table 8. The corresponding half-life for disappearance of OA from the rumen was 4.2 times faster in sheep fed hay ($T_{\frac{1}{2}}$=0.63 h) than fed grain ($T_{\frac{1}{2}}$=2.67 h) ($p<0.001$). The apparent relative bioavailability of OA, in terms of area under the serum concentration-time data curve for OA, was 4.3 times greater in sheep fed grain than sheep fed hay ($p<0.001$). This indicates that more OA reached in the circulatory system in grain-fed as compared to hay-fed sheep.

Figure 7. Mean rumen concentration of OA in sheep given a single oral dose of OA (0.5 mg/kg body weight), (Experiment Three). HAY: hay fed sheep (values represent mean±SE of four sheep); GRAIN: grain fed sheep (values represent mean±SE of four sheep).


1500
1000
500
0
RUMEN CONCENTRATION OF OA
(ng/ml)
GRAIN
HAY
2
4
6
8
10
12
TIME AFTER DOSING (h)

The pattern of the formation of Oα was influenced by the dietary treatment (Figure 8). As shown in Table 8, Oα reached a maximum concentration of 220 ng/ml in the rumen at 1.4 h after dosing in the hay fed sheep, whereas in the grain fed sheep it reached a corresponding maximum concentration of 88 ng/ml at 7 h after dosing. After reaching the peak concentration Oα disappearance followed a bi-exponential decline in sheep fed hay, whereas it followed a mono-exponential decline in sheep fed grain. The AUC for Oα obtained from the rumen concentration-time data was still 1.5 times greater in sheep fed hay compared to those fed grain ($P<0.02$). The half-life for the formation of Oα in the rumen of sheep fed hay or fed grain was 0.9 and 1.93 h, respectively. The half-life for the disappearance of Oα from the rumen was not affected by the dietary treatment ($P>0.2$), suggesting the flow rate of rumen content to the lower gut and/or rate of Oα absorption may not be affected by type of diet.

Figure 8. Mean rumen concentration of Oα in sheep after given a single oral dose of OA (0.5 mg/kg body weight), (Experiment Three). HAY: hay fed sheep (values represent mean±SE of four sheep); GRAIN: grain fed sheep (values represent mean±SE of four sheep).

