# THE METABOLISM OF THE MYCOTOXIN ZEARALENONE IN THE RAT

BY

ABDULLAH MAHOMED HASSEN

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presented to the University of Manitoba
in partial fulfilment of the requirements for the degree of
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in
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**ABSTRACT**

Although there are numerous reports on zearalenone (ZEN) toxicity, there are limited data on absorption, distribution and elimination of ZEN in animals. Experiments were undertaken to further document the *in vitro* and *in vivo* metabolism of ZEN and to investigate its pharmacokinetics.

ZEN dose level had little effect on its metabolism. After 96 h, animals excreted 15% and 64% of dose in urine and feces. Animals fed 7.5 and 15% protein diets were more sensitive to toxic effects of ZEN, than those fed 45% protein. Dietary ZEN increased uterine weight in 7.5 and 15% protein groups, however, only the 7.5% protein animals receiving the 400 ppm ZEN diet had significantly larger uteri. Dietary protein affected ZEN metabolism, affecting both route of excretion and metabolites formed. More conjugated ZEN was excreted in urine and less $\alpha$-zearalenol (ZOL) formed in 45% compared to 7.5 or 15% protein groups.

UDP-glucuronyltransferase (GT) enzyme activity declined after 14 days storage at both -20 and -80°C. Postnatal developmental pattern of GT activity is substrate, gender and age specific. Immature female rats exhibit a greater hepatic conjugation than males, while adults male rats exhibit greater GT activity than females. $GT_1$ isoenzyme activity is greater

than $GT_2$ activity in all age groups. Dietary ZEN induced both $GT_1$ and $GT_2$ activity including activity towards itself. Developmental, kinetic and induction studies suggests that ZEN behaves like a $GT_2$ substrate.

Hepatic GT activity was greater in 250 ppm ZEN fed rats compared to controls while 3$\alpha$-hydroxysteroid dehydrogenase (HSD) activity, measured by androsterone reduction, was unaffected. Phenobarbital pretreatment induced NADPH-cytochrome $P_{450}$ reductase and depressed HSD activities, while increasing urinary ZOL excretion. When hepatic ZEN reduction was measured with ZEN as substrate an increase in ZOL production was observed. The data suggest that HSD enzyme activity is a poor index of hepatic ZEN reduction.

GT activity was increased in hepatic, intestinal and renal microsomes in rats fed 250 ppm ZEN. ZOL synthetic activity was increased in intestinal preparations in rats fed 250 ppm ZEN. This data indicates that extrahepatic ZEN metabolism is significant.

Systemic elimination of ZEN and metabolites occurs through biliary and urinary routes, with biliary excretion the major route and ZEN glucuronide the major excretory product. ZEN undergoes enterohepatic recirculation which increases estrogen receptors exposure to and contribute to ZEN toxicity.

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# CHAPTER 1

## INTRODUCTION

Mycotoxins are secondary metabolites of mold produced during fungal growth. Cereal grains, corn and other field crops provide an excellent substrate for mold growth, therefore when environmental conditions are suitable, fungal growth and mycotoxin synthesis will occur. Mycotoxins may cause toxicity to humans or animals upon ingestion, and contamination of foods and feeds continues to be a problem (Jelinek *et al.*, 1989). It has been suggested that mycotoxins, apart from their overt effects, predispose livestock to secondary diseases, such as infectious diseases, resulting in decreased productivity as a result of sub-clinical levels of mycotoxin intake (Mirocha *et al.*, 1977; Richard *et al.*, 1978). They are recognized as an important problem in livestock production, and apart from the economic importance, are of concern because of the potential for mycotoxins to enter the human food chain.

There are in excess of one hundred different mycotoxins that can occur in cereal grains, feedstuffs and mixed feeds (Mirocha and Christenson, 1974; Heseltine, 1974). Relatively few of these compounds have been investigated for their

effects on animals. Generally speaking, the mycotoxins of greatest concern to the animal industry are the aflatoxins, T-2 toxin, deoxynivalenol, ochratoxin and zearalenone (ZEN). The pathological alterations due to mycotoxicoses are variable depending on the toxin and exposure. For example, aflatoxin produces hepatic necrosis, ochratoxin produces nephroses, T-2 toxin is a dermonecrotic toxin and zearalenone, is an estrogenic toxin (Pier, 1973).

*Fusarium* mycotoxins are a major concern in Canada and other countries in temperate climates (Tanaka *et al.*, 1988). The focus of this dissertation will be on the mycotoxin ZEN, produced by a number of species of *Fusarium* (Caldwell *et al.*, 1970) when hosted on corn and other cereal crops. Estrogenic compounds in the food chain are a significant problem. Over 300 plants have been shown to contain phytoestrogens (Farnsworth *et al.*, 1975), such as the isoflavones in soybeans and clovers, and coumestans in clovers and alfalfa. Agro-chemicals such as the dichlorodiphenyltrichloroethane analogue methoxychlor and chlordecone, as well as some polychlorinated biphenyls, are known to be weakly estrogenic and agricultural commodities could contain residues of these chemicals (Bulger and Kupfer, 1985). The presence of an estrogenic mycotoxin such as ZEN in edible plants constitutes another potential source of estrogenic exposure to both animals and man. Where residue levels in edible plants are uncontrolled, food products derived from animals exposed to fungal estrogens,

phytoestrogens and anabolic agents with estrogenic activity such as diethylstilbesterol, used as a growth promoter in farm animals, could increase the estrogenic burden in the human food chain. In Puerto Rico, ZEN was implicated as a causal agent for the outbreak of precocious pubertal changes in thousand of young children (Schoental, 1983; Sáenz de Rodríguez *et al.*, 1985). Therefore, ZEN is one of the estrogenic burdens we face, and it is of significance.

Although there are numerous reports on ZEN mycotoxicoses, there are limited data on the absorption, distribution and elimination of ZEN in animals. The experiments presented in this thesis were, therefore, undertaken to further document the *in vitro* and *in vivo* metabolism of ZEN and to investigate its pharmacokinetics.

# CHAPTER 2

# LITERATURE REVIEW

## History

In the early 1900s, American swine producers suspected contaminated feedstuffs caused false heat, pseudopregnancy and infertility in their herds (Mirocha *et al.*, 1977). The consumption of moldy feed was implicated as the cause of this hyperestrogenism in swine (Buxton, 1927; McNutt *et al.*, 1928). However, initial evidence was anecdotal in nature, typical of the initial observations of mycotoxicoses, and little attention was paid to these reports until the specific estrogenic compound F-2, known today as ZEN, was isolated from the mycelia of the fungus *Gibberella Zeae (Fusarium graminearum)* and identified as the causal agent (Stob *et al.*, 1962). ZEN's structure and physicochemical properties were determined by Urry and co-workers (1966) (Table 1). This mold secondary metabolite induced an hyperestrogenic condition in young swine identical to that described by McNutt *et al.*, (1928) as 'vulvovaginitis'.

ZEN and its estrogenic metabolites $\alpha$- and $\beta$-zearalenol (ZOL), promote estrus in rats, mice and swine (Mirocha and Christenson, 1974; Mirocha *et al.*, 1968a; Chang

**TABLE 1.** Physicochemical Properties of Zearalenone

| | |
|---|---|
| Chemical Abstract Name: | [S-(E)]-3,4,5,6,9,10-hexahydro-14, 16 dihydroxy-3-methyl-1H-2-benzoxacyclotetradecin-1,7 (8) -dione |
| Chemical Synonym: | 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-ß-resorcylic acid lactone; |
| Molecular Weight: | 318.4 |
| Empirical Formula: | $C_{18}H_{22}O_5$ |
| Melting-Point: | 164-165°C |
| UV Absorption | (in methanol) at 236 ($\epsilon$ = 29,700), 274 ($\epsilon$ = 13,909), and 316 ($\epsilon$ = 6,020) nm (Mirocha *et al.*, 1977) |
| Optical Rotation: | $[\alpha]_{546}^{25}$ -170.5° (c = 1.0 in methanol) |
| Solubility: | Solubilities at 25°C in % by weight are: water, 0.002; n-hexane, 0.05; benzene, 1.13; acetonitrile, 8.6; dichloromethane, 17.5; methanol, 18; ethanol, 24; and acetone, 58 (Hidy *et al.*, 1977) |
| Stability: | Stable in solid state; Stable to hydrolysis |

From Urry *et al.*, (1966) and Windholz (1976) unless otherwise stated.

*et al.*, 1979). ZEN, in addition to its hyperestrogenic effects, has been shown to be a growth promoter in farm animals (Stob *et al.*, 1962). A metabolite of ZEN, zearalenol (Ralgro), is used as growth promoter in cattle and sheep (Hurd, 1977; Ralston, 1978). Since diethylstilbesterol was found to be carcinogenic and banned, several investigators have conducted a systematic study of the biological activities of ZEN and several of its synthetic analogues.

**Animal Toxicity**

The presence of ZEN in feed causing hyperestrogenism in swine has been a long standing animal husbandry problem. Swine are probably the most sensitive animals to the estrogenic effects of ZEN and the hyperestrogenism is most pronounced in prepubertal gilts (Kurtz *et al.*, 1969). Typical signs of hyperestrogenism are prolonged estrus, anestrus, changes in libido, infertility, increased incidence of pseudopregnancy, increased udder or mammary gland development, and abnormal lactation (Mirocha *et al.*, 1968a; Mirocha and Christenson, 1974). Stillbirths, neonatal mortality, abortions, mastitis, vulvovaginitis and rectal/vaginal prolapses are secondary complications associated with ZEN ingestion (Kurtz *et al.*, 1969; Miller *et al.*, 1973; Chang *et al.*, 1979; Sharma *et al.*, 1974; Sundlof and Strickland, 1986). Clinical signs of hyperestrogenism may be induced in prepubertal gilts at low exposures of ZEN. Immature gilts

given 1 mg ZEN per day developed tumefaction of the vulva, and 5 mg per day caused an increase in uterine weight (Chang *et al.*, 1979). Large amounts of ZEN in feed, 100 ppm ZEN, has pronounced effects on cycling animals, including nymphomania, pseudopregnancy, ovarian atrophy and morphologic changes in the endometrium (Chang *et al.*, 1979). Young males underwent a feminizing effect with atrophy of the testes, swelling of the prepuce and enlargement of the mammary glands (Mirocha and Christenson, 1974).

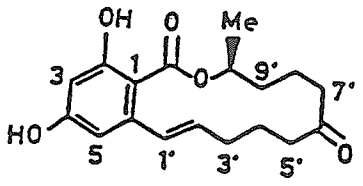
Cattle appear to be less sensitive than swine to the estrogenic effects of ZEN. Although there have been reports of effects of ZEN on cattle, there appears to be no conclusive evidence linking dietary ZEN with hyperestrogenism in cattle. There have been reports linking infertility in dairy cattle (Mirocha *et al.*, 1968) and early abortions in cows (Kallela and Ettala, 1984) to ZEN, however, no cause and effect relationship has been demonstrated.

Both growing turkeys and finishing broiler chickens can tolerate high levels of estrogens in their diets and appear to be less susceptible to the effects of ZEN (Allen *et al.*, 1981). Feeding 800 mg $kg^{-1}$ of ZEN in the diet resulted in minimal effects on performance in 0 to 3 week-old broiler chickens (Chi *et al.*, 1980). High levels of dietary ZEN caused swelling of the vent in turkey poults (Mirocha *et al.*, 1971).

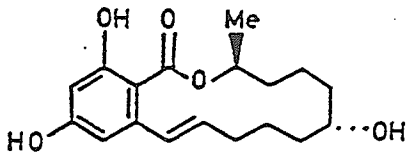
Rats appear to be moderately sensitive to ZEN toxicoses. Orally administered ZEN, at a dose of 1 mg $kg^{-1}$ BW, was shown to cause uterine hypertrophy (Ueno *et al.*, 1974). Kiritsy *et al.* (1978) demonstrated uterine hypertrophy in rats fed diets containing 50 ppm, a daily expsoure less than 1 mg $kg^{-1}$BW.

**Chemistry**

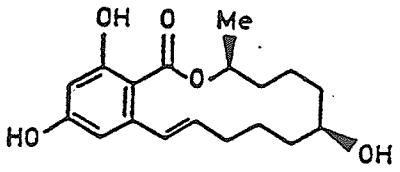
The chemistry of ZEN has been reviewed by Shipchandler (1975) and Pathre and Mirocha (1976). The estrogenic activity and potency of ZEN and its derivatives (Fig. 1) can be partially attributed to their binding affinity to estrogen receptors in target tissues (Katzenellenbogen *et al.*, 1979). The relative estrogenic binding affinity for hepatic and uterine estrogen receptor has been observed in a number of species with the order of binding affinity being α-ZOL > ZEN > ß-ZOL (Katzenellenbogen *et al.*, 1979; Powell-Jones *et al.*, 1981; Fitzpatrick *et al.*, 1989). Because both ZEN and its derivatives, α- and ß-zearalenol (ZOL) possess estrogenic activity (Peters, 1972; Mirocha and Christenson, 1974), the reaction of particular interest is the reduction of the 6'-ketone to a secondary alcohol concomitant with the reduction of the 1'-2' double bond to yield 2 diastereoisomeric zearalenols (ZOL). α-Reduction of the 6'-keto group has generally been found to increase binding affinity to estrogen receptors, however, the ß-epimer is much less active (Fitzpatrick *et al.*, 1989).




Zearalenone




α-Zearalenol (α-ZOL)




β-zearalenol (β-ZOL)

**FIGURE 1.** Structure of zearalenone and its derivatives

**Receptor Binding in Target Tissues**

Similarities between ZEN physiological activities and estrogens prompted numerous studies on the interaction of ZEN and its derivatives with estrogen receptors. The estrogenic activity of ZEN and its derivatives may attributed, in part, to their ability to bind to estrogen receptors in target tissues (Greenman *et al.*, 1979; Boyd and Wittliff, 1978; Kiang *et al.*, 1978; Katzenellenbogen *et al.*, 1979; Powell-Jones *et al.*, 1981; and Fitzpatrick *et al.*, 1989). The traditional concept of the estrogen receptor model is a two step hypothesis. The estrogenic compounds enter the target cells and bind to receptor molecules in the cytoplasm. The binding of estrogens to its receptor promotes a translocation of the occupied receptor into the nucleus where the receptor-hormone complex binds to chromatin. In the nucleus the estrogen-receptor complex stimulates the RNA polymerase I and II enzymes (Stormshak, 1979) resulting in increased protein synthesis and uterine growth. ZEN and it derivatives, $\alpha$- and ß-ZOL, have been shown to induce synthesis of protein in the rat uterus (Kiang *et al.*, 1978; Katzenellenbogen *et al.*, 1979). Competitive binding assays show that ZEN and ZOL are capable of inhibiting the binding of estradiol to cytosolic estrogen receptor, indicating that these compounds are capable of binding to estrogen receptors and that both ZEN and $\alpha$- and $\beta$-ZOL stimulate the translocation of cytoplasmic estrogen receptor into the nucleus as shown by *de novo* protein

synthesis assays (Katzenellenbogen *et al.*, 1979).

Interspecies sensitivities to dietary ZEN may be explained, in part, by differences in relative binding affinity (RBA) of ZEN and its metabolites to estrogen receptors. The RBA of α-ZOL was 10-20 times greater than that of ZEN, and 100 times greater than that of β-ZOL (Tashiro *et al.*, 1980; Fitzpatrick *et al.*, 1989). Therefore, the reduction of ZEN producing α-ZOL appears to be an activation reaction, while the formation of the beta epimer appears to be an inactivation reaction. In swine, the predominant metabolic pathway is α reduction, producing the more potent metabolite (Olsen and Kiessling, 1983), while in poultry, more β-ZOL is formed. Additionally, the binding of ZEN, α- and β-ZOL is greater in pigs than in rats than in chickens (Fitzpatrick *et al.*, 1989). Therefore the observed interspecies differences in ZEN toxicity may be attributed to binding affinity to estrogen receptors and *in vivo* metabolites formed.

ZEN, its derivatives and other resorcyclic acid lactones possess a phenolic structure (Hacking, 1983) that appear an unlikely ligand for the estrogen receptor molecule. A plausible explanation for this binding phenomenon is the relatively close spatial similarities that exist between estradiol and ZOL (Hurd, 1977; Lindsay, 1985). Both α- ZOL and estradiol are 10-11 A° in length (Lindsay, 1985). In addition, both ZEN and α-ZOL possess similar oxygen-containing functions at the ends of their ring systems to that of

estradiol (Hurd, 1977). Thus, it appears that the greater the similarity in molecular arrangement and spatial orientation a compound possesses to estradiol, the greater the binding and the greater its estrogenic potential. Differences in estrogenicity may be attributed to variations in spatial and molecular arrangement of estrogenic compounds. For example, the *in vitro* uterotropic activity of ZEN is only 25% of ZOL which can be explained by α-ZOL having a double bond at the C-11/C-12 position and a carbonyl instead of an hydroxyl group at C-7 (Lindsay, 1985). These differences in molecular structure and arrangement alters the three-dimensional structure of α-ZOL in a manner that leads to greater affinity or 'fit' for the estrogen receptor when compared to ZEN. However, ZEN and ZOLs are less potent than estradiol because of their phenolic functional group.

**Metabolism**

There have been numerous studies characterizing ZEN metabolism *in vitro* and *in vivo*. However, there are discrepancies in the literature in the characterization of the *in vitro* enzyme activities responsible for ZEN biotransformation and conflicting reports concerning the route of excretion and the metabolites formed (Ueno *et al.*, 1977; Smith, 1980a; Smith, 1982b; Kiritsy *et al.*, 1987). This may be accounted for by the nature of previous reports, that is, *in vitro* studies focussed on hepatic metabolism of ZEN,

however, the liver is not the sole metabolic organ. Extrahepatic contribution to the metabolism of any xenobiotic can be substantial and should be determined for a complete understanding of the metabolism of a compound. Moreover, differences in experimental design, dose level, analytical technique and experiment duration contribute to inconsistencies in the literature. *In vivo* and *in vitro* studies, not only complement each other but provide important information on the mechanism of toxic expression of the compound in the various target tissues; they also provide us with a rationale for intervention to ameliorate the toxicity of xenobiotics.

**Zen Metabolism *in Vivo***

There have been limited reports on the metabolic disposition of ZEN in various species including man. However, pharmacokinetic evaluation of ZEN is either lacking or incomplete in most species. There are conflicting reports concerning the excretion routes of ZEN and the major metabolites formed (Ueno 1977; Smith 1980b; Smith 1982a; Kiritsy *et al.*, 1987; Olsen *et al.*, 1985). Oral administration of 10 mg $kg^{-1}$ BW $^{3}$H-ZEN to female rats resulted in 4-6% and 40-60% of the administered dose excreted in urine and feces, respectively (Ueno *et al.*, 1977). In the feces major excretory product was free ZEN (38-56%) and only 4-6% as $\alpha$-ZOL. Smith (1980b), using Ueno's catalytic exchange method

to produce $^{3}$H-ZEN dosed rats with 10 mg $^{3}$H-ZEN 100 $g^{-1}$ BW and recovered 31% of the radioactivity in urine and 62% in feces. In a subsequent report, Smith (1982a) dosed rats at a level of 10 mg ZEN 100 $g^{-1}$BW, recovered only 3% of the administered dose in urine and 22% in feces with a total recovery was only 25% of oral dose. Clearly, these conflicting reports on the excretory profiles of ZEN and its metabolites do not permit one to evaluate the disposition and excretion ZEN. Discrepancies in these reports may be attributed to differences in the ZEN dose employed as well as different recoveries of the administered dose. Pharmacological doses, as opposed to physiological levels, may result in saturation of metabolic pathways, alteration in the ability to detoxify the xenobiotic resulting in alterations in the flux of the parent compound or its metabolites through different metabolic pathways and subsequent changes in excretion profiles (Krayhill, 1977; Mirocha *et al.*, 1983).

There is very limited data in the literature on ZEN analysis in blood samples. Olsen *et al.*, (1985) demonstrated that ZEN and its metabolites in one prepubertal gilt were present in plasma and excreted in urine predominantly as the glucuronide. In a disposition study in pigs, Farnworth and Trenholm (1983) recognized that due to the limited blood and urine sampling, their data was insufficient to establish metabolic routes for ZEN metabolism. No blood or bile samples were obtained in the above studies to compare excretory

profiles of ZEN and its metabolites (Smith 1980b; Smith 1982a; Kiritsy *et al.*, 1987).

**Zen Metabolism *in Vitro***

ZEN metabolism has been reported to occur in a typical biphasic manner in hepatic tissue: phase I reduction, to produce α- and ß-ZOL and a phase II conjugation of ZEN and its phase I metabolites to their glucuronides, catalyzed by UDP-glucuronyltransferase (GT) (Kiessling and Pettersson, 1978). The dehydrogenase enzyme responsible for reduction of ZEN to ZOL was reported to be 3α-hydroxysteroid dehydrogenase (HSD) in rat liver by Olsen *et al.*, (1985). Androstanedione and ZEN have been observed to mutually inhibit reduction of each other, indicating involvement of HSD in ZEN metabolism (Olsen *et al.*, 1981; Stangroom and Smith, 1984). HSD is involved in the metabolism of steroids, and the estrogenic effects of ZEN has been attributed, in part, to disturbance of normal steroid metabolism by ZEN (Thouvenot and Morfin, 1980). Olsen *et al.* (1981) reported that ZEN reduction is catalyzed by HSD, however, the belief that HSD is the primary reducing enzyme for ZEN is not universally held (Ueno *et al.*, 1983; Tashiro *et al.*, 1983). Tashiro *et al.*, (1983) suggested that the microsomal ZEN reductase is distinct from this steroid dehydrogenase. This issue remains unresolved.

The effect of dietary ZEN on HSD activity has not been clearly demonstrated. James and Smith (1982) observed that

dietary ZEN decreased HSD activity, however, work by Kiritsy *et al.*, (1987) demonstrated that their enzyme assay conditions were not saturated and suggested that James and Smith's observations were erroneous. Strangroom and Smith (1984), using saturating conditions for enzyme analysis, reported an induction of HSD activity by ZEN. Kiritsy *et al.*, (1987) observed that feed restriction significantly increased HSD enzyme activity, however, no increase in $\alpha$ and $\beta$ ZOL synthesis were observed *in vivo* and the role of HSD in ZEN metabolism was questioned. In our laboratory, rats pretreated with phenobarbital increased excretion of conjugated ZEN and $\alpha$-ZOL (Pandey *et al.*, 1989), indicating an enhancement of ZOL synthesis by a known inducer of cytochrome $P_{450}$ enzyme activity.

Conflicting reports on the effect of dietary ZEN on HSD activity may be due to analytical methods used. Hepatic HSD is a multifunctional enzyme present in both microsomal and cytosol fractions with potentially different substrate affinities and specific activities towards different substrates for each sub-cellular isoenzyme (Hurlock and Talalay, 1959). In their studies, ZEN reduction was measured indirectly using androsterone as substrate, and it was assumed that androsterone reduction reflected ZEN reduction (James and Smith, 1982; Stangroom and Smith, 1984; Kiritsy *et al.*, 1987). Our current understanding of ZEN metabolism makes this assumption questionable. The need to characterize the

enzyme(s) responsible for reduction of ZEN becomes obvious when one considers that α-ZOL is the most potent estrogenic metabolite of ZEN and that much of ZEN's toxicity is probably due to the greater estrogenic potential of this metabolite.

Glucuronidation is an important pathway in the detoxification of endogenous and exogenous compounds (Dutton, 1980). The addition of the glucuronide moiety from UDP-glucuronic acid to the aglycone acceptor enhances the excretory processes for the acceptor compounds. Conjugation, therefore, represents a major detoxification mechanism for xenobiotics.

GT enzyme activity is mediated by a family of isoenzymes that can be selectively induced by various xenobiotics (Finley *et al.*, 1986). The induction of GT activity by PB increased the urinary excretion of conjugated ZEN and α-ZOL (Pandey *et al.*, 1989). It has been suggested, by Kiritsy *et al.* (1987), that conjugation of ZEN and its metabolites act to reduce the toxic expression of ZEN by increasing urinary excretion, thus limiting the potential for enterohepatic recirculation (Smith, 1980a).

Although the liver appears to be the main organ responsible for ZEN metabolism from *in vitro* studies, numerous reports have shown that a variety of other tissues including the kidney (Tomkins, 1956), ovary (Jarrell and Robaire, 1982), and intestine (Nienstedt *et al.*, 1972) exhibit HSD activity. GT activity has also been reported to be widely distributed in

the body (Aitio and Marniemi, 1979; Bottoms *et al.*, 1977; Olsen *et al.*, 1987). Olsen *et al.*, (1987), using direct measures of ZEN conjugation and ZOL formation, reported extensive glucuronidation and reducing activities in the sow intestinal mucosa. The fact that both these metabolizing enzymes are widely distributed throughout the body suggests extrahepatic metabolism and tissue uptake of the mycotoxin is highly probable, although it is not clear from the literature to what extent it contributes to the overall metabolism of ZEN in the rat.

There have been numerous studies on the effect of dietary manipulation on ZEN toxicity (Smith, 1980a; James and Smith, 1982; Smith, 1982a; Stangroom and Smith, 1984; Kiritsy *et al.*, 1987). Smith (1980a) reported that 25% alfalfa and 25% oats fed to female weanling rats on diets containing 250 ppm ZEN, improved feed efficiencies, feed consumption and body weights. Increasing dietary protein also promoted growth in ZEN treated rats and the synthetic anion exchange zeolite was effective in alleviating ZEN toxicoses (Smith, 1980a). In a subsequent study, Smith (1982a) reported that rats fed high-protein diets excreted more free ZEN and $\alpha$-ZOL in urine compared to control rats. The feeding of protein+alfalfa also resulted in increased urinary ZEN excretion and increased fecal excretion of ZEN, $\alpha$- and $\beta$-ZOL while a 5% anion-resin diet resulted in a reduction in urinary excretion of conjugated ZEN and $\alpha$-ZOL, probably due to increased biliary excretion (Smith, 1982a).

