Nutritional and Toxicological Effects of Fusarium Metabolites: Zearalenone Metabolism in the Rat

by

Mary Catherine Kiritsy

A thesis
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ABSTRACT

Zearalenone is a common contaminant of mold origin found in animals feeds. Consumption of zearalenone is associated with reduced feed intake. Our experiment was designed to study the effect of this reduced intake on the metabolism and toxic expression of zearalenone. Weanling female Sprague-Dawley rats were fed a standard diet either ad libitum or 75% ad libitum intake for 14 days. In the Enzyme Study, the enzyme activity of 3αhydroxysteriod dehydrogenase (HSD) and uridine diphosphate (UDP) glucuronyl transferase, the zearalenone metabolizing enzymes, were determined. Feed restriction altered liver enzyme activity, 3αHSD activity increased 28% (7.7 to 9.9 nM/min/mg protein), while UDP glucuronyl transferase activity increased 79% (52.5 to 93.8nM/hr/mg protein). In the Metabolic Study, animals were orally dosed with 1mg zearalenone/kg body weight. Zearalenone and its metabolites were measured in the urine and feces. Alterations in enzyme activity were reflected in urinary and fecal metabolites. Feed restriction resulted in more free and conjugated zearalenone being excreted in the urine, with a corresponding decrease in αzearalenol and fecal metabolites produced. No conjugated metabolites were found in the feces. In the Toxicity Study, animals were fed diets containing 0 to 150ppm zearalenone for 3 weeks.

Growth and organ weights were determined. Feed restriction ameliorated zearalenone's toxic expression. These results indicate that nutritional regime effects zearalenone metabolism, excretion and toxicity.

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Chapter I

REVIEW OF LITERATURE

1.1 INTRODUCTION TO MYCOTOXINS AND MYCOTOXICOSIS

1.1.1 General Characteristics

The term mycotoxin refers to a large number of secondary metabolites produced by molds. They are toxic upon ingestion and the respective illnesses attributed to their consumption by man and animals are mycotoxicosises (Wilson, 1982). The severity of the disease ranges from reduced feed consumption, to productivity declines, to death in extreme cases (Mirocha et al., 1971). Mycotoxicosis are characterized by the following features:

1. The disease is nontransmissible.
2. Treatment with antibiotics has little effect.
3. The disease is often seasonal, since certain conditions favor mold growth.
4. The disease is usually associated with the consumption of a specific feedstuff.
5. The suspected feed usually shows signs of moldiness, which may be less apparent after processing (Harwig and Munro, 1975).

The molds that produce toxins are widespread in nature and only need suitable environmental conditions to grow. Molds may grow on crops in the field or in storage after harvesting, and during this growth process may produce mycotoxins. Mold growth results in changes in the physical consistency of the plant and causes decreased nutritional value of the infested crop. The presence of visible mold growth does not mean that there are mycotoxins present. However, there may be mycotoxins in feed even though mold is not visible. Mycotoxin production depends on the presence of mold spores, available substrate and specfic environmental conditions (Wilson, 1982; Harwig and Munro, 1975).

1.1.2 Documentation of Mycotoxicosis

Contamination of feeds and foodstuffs by mycotoxins is not a new problem, although it has become better documented in the last two decades. One of the first recorded incidents of mycotoxicosis was ergotism. It was characterized by gangrenous degeneration of the lower extremities and hallucenogenic mental aberations, leading to its common name St. Anthony's Fire. Ergotism occurred when rye grain contaminated with Claviceps purpurea were ingested (Wilson, 1982).

Joffe (1971) described outbreaks of alimentary toxic aleukia (ATA) during the 1940's in Russia. Crops were left

over the winter in the fields and were invaded by Fusarium molds. Consumption of these crops resulted in thousands of deaths.

In 1960, an outbreak of mycotoxicosis in England awakened interest in molds and feed saftey. The disease, called Turkey X Disease, resulted in the death of over 100,000 turkey and chicken poults who had consumed contaminated peanut meal. We now know that these deaths were due to aflatoxins present in the diet (Buckle, 1983).

Mycotoxin outbreaks have occurred in Canada in recent years. A large Fusarium mold problem in Southern Ontario and Quebec in 1980 resulted in the downgrading of the wheat crops and livestock death (Trenholm et al., 1981a). The mycotoxin deoxynivalenol (DON) was found in Manitoba wheat in the 1984 and the 1985 crop.

Recently, Schoental (1983) observed an increase of precocious sexual development in children in Puerto Rico. He suggested that it may be due to the consumption of the mycotoxin zearalanol found in agricultural products.

1.2 INTRODUCTION TO ZEARALENONE

1.2.1 Chemical and Physical Properties

Zearalenone is a naturally occurring mycotoxin produced by Fusarium mold species roseum, and other species in the Gibberella zea complex: F. tricinctum, F. gibosum, F.

roseum equiseti, F. roseum culmorum and F. roseum graminearum (Mirocha et al., 1977). Zearalenone acts as a hormone. It regulates the sexual stage in F. roseum acting as a primary messenger, binding to a cytosolic protein, releasing cyclic 3'-5'-adenosine monophosphate (c-AMP), the normal secondary messenger in the reproductive stage.

Zearalenone (Figure 1) has a molecular weight of 318 and a melting point of 164-165°C. Zearalenone is insoluble in polar solvents and soluble in nonpolar solvents. In spite of zearalenone's large lactone ring, it is remarkably resistant to hydrolytic cleavage. This may be due to the methyl group, which hinders nucleophillic attack on the lactone carbonyl. The double bond between the C-1', C-2' is stable under bromination, epoxidation and hydroboration (Mirocha, 1971).

Reduction of zearalenone results in two isomers of zearalenol, with the C-6' hydroxyl group in the α or β configuation. The melting point of αzearalenol is 168-169°C and βzearalenol is 174-176°C. A man made derivative, zearalanol, is manufactured from zearalenone and is used as a growth promoting implant for beef cattle. Zearalanol has no double bond between C1'-C2' and is found in both the α and β configuration (Mirocha, 1971).

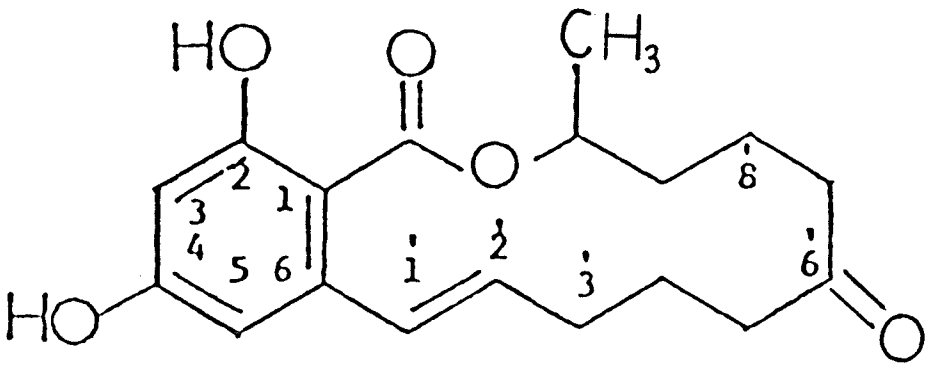


Figure 1: Zearalenone 6-(10-hydroxy-6-oxo-trans-1-undecenyl) ßresorcylic acid lactone

1.2.2 Occurrence and Optimum Growth Conditions

Zearalenone has been observed worldwide. It has been detected in corn, barley, oats, sorghum, sesame seed, cornflakes, white corn meal, maize beer, hay, corn silage and commercial rations. Corn is the most commonly contaminated product (Mirocha, 1971).

The temperate Canadian climate provides excellent growing conditions for Fusarium molds and acts to stimulate zearalenone production. In a moist environment, low temperatures or alternately moderate and low temperatures stimulate Fusarium mold growth and toxin production. Mirocha et al. (1977) observed that the key enzyme system for zearalenone production was turned on at temperatures of 12-14°C. Under laboratory conditions, corn was incubated at 24-27°C for one week to ensure mold growth. Mycotoxins were produced when the mold was then incubated at lower temperatures (12-14°C) for a few more weeks. However, once the enzyme system was activated, optimum toxin production could be attained at higher temperatures (27°C).

The environmental conditions that maximize zearalenone growth in the lab are similar to conditions that often occur in the field. In the corn belt areas of the midwestern United States and Eastern Canada, corn is normally harvested dry. It is stored on the cob in open wire mesh cribs where it is exposed to the elements. Sutton et al. (1979)

observed that zearalenone contamination of corn was was proportional to the rainfall. The higher the rainfall, the greater the zearalenone infestation. If there is a wet mild autumn, or if snow reaches overwintered corn, the moisture content of the corn may increase to as much as 22-30%. This allows fungi to proliferate within the tissue.

Temperature also plays a role in zearalenone production. With decreasing autumn temperatures, optimum conditions exist for zearalenone biosynthesis or the induction of zearalenone synthesizing enzymes. Cool autumn night temperatures promote the induction of the zearalenone synthesizing enzymes, while the warm days act to maximize zearalenone production (Mirocha et al., 1971).

1.3 BIOLOGICAL PROPERTIES OF ZEARALENONE

1.3.1 Estrogenicity in Animals

Ingestion of zearalenone contaminated feed by swine causes an estrogenic syndrome called vulvovaginitis. The physical symptoms associated with vulvovaginitis are; edema; swelling and reddening of the vulva; increased secretion in the vagina; prolapse of the vagina, which may cause prolapse of the rectum in severe cases; metrorrhagia; increased secretion and size of the uterus; and lactation of the mammary glands in immature, as well as ovarectomized females and castrated males (Mirocha et al., 1971).

This estrogenic syndrome may also result in reduced litter size, neonatal mortality and infertility (Nelson et al., 1973). There may be inhibition of ovulation, implantation and suppression of gonadotrophic hormone release (Hidy et al., 1977 ; Hobson et al., 1977). Male swine may undergo a feminizing effect, including atrophy of the testes and mammary gland enlargement. Stunting of growing pigs, both male and female has occurred when animals are fed zearalenone at extremely high levels (500-600ppm, Nelson, 1979).