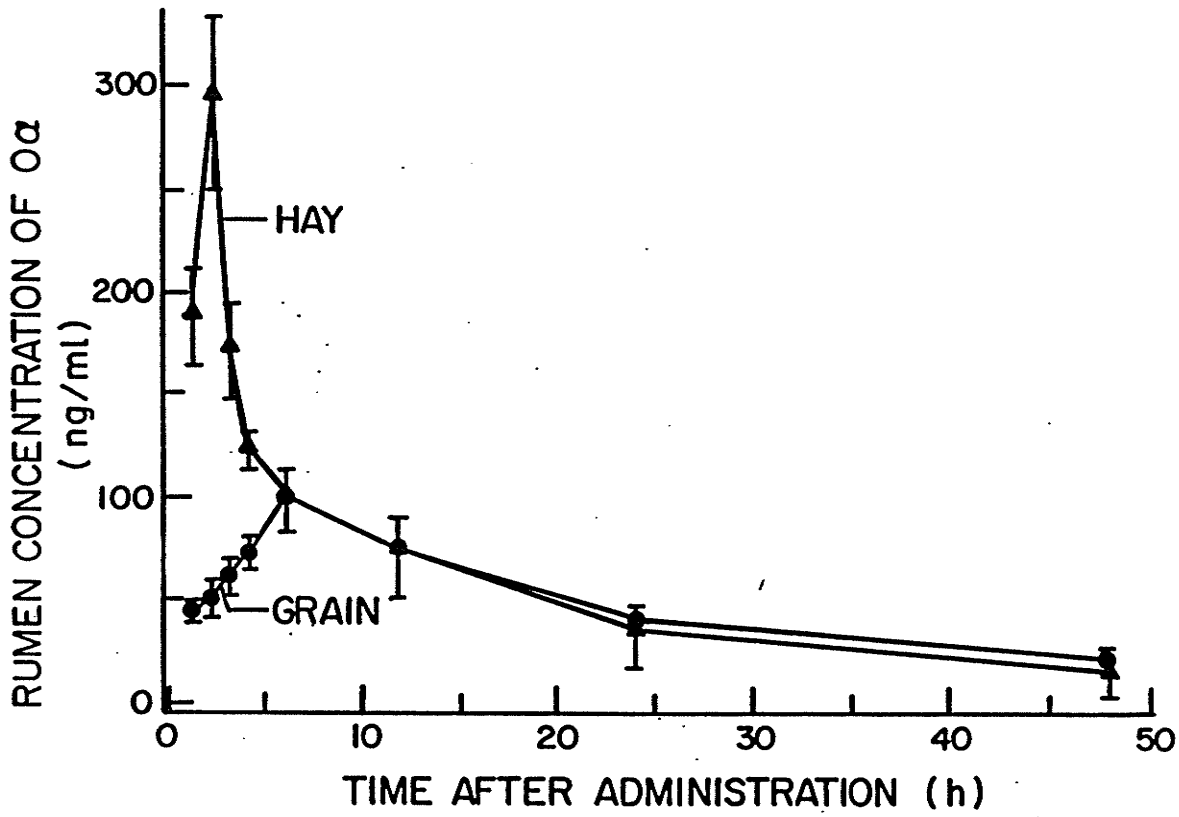

RUMEN CONCENTRATION OF Oα
(ng/ml)
300
200
100
0
HAY
GRAIN
0
10
20
30
40
50
TIME AFTER ADMINISTRATION (h)

Table 8. Pharmacokinetic parameters obtained for OA and Oα following oral administration of OA (0.5 mg/kg body weight) to sheep fed diets containing grain or hay (Experiment Three).

	Treatment			
	Hay	Grain	SE	Significance level (P)
OA				
$T_{½}$[1] disappearance	0.63	2.7	0.25	0.0004
AUC[2]	1456	6495	620	0.002
Oα				
$T_{½}$[3] formation	0.90	1.90	0.26	0.008
$T_{½}$[4] disappearance	8.7	13.9	1.86	0.21
C_{max}[5]	220	88	28	0.003
T_{max}[6]	1.4	7.0	0.56	0.002
AUC[2]	3086	2176	350	0.02
AUC[2]	494	196	110	0.15

1. Half-life (h) for disappearance of OA or Oα from the rumen.
2. Area under the serum concentration-time data curve for OA and Oα (ng.h/ml).
3. Half-life (h) for formation of Oα in the rumen.
4. The maximum concentration (ng/ml) of Oα found in the rumen.
5. The time (h) to reach maximum concentration of Oα in the rumen
6. Area under the rumen concentration-time data curve for Oα (ng.h/ml).

SE: Standard error of the difference in means.

4.4. Discussion.

This study demonstrates that the rumen of sheep is able to efficiently hydrolyze OA and that the type of diet is an important factor affecting the hydrolysis of OA and therefore the bioavailability of OA in sheep. In the experiment involving intravenous injection of OA (0.2 mg/kg body weight), OA was relatively slowly distributed to the peripheral tissues with a half-life of 1.49 h. Oα and the hydroxy metabolite of OA were not detected in the blood serum and the cumulative excretion data also indicated that no substantial biotransformation of OA had occurred in liver and other tissues. Only a trace amount of OA was excreted as Oα in the urine and feces. Oα may have been formed in the gastrointestinal tract after the secretion of OA into the intestinal tract followed by hydrolysis of the OA by the intestinal enzymes (Pitout, 1969). Doster and Sinnhuber (1972) demonstrated that Oα was formed when OA was incubated with an homogenate of a small intestine preparation, whereas only trace amounts of Oα was formed in the presence of liver homogenate. The results of the present study, however, would suggest that either limited biliary recycling occurs or that OA is rapidly reabsorbed before being hydrolyzed by the intestinal microflora or enzymes. These results are in contrast to a earlier study in our laboratory with young calves, in which approximately 75% of OA was found in feces

after intravenous injection of OA (0.25 mg/kg body weight) (Sreemannarayana et al, 1988).