Feed restriction to 75% of *ad libitum* diets, influenced both the route of excretion as well as the metabolite profile in rats (Kiritsy *et al.*, 1987). There were greater urinary losses of conjugated ZEN and less α-ZOL. The authors concluded that animals on an ad libitum diet were more sensitive to the toxic effects of ZEN compared to feed restricted animals due to an increased GT activity and subsequent increase in formation of the inactive glucuronide in feed restricted animals.

# CHAPTER 3

# ZEARALENONE METABOLISM AND EXCRETION IN THE RAT: EFFECT OF DIFFERENT DOSES

## INTRODUCTION

Zearalenone (ZEN) [6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid-lactone] is a naturally occurring mycotoxin synthesized by *Fusarium* mold species endemic to the temperate Canadian climate (Hardwig and Munro, 1975). ZEN and its metabolites α- and β-ZOL belong to a rare class of natural products which are capable of binding to estrogen receptors because of their chemical similarity to estradiol (Hurd, 1977; Katzenellenbogen *et al.*, 1979). ZEN toxicity may occur when mold-contaminated feed is ingested by many species of livestock and experimental animals (Mirocha *et al.*, 1977). Dietary ZEN acts systemically producing an 'estrus syndrome' in swine, cattle and laboratory animals (Chang *et al.*, 1979). The clinical effects of subacute ZEN mycotoxicosis include decreased intake, reduced growth and decreased reproduction; ZEN has also been implicated in teratogenesis and carcinogenesis (Mirocha *et al.*, 1980).

ZEN is a hydrophobic xenobiotic which is metabolized by two different reactions, a reduction to an alcohol and a

conjugation with glucuronic acid (Kiessling and Pettersson, 1978). Reduction and conjugation of ZEN increase its solubility and may promote its excretion in the urine. Conjugation of ZEN increases its molecular weight which acts to promote biliary excretion (Mulder *et al.*, 1981). Conflicting reports concerning the route of ZEN excretion and the major metabolites formed have been published (Ueno *et al.*, 1977; Smith, 1980a; Smith, 1982a; Kiritsy *et al.*, 1987). These discrepancies may be due to basic differences in experimental design, such as dose level, and/or analytic technique. For example, overdosing animals results in metabolic overload, the ability to detoxify the compound is exceeded, consequently the rate and route of metabolism, and excretion may be significantly altered (Krayhill, 1977; Mirocha *et al.*, 1983).

The purpose of this study was to examine the effect of ZEN dose level on the metabolites formed and the route of excretion.

## MATERIALS AND METHODS

**Animals and Treatment:** Use of experimental animals conformed to the guidelines of the Canadian Council on Animal Care. Twenty young female Sprague Dawley rate (50-60 g) were purchased from the University of Manitoba central breeding facility. Animals were housed in individual cages and kept on a 14-10 hour light-dark cycle. The room temperature was maintained at 21 ± 1°C with a relative humidity of 50%. Rats were fed a commercial chow diet (Ralston Purina Ltd., St. Louis, MO) for 2 days and then switched to a standard semipurified diet (Kiritsy *et al.*, 1987). After 2 weeks on the dietary regimen animals were randomly assigned to experimental treatment and received either 1 (low dose, LD) or 100 (high dose, HD) mg ZEN $kg^{-1}$ BW by gavage. Rats were transferred to metabolic cages and maintained on their dietary regimen. Urine and feces were collected every 24 hours for 4 days and were kept frozen until analyzed.

**Preparation of Samples:** Modifications were made to the method described by Farnworth and Trenholm (1983) for the analysis of ZEN, $\alpha$- and $\beta$-ZOL. Urine samples were allowed to thaw and mixed on a vortex. One hundred ul urine, 100 ul $\beta$-glucuronidase enzyme (Sigma Chemical Co., St. Louis, MO., Type

VIII, 458 units/100 ul in 50% glycerol) and 1.8 ml glass distilled water were pipetted into an 8 ml screw top test tube. Tubes were loosely capped and the mixture was incubated overnight at 37°C in an agitating water bath. To the cooled incubation mixture 2 ml of 10% 2-propanol in ether was added, the mixture was vortexed and then centrifuged at 1500 g for 5 minutes. The top layer was transferred to a clean 8 ml screw top test tube, the extraction was repeated on the original tube and the two extractions were combined. The samples were kept on ice for the remainder of the procedure. To the cold sample, 2 ml of chilled 0.184 M NaOH was added, vortexed thoroughly centrifuged for 2 min at 1500 g, and the top layer was discarded. The aqueous layer was washed twice with 2 ml of benzene by vortexing, centrifuging for 2 min at 1500 g and the top layer was discarded. The sample was neutralized with chilled 0.5 N acetic acid and extracted three times with 2 ml of benzene by vortexing, centrifuging for 2 min at 1500 g, the top layer was retained from each extraction, combined and evaporated to dryness. Samples were taken up in a known amount of HPLC solvent. For the determination of free ZEN, $\alpha$- and $\beta$-ZOL in urine, the $\beta$-glucuronidase enzyme incubation step was omitted and 1.9 ml of distilled water was added to the sample.

Total fecal samples were allowed to thaw and transferred to a glass homogenizing tube containing 9 volumes of distilled water. A polytron homogenizer was used to blend feces to a

uniform consistency. A 100 ul aliquot was taken, cleaned up and extracted for ZEN, α- and β-ZOL using the method detailed above for urine. Recoveries of standards added to feces were (means ± SD for 10 observations) 97.1 ± 5.1% for ZEN, 95.6 ± 4.6% for α-ZOL and 96.7 ± 4.2% for β-ZOL. Therefore, the acid-base clean up and extraction technique was considered quantitative and all values reported here were not corrected for recovery.

**HPLC Analyses:** ZEN, α- and β-ZOL standards were obtained from Commercial Solvents Corp., (Terre Haute, IN). Solvents were HPLC grade; other chemicals were reagent grade; and all were obtained from Fisher Scientific (Ottawa, Ont).

High performance liquid chromatography was performed using a Beckman Model 110A pump with Model 420 controller (Toronto, Ont). A Whatman CO:PELL ODS precolumn (Terochem, Edmonton, Alta.) and an Ultrasphere ODS (5 u, 250 x 4.6 mm ID) analytical column (Beckman, Toronto, Ont) was used. Detection was accomplished using a Schoeffel Model FS-970 fluorometer with excitation wavelength set at 280 nm filter. A Bausch and Lomb VOM7 recorder (Fisher Scientific, Ottawa, Ont) and a Hewlett Packard 3390A integrator (Edmonton, Alta) were used to record and integrate the detector signals. The flow rate was 1 ml $min^{-1}$ and the solvent system employed was water:methanol:acetonitrile 43:42:15 + 0.2% phosphoric acid.

**Statistical analysis:** Means were calculated on treatment groups and compared using a student's t-test (SAS, 1985).

## RESULTS

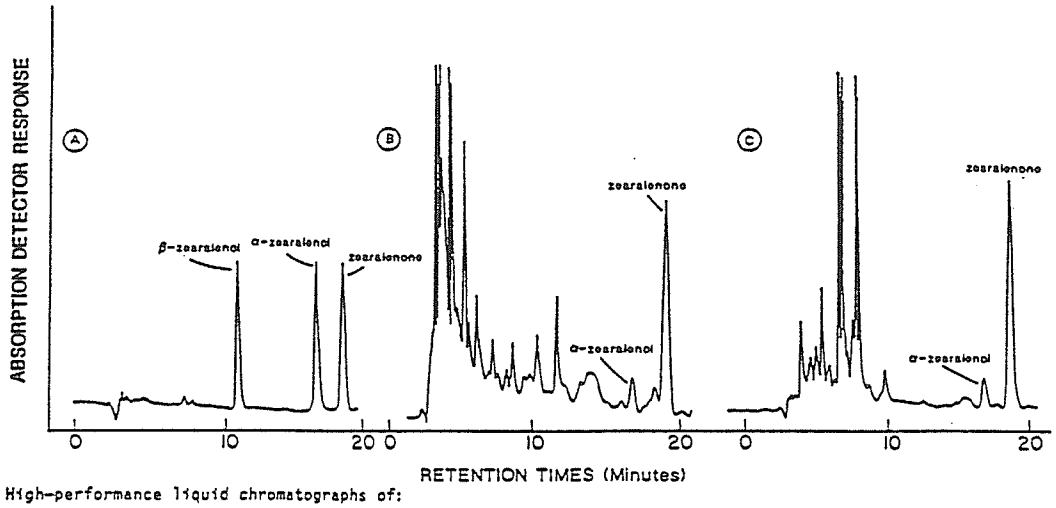
The HPLC method described is quite straight forward, and requires less time for sample extraction and clean-up than methods requiring column chromatographic clean up (Smith, 1982a). It permits the quantitative determination of both the free and glucuronide-bound forms of ZOL and α-ZOL in urine and feces (Table 2) without the use of an internal standard (James *et al.*, 1982; Smith, 1982a). The use of both absorbance and fluorescence detectors (Fig. 2 and 3) minimized the effect that interfering peaks may have on analysis.

ZEN dose had little effect on its metabolism, neither the route of excretion nor the metabolites formed were affected (Table 2). As the ZEN dose increased from 1 to 100 mg $kg^{-1}$ BW, there was an increase in the proportion of ZEN excreted in the feces (40 to 54%), however this was the only significant difference observed. The quantity of ZEN and metabolites excreted in feces in 96 h was about 2-4 times greater than that excreted in urine. In both the LD and HD animals, free fecal ZEN was the major metabolite, 40 and 54% of the administered dose, with a total of 52 and 64% of ZEN and metabolites recovered in the feces, respectively. The urine was a significant route of excretion accounting for 20 and 15%

**TABLE 2.** Effect of Dose on 96-h Excretion of Zearalenone and α-Zearalenol in the Urine and Feces, as a Percent of the Administered Dose

| | ZEARALENONE | |
|---|---|---|
| | LOW DOSE 1 mg $kg^{-1}$ BW | HIGH DOSE 100 mg $kg^{-1}$ BW |
| Free Urinary Zearalenone | 16.2 ± 2.0 | 11.5 ± 1.9 |
| Conjugated Urinary Zearalenone | 2.3 ± 0.7 | 1.9 ± 0.9 |
| Fecal Zearalenone | 40.3 ± 4.3 | 54.3 ± 3.6* |
| Free Urinary α-Zearalenol | 1.4 ± 0.3 | 1.1 ± 0.2 |
| Conjugated Urinary α-Zearalenol | 0.4 ± 0.1 | 0.2 ± 0.1 |
| Fecal α-Zearalenol | 11.5 ± 1.3 | 9.8 ± 1.1 |
| Total Recovery | 72.1 ± 3.9 | 78.8 ± 3.7 |

N = 10, mean ± SEM
*Significantly different ($p<0.05$)



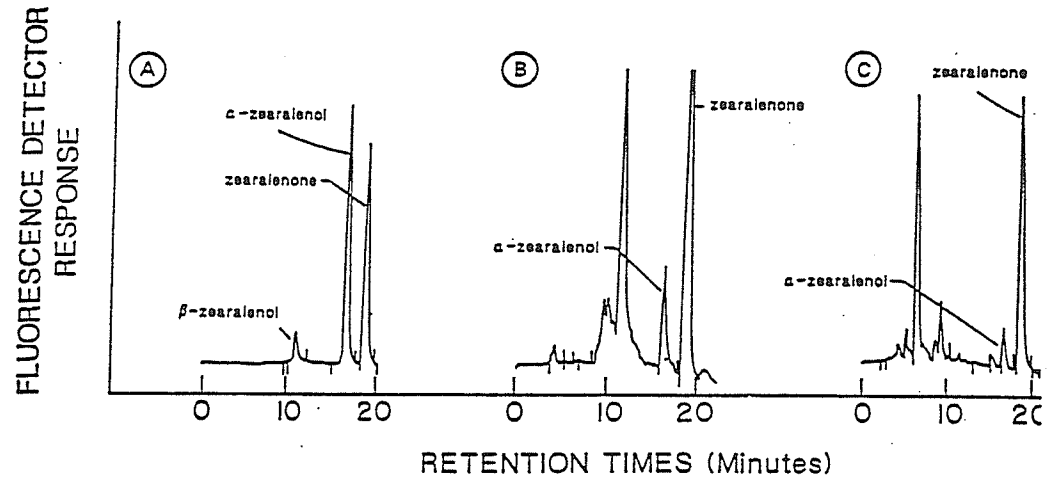

High-performance liquid chromatographs of:

A) Standard solution containing zearalenone (25 ng), α-zearalenol (25 ng) and β-zearalenol (25 ng).

B) Urine from rats dosed with 1 mg zearalenone $Kg^{-1}$ body weight.

C) Feces from rats dosed with 1 mg zearalenone $Kg^{-1}$ body weight.

**FIGURE 2.** HPLC chromatographs showing retention times using absorbance detector




High-performance liquid chromatographs of:

A) Standard solution containing zearalenone (25 ng), α-zearalenol (25 ng). and β-zearalenol (25 ng).

B) Urine from rats dosed with 1 mg zearalenone $Kg^{-1}$ body weight.

C) Feces from rats dosed with 1 mg zearalenone $Kg^{-1}$ body weight.

**FIGURE 3.** HPLC chromatographs showing retention times using flourescence detector

of the oral dose in LD and HD animals. Similar amounts of conjugated metabolites (3 and 2%) and α-ZOL (13 and 11%) was observed in LD and HD animals, respectively.

## DISCUSSION

The production of α-ZOL is metabolically significant, in that α-ZOL is the most potent ZEN metabolite (Hurd, 1977). A comparison of relative binding affinities of ZEN, α-ZOL, β-ZOL and diethylstilbestrol (DES) for cytosolic estrogen receptors indicated that DES and α-ZOL had similar receptor affinities, approximately 15 times greater than that of ZEN (Fitzpatrick *et al.*, 1989). Therefore, the reduction of ZEN to yield α-ZOL appears to be an activation reaction which may, in part, account for the estrogenic effect of dietary ZEN.

Significant quantities of ZEN and metabolites, 15-20% of the oral dose, were excreted in the urine. This agrees with other reports from our laboratory (Kiritsy *et al.*, 1987), but is in contrast to the finding of Smith (1982a). This discrepancy may be due to differences in recoveries, for example, Smith dosed his animals with 100 mg ZEN $kg^{-1}$ BW and recovered, after 48 h, 3% of the total dose in the urine and 22% in the feces. However, when tritiated ZEN was orally administered to rats (Smith, 1980b), 31% of the radioactivity was recovered in the urine and 62% in the feces. In the experiment reported here, HD animals were dosed with 100 mg ZEN $kg^{-1}$ BW, and 79% of the dose was recovered, 15% in the

urine and 64% in the feces after 96 h. ZEN dose level had little effect on its metabolism.

# CHAPTER 4

# EFFECT OF DIETARY PROTEIN ON ZEARALENONE METABOLISM AND TOXICITY IN THE RAT

## INTRODUCTION

Zearalenone (ZEN) is a naturally occurring mycotoxin synthesize by *Fusarium* mold species endemic to the temperate Canadian climate (Hardwig and Munro, 1975). ZEN and its metabolites $\alpha$- and $\beta$-zearalenol (ZOL), belong to a rare class of natural product, the $\beta$-resorcyclic acid-lactones, which are capable of binding to estrogen receptors because of their chemical similarity to estradiol (Hurd, 1977; Katzenellenbogen *et al.*, 1979). Dietary ZEN acts systemically producing an 'estrus syndrome' in swine, cattle and laboratory animals (Chang *et al.*, 1979). The clinical effects of subacute ZEN mycotoxicosis include decreased feed intake, reduced growth and decreased reproduction; ZEN has also been implicated in teratogenesis and carcinogenesis (Mirocha *et al.*, 1980).

Diet composition affects xenobiotic metabolism (Dickerson, 1978). The persistence and severity of the pharmacological action of many compounds is determined by the capabilities of the catabolic enzyme systems to metabolize the compound (Hathcock, 1982). This ability depends upon

nutritional factors (Park and Ionnides, 1981) and exogenous and endogenous chemicals. The effect of undernutrition on the oxidative metabolism of foreign compounds (Dickerson, 1978) and hepatic conjugation (Kato *et al.*, 1969; Merril and Bray, 1982) has been documented.

Some interrelationships between diet composition and ZEN toxicosis and metabolism have been investigated in the rat (Smith, 1980a; Smith, 1982a; Smith, 1980b; James and Smith, 1982; Stangroom and Smith, 1984; Kiritsy *et al.*, 1987). Dietary supplements of protein, dehydrated alfalfa meal and anion-exchange resin were shown to overcome ZEN toxicosis. This protective effect may be due to a binding of ZEN in the intestinal lumen which reduces the absorption of the toxin (James and Smith, 1982) or to a sequestering of bile salts by these dietary constituents (Kritchevsky, 1978) which disrupts enterohepatic circulation of ZEN.

The purpose of our experiment was to examine the effect of dietary protein on ZEN metabolism; specifically, to determine the effect of dietary protein on the activities of the ZEN metabolizing enzymes, ZEN metabolism and toxicity in the rat.

## METHODS AND MATERIALS

**Animals and Treatment:** Use of experimental animals conformed to the guidelines of the Canadian Council on Animal Care. Two hundred young female Sprague Dawley rats (50-60 g) were purchased from the University of Manitoba central breeding facility. Each of the three groups of animals was received when required for the three separate experimental studies. All animals were housed separately in galvanized steel cages and kept on a 14-10 h light-dark cycle. The room temperature was maintained at 21 ± 1°C with a relative humidity of 50%. Rats were fed a commercial chow diet (Ralston Purina Co., St. Louis, MO) for 2 days and then animals were randomly assigned to standard semipurified diets containing 7.5, 15 or 45% protein (Table 3), formulated according to the National Research Council's guidelines. These diets were fed daily and animals were weighed weekly.

**Enzyme Study:** After 2 weeks on the dietary regimen animals were killed by decapitation, their livers were excised and immediately assayed for 3α-hydroxysteroid dehydrogenase (HSD) and uridine diphosphate glucuronyl transferase (GT) enzyme activity, the ZEN metabolizing enzymes (Kiessling and Pettersson, 1978). A modification of the spectrophotometric

**TABLE 3.** Composition of Diets of Different Protein Concentrations (% wt.)

| | Low Protein 7.5% | Standard Protein 15% | High Protein 45% |
|---|---|---|---|
| Casein* | 8.62 | 17.24 | 51.72 |
| DL Methionine* | 0.15 | 0.30 | 0.90 |
| Glucose** | 35.76 | 31.38 | 13.84 |
| Cornstarch*** | 35.76 | 31.38 | 13.84 |
| Corn Oil+ | 5.00 | 5.00 | 5.00 |
| Lard++ | 5.00 | 5.00 | 5.00 |
| Fiber (Alphacel)+++ | 5.00 | 5.00 | 5.00 |
| Mineral Mix+++ | 3.50 | 3.50 | 3.50 |
| Vitamin Mix+++ | 1.00 | 1.00 | 1.00 |
| Choline Bitartrate* | 0.20 | 0.20 | 0.20 |

*United States Biochemicals, Cleveland, OH.
**The R Wine Baril, Winnipeg, MB.
***Canada Starch, Cardinal, ON.
+Best Foods, Montreal, PQ.
++Canada Packers, Toronto, ON.
+++ICN Pharmaceuticals Inc., Cleveland, OH.

method of James and Smith (1982) was used to determine the activity of HSD. Approximately 2 g of fresh liver was homogenized for 1 min at 0°C in 38 ml of 0.01 M potassium phosphate buffer at pH 7.2. The homogenate was centrifuged at 25,000 x g for 10 min at 4°C. The reaction mixture contained 0.1 ml supernatant, 0.6 ml 0.1 M sodium pyrophosphate buffer at pH 8.9, 2.0 ml glass distilled water and 0.2 ml 0.01 M NAD at 25°C. To initiate the reaction, 0.1 ml of 0.01 M androsterone in methanol was added. Assays were performed in triplicate. The protein content in each homogenate was determined according to Lowry *et al.*, (1951). The activity of HSD was measured as the change in optical density at 340 nm and was expressed as nmol NADH produced $min^{-1}$ $mg^{-1}$ protein. GT enzyme activity was determined by the spectrophotometric method of Hall and Esbenshade (1984). The activity of GT was measured as the change in optical density at 400 nm and was expressed as nmol p-nitrophenol conjugated $h^{-1}$ $mg^{-1}$ protein.

**Metabolism Study:** After 2 weeks on the dietary regimen, animals were dosed with ZEN, 1 mg $kg^{-1}$ BW, by stomach intubation. Rats were transferred to stainless steel metabolic cages and maintained on their dietary regimen. Urine and feces were collected every 24 hours for 4 days and were kept frozen until analyzed. ZEN and its metabolites were extracted and quantified by HPLC as previously described.

**Toxicity Study:** Within each dietary treatment group, 10 animals were assigned to the semi-purified diet containing 0, 50, 150 or 400 ppm pure ZEN. After 3 weeks on this dietary regimen, animals were humanely killed by decapitation, liver and uterine weights were determined.

**Statistical Analysis:** Data were analyzed using the Statistical Analysis System (SAS, 1985). A one-way analysis of variance (ANOVA) was used to determine the effect of treatment (dietary protein) in the enzyme and metabolic studies. A two-way ANOVA was used in the toxicity study to determine the independent effect of feed restriction and dietary ZEN on weight gain, liver and uterine weight. Group comparisons were made as orthogonal contrasts where necessary. A difference was accepted as significant when $p<0.05$. All data are presented as mean $\pm$ SEM.

## RESULTS

**Enzyme Study:** Dietary protein affected feed intake, weight gain, feed efficiency and the activity of GT (Table 4). Total feed intake was significantly less in the 45% protein group than both the 15 or 7.5% protein groups. The diet containing 15% dietary protein supported the best weight gain, while no significant difference in weight gain between the 45 and 7.5% protein diets was observed. Feed efficiency was calculated and found to be significantly different for all three levels of dietary protein, with high protein animals having the greatest efficiency and low protein animals having the least efficiency. The activity of GT was measured and found to be significantly greater in the 7.5% dietary protein group than either the 15 or 45% protein group; the activities were 60.5, 49.9 and 41.7 nmol of p-nitrophenol conjugated $h^{-1}$ $mg^{-1}$ protein, respectively.

**Metabolism Study:** There was a significant effect of dietary protein on the metabolism of ZEN, with both the route of excretion and the metabolites formed being affected (Table 5). In the 15 and 7.5% protein groups, free fecal ZEN was the major excretory metabolite accounting for 31 and 26.5% of the administered dose, with 38.4 and 33.5% of the ZEN and its

**TABLE 4.** Enzyme study. Effect of Dietary Protein on Final Body Weight, Gain/Feed Ratio, Hepatic 3α-hydroxysteroid Dehydrogenaseand Hepatic Uridine Diphosphate Glucuronyl Transferase Activity in Rats

| | Low Protein | Standard Protein | High Protein |
|---|---|---|---|
| Feed intake (g) | 190 ± 4.3[a] | 179 ± 4.7[a] | 131 ± 3.3[b] |
| Final body weight (g) | 110 ± 2.6[a] | 119 ± 3.6[b] | 104 ± 3.3[a] |
| Weight gain/ feed consumed | 0.31 ± 0.01[a] | 0.38 ± 0.01[b] | 0.43 ± 0.01[c] |
| Activity of 3α-hydroxysteroid dehydrogenase* | 2.3 ± 0.1[a] | 2.5 ± 0.2[a] | 2.4 ± 0.2[a] |
| Activity of uridine diphosphate glucuronyl transferase+ | 60.5 ± 3.5[a] | 49.9 ± 3.1[b] | 41.7 ± 4.1[b] |

N=15, mean ± SEM
Means not designated with the same letter are significantly different $p<0.05$, Tukey's Studentized Range.
*Namomoles NADH formed/min/mg protein
+Nanomoles p-nitrophenol conjugated/h/mg protein

**TABLE 5.** Metabolism study. Effect of Dietary Protien on 96 h Excretion of Zearalenone and α-zearalenol in Urine and Feces, Expressed as a % of the Administered Dose*

| | Free Urinary | Conjugated Urinary | Free Fecal | Total Distribution |
|---|---|---|---|---|
| Zearalenone Excretion | | | | |
| Low Protein | 20.1 ± 2.1[a] | 3.2 ± 1.1[a] | 26.5 ± 2.3[a] | 49.7 ± 2.3[a] |
| Standard Protein | 21.5 ± 2.6[a] | 2.3 ± 0.7[a] | 31.0 ± 6.1[a] | 54.7 ± 5.0[a] |
| High Protein | 41.0 ± 5.0[b] | 7.9 ± 2.9[a] | 13.0 ± 2.0[b] | 62.0 ± 3.6[a] |
| α-zearalenol Excretion | | | | |
| Low Protein | 1.4 ± 0.3[a] | 0.6 ± 0.1[a,b] | 7.0 ± 0.8[a] | 9.0 ± 1.0[a] |
| Standard Protein | 1.1 ± 0.4[a] | 0.3 ± 0.1[a] | 7.4 ± 1.4[a] | 8.9 ± 1.4[a] |
| High Protein | 1.4 ± 0.3[a] | 1.1 ± 0.2[a] | 2.3 ± 0.2[b] | 4.7 ± 0.5[b] |

N=10, mean ± SEM
Means not designated with the same letter are significantly different $p<0.05$, Scheffe's test.
*Total recovery for the low protein diet group was 58.8 ug of zearalenone and metabolites, 59% of the oral dose.
Total recovery for the standard protein diet group was 63.5 ug of zearalenone and metabolites, 64% of the oral dose.
Total recovery of the high protein diet group was 66.6 ug of zearalenone and metabolites, 67% of the oral dose.

metabolites recovered in the feces, respectively. In the 45% protein group, free urinary ZEN (41%) was the major excretory metabolite and 51.4% of the ZEN dose was recovered as ZEN or its metabolites in the urine. Animals on the 7.5 and 15% protein diets excreted more total α-ZOL (8.9-9 vs 4.7%, $p<0.05$) than did animals on the high protein diet due to greater fecal excretion of α-ZOL.

