Metabolism and Toxicity of Trichothecenes

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Abstract

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Trichothecenes are mycotoxins commonly found in cereals. The toxins are mainly produced by *Fusarium* fungi, which infect cereals in the field. Trichothecenes have been associated with outbreaks of diseases both in humans and in farm animals. Trichothecenes are closely related toxins based on a C-15 skeleton with an epoxide ring. The microbiological transformation of trichothecenes to the less toxic de-epoxide in the gut has been studied in this thesis. It was found that the micro-organisms in faeces from pigs in the Uppsala area had the ability to transform the trichothecenes nivalenol (NIV) and deoxynivalenol (DON) to their corresponding de-epoxy metabolites. The pig intestinal micro-organisms did not acquire the de-epoxidation ability during a seven week long exposure to low levels of DON in the feed, but the ability was transferred between animals by spreading faeces from pigs with an intestinal de-epoxidation ability in the pens of pigs lacking this ability. The faecal micro-organisms in humans were not able to transform trichothecenes to their de-epoxy metabolite. The de-epoxides of NIV and DON were shown to be 51 and 24 times less cytotoxic than the corresponding toxin with an intact epoxide ring, verifying that the de-epoxidation is a detoxification of trichothecenes.

The absorption, metabolism and excretion of 3-acetylDON in pigs with a known intestinal de-epoxidation ability were also studied. The toxin was rapidly deacetylated and absorbed into the blood. DON was detected in plasma from 20 minutes after feeding. A maximum plasma concentration of 51.9 ± 6.8 ng/ml was reached three hours after feeding. The toxin was rapidly excreted and the DON concentration in plasma declined rapidly after reaching the peak concentration. No accumulation occurred in plasma during the three days exposure period. No de-epoxy DON was present in plasma or urine, indicating that the de-epoxidation of trichothecenes occurs in the distal parts of the gastrointestinal tract where the absorption is low. DON and conjugated DON were the only metabolites detected in plasma and urine, while de-epoxy DON and DON were detected in faeces.

Studies where the trichothecenes DON, NIV and T-2- toxin were given to farm animals via their feed have been reviewed. It was concluded that pigs are more sensitive to trichothecenes in feed than other farm animals. Guideline limits of 0.3 mg DON and 0.2 mg T-2/HT-2 toxins/kg feed are proposed to ensure that no negative effects will occur in pigs. Similarly, guideline values of 0.5 mg T2/HT-2 toxins and 2.5 mg DON/kg feed are proposed in chicken feed. Ruminants are more resistant to trichothecenes and no guideline value is proposed for feed for ruminants.

Keywords: cell toxicity, de-epoxidation, detoxification, metabolism, risk assessment, trichothecenes,
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List of original papers

The present thesis is based on the following papers which will be referred to by their Roman numbers.

I. Eriksen, G.S., Pettersson, H., Johnsen, K and Lindberg, J.E. 2002. Transformation of trichothecenes in ileal digesta and faeces from pigs. Archives of Animal Nutrition, **56**, 263-274.

II. Eriksen, G.S. and Pettersson, H. 2003. Lack of de-epoxidation of type B trichothecenes in incubates with human faeces. Accepted for publication in Food additives and contaminants.

III. Eriksen, G.S., Pettersson, H. and Lindberg, J.E. 2003. Absorption, metabolism and excretion of 3-acetyl DON in pigs. Submitted for publication in Archives of Animal Nutrition.

IV. Eriksen, G.S., Pettersson, H. and Lundh, T. 2003. Cytotoxicity of four trichothecenes and their de-epoxy metabolites. Manuscript.

V. Eriksen, G.S. and Pettersson, H. 2003. Risk assessment of trichothecenes in animal feed. Submitted for publication in Animal Feed Science and Technology.

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List of abbreviations

3T3	3T3 Swiss albino mouse fibroblasts
3-aDON	3-acetyldeoxynivalenol
15-aDON	15- acetyldeoxynivalenol
ATA	alimentary toxic aleukia
BrdU	5-bromo-2'-deoxyurine
DAS	diacetoxyscirpenol
De-ep DON	de-epoxy deoxynivalenol
De-ep NIV	de-epoxy nivalenol
DMSO	dimethyl sulfoxide
DON	deoxynivalenol
ECD	electron capture detector
EDTA	ethylenedinitrilotetraacetic acid
FAO	Food and Agricultural Organisation of the United Nations
FCS	foetal calf serum
Fus X	fusarenon X
GC	gas chromatography
HPLC	high performance liquid chromatography
IARC	International Agency for Research on Cancer
i.p.	intraperitoneal
JECFA	Joint FAO/WHO expert committee on Food additives and Contaminants
MS	mass spectrometry
NIV	nivalenol
PBS	phosphate-buffered saline
ppb	parts per billion (µg/kg)
SCF	Scientific Committee for Food, in the European Union
UV	ultraviolet
WHO	World Health Organisation

Introduction

Background

Trichothecenes are mycotoxins produced by a range of different fungi. Fungal species of the *Fusarium* genus are the main producers of trichothecenes (Scott, 1989). *Fusarium* species are field fungi with a world-wide occurrence and the genus is probably the most important toxin-producing fungi of the northern temperate regions (Ueno, 1983). The fungi infect cereals in the field. The degree of infection will depend on various factors, for example temperature, humidity, rainfall during anthesis (flowering) and at crop harvest, soil treatment and crop rotation. *Fusarium* fungi produce a range of different toxins and the *Fusarium* toxins most likely to affect human and animal health are probably trichothecenes, fumonisins and zearalenone. Deoxynivalenol (DON) is the most abundant trichothecene in cereals but other trichothecenes, such as nivalenol (NIV), T-2 toxin and HT-2 toxin also occur frequently in cereals (Scott, 1989; Placinta, Mello & Macdonald, 1999; JECFA, 2001b).

Diseases, feed refusal and reduced growth in livestock have been associated with mouldy feed at least since the end of the 19th century (review in Trenholm *et al.*, 1989). The first trichothecene to be isolated, trichothecin, was isolated from *Trichothecium roseum* and described in 1949 by Freeman & Morrison (1949). The isolation and characterisation of trichothecin was followed by the isolation and description of other trichothecenes such as diacetoxyscirpenol (DAS) (Brian *et al.*, 1961), T-2 toxin (Bamburg, Riggs & Strong, 1968), NIV (Tatsuno *et al.*, 1968) and DON (Vesonder, Ciegler & Jensen, 1973; Yoshizawa & Morooka, 1973). More than 180 different trichothecenes have now been isolated and described.

Following the isolation of trichothecenes from mouldy grain, associations between the toxins and outbreaks of human diseases were made. A possible role of trichothecenes in the human disease Alimentary Toxic Aleukia (ATA) in Russia was reported (Joffe, 1974). The disease has been reported since the 19th century, and a severe outbreak occurred in the Orenburg district during 1942-1947 where 100 000 people died. The disease was attributed to the consumption of *Fusarium* infected wheat overwintered outdoors (Joffe, 1986). The disease was characterised by spots on the skin, necrotic angina, extreme leukopenia and multiple haemorrhages and exhaustion of the bone marrow. Isolates of *Fusarium sporotrichioides* and *F. poae* from the wheat were later shown to produce T-2 toxin and related trichothecenes (Yagen & Joffe, 1976). Symptoms similar to those reported from patients suffering from ATA have later been observed in animals exposed to acute toxic doses of T-2 toxin (Joffe, 1974; Joffe & Yagen, 1978; Joffe, 1986). Trichothecenes, including DON and NIV, have also been associated with outbreaks of human gastrointestinal disorders in Japan (Yoshizawa, 1983), India (Bhat *et al.*, 1989) and China (Luo, 1988).