McNutt et al. (1928) were the first to link molded corn to vulvovaginitis in swine. However, it was not until Stob et al. (1962) reported their findings that zearalenone contaminated feed caused vulvovaginitis, that the toxic agent of the mold was identified. They isolated zearalenone from cultures of Gibberella zea and observed that it had marked anabolic and uterotrophic activity when given to mice. Administration of zearalenone caused estrogenism in laboratory animals. Swine are the most sensitive animal tested to zearalenone toxicosis (Mirocha, 1977).

1.3.2 Biological Activites

Zearalenone is a phenol, but acts to produce the same biological responses of the natural sex and corticoid hormones (Mirocha, 1977). The biological potency of zearalenone and its metabolites has been measured in vivo.

When compared to other estrogens, zearalenone has been classified as moderately estrogenic in swine, weakly estrogenic in mice and relatively nonestrogenic in poultry (Stob et al., 1962; Allen et al., 1981; Stob, 1983).

In a study by Greenman et al. (1979) using cell-free preparations of uteri from immature BALB/c mice, both zearalenone and zearalenol inhibited [H^3] estradiol-17β binding to specfic sites in the cytosol. When intact uteri were incubated, these compounds caused the translocation of specfic estrogen binding sites into the nuclei that were exchangeable with the estradiol. These findings suggest that the uterotrophic effects of zearalenone and its derivatives are mediated through their association with estogen receptors in the uterus.

Similar studies by Katzenellenbogen et al. (1979), using epimeric zearalenone and zearalenols, looked at the direct estrogen receptor interactions and the biological activites of these compounds in the immature rat uteri. All compounds were found to compete with estradiol for cytoplasmic receptor binding and they caused translocation of estrogen receptor sites into the nucleus. Zearalenol is four times as estrogenic as the other zearalenone metabolites (Mirocha, 1977). Zearalenol was more effective in inhibiting estradiol binding and stimulating the translocation of estrogen binding sites into the nuclei than was zearalenone. This may account for the difference in biopotencies between the compounds.

Zearalenone and the zearalenols act to increase the permeability of the uterine cells which allows for an increased uptake of amino acids and induces DNA and RNA synthesis (Katzenellebogen et al., 1979; Ueno and Yagasaki, 1975). They act to alter the utilization of dietary amino acids by promoting the synthesis of uterine protein, increasing uterine size. Zearalenone appears to alter protein utilization by diverting amino acids from muscle protein synthesis to protein in the uterus. It is unknown if liver and kidney enlargement in rats is due to a direct anabolic action of zearalenone (Smith, 1982b).

A molecular basis for the mycotoxin receptor interaction has been postulated by Powell-Jones et al. (1981). The researchers suggested that there is a folding of the toxin molecule such that hydroxyl or potential hydroxyl groups become oriented to facilitate binding to unoccupied receptor sites.

These studies indicate that the resorcylic acid lactones have similar biological and biochemical responses as the estrogen estradiol. Zearalenone mediates its toxic effects by competing with estrogen for receptor sites and increasing protein synthesis.

1.4 METABOLISM OF ZEARALENONE

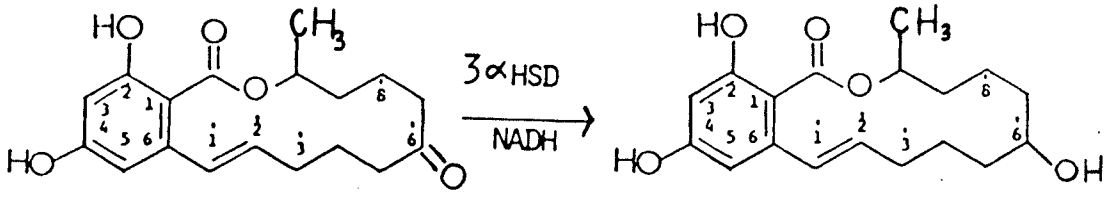
1.4.1 Molecular Weight Basis for Metabolism

The molecular weight of a compound effects its metabolism and excretion (Matthews, 1981). There appears to be a threshold for urinary excretion of 325±50 in rats. Compounds with molecular weights below this threshold are excreted in the urine and compounds above this threshold are excreted in the bile. Zearalenone has a molecular weight of 318. Since zearalenone has a molecular weight near the threshold it should be found in both the urine, and the feces via biliary excretion. However, the low solubility of zearalenone makes it an unlikely candidate for urinary excretion (Hidy et al., 1977).

Zearalenone is metabolized by two different reactions, a reduction to an alcohol and a conjugation with a glucuronic acid (Kiessling and Pettersson, 1978). Reduction and conjugation of zearalenone increases its solubility and may promote its excretion in the urine. Conjugation of zearalenone also increases its molecular weight, promoting biliary excretion. The metabolism of xenobiotics differs with sex, age and species (Ueno et al., 1983; Figure 2).

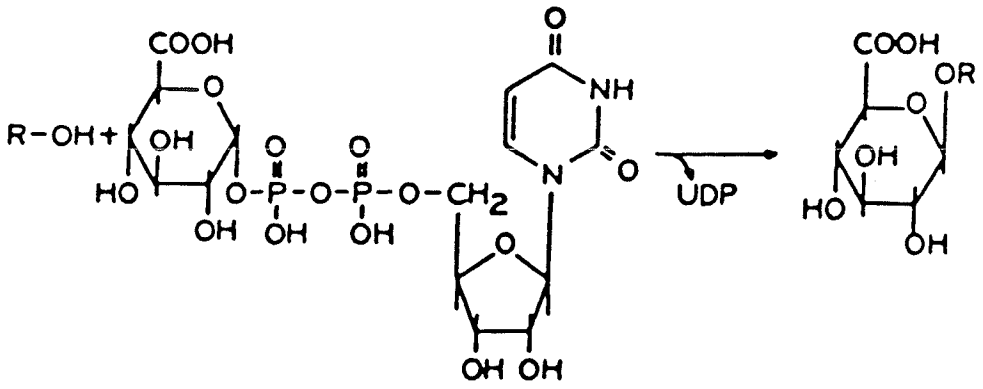
ZEARALENONE IS METABOLIZED BY TWO DIFFERENT ROUTES:

1. REDUCTION OF ZEARALENONE TO ZEARALENOL, A REACTION CATALYZED BY A NADH DEPENDENT 3α HYDROXYSTEROID DEHYDROGENASE



ZEARALENONE ZEARALENOL

2. CONJUGATION OF ZEARALENONE BY A GLUCURONIC ACID (UDP GLUCURONIC ACID) TO MAKE ZEARALENONE MORE SOLUBLE



ZEARALENONE + UDP GLUCURONIC ACID ⟶ UDP + R-O-GLUCURONIC ACID

KIESSLING AND PETTERSSON, 1978

Figure 2: Metabolism of Zearalenone

1.4.2 Reduction

The reduction of zearalenone to zearalenol takes place at the keto group in the 6' position. This reaction is similar to the steroid reaction (androsterone to 5*α*androsterone-3-17-dione) by 3*α*hydroxysteroid dehydrogenase (3*α*HSD) in the presence of NAD (Kiessling and Pettersson, 1978). In a mixed substrate assay, the strong inhibition of the formation of zearalenol from zearalenone by androsterone indicates that zearalenone reduction is catalyzed by the same enzyme (Olsen et al., 1981).

Several enzymes have the ability to reduce zearalenone to zearalenol. Three *α*HSD is not one single well defined enzyme (Olsen et al., 1981). Ueno et al. (1983) observed two reductase enzymes, one active at pH 4.5 and the other active at pH 7.4. In vitro, the enzyme at the acidic pH is most active and it is found in the microsomal portion of the cell. The cytosolic fraction contains the enzyme active at the physiological pH. Ueno et al. (1983) speculated that 90% of the zearalenone reductase activity was tightly bound to the microsomes. The microsomal enzyme reduces zearalenone to *α*zearalenol only, while the cytosolic enzyme reduces zearalenone to both *α* and *ß* configurations. Hoff and Schiefers (1973) observed that there are two different cytosolic and three different microsomal 3*α*HSDs. One of the cytosolic and two of the microsomal enzymes found in males are different from those found in females.

A study by Olsen and Kiessling (1983) showed that the reducing capacity of the liver differs between species and is dependent on the presence of NAD. They observed swine to have the greatest capacity to reduce zearalenone. The reduction pathway is of lesser quantitative importance than the conjugation route, but of great physiological importance, since the zearalenols exhibit greater biological activity than zearalenone (James et al., 1981).

1.4.3 Conjugation

Uridine diphosphate (UDP) glucuronyl transferase catalyzes the transfer of glucuronic acid from UDP glucuronic acid to an acceptor alcohol. This enzyme is nonspecific, it catalyzes the glucuronidation for many endogenous and xenobiotic compounds. They are formed directly from UDP glucuronic acid and an acceptor by the action of the enzyme. The enzyme is both activatable, essentially an in vitro phenomenon, and inducible by administration of a xenobiotic (Mulder et al., 1981). Zearalenone and the reduced forms of zearalenone can be conjugated. There is a minor competition for zearalenone between conjugation and reduction enzymes (Kiessling and Pettersson, 1978).

UDP glucuronyl transferase is tightly bound to liver endoplasmic reticulum and exhibits latency in fresh preparations. Two models have been proposed to explain the

release of the enzyme from the membrane (Bingham et al., 1982). Model one assumes that the enzyme is embedded in the luminal face of the endoplasmic reticulum. Detergent activation destroys the membrane allowing glucuronic acid to gain access to the enzyme via a thiol-dependent permease. Model two assumes the enzyme is noncompartmentalized and allosteric, but constrained by phospholipids in such a way that its activity is low. During membrane perturbation, the positive allosteric effectors convert the enzyme into its activated conformation. Activations of the enzyme are not additive and increasing perturbation leads to optimal activation, then to inactivation (Dutton et al., 1981).