**Toxicity Study:** The analysis of the feed intake data indicated an interaction between dietary treatment and ZEN level (Table 6, $p<0.0001$). Total feed intake was significantly less in the 45% protein group fed 400 ppm ZEN than both the 15 to 7.5% protein groups. However, the inclusion of ZEN in the diet did not reduce feed intake in the 45% protein group, while feed intake declined significantly in the 15 and 7.5% protein groups, a decrease of 31 and 40% respectively. A significant interaction between dietary treatment and ZEN level (Table 6, $p<0.0001$) was observed also for feed efficiency and final body weights. The inclusion of ZEN in the diet did not reduce feed efficiency or affect final body weight in the 45% protein group. However, in the 15 and 7.5% dietary protein groups there was a significant decrease in feed efficiency and final body weight observed as the level of dietary toxin increased. These results suggest that animals receiving ZEN in a high protein diet are less sensitive to the toxic effects of ZEN than are animals on a normal or low protein diet.

**TABLE 6.** Toxicity Study. Effect of Dietary Protein and Dietary Zearalenone on Feed Intake, Final Body Weight and Gain: Feed Ratio in Rats

| Zearalenone (ppm diet) | Low Protein | Standard Protein | High Protein |
|---|---|---|---|
| | | Feed Intake, g | |
| 0 | $315 \pm 15^{a}_{I}$ | $307 \pm 5^{a}_{I}$ | $250 \pm 7^{a}_{II}$ |
| 50 | $264 \pm 8^{b}_{I}$ | $281 \pm 7^{a,b}_{I}$ | $250 \pm 7^{a}_{I}$ |
| 150 | $241 \pm 19^{b}_{I}$ | $241 \pm 10^{b,c}_{I}$ | $234 \pm 5^{a}_{I}$ |
| 400 | $187 \pm 8^{c}_{I}$ | $210 \pm 8^{c}_{I}$ | $228 \pm 7^{a}_{I}$ |
| | | Body Weight, g | |
| 0 | $158 \pm 9^{a}_{I}$ | $145 \pm 2^{a,b}_{I}$ | $160 \pm 3^{a}_{I}$ |
| 50 | $133 \pm 4^{b}_{I}$ | $162 \pm 4^{a}_{II}$ | $154 \pm 4^{a}_{I,II}$ |
| 150 | $119 \pm 6^{b}_{I}$ | $126 \pm 5^{b,c}_{I}$ | $148 \pm 4^{a}_{II}$ |
| 400 | $93 \pm 4^{c}_{I}$ | $111 \pm 3^{c}_{I}$ | $141 \pm 4^{a}_{II}$ |
| | | Gain:Feed | |
| 0 | $0.31 \pm 0.01^{a}_{I}$ | $0.28 \pm 0.01^{a}_{I}$ | $0.41 \pm 0.01^{a}_{II}$ |
| 50 | $0.27 \pm 0.01^{a}_{I}$ | $0.36 \pm 0.01^{b}_{II}$ | $0.39 \pm 0.01^{a}_{II}$ |
| 150 | $0.24 \pm 0.01^{a,b}_{I}$ | $0.27 \pm 0.01^{a}_{I}$ | $0.40 \pm 0.03^{a}_{II}$ |
| 400 | $0.18 \pm 0.01^{b}_{I}$ | $0.24 \pm 0.01^{a}_{II}$ | $0.35 \pm 0.02^{a}_{III}$ |

N=10, mean ± SEM
Means within columns not designated with the same letter superscript are significantly different $p<0.05$, Tukey's Studentized Range.
Means within rows not designated with the same numerical subscript are significantly different $p<0.05$, Tukey's Studentized Range.

When uterine weights were expressed as a percentage of body weight, a significant interaction between dietary treatment and ZEN level was observed (Table 7, $p<.0005$). Dietary ZEN did not affect uterine weight in the 45% protein group, whereas in the 15 to 7.5% protein animals, uterine enlargement was observed. However, only the 7.5% protein animals receiving the 400 ppm ZEN diet had significantly larger uteri than other experimental animals. No significant differences in liver weights were observed when liver weights were expressed as a percentage of body weight (Table 7).

**TABLE 7.** Toxicity Study. Effect of Dietary Protein and Dietary Zearalenone on Uterine Weight and Liver Weight in Rats

| Zearalenone (ppm diet) | Low Protein | Standard Protein | High Protein |
|---|---|---|---|
| | Uterine Weight, % BW | | |
| 0 | $0.19 \pm 0.01^{a}_{I}$ | $0.21 \pm 0.02^{a}_{I}$ | $0.19 \pm 0.02^{a}_{I}$ |
| 50 | $0.23 \pm 0.01^{a,b}_{I}$ | $0.20 \pm 0.01^{a}_{I}$ | $0.18 \pm 0.01^{a}_{I}$ |
| 150 | $0.22 \pm 0.02^{a,b}_{I}$ | $0.21 \pm 0.02^{a}_{I}$ | $0.17 \pm 0.01^{a}_{I}$ |
| 400 | $0.29 \pm 0.02^{b}_{I}$ | $0.24 \pm 0.02^{a}_{I,II}$ | $0.18 \pm 0.01^{a}_{II}$ |
| | Liver Weight, % BW | | |
| 0 | $6.5 \pm 0.32^{a}_{I}$ | $5.8 \pm 0.27^{a}_{I}$ | $6.1 \pm 0.34^{a}_{I}$ |
| 50 | $6.3 \pm 0.25^{a}_{I}$ | $5.3 \pm 0.11^{a}_{I}$ | $6.2 \pm 0.32^{a}_{I}$ |
| 150 | $6.1 \pm 0.41^{a}_{I}$ | $6.0 \pm 0.30^{a}_{I}$ | $6.3 \pm 0.36^{a}_{I}$ |
| 400 | $6.4 \pm 0.52^{a}_{I}$ | $6.7 \pm 0.24^{a}_{I}$ | $6.4 \pm 0.30^{a}_{I}$ |

N=10, mean ± SEM
Means within columns not designated with the same letter superscript are significantly different $p<0.05$, Tukey's Studentized Range.
Means within rows not designated with the same numerical subscript are significantly different $p<0.05$, Tukey's Studentized Range.

## DISCUSSION

The dietary regimen had a significant effect on ZEN toxicity. Reduced feed intake, poor growth and feed inefficiency are symptoms of ZEN toxicosis (Mirocha *et al.*, 1980; Kiritsy *et al.*,(1987). In the present study, animals fed the 15 and 7.5% protein diets were more sensitive to these toxic effects of ZEN, than were animals fed the 45% protein diet. A ZEN induced increase in uterine weight was observed in animals consuming the 15 and 7.5%, however, only the 7.5% protein animals receiving the 400 ppm ZEN diet had significantly larger uteri than other experimental animals. These data support the concept that a high protein diet ameliorates the toxic effects of dietary ZEN (Smith, 1980a and Smith, 1982b).

The low protein diet resulted in a significant increase in GT enzyme activity. This observation is in general agreement with several other reports (Merril and Bray, 1982; Wood and Woodcock, 1970). However, this enhanced enzyme activity in animals on a low protein diet did not result in an increased urinary excretion of bound ZEN nor diminish the toxic effects of dietary ZEN as previously reported (Kiritsy *et al.*, 1987). It has been suggested that free animo acids in

the blood compete with ZEN for renal reabsorption, thereby promoting urinary losses of ZEN (Smith, 1982b). Therefore, the direct effect of free animo acids on renal reabsorption may account for this discrepancy.

Significant quantities of ZEN and its metabolites, 25-51% of the oral dose, were excreted in the urine. This agrees with previous studies in our laboratory (Kiritsy *et al.*, 1987; Chapter 3) but is in contrast to the findings of Smith (1982a; 1982b). This apparent discrepancy may be due to differences in dose level and recoveries. The use of pharmacological doses in toxicity experiments may result in erroneous conclusions. Overdosing animals results in metabolic overload, the ability to detoxify the compound is exceeded, consequently the rats and route of metabolism and/or excretion may be significantly altered (Smith, 1982b; Krayhill, 1977). For example Smith (1982a) dosed his animals with 10 mg ZEN per 100 g body weight and recovered 3% of this total dose in the urine and 22% in the feces. However, when tritiated ZEN was orally administered to rats, 31% of the radioactivity was recovered in the urine and 62% in the feces (1980b). In the experiment reported here, animals were dosed with 1 mg ZEN per kg BW and for the 15% dietary protein animals, 64% of the oral dose was recovered, 26% in the urine and 38% in the feces.

In the present work, there was an effect of dietary protein on the metabolism of ZEN with both the route of excretion and the metabolites formed affected. In 45% dietary

protein animals there was more conjugated ZEN excreted in the urine and less α-ZOL formed than in the 15 or 7.5% dietary protein animals. Since α-ZOL is the most potent ZEN metabolite (Hurd, 1977), diminished production would reduce the toxicity of the ZEN dose. Conjugation inactivates ZEN, while the increased urinary excretion would act to limit enterohepatic circulation (Smith, 1982a; Kiritsy *et al.*, 1987), with a subsequent reduction in the toxic expression of ZEN.

# CHAPTER 5

# UDP-GLUCURONYLTRANSFERASE CONJUGATION OF ZEARALENONE

## INTRODUCTION

Glucuronide formation represents an important process whereby endogenous substances and xenobiotics, are converted to more water soluble products, which are more readily excreted than the parent compound. Conjugation of endogenous and exogenous compounds with glucuronic acid is catalyzed by a family of inducible enzymes, uridine diphosphate glucuronyltransferases (GT - EC 2.4.1.17) (Dutton, 1980). This enzyme system is active in lungs, intestinal and renal tissues (Koster *et al.*, 1986), but is most active in the liver (Bock *et al.*, 1980).

GT isoenzymes are associated with endomembranes of the endoplasmic reticulum (Antoine *et al.*, 1983). Because of the close proximity of GTs on the endoplasmic reticulum with cytochrome P-450 enzymes, the phase I enzyme system capable of converting aliphatic and aryl xenobiotics to hydroxylated and/or demethylated metabolites that can then serve as substrates for GT isoenzymes, and it has been suggested that glucuronidation serves as a second phase of a two-step

disposition process for xenobiotics (Dutton, 1980). Numerous xenobiotics, ZEN, phenols and morphine, for example serve as substrates for GTs without having to undergo hydroxylation or dealkylation. Therefore, glucuronidation can be either be a phase I or a phase II disposition mechanism.

Several GT isoenzymes or 'clusters' have been described based on the criteria of temporal development (Wishart, 1978a; Lucier *et al.*, 1977), purification (Falany and Tephly, 1983; Burchell, 1977) or differential induction (Wishart, 1978b; Lilienblum *et al.*, 1982; Watkins *et al.*, 1982). Induction studies have enabled the characterization of at least two (Wishart, 1978b), possibly three (Lilienblum *et al.*, 1982) GT isoenzymes with restricted specificity for aglycones. In 1979, Bock *et al.*, classified two forms of GT in rat liver based on differential induction either as $GT_1$, induced by 3-methylcholanthrene and $GT_2$, induced by phenobarbital. The concept of GT-heterogeneity was strongly supported by the observation of a GT development pattern (Wishart, 1978a), and substrate geometry specificity (Okulicz *et al.*, 1981) for $GT_1$ the late fetal isoenzyme, specific for flat aglycones, and $GT_2$, the neonatal isoform, accepting thick, bulky aglycones. The conjugation velocity of GT correlated with the chemical nature of the aglycone (Boutin *et al.*, 1983). High conjugation velocities were observed towards p-nitrophenol, 4-methylumbelliferone, 1-naphthol, p-bromophenol, and eugenol, all planar molecules, and classified as $GT_1$ substrates. The

conjugation velocities of a second group of substrates, classified as $GT_2$ substrates, including 4-hydroxybiphenyl, morphine and chloramphenicol, all bulky molecules and classified as $GT_2$ substrates, were 5-7 times lower (Boutin *et al.*, 1983).

Developmental studies in the perinatal period in rats clearly demonstrate that substrates of GT isoenzymes can be subdivided into at least two groups (Wishart, 1978a). Wishart characterized a 'late foetal' group of GT substrates which glucuronidation was preferentially stimulated by 3-methylcholanthrene ($GT_1$), and a 'neonatal group' which was stimulated by phenobarbital ($GT_2$). In the late foetal group substrates, adult GT activity values were reached 16-20 days of gestation while adult values for the neonatal group substrates were reached by the second postnatal day. While developmental studies have provided information on the expression and development of GT isoenzymes during the prenatal and perinatal periods, there is a paucity of information on the influence of age and gender on GT activity. Studies on the effect of age and gender on GT activity towards ZEN have not been undertaken.

It has been demonstrated that the actions of a variety of drugs and xenobiotics are more pronounced and persist longer in female than in male rats (Kato, 1974). Sexual dimorphism in drug metabolism has been demonstrated to exist for a variety of drugs and enzymes. In 1958, Quinn and co-workers,

provided an explanation for at least some of these sex related differences. They demonstrated that the activities of the hexobarbital, aminopyrine, and anti-pyrine metabolizing enzymes of the liver were lower in female than in male rats, resulting in significantly longer biological half-lives in females. GT activity towards 4-hydroxyamphetamine was higher in microsomes from female rats (Dingall *et al.*, 1974) and excreted 4-hydroxyamphetamine as 30% glucuronide while male rats excreted the parent unchanged (Sever *et al.*, 1973). Sex differences in p-nitrophenol glucuronidation was found to occur at maximal activation of GT with females exhibiting lower conjugation capacity than male rats (Chabbra and Fouts, 1974). Holck *et al.*, (1937) reported that the sex-related difference to hexobarbital or pentobarbital anaesthesia was a characteristic of adult rats. The adult female rats slept longer than male rats, a phenomenon that was not observed in immature rats of 3 to 4 weeks of age. Furthermore, castration of adult rats lengthened barbiturate-induced anaesthesia, approximating the duration observed in females, suggesting that sex differences are dependent male sex hormones. Additionally, *in vitro* hepatic metabolism studies have shown that sex differences depend on the individual substrate (Kato and Gillette, 1965). Hartiala and Pulkkinen (1964) reported no sex differences in GT activity towards o-aminophenol, but GT activity in 4-month old male rats was 66% of the month value and 42% of 1 month-old GT activities in female rats.

While there is a paucity of published data on the effect of storage on enzyme activity, GT has been reported to be stable under a variety of conditions (Falany and Tephly, 1983). GT activity has been reported to be dependent on storage temperature (Burchell *et al.*, 1975), although the effect of storage conditions on GT stability in the literature are not clearly defined. That is the stability of GT activity under various conditions is either not reported or differ markedly. When stored at 0°C for one hour, liver homogenate GT activity was reported to be negligible (Dutton, 1966). Gregory and Strickland (1973) reported that GT activity in homogenate pellets stored at 5°C was stable for several months. Bock *et al.* (1984), stored microsomes at -80°C from 6 months to 2 years and reported that GT activities and their latency characteristics appear to be well preserved during storage, however they did not provide any evidence to support this conclusion. In other reports microsomes were stored at -20°C (Lilienblum *et al.*, 1982) and -70°C (Coughtrie *et al.*, (1986) and assayed for GT activity within 4 weeks. The importance of evaluating stability of GT activity under different conditions, therefore, cannot be overemphasized.

The effects of age and gender as well as storage conditions on GT activity towards ZEN have not been undertaken. In addition the GT isoenzyme responsible for ZEN conjugation has not been characterized using kinetic studies, differential induction or any other technique. Stimulation of

GT activity can be demonstrated after pretreatment with certain aglycones, substrates of the enzymes. Conney *et al.*, (1960) suggested that the administration of certain drugs enhances hepatic microsomal metabolism of the same or a closely related compound. Therefore studies were designed with the following main objectives:

(a) to determine the effect of storage temperature (-20°C vs -80°C) and storage time on the stability of hepatic microsomal GT activity towards ZEN, NA and HBP.

(b) to determine the effect of age and gender on the pattern of GT activity towards known $GT_1$ (α-Naphthol, NA) and $GT_2$ (4-hydroxybiphenyl, HBP) substrates and ZEN;

(c) to characterize the isoenzyme responsible for ZEN conjugation by kinetic and induction studies. The kinetic parameters ($K_M$ and $V_{MAX}$) of ZEN , NA and HBP will be compared. In the induction study, the effect of dietary ZEN on induction of hepatic microsomal GT activity towards itself, NA, and HBP.

## METHODS AND MATERIALS

**Animals and Treatment:** The use of animals conformed to the Guidelines of the Canadian Council of Animal Care. All animals were purchased from the University of Manitoba central breeding facility in separate groups conforming to the experiments conducted. All animals were housed in individual cages. The room temperature was maintained at 21 ± 1°C with a relative humidity of 50% and a 14/10 h light-dark cycle. All experimental animals were fed a commercial chow diet (Ralston Purina Co., St Louis, Mo.) with the exception of the rats in the induction study that received a semi-purified diet containing either 0 or 250 ppm ZEN for 14 days (Kiritsy *et al.*, 1987). For the storage study, 6 female Sprague Dawley rats (225 g) were used. In the age-gender study 8 male and 8 female Sprague Dawley rats from 3 age groups (35, 56 and 112 days) were used. In the induction study 16 female weanling Sprague Dawley rats (55-65 g) were randomly assigned to diets containing either 0 or 250 ppm ZEN after a 2-day acclimation period. After 14 days on the diet rats were killed by decapitation, livers removed and microsomes prepared. For the kinetic study, 8 female Sprague Dawley rats (200 - 250 g) were used.

**Preparation of microsomes:** Animals were killed by decapitation and the livers removed immediately and kept on ice. All further manipulations were carried out at 0-4°C. Livers were homogenized in two volumes of 0.25M - sucrose - 0.154M - KCL buffer in a Potter-Elvejhem Homogenizer for 90 s. The homogenates were transferred to polycarbonate centrifuge tubes and centrifuged for 20 min (Beckman L5-50B Centrifuge, Rotor 50Ti) at 10,000 x g to remove cellular debris, nuclei and mitochondria. The postmitochondrial supernatant was centrifuged at 105,000 x g for 60 min to yield a particle-free cytosol and the microsomal pellet. The microsomal pellet was washed by resuspending the pellet in the buffer, homogenized and resedimented at 105,000 x g for 60 min. The microsomes were then resuspended in the same buffer, homogenized and protein determined according to Lowrey *et al.* (1951). The microsomal protein was adjusted to 10 mg $ml^{-1}$ prior to further dilution and activation with the detergent, 0.1% Triton X-100 in 0.154M KCL (w/v). For the storage study, microsomes were stored at -20°C and -80°C and GT activity determined after overnight storage, and after 1, 2, 4, 6 and 8 weeks.

**GT Assay:** GT activity was assayed according to the linked method of Mulder and van Doorn (1975) towards three aglycones: zearalenone, $\alpha$-naphthol (a prototype $GT_1$ substrate) and 4-hydroxybiphenyl (a prototype $GT_2$ substrate). Microsomes were incubated in 0.1% Triton-X-100 in 0.154 M KCl for at least 30

min at 0-4°C prior to assay. Assays were performed at 37°C in a total volume of 2 ml containing: 75 mM-Tris-HCl buffer, pH 7.3, 5.0 mM $MgCl_2$; 0.2 mM phosphoenolpyruvate; 0.2 mM NADH; 0.625 U lactate dehydrogenase $ml^{-1}$; 5.0 U pyruvate kinase $ml^{-1}$; 1.5 mM UDP-glucuronate and 0.3 mM acceptor substrate (ZEN, HBP or NA) dissolved in ethanol-water (1:1, v/v) and 200 ug of microsomal protein. The reaction was started by the addition of 0.5 ml microsomal suspension after pre-incubation of the reaction mixture at 37°C for 5 min. The change in optical density at 340 nm was monitored continuously for 5 min against a water blank at 31°C (Milton Roy Spectrophotometer SP300, Rochester, NY). In substrate blanks, UDP-glucuronate was omitted in the incubation medium. The analyses were performed in duplicate samples. GT activity was expressed as nmol substrate conjugated $mg^{-1}$ protein $h^{-1}$. In the kinetic study final incubation substrate concentrations were as follows: HBP:0.05 - 4.0 mM; NA: 0.0375 - 1.2 mM; ZEN: 0.05 - 1.5 mM and reaction velocities, nmol $min^{-1}$ mg $protein^{-1}$, were calculated from initial rates. Optimization of microsomal protein concentration and Triton X-100 activation of GT was predetermined in separate experiments, each involving 6 female rats (200-250 g BW). In preliminary experiments the reaction was determined to be linear up to 10 min.

**Statistical analyses:** Data were analyzed using the Statistical Analysis System (SAS, 1985). All data are presented as Means ± SEM. A difference was accepted as significant when $p<0.05$.

**Age-gender and storage study:** Split-plot analyses were used to determine age, sex, substrate and substrate*age*sex effects. The Students' t-test was used to determine the effect of storage time on GT activity. A paired t-test was used to compare GT activity in microsomes stored at -20°C and -80°C.

**Induction study:** Two-way analysis of variance procedure was used to compare treatment groups and multiple comparisons were made using Tukeys multiple range test.

**Kinetic study:** Pairwise contrasts of $K_m$ and $V_{max}$ were done by applying a power transformation of the Michaelis-Menten model, a cubed-root transformation of both sides (TBS) of the Michaelis-Menten model, using the statistical programming language S-Plus. The conventional Lineweaver-Burke model is essentially a TBS model, however, there was a lack of homogeneity of variance even after linearization of the data, therefore, we were unable to compare the parameters due to a violation of the underlying assumption for homogeneity of variance. Heterogeneity of variance was removed by applying a cube-root transformation of both sides of the Michaelis-

Menten model which permitted comparison without affecting interpretation of the original model. The cube-root transformation was applied because it exhibited the 'best' residual behaviour, no indications of variance heterogeneity was present, consistent with model assumptions.

## RESULTS

**Age-gender and storage study:** Enzymatic activity towards acceptor substrates in microsomal preparations increased as the concentration of detergent, Triton X-100 increased (Fig. 4). The observed GT activity towards ZEN increased from 1.7 to 10.4 nmol mg protein$^{-1}$ min$^{-1}$ with the addition of 0.1% Triton X-100 (W/V) to the assay medium. The detergent-induced activation decreased at higher concentrations. GT activity was linear with respect to protein concentration and incubation time, up to 250 $\mu$g protein ml$^{-1}$ and 20 min (Fig. 5).