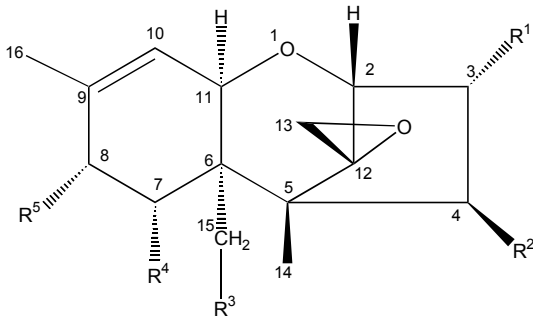

Figure 1. Chemical structure of type A and type B trichothecenes. Substituents R^1 – R^5 are given in table 1.

Table 1 Chemical structures of the substituents R^1 – R^5 are type A and type B trichothecene

Trichothecene	R1	R2	R3	R4	R5
		Type A			
HT-2 toxin	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
T-2 toxin	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
Diacetoxyscirpentriol	OH	OAc	OAc	H	H
		Type B			
Deoxynivalenol	OH	H	OH	OH	O
3-acetyl- deoxynivalenol	OAc	H	OH	OH	O
15-acetyl- deoxynivalenol	OH	H	OAc	OH	O
Nivalenol	OH	OH	OH	OH	O
Fusarenon X	OH	OAc	OH	OH	O

Chemical structure of trichothecenes

Trichothecenes are closely related sesquiterpenoids with a 12,13 epoxide ring and a variable number of hydroxyl or acetoxy groups (Fig. 1 and Table 1). Some commonly found trichothecenes only differ by one acetyl group. Acetylated DON, NIV and HT-2 are produced at the same time by the fungi as the corresponding toxins lacking the acetyl group and the acetylated and non-acetylated form of the toxin may co-occur. The 12,13-epoxide ring is considered to be essential for the toxicity of the trichothecenes. Most trichothecenes also have a C-9,10 double bond, which is also important for the toxicity (Ehrlich & Daigle, 1987).

Trichothecenes are divided into four categories according to functional groups (Ueno, 1977). Type A has a functional group other than a keto group at C-8. This is the largest group and includes toxins like T-2 toxin, HT-2 toxin and DAS. Type B trichothecenes has a keto group at C-8 and includes the most widespread trichothecene DON and toxins like 3-aDON, NIV, and Fusarenon-X. The third category (Type C) has a second epoxide ring at C-7,8 or C-9,10 and toxins from the fourth group (Type D) contains a macrocyclic ring between C-4 and C-15 with two ester-linkages. Type C and type D trichothecenes are not produced by *Fusarium* fungi and are not discussed any further.

Trichothecenes are secondary metabolites and the first step of the synthesis is enzymatic cyclisation of the intermediate farnesyl pyrophosphate to trichodiene.

The formation of trichodiene is followed by species-dependent sequences of oxygenations, isomerizations, cyclizations and esterifications to the different trichothecene toxins (Desjardins, Hohn & McCormick, 1993).

The solubility of trichothecenes varies with the number of polar groups. Most trichothecenes are soluble in solvents like acetone, chloroform and ethylacetate, but highly hydroxylated trichothecenes like DON and NIV are also soluble in more polar solvents like acetonitrile, methanol, ethanol and water (Ueno, 1987).

The trichothecene skeleton is chemically stable and the 12,13 epoxide ring is stable to nucleophilic attacks. Furthermore, trichothecenes are heat stable and are not degraded during normal food processing. Trichothecenes are also stable at neutral and acidic pH (Ueno, 1987) and are consequently not hydrolysed in the stomach after ingestion.

Natural production of trichothecenes

Trichothecenes are commonly found in cereals such as wheat, rye, oat, maize and barley world-wide (Scott, 1989; Placinta *et al.*, 1999; JECFA, 2001a). *Fusarium* fungi are the most widespread natural producers of trichothecenes and different species have different toxin production profiles (Table 2). The fungi are commonly occurring soil fungi where they grow and sporulate both in soil and on plant material. Some of the *Fusarium* species are also plant pathogens causing different plant diseases and the infection of the plant by Fusarium fungi may reduce the crop yield significantly (Snijders & Perkowski, 1990; Mesterhazy *et al.*, 1999).

Table 2. Toxins produced by cultures of Fusarium species (from Eriksen & Alexander, 1998*)*

Species [a]	Toxin produced [b]
F. acuminatum	**T-2, HT-2,** DAS, MAS, MON, NEO
F. avenaceum	**MON,** FUS C
F. cerealis (F. crokwellense)	**NIV, FUS X, ZEN,** FUS C
F. culmorum	**DON, ZEN,** NIV, FUS X, FUS C, A-DON
F. equiseti	**DAS, ZEN, FUC**
F. graminearum	**DON, A-DON, ZEN,** NIV, FUS X
F. oxysporum	MON
F. poae	**DAS, MAS, NIV, FUS X,** T-2, HT-2, FUS C
F. proliferatum	**FUM, MON**
F. sacchari (F. subglutinans)	**MON**
F. sambucinum	**DAS,** MAS
F. torulosum	**WOR**
F. semitectum (F. incarnatum)	**ZEN**
F. sporotrichioides	**T-2, HT-2,** DAS, NEO, FUS C
F. tricinctum	FUS C
F. verticilloides (F. moniliforme)	**FUM,** FUS C

[a] Alternative name in brackets.

[b] Main toxins produced in bold. Abbreviations: A-DON: 3- and 15-acetyldeoxynivalenol, DAS: diacetoxyscirpenol, DON: deoxynivalenol, FUC: fusarochromanone, FUM: fumonisins, FUS C: fusarin C, FUS X: fusarenon X, MAS: monoacetoxyscirpenol, MON: moniliformin, NEO: neosolaniol, NIV: nivalenol, WOR: wortmannin, ZEN: zearalenone.

Occurrence of trichothecenes

Deoxynivalenol

DON is probably the most frequently detected trichothecene and the toxin is mainly produced by *F. graminearum* and *F. culmorum* (WHO, 1990). The two *Fusarium* species are plant pathogens and cause outbreaks of fusarium head blight (also called wheat scab). The most serious outbreaks of the disease occur in years with heavy rainfall during the flowering season. *Fusarium* infections of cereals lead to severe yield loss and reduced kernel quality in addition to the occurrence of toxins (McMullen, Jones & Gallenberg, 1997). *F. culmorum* has an optimal growth at 21°C and is commonly found in northern areas like Scandinavia and Northern Europe, while *F. graminearum* has an optimal growth at 25°C and is more widespread in warmer climates, e.g. North America, China and Japan.

The occurrence of DON has been the subject of various reviews (Scott, 1989; WHO, 1990; Placinta *et al.*, 1999). A comprehensive compilation of the data from surveys of DON in random samples of cereals has recently been prepared by JECFA (2001). In an world-wide average, DON has been found in 57% of the wheat samples, 40% of the maize samples, 68% of the oats samples, 59% of barley samples, 49% of rye samples and 27% of the rice samples analysed. DON was also found in wheat and maize products, for example flour, bread and breakfast cereals. The concentrations of DON in random cereal samples showed a large annual variation, concentrations ranging from below the detection limits (5-50 µg/kg) to more than 30 mg/kg (JECFA, 2001b).

According to Trucksess *et al.* (1995), about 40% of the wheat samples and 57% of the barley samples from the 1993 harvest in USA had DON-levels exceeding the US advisory levels for wheat intended for human consumption. DON often occur together with the 3- or 15- acetyl-DON as well as other *Fusarium* toxins (Scott, 1989).

Nivalenol

Fusarium cerealis and *F. poae* are the main producers of nivalenol, but isolates of *F. culmorum* and *F. graminearum* are also able to produce nivalenol (Eriksen & Alexander, 1998). *F. poae* is the main producer of nivalenol in Sweden (Pettersson, 1991; Pettersson *et al.*, 1995). In contrast to DON, NIV occurs more frequently in years with dry and warm growing seasons (Pettersson, 1995). Nivalenol is more common in Europe, Australia and Asia than in America, where the occurrence of NIV is limited. Both mean levels and incidence of positive samples of NIV are lower than for DON even in the Nordic countries and Europe (Eriksen & Alexander, 1998). NIV may occur together with fusarenon X (Fus X), the C-4 acetylated derivative of NIV, and other toxins produced by *Fusarium* fungi.