1.5 METABOLIC STUDIES INVOLVING ZEARALENONE

1.5.1 Physiologoical Versus Pharmacological Dosing

The use of pharmacological doses in toxicity experiments may result in erroneous conclusions. Overdosing animals results in metabolic overload, the detoxification enzymes are swamped, consequently the rate and the route of metabolism and/or excretion may be significantly altered resulting in the toxic expression of the test compound (Kraybill et al., 1977; Mirocha et al., 1983). The route of dosing affects xenobiotic metabolism. The LD_{50} for zearalenone after intraperitoneal injection in the rat is 5.5g/kg body weight and greater than 10g/kg body weight for oral administration (Hidy et al., 1977).

Conflicting reports concerning the route of zearalenone excretion and the major excretory metabolites have been made. This disagreement may be due to different analytical methods employed, each with its own limitations; problems with the stability of radiolabelled compounds; different dose levels for cold studies and differences between the sex, age and species studied (Trenholm et al., 1981b; Smith, 1982a; Olsen and Kiessling, 1983).

1.5.2 Studies Involving Rats

Table 1 summarizes studies on zearalenone metabolism in rats. In a study by Mirocha et al. (1976), 8 rats were given a pharmacological dose, 10mg/kg body weight of zearalenone (1mg/rat), by stomach intubation. Urine was collected every 24 hours for 5 days. Only free (27.5%) and conjugated (40.95%) zearalenone were found, with 67.4% of the total dose recovered. Most of the dose was excreted within the first 48 hours.

Additional experiments conducted by Mirocha et al. (1975) used [H^3] zearalenone to determine the distribution of the mycotoxin in the tissue and excreta. Animals were given 1mg of zearalenone by stomach intubation and sacrificed at 0.5, 2, 6, 12 and 24 hours. No appreciable amounts of radioactivity were found in the brain, kidney, spleen, ovary or uterus. The stomach and the intestine contained the bulk of the radioactivity during the first 6 hours and was later

Table 1 Studies Involving Rats

Author	n	Route of Dose	Amount of Dose	Urinary Excretion (%) Free	Bound	Total	Fecal Excretion (%) Total	Time (hrs)	Label	Recovery (%)
Mirocha et al. (1976)	8	oral	10mg/kgbw	27.6	40.95			120	no	67.4
Mirocha et al. (1975)	?	oral	10mg/kgbw			60.5	4.7	24	H^3	65.2
Ueno et al. (1977)	2	oral	10mg/kgbw			4-6	40-60	120	H^3	44-66
Baldwin et al. (1977)	?	oral	?			20-30	70-80	48	C^{14}	?
James et al. (1981)	6	oral	10mg/100gbw	71	29			48	no	50-80
Smith (1980a)	12	oral	10mg/100gbw			31.1	62.3	96	H^3	93.4
Smith (1982b)	10	oral	10mg/100gbw	2	1		22	48	no	50-80

recovered in the urine. After 24 hours, 60.5% of the dose was recovered in the urine and 4.7% in the feces. They attributed the radioactivity found in the feces to be the result of contamination with the urine.

The findings of Ueno et al. (1977) were not in agreement with the Mirocha et al. (1976) study. They intubated 2 rats with 10mg/kg body weight of [H^3] zearalenone (1mg/rat) and found 4-6% of the dose recovered in the urine with 40-60% in the feces. In the feces, the major metabolite was identified as zearalenone (38-56%) and the minor metabolite as zearalenol (4-6%). Conjugated and free zearalenone derivatives were also found in the urine. Conjugated zearalenone was the major urinary metabolite. The [H^3] labeling procedure did not allow for quantification of metabolites. Baldwin (1977) reported that 70-80% of an oral C^{14} labelled zearalenone dose to rats was excreted in the feces with 20-30% found in the urine.

In a metabolic study by James et al. (1981) 6 rats were intubated with a massive dose, 10mg/100g body weight of zearalenone (10mg/rat). Urine was collected for 48 hours. The major urinary component was free zearalenone (56.5%), followed by conjugated zearalenone (25.6%) and free αzearalenol (9.3%). The free forms exceeded the conjugated forms for all metabolites by at least 100%. The free metabolites constituted 71% of the dose recovered, while the conjugated forms made up 29%. These results must be viewed

with caution. The authors state that their recovery rate ranged from 50-80% and less than 3% of the total dose given was recovered. Variability between animals was large and was increased due to difficulties ensuring total collection of urine over the entire collection peroid. The finding that zearalenone is metabolized to conjugated zearalenone more than to the free or conjugated alcohol forms agrees with enzyme studies done by Kiessling and Pettersson (1978).

Smith (1982a) orally dosed 10 rats with 10mg/100g body weight of zearalenone. Urine and feces were collected for 48 hours. They found that urinary free zearalenone and metabolites exceeded the conjugated forms, with free zearalenone being the major urinary metabolite (56.6%) followed by conjugated zearalenone (25.6%). Excretion of zearalenone and metabolites was 10 times greater in the feces than in the urine. Almost all of the metabolites recovered in the feces were free zearalenone. No conjugated zearalenone nor metabolites were observed in the feces. Only 3% of the total dose was recovered in the urine and 22% in the feces. These findings were different from previously published [H^3] zearalenone studies (Smith, 1980a).

In his 1980a study, Smith orally dosed 12 rats with 10mg/100g body weight of [H^3] zearalenone prepared using Mirocha et al. (1977) methods. Urine and feces were collected for 4 days and he observed 62.3% of the dose in the feces and 31.1% of the dose in the urine. In this

study, zearalenone metabolites in free and conjugated forms were not anaylzed for. They attributed the discrepancy in the excretion profiles to the exchangeability of the [H^3] label (Smith, 1982a).

In an autoradiographic study of [H^3] zearalenone, 2mg/100g body weight was given to mice by iv injection (Applegren et al., 1982). The study showed a rapid excretion of radioactivity into the bile as well as the urine, 20 minutes after injection. The significance of these findings is that zearalenone can be found in the feces through biliary excretion.

1.5.3 Studies Involving Swine

Studies on zearalenone metabolism involving swine are summarized in Table 2. Olsen et al. (1985) fed one prepubertal gilt 192ug of zearalenone/kg body weight/day for 4 days and plasma and incomplete urine collections were made over the dosing period. They observed zearalenone to be rapidly metabolized to the α alcohol form with plasma levels of this derivative to be 3-4 times greater than zearalenone. Urinary excretion of zearalenone and αzearalenol were approximately the same and traces coud be found in the urine for 4 days after the treatment had stopped. Almost all the compounds found in both the urine and plasma were conjugated to glucuronic acid. No βzearalenol was found in urine nor plasma.

Table 2 Studies Involving Swine

Author	n	Route of Dose	Amount of Dose	Time (hrs)	Label	Recovery (%)
Olsen et al. (1985)	1 prepubertal gilt	oral	192 ug/kgbw daily	96	no	?
Mirocha et al. (1981)	1, 3 week old piglet	oral	90 mg/kgbw	96	no	5
Farnworth and Trenholm (1981)	12 young male and female pigs	oral	0, 3.5, 7.5 and 11.5 mg/kgbw	72	no	83.7 for urine 56 for feces
Farnworth and Trenholm (1983)	24 young male and female pigs	oral	0, 5, 10 and 15 mg/kgbw daily	96	no	?

The work of Olsen et al. (1985) does not confirm the earlier work reported by Mirocha et al. (1981). They intubated 1, 3 week old pig with 90mg/kg body weight zearalenone, and urine was collected daily for 4 days. Most of the zearalenone and metabolites, in free and conjugated forms, were excreted during the first 48 hours. Free zearalenone was found to be the major urinary constituent (43%), followed by conjugated αzearalenol (28%) and conjugated zearalenone (20%). Of the total urinary zearalenone, 31% was in the conjugated form. With both zearalenol diasteriomers, the conjugated forms predominated (87% for α and 79% for ß). However, less than 5% of the total zearalenone administered was recovered. Final urinary distribution of metabolites was 63% zearalenone, 32% αzearalenol and 5% ßzearalenol.

Differences betwen these two studies can be attributed to the fact that Mirocha et al. (1981) used a piglet and Olsen et al. (1985) used an adult female pig. While no work has been done in pigs, work in rats indicates that sexually immature rats have lower activity of 3αHSD than mature rats. This may due to lower levels of circulating steroids (Hoff et al., 1977).

Farnworth and Trenholm (1981) looked at the effects of a single oral dose of zearalenone on excretion in 12, young, 20-30kg female pigs. Dose levels were more realistic in terms of contamination levels in the farm situation, but

still able to produce detectable levels of zearalenone in the body. Doses of 0, 3.5, 7.5 and 11.5mg/kg body weight (corresponding to 0, 70, 150 and 230ppm zearalenone in the field) were given to 3 pigs at each dose level. The study analyzed for only free zearalenone in the feces and free zearalenone and free αzearalenol in the urine.

The data for 3 animals indicates that zearalenone was probably rapidly absorbed, reaching maximum blood levels 20-30 minutes after dosing. Then the zearalenone was quickly redistributed, metabolized and/or excreted, as suggested by the rapid decrease in plasma concentrations of zearalenone after 60-90 minutes. The remaining animals in the study showed delayed absorbtion. The differences in the absorbtion patterns have been attributed to the fact that animals were allowed free access to food before and after dosing. The unequal dissolving of the gelatin capsule used to administer the zearalenone could have also had an effect (Farnworth and Trenholm, 1981).

Much of the original dose was recovered in the feces. Free zearalenone was found in the feces only at 24 hours. They speculated that high levels of zearalenone in the feces were a combination of unabsorbed zearalenone and zearalenone excreted in the bile. The main excretory metabolite in the urine was free αzearalenol. Free zearalenone was also found in the urine along with detectable, but not measurable amounts of βzearalenol. The recovery rate for the urine was

83.7%. Limitations of this study include only a 56% recovery rate for zearalenone in the feces, and that the urine data is based on collections from only 2 animals at three different times (Farnworth and Trenholm, 1981).

Farnworth and Trenholm (1983) dosed 12 male pigs (9-13kg) and 12 female pigs (7-20kg) orally with 0, 5, 10 or 15mg/kg body weight zearalenone. In all but 1 animal, zearalenone was found in the plasma 1 hour after dosing. After 1-2 hours, the concentrations of zearalenone in the plasma exceeded that of αzearalenol. Peak plasma zearalenone occurred at 4 hours and subsequently decreased, while αzearalenol plasma concentrations continued to increase up until 7 hours post dosing. No βzearalenol was found in the plasma. Except for the 5mg group, female animals had lower levels of conjugated zearalenone than males receiving the same dose.