In the initial storage study, microsomal GT enzyme activity towards ZEN declines significantly after 7 days storage at both temperatures, with GT enzyme activity generally higher in microsomes stored at -20 than at -80°C (Fig. 6). In a repeat storage study where enzyme activity towards all three substrates was measured, microsomal GT enzyme activity declined significantly with storage (Fig. 7). GT activity towards NA and ZEN was stable for 28 days as opposed to 14 days for HBP, after which significant declines in enzyme activity was observed. After 42 days of storage, GT activity decreased by 14 - 21% towards NA (Fig. 7C) and by 36

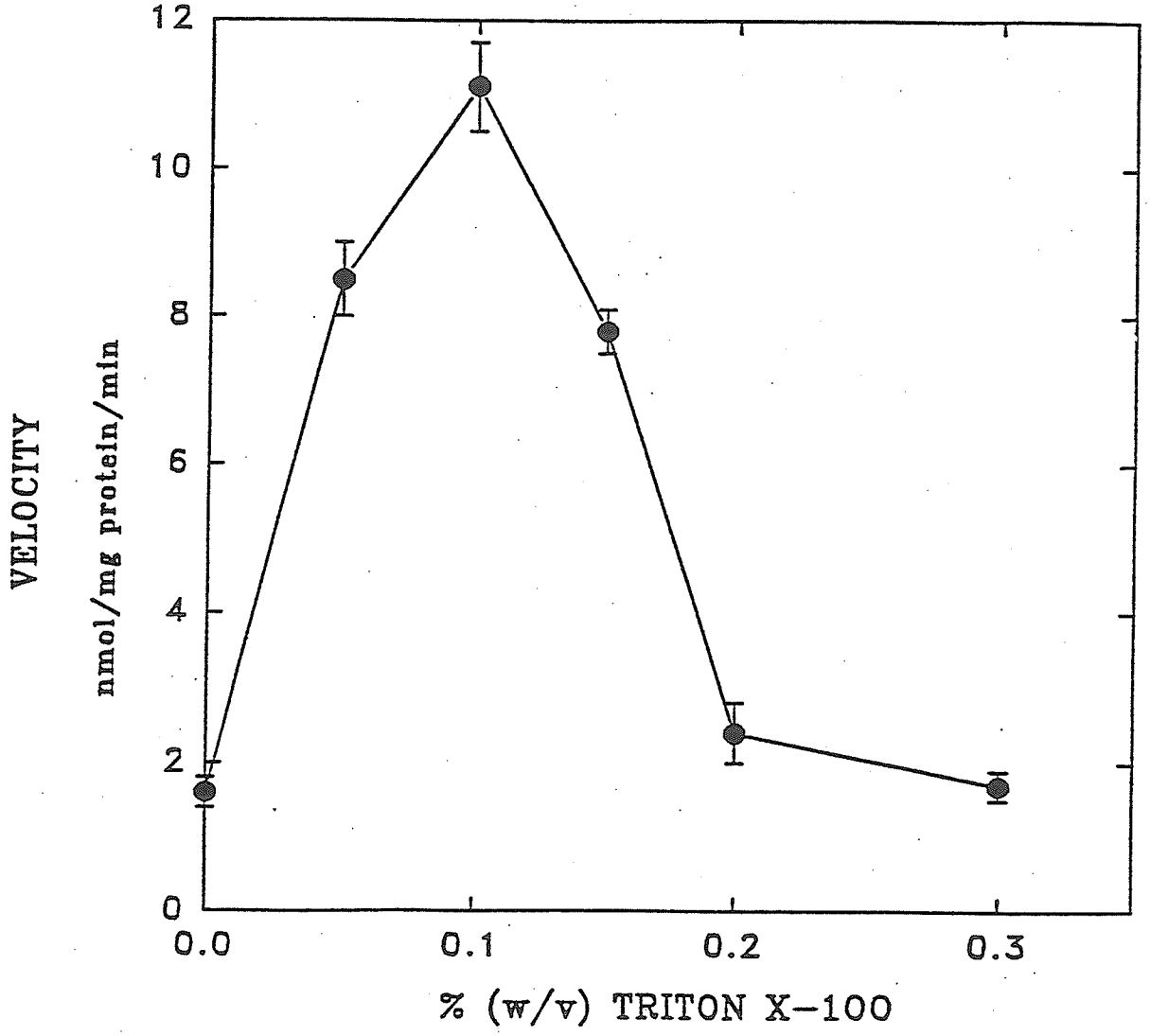



**FIGURE 4.** Effect of Triton X-100 concentration on hepatic microsomal GT enzyme activity towards ZEN (nmol $mg^{-1}$ protein $min^{-1}$). Data presented as Means ± SEM; (N=6)

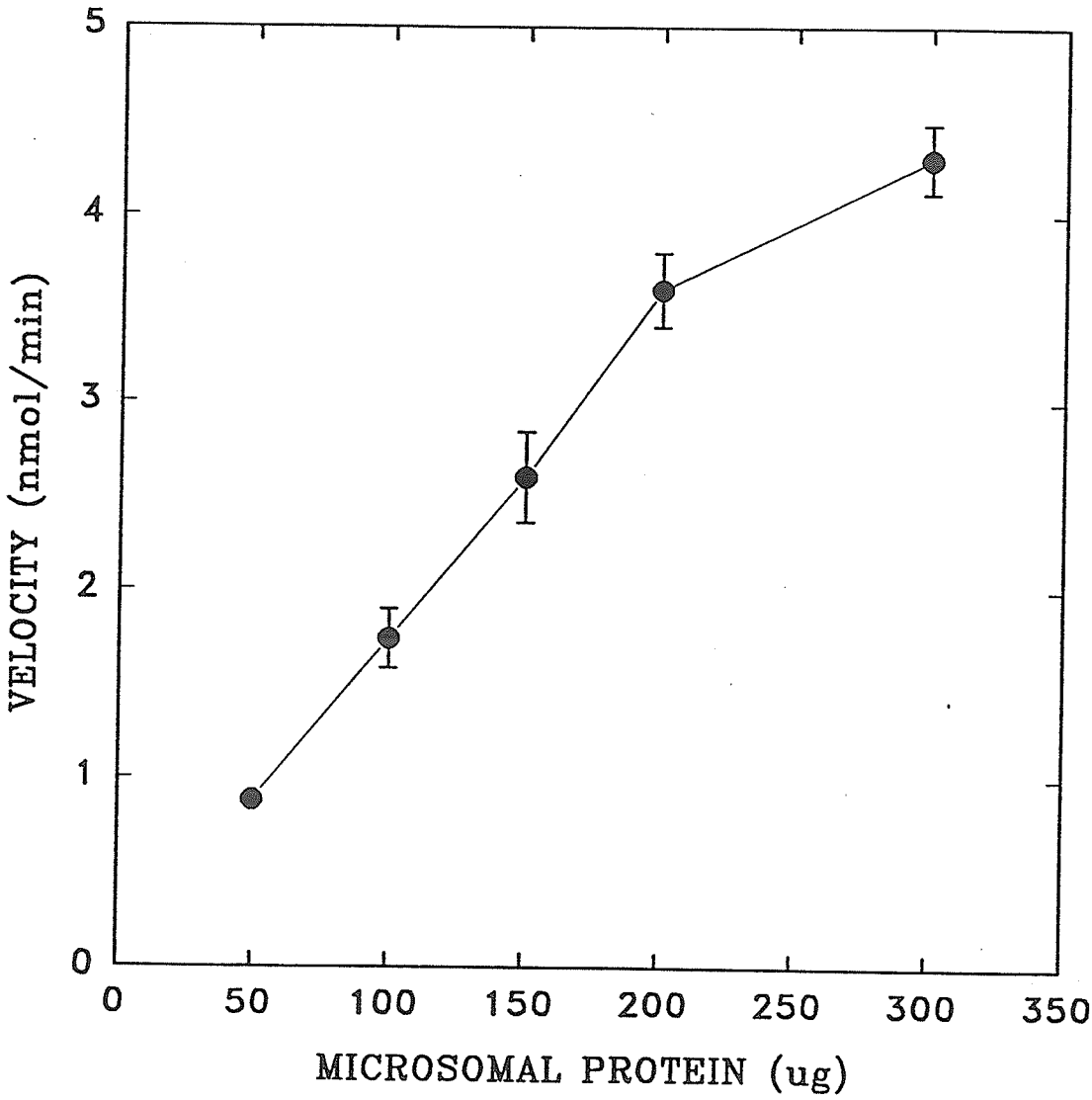



**FIGURE 5.** Effect of hepatic microsomal protein concentration on GT enzyme activity (nmol $mg^{-1}$). Data presented as Means ± SEM; (N=6)

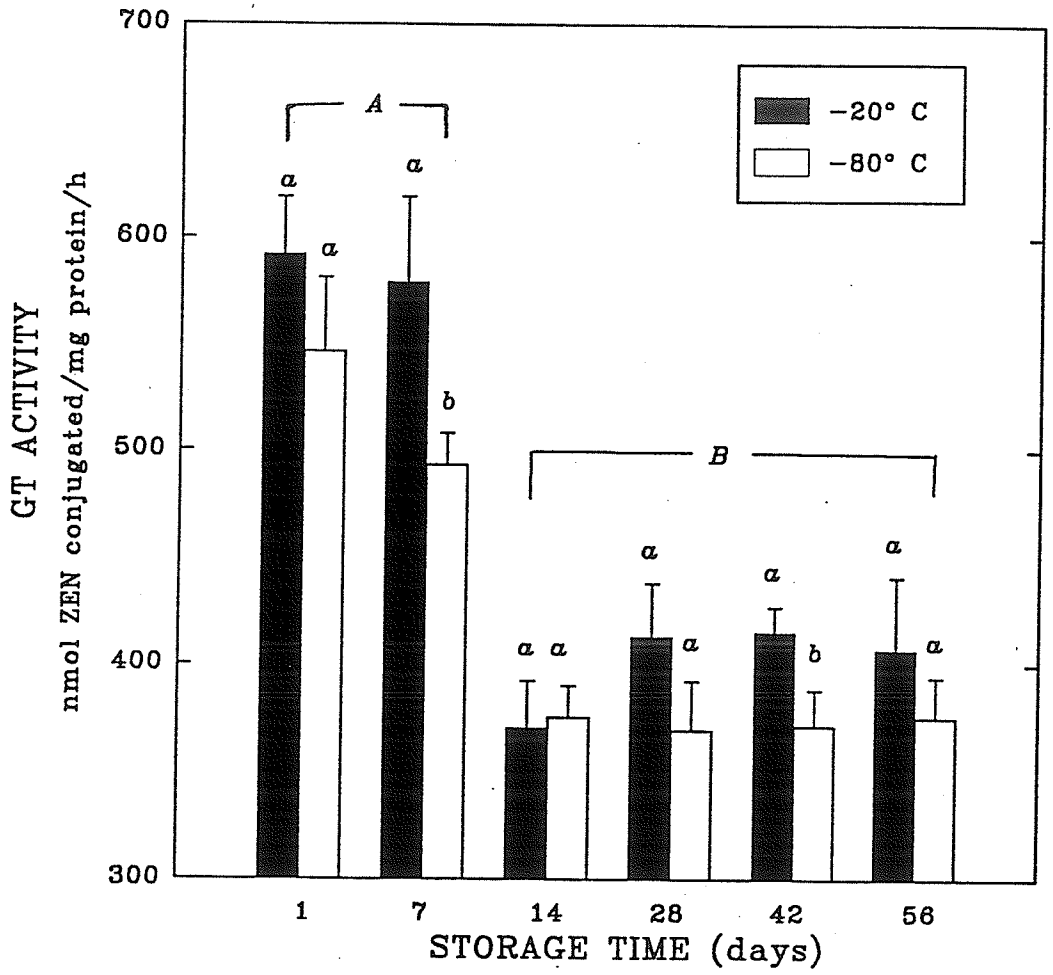



**FIGURE 6.** Effect of storage time and temperature on hepatic microsomal GT activity towards ZEN. Differences between microsomes stored at -20 and -80°C are designated by lowercase letters. Differences between microsomes stored at different times are designated by uppercase letters. Means having different letters are significantly different, $p<0.05$. Data presented as Means $\pm$ SEM; (N=6)

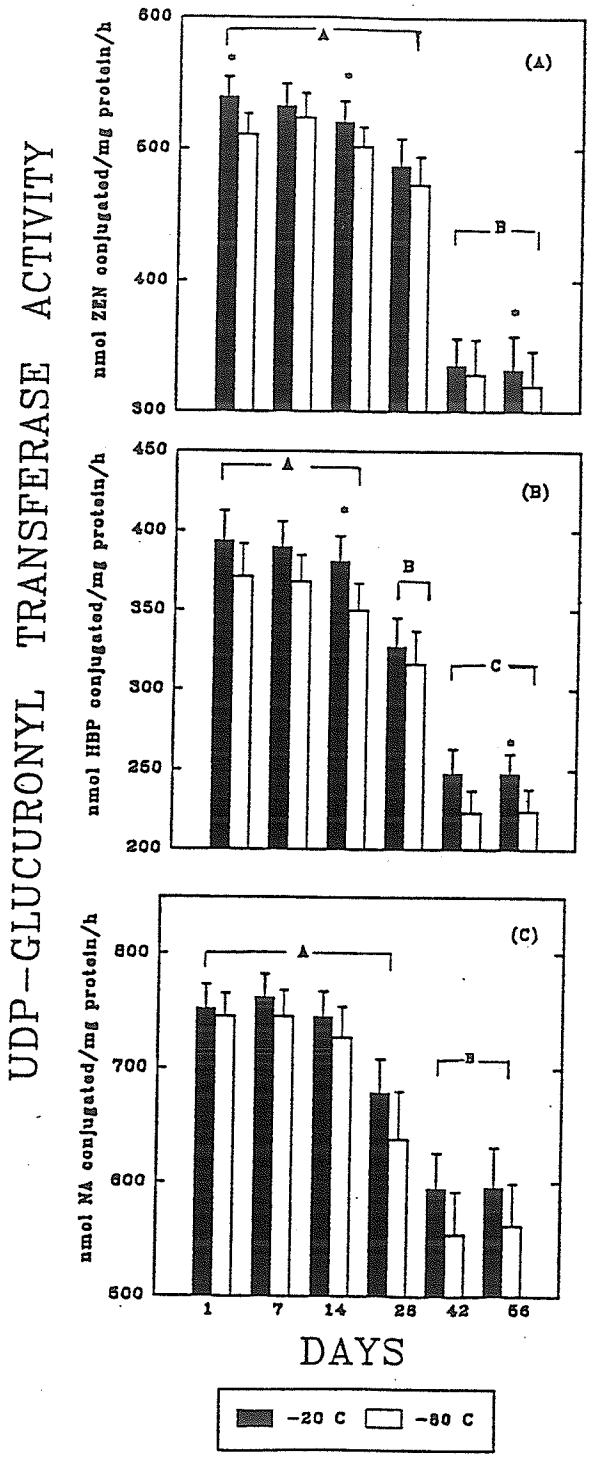



**FIGURE 7.** Effect of storage time and temperature on hepatic microsomal GT activity towards (A) ZEN, (B) HBP and (C) NA. An * indicates differences between microsomes stored at -20 and -80°C within the same storage period, $p<0.05$. Means having different letters are significantly different, $p<0.05$. Data presented as Means $\pm$ SEM; (N=6)

- 38% towards ZEN (Fig. 7A), at either -20 or -80°C. After 14 days, GT activity towards HBP (Fig. 7B)) decreased by 15 - 17% at either -20 or -80°C. Generally, no differences in GT activity between microsome stored at the two temperatures. However, where differences were observed, enzyme activity was higher in microsomes stored at -20 compared to -80°C. After 42 days storage, GT activity towards NA and ZEN was similar in both temperature storage groups. After 14 days of storage, GT activity towards HBP and ZEN was significantly greater at -20 compared to -80°C. Therefore, the liver microsomes used in the age-sex and subsequent studies were stored at -20°C and analyzed within 14 days of preparation.

Hepatic $GT_1$ enzyme activity was greater than $GT_2$ activity and ZEN in all age groups in both sexes (Fig. 8) . Young female 35 day-old rats had greater $GT_1$ and $GT_2$ enzyme activity, 1687 and 800 nmol $mg^{-1}$ protein $h^{-1}$, than comparable males, 1397 and 465 nmol $mg^{-1}$ protein $h^{-1}$ respectively (Fig. 8A & 8B). Similarly, for ZEN, young female rats had greater glucuronidation rates than male rats, 1006 and 425 nmol $mg^{-1}$ protein $h^{-1}$, respectively (Fig. 8C). GT enzyme activity diminished with age, with a 75% reduction in enzyme activity observed in 112-day old females for both $GT_1$ and $GT_2$ activity, and a 30% reduction in enzyme activity observed in 112 day-old males compared to 35 day-old rats. Old male rats had greater conjugation capacity, $GT_1$ and $GT_2$ activity 997 and 315 nmol

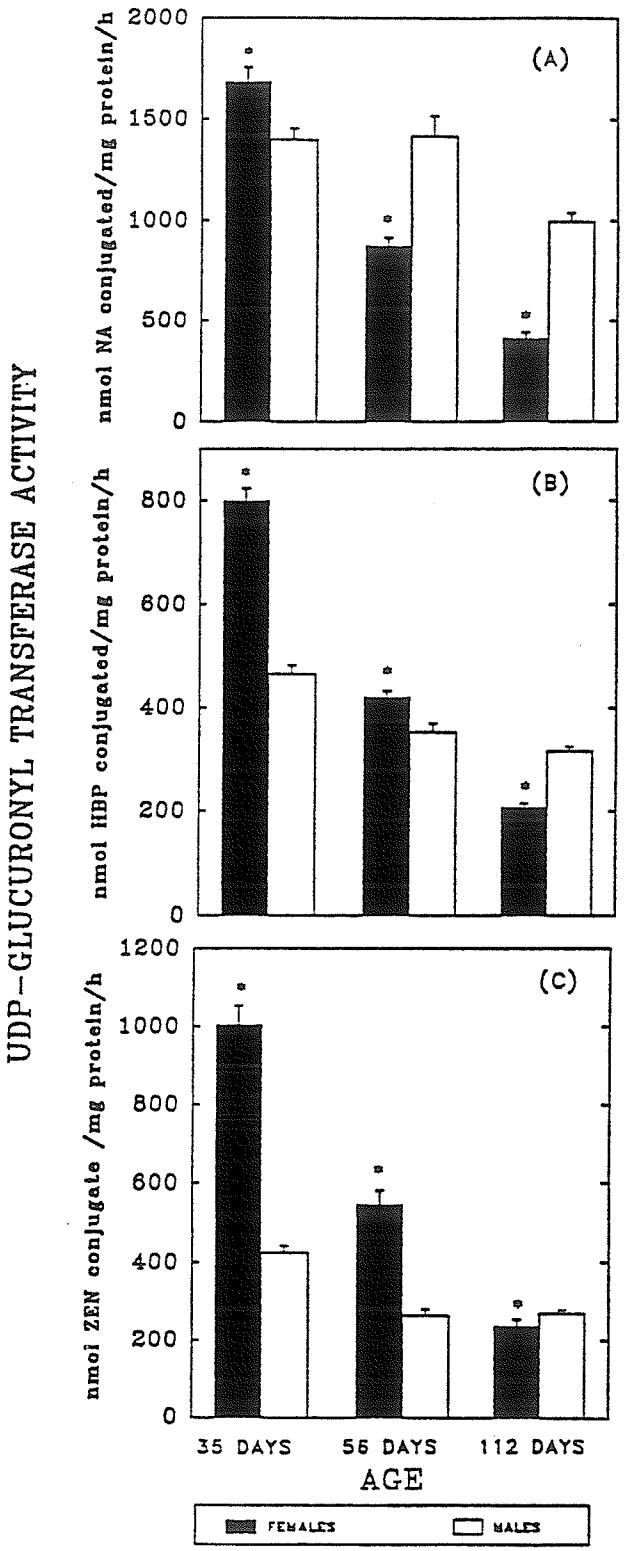



**FIGURE 8.** Effect of age and gender on hepatic microsomal GT activity towards (A) NA, (B) HBP and (C) ZEN. Means designated by an * are significantly different, $p<0.05$. Data presented as Means $\pm$ SEM; (N=8)

substrate conjugated $mg^{-1}$ protein $h^{-1}$, than their female counterparts, 411 and 207 nmol substrate conjugated $mg^{-1}$ protein $h^{-1}$, respectively (Fig. 8A & 8B). Similarly, glucuronidation rates of ZEN, was higher in old male rats compared to females, 269 and 237 nmol substrate conjugated $mg^{-1}$ protein $h^{-1}$, respectively (Fig. 8C). The rate of decline in GT activity with age is less in males than females for all three substrates.

**Induction study:** Feed efficiency was significantly depressed in ZEN treated rats, 0.28 ± 0.01, compared to control animals 0.38 ± 0.01 ($p<0.05$). Dietary ZEN significantly enhanced GT activity towards all three substrates (Fig. 9). Compared to untreated rats, GT activity was induced by 47%, 51% and 50% towards ZEN, HBP and NA respectively. $GT_1$ activity, determined from NA conjugation, was significantly greater than $GT_2$ activity, determined from HBP conjugation and ZEN, in both control and treated animals. No differences were detected between $GT_2$ activity and ZEN in both control and untreated animals.

**Kinetic study:** The kinetic parameters are summarised in Table 8. Glucuronidation of NA is characterised by a significantly greater $V_{MAX}$ than either HBP or ZEN, while no differences were detected in $V_{MAX}$ between HBP and ZEN. There was a significant difference between the apparent $K_M$ for NA and HBP, while no differences were observed in $K_M$ values

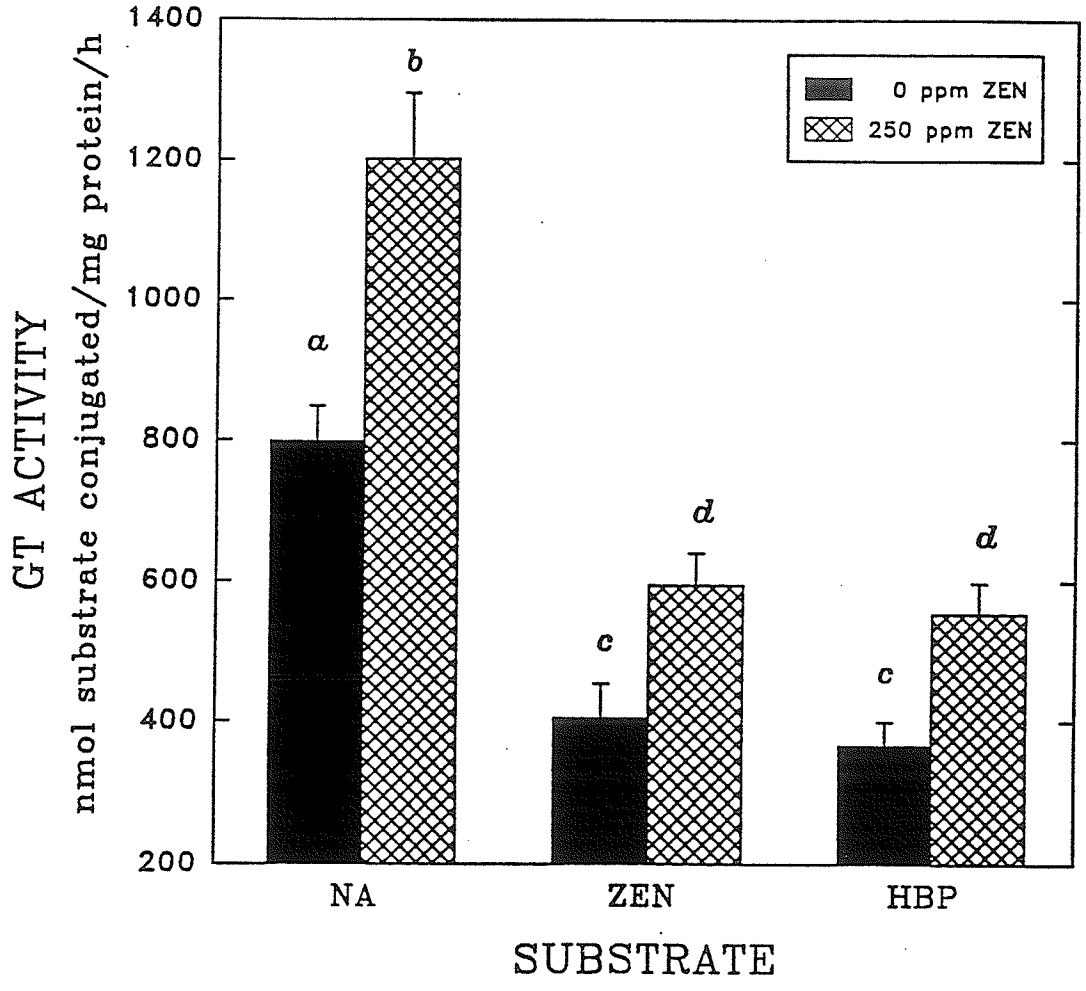



**FIGURE 9.** Effect of dietary zearalenone on induction of hepatic GT isoenzyme activity towards NA, ZEN and HBP. Means designated different letters are significantly different $p<0.05$. Data presented as Means $\pm$ SEM (N=8)

**TABLE 8.** Kinetic study: $V_{max}$ and $K_m$ estimates for NA, HBP and ZEN.

| | $V_{max}$ | $K_m$ |
|---|---|---|
| | nmol $min^{-1}$ mg $protein^{-1}$ | uM |
| NA | 640 ± 26[a] | 0.132 ± 0.014[a] |
| HBP | 420 ± 32[b] | 0.185 ± 0.027[b] |
| ZEN | 454 ± 36[b] | 0.184 ± 0.032[a,b] |

Means with different letters down a column are significantly different from each other; $p<0.05$. Data presented as Means ± SEM; (N=6).

between NA and ZEN. No differences in $K_M$ were detected between HBP and ZEN.

## DISCUSSION

In freshly prepared homogenates or microsomes, GT activity is latent. Membrane perturbations such as mechanical disruption, freeze-thawing, or exposure to organic solvents, detergents, proteases and phospholipases serve to disrupt microsomal membrane structure and activate the enzyme (Dutton, 1980). The detergent, Triton X-100, acts to reduce barriers that restrict access to the catalytic site, embedded in the hydrophobic membrane, enhancing substrate binding and access to intramembrane catalytic sites. (Dutton, 1980). Membrane phospholipids constrain the enzyme in a low activity or latent conformation and membrane perturbants such as Triton X-100 remove latency by affecting lipid-enzyme interactions, producing a variety of high activity conformers, each with individual kinetic and regulatory properties (Zakim and Vessey, 1976). It is therefore imperative that optimization of enzyme activity *in vitro* with detergents is performed to ensure measurement and comparison of enzyme activities is done at maximal enzyme velocities.

While there is a paucity of data on GT enzyme activity stability during storage, much of the information on GT enzymes activity to date come from studies which assumed that enzyme activity was stable under controlled storage conditions

(Dutton, 1966; Gregory and Strickland, 1973; Bock *et al.*, 1984). Our observations suggest that GT activity is not stable as previously assumed. Specifically GT enzyme activity declined significantly with time and that microsomes stored at -20°C had higher activity than those stored at -80°C. In all subsequent studies liver microsomes were stored at -20°C and analyzed for enzyme activity within 7 days.

Wishart and Campbell (1979) distinguished two groups of GTs on the basis of onset of development, with $GT_1$ activities reaching adult values during the late fetal period and $GT_2$ reaching near adult values neonatally. An underlying assumption of these authors was that adult values were relatively constant (Wishart, 1978; Wishart and Campbell, 1979). The data from this study demonstrates that maximal GT activity was reached in young rats, and that there was a progressive decline in both $GT_1$ and $GT_2$ enzyme activities with age, in both sexes. The age-dependent decline in GT activity was more pronounced in females, with older male rats having a greater glucuronidation capacity than females. This finding is consistent with reports that drugs produce exaggerated effects in female rats (Watanabe *et al.*, 1988), and that there are sex differences in hepatic drug-metabolizing enzyme activity (Colby *et al.*, 1980). The developmental pattern of GT enzyme activity reported here demonstrates that substrate-specific sex differences are evident in the various age-

groups. The developmental pattern of GT activity towards ZEN and HBP show great similarity.