T-2 and HT-2 toxins

T-2 toxin and its deacetylated form, HT-2 toxin, normally occur together in cereals. The toxins are mainly produced by *F. sporotrichioides*, but both *F. acuminatum* (Logrieco *et al.*, 1992) and a new species previously considered to be

a special form of *F. poae* (Torp & Langseth, 1999) have also been shown to produce T-2 toxin and HT-2 toxin. The occurrence of *F. sporotrichioides* in cereals is mainly a result of water damage to grains occurring when the cereals remain for extended periods on the field at or after harvest or when the grain is wet during storage (JECFA, 2001c). The main producer of T-2 and HT-2 toxins in Norway is probably a newly described *F. poae*-resembling species (Torp & Langseth, 1999). High levels of T-2 and HT-2 toxins in Norway have been observed in warm and dry summers (Langseth & Rundberget, 1999.

The available data on the occurrence of T-2 and HT-2 toxins from randomly selected samples world-wide has been prepared by JECFA (2001c). The mean values and incidence of positive samples are very dependent on the detection limit of the method used in the study. In studies with a low detection limit, most positive samples have a concentration <100 µg/kg cereal. The average concentration of the toxins is difficult to establish since so many samples are below the detection limit, but the average concentrations of the toxins are below <100 µg/kg. According to JECFA (2001c), is it not possible to draw any conclusions about what climate is associated with increased levels of T-2 and HT-2 toxins in cereals, probably due to the differences in optimal growth conditions for the T-2 and HT-2 toxin producing species.

Toxicity of trichothecenes

The toxicity of trichothecenes has been subject of several reviews (Beasley, 1989; WHO, 1990; IARC, 1993b; Rotter, Prelusky & Pestka, 1996; JECFA, 2001a). Trichothecenes are toxic to all tested animal species, but the sensitivity varies considerably between species and also between the different trichothecenes. T-2 toxin is the most toxic trichothecene produced by *Fusarium* fungi.

Hydrolisation of the C-9,10 double bond decreases the toxicity of trichothecenes. Type A trichothecenes, having a functional group other than carbonyl in the C-8 position, are generally more toxic than type B trichothecenes with a carbonyl in this position. The toxicity of type A trichothecenes decreases in the order of isovaleryl > hydrogen > hydroxyl groups in the C-8 position. The toxicity of type B trichothecenes is influenced by the substitutions in the C-4 position. In lymphocytes, the toxicity of the type B trichothecenes decreases in the order from acetyl > hydroxyl > hydrogen in the C-4 position. Loss of side chains in the C-4, C-8 or C-15 positions decreases the toxicity and the loss of more than one side chain in these positions further decrease the toxicity (Betina, 1989; Feinberg & McLaughlin, 1989).

Biochemical and cellular toxicity

Trichothecenes have been shown to be potent inhibitors of the eucaryote protein synthesis (Ueno, 1968; Feinberg & McLaughlin, 1989). Trichothecenes bind to the 60S subunit of the ribosomes and inhibit the peptidyl transferase activity (Wei *et al.*, 1974; Wei & McLaughlin, 1974). Trichothecenes with substituents in both C-3 and C-4 positions mainly inhibit the initiation of the protein synthesis, while

trichothecenes lacking substituents at either position inhibit the elongation step (Wei *et al.*, 1974; Ehrlich & Daigle, 1987).

Cellular effects on DNA synthesis, DNA breakage and membrane integrity have been considered to be secondary effects of the inhibited protein synthesis (Feinberg & McLaughlin, 1989; WHO, 1990). Cells depending on high protein synthesis, such as lymphocytes and epithelial cells, have been considered to be most susceptible to trichothecenes.

Trichothecene exposure leads to apoptosis both in vitro (Pestka, Yan & King, 1994; Ueno *et al.*, 1995; Sun *et al.*, 2002) and in vivo in several organs such as lymphoid organs (Shinozuka *et al.*, 1997; Islam *et al.*, 1998; Murshedul *et al.*, 2000; Poapolathep *et al.*, 2002.), haematopoietic tissues (Shinozuka *et al.*, 1998), liver (Ihara *et al.*, 1997) and intestinal crypts (Li *et al.*, 1997). In the human HL-60 cell line, apoptosis was induced with up to 100 ng T-2 toxin/ml for 2 hours, while no inhibition of the protein synthesis could be detected (Yoshino *et al.*, 1996).

It has been shown that the apoptosis is preceded by cytochrome C release from the mitochondria and activation of the caspases (Nagase *et al.*, 2001; Miura, Aminova & Murayama, 2002). Apoptosis is mediated by activation of caspases, but the factor triggering this activation is not known. Increased cellular concentration of Ca^{2+} has been suggested (Yoshino *et al.*, 1996; Miura *et al.*, 2002; Holme *et al.*, 2003). There are also indications that different MAP kinases may be involved in the induction of apoptosis, but it seems not to be mandatory (Shifrin & Anderson, 1999; Yang *et al.*, 2000; Holme *et al.*, 2003).

Trichothecenes also interfere with the cell membranes. T-2 toxin affected the permeability of cell membranes of L-6 myoblasts *in vitro* at concentrations of 0.4 pg/ml. The effect occurred within 10 minutes, indicating that the mechanism is different from inhibition of protein synthesis since most short-lived proteins have half lives measured in hours (Bunner & Morris, 1988). A recent study has shown that less than 10µmol/ml DON (<0.296 µg/ml) selectively inhibited some intestinal transport proteins in human intestinal epithelial cells when the cells were incubated with the toxin for 24 and 48 hours. The transport of palmitate across the membrane was increased. The effects were mimicked both by cycloheximide, an inhibitor of the protein synthesis, and deoxycholate, an inducer of apoptosis, a finding which according to the authors indicated that inhibition of the protein synthesis and apoptosis are the main mechanisms for DON toxicity in the cells (Maresca *et al.*, 2002). T-2 toxin also caused changes in the phospholipid turnover in bovine platelets (Grandoni *et al.*, 1992) and haemolysis of erythrocytes in vitro (Rizzo *et al.*, 1992; Rizzo *et al.*, 1994). Antioxidants like ascorbic acid, α-tocopherol and selenium (Tutelyan *et al.*, 1990; Rizzo *et al.*, 1994) and glutathione precursors (Fricke & Jorge, 1991) protect against lipid peroxidation induced by trichothecenes in vivo and against the cytotoxicity of T-2 toxin (Shokri *et al.*, 2000).

Effect on feed intake and growth

Trichothecenes in feed cause a reduced feed intake in exposed animals. Pigs are especially sensitive to trichothecenes in feed (Trenholm *et al.*, 1989, Rotter *et al.*, 1996). The difference in toxicity between DON and T-2 toxin is apparently less in

respect of feed intake than in most other toxic effects of trichothecenes. Both DON and T-2 toxin change the levels of dopamine, tryptophan, serotonine and serotonine metabolites in the brain of rodents and pigs (MacDonald, Cavan & Smith, 1988; Prelusky *et al.*, 1992; Prelusky, 1993). The brain level of these substances is also altered by other anorectic substances (Rotter *et al.*, 1996). The reduced feed intake is considered to be, at least partly, a result of the altered levels of neurotransmitters in the brain (Prelusky & Trenholm, 1993; Rotter *et al.*, 1996), but even involvement of the peripheral nervous system has been proposed (Fioramonti *et al.*, 1993). Fusaric acid, a non-trichothecene mycotoxin produced by *Fusarium* fungi, also changed the levels of these transmitters in pig brain (Smith & MacDonald, 1991) and this toxin may influence the effect of trichothecenes on feed intake.