Urine samples were collected only from male pigs. Only zearalenone and αzearalenol were found, although a small peak with a retention time similar to βzearalenol appeared on some samples. This peak was probably due to the addition of phenol red during the assay extraction procedure. Both free and conjugated zearalenone concentrations exceeded those of the αzearalenol counterpart for the entire collection period. It was clear that these compounds were increasing in concentration throughout the observation period. The limited number of samples made it impossible to

establish the percentages of these compounds in the urine, or to relate metabolites in the plasma to those in the urine. Other limitations of this study include the collection of urine from male pigs instead of female pigs.

This study was not sufficent to establish differences in zearalenone metabolism between male and female pigs. However, it should be noted that only at the low dose level, the females had higher plasma zearalenone and metabolites than males at the same dose (Farnworth and Trenholm, 1983). Young female pigs are more sensitive even to relatively low levels of zearalenone in a single oral dose, due to a limited ability to metabolize zearalenone, and the estrogenic effect of zearalenone (Ruhr et al., 1983).

The formation of the zearalenone glucuronide, followed by excretion via the urine, appears to be the main route of elimination in pigs, since both plasma and urinary levels of conjugated zearalenone exceeded conjugated αzearalenol. This would be consistent with the metabolism of estrogen, where the conjugated forms predominate in the urine (Levitz and Young, 1977).

1.6 NUTRITIONAL TOXICOLOGY

1.6.1 Introduction

Nutritional toxicology is an area of research which focuses on toxic agents in the diet and their interrelations with nutrition. This includes the diet as a source of toxins, the effects of toxins on nutrients and nutritional processes, the effects of nutrients and normal metabolism on toxins and the scientific basis for regulatory decisions affecting toxicological saftey of dietary components. Toxicants may change nutrient intake, digestion, absorbtion, transport, activation, function, metabolism or elimination. On the other hand, food consumption, meal timing, nutrient intake and nutritional status may alter the actions, potencies and detoxification of toxins (Hathcock, 1982).

Nutritional adequacy is needed for the normal functioning and the metabolic detoxification of foreign compounds. Deficiencies of proteins, lipids (total and essential fatty acids) and vitamins and minerals, reduce the body's ability to detoxify and excrete xenobiotics and act to increase the toxic expression of these compounds. Other nutritional deficiences increase xenobiotic metabolism, such as energy, iron and thiamin, and therefore reduce toxicity (Hathcock, 1982).

Along with altered detoxification or biotoxification of compounds, nutritional deficiences may alter the primary

susceptability of cells to toxicants. A decrease in the biochemical or structural integrity makes cells more easily damaged by toxicants. Deficiencies cause decreased rates of cellular replacement, decreased enzyme synthesis and activation, and decreased coenzyme synthesis resulting in increased susceptibility to toxins (Wattenberg, 1975).

Many toxins can decrease food intake. Most toxic responses include growth depression, lethargy, metabolic inhibition and anorexia (Hathcock, 1982).

The detoxification process takes place in two steps: phase I which is primarily oxidative, where polar groups are introduced to the molecule; and phase II, predomiately conjugational, in which the polar metabolic or original products are linked with highly water soluble endogenous products (Ariëns and Simonis, 1982). These products are then excreted via the urine, bile and feces (Kasper and Henton, 1980).

The toxic effects of mycotoxins are based on a reversible interaction between the molecules of the toxicant and the molecular sites of action. The reversibilty of this type of action implies that the effect is usually related to the contamiation of the drug in the body fluids and disappears when the toxin is eliminated from the body. The effects can be acute or subacute, and the potency of the toxic effect depends upon the dose, the rate of uptake and the rate of

elimination. A dose given at one time may be highly toxic or lethal, while the same dose divided up and given over a longer period of time will be less toxic or even harmless. Molecular sites of action are usually specific and are indicated as specfic receptor sites for the agent (Ariëns and Simonis, 1982).

1.6.2 Basic Toxin-Diet Interaction

Another area of investigation with zearalenone involves the basic toxin-diet interaction. The toxicity of many coumpouds is influenced by the persistence of a pharmacological action which is determined by the capability of the catabolic enzyme system to metabolize the compound. This ability depends upon nutritional factors and other exogenous and endogenous compounds (Clinton et al., 1977 ; Brit et al., 1983).

Deficiencies and excesses of dietary protien have great effects on the ability of the body to metabolize foreign compounds (Cambell and Hayes, 1974). Because of this, protein has been the most studied nutrient in terms of its effect on mycotoxicosis. Since mycotoxicoses are often characterized by changes in the metabolic utilization of dietary protien, mycotoxicosis may be overcome with dietary supplements of protein (Smith, 1982b).

1.6.3 Protein Deficiency

Studies have shown increased toxicity of drugs during protein deficiency (Drill, 1952). Hayes and Schiefer (1980) reported that low protein diets exacerbated T-2 toxicosis. Mice were given 20ug/g feed of T-2 toxin in diets containing 8, 12 and 16% protein for 1, 2, 3, or 4 weeks. Intestinal hemorrhaging was present in the rats with deficient protein diets. They observed that dietary T-2 toxin was more toxic in diets containing the 2 lower protein levels.

Mayura et al. (1983) suggested that dietary protein deficiency increases the susceptibility of rats to the teratogenic effects of ochratoxin A. Weanling female rats were fed one of 3 diets; a 5% protein diet for 4 weeks, followed by a normal protein diet (27% protein); a 10% protein diet for the entire experimental period or a 27% protein diet for the entire experimental period. Rats were given a single subcutaneous dose of 1.75mg/kg body weight of ochratoxin A on day 6 of gestation. In animals fed the 10% protein diet, 27% failed to mate and 37% failed to fertilize. Pregnant rats were killed on day 20 of gestation. The highest incidence of skeletal defects occurred in the fetuses from mothers on the 10% protein diet. In this group, 75% of the fetuses had some kind of skeletal defect, whereas fetuses in the 5% and 27% protein diets had 36 and 42% defects respectively. Under this experimental regime, the 10% protein diet made the animals more susceptible to the teratogenic effects of ochratoxin A.

Adejuwon et al. (1982) studied the effect to which nutritional factors alter the body's capacity to conjugate estradiol. Male and female albino rats were made a protein calorie malnutrition group (pcm) or a starvation group. The pcm rats consumed a 3% protein diet for 3 weeks and the starvation group received no food or water for 4 days. Both of these treatments took place immediately prior to the start of the experiment. Conjugation in pcm rats was significantly lower than in the controls, who were fed an 18% protein diet (9.7 and 12.3% conjugation, $p<0.05$). However, conjugation was not significantly different from the control animals in the starvation group (13.8 and 11.9% conjugation). This study indicates that starvation for 4 days does not effect the rate at which the adult liver conjugates estradiol 17β. In the starvation state, however, there is no gross enzymatic effect on the liver like that seen in pcm. The extent to which pcm affects liver enzymes is not known. There may be a reduced synthesis of UDP glucuronyl transferase

Adejuwon et al. (1982) did not take into consideration the amount of activity with regard to the protein content of the liver. Their observations would become markedly different if their results had been expressed on a per mg protein basis. With less protein in the livers of the pcm and starved rats, there would be an increased activity of the conjugating enzyme.

1.6.4 Dietary Fiber

Dietary fiber has been shown to be a protective agent against zearalenone toxicosis (James and Smith, 1982). James and Smith (1982) observed that the incorporation of alfalfa into feed containg 250ug of zearalenone/g feed reduced the inhibitory effects of zearalenone on growth and feed consumption, and minimized zearalenone induced enlargement of the liver. It also increased hepatic 3αHSD activity, which normally decreased on zearalenone feeding. The mechanism by which alfalfa, alleviates zearalenone toxicosis, is, as of yet, unknown. It may well be due to the bile acid sequestering effects by dietary fiber, interrupting enterohepatic circulation, resulting in increased fecal excretion (Ershoff et al., 1956). Alfalfa contains a phytoestrogen (coumesterol) which may be degraded by 3αHSD. Therefore, high levels of ingested phytoestrogens could induce the activity of the enzyme and increase the rate of breakdown and excretion of zearalenone (Kiessling and Pettersson, 1978).

1.6.5 Conclusion

Decreased feed intake is a symptom of Fusarium mycotoxicosis, therefore the effect of nutritional deprivation on zearalenone metabolism should be studied. If diet restriction changes the liver's capacity to metabolize zearalenone, there should be an alteration in the biological

clearance rate which would enhance or diminish zearalenone's physiological action. James and Smith (1982) have shown an unexplainable reduction of 3αHSD activity when animals were given zearalenone, while Stangroom and Smith (1984) have observed an increase in activity. If this enzyme is affected by a restricted intake, then the potential effects on the excretion profile are unknown. Merrill and Bray (1982) found that UDP glucose dehydrogenase, the enzyme which synthesizes UDP glucuronic acid and UDP glucuronyl transferase were not influenced by supplementations of protein, but increased activity was observed with protein deficiency. The effect of decreased caloric intake on UDP glucose dehydrogenase and on UDP glucuronyl transferase and 3αHSd should be studied to determine if there is an increase in the toxic action of zearalenone in this state.

Chapter II

MATERIALS AND METHODS

2.1 ANIMALS

Female weanling Sprague-Dawley rats (50-60g) were randomly assigned to control or the experimental groups. Animals were housed separately in stainless steel cages and kept on a 14-10 hour light-dark cycle. Animals were fed a commercial chow diet (Ralston Purina Co., St. Louis, MO) for 2 days. Animals were then switched to a standard semi-purified diet, fed daily and weighed weekly. The control animals received the diet ad libitum while the pair-fed experimental animals recieved 75% of their control partner's intake.

2.2 DIET FORMULATION

Diets were formulated according to the National Research Council's guidelines set forth in the Nutrient Requierments of Laboratory Animals (1978). The level of corn oil in the diet was maintained at 5% in accordance with the recommendation of the American Institute of Nutrition (1977), to ensure that the diet was sufficient in essential fatty acids. Specfic diet formulation in Table 3.