To date, ZEN has not been classified in any of the GT isoenzyme clusters. The observation that dietary ZEN, a bulky substrate, resulted in enhanced glucuronidation of itself as well as NA and HBP, $GT_1$ and $GT_2$ substrates, respectively suggests cross-induction. Boutin *et al.* (1983) reported induction by HBP pretreatment of both $GT_1$ and $GT_2$ isoenzyme activity. This was unanticipated because HBP is a bulky substrate, and specific for $GT_2$ isoenzymes, therefore, no effect on planar $GT_1$ metabolism substrates would be expected. The data show that ZEN and HBP have similar glucuronidation rates and both rates are significantly different from that of NA. This finding supports the observation of our age-gender study, where we suggested that HBP and ZEN belong to the same GT cluster and are therefore substrates for the same GT isoform. Furthermore, that cross-induction of GT isoenzyme activity is more common than the literature suggests (Wishart and Campbell, 1979; Bock *et al.*, 1979; Lilienblum *et al.*, 1982). The data is in agreement with other reports (Boutin *et al.*, 1983; Pandey *et al.*, 1990) that there is cross-induction of GT isoenzyme activity.

Additional evidence to support the classification of ZEN as a $GT_2$ substrate is provided by the kinetic data, with ZEN and HBP exhibiting similar Michaelis-Menten behaviour. Kinetic data supports the concept of separate isoenzymes for

$GT_1$ and $GT_2$ substrates based on $K_m$ and $V_{MAX}$ values. While no difference in $V_{MAX}$ and $K_M$ between HBP and ZEN were observed, suggesting that ZEN is a $GT_2$ substrate; we did not detect a statistical difference in the apparent $K_M$ between NA and ZEN, suggesting both are $GT_1$ substrates. This lack of statistical difference may be attributed to an experimental 'flaw'. In a pilot range finding experiment, the concentrations were thought to include substrate concentrations in excess of $V_{MAX}$ values for NA, however, the final substrate concentration range was not great enough to accurately allow estimation of the asymptotic $V_{MAX}$ value for NA. That is, the velocity versus substrate concentration curve did not plateau, resulting in an underestimation of $V_{MAX}$. Since the estimation of $K_M$ is dependent on $V_{MAX}$, our kinetic data suggests that under proper conditions, the estimated $K_M$ for NA would be lower than estimated in this experiment, resulting in increased sensitivity. The kinetic data, while not providing conclusive evidence to implicate separate isoenzymes for NA and ZEN, suggests that they are different. In addition, age-gender and induction studies suggest that ZEN behaves in a similar manner to HBP. Further experimentation with a broader substrate range for NA would be warranted to clearly demonstrate that ZEN is a $GT_2$ substrate using kinetic studies. However, all indications are, from the three experiments discussed in this chapter that ZEN bears a closer resemblance to $GT_2$ than $GT_1$ substrates.

In summary, our data indicates that GT activity is not stable as previously thought. The post-natal developmental pattern of GT activity is substrate, gender and age specific. Immature female rats exhibit a greater capacity for hepatic conjugation compared to males. However, this trend is reversed in adult rats where males exhibit greater GT activity. The age-dependent decline is gender specific, with glucuronidation in female rats decreasing at greater rates than males, that is the decline males is not as pronounced as that observed in females. The development pattern is substrate dependent i.e. enzyme activity towards $GT_1$ substrates is greater than $GT_2$ substrates in all age groups. The data from the developmental, kinetic and induction study suggests that ZEN can be classified as a $GT_2$ substrate because of its similar behaviour to HBP, a substrate belonging to the $GT_2$ cluster.

# CHAPTER 6

# ZEARALENOL SYNTHESIS - REDUCTION OF ZEARALENONE

## INTRODUCTION

Zearalenone (ZEN) is a hydrophobic xenobiotic which has been reported to be metabolized by two different reactions: a reduction to an alcohol and a conjugation with glucuronic acid. When measured simultaneously in liver homogenates, Kiessling and Pettersson (1978) reported there was a minor competition for ZEN between the reduction and conjugation pathways, and that the reduction reaction was of minor quantitative significance. In *in vivo* metabolism studies we have demonstrated that the production of $\alpha$-ZOL is greater than 12% of the oral dose and, therefore quantitatively significant (Chapter 3). Olsen *et al.* (1981), based upon the observation that androstanedione and ZEN mutually inhibited each other's reduction, suggested a common enzyme system for metabolism. That is, ZEN and androstanedione were both reduced by HSD (Olsen *et al.*, 1981; Smith, 1982a; Stangroom and Smith, 1984).

HSD is primarily involved in the metabolism of steroids, and the estrogenic effects of ZEN has been attributed to disturbances in steroid metabolism (Thouvenot and Morfin, 1980). Hepatic HSD is a multifunctional enzyme present in

both microsomal and cytosol fractions with potentially different substrate affinities and specific activities towards different substrates for each sub-cellular isoenzyme (Hurlock and Talalay, 1959). However, the belief that HSD is the primary reducing enzyme for ZEN reduction not universally held (Ueno *et al.*, 1983; Tashiro *et al.*, 1983). Tashiro *et al.*, (1983) suggested that the microsomal ZEN reductase is distinct from this steroid dehydrogenase. Conflicting reports on the effect of dietary ZEN on HSD activity may be due to different analytical methods. This issue remains controversial at the moment.

The effect of dietary ZEN on HSD activity has not been clearly demonstrated. James and Smith (1982) observed that dietary ZEN decreased HSD activity whereas, Strangroom and Smith (1984), using different conditions for enzyme analysis, reported an induction of HSD activity by ZEN. Kiritsy *et al.* (1987) observed that dietary restriction increase hepatic HSD enzyme activity 27%, however *in vivo* synthesis of ZOLs decreased 25%. Additionally, increasing dietary protein had no effect on HSD and decreased urinary $\alpha$-ZOL (Chapter 4). Thus the *in vitro* incubation assay does not reflect the *in vivo* metabolites formed. Phenobarbital (PB) is frequently used as a mixed-function oxidase inducer but is also an effective inducer of Phase II biotransformation enzymes (GT) (Thompson *et al.*, 1982). The induction of GT activity by PB

pretreatment has been shown to significantly increase urinary excretion of conjugated α-zol (Pandey *et al.*, 1990).

In this chapter experiments are described with the following objectives in mind:

(a) to characterize the subcellular distribution of hepatic HSD and zearalenol syntheses activities;

(b) to determine the effect of PB pretreatment on hepatic microsomal HSD and NADPH-cytochrome $P_{450}$ reductase enzyme activities and

(c) to determine the effect of PB pretreatment on the 48-hour excretion of total urinary ZEN and ZOL.

## METHODS AND MATERIALS

**Animals and Treatment:** The use of animals conformed to the Guidelines of the Canadian Council of Animal Care. Sprague-Dawley rats were purchased from the University of Manitoba central breeding facility. Each group of animals was received when required in the three separate studies. All animals were housed in individual cages. The room temperature was maintained at 21 ± 1°C with a relative humidity 50% and a 14-10-h light-dark cycle. For the ZEN reduction-HSD characterization study, 6 female rats (200-250 g) were used. For the PB-enzyme induction study, 20 female rats (55-60 g) were randomly assigned to either PB or saline treatment groups (10 per group). Similarly in the metabolism study, 20 female rats were randomly assigned to either PB or saline treatment groups (10 per group).

**Enzyme Induction Study:** The rats received intraperitoneal treatment with phenobarbital (Abbot Laboratories Ltd., Toronto, Ont), or the saline control vehicle, as follows: PB, an initial dose of 30 mg $Kg^{-1}$ BW on day 1 and 60 mg $Kg^{-1}$ BW on days 2 and 3, while control rats received saline on all 3 days. Animals were killed 4 days after the initial treatment,

their livers excised and microsomes prepared and stored at -20°C for HSD and NADPH-cytochrome $P_{450}$ assays.

**Metabolism Study:** The PB dose regimen was identical to that described in the enzyme induction study. On day 4, rats were dosed with 1 mg ZEN $kg^{-1}$ BW, by stomach intubation. Animals were kept in polycarbonate metabolism cages to facilitate urine collection. Urine was collected for 48 h, pooled and kept frozen at -20°C until analyzed.

**Tissue Preparation:** Rats were killed by decapitation, the livers excised and chilled ice. All further manipulations were performed at 0-4°C. Unless otherwise specified all reagents and purified enzymes used were obtained from Sigma Chemical Co. (St. Louis, MO). Livers were homogenized for 90 s at maximum speed in a Potter-Elvejhem homogenizer. For the spectrophotometric HSD method, samples were homogenized in 19 and 10 volumes of 0.01 M $K_2HPO_4$ pH 7.2, for homogenate and microsomal preparations, respectively. For the direct HPLC determination of ZOL formation, livers were homogenized in 10 and 3 volumes of 0.25 M-sucrose-5 mM-tris-1 mM-EDTA buffer, pH 7.2, for preparation of homogenates and microsomal fractions respectively. Homogenates were prepared by centrifuging the samples at 25,000 x g for 10 min. Microsomes were prepared by centrifuging the crude homogenates for 20 min at 10,000 x g and the supernatant spun at 105,000 x g for 60 min (Beckman L5-50 B Ultracentrifuge, Rotor 50 Ti) yielding the cytosolic

fraction and the microsomal pellet. The pellet was washed in the homogenizing buffer and centrifuged at 105,000 x g for 60 min. The pellet was suspended in 3 ml buffer $g^{-1}$ wet tissue. Microsomes for cyt $P_{450}$ assay were prepared in 0.25 M sucrose-5mM tris, pH 7.4. Protein measurement in all fractions was determined according to Lowry *et al.* (1951).

**HSD Assay:** An indirect spectrophotometric method, with androsterone as the enzyme substrate, was used to determine HSD enzyme activity (Kiritsy *et al.*, 1987). The incubation medium, consisting of 0.7 mM NAD, 0.2 M sodium pyrophosphate buffer and 100 $\mu$l protein was preincubated at 32°C for 5 min. The reaction was started by the addition of 100 $\mu$l of 1.5 mM androsterone or 100 $\mu$l methanol for the enzyme blanks. The reaction was monitored for 10 min at 340 nm in a Milton Roy Spectrophotometer SP3000 (Rochester, NY). Protein concentration of homogenate, microsomal and cytosolic preparations were adjusted to 4, 5 and 6 mg $ml^{-1}$, respectively. At these protein concentrations, the reactions were linear for at least 20 min. Analyses were performed in triplicate and HSD enzyme activity was expressed as nmol NADH produced $mg^{-1}$ protein $min^{-1}$.

**Determination of zearalenone reducing activity:** The activity of ZEN reduction to $\alpha$- and ß-ZOL was measured by measuring the rate of production of ZOL (Olsen *et al.*, 1981). Microsomal ZEN reduction activity was determined by incubating 0.5 ml

microsomal suspension with 100 $\mu$l ZEN (100 $\mu$g $ml^{-1}$ of methanol), 150 $\mu$l of 20 mM NADH + NADH regenerating system [150 $\mu$l 100 mM ethanol and 5 $\mu$l alcohol dehydrogenase (Stock 30,000 U reconstituted to 71 U $ml^{-1}$). ZEN reduction activity in the cytosolic fraction was determined by incubating 0.5 ml cytosolic fraction with 100 $\mu$l ZEN (100 $\mu$g $ml^{-1}$ of methanol), 150 $\mu$l of 20 mM NADPH + NADPH regenerating system [150 $\mu$l 100 mM glucose-6-phosphate and 5 $\mu$l glucose-6-phosphate dehydrogenase, 250 U $ml^{-1}$] and sucrose-(0.25M)-tris buffer (5 mM, pH 7.4)-EDTA (1 mM) to a final volume of 3.0 ml. Protein concentrations of homogenate, microsomal and cytosolic preparations were 4, 4 and 7 mg $ml^{-1}$, respectively. Incubations were carried out at 37°C in a shaking water bath, and 100 ul aliquots of the reaction mixture were removed after 5, 10 and 20 min into screw-top centrifuge tubes containing 1.9 ml of 10% propanol in ether. After the mixture was vortexed for 20 s, 2.0 ml of distilled water was added to the tube, vortexed for 30 s and centrifuged at 1500 x g for 2 min. The ethereal layer was transferred to a clean screw-top tube and the extraction was repeated once more. The combined ethereal layers were evaporated to dryness under a steady stream of nitrogen in a 35-40°C evaporator. Samples were taken up in 5.0 ml mobile phase (water:methanol:acetonitrile 43:42:15) analyzed by HPLC.

**HPLC Analytical Protocol:** High-performance liquid chromatography was performed using a Beckman Model 110A pump with Model 420 controller (Toronto. Ont). The analytical column was an Ultrasphere ODS, column (250 x 4.6 mm ID, 5 $\mu$m particle size) (Beckman Toronto, Ont). A precolumn was used to protect the column (Whatman Co:PELL ODS). Detection was accomplished using a Beckman 160 Absorbance Detector (Toronto, Ont) using a 254 nM filter. A Hewlett Packard 3390A integrator (Edmonton, Alta) was used to integrate the detector signals. The flow rate was 1 ml $min^{-1}$ and the solvent system employed was water:methanol:acetonitrile 43:42:15 with 0.2% phosphoric acid. Analyses were performed in duplicate and enzyme activity was expressed as nmol ($\alpha$+$\beta$)-ZOL formed $mg^{-1}$ protein $h^{-1}$.

**NADPH-cytochrome $P_{450}$ reductase:** Microsomal cyt $P_{450}$ activity was determined by measurement of the rate of cytochrome c reduction via increased absorbance at 550 nm according to a modification of the method of Jeffrey *et al.*, (1977). The incubation system contained 50 mM Tris, 150 mM KCl, 10 mM $MgCl_2$ with 200 mM oxidized cytochrome c (Sigma III, from horse heart) and 100 ug microsomal protein. The system was pre-incubated for 2 min at 37°C. The reaction was started by the addition of 100 ul 40 mM NADPH and the change in absorbance monitored continuosly for 5 min at 37°C in a Milton Roy Spectrophotometer 3000 (Rochester, NY). The final incubation

volume was 2 ml. An extinction coefficient of 0.021 $nmol^{-1}$ $cm^{-1}$ was used to determine the rate of cytochrome c reduction. Cyt $P_{450}$ activity was expressed as nmol cytochrome c reduced $mg^{-1}$ protein $min^{-1}$.

**Urinary Total ZEN and ZOL:** HPLC analysis of total ZEN, $\alpha$- and ß-ZOL in urine samples was determined after incubation of aliquots with ß-glucuronidase (Chapter 3). Hydrolysis of glucuronides to release ZEN and the phase I metabolites was accomplished by incubation with ß-glucuronidase (E. Coli, Type VIII, Sigma; 500 units/100$\mu$l in 50% glycerol). Diluted urine samples (100 $\mu$l), and ß-glucuronidase (100 $\mu$l) and 200 $\mu$l 0.05 M Na acetate buffer, pH 5.5 were pipetted into 8 ml screw-top centrifuge tubes and diluted to 2.0 ml with distilled water. The tubes were capped loosely and incubated in a 37°C shaking water bath for 18 h. The tubes were cooled to room temperature, 2.0 ml of 10% 2-propanol in ether was added, and the acid-base cleanup procedure described above for unconjugated substrates was performed. Duplicate 100 $\mu$l urine samples plus 1.9 ml of distilled water and 2.0 ml of 10% 2-propanol in diethylether were pipetted into 8 ml screw-top centrifuge tubes, mixed for 20 s and centrifuged at 1500 x g for 5 min. The ethereal layer was transferred to clean 8 ml screw-top tubes. The extraction procedure was repeated, with the aqueous phase and the two organic layers combined and kept on ice for 10-15 min. The samples were kept on ice throughout

the remainder of the extraction procedure. 2 ml of chilled 0.184 M NaOH was added to the tubes, mixed for 30 s, chilled and mixed for a further 30 s and recentrifuged for 2 min at 1200 x g. The resultant organic layer was removed and discarded, and the aqueous layer was washed with 2.0 ml benzene, mixed for 10 s and centrifuged for 2 min at 1200 x g. The resultant benzene layer was discarded. After repeating the washing procedure with benzene, the aqueous (basic) layer was neutralized with chilled 0.5 N acetic acid and mixed for 10 s. 2.0 ml benzene was added to the neutralized samples, mixed for 30 s, and centrifuged for 2 min at 1500 x g. Using a clean glass pipet, the benzene layer was transferred to screw-top glass vials and the extraction process repeated two more times. The combined benzene extracts were evaporated to dryness under a gentle stream of $N_2$ in a 35-40°C water bath. After cooling the vials, 400 $\mu$l of solvent (water:methanol:acetonitrile 43:42:15) was added to the evaporated samples and subjected to HPLC analyses.

**HPLC Analytical Protocol:** High-performance liquid chromatography was performed using a Beckman Model 116M Solvent Delivery Module liquid chromatograph (System Gold, Beckman Toronto, Ont). The analytical column was an Ultrasphere ODS, column (250 x 4.6 mm ID, 5 $\mu$m particle size) (Beckman Toronto, Ont). A precolumn was used to protect the column (Whatman Co:PELL ODS). Detection was accomplished

using a Beckman Programmable 166 Detector using a 254 nM filter. The flow rate was 1 ml $min^{-1}$ and the solvent system employed was water:methanol:acetonitrile 43:42:15 with 0.2% phosphoric acid. Amount of total ZOL excreted was expressed as the sum of $\alpha$- and ß-ZOL.

**Statistical Analysis:** Data were analyzed using the Statistical Analysis System, Inc. (SAS, 1985). Means were compared by using a Students' t-test. A difference was accepted as significant when $p<0.05$. All data are presented as mean ± SEM.

## RESULTS

Reduction activity in the homogenate, microsomal and cytosolic preparations depends on the available substrate. A graphical representation of HSD enzyme activity and total ZOL synthesis is shown in (Fig. 10). The rate of ZOL production was significantly less than HSD enzyme activity in all liver preparations. HSD enzyme activity in the homogenate, microsomal and cytosolic fractions were 648, 288 and 582 nmol $mg^{-1}$ protein $h^{-1}$, respectively compared to ZOL production of 16.2, 34.5 and 2.7 nmol $mg^{-1}$ protein $h^{-1}$ in those same fractions (Fig. 10).

In the enzyme induction study with PB, a potent inducer of xenobiotic metabolizing enzymes, hepatic microsomal Cyt $P_{450}$ enzyme activity was significantly enhanced, 24.7 and 20.5 nmol cytochrome c reduced $mg^{-1}$ protein $min^{-1}$, in PB treated and saline controls, respectively, while HSD enzyme activity was significantly reduced in PB treated rats, 4.8 nmol $mg^{-1}$ protein $h^{-1}$, compared to, 6.4 nmol $mg^{-1}$ protein $h^{-1}$ in controls (Fig. 11).

Excretion of total urinary ZEN and ZOL was significantly increased after PB pretreatment, 34.2 and 4.2 ug respectively, while control rats excreted 17.1 and 1.7 ug, respectively (Fig. 12).

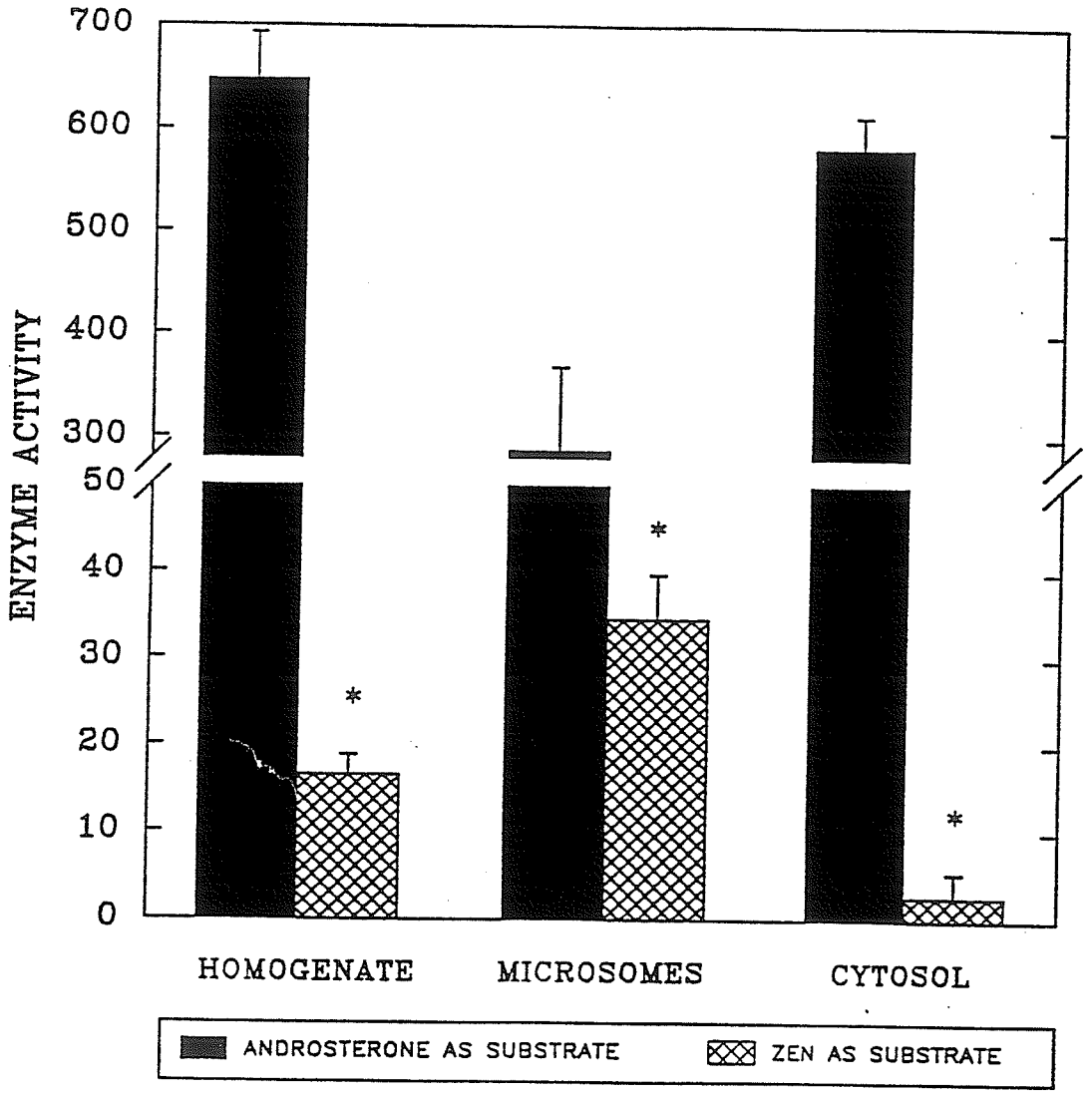



**FIGURE 10.** Subcellular distribution of hepatic HSD activity towards Androsterone (nmole $mg^{-1}$ protein $h^{-1}$) and Zearlaenol synthesis (nmol $mg^{-1}$ protein $h^{-1}$). Means designated by an * are significantly different, $p<0.05$. Data presented as Means $\pm$ SEM; (N=6)

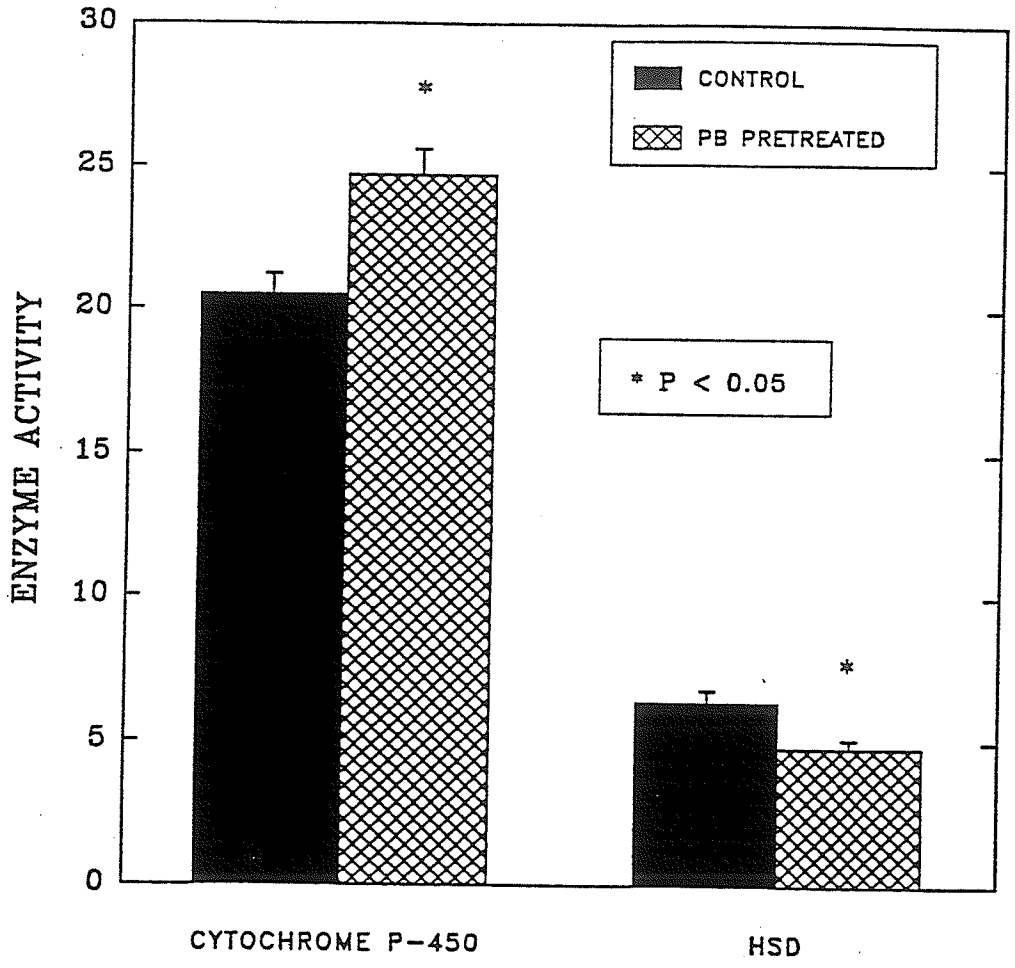



**FIGURE 11.** Effect of PB-pretreatment on hepatic HSD enzyme activity expressed as nmol $mg^{-1}$ protein $h^{-1}$, and NADPH-cytochrome reductase activity, expressed as nmol $mg^{-1}$ protein $min^{-1}$. Means designated by an * are significantly different, $p<0.05$. Data presented as Means $\pm$ SEM; (N=10)