Effects on the immune system

Trichothecenes have multiple effects on the immune system of exposed animals and the effects have been reviewed by Taylor, Pang & Beasley (1989) and Bondy & Pestka (2000). Trichothecenes impair the immune system, increase the production of IgA and reduce experimental animals' resistance towards bacterial infections. Low doses DON and T-2 toxin may even stimulate the mitogen activated proliferation of lymphocytes and increase the resistance to infections, depending on the administration of the toxin, the timing and the dose. The decreased response of the immune system is easily explained by the inhibition of the protein synthesis and the apoptosis caused by trichothecenes (Pestka & Bondy, 1994; Bondy & Pestka, 2000). The stimulatory effect has been more difficult to explain, but recent studies indicate that induction of cytokines is involved, possibly upregulated via induction of MAP-kinases leading to increased levels of mRNA expression (Bondy & Pestka, 2000; Zhou, Islam & Pestka, 2003).

Significance of the epoxy group

The 12,13 epoxy group is considered to be essential for the toxicity of trichothecenes (Betina, 1989; Rotter *et al.*, 1996). It has been shown that the de-epoxy metabolites of T-2 toxin had a significantly higher LC_{50} in brine shrimp assay than the corresponding epoxy analogues (Swanson *et al.*, 1987b). Furthermore, de-epoxy T-2 toxin was 400 times less toxic than T-2 toxin in the rat skin irritation assay. No clinical symptoms were observed in rats injected a 5 times higher dose of de-epoxy T-2 toxin (60 mg/kg bw) than a dose that killed 5 of 7 rats given 12 mg T-2 toxin/kg bw (Swanson *et al.*, 1988).

Cattle are less sensitive towards trichothecenes than most monogastric species (Beasley, 1989). Trichothecenes are to a large extent de-epoxidised in the rumen of the cattle before absorption to the blood (Cote, *et al.*, 1986; Swanson & Corley, 1989), which is regarded as a detoxification. De-epoxides have also been found in urine from rats, while no information is available from mice.

Risk assessments of trichothecenes

Several risk assessments of trichothecenes in human food have been published in recent years (Eriksen & Alexander, 1998; Scientific committee for food (SCF), 1999: http://europa.eu.int/comm/food/fs/sc/scf/out44_en.pdf. Accessed 2002-02-03; 2000: http://europa.eu.int/comm/food/fs/sc/scf/out74_en.pdf. Accessed 2002-02-03; 2001: http://europa.eu.int/comm/food/fs/sc/scf/out88_en.pdf. Accessed 2002-02-03; 2002: http://europa.eu.int/comm/food/fs/sc/scf/out123_en.pdf. Accessed 2002-02-03.

JECFA, 2001a). The International Agency for Research on Cancer (IARC) has evaluated the carcinogenic effects of trichothecenes and concluded that T-2 toxin, DON, NIV and Fus X were not classifiable as to their carcinogenicity to humans (IARC, 1993a). Based on these risk assessments, JECFA and EU established provisional maximum tolerable daily intakes for humans of 60 ng T-2 and HT-2 toxins/kg bw and 1 μg DON/kg bw. In addition, EU also established a temporary tolerable daily intake of 0.7 μg NIV/kg bw, which was not evaluated by JECFA.

Objective

The main objectives of this thesis were:

- To study the ability of pig faecal micro-organisms to metabolise trichothecenes to the much less toxic de-epoxy metabolites at commercial pig farms.

- to study the possibility of gastrointestinal micro-organisms in pigs to acquire the de-epoxidation ability when pigs are exposed to low levels of trichothecenes in the feed. The possibility to use the pig faecal de-epoxidation ability as a biomarker of recent exposure was also investigated.

- To compare the transformation of trichothecenes to their de-epoxy metabolites in human faeces with the transformation in pig faeces and other species.

- To study the absorption, metabolism and excretion of DON in pigs with a known gastrointestinal de-epoxidation ability.

- To propose guidelines values for trichothecene concentrations in feed for farm animals based on available feeding studies.

Materials and Methods

Animals and feeding (Paper I and III)

Castrated male pigs were given feed contaminated with either 0.8-1.2 mg DON/kg feed for seven weeks (Paper I) or 2.5 mg 3-aDON/kg feed for 2.5 days (Paper IV). The pigs were fed twice daily with a total allowance corresponding to 4% of their body weight and day. The doses of DON and 3-aDON corresponded to of 0.04 – 0.05 and 0.1 mg/kg bw and day, respectively. Ileal fistulas were inserted to the pigs used in the first experiment (Paper I) and catheters were inserted into the jugular vein in the other experiment (Paper III). The ability of the gastrointestinal microflora to transform trichothecenes was examined in the first study and samples of ileal digesta and faeces were collected weekly and incubated with DON or NIV as described below. Straw mixed with faeces from a pig farm where a microbial de-epoxidation ability in the faeces had been found just prior to the collection of faeces, was distributed in the pens at the end of the exposure period. The straw and faeces were left in the pens for 24 hours. The pigs were fed an uncontaminated feed for the following two weeks. The objective of the second animal experiment (Paper IV) was to study the absorption and metabolism of DON in pigs with a gastrointestinal flora having the ability to transform DON to the de-epoxy metabolite. Plasma urine and faeces samples were collected from before the exposure started to 48 hours after feeding the pigs the last ration of contaminated feed.

Faecal incubations of trichothecenes (Paper I and II)

For *in vitro* studies of gastrointestinal de-epoxidation ability (Paper I and II), faeces was collected within one minute of defecation and anaerobically transferred to a degassed McDougals buffer solution. DON or NIV was added to the tubes which were then flushed with CO_2 and incubated under anaerobic conditions for 24 or 48 hours at 37°C. Tubes where the toxins were added after the incubation and tubes without any added toxin were prepared and treated the same way as the sample incubations. The last tubes were used for control and as blank.

Bacterial DNA profile (Paper I)

The terminal restriction fragment length polymorphism (T-RFLP) profiles of the DNA from the faeces and ileum content were produced from four out of the five pigs used in the feeding experiment in Paper I. Bacterial DNA was isolated and purified from the ileum content and faeces. The bacterial DNA was then amplified by polymeric chain reaction (PCR). Fluorescently labelled terminal restriction fragments (T-RFs) of the amplified DNA were analysed by electrophoresis and similarities between samples were compared by similarity coefficients.

Extraction and analysis of samples (Paper I, II, III)

The faecal incubations and faeces samples were extracted with ethylacetate/acetonitrile (9/1) while plasma and urine samples were extracted with ethylacetate only. All samples were extracted twice. All faeces-containing samples were washed with hexane. The solvent was evaporated and the samples were derivatised with Trisil®TBT. Gas chromatography was performed by use of capillary column with temperature programming and a ^{63}Ni electron capture detector. Chromatograms from the samples were compared with chromatograms from blank samples and control samples with a known amount of toxin added. The retention time for metabolite formed was compared with retention time for the de-epoxy metabolite of the toxin. The amounts of de-epoxy NIV and de-epoxy DON were estimated by using the response factors for DON and NIV, respectively.

The presence of the de-epoxy metabolites in some of the samples from paper I was verified by GC-MS both with full scan for fragments and with selective ion monitoring set to detect DON and NIV and the de-epoxy metabolites of the two toxins. The retention time was also compared with the retention times for two substances previously shown to be de-epoxy metabolites of DON and NIV.

Production of de-epoxy deoxynivalenol and de-epoxy nivalenol (Paper IV)

De-epoxides of DON and NIV were produced by incubating the toxins for 24 hours at 37°C with faeces from sows with a proven gastrointestinal ability to transform trichothecenes to their corresponding de-epoxide. The incubates were extracted with ethylacetate and the extract was washed with hexane. The solvent was evaporated and the de-epoxides were purified by reverse phase HPLC, using a Rad-Pack C-18 column with acetonitrile/water (10/90) as mobile phase. The mobile phase was evaporated from the collected fractions and the de-epoxides dissolved in acetonitrile and stored in –20°C.