TABLE 3

Diet Composition (%)

	BASAL CARBOHYDRATE
Corn Starch	30%
Glucose	35%
Casein	20%
DL-Methionine	0.3%
Corn Oil	5%
Fiber	5%
AIN Mineral Mix	3.5%
AIN Vitamin Mix	1%
Choline Bitartrate	0.2%
Energy	15.9Joules/g

2.3 EXPERIMENTAL DESIGN

TABLE 4

Experimental Population

	Control Group	Experimental Group
Enzyme Study	15	15
Metabolic Study	6	6
Toxicity Study	40	40

2.4 EXPERIMENTAL PROTOCOL

2.4.1 Enzyme Study

2.4.1.1 Animals

After 2 weeks on the dietary regime, the animals were humanely killed, livers excised, and assayed for 3αHSD and UDP glucuronyl transferase enzymes.

2.4.1.2 3αHSD Enzyme Assay

Method (Sigma Co. Product Information Sheet for 3αHSD ; James and Smith, 1982). (Appendix A)

2.4.1.3 UDP Glucuronyl Transferase Enzyme Assay

Method (Hall and Esbenshade, 1984) (Appendix B)

2.4.2 Metabolic Study

2.4.2.1 Animals

After 2 weeks on the dietary regime, the animals were dosed with 1mg/kg body weight of zearalenone by stomach intubation. Animals were transfered to metabolic cages and urine and feces were collected every 24 hours for 4 days.

2.4.2.2 HPLC Methods

The method used in this study was an adaptation of the ß-glucuronidase hydrolysis proceedure of James et al. (1981). The extraction procedure and HPLC operating conditions were those of Trenholm et al. (1981b). (Appendix C)

2.4.3 Toxicity Study

2.4.3.1 Animals

Within each treatment group, each animal was assigned to a semi-purified diet containing 0, 50, 100 or 150ppm pure zearalenone. After 3 weeks on this dietary regime, the animals were humanely killed and and liver and uterine weights were determined.

2.4.4 Statistical Analysis

Data was analyzed using the Statistical Analysis System (SAS), 1982 version. A one way analysis of variance (ANOVA) was used on the Enzyme and Metabolic Studies to determine the effect of treatment (control versus experimental). A two way ANOVA was used on the Toxicity Study to determine the independent effects of ppm and treatment.

Chapter III

RESULTS AND DISCUSSION

3.1 ENZYME STUDY

In the Enzyme Study, animals on the restricted dietary regime consumed 75% of their control animals ad libitum feed intake. There was a significant difference observed in final body weights between the control and experimental animals, with the experimental animals supporting lower body weights than control animals (Table 5, $p<0.0001$). Cumulative feed efficiency was also significantly lower in experimental animals ($p<0.0001$). The differences in growth and feed efficiceny were a reflection of the dietary restriction placed on the experimental animals.

Feed restriction resulted in a significant difference in 3αHSD enzyme activity. There was a difference in enzyme activity of 27.5%, from 7.75 to 9.88nM of NADH produced/minute/mg protein in control and feed restricted animals respectively (Table 6, $p<0.0038$).

There are no other results on the effect of diet per se on 3αHSD activity reported in the literature. Stangroom and Smith (1984) observed the effect of substrate on the 3αHSD activity. They observed that rats given zearalenone

Table 5 Enzyme Study:
The Effect of Feed Restriction on Body Weight (g) and Cumulative Feed Efficiency (Total Weight Gain (g)/Total Food Intake (g))

	n	Body Weight		Cumulative	Cumulative
		Initial	Final	Food Intake (g)	Feed Efficiency
Control	15	143.93 ± 2.95	207.93 ± 5.05	260.57 ± 7.28	0.256 ± 0.010
Experimental	15	138.47 ± 2.02	180.07 ± 3.14	197.79 ± 5.33	0.205 ± 0.006
ANOVA (p)		NS	0.0001	0.0001	0.0001

mean ± SEM

Table 6 Enzyme Study:

The Effect of Feed Restriction on 3αHSD (nmol/NADH produced/minute/mg protein) and UDP glucuronyl transferase (nmol conjugated/hr/mg protein) Activity

	n	3αHSD	UDP Glucuronyl Transferase
Control	15	7.75 ± 0.46	52.567 ± 3.869
Experimental	15	9.88 ± 0.49	93.758 ± 7.686
ANOVA (p)		0.0038	0.0001
mean ± SEM			

(250ug/g feed) had significantly higher (5.3%) 3αHSD enzyme activities when compared to control animals (61.1 to 64.5 nM of NADH produced/minute/mg protein, $p<0.01$). However, they could not reproduce these effects in subsequent experiments reported in the same document. Futhermore, this increase in the activity of 3αHSD after zearalenone administration was in contrast to their earlier report (James and Smith, 1982). In this study, the enzyme activity of the zearalenone treated animals decreased 30.9%, from 4.2 to 2.9 nM of NADH produced/minute/mg protein, $p<0.05$). The authors attributed the 10 fold difference in activity of 3αHSD between the studies to the nonsaturated substrate levels in the 1982 study (Stangroom and Smith, 1984). These reports by Smith and coworkers (James and Smith, 1982; Stangroom and Smith, 1984) must be cautiously interpreted. Neither of these enzyme assay conditions reported could be shown to produce a linear response nor a response proportional to the amount of protein in our laboratory.

The following two studies were conducted to characterize the pH optimum and cellular location of the zearalenone reducing enzyme and not to establish maximum reaction rates. These studies have produced data on the activity of 3αHSD in rat liver. Tashiro et al. (1983) measured the in vitro conversion of zearalenone to zearalenol, and observed the activity to be 3.0pM of NADH produced/minute/mg protein. In a similar study, Ueno et al.(1983) observed an enzyme

activity of 17.2pM of NADH produced/minute/mg protein for 3αHSD. Both Tashiro et al. (1983) and Ueno et al. (1983) observed pmolar activity, a very small amount of activity when compared to this study and others (James and Smith, 1982; Stangroom and Smith, 1984).

Varying results have been reported in the literature on the measurement of 3αHSD enzyme activity. The differences observed can be attributed to differences in analytical methods used, sex of animals, age of animals and the fractions of the liver used in the enzyme preparation.

Feed restriction resulted in a significant difference in UDP glucuronyl transferase enzyme activity between ad libitum and restricted animals. A difference of 78.7% was observed between control and experimental animals, with UDP glucuronyl transferase activities of 52.5 to 93.8nM of p-nitrophenol conjugated/hour/mg protein respectively (Table 6, $p<0.0001$).

This is in general agreement with other reports in the literature. Wood and Woodcock (1971) studied the effects of dietary protein deficiency on the conjugation of foreign compounds in the rat liver. They fed immature male rats an 18% protein or protein-free diet for 7 days and measured UDP glucuronyl transferase activity. A marked difference of 115% in enzyme activity was observed in the protein deficient animals (85 to 183 nM of p-nitophenol

conjugated/hour/mg protein, $p<0.01$). Protein deficiency alone increases UDP glucuronyl transferase activity, but when this is accompanied by a subnormal caloric intake, a much smaller increase of enzyme activity was observed. The authors suggest that this effect was not due to the synthesis of a new enzyme protein, since protein synthesis is minimal in deficient animals. The alterations in the composition and/or or structure of the endoplasmic reticulum or both was the cause of the increased enzyme activity.

In a recent study by Merrill and Bray (1982) chloral hydrate induced sleeping time was used as a measure of glucuronide formation in vivo. UDP glucuronyl transferase enzyme conjugates the main by-product of chloral hydrate rendering it inactive. Male wistar rats were fed diets of 7.5, 15 and 45% protein for 16 days. Animals fed the 15% protein diet best represented normal growth patterns. Feed efficiency was significantly less on the 7.5% protein diet, but no difference was seen between the 15 and 45% groups. The in vitro activity of UDP glucuronyl transferase was significantly higher in the 7.5% group (100 and 108%) with an activity of 38.37 nM conjugated/hour/mg protein when compared to the 15% (19.19nM) and the 45% (18.44nM) protein groups ($p<0.05$). Chloral hydrate induced sleeping time was inversely related to the in vivo UDP glucuronyl transferase activity. Animals in the protein deficient group had sleeping times of 58.4 minutes, 60 and 66% shorter than the

15 and 45% protein groups at 93.2 and 97.2 minutes, respectively.

They concluded that low dietary protein intake caused an increase in UDP glucuronyl transferase activity. Merrill and Bray (1982) hypothesized that the diet caused structural changes in the endoplasmic reticulum, lowering the permeability barrier. A decrease in dietary protein may have caused a decreased phospholipid content and altered the composition of the phospholipid present.

These studies support the data presented in this experiment, that a decreased caloric intake causes an increase in the activity of UDP glucuronyl transferase. The activity of the enzyme was well within the normal range of 20 to 80 nm conjugated/hour/mg protein for untreated rats.

3.2 METABOLIC STUDY

In the Metabolic Study, animals on the restricted diet had significantly different cumulative feed intakes ($p<0.0001$) and cumulative feed efficiencies (Table 7, $p<0.0217$). Experimental animals had both lower cumulative food intakes and cumulative feed efficiencies than control animals. The initial experimental population were selected so that after two weeks of feed restriction no difference would be observed in body weights between control and experimental animals.

Table 7 Metabolic Study:

The Effect of Feed Restriction on Body Weight (g) and Cumulative Feed Efficiency (Total Weight Gain (g)/Total Food Intake (g))

	n	Body Weight		Cumulative Food Intake (g)	Cumulative Feed Efficiency
		Initial	Final		
Control	6	78.00 ± 2.45	152.00 ± 4.38	215.17 ± 3.04	0.344 1 0.015
Experimental	6	89.67 ± 5.89	138.33 ± 4.99	165.33 ± 2.74	0.295 ± 0.010
ANOVA (p)		NS	NS	0.0001	0.0217

mean ± SEM

There was a significant effect of feed restriction on the percentages of cumulative excretion of zearalenone and metabolites in the urine and feces. There were differences in both the route of excretion and the form of metabolites excreted (Table 8). The restricted animals excreted 15.34% more bound urinary zearalenone than control animals (11.03 to 26.37%, $p<0.0292$). There was a corresponding difference of 21.67% in free fecal zearalenone (38.34 to 16.67%, $p<0.0459$) and free fecal αzearalenol of 4.28% (9.72 to 5.44%, $p<.0.0354$) in restricted animals excreting less than their matched controls. These differences were reflected in the total distribution of metabolites. Restricted animals excreted 4.47% more total zearalenone (81.85 to 86.32%, $p<0.0129$) and 3.65% less total αzearalenol (15.28 to 11.63%, Table 8, $p<0.05$). Only small amounts of ßzearalenol were excreted, and these were to variable to detect differenecs in excretion between control and experimental animals.