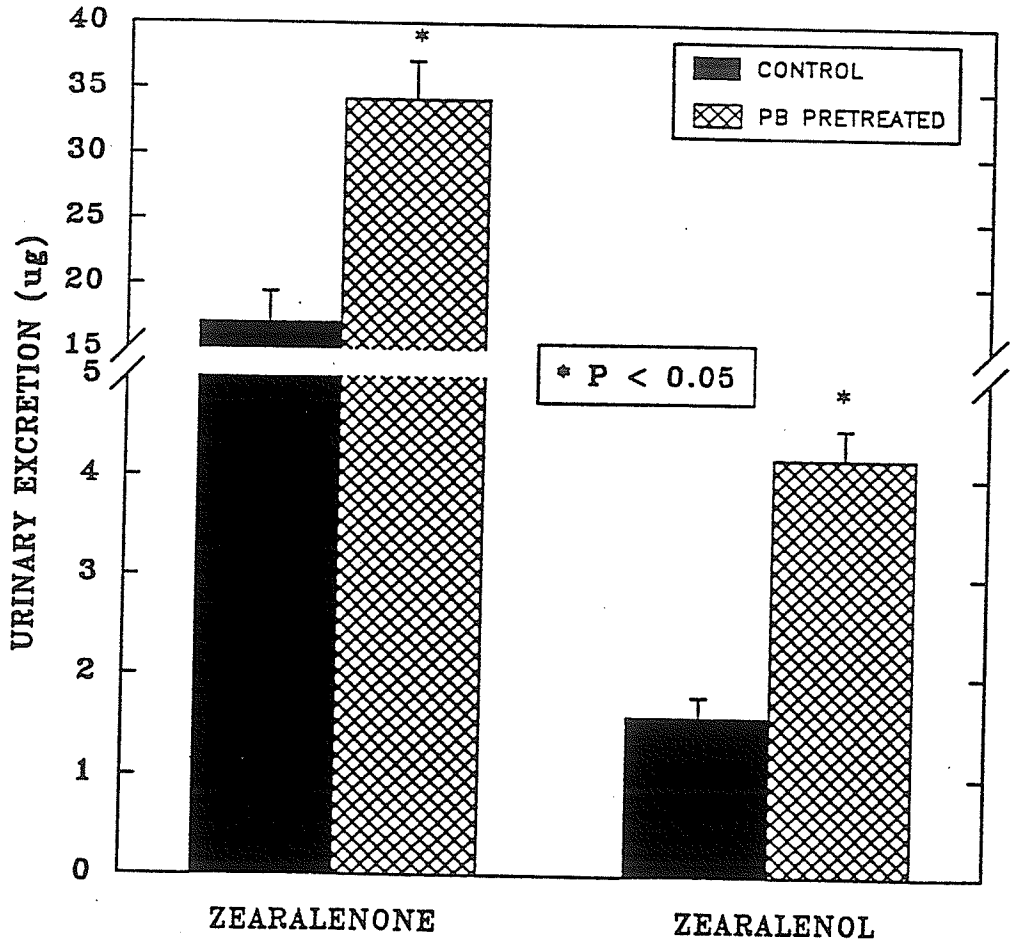



**FIGURE 12.** Effect of PB-pretreatment on 48-hour urinary excretion of total ZEN and ZOL in rats dosed ZEN (1 mg $kg^{-1}$ BW). Means designated by an * are significantly different, $p<0.05$. Data presented as Means $\pm$ SEM; (N=10)

## DISCUSSION

Taken collectively, these data suggest that HSD enzyme activity is not an accurate measure of ZEN reduction *in vivo* and that another enzyme or enzyme system, such as cytochrome $P_{450}$, a mixed-function oxidase, may be responsible for ZOL production. The evidence supporting the notion that HSD is the primary enzyme for ZOL formation is largely circumstantial and does not preclude the possibility that other dehydrogenase/reductase reactions are involved in ZEN metabolism. James and Smith (1982) reported that the dietary ZEN caused a reduction in the activity of HSD. However, in a later report, Stangroom and Smith (1984) reported that dietary ZEN increased the hepatic HSD activity. Kiritsy *et al.* (1987) observed that dietary manipulations, a feed restricted dietary regimen, which produced significant increases in hepatic HSD activity, did not result in increased excretion of $\alpha$-ZOL which suggested that HSD enzyme activity was not an accurate measure of ZEN reduction. A second dietary manipulation, a high protein diet had no effect on hepatic HSD activity, resulted in a decreases in excretion of $\alpha$-ZOL (Chapter 4). When HSD activity, using androsterone as substrate, was compared to ZEN reduction to ZOL, the rate of ZOL synthesis was significantly less in all subcellular preparations, providing further

support on the inadequacy of using the HSD assay to quantify ZOL synthetic activity. Since PB pretreatment depressed microsomal HSD, the increase in urinary excretion of ZOL cannot be attributed to HSD enzyme activity. This finding raises the possibility that a 'reductase' enzyme, that may be a component of the mixed function oxidase enzyme system, could be involved in ZEN reduction. The observed increase in ZEN excretion in the urine is due the induction of GT by PB (Pandey *et al.*, 1990).

The findings of these studies, therefore, strongly suggest that HSD enzyme activity, as measured by using androsterone as a substrate, is a poor and unreliable indicator of hepatic ZEN reduction to ZOL. This may explain, in part, the lack of correlation between the *in vivo* metabolite profile and the *in vitro* HSD enzyme activity. However, extrahepatic reduction of ZEN may also contribute to the discrepancy. It is therefore, important to assess the contribution of the gut to overall ZOL formation.

# CHAPTER 7

# EFFECT OF DIETARY ZEARALENONE ON *IN VITRO* ENZYME ACTIVITY

## INTRODUCTION

HSD is an androgen-dependent enzyme that can be induced in prepubertal rat liver after testosterone administration (Hoff *et al.*, 1977). Kiessling and Pettersson (1978) suggested ZEN reduction is catalyzed by a hydroxysteroid dehydrogenase (HSD). They suggested that the estrogenic effects of ZEN is due to a disturbance of steroid metabolism. That is, dietary ZEN competes with the metabolism of endogenous steroids, resulting in the expression of estrogenic effects of ZEN.

Studies on the effect of dietary ZEN on hepatic HSD activity are inconsistent. James and Smith (1982) reported that the inclusion of ZEN in the diet caused a reduction in the activity of HSD. However, Stangroom and Smith (1984) reported that dietary ZEN increased the hepatic HSD activity, although this was only observed in one of their four trials. Dietary manipulations, a feed restricted dietary regimen, which produced significant increases in hepatic HSD activity, did not result in increased excretion of $\alpha$-ZOL (Kiritsy *et al.*, 1987), which suggested that HSD enzyme activity was not

an accurate measure of ZEN reduction, in agreement with our findings from chapter 4, where dietary manipulation, a high protein diet had no effect on hepatic HSD activity, resulted in a decrease in excretion of α-ZOL. When HSD activity using androsterone as substrate was compared to ZOL syntheses, the rate of ZOL syntheses was significantly less than HSD activity in all hepatic subcellular preparations. Additionally, studies to date have been limited to hepatic metabolism of ZEN. The lack of correlation between the *in vitro* enzyme activity and the *in vivo* metabolites formed may be due to the involvement of extrahepatic sites in ZEN metabolism.

The relative binding affinity for estrogen receptors of α-ZOL is 20 times that of the parent, therefore, the reduction of ZEN producing α-ZOL is an activation process and metabolically significant (Fitzpatrick *et al.*, 1989). It is, therefore, important to evaluate the extent of ZEN reduction to ZOL because of its potential estrogenic effects.

The main objectives of the experiments outlined in this chapter are:

(a) to investigate the effect of dietary ZEN on hepatic HSD and GT activities using the conventional HSD assay and to quantify ZEN reduction activity directly using ZEN as substrate and,

(b) to investigate the effect of dietary ZEN on hepatic and extrahepatic ZEN metabolizing activity.

## METHODS AND MATERIALS

**Animals and Treatment:** The use of animals conformed to the Guidelines of the Canadian Council of Animal Care. Sprague-Dawley rats were purchased from the University of Manitoba central breeding facility in 3 separate groups conforming to the experiments conducted. All animals were housed in individual cages. The room temperature was maintained at 21 ± 1°C with a relative humidity 50% and a 14-10 h light-dark cycle.

**Effect of dietary ZEN on hepatic HSD and GT activity:** 30 female rats (55-60 g) were used. Rats were maintained on laboratory chow (Ralston Purina, St Louis, MO) for a 2 days acclimation period, then randomly assigned to standard semipurified diets (Kiritsy *et al.*, 1987) with and without 250 ppm ZEN, for 2 weeks. Feed intake and body weight was monitored throughout the feeding period. At the end of the feeding period, the rats were killed by decapitation, livers excised and microsomes prepared for GT assay (Chapter 5) and HSD enzyme determination (Chapter 6).

**Effect of dietary ZEN on hepatic ZEN reduction and GT activity:** 30 female rats (55-60 g) were used. Rats were maintained on laboratory chow (Ralston Purina, St Louis, MO) for a 2 days acclimation period, then randomly assigned to standard semipurified diets (Kiritsy *et al.*, 1987) with and without 250 ppm ZEN for 2 weeks. Feed intake and body weight was monitored throughout the feeding period. At the end of the feeding period, the rats were killed by decapitation, livers excised and microsomes prepared for the GT enzyme assay, as described earlier in Chapter 5 and ZOL syntheses determination in Chapter 6.

**Effect of dietary ZEN on renal, hepatic and intestinal ZEN reduction and GT activity:** 16 male Sprague Dawley rats were randomly assigned to semipurified diets containing either 0 or 250 ppm zearalenone, fed for 14 days.

**Tissue Preparation:** Liver microsomes for the GT and ZEN reduction assays were prepared as described previously (Chapters 5 and 6). In the third study, after 14 days on the diets, animals were killed by decapitation, livers and kidneys were quickly removed and kept on ice. An abdominal incision was made, the distal end of the large intestine was ligated and a loose tie was made at the duodenal end. The intestine was filled with ice-cold isotonic KCL solution containing 0.05 M Tris-HCl buffer, pH 7.8. The duodenal tie was secured, the intestine was carefully removed and the intestinal contents

were flushed twice with ice-cold buffer and the intestine was kept in ice-cold saline. The following procedures were performed in a cold-room at 4°C. After opening the intestinal strips (3 x 20 cm), mucosal scrapes, proximal to distal, were obtained using a glass slide. The mucosal scrapes were pooled, weighed and suspended in 20 ml of ice-cold isotonic KCL solution containing 0.05 M Tris-HCl buffer pH 7.8. Trypsin inhibitor (5 mg $g^{-1}$ of mucosa), glycerol (20% v/v), and heparin (3 U $ml^{-1}$) were added to this suspension. The mixture homogenized in a Polytron homogenizer for 60 s and microsomal and cytosolic fractions prepared. Kidneys were chopped into small pieces and homogenized in 3 volumes of 0.25 M sucrose buffer $g^{-1}$ wet weight of kidney tissue.

**Microsomal Preparation:** Intestinal, renal and hepatic microsomes were prepared by centrifuging the respective homogenates as previously described (Chapter 5). The intestinal microsomal pellets were suspended in 4.0 ml 0.25 M sucrose buffer $g^{-1}$ mucosa scrapings. The renal microsomal pellets were resuspended in 3.0 ml homogenizing buffer $g^{-1}$ wet weight kidney. The microsomal and cytosolic preparations were stored at -20°C until assayed for GT enzyme and ZEN reduction activity. Protein content of the various fractions were determined according to Lowry *et al.* (1951).

**Hepatic HSD Assay:** The indirect spectrophotometric method, with androsterone as the enzyme substrate, was used to

determine HSD enzyme activity as previously described (Chapter 6). The reaction was monitored for 10 min at a wavelength of 340 nm and 37°C in a Milton Roy Spectrophotometer 3000 (Rochester, NY).

**Hepatic and Renal Microsomal GT Assay:** GT activity in renal and hepatic microsomes was assayed according to the linked method of Mulder and van Doorn (1975) as previously described (Chapter 5).

**Intestinal Microsomal GT Assay:** GT activity in intestinal microsomes was assayed according to a modification of the linked method of Mulder and van Doorn (1975) to enable GT activity determination directly by HPLC analysis. The spectrophotometric method was unsuitable for determination of GT activity in intestinal microsomes due to excessive interference and high blanks. The Triton X-100 concentration for maximal GT activity in intestinal microsomes was determined to be 0.0125%. Microsomes were incubated in 0.01251% Triton-X-100 in 0.154 M KCl for at least 30 min at 0-4°C prior to assay. The assay medium contained: 75 mM-Tris-HCl buffer, pH 7.3, 5.0 mM $MgCl_2$; 0.2 mM phosphoenolpyruvate; 0.2 mM NADH; 0.625 U lactate dehydrogenase $ml^{-1}$; 5.0 U pyruvate kinase $ml^{-1}$; 1.5 mM UDP-glucuronate, 10 mM saccharo-1-4-lactone and 0.3 mM acceptor substrate (ZEN) dissolved in ethanol-water (1:1, v/v). The reaction mixture was preincubated for 5 minutes and the reaction started by addition of microsomal

suspension yielding a final protein concentration 200 $\mu$g ml$^{-1}$. Incubations were carried out at 37°C in a shaking water bath, and 100 ul aliquots of the reaction mixture were removed after 5, 10, and 20 min into screw-top centrifuge tubes containing 1.9 ml of 10% propanol in ether. After the mixture was vortexed for 20 s, 2.0 ml of distilled water was added to the tube, vortexed for 30 s and centrifuged at 1500 x g for 2 min. The ethereal layer was transferred to a clean screw-top tube, and the extraction was repeated once more. The combined ethereal layers were evaporated to dryness under a steady stream of nitrogen in a 35-40°C evaporator. Samples were taken up in 5.0 ml mobile phase (water:methanol:acetonitrile 43:42:15) and subjected to HPLC analysis.

**HPLC Analytical Protocol:** High-performance liquid chromatography was performed using a Beckman Model 110A pump with Model 420 controller (Toronto, Ont). The analytical column was an Ultrasphere ODS, column (250 x 4.6 mm ID, 5 $\mu$m particle size) (Beckman Toronto, Ont). A precolumn was used to protect the column (Whatman Co:PELL ODS). Detection was accomplished using a Beckman 160 Absorbance Detector (Toronto, Ont) using a 254 nM filter. A Hewlett Packard 3390A integrator (Edmonton, Alta) was used to integrate the detector signals. The flow rate was 1 ml min$^{-1}$ and the solvent system employed was water:methanol:acetonitrile 43:42:15 plus 0.2% phosphoric acid. Analyses were performed in duplicate and GT

enzyme activity was expressed as nmol ZEN conjugated $mg^{-1}$ protein $h^{-1}$.

**Renal, Hepatic and Intestinal ZEN reducing activity:** The activity of ZEN reduction to $\alpha$- and ß-ZOL was measured by measuring the rate of production of ZOL according to a modification of the method of Olsen *et al.* (1981) as previously described (Chapter 6).

**HPLC Analytical Protocol:** The HPLC analytical protocol described previously in Chapter 6 was used.

**Statistical Analyses:** Data were analyzed using the Statistical Analysis System, Inc. (SAS, 1985). Means were compared using the Students' t-test. A difference was accepted as significant when $p<0.05$. All data are presented as mean ± SEM.

## RESULTS

Dietary ZEN, significantly reduced feed efficiency compared to control animals. Total feed intake was less in the 250 ppm ZEN treated group, resulting in decreased weight gain, reduced intake and reduced feed efficiency (Table 9). The effect of dietary ZEN on hepatic HSD and GT activities is represented graphically in Figure 13. Microsomal GT activity was significantly greater in the 250 ppm ZEN treated rats compared to controls, 706 ± 49 and 495 ± 32 nmol $mg^{-1}$ protein $h^{-1}$ respectively. Hepatic HSD activity, measured by androsterone reduction reaction, was unaffected by dietary ZEN.

In the second feeding study, rats fed 250 ppm ZEN, exhibited significantly reduced feed efficiency, 0.33 ± 0.2, compared to control animals, 0.46 ± 0.02. When hepatic ZEN reducing capacity was measured directly, using ZEN as substrate, a significant elevation of both microsomal and cytosolic ZOL synthetic activities was observed (Fig. 14). Hepatic microsomal and cytosolic ZOL syntheses was 32.6 and 4.4 in ZEN treated rats compared to 20.2 and 2.3 nmol $mg^{-1}$ protein $h^{-1}$ in control rats, respectively. Hepatic microsomal GT activity was significantly higher in ZEN treated rats

**TABLE 9.** Effect of Dietary Zearalenone on Feed Intake, Body Weight and Feed Efficiency

| | DIETARY ZEARALENONE | |
|---|---|---|
| | 0 ppm | 250 ppm |
| Feed Intake (g) | 293 ± 4[a] | 212 ± 6[b] |
| Weight Gain (g) | 119 ± 3[a] | 73 ± 3[b] |
| Feed Efficiency (Gain/Feed Ratio) | 0.41 ± 0.01[a] | 0.30 ± 0.02[b] |

Means with different letters are significantly different, $p<0.05$, Students t test. All data presented as Mean ± SEM; (N=15).

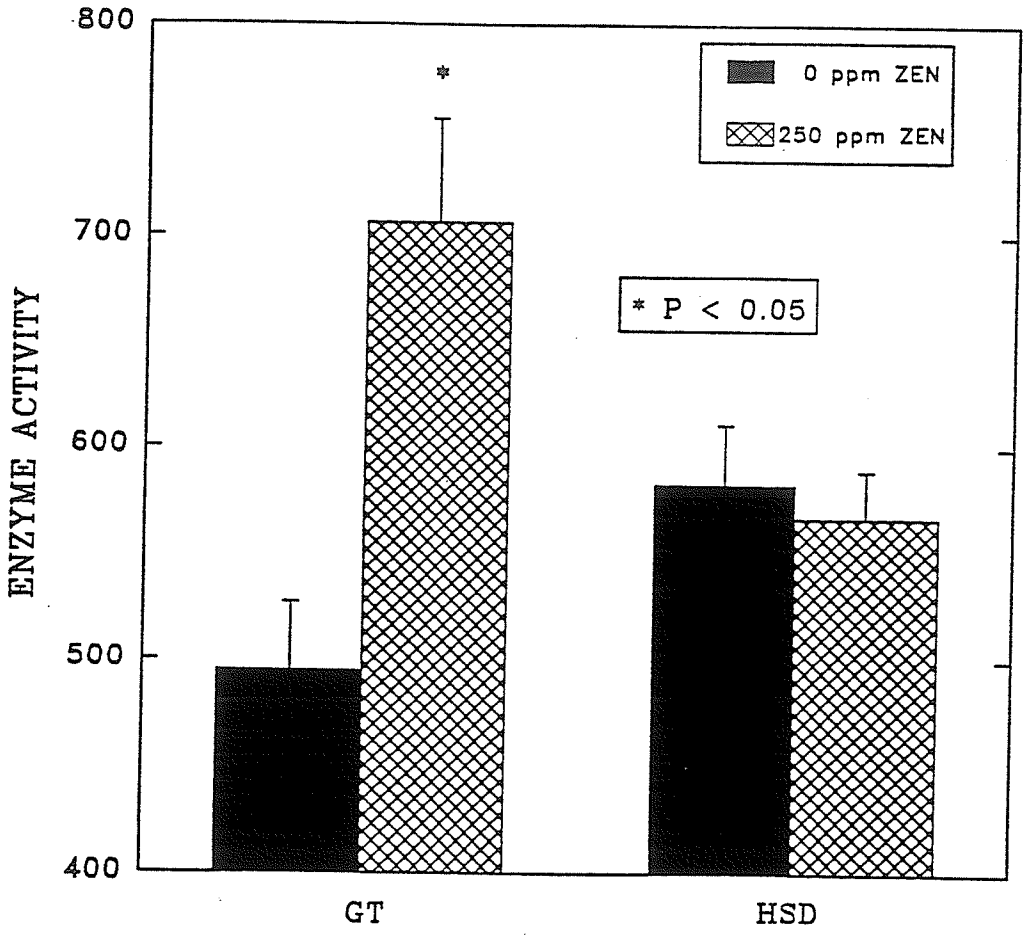



**FIGURE 13.** Effect of dietary ZEN on hepatic HSD and GT activities in rats. HSD activity is measured using androsterone as substrate and expressed as nmol $mg^{-1}$ protein $h^{-1}$. GT activity towards ZEN is expressed as nmol $mg^{-1}$ protein $h^{-1}$. Means designated by an * are significantly different, $p<0.05$. Data presented as Means ± SEM; (N=15)

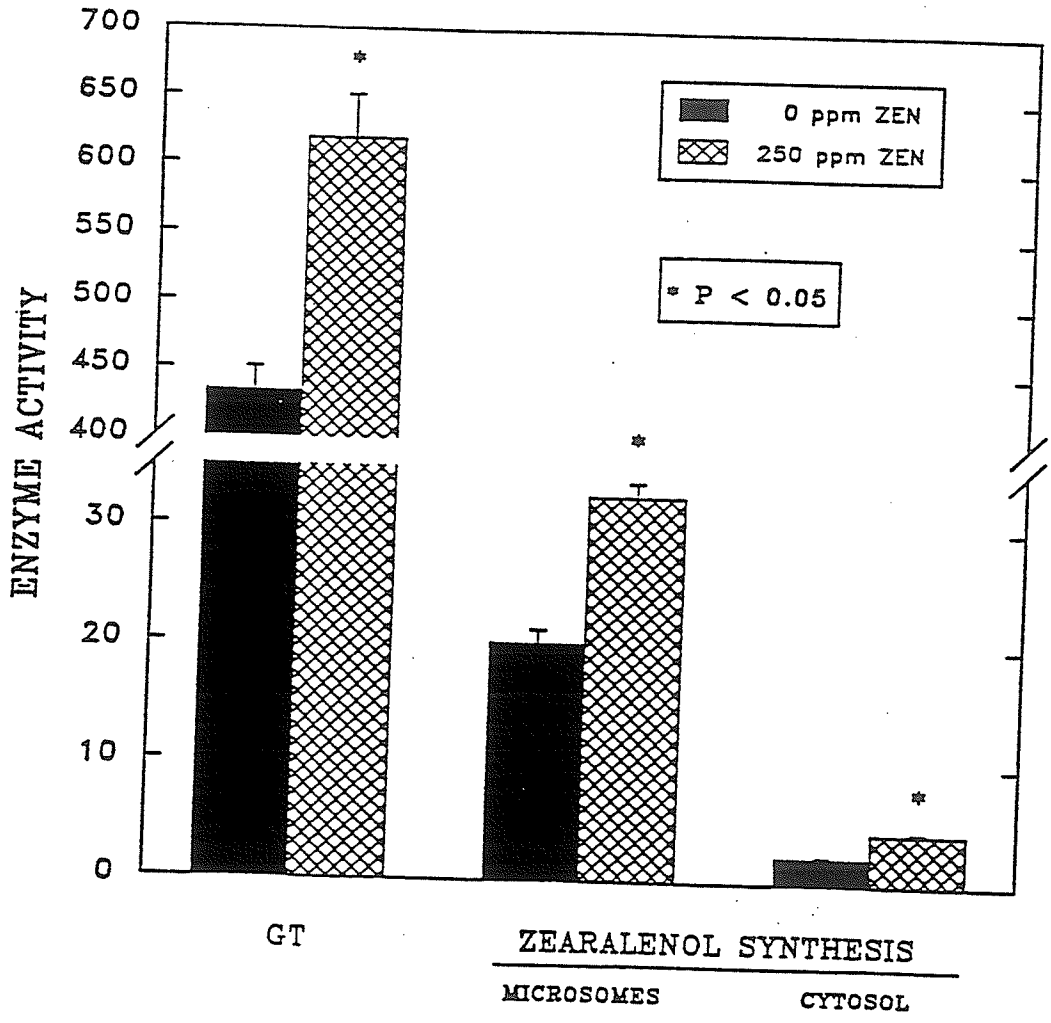



**FIGURE 14.** Effect of dietary ZEN on hepatic ZOL synthesis and GT activity in rats. ZOL syntheses in microsomal and cytosolic fractions measured directly using ZEN as substrate and expressed as nmol $mg^{-1}$ protein $h^{-1}$. GT activity towards ZEN is expressed as nmol $mg^{-1}$ protein $h^{-1}$. Means designated by an * are significantly different, $p<0.05$. Data presented as Means ± SEM; (N=15).

compared to controls, 619 and 434 nmol ZEN conjugated $mg^{-1}$ protein $h^{-1}$ respectively.

In rats fed 250 ppm ZEN, GT activity was significantly higher in hepatic, intestinal and renal microsomes than control rats (Fig. 15). Hepatic, intestinal and renal GT activity was 503, 391 and 123 in ZEN treated rats, compared to 335, 248 and 72 nmol ZEN conjugated $mg^{-1}$ protein $h^{-1}$ in controls, respectively. ZOL synthetic capacity was significantly enhanced in both intestinal microsome and cytosol preparations from rats fed 250 ppm ZEN compared to control rats (Fig. 16). Intestinal microsomal ZOL syntheses was 11.3 and 6.7 nmol ZOL formed $mg^{-1}$ protein $h^{-1}$ in ZEN fed and control rats respectively. Intestinal cytoslic ZOL syntheses was 1.5 and 0.8 nmol ZOL formed $mg^{-1}$ protein $h^{-1}$ in ZEN fed and control rats respectively. No effect of dietary ZEN on ZEN reducing capacity in renal microsomes was observed while cytosolic ZEN reduction was undetectable.