Cell toxicity test (Paper IV)

Cell culture

Swiss mouse fibroblasts (3T3 cells) were used in the cell toxicity assay. The cells were cultured in cell growth medium supplemented with glutamine and antibiotics (penicillin and streptomycin). The cells were cultured in monolayers in cell culture flasks in a modified atmosphere (80% humidity and 5% CO_2) at 37°C. The cells were subcultivated twice weekly. Cells were grown for 24 hours in wells in a 96- well microtiter plate before the toxicity testing.

Cell toxicity assay

Cell toxicity was measured using the BrdU (5 bromo-2'-deoxyuridine) assay, which measures incorporation of BrdU in DNA in proliferating cells. The assay gives a measure of the DNA synthesis in the cells. Trypsin, EDTA and phosphate-buffered saline were used to release the cells from the cell culture flask.

Fusarenon-X, NIV, de-epoxy NIV, 3-aDON, 15-aDON, DON and de-epoxy DON were dissolved in medium containing 2% DMSO and 2% ethanol. Two-fold dilution series of Fus x (78 – 2500 ng/ml), NIV (78 – 2500 ng/ml), de-ep NIV (3606 – 125000 ng/ml), 3-aDON (156 – 5000 ng/ml) 15-aDON (313 – 10000 ng/ml), DON (78 – 2500 ng/ml) and de-ep DON (3606 – 125000 ng/ml) were added in triplets to 3T3-cells cultivated in wells on a 96-well microtiter plate and left in a modified atmosphere with humidity of 80% and 5% CO_2 at 37°C for 24 hours. The absorbance was measured with a microtiter reader after addition of a substrate to the enzyme linked to BrdU-antibodies added to the wells.

Results

Transformation of trichothecenes by the gastrointestinal microflora (Paper I and II)

No trace of 3-acetylDON was detected in any incubation with pig faeces. Neither was any peak that could correspond to a 3-acetyl de-epoxy metabolite observed in the chromatograms. It was therefore concluded that 3-aDON was completely deacetylated in all incubations with pig faeces.

Most of the added 3-aDON and NIV (>90%) was transformed to the corresponding de-epoxy metabolite during the incubation period in all incubations with faeces collected at commercial pig farms. In incubations with faeces from one of the experimental farms, no de-epoxides were formed in incubates with faeces from two of the four pigs, while more than 90% of the toxins were de-epoxidised in the faecal incubates from the other two pigs. In faecal incubations from the other experimental farm significant amounts of toxins with an intact epoxy group were present in incubates with faeces from two out of the five pigs (20-60%), but de-epoxides of the two toxins were detected in all incubations.

The gastrointestinal flora in young pigs fed low levels of DON in the feed for seven weeks did not acquire the de-epoxidation ability in response to the trichothecene in feed, neither in faeces nor in ileal digesta. When faeces from pigs with a known de-epoxidation ability were spread in the pens, the gastrointestinal flora of the pigs acquired the ability to transform trichothecenes to their de-epoxy metabolites. The de-epoxidation ability was found in both faeces and ileal digesta one week after the faeces had been spread in the pens. This acquirement of the de-epoxidation ability was not accompanied by any detectable change in the bacterial terminal restriction fragments of the DNA in the faeces or ileum of the pigs.

In contrast to the incubations with pig faeces, no de-epoxidation occurred in incubations with human faeces and only 78 ± 30% of the 3-aDON added to the incubations was deacetylated during the incubation period.

Cytotoxicity of trichothecenes and their de-epoxy metabolites (Paper III)

The cytotoxic effect of NIV and DON, given as the concentration inhibiting 50% of the DNA synthesis (IC_{50} values) in the BrdU assay, was in the same range as previously reported. This shows that the reproducibility of the bioassay is high. The IC_{50} values for 15-acetylDON, 3-acetylDON, DON and de-epoxy DON were 510, 4888, 444 and 23 266 ng/ml respectively and the IC_{50} values for Fusarenon X, NIV and de-epoxy NIV were 255, 373 and 19029 ng/ml. The results verify that the de-epoxidation of trichothecenes is a detoxification reaction. Furthermore, it shows that the acetylated derivatives of DON are less toxic than DON in the assay, while the cytotoxicity of Fusarenon X, the acetylated derivative of NIV, is similar to the toxicity of NIV.

Absorption, metabolism and excretion of 3-acetylDON (Paper IV)

No 3-aDON was detected in samples of blood plasma, urine or faeces from pigs exposed to 3-aDON in the feed.

DON was found in blood plasma as early as 20 minutes after the contaminated diet first was given to the pigs. The DON plasma concentration reached a maximum 3 h after feeding, and decreased rapidly thereafter. Only low concentrations of DON were found in plasma 8 h after feeding. No increase of DON concentration in plasma occurred during the feeding period and the maximum concentration of DON in plasma on the third day of feeding was not higher than on day 1 ($p= 0.57$).

DON and the glucuronide conjugate were the only metabolites found in plasma and urine, while both de-epoxy DON (51.9 ± 15.2%) and DON (48.1 ± 15.2%) were found in faeces.

A significant proportion of the DON present in plasma (42.1 ± 6.9) and urine (33.2 ±10.7%) was glucuronide conjugated. No other metabolite could be detected in plasma or urine samples.

DON was mainly excreted in the urine. Only 1.5 ± 0.4% of the DON ingested by the pigs was recovered in faeces, while 45 ± 26% of the toxin ingested was recovered as metabolites of 3-aDON in urine. The total recovery of the DON ingested by the pigs was 46 ± 25% and the fate of the rest of the toxin given to the pigs remains unknown. DON was still present in the last urine and faeces collected from the pigs and some of the toxin unaccounted for was still not excreted. The concentrations in faeces and urine at the time were low, and it is not likely that most of the missing toxin still remained within the animal.

Risk assessment of trichothecenes in feed (Paper V)

Domestic animals are affected by trichothecenes in feed. The main effects are impairment of the immune system, reduction of feed intake and gastrointestinal disturbances. Different species are not equally sensitive towards trichothecenes in feed.

The available toxicological information from feeding studies with trichothecenes in ruminants is not sufficient to allow a scientifically based risk assessment. The available studies of the metabolism of trichothecenes in cattle have showed that the toxins to a large extent are de-epoxidised in the rumen before absorption. Furthermore, no effects have been found on milk production or feed intake even at levels up 12 mg DON/kg feed in the few available studies of the toxic effects of DON in ruminant feed. However, no studies of the effect of DON on the immune system in cattle are available.

Feed refusal, diarrhoea, decreased body and organ weights and decreased plasma concentration of IgA and IgM have been found in cattle given 10 mg T-2 toxin/kg feed. These levels are high and unlikely to occur in normal feed unless it is visibly damaged by mould. There are no indications of presence of trichothecenes in milk under normal production conditions, and only the de-epoxy metabolites of trichothecenes have been found in milk from cows given high levels of trichothecenes under experimental conditions. The presence of trichothecenes in milk is therefore unlikely to pose any risk to the milk consumer.

No data are available for nivalenol in ruminants.

It is concluded that it is unlikely that the concentrations of trichothecenes in feed would reach the levels likely to have any negative effect on ruminants or the food quality of milk or meat from cattle. A maximum limit for trichothecenes in feed for ruminants is probably not needed.