The control animals excretion profile showed free fecal zearalenone (38.34%) as the major excretory metabolite followed by free urinary zearalenone (34.45%), bound urinary zearalenone (11.03%), free fecal αzearalenol (9.72%), the urinary forms of αzearalenol and all ßzearalenols (Table 8). The major excretion product in the restricted animals was free urinary zearalenone, which constituted 43.28% of the dose recovered in this group. Bound urinary zearalenone (26.37%), free fecal zearalenone (16.67%) and free fecal

Table 8 Metabolic Study:

The Effect of Feed Restriction on the Cumulative Excretion of Zearalenone and Metabolites in the Urine and Feces (%)*

	n	Free Urinary	Bound Urinary	Free Fecal	Total Distribution	Recovery (ng)	Total Recovery (ng)	
zearalenone								
control	6	32.49 ± 9.39	11.03 ± 2.39	38.34 ± 8.11	81.85 ± 1.14	80276.85	Control	98206.80
experimental	6	43.28 ± 5.94	26.37 ± 5.34	16.67 ± 4.97	86.32 ± 0.95	76456.27	Experimental	89117.92
α zearalenol								
control	6	3.44 ± 0.78	2.10 ± 0.51	9.72 ± 1.05	15.28 ± 1.18	15310.84		
experimental	6	3.61 ± 0.39	2.58 ± 0.37	5.44 ± 1.42	11.63 ± 1.14	11031.42		
β zearalenol								
control	6	1.65 ± 0.41	0.45 ± 0.22	0.79 ± 0.22	2.89 ± 0.37	2619.11		
experimental	6	1.07 ± 0.40	0.57 ± 0.21	0.41 ± 0.20	2.05 ± 0.36	1630.23		
ANOVA (p)								
zearalenone		NS	0.0292	0.0459	0.0129			
α zearalenol		NS	NS	0.0354	0.0515			
β zearalenol		NS	NS	NS	NS			

mean ± SEM

*corrected for recovery

αzearalenol (5.44%) made up the majority of the other metabolites excreted in restricted animals. Small amounts of urinary αzearalenol and urinary and fecal βzearalenol were recovered. In both control and experimental animals the percentages of the total distribution of metabolites were similar. However, when free and bound forms were taken into consideration differences in the major excretory metabolites appeared (Table 9).

TABLE 9

Metabolic Study: The Summary of the Major Excretory Metabolites (%)

Control	
Free Fecal Zearalenone	38.34
Free Urinary Zearalenone	34.45
Bound Urinary Zearalenone	11.03
Free Fecal αZearalenol	9.72
Experimental	
Free Urinary Zearalenone	43.28
Bound Urinary Zearalenone	26.37
Free Fecal Zearalenone	16.67
Free Fecal αZearalenol	5.44

The urine method had a recovery rate of 93% and the recovery rate for the fecal method was 80%. Data was corrected for method recovery. In this study, 62.5% of the total dose given was recovered. The daily excretion profiles presented (Tables 10, 11 and 12) were converted to percentages of cummulative excretion for the purposes of this discussion.

In the control animals, 48.85% of the dose recovered was excreted in the feces and 51.16% in the urine (Table 13). Experimental animals had 22.52% fecal excretion and 77.48% urinary excretion. When these percentages are corrected for recovery, the control animals excreted 30.53% of the dose in the feces and 31.79% in the urine. Experimental animals had fecal excretions of 14.08% of the dose and urinary excretions of 47.98%.

In a 1976 study by Mirocha and coworkers on urinary excretion in rats, they observed 27.5% as free metabolites and 39.8% as conjugated metabolites. Of the total dose given, 67.4% was recovered.

James et al. (1981) developed a HPLC method to quantify zearalenone and metabolites in rat urine. The authors observed 71% of the dose as free metabolites and 29% as bound metabolites. The recovery rate for this method was 50 to 80%, and only 3% of the total does was recovered. When Smith (1982a) used this method, urinary recovery of

Table 10 Metabolic Study:

The Effect of Feed Restriction on the Daily Excretion Profile of Free Zearalenone and Free Metabolites in the Urine (ng)*

	n	Day 1	Day 2	Day 3	Day 4	Total
Control						
zearalenone	6	15804.28 ± 4848.25	6265.27 ± 1185.88	2443.14 ± 354.79	865.40 ± 124.05	26317.13 ± 5940.17
α zearalenol	6	1095.56 ± 470.67	946.92 ± 124.10	633.30 ± 66.38	354.66 ± 94.12	3030.43 ± 552.91
β zearalenol	6	370.67 ± 101.48	530.84 ± 112.11	389.12 ± 82.05	106.27 ± 49.99	1396.63 ± 277.63
Experimental						
zearalenone	6	16432.00 ± 3835.97	10687.12 ± 1218.50	2878.80 ± 327.58	878.43 ± 108.43	36020.95 ± 5885.06
α zearalenol	6	1330.31 ± 554.46	1262.65 ± 177.05	457.67 ± 52.46	164.57 ± 69.72	3215.20 ± 772.84
β zearalenol	6	276.04 ± 75.15	280.63 ± 103.05	196.31 ± 76.97	0.0	752.98 ± 180.54

mean ± SEM

*corrected for recovery

Table 11 Metabolic Study:

The Effect of Feed Restriction on the Daily Excretion Profile of Bound Zearalenone and Bound Metabolites in the Urine (ng)*

	n	Day 1	Day 2	Day 3	Day 4	Total
Control						
zearalenone	6	9437.74 ± 3074.14	1815.20 ± 529.22	382.17 ± 106.14	135.86 ± 69.74	11770.97 ± 3395.48
α zearalenol	6	1375.81 ± 498.54	337.27 ± 37.42	160.14 ± 87.87	97.10 ± 72.09	2113.35 ± 652.77
β zearalenol	6	87.58 ± 41.55	52.90 ± 34.65	154.30 ± 99.70	47.42 ± 30.57	342.20 ± 137.98
ANOVA (p)						
Experimental						
zearalenone	6	19061.13 ± 6126.32	3583.96 ± 1039.22	652.55 ± 220.14	225.63 ± 61.27	23523.26 ± 6383.73
α zearalenol	6	1239.12 ± 313.95	651.79 ± 124.08	210.34 ± 70.17	72.90 ± 15.08	2174.16 ± 404.51
β zearalenol	6	67.27 ± 27.77	115.22 ± 25.14	139.03 ± 81.72	31.52 ± 19.94	430.09 ± 165.92
ANOVA (p)						

mean ± SEM

*corrected for recovery

Table 12 Metabolic Study:

The Effect of Feed Restriction on the Daily Excretion Profile of Free Zearalenone and Free Metabolites in the Feces (ng)*

	n	Day 1	Day 2	Day 3	Day 4	Total
Control						
zearalenone	6	23906.79 ± 10985.97	13585.08 ± 1863.40	3937.52 ± 1503.16	759.35 ± 221.59	42188.75 ± 13196.00
α zearalenol	6	2976.67 ± 1235.36	4663.67 ± 874.89	1985.56 ± 675.69	541.17 ± 146.01	10167.06 ± 2255.05
β zearalenol	6	288.48 ± 133.83	312.69 ± 107.95	173.05 ± 59.03	20.31 ± 15.16	880.27 ± 292.80
Experimental						
zearalenone	6	4036.35 ± 2690.98	9307.23 ± 2771.27	2739.48 ± 657.44	829.00 ± 152.17	16912.06 ± 5628.38
α zearalenol	6	415.13 ± 223.79	3231.73 ± 1181.34	1526.60 ± 377.56	468.60 ± 137.23	5642.06 ± 1787.27
β zearalenol	6	22.19 ± 22.19	286.77 ± 140.15	138.21 ± 57.49	0.0	447.17 ± 209.34

mean ± SEM

*corrected for recovery

TABLE 13

Metabolic Study: The Summary of the Urinary and Fecal Distribution of Metabolites (%)

	Urine	Feces
Control	51.16	48.85
Experimental	77.48	22.52
Corrected For Recovery		
Control	31.79	30.53
Experimental	47.98	14.08

zearalenone revealed 2% of the total dose as free urinary zearalenone and metabolites and 1% as bound urinary metabolites. With a 50 to 80% recovery rate, the amount of zearalenone recovered was minimal, especially when considering the size of the dose (10mg/100g body weight). They also measured fecal excretion and observed 22% of the dose in the feces. They did not state the recovery rate for the fecal method. The major urinary and fecal metabolites were free zearaleneone, 82.2 to 99.6% respectively.

Smith (1980a) conducted a [H^3] labelled study to determine the excretion profile of zearalenone and metabolites in rats. Of the 93.4% of the dose recovered, 31.1% was found in the urine and 62.3% as fecal metabolites. Quantification of the metabolite forms was not carried out in this experiment.

Mirocha et al. (1975) observed 60.5% of a labelled zearalenone dose excreted in the urine, with 4.7% in the feces. However, both Ueno et al. (1977) and Baldwin et al. (1977) observed the majority of a radiolabelled dose of zearalenone to be excreted in the feces 40 to 60% and 70 to 80%, respectively. There is no agreement in the literature as to the rate, route and form of zearalenone metabolites excreted.

3.3 TOXICITY STUDY

A significant decrease was observed in cumulative feed intake between control and experimental animals in the Toxicity Study (Table 14, $p<0.0001$). This difference was due to the dietary restriction placed on experimental animals, which was part of the experimental design.