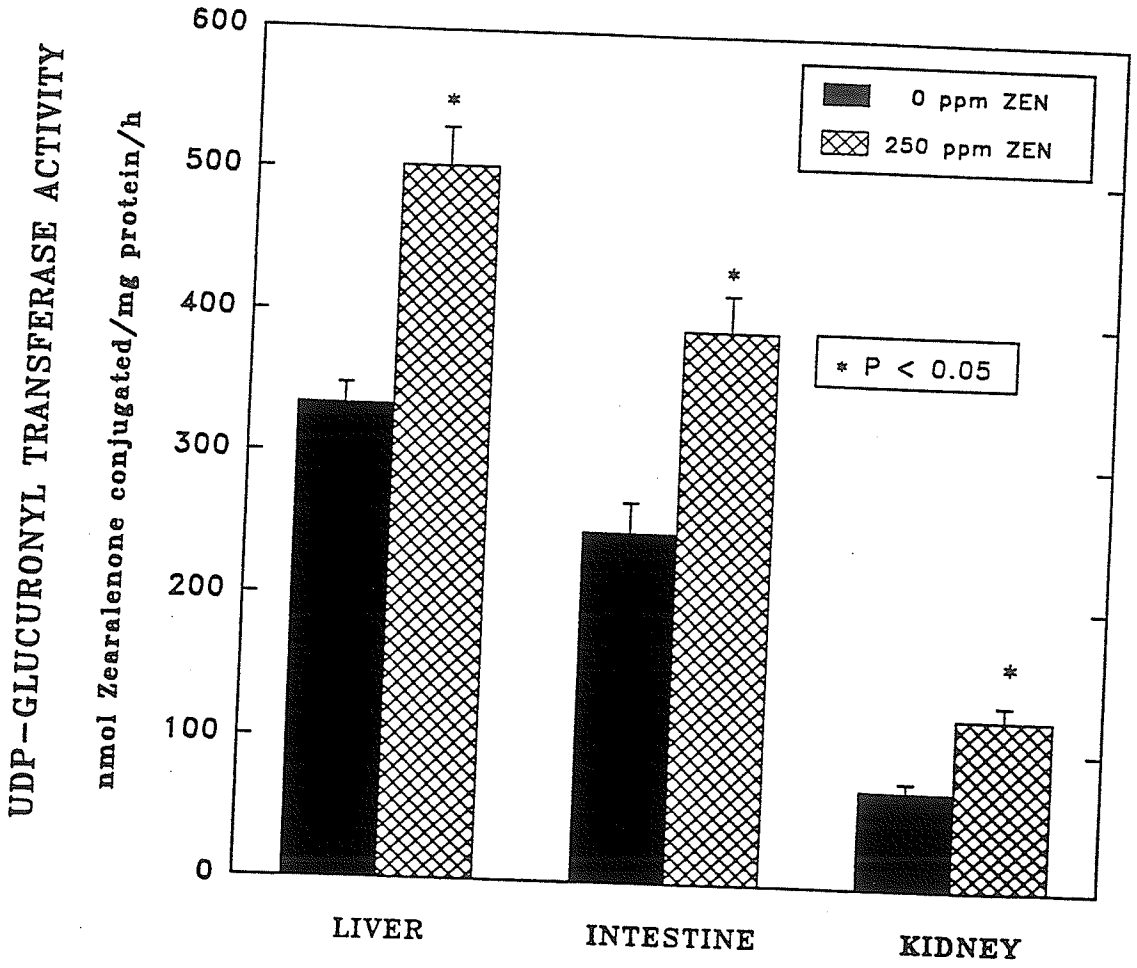



**FIGURE 15.** Effect of dietary Zen on rat hepatic intestinal and renal GT activity. GT activity towards ZEN is expressed as nmol $mg^{-1}$ protein $h^{-1}$. Means designated by an * are significantly different, $p<0.05$. Data presented as Means $\pm$ SEM; (N=8)

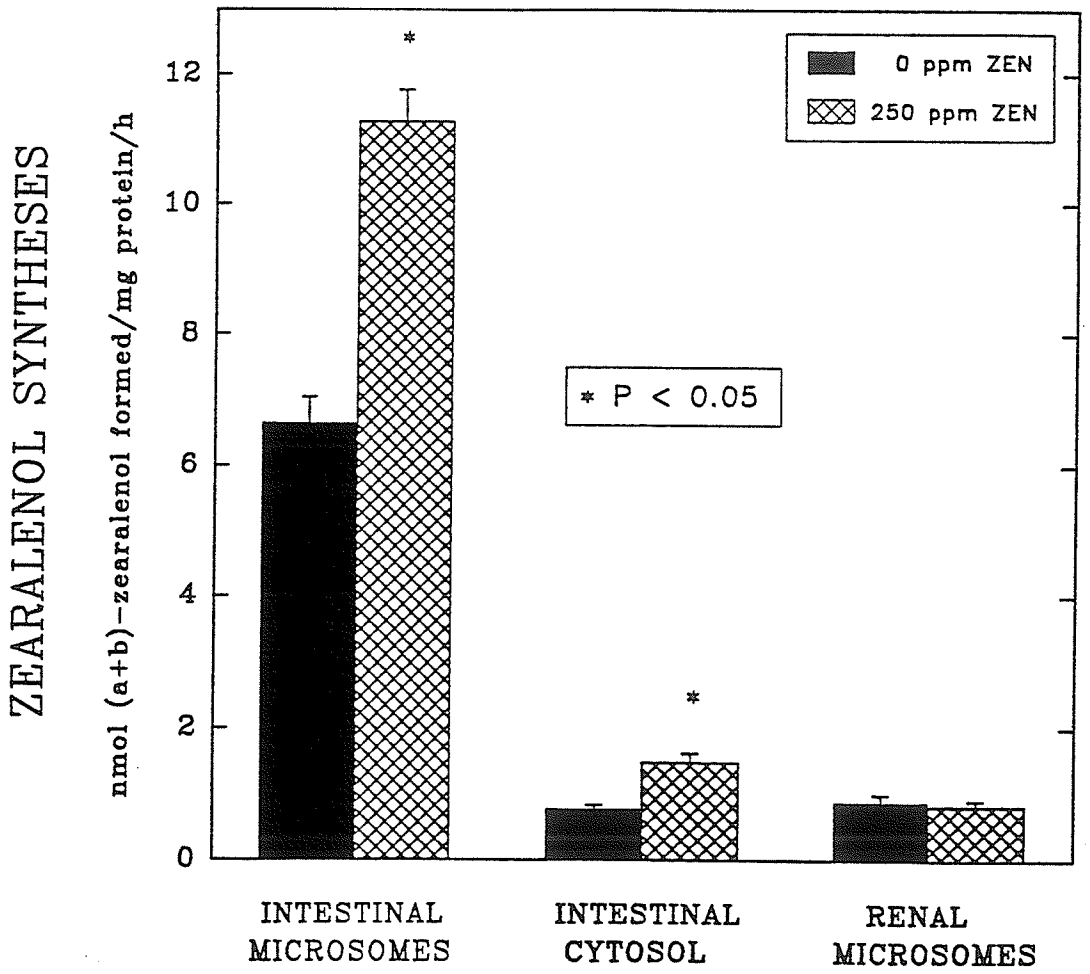



**FIGURE 16.** Effect of dietary ZEN on rat intestinal and renal ZOL syntheses. ZOL syntheses in microsomal and cytosolic fractions was measured directly sing ZEN as substrate and expressed as nmol $mg^{-1}$ protein $h^{-1}$. Means designated by an * are significantly different, $p<0.05$. Data presented as Means $\pm$ SEM; (N=8)

## DISCUSSION

Feed intake and efficiency was reduced in rats fed 250 ppm ZEN. Reduced feed intake and efficiency is consistent with ZEN toxicity and the extent of this effect dependent on the amount of dietary ZEN (Kiritsy *et al.*, 1987). Dietary ZEN acts systemically by binding to estrogen receptors. Resch and Simpson (1985) demonstrated that ZEN directly implanted in the brain reduced food intake and body weight gain, mimicking estrogen.

Glucuronidation is an important pathway in the detoxification of endogenous and exogenous compounds (Dutton 1980). GT enzyme activity is mediated by a family of isoenzymes that can be selectively induced by various xenobiotics (Finley *et al.*, 1986). The induction of GT activity by PB increased the urinary excretion of conjugated ZEN and $\alpha$-ZOL (Pandey *et al.*, 1989). We have suggested, (Kiritsy *et al.*, 1987; Chapter 3) that conjugation of ZEN and its metabolites act to reduce the toxic expression of ZEN by increasing urinary excretion, thus limiting the potential for enterohepatic recirculation (Smith, 1980a). Therefore, the observation that dietary ZEN induces GT enzyme activity is consistent our earlier reports and the literature.

The relative binding affinity for estrogen receptors of α-ZOL is 20 times that of the parent, therefore, the reduction of ZEN producing α-ZOL is an activation process and metabolically significant (Fitzpatrick *et al.*, 1989). The activity of HSD *in vivo* was not affected by dietary ZEN. This is in contrast to earlier reports by Smith and co-workers (James and Smith, 1982, Stangroom and Smith, 1984) who had reported that dietary ZEN had influenced HSD activity. Given that HSD activities were not measured under optimal reaction conditions, the lack of agreement between our results and those previously published is understandable. The lack of measurable effects of ZEN on HSD suggests that the enzyme is not a likely contributor in ZEN metabolism.

In a subsequent ZEN feeding trial, in which hepatic ZEN reduction was measured directly, an increase in ZOL synthesis was observed. Taken collectively, with our previous observations on the subcellular distribution of HSD and ZOL synthesis activity (Chapter 6). It is apparent that HSD enzyme activity is a poor and unreliable index of ZEN reduction. Additionally, the observation that PB-pretreatment increased *in vivo* α-ZOL synthesis, without affecting HSD enzyme activity (Chapter 6) further supports this notion. That is PB-induced cyt $P_{450}$ enzyme activity, may account for the increased *in vivo* synthesis of α-ZOL.

Most *in vitro* studies on ZEN focussed on hepatic metabolism, and may have contributed to the poor correlation

between the *in vitro* enzyme profile and the *in vivo* metabolites formed. We have suggested that this lack of correlation may be due, in part, to the fact that extrahepatic reduction and conjugation sites for ZEN metabolism exist and may contribute substantially to overall ZEN metabolism. Indeed, we detected extensive induction of GT in intestinal and renal microsomes, while ZOL reduction activity was enhanced in intestinal microsomal and cytosolic fractions and not in the kidney. This data indicates that while the primary organ responsible for ZEN metabolism is the liver, there is a substantial contribution by extrahepatic sites to the overall metabolism of ZEN and that these sites should be considered when evaluating the metabolism and toxicity of a xenobiotic such as ZEN. The *in vitro* metabolism data may provide important clues on the possible metabolic pathways and routes of excretion. However, metabolic pathways and routes of excretion can not be confirmed by *in vitro* studies alone and can only be substantiated with *in vivo* studies to determine the absorption, elimination and excretion of any compound or pharmacokinetic analyses.

# CHAPTER 8

# PHARMACOKINETICS OF ZEARALENONE

## INTRODUCTION

Zearalenone (ZEN) [6-(10-hydroxy-6-oxo-trans-1-undecenyl)-ß-resorcyclic acid-lactone] is an estrogenic mycotoxin produced by several species of *Fusarium* fungi endemic to temperate climates (Mirocha *et al.*, 1977). ZEN and it metabolites $\alpha$- and ß-zearalenol (ZOL) belong to a rare class of natural products, the ß-resorcylic acid-lactones, which are capable of binding to estrogen receptors because of their chemical similarity to estradiol (Hurd, 1977; Katzenellenbogen *et al.*, 1979). At low levels, dietary ZEN and its metabolites act as anabolic hormones promoting growth, however, at high doses these compounds become toxic (Lindsay, 1985). ZEN toxicity may occur when mold-contaminated feed is ingested by livestock and experimental animals (Mirocha *et al.*, 1977). The clinical effects of subacute mycotoxicosis include decreased food intake, reduced growth and reproductive effects. Dietary ZEN acts systemically producing an estrous syndrome in swine, cattle and laboratory animals.

ZEN metabolism occurs in a typical biphasic manner (Kiessling and Pettersson, 1978). Phase I reduction, catalyzed by 3$\alpha$-hydroxysteroid dehydrogenase (HSD), produces

α- and ß-ZOL. The relative binding affinity for estrogen receptors of α-ZOL is 10-20 times greater than that of ZEN, and 100 times greater than ß-ZOL, therefore, reduction of ZEN to α-ZOL is an activation process, while synthesis of ß-ZOL is an inactivation reaction (Fitzpatrick *et al.*, 1989). Phase II conjugation of ZEN and the phase I metabolites to their glucuronides, catalyzed by UDP-glucuronyltransferase (GT), is an inactivation process. Reduction and conjugation of ZEN increases its solubility and may promote its excretion in the urine (Kiritsy *et al.*, 1987). Conjugation of ZEN and ZOL, increases their molecular weight which acts to promote biliary excretion (Kiritsy *et al.*, 1987; Mulder *et al.*, 1981).

Though *in vitro* studies indicate that the liver may be the main organ responsible for ZEN metabolism, numerous studies have demonstrated that other tissues, such as kidney (Tomkins, 1956), ovary (Jarrell and Robaire, 1982), and intestine (Nienstedt *et al.*, 1972), exhibit HSD activity. GT activity has also been reported to be widely distributed in the body (Aitio and Marniemi, 1979; Bottoms *et al.*, 1977; Olsen *et al.*, 1987). While the enzyme distribution suggests that extrahepatic metabolism and tissue uptake of the mycotoxin is probable, the contribution of extrahepatic tissue to the metabolism of ZEN is unclear. There have been numerous studies characterizing ZEN metabolism *in vitro*, however, there is little, or incomplete information on its metabolic fate (Dailey *et al.*, 1980; Smith 1982a; Farnworth and Trenholm,

1983; Olsen *et al.*, 1985). To our knowledge the pharmacokinetics of ZEN have not been evaluated in the rat.

The objective of the present study was to investigate the pharmacokinetics of ZEN as a function of two routes of administration (intravenous and intragastric) in bile-duct cannulated and intact rats. These disposition studies enabled us to test the working hypothesis that the mycotoxin undergoes extensive presystemic metabolism, biliary excretion and subsequent enterohepatic recirculation (EHC). The persistence of EHC result in an elevation of plasma ZEN and $\alpha$-ZOL concentrations and exacerbation of the toxic effects of the mycotoxin.

## MATERIALS AND METHODS

**Experimental Design:** The use of experimental animals conformed to the guidelines of the Canadian Council on Animal Care. Eighteen female Sprague Dawley rats (250-300 g BW) were purchased from the University of Manitoba central breeding facility. The animals were assigned to four ZEN treatment groups: (1) iv intact: intravenous non-bile-duct cannulated (n=3); (2) iv cannulated: intravenous bile-duct cannulated (n=6); (3) ig intact: intragastric non-bile-duct cannulated (n=3) and (4) ig cannulated: intragastric bile-duct cannulated (n=6). More animals were used in the bile-duct cannulated groups due to the potential for greater variation do to surgical intervention. Experiments were carried out in female rats because of their susceptibility to the estrogenic effects of the mycotoxin.

**Animal Treatment and Surgical Procedure:** After an overnight fast, rats were anaesthetized with pentobarbital (Nembutal, 50 mg $kg^{-1}$ BW ip). The animals were placed on a thermostatically controlled heating blanket. A rectal thermometer connected to a Harvard Animal Blanket Control Unit was used to maintain body temperature at 37.5°C. A tracheotomy was performed. The left carotid artery was cannulated with a polyethylene

catheter (PE50) to obtain arterial blood samples and for the measurement of blood pressure (Statham Pressure Transducer, Model P23Dc) connected to a Grass Polygraph Model V. The left jugular vein was cannulated with two lines; (PE160) for the continuous infusion of either saline (97 $\mu$l $min^{-1}$) in rats where no bile was collected, or bile salt (4.0 mM Na taurocholate, 12 ml $kg^{-1}/h^{-1}$, and heparin (10 U $ml^{-1}$) in saline (total flow rate of 97 $\mu$l $min^{-1}$).

To cannulate the bile duct a 2 cm midline incision was made in the abdomen just below the liver. A loose surgical knot was placed around the exposed bile duct above the juncture with the duodenum and kept taut to straighten the duct. Approximately 10 mm from the hilus of the liver a small incision was made, a polyethylene cannula (PE10) was inserted until it was 2 to 3 mm from the hilus and the cannula secured to the bile duct with the surgical suture. The original knot was released and the abdomen was closed with surgical suture.

To cannulate the urinary bladder, a midline incision of about 2 cm was made along the linea alba in the lower abdomen of the rat and the apex of the bladder was gently pulled above the plane of the cut. A 3 mm incision at the apex was made and the catheter was inserted through the opening into the bladder and secured with a purse-string suture.

Following a 45-min stabilization period, a bolus of ZEN, 1 mg $kg^{-1}$ BW, dissolved in 50% ethanol (final volume 20-30 $\mu$l), was administered either iv or ig. In iv bile-duct cannulated

rats, complete bile and urine samples were collected in preweighed Eppendorf tubes at 10, 20, 30, 40, 50, 60, 90, 120, and 160 and 200 min after ZEN. In ig dosed bile-duct cannulated, ig intact and iv intact rats, complete bile and urine samples were obtained at 20, 40, 60, 80, 100, 120, 140, 160, 190, 220, 250 and 280 min after ZEN. Blood samples of approximately 200 $\mu$l were obtained in heparinized microcentrifuge tubes at midpoints of urine and bile collections for all treatment groups. An equal volume of heparinized saline to blood volume withdrawn was injected in the arterial line to replace blood volume. All bile and urine samples were kept on ice and stored at -80°C while blood samples were centrifuged immediately for 5 min on a bench top microcentrifuge (15,000 x g) and the plasma transferred to preweighed microcentrifuge tubes and stored at -80°C. Bile, urine and plasma volumes were determined gravimetrically. The blood pressure and heart rate were monitored throughout the duration of the experiment.

**Sample Preparation:** Duplicate diluted bile, urine and plasma samples (100 $\mu$l) plus 1.9 ml of distilled water and 2.0 ml of 10% 2-propanol in diethylether were pipetted into 8 ml screw-top centrifuge tubes, mixed for 20 s and centrifuged at 1500 x g for 5 min. The ethereal layer was transferred to clean 8 ml screw-top tubes. The extraction procedure was repeated with the resultant aqueous phase and the two organic layers

combined and kept on ice for 10-15 min. The samples were kept on ice throughout the remainder of the extraction procedure. 2.0 ml of chilled 0.184 M NaOH was added to the tubes, mixed for 30 s, chilled and mixed for a further 30 s and centrifuged for 2 min at 1200 x g. The resultant organic layer was removed and discarded, and the aqueous layer was washed with 2.0 ml benzene, mixed for 10 s and centrifuged for 2 min at 1200 x g. The resultant benzene layer was discarded. After repeating the washing procedure with benzene, the aqueous (basic) layer was neutralized with chilled 0.5 N acetic acid and mixed for 10 s. Benzene (2.0 ml) was added to the neutralized samples, mixed for 30 s, and centrifuged for 2 min at 1500 x g. Using a clean glass pipet, the benzene layer was transferred to screw-top glass vials and the extraction process repeated two more times. The combined benzene extracts were evaporated to dryness under a gentle stream of $N_2$ in a 35-40°C water bath. After cooling the vials, 200-400 $\mu$l of solvent (water:methanol:acetonitrile 43:42:15) were added to the evaporated samples and subjected to high performance liquid chromatography (HPLC) analyses.

Hydrolysis of glucuronides to release ZEN and the phase I metabolites was accomplished by incubation with ß-glucuronidase (500 units $100\mu l^{-1}$ in 50% glycerol, E. Coli, Type VIII, Sigma, St. Louis, Mo). 100 $\mu$l of diluted bile, urine and plasma samples (100 $\mu$l), and ß-glucuronidase, 100 $\mu$l for urine and 200 $\mu$l for bile and plasma samples, and equal

volumes of 0.05 M Na acetate buffer, pH 5.5 were pipetted into 8 ml screw-top centrifuge tubes and diluted to 2.0 ml with distilled water. The tubes were capped loosely and incubated in a 37°C shaking water bath for 18 h. The tubes were cooled to room temperature, 2.0 ml of 10% 2-propanol in ether was added, and the same acid-base cleanup procedure described above for unconjugated substrates was performed. The identity of the glucuronide was confirmed by selective inhibition of hydrolysis of ß-glucuronidase activity with 10 mM saccharo-1,4,-lactone. Efficiency of the acid-base extraction procedure was determined by recovery checks of ZEN and all phase I and II metabolites from spiked bile, urine and plasma samples with 200 ng of α-ZOL, ß-ZOL and ZEN. There were 6 replicates for each biological fluid.

**HPLC Analytical Protocol:** High-performance liquid chromatography was performed using a Beckman Model 116M Solvent Delivery Module liquid chromatograph (System Gold, Beckman, Toronto, Ont). The analytical column was an Ultrasphere ODS, column (250 x 4.6 mm ID, 5 μm particle size) (Beckman, Toronto, Ont). A precolumn was used to protect the column (Whatman Co:PELL ODS). Detection was accomplished using a Beckman Programmable 166 Detector using a 254 nM filter. The flow rate was 1 ml $min^{-1}$ and the solvent system employed was water:methanol:acetonitrile 43:42:15 with 0.2% phosphoric acid.

**Treatment of Data:** The area under the plasma concentration versus time curves (AUC) for each experimental animal was determined by the trapezoidal method from time t=0 to the last measured concentration. The extrapolation to infinity was calculated by adding the estimated residual area with the term $C_t/ß$ where $C_t$ is the last measured concentration and ß is the slope of the terminal disposition phase, determined by least squares regression of the ln plasma concentration vs time data ($AUC_{0\to\infty} = AUC_{(0\to t)} + C_t/ß$). The terminal disposition constant, ß, was obtained using a non-linear regression program (ESTRIP), from plasma concentration-time profiles of each rat (Brown and Manno, 1978). The major pharmacokinetic parameters were calculated using the following model-independent equations:

(a) Total plasma clearance: $CL_p$ (for iv) and $CL_{po}$ (for ig) = $DOSE/AUC_{0\to\infty}$. Apparent volume of distribution: $V_d$ or $V_d/f = CL_p/ß$.

(b) Renal and Biliary Clearance: $CL_{R \text{ or Bile}} = X_{(0\to t)}/AUC_{(0\to t)}$, where X is the amount of the parent or metabolite excreted in urine or bile and $AUC_{(0\to t)}$, the respective AUC in plasma for the same substrate and time interval.

(c) Metabolic Clearance to Urine: $CL_{M(urine)} = X_{u(0\to t)}/AUC_{ZEN(0\to t)}$, where $X_u$ is the amount of the metabolite excreted in urine and $AUC_{ZEN(0\to t)}$, the respective AUC of the parent compound in plasma for the same time interval.

(d) Metabolic Clearance to Bile: $CL_{M(bile)} = X_{b(0\to t)}/AUC_{ZEN(0\to t)}$, where $X_b$ is the amount of the metabolite excreted in bile and

$AUC_{ZEN(0\rightarrow t)}$, the respective AUC of the parent compound in plasma for the same time interval. The calculations from metabolic clearance assumes that the rate limiting step is metabolic production.

**Statistical Analysis:** Data were analyzed using the Statistical Analysis System, Inc. (SAS, 1985). Two-way analysis of variance procedure was used to compare treatment groups and multiple comparisons were made using the Tukeys' Studentized Range test. Within treatment mean comparisons were made using the paired t-test procedure. A difference was accepted as significant when $p<0.05$. All data are presented as mean ± SEM.

## RESULTS

No differences were observed in the efficiency of the acid-base extraction procedure among plasma, urine and bile samples for all substrates (Table 10). Therefore, sample means for plasma, urine and bile samples were pooled to obtain an overall efficiency mean of 89.2%. An overall correction factor of 1.12 was applied in order for the data to be quantitative.

The recovery of administered dose, expressed as % of dose, in bile and urine is presented in Table 11, the cumulative excretion of ZEN and ZEN glucuronide are represented graphically in Figure 17. Urinary excretion of ZEN and phase I metabolites ranged from 0.009-0.142%. Intact rats excreted greater amounts of these compounds in urine compared to bile-duct cannulated rats for both routes of administration. Urinary excretion of glucuronides ranged from 0.065-2.020% of the dose. Intact rats excreted greater amounts of all glucuronides in urine compared to bile-duct cannulated rats for both routes of administration. Biliary excretion of ZEN and phase I metabolites ranged from 0.003-0.129%. Biliary excretion of ß-ZOL and α-ZOL glucuronide was 1.6 and 0.8% and 2.4 and 3.9% for iv and ig dosed rats, respectively. Biliary excretion of ZEN glucuronide was 86%

**TABLE 10.** Efficiency of acid-base extraction procedure.

| | % RECOVERY | | |
|---|---|---|---|
| | PLASMA | BILE | URINE |
| ß-Zol | 86.7 ± 2.1 | 87.0 ± 3.2 | 89.8 ± 3.7 |
| α-Zol | 89.0 ± 2.4 | 86.0 ± 3.4 | 91.0 ± 2.3 |
| ZEN | 90.8 ± 2.3 | 89.5 ± 1.9 | 91.8 ± 3.2 |
| ß-Zol glucuronide | 88.3 ± 4.3 | 89.0 ± 2.6 | 87.8 ± 2.9 |
| α-Zol glucuronide | 90.2 ± 2.2 | 88.0 ± 2.6 | 88.3 ± 2.0 |
| ZEN glucuronide | 91.5 ± 2.4 | 90.7 ± 2.0 | 89.7 ± 3.4 |

Plasma, urine and bile samples were spiked with 200 ng α-Zol, ß-Zol and ZEN and efficiency of acid-base extraction procedure was determined by HPLC as described in "Methods and Materials" section.
Mean ± SD, N=6;
No differences in recoveries were detected among plasma, bile and urine samples for all compounds.
Mean recoveries for all fluids were pooled (mean 89.2 ± 1.0) and an overall correction factor of 1.12 was therefore applied in order to make data quantitative.