Poultry are more sensitive for trichothecenes in feed than ruminants. Altered organ weights were found in chickens given 9 mg DON/kg feed, but not in chickens given 5 mg DON/kg bw. Feed refusal and reduced weight gain have been found when the concentration of DON reached 16-20 mg/kg feed. Effects of DON on the immune system in poultry have been observed at a concentration of 18 mg DON/kg feed. Based on these findings, we propose that a safety factor of 2 is adequate. A guideline value of 2.5 mg DON/kg poultry feed is therefore proposed. This limit should apply to the sum of DON and acetylated derivatives that may be present (3-acetylDON or 15-acetylDON).

The available database of the effects of NIV on chickens is very sparse and does not allow any scientific assessment of the risk of NIV in poultry feed. Nivalenol caused some minor pathological changes in chickens given 1 mg NIV/kg feed, indicating that nivalenol may be more toxic to chickens than DON.

The more toxic T-2 toxin caused oral lesions in chickens and hens given a feed containing 1.0 mg T-2 toxin/ kg feed, while reduced feed intake and weight gain was observed in chickens given a feed containing 2.0 mg T-2 toxin/kg feed. A safety factor of 2 is also proposed for T-2 toxin since the oral lesions are observed at 1.0 mg/kg feed and reduced weight gain is found at 2 mg T-2 toxin/kg feed or more. The concentration of T-2 toxin in poultry feed should not exceed 0.5 mg /kg feed. This guideline should apply to the sum of T-2 toxin and HT-2 toxin, since T-2 toxin is rapidly metabolised to HT-2 toxin *in vivo*, and the toxic effects of the two toxins can not easily be separated in animal experiments.

Pigs are more sensitive to trichothecenes than other farm animals. DON causes reduction in the feed intake in pigs in concentrations from 0.6 mg DON/kg feed in studies where naturally infected cereals have been mixed into the diet. In studies where pure DON has been added to the feed, a temporary feed refusal has been observed in pigs given 3-6 mg DON/kg feed, vomiting has been found in pigs

given 20 mg DON/kg feed and complete refusal at 12 mg DON/kg feed. No lower dose has been used in these studies, and a No Observed Effect Level can therefore not be set based on these experiments. Due to the varying results in feeding studies with cereals infected with *Fusarium*, a clear dose-response relationship can not be established from the available data. The reduction in feed intake and weight gain was only temporary at the lowest toxin levels, but the loss in body weight gain was not recovered before slaughter. Feed containing 3 mg pure DON/kg would therefore lead to an economic loss for the farmer. A reduced immune response towards tetanus toxoid was found in pigs fed from 1.8 mg DON/kg feed and a significant decreasing trend in serum α-globulin was found with increasing DON-levels in pigs given 0-3 mg DON/kg. Effects on the level of plasma globulins have been observed in pigs given feed containing from 3 mg DON/kg bw. Reduced feed intake and weight gain are the effects occurring at the lowest level of DON in feed. Since the reduction in feed intake is transient, a safety factor of 2 is used and a guideline of 0.3 mg DON/kg feed is proposed. This guideline value may be lower than necessary for some pigs or some feeds, but the guideline value could apply until more information about factors having an influence on the toxic effects of DON in pigs is available.

Very little information is available about the effects of NIV in pig feed. There are no studies available with pigs fed pure nivalenol in feed given ad libitum. Consequently, there is no information about reduction in feed intake. Reduced feed intake was observed in pigs given a naturally contaminated diet containing 5.8 mg NIV but not in pigs given 2.9 mg/kg feed. The feed used in these studies also contained other mycotoxins and the possibility of interference from these other substances can not be ruled out. Pathological lesions have been found in pigs fed 2.5 mg pure NIV/kg feed. A dose-dependent decrease in spleen cell number and CD4+ and CD+ was found in pigs fed 0, 2.5 or 5 mg NIV/kg bw, but no information about the immune response towards an infection or an antigen is available for NIV.

The available database does not allow any guideline value to be set for NIV in pigs.

Reduced feed intake and weight gain and impairment of the immune system are the effects found at the lowest levels of T-2 toxin in pig feed. A concentration of 0.5 mg T-2 toxin /kg feed caused both a reduction in feed intake and a reduction in various parameters of the immune system in exposed pigs. Higher levels of T-2 toxin have caused complete feed refusal and decreased reproduction in pigs. No effect on the immune system was reported from other studies with pigs given the same levels of T-2 toxin in feed. Based on these facts, a guideline value of 0.2 mg T-2 toxin /kg feed is proposed in pig feed. This guideline should apply to the sum of T-2 toxin and HT-2 toxin.

Discussion

Gastrointestinal transformation to the de-epoxy metabolite

The micro-organisms in faeces from pigs did not acquire the de-epoxidation ability during a seven week long exposure period to low levels of DON in the feed (0.8 mg DON/kg feed for 3 weeks, followed by 1.2 mg/kg feed for 4 weeks, Paper I). When the exposure ceased, the de-epoxidation ability was transferred to the experimental pigs from pigs with a known faecal de-epoxidation ability by spreading faeces in the pens. This finding indicates that the faecal de-epoxidation ability is easily transferred between pigs. In a previous study in our laboratory, de-epoxy-metabolites of DON and NIV were found in faeces incubates from pigs exposed to nivalenol in the diet for one or three weeks, while these metabolites were not detected in incubations with faeces collected prior to the exposure period (Hedman & Pettersson, 1997). It was suggested that the de-epoxidation ability may be used as an indicator of recent exposure to trichothecenes (Hedman, 1996). The transfer of the de-epoxidation ability between pigs and the fact that the faecal micro-organisms did not acquire the ability during the seven week exposure period indicate that de-epoxidation ability is not a good biomarker for recent trichothecene exposure in pigs.

The 12,13 epoxide ring has been considered to be essential for the toxicity of the trichothecenes (Wei *et al.*, 1974; Ehrlich & Daigle, 1987; Betina, 1989; Rotter *et al.*, 1996). The de-epoxides of DON and NIV were 24 and 51 times less toxic in the cell toxicity test than the corresponding toxin (Paper III). This reduction in toxicity after de-epoxidation verifies previous findings that the de-epoxides are less toxic than the trichothecenes with an intact epoxide ring. It has been shown that the de-epoxides are much less acutely toxic than the corresponding trichothecenes in a brine shrimp LC_{50} test (Swanson *et al.*, 1987b). Furthermore, no effect was observed in rats after i.p. injection of 60 mg de-epoxy T-2 toxin/kg bw, while seven out of nine rats injected with 12 mgT-2 toxin/kg bw died. De-epoxy T-2 toxin was also 400 times less toxic than T-2 toxin in the rat skin irritation assay (Swanson *et al.*, 1988).

Since the de-epoxidation is a detoxification reaction, any differences in the ability to transform trichothecenes to their corresponding de-epoxy metabolite may influence the toxicity of trichothecenes. De-epoxy metabolites of trichothecenes have been reported from urine and plasma from rats (Yoshizawa, Takeda & Ohi, 1983; Lake *et al.*, 1987), cattle (Cote, *et al.*, 1986; Yoshizawa *et al.*, 1986) and to a lesser extent in sheep (Prelusky, Veira & Trenholm, 1985; Prelusky *et al.*, 1987). Several studies have shown that the gastrointestinal micro-organisms of these species have the de-epoxidation ability (Westlake, Mackie & Dutton, 1987; Swanson *et al.*, 1987a, 1988). Trichothecenes are also transformed to the de-epoxy metabolites in incubations with faeces from some pigs (Swanson *et al.*, 1988; Kollarczik, Gareis & Hanelt, 1994; Paper I), but not in faecal incubations from other pigs (He, Young & Forsberg, 1992; Paper I). No de-epoxidation occurred when trichothecenes were incubated with liver homogenates (Ohta, Ishii & Ueno, 1977; Cote, Buck & Jeffery, 1987). It has therefore been assumed that the de-epoxidation reaction occurs in the rumen of cattle or in the gastrointestinal tract of

monogastrics before the absorption (Swanson & Corley, 1989; Rotter *et al.*, 1996). Recently, DON and conjugated DON were detected in human urine (Meky *et al.*, 2003). Other metabolites were not looked for in human urine in the study. No de-epoxidation of DON or NIV occurred in incubations of trichothecenes with human faeces, indicating that trichothecenes are not de-epoxidised in humans (Paper II).