The measurement of feed efficiency estimates the effect of treatment on the diet utilization and its impact on growth as measured by weight gain per unit nutrient consumed. Analysis of the cumulative feed efficiency data indicated the presence of an interaction between dietary treatment and zearalenone level (Table 14, $p<0.003$). Feed efficiences in feed restricted animals given zearalenone at 50, 100 and 150ppm, while not significantly different from each other, showed a significant increase when compared to control animals at the same dietary zearalenone level (12.9 to 26.3%). No difference in feed efficiency was observed

Table 14 Toxicity Study:

The Effect of Feed Restriction on Body Weight (g) and Cumulative Feed Efficiency (Total Weight Gain (g)/Total Food Intake (g))

		Body Weight							
		Initial		Final		Cumulative Food Intake (g)		Cumulative Feed Efficiency	
	n	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
0 ppm	10	63.50±0.82	62.25±1.56	178.10±3.33	148.89±3.94	311.86±2.59	231.70±5.89	0.360±0.006	0.360±0.007
50 ppm	10	58.57±1.41	66.60±1.58	152.22±2.24	146.33±2.12	289.00±11.34	218.40±8.69	0.289±0.005	0.347±0.009
100 ppm	10	63.90±2.17	65.50±2.02	150.44±4.47	140.10±1.67	283.89±8.01	220.50±6.92	0.297±0.012	0.341±0.012
150 ppm	10	65.20±2.57	59.00±1.72	135.89±3.57	127.11±3.86	291.44±10.43	221.44±8.31	0.250±0.010	0.339±0.008
ANOVA (p)									
PPM Error				0.0001		NS		0.0001	
Treatment Error				0.0001		0.0001		0.0001	
PPM*Treatment Error				0.0024		NS		0.0003	
mean ± SEM									

between experimental and control animals at the 0ppm level. In control animals only, there was a trend towards a decrease in cumulative feed efficiency as the level of toxin increased (17.5 to 30.6%). It is evident that the dietary restriction along with the administration of zearalenone overcomes some of the deleterious effects of the toxin by increasing feed efficiency. Without a decreased feed intake, zearalenone causes a dose dependent derease in feed efficiency. Smith (1980a) also observed that feed efficiency was reduced in animals fed zearalenone at 250ug/g feed when compared to control animals ($p<0.01$).

A significant interaction between dietary treatment and zearalenone level was observed for final body weights, with restricted animals significantly lower than controls (Table 14, $p<0.0024$). Experimental animals at the 0ppm level showed a greater decrease in body weight (16.4%), than other experimental animals receiving toxin when compared to control animals (3.9 to 6.9%). This result suggests that animals receiving zearalenone, but allowed to consume feed ad libitum, are more sensitive to the toxic effects of zearalenone than animals on a restricted regime.

No difference was observed in organ weights when animals were fed zearalenone at levels of 0, 50, 100 and 150ppm (Table 15). There was an interaction between dietary treatment and zearalenone level in uterine weights between control and experimental animals ($p<0.0347$). Restricted

animals had significantly lower uterine weights than their matched controls (5.0 to 3.31%, $p<0.0002$), although the weights among the restricted animals were not significantly different from each other. Uterine weights of control animals decreased with increasing zearalenone levels (14.5 to 22.8%). However, no significant differences were observed when uterine weights were expressed as a percentage of body weight (Table 16).

An interactive effect between dietary treatment and zearalenone level was observed for liver weights (Table 15, $p<0.0411$). Restricted animals, only at the 0 and 100ppm level showed significant decreases in liver weight (9.9 to 17.9%, $p<0.001$). In control animals there was a trend toward decreasing liver weight as the toxin level increased (14.5 to 24.5%, $p<0.0002$). When liver weight is expressed as a percentage of body weight, experimental animals reccieving 150ppm zearalenone had signifiantly higher liver weights than other experimental animals (8 to 12 %, Table 16, $p<0.0181$).

Smith (1980b) fed rats 0, 50, 100, 250 and 500ug zearalenone/g feed (0, 50, 100, 250 and 500ppm zearalenone) over a 14 day period. Uterine and liver weight increases were only observed in the 250 and 500ug groups ($p<0.05$). A later experiment by James and Smith (1982) reported no increase in uterine weights in rats fed 250ug zearalenone/g feed for 2 weeks. They attributed the lack of zearalenone-

Table 15 Toxicity Study:

The Effect of Feed Restriction on Uterine Weight (g) and Liver Weight (g)

	n	Uterine Weight		Liver Weight	
		Control	Experimental	Control	Experimental
0 ppm	10	0.435 ± 0.037	0.291 ± 0.029	8.29 ± 0.19	6.81 ± 0.37
50 ppm	10	0.372 ± 0.027	0.324 ± 0.017	6.96 ± 0.27	6.47 ± 0.24
100 ppm	10	0.340 ± 0.012	0.323 ± 0.013	7.17 ± 0.25	6.46 ± 0.17
150 ppm	10	0.336 ± 0.015	0.288 ± 0.015	6.26 ± 0.29	6.34 ± 0.29

ANOVA (p)

PPM Error	NS	0.0002
Treatment Error	0.0002	0.0010
PPM*Treatment Error	0.0347	0.0411

mean ± SEM

Table 16 Toxicity Study:
The Effect of Feed Restriction on Uterine Weight and Liver Weight expressed as a % of Body Weight

	n	Uterine Weight (% of body weight) Control	Uterine Weight (% of body weight) Experimental	Liver Weight (% of body weight) Control	Liver Weight (% of body weight) Experimental
0 ppm	10	0.245 ± 0.021	0.223 ± 0.033	4.79 ± 0.11	4.45 ± 0.12
50 ppm	10	0.251 ± 0.019	0.236 ± 0.015	4.53 ± 0.14	4.48 ± 0.093
100 ppm	10	0.221 ± 0.008	0.230 ± 0.008	4.82 ± 0.06	4.60 ± 0.13
150 ppm	10	0.241 ± 0.013	0.248 ± 0.015	4.60 ± 0.14	4.97 ± 0.13
ANOVA (p)					
PPM Error		NS		NS	
Treatment Error		NS		NS	
PPM*Treatment Error		NS		0.0181	

mean ± SEM

induced uterine weight enlargement as being masked by the effect of endogenous estrogen production. They stated that rats in the 1982 study were weanling animals who were approaching puberty, while the earlier study used immature 23 day old rats.

Kiessling (1982) administered daily oral doses of zearalenone at 1.25 or 3.75mg/kg body weight to female rats for 8 weeks. He reported liver enlargement when results were expressed either as a percentage of body weight or carcass weight ($p<0.05$), but no increase in uterine weight was observed.

A dose and time dependent increase in uterine weight in immature and ovarectomized mice and rats has been shown by Ueno et al. (1974). In this study mice were given oral, subcutaneous and/or intraperitoneal doses of 5 and 10mg/kg body weight zearalenone. All doses and routes produced an increase in the uterine weight of immature mice. The authors also orally dosed mice and rats with 0.5 to 2.0mg/kg body weight of zearalenone daily for 8 days. Increased uterine weight was observed, however liver, kidney, spleen and total body weight did not increase. The researchers noted that the uteri of ovarectomized mice were much more sensitive to the effects of zearalenone when compared to intact animals.

In the present study several factors contributed to the lack of uterine and liver weight enlargement. The use of weanling animals, as in the James and Smith (1982) study, may have had a masking effect on uterine weight enlargement, since these animals were endogenously producing large amounts of estrogens for growth purposes. The levels of zearalenone used in this study were low, and have not been shown by other researchers to cause uterine nor liver weight increases.

Control and experimental animals did not receive the same amount of toxin throughout the study. The diet of the experimental animals could have been adjusted in accordance with the dietary restriction to allow both control and experimental animals to consume the same amount of toxin per gram of feed consumed. Since the control animals received more toxin than the experimental animals, this may account for the stronger toxic effects on the control animals as exhibited through a decrease in cumulative food efficiency, final body weight and organ weight. This study revealed that a decreased food intake has a beneficial effect on zearalenone toxicosis at these zearalenone levels.

Chapter IV

CONCLUSION

This study revealed that diet has a significant effect on zearalenone metabolism. The effect of feed restriction altered the metabolism of zearalenone by inducing the activity of the zearalenone metabolizing enzymes, which altered the rate and route of excretion of zearalenone metabolites, and apparently lessened the toxic effects of zearalenone.

The restricted animals showed a greater activity of both 3αHSD and UDP glucuronyl transferase activity when compared to their matched controls. Since feed refusal is a symptom of mycotoxicosis, the decrease feed intake has a beneficial effect on enzyme induction. Restricted animals have a greater enzyme activity and are hence able to metabolize the toxin.

This alteration of in enzyme activity is reflected in the excretion of zearalenone. There was a significant effect of feed restriction on the percentage of cumulative excretion of zearalenone and metabolites in the urine and feces. There were differences in both the route of excretion and the form of metabolites excreted. Restricted animals excreted more bound urinary zearalenone and less free fecal zearalenone and free fecal αzearalenol than control animals.

The toxicity study demonstrated that zearalenone, when fed in association with a restricted dietary regime, prevented the toxic expression of zearalenone.

Therefore, reduced feed intake is a beneficial mechanism to overcome zearalenone toxicosis.

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Appendix A

3α HYDROXYSTERIOD DEHYDROGENASE ENZYME ASSAY

1. Method Development:

The method of James and Smith (1982) for 3αHSD enzyme activity was first tested, however a linear response could not be produced using their method. Different liver dilutions and substrate concentrations were tested to find optimum conditions for the enzyme assay, but the response was still not linear. In a later paper by the same authors, they stated that their earlier study on 3αHSD was not conducted under optimal conditions. (Stangroom and Smith, 1984). They slightly modified the method and obtained an increased enzyme activity. Stangroom and Smith (1984) concluded that the time versus velocity curve reached a plateau after approximately 1.5 minutes, therefore to ensure measurement of the reaction velocities during the linear portion of the curve, their incubations were terminated at 1.5 minutes. When the new method was tested in our lab, a similar reaction curve to the one obtained with the 1982 method was produced. This method did not produce a linear maximum reaction rate, nor was it

proportion to the amount of enzyme (protein) in the incubation mixture. Modifications to Stangroom and Smith's (1984) method could not produce a linear response for enzyme activity.