Results from the experiments in which OA was administered orally suggest that the rumen of sheep may play an important role in the detoxification of OA. Cumulative excretion data from Experiment Two indicated that about 90 to 99% of the toxin was converted to the non-toxic metabolite, Oα, and that only 0.5 to 3% of the original dose of OA was found in the urine. The amount of OA reaching the circulatory system was also influenced by the type of diet. The overall systemic exposure to OA, when expressed in terms of AUC(0-120h), was found to be only 25.6% as extensive in sheep fed hay as compared to those fed grain with the same oral dose of OA (0.5 mg/kg body weight). The effects of diet on the hydrolysis of OA and subsequent reduction in the bioavailability of OA were more clearly shown in Experiment Three. The results demonstrated that after receiving a single oral dose of OA (0.5 mg/kg body weight), the rate of disappearance of OA from the rumen was 4.2 times faster in sheep on hay than sheep on grain, with the apparent relative bioavailability of OA in sheep on grain being 4.3 times greater than sheep on hay. In general, there was a recipical relationship between the rate of OA hydrolysis in the rumen and relative amount in the circulatory system, indicating the rumen played an important role in the detoxification of OA. It was also shown that the rate of formation of the OA degradation product, Oα, was much

greater in sheep fed hay compared to those fed grain. The time to reach the maximum concentration of Oα occurred in 1/5 the time it occurred in sheep fed hay as compared to sheep fed grain. The disappearance of Oα from the rumen of hay-fed sheep followed a bi-exponential decline.

It is well known that diets that promote rapid fermentation in the rumen result in rapid production of volatile fatty acid which is usually associated not only with a reduction in pH of the rumen but also with a change in the species and population of microflora, particularly protozoa in the rumen (Jounay et al, 1988). The results from the current study would suggest that diet influenced both the pH and the type of microflora that developed in the rumen. Presumably, the type or number of microflora, particularly the population of protozoa (Eadie and Mann, 1977) was affected by diet, and consequently the rate of hydrolysis of OA (Kiessling et al, 1984) was reduced in sheep fed grain compared to those fed hay. From a practical point of view, this may be of considerable importance as OA is usually produced by fungi that grow on grain but not hay (Chu, 1974). The feeding of grain contaminated with OA could therefore result in conditions that would decrease the rate at which OA is hydrolyzed and thereby increase its toxicity to animals. Results from the present study would suggest that only small amounts of grain that is contaminated with OA should be fed

to animals. This would optimize conditions in the rumen for the rapid hydrolysis of OA.

Although the diet may affect the rate of hydrolysis of OA, it did not affect the half-life for overall absorption and elimination of OA. Evidence for direct absorption of OA and Oα across the rumen epithelia was not obtained. The very long persistence of both OA in the grain fed sheep and Oα in both grain and hay fed sheep would suggest that the across-the-rumen absorption dose not occur or that it is a very slow process. In contrast to these results, Oα but not OA appeared to be rapidly eliminated from the blood of sheep as in all cases the concentration of Oα in the blood was relatively low but accumulation of Oα in the urine was relatively high as compared to OA. Apparently Oα was cleared at a sufficiently rapid rate so as to prevent an accumulation in the blood. Finally, although organ toxicity of OA to the ruminant was not established in this study, it was observed that a single small dose of OA did increase the volume of urinary excretion. The continued administration of high dose of OA probably would have resulted in a more severe problem. Further studies should be carried out to solve these problems and the establish role of biliary recycling.

In summary, it can be concluded that the rate of hydrolysis of OA by the rumen microflora is a major factor contributing

to the reduction in the bioavailability of OA and thus the systemic toxicity of OA. The type of diet may change the microflora of the rumen, and therefore the rate of degradation of OA. The rate of hydrolysis of OA in the rumen of sheep fed grain was much slower than for sheep fed hay. As a result, OA may be more toxic to grain- as compared to hay-fed sheep.

5. SUMMARY AND CONCLUSIONS.

1. The rate of hydrolysis of OA with the corresponding formation of Oα by rumen fluid is 2 to 5 times greater for sheep fed hay than for sheep fed grain.

2. The particulate fraction of rumen fluid is responsible for most of the hydrolysis of OA in the rumen.

3. The rumen pH values decrease significantly in sheep fed grain (pH 5.5 0.1) compared to sheep fed hay (pH 7.0 0.1).

4. Systemic metabolism of OA is not significant as 95% of OA was excreted unchanged in the urine of sheep injected intravenously with OA.

5. The type of diet (grain or hay) does not influence the rate of absorption and disposition of OA in sheep.

6. The disappearance of OA from the rumen of sheep fed hay is faster and the formation of Oα in the rumen occurs earlier for sheep fed hay compared to sheep fed grain.

7. The apparent relative bioavailability of OA in sheep fed hay is only 20% of that in sheep fed grain, a result of the different rates of OA hydrolysis in the rumen.

8. The difference in rate of hydrolysis of OA between grain-fed and hay-fed sheep is not attributed to a pH effect on the activity of the hydrolytic enzymes, but to a difference in type or population of rumen microflora.

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