Even if the de-epoxidation is a detoxification, the toxicological significance of the gastrointestinal de-epoxidation ability in monogastrics is not known. If a significant proportion of the trichothecenes is de-epoxidised prior to absorption or before any damage occurs on the epithelial layer in the gastrointestinal tract, the ability may significantly reduce the toxicity of trichothecenes. The reduction in feed intake in pigs is normally largest the first days and decreases after 3-7 days. The acquirement of the de-epoxidation ability after an exposure period observed earlier (Hedman & Pettersson, 1997) could explain the adaptation to DON in feed observed in pigs. However, the results from Paper I indicate that exposure to low dietary levels of DON is not sufficient for the gastrointestinal micro-organisms to acquire the de-epoxidation ability. The adaptation to trichothecene contaminated feed observed in pigs is therefore probably not the explanation to the apparent adaptation of trichothecene contaminated feed.

No de-epoxidised DON was detected in plasma and urine of pigs with a known faecal de-epoxidation ability given 3-acetylDON in the feed (Paper IV). Since de-epoxy DON is a small molecule, less polar than the parent trichothecene, it is not likely that the de-epoxy trichothecene is less absorbed from the gut than the parental toxin. A more likely explanation to the lack of de-epoxide in plasma and urine is that the de-epoxidation is too slow or occurs too far down in the intestinal tract to have any significant influence on the absorption of trichothecenes in the pig. The observed adaptation to trichothecene contaminated feed should therefore have another explanation.

A gastrointestinal de-epoxidation in the gut before absorption in some species, but not in all species, could contribute to species-differences in sensitivity towards trichothecenes. Bacteria in the rumen of cattle, which are rather resistant towards trichothecenes (Charmley *et al.*, 1993; Danicke *et al.*, 2002), are able to de-epoxidate trichothecenes (Hedman & Pettersson, 1997; Westlake *et al.*, 1987). This de-epoxidation ability in the rumen has been regarded as an important factor for cattle being relative resistant towards trichothecenes compared to other species of farm animals (Trenholm *et al.*, 1989). The risk assessments of trichothecenes in human food are based on studies in pigs and rodents (Eriksen & Alexander, 1998; SCF 1999; 2000; 2001; 2002; JECFA, 2001a). Extrapolation of data from rodents to humans may be uncertain if a significant proportion of the toxins is detoxified before absorption in rodents but not in humans. Rodents are known to be cophrophagous and no restrictions of the ingestion of faeces were described in the studies of trichothecene metabolism in rats. The presence of de-epoxidised trichothecenes in the plasma and urine of rats may be a result of the ingestion of faeces containing de-epoxidised trichothecenes, and not a de-epoxidation in the gut prior to absorption after ingestion of contaminated feed. DON and DON-glucuronide conjugate have recently been found in human urine (Meky *et al.*, 2003). The de-epoxide was not looked for in human urine in the study by Meky *et al.* (2003), but no de-epoxidation occurred in incubates with human faeces (Paper II).

Absorption, metabolism and excretion of 3-aDON in pigs

No 3-aDON was found in plasma, urine or faeces (Paper IV). Free and conjugated DON were the only metabolites detected in plasma and urine. Taken together with the faecal incubations where no acetylated toxin was found, the results indicate that acetylated trichothecenes are rapidly deacetylated. Acetylated NIV and DON are precursors of the toxin in the fungi and may occur together with the trichothecene, but the levels of the acetylated derivatives are normally rather low compared to the levels of NIV or DON. Any guideline for maximum trichothecene concentration of trichothecenes in feed should therefore apply to the sum of the toxin and the acetylated derivative, since the acetylated toxins seem to be rapidly deacetylated in the gut.

The plasma concentration of DON reached a maximum of 44-64 ng DON/ml plasma 3 hours after the start of the feeding with contaminated feed both on day 1 and day 3 of exposure. DON did not accumulate in plasma during the 3 day feeding period. The maximum plasma concentration of DON was < 25% of the maximum levels in pigs given one single intragastric dose, corresponding to twice the dose given in this study (136-325 ng DON/ml plasma, Prelusky *et al.*, 1988). The maximum plasma concentration was reached 15-30 min after exposure in the latter study.

The different method of administration may explain the rapid absorption in that study. An intra-gastric administered dose of toxin may be more easily available for absorption than toxin mixed into the feed, especially if the stomach and upper part of the gut are empty at the time of intragastric dosing. A short time from exposure to maximum plasma concentrations was also found in pigs given a single intragastric dose of 2 mg DAS/kg bw where the maximum plasma concentration of DAS and the deacetylated metabolites was found one hour after dosing (Bauer *et al.*, 1985). The maximum concentration of DAS including the deacetylated metabolites was 13-41 ng/ml plasma. Other metabolites, including conjugates, were not determined in the study. The DAS concentration in plasma relative to the dose given was low compared to the studies with DON. The high dose of DAS induced salivation and vomiting, which probably reduced the amount of toxin available for absorption. The absorption in that study is therefore difficult to compare to the other studies where no vomiting occurred.

The maximum plasma concentration of 3-6 ng /ml of NIV in pigs given 2.5 mg NIV/kg feed was reached after 2.5-4.5 hours after feeding. The absorption was prolonged and NIV was still being absorbed in pigs 16 hours after exposure (Hedman, Pettersson & Lindberg, 1997). The maximum concentration of NIV in the latter study was only about 10% of the maximum concentration of DON given the same concentration of 3-aDON in the feed (Paper IV). The apparently slower absorption NIV compared to DON may be a result of NIV being the more polar of the two toxins. The concentration of DON decreased rapidly after reaching peak values (Prelusky *et al.*, 1988; paper IV), while the concentration of NIV in plasma levelled out near the peak concentration. The slow decrease of NIV in plasma relative to DON may be caused by a slow, but prolonged absorption from the gut. It is also possible that the glucuronidation of DON facilitated the excretion of DON compared to NIV, which was not glucuronide conjugated to any detectable

extent (Hedman *et al.*, 1987). In contrast to NIV, DON did not accumulate in plasma. No difference in maximum plasma concentration between day 1 and day 3 was found, even if a low concentration of DON was found before feeding with DON at day 3.

The difference between species in sensitivity towards trichothecenes can partly be explained by differences in absorption. The absorption of DON in pigs was estimated to about 50% (Prelusky *et al.*, 1988) and the absorption of NIV was 11-43% during the first 7.5 hours after feeding (Hedman *et al.*, 1997). The absorption in less sensitive species like poultry (Prelusky *et al.*, 1986a), sheep (Prelusky, Veira & Trenholm, 1985; Prelusky *et al.*, 1986b) and cows (Prelusky *et al.*, 1984) is lower. There is no available kinetic study from rodents, but 25 % of an oral dose was recovered in urine, indicating that the absorption is higher than in ruminants.