The product information sheet for 3αHSD (Sigma Chemical Co.) contained the method used in this study. It could be demonstrated that the linear response fit the theoretical model (1 unit of enzyme oxidized 1.0 uM of androsterone/minute/mg of protein). A range of enzyme dilutions and substrate concentrations were tested to validate the enzyme reaction conditions. No modifications of the substrate, buffer nor pH were necessary since the reaction proceeded at optimal rates, however more substrate was added to enable the reaction to proceed for a longer time.

2. Principle: Androsterone + β-NAD + H^+ $\xrightarrow{3\alpha HSD}$ 5αandrosterone-3-17-dione + β-NADH.

One unit of enzyme will oxidize 1.0uM of androsterone per minute at pH 8.9 at 25°C in the presence of β-NAD. This is the same enzyme that oxidizes zearalenone to its alcohol forms.

3. Preparation of Homogentate:

Livers were excised, weighed and chilled on ice. Two grams of liver was cut into pieces with scissors

and homogenized in a glass homogenizing tube with a Teflon pestle with 38ml of ice cold 0.01M potassium phosphate buffer, at pH 7.2. The sample was homogenized 1 minute on ice. The homogenate was centrifuged at 4°C for 10 minutes at 14,000rpm (25,000g). Supernatant was extracted with a pipet to exclude the fat layer and kept on ice.

4. Assay:

The following reagents were pipetted into a cuvet with a 1cm light path. The sample cuvet contained 0.6ml 0.1M sodium pyrophosphate buffer at pH 8.9, 2ml glass distilled water, 0.2ml 0.01M NAD. All reagents were at 25°C. Supernatant was added and the cuvet was mixed by inverting 4 times. The sample was monitored at A_{340}nm until constant (5 minutes) using a thermostated spectrophotometer with a cuvet containing glass distilled water as the reference. To initiate the reaction, 0.1ml 0.01M androsterone was added to the sample cuvet and immediately mixed by inversion 4 times. The reaction was monitored for 5 minutes.

5. Determination of Activity:

The $^{\Delta}A_{340}$nm/minute was recorded. Activity was calculated through the equation: um NADH produced/minute/mg protein $=^{\Delta}A_{340}$nm/minute x 3.0ml reaction volume/6.22.

Determination of protein in the cuvet was performed by the Lowry Method (1951) on the supernatant. Each sample was analyzed in triplicate.

Appendix B

UDP GLUCURONYL TRANSFERASE ASSAY

1. Method Development:

The method by Hall and Ebenshade (1984) was followed. Validation of the method was carried out to determine if the enzyme system was saturated and that the protein concentration used was appropriate. After testing, it was concluded that the method was working at optimum conditions and no modifications were made.

2. Principle:

p-nitrophenol +UDP glucuronic acid UDP glucuronyl transferase UDP + p-nitrophenol glucuronic acid.

This assay measured the disappearence in color as p-nitrophenol is conjugated. This is the same enzyme that conjugates zearalenone and derivatives.

3. Preparation of Homogenate:

Livers from female rats were excised, weighed and chilled on ice. Three grams of liver was cut into pieces with scissors and homogenized in a glass homogenizing tube with a Teflon pestle with 12ml of ice cold 0.154M isotonic KCL, at pH 7.4. The sample

was homogenized for 1 minute on ice. The homogenate was centrifuged at 4°C for 20 minutes at 4,000rpm (2,000g) and the supernatant was extracted with a pipet to exclude the fat layer and kept on ice.

4. Assay:

The assay was perfomed in 13 x 100mm glass test tubes at 37°C in a shaking water bath (40 strokes/minute). Each tube contained 500ul of the supernatant and the final volume in the tubes was brought to 2.5ml with 75mM maleate-malic acid buffer (pH 6.4) containing 0.05% triton x-100, 0.5 mM p-nitrophenol and 1mM UDP glucuronic acid. The reaction was initiated with the addition of the maleate-malic acid buffer. Two 250ul aliquots were removed at time 0, 5, 10, 15 and 20 minutes of incubation. Each aliquot was added to 2ml of 75mM Tris-HCL buffer ice cold, at pH 9.0, to stop the reaction. Absorbtion of the aliquots at 400nm were averaged for each time interval. Duplicate assays were performed for each animal, and the rate of change in absorbtion was averaged for each animal. Control assays omitted UDP glucuronic acid from the assay, and were performed for each animal.

5. Determination of Acitivity

The ΔA_{400}nm was plotted for sample and control. The absorbtion from the graphs were correlated to a

standard curve prepared by measuring known quantities of p-nitrophenol (25, 50, 100 and 150nM). The amount of enzyme activity (nM p-nitrophenol conjugated/minute/mg of protein) was then determined. Determination of protein in the cuvet was performed by the Lowry Method (1951) on the homogenate.

Appendix C

HPLC ASSAYS FOR ZEARALENONE AND METABOLITES

6. Method Devleopment:

Slight modifications were made to the Trenolm et al.(1981b) method for the analysis of urine. Adjustments were made to the solvent to shorten the retention time between the α and ßzearalenol peaks. The flow rate was changed to 1ml/minute and standards contained 50ng of zearalenone, αzearalenol and ßzearalenol. The addition of phenol red to the samples caused a peak to appear at the same postion as ßzearalenol. An additonal sample was run through the procedure to determine the amount of acid needed to neutralize the samples.

The fecal method used by Trenholm et al. (1981b) called for a washing of the ethyl acetate sample with hexane. Since hexane and ethyl acetate are miscible, this procedure could not be used. Freeze dried feces were soxhleted with ethyl acetate for 16 hours and compared to fresh feces homogenized in water for percentage recovery of zearalenone and metabolites. The recovery from the soxhleted samples was only 70-80% of the metabolites recovered from the homogenized feces.

For determination of bound metabolites, 457 units of enzyme were incubated with the sample. No difference in recovery of bound metabolites was found when a range of ratios of sample to enzyme were tested (1:1, 1:2 and 1:5). When clean samples with added standard were tested for percentage of recovery, there was 93% urinary and 80% fecal recovery of zearalenone and metabolites.

7. Urine Metabolites Hydrolysis and Extraction:

A 0.1ml urine sample and 1.8ml glass distilled water were pipetted into an 8ml screw top test tube. A ß-glucuronidase solution (Type B-1 III Sigma Chemical Co.), 0.1ml, in glycerol was added. Tubes were loosely capped and the mixture was incubated overnight at 37°C in a water bath. Two mls of 10% 2-propanol in ether was added to the cooled incubated tubes. The mixture was vortexed, layers allowed to seperate and the top layer was removed. This step was repeated. The two extraction layers (ether) were combined. The ether layer was placed on ice for 10-15 minutes, and adjusted to pH 12 with the addition of 2.0ml of chilled 0.184M NaOH. The mixture was vortexed and then chilled on ice. The layers were separated and the top layer was discarded. The cold aqueous layer was washed with 2ml portions of benzene. To avoid the formation of

an emulsion the tubes were gently inverted while mixing. The samples were chilled and the top (benzene) layer was discarded. This step was repeated. Phenolphathalein indicator (3 drops) was added to neutralize the sample to pH 7.0-8.0 range by adding 0.5N chilled acetic acid. The indicator turned from red to slightly yellow. The sample was chilled and extracted 3 times with 2.0ml of benzene, vortexed 30 seconds and chilled. The extracts were combined and evaporated to dryness. Samples were taken up in an known volume of the mobile phase without phosphoric acid. For the determination of free zearalenone, α and ßzearalenol in urine, the incubation step was omitted and 1.9ml of water was added to the sample. Standards of known concentration were used to establish that the recoveries from the hydrolysis, clean up and extraction were quantitative.

8. Fecal Metabolites Hydrolysis and Extraction:

The total fecal sample was thawed and transfered to a glass homogenizing tube with 10ml of glass distilled water. A polytron homogenizer was used to blend the feces to a uniform consistency. Aliquots of 0.1ml were taken from this mixture and analyzed for free and conjugated metabolites by the same procedure which determined urinary metabolites. The

only change was centrifugation of the samples between the ether extractions.

9. Mobile Phase:

   Water:Methanol:Acetonitrile and Phosphoric Acid (45:42:13 + 0.2%). Flow rate 1.0ml/minute.

10. Standards:

    Zearalenone, αzearalenol, and βzearalenol (International Minerals Chemical Corp., Terre Haute, IN).

Appendix D

NUTRIENT SUPPLIERS

TABLE 17

Nutrient Suppliers

CARBOHYDRATE	
Corn Starch	Casco Brand, Canada Starch Co. Cardinal, Ontario K0E 1E0
Alphacel Non-Nutritive bulk	ICN Pharmaceticals Inc. Life Scienes Group 26201 Mills Road Cleveland, OH 44128
Dextrose Sugar	The R. Wine Baril Winnipeg, Manibtoba
PROTEIN	
Vitamin Free Casein "Vita Free"	USB (United States Biochemicals) 21000 Miles Parkway Cleveland, OH 44128
DL-Methionine	USB
Mazola Corn Oil	Best Foods (Division of Canada Starch Inc.) CP 129 Station 'A' Montreal, Quebec H3C 1C5
VITAMINS AND MINERALS	
AIN Vitamin Mixture 76	ICN Pharmacueticals Inc.
AIN Mineral Mixture 76	ICN Pharmaceuticals Inc.
Choline Bitarate	USB

Appendix E

LIST OF MAJOR EQUIPMENT

1. Sp6-300 Spectrophotometer, Pye Unicam
2. Sp8-400 UV/VIS Spectrophotometer, Pye Unicam
3. International Centrifuge, International Equipment Co.
4. International Refrigerated Centrifuge, International Equipment Co.
5. Meyer N-Evap Analytical Evaporator
6. Caframo Stirrer Type R2R1-64 Homogenizer, Canlab Polytron
7. Water-bath Shaker, Eberbach Corp.
8. HPLC
   a) Pump, Beckman 110A
   b) Ultraviolet Absorbance Detector 254nm, Beckman 160
   c) Column, Hibar RP-18, 10um (250 x 4mm) with a Whatman precolumn
   d) Recorder, Bausch and Lomb 500 UV Vom 7