Risk assessment of trichothecenes in animal feed

Trichothecenes are toxic to most or all farm animals, but the sensitivity towards trichothecenes varies significantly between species. The toxic response also depends on which toxin the animals are exposed to, the duration of the exposure and the dose (Trenholm *et al.*, 1989; Prelusky, Rotter & Rotter, 1994). The pig is the farm animal most sensitive to trichothecenes. Reduced feed intake at low doses, followed by vomiting and complete feed refusal at higher doses are observed in pigs given trichothecenes in the feed. In pigs, the effects occurring at the lowest levels of trichothecenes in feed are reduced feed intake and changes in the immune system. DON is less acutely toxic than other trichothecenes such as T-2 toxin and DAS, but the reduction in feed intake occurs at the same toxin level as for T-2 toxin in feed. Pigs reduce the feed intake at a lower level of trichothecenes in feed than the other species. The biochemical explanation for the reduction in feed intake is not clarified. It has been shown that both DON and T-2 toxin alter the brain levels of serotonine and metabolites thereof in various species, including rat and pig (Fitzpatrick, Boyd & Watts, 1988; Prelusky, 1993; Wang, Fitzpatrick & Wilson, 1998). No such changes in the seritonergic signalling system in the brain were detected in the chicken brain as opposed to rats (Fitzpatrick *et al.,* 1988). In chickens, the DON treatment resulted in a decrease in norepinephrine in the hypothalamus and hippocampus, and a decrease in dopamine in the pons and medulla oblongata region. The results suggested that DON influences brain biogenic amine metabolism, and that there may be interspecies differences in the central effects of this mycotoxin (Fitzpatrick *et al.*, 1988). These differences may explain the differences in tolerance towards the effect on feed intake between different species (Rotter *et al.*, 1996). The reduced feed intake at the lowest levels of trichothecenes is a temporary effect, while the effect is permanent at higher toxin levels. Reduced feed intake occurs at lower levels of DON in feed when naturally contaminated feed is used than in studies where pure DON is added to the feed (Paper V). The explanation for this is not known. Acquirement of a gastrointestinal de-epoxidation ability was a possible explanation, but exposure of pigs to low levels of DON failed to lead to an acquirement of the de-epoxidation ability (Paper I). Furthermore, the gastrointestinal de-epoxidation ability is probably present in the lower parts of the gut, having no major influence on the toxicity of trichothecenes (Paper IV). Another possible explanation of the

difference in toxicity between naturally contaminated feed and pure toxin is a difference in nutritional value. Growth of *Fusarium* fungi has been shown to change the nutritional values of the feed (Williams *et al.*, 1992a; Williams *et al.*, 1992b). A difference in nutrition of the pigs may have an influence on the results from the feeding studies. Furthermore, there are indications that the effects of DON on feed intake in pigs may be influenced by factors such as age, body weight, sex, feeding period, feeding system, diet, etc (Lauber, Dillenburger & Drochner, 2001). Pigs are also more genetically heterogeneous than rodents, which may give larger individual differences. Individual differences will influence the results in pig experiments since the number of animals used is normally low, due to practical and economical reasons. It is also possible that other substances produced by the fungi influence the toxicity of DON, either some undetected toxic substance present in the feed, or some non-toxic substance that may enhance the effects of DON. It is interesting to note that high doses of fusaric acid, which is also produced by *Fusarium*, also cause an increase in brain serotonine and serotonine metabolites as observed for DON (Smith & MacDonald, 1991). It was also shown that the feed intake in pigs decreased with increasing levels of fusaric acid in a DON contaminated pig feed. The authors concluded there was a synergistic effect of the two substances on feed intake and weight gain in pigs (Smith, McMillan & Castillo, 1997). The effect of other anorexic compounds with a central seritonergic mechanism also diminishes with time (Silverstone, 1992).

Ruminants are rather resistant towards trichothecenes compared to monogastric animals. It is generally considered that the de-epoxidation activity in the rumen plays a significant role in the protection of cows to trichothecenes (Prelusky, Rotter & Rotter, 1994; Paper V). In the available feeding experiments with cows, effects of trichothecenes have only been found at high concentrations of toxins in the feed. Field outbreaks of a haemorrhagic disease in cattle were associated with T-2 toxin in the feed. The symptoms included feed refusal, decreased milk yield, hair loss, delayed clotting and prothrombin times, bloody diarrhoea and haemorrhages of the mucous membrane (Hsu *et al.*, 1972; Petrie, Robb & Stewart, 1977). The symptoms have been reproduced experimentally when acute toxic doses are used, and not in experiments using concentrations that may realistically be found in feed (review in Prelusky, Rotter & Rotter, 1994). The haemorrhagic problems have only occurred in the field and it is probable that other trichothecenes than the reported T-2 toxin or some other unknown substance were involved in the outbreaks.

It is concluded that ruminants are quite tolerant towards trichothecenes and unless the feed or feed ingredients are visibly damaged by mould, it is not likely that any toxic effect of trichothecenes will occur.

Chickens are more sensitive to trichothecenes than ruminants. Turkeys may be more sensitive towards trichothecenes than chickens (Richard *et al.*, 1978). Conflicting results have been reported from studies of the ability of gastrointestinal micro-organisms from chickens to de-epoxidate trichothecenes. He *et al.* (1992 and Swanson *et al.*, (1988) found that the trichothecenes were de-epoxidised in incubations with chicken faeces, while no de-epoxides of T-2 toxin or metabolites were found in excreta from chickens exposed to T-2 toxin (Visconti & Mirocha, 1985). More than 50% of the NIV found in faeces from hens fed pure NIV in the feed was de-epoxidised (Garaleviciene, Pettersson & Elwinger, 2002).

Neither NIV nor de-epoxidised NIV was detected in plasma from the hens. The toxicological significance of the gastrointestinal de-epoxidation ability in poultry has not been verified. Symptoms found in chickens and hens after exposure to trichothecenes include reduced feed intake and weight gain, oral lesions, necrosis of lymphoid, haematopoietic, and mucosal tissues, possible neural disorders expressed as abnormal wing positioning, hysteroid seizures, impaired righting reflex and abnormal feathering.

Conclusions

- The ability of gastrointestinal micro-organisms to transform trichothecenes to their de-epoxy metabolite is common in pigs at commercial pig farms in the Uppsala area, at least it was in the year of investigation.

- Pigs do not acquire the gastrointestinal de-epoxidation ability after exposure to low levels of DON in the feed. The gastrointestinal de-epoxidation ability is easily transferred with bacteria between pigs. The ability of faecal micro-organisms from pigs to de-epoxidate trichothecenes is therefore not a good indicator of recent trichothecene exposure.

- No de-epoxidation activity was found when samples of human faeces were incubated with trichothecenes, but faeces from more individuals need to be screened to conclude about the presence of a human de-epoxidation ability. Factors such as the diet may have an influence on the results.

- De-epoxy metabolite are not present in detectable concentrations in urine or plasma samples even when pigs with a gastrointestinal microflora able to de-epoxidate trichothecenes are exposed to trichothecenes.

- The maximum plasma concentration of type B trichothecenes in pigs is reached about 3 hours after feeding. Acetylated DON is rapidly deacetylated to DON and 3-aDON is not present plasma, urine or faeces in pigs fed 3-aDON. DON and glucuronide-conjugated DON are the only known metabolites in urine and plasma, but de-epoxidised DON can be found in addition to DON in faeces. DON does not accumulate in pig plasma during a three days feeding period.

- The de-epoxides are considerably less cytotoxic than the corresponding toxins with an intact epoxide ring.

- Ruminants are quite resistant towards trichothecenes and no toxic effects are expected when the animals are not given visibly damaged feed. There are probably no need for a guideline value for trichothecenes in feed for ruminants

- Altered organ weights have been found in chickens given a feed containing 9 mg DON/kg feed, while no effect was found in chickens given 5 mg DON/kg feed. Oral lesions have been observed in chickens given 1.0 mg T-2 toxin/kg feed. Based on these studies we propose a guideline of 2.5 mg DON and 0.5 mg T-2 toxin /kg chicken feed.

- Reduced feed intake in pigs has been observed at DON concentration in feed from 0.6 mg DON/kg, while the reduction in feed intake occur at DON levels from 3-6 mg DON/kg feed when pure DON has been added to the diets. Reduced immune response has been found in pigs given 1.8 mg DON/kg feed. At concentration of 0.5 mg T-2 toxin/kg feed both decreased feed intake and reduced immune response in pigs. Guideline values of 0.3 mg DON/kg feed and 0.2 mg T-2 toxin/kg feed is proposed.

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