**TABLE 11.** %DOSE RECOVERY IN BILE AND URINE

| | % DOSE RECOVERY | | | | | |
|---|---|---|---|---|---|---|
| | INTRAVENOUS | | | INTRAGASTRIC | | |
| | Intact | Bile-duct Cannulated | | Intact | Bile-duct Cannulated | |
| | Urine | Urine | Bile | Urine | Urine | Bile |
| ß-ZOL | 0.037±0.004 | 0.009±0.001 | 0.004±0.002 | 0.076±0.012 | 0.020±0.002 | 0.005±0.003 |
| α-ZOL | 0.045±0.005 | 0.009±0.001 | 0.013±0.006 | 0.056±0.003 | 0.026±0.004 | 0.003±0.002 |
| ZEN | 0.049±0.021 | 0.037±0.003 | 0.119±0.018 | 0.142±0.010 | 0.086±0.009 | 0.129±0.048 |
| ß-ZOL glucuronide | 0.151±0.015 | 0.065±0.005 | 1.583±0.255 | 0.152±0.012 | 0.080±0.008 | 0.799±0.056 |
| α-ZOL glucuronide | 0.184±0.032 | 0.088±0.003 | 2.405±0.342 | 0.177±0.012 | 0.087±0.011 | 3.918±0.765 |
| ZEN glucuronide | 2.020±0.110 | 0.807±0.080 | 85.765±3.494 | 1.481±0.129 | 1.072±0.045 | 53.456±1.954 |
| **TOTAL:** | **2.486** | **1.015** | **89.889** | **2.084** | **1.371** | **58.310** |
| | | **90.904** | | | **59.581** | |

Mean ± SEM; N=3 intact; N=6 bile-duct cannulated;

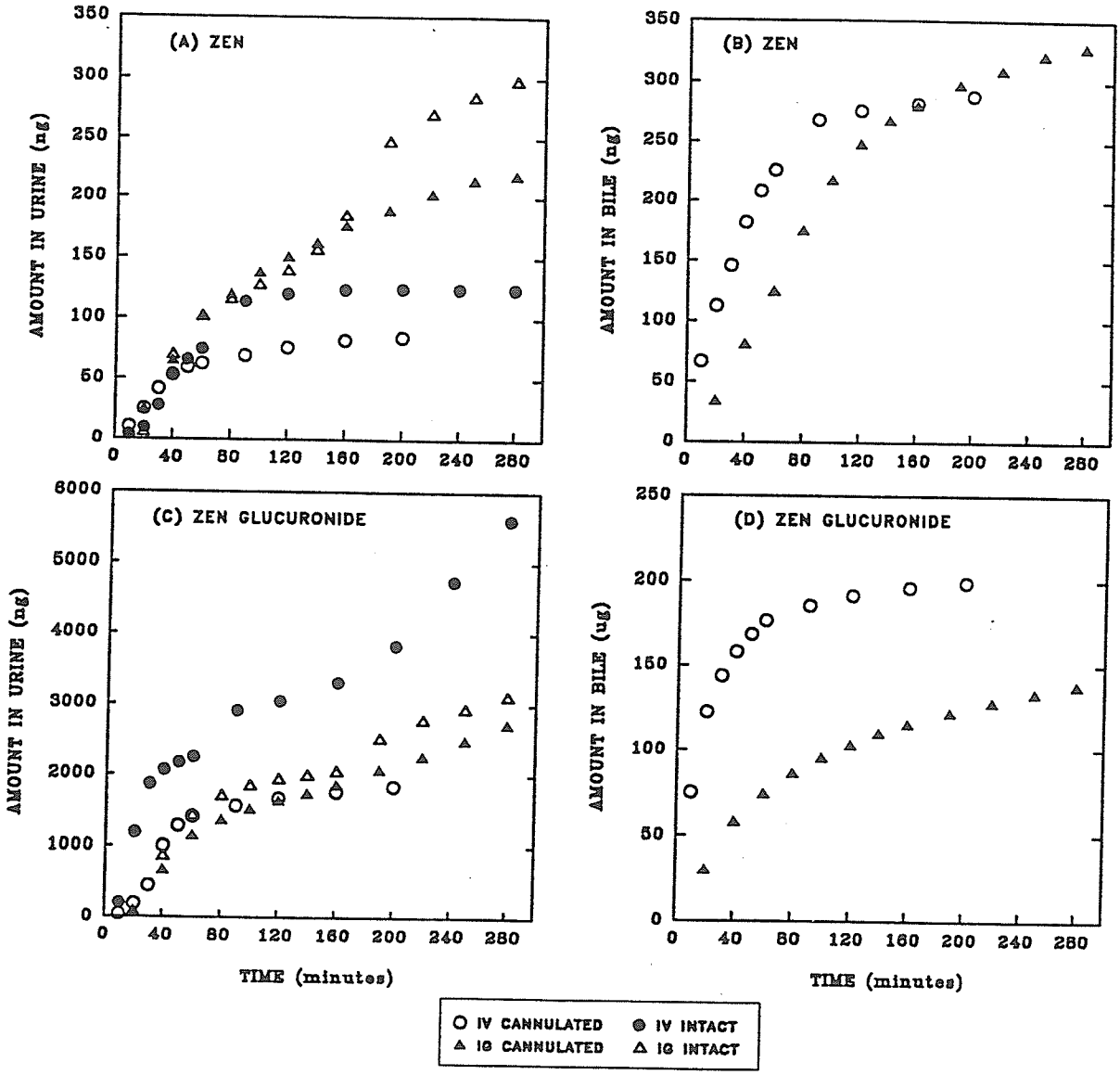



**FIGURE 17.** Mean cumulative excretion versus time plots of (A) zearalenone in urine, (B) zearalenone in bile, (C) zearalenone glucuronide in urine and (D) zearalenone glucuronide in bile

for iv and 53% for ig dosed rats. Total recovery in bile and urine was 91% in iv and 60% ig dosed rats.

Plasma concentration of ZEN and ZEN glucuronide versus time plots are shown in Figure 18. Peak plasma concentration, $C_p$, was greater in intact rats compared to bile-duct cannulated rats for both routes of administration (Fig. 19). The AUC was lower in ig compared to iv dosed rats (Fig. 20). Biliary cannulation resulted in a significant reduction in the plasma AUC in both routes of administration. The AUC in bile-duct cannulated ig dosed rats was only 10% of intact iv dosed rats compared to 45% in bile-duct cannulated iv dosed rats. The AUC in intact ig dosed rats was 20% of intact iv rats, which is twofold greater than bile-duct cannulated ig dosed rats. In bile-duct cannulated iv dosed rats the AUC was 47% of intact iv dosed rats, approximately 2.3 times greater than intact ig dosed rats. In bile-duct cannulated rats, $CL_{po}$ was higher in ig dosed rats than $CL_p$ iv dosed rats (Fig. 21). For both routes of administration, biliary cannulation resulted in higher plasma clearances. $V_d/f$, in ig dosed bile-duct cannulated and intact rats is greater than $V_d$ observed in iv dosed rats (Fig. 22). ß was lower in iv dosed intact rats compared to all other groups. Biliary cannulation had no effect on ß in ig dosed rats.

Biliary clearance data are shown in Figure 23. The route of administration had no effect on $CL_{bile}$ of phase I

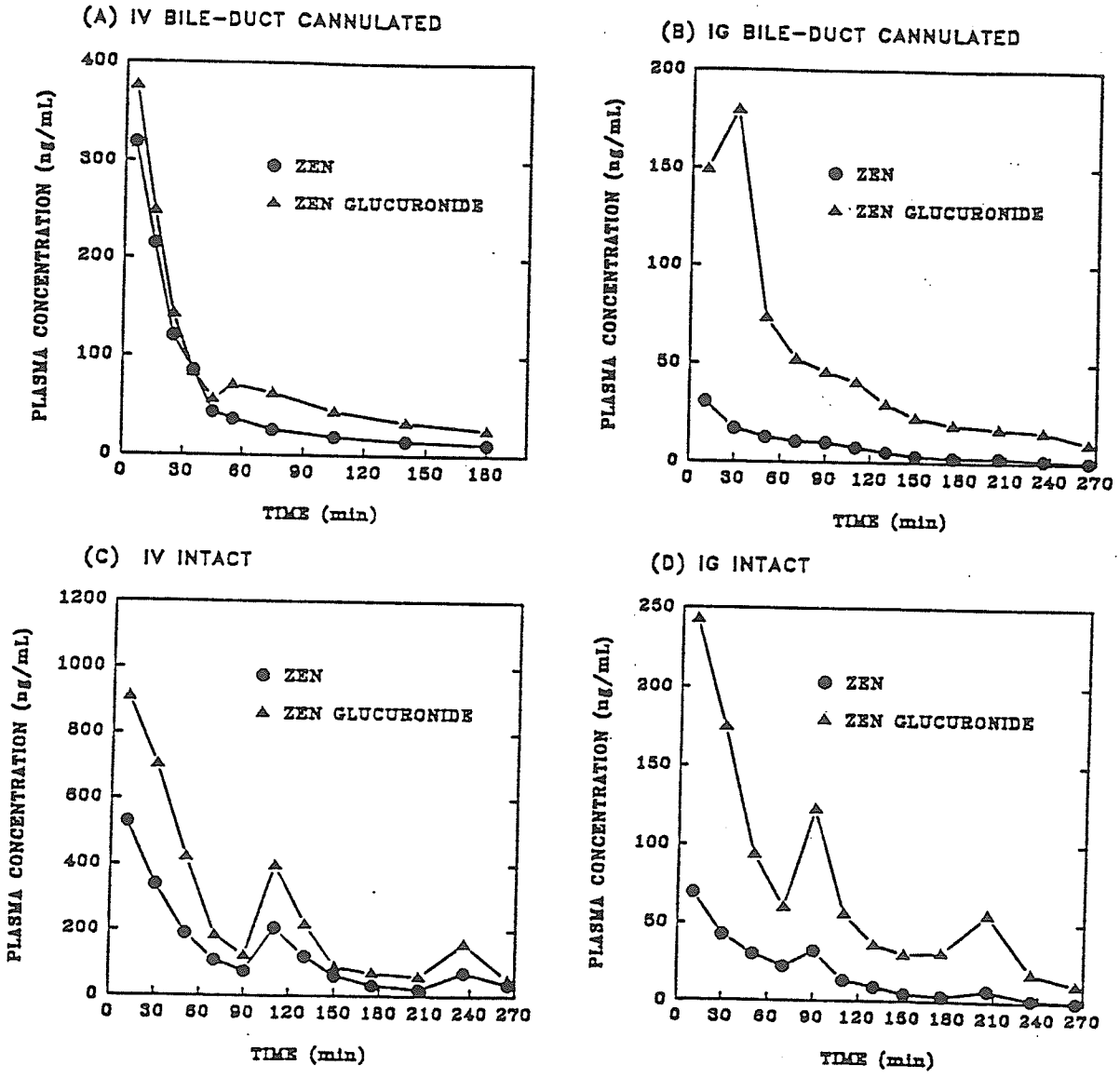



**FIGURE 18.** Mean plasma concentration of zearalenone and zearalenone glucuronide versus time curves for (A) iv bile-duct cannulated, (B) ig bile-duct cannulated, (C) iv intact and (D) ig intact rats

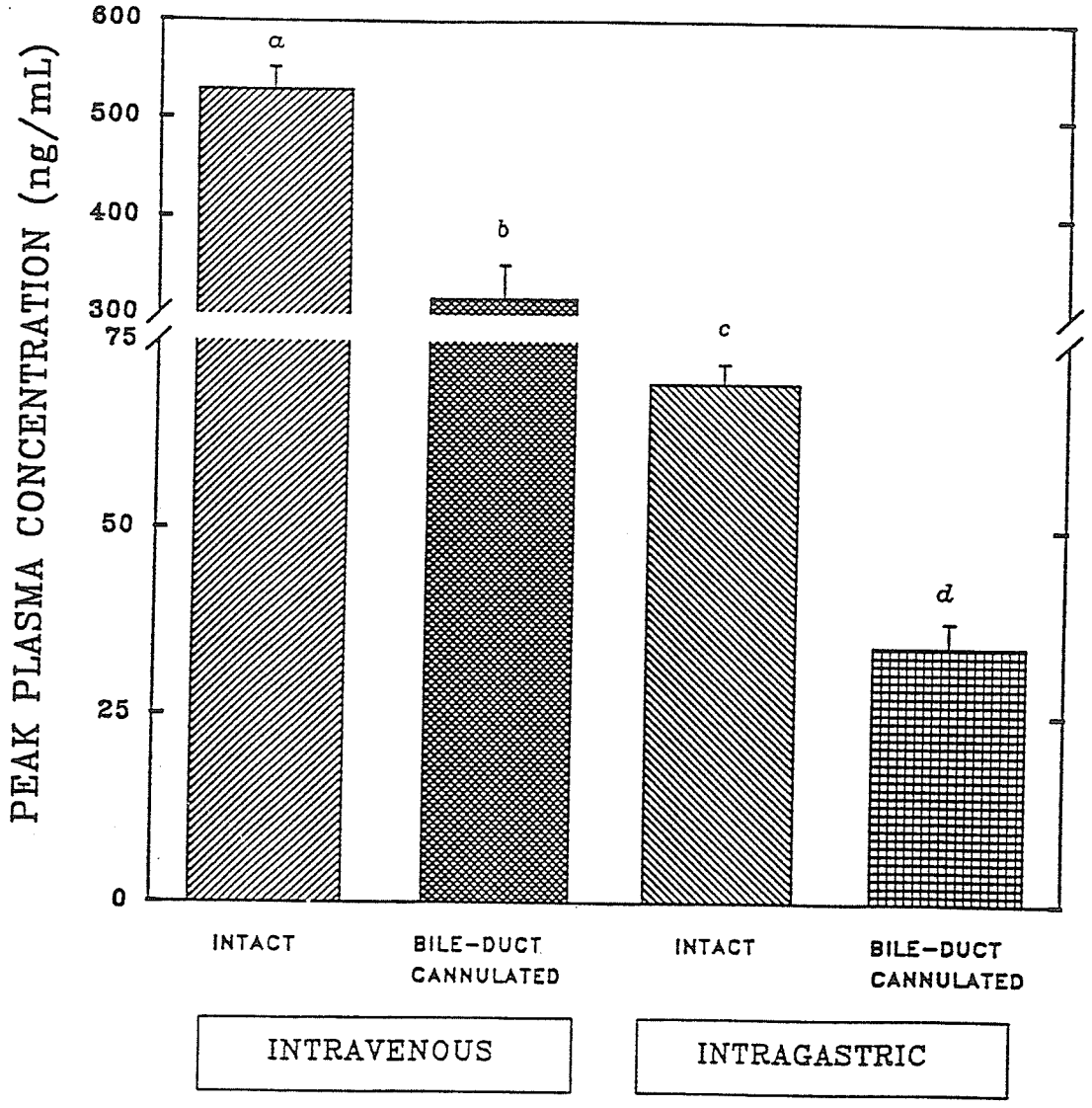



**FIGURE 19.** Peak plasma zearalenone concentration reached post-dosing in iv and ig dosed intant and bile-duct cannulated rats. Data presented as Means ± SEM. Means with different letters are significantly different, $p<0.05$

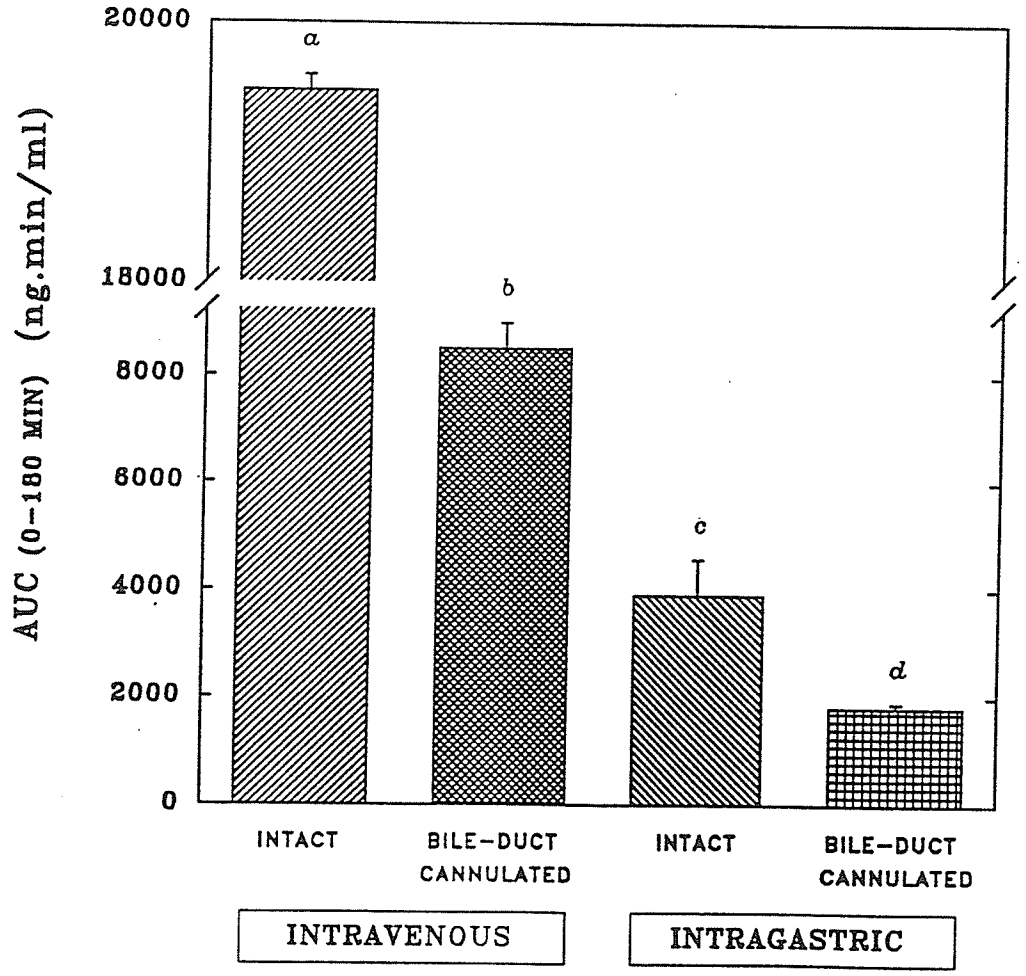



**FIGURE 20.** The area under the plasma concentration vs time curve to sampling time 180 min ($AUC_{0\text{-}180\,min}$) in iv and ig dosed intact and bile-duct cannulated rats. Data presented as Means $\pm$ SEM. Means designated with different letters are significantly different, $p<0.05$

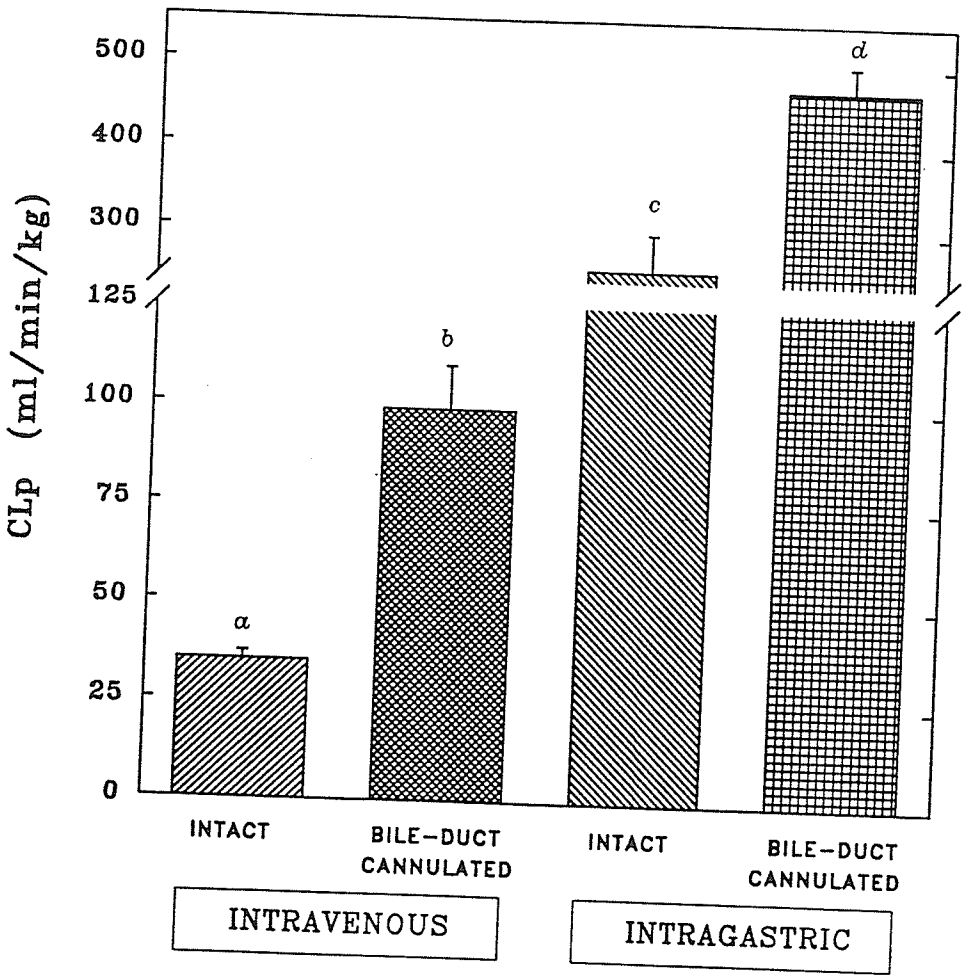



**FIGURE 21.** Plasma clearance in iv and ig dosed intact and bile-duct cannulated rats. $CL_p$ (iv) or $CL_{po}$ (ig) = $DOSE/AUC_{0\to\infty}$, where $AUC_{0\to\infty}$ is the area under the plasma concentration vs time curve to the last sampling time plus $C_t/\beta$, where $C_t$ is the last measured plasma concentration and $\beta$ is the terminal disposition constant. Data presented as Means ± SEM. Means designated with different letters are significantly different, $p<0.05$

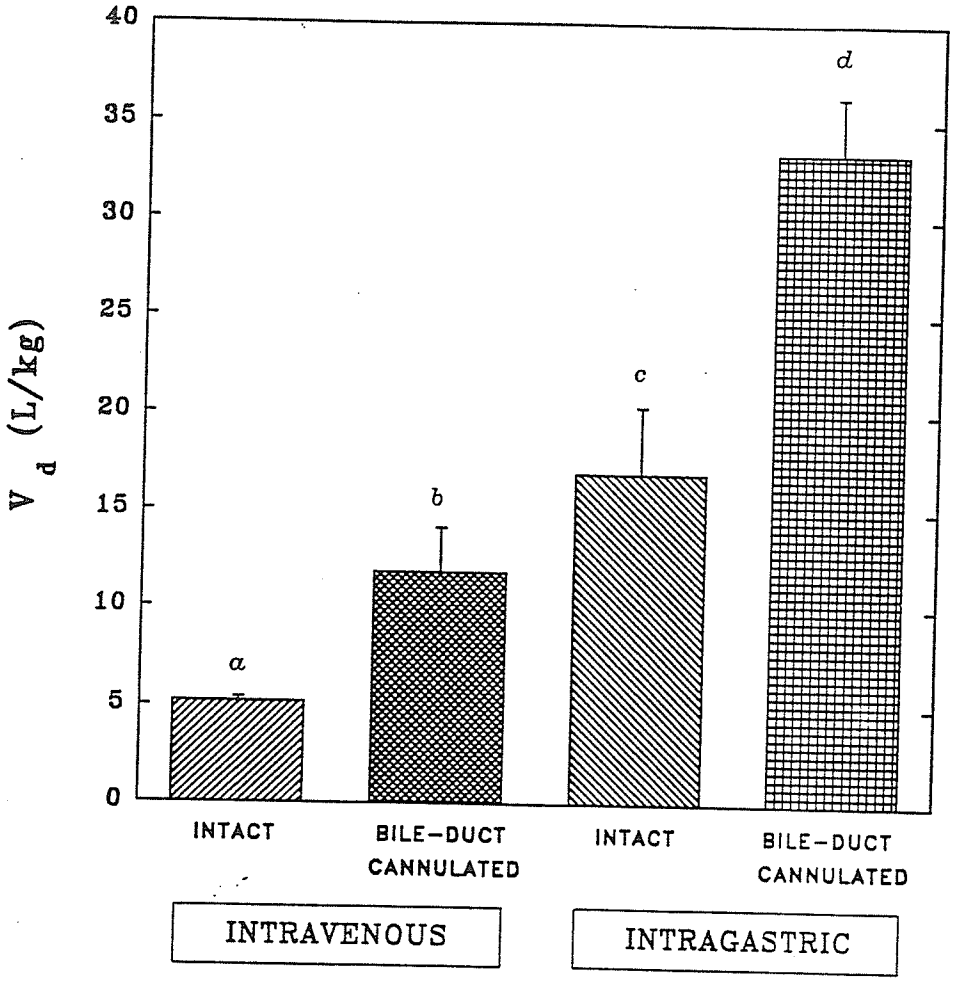



**FIGURE 22.** $V_d$, the apparent volume of distribution in iv and ig dosed intact and bile-duct cannulated rats. $V_d = CL_p / \beta$, $V_d$ after ig dose is actually $V_{d/f}$, where f = bioavailability). Data presented as Means ± SEM. Means designated with different letters are significantly different from each other, $p<0.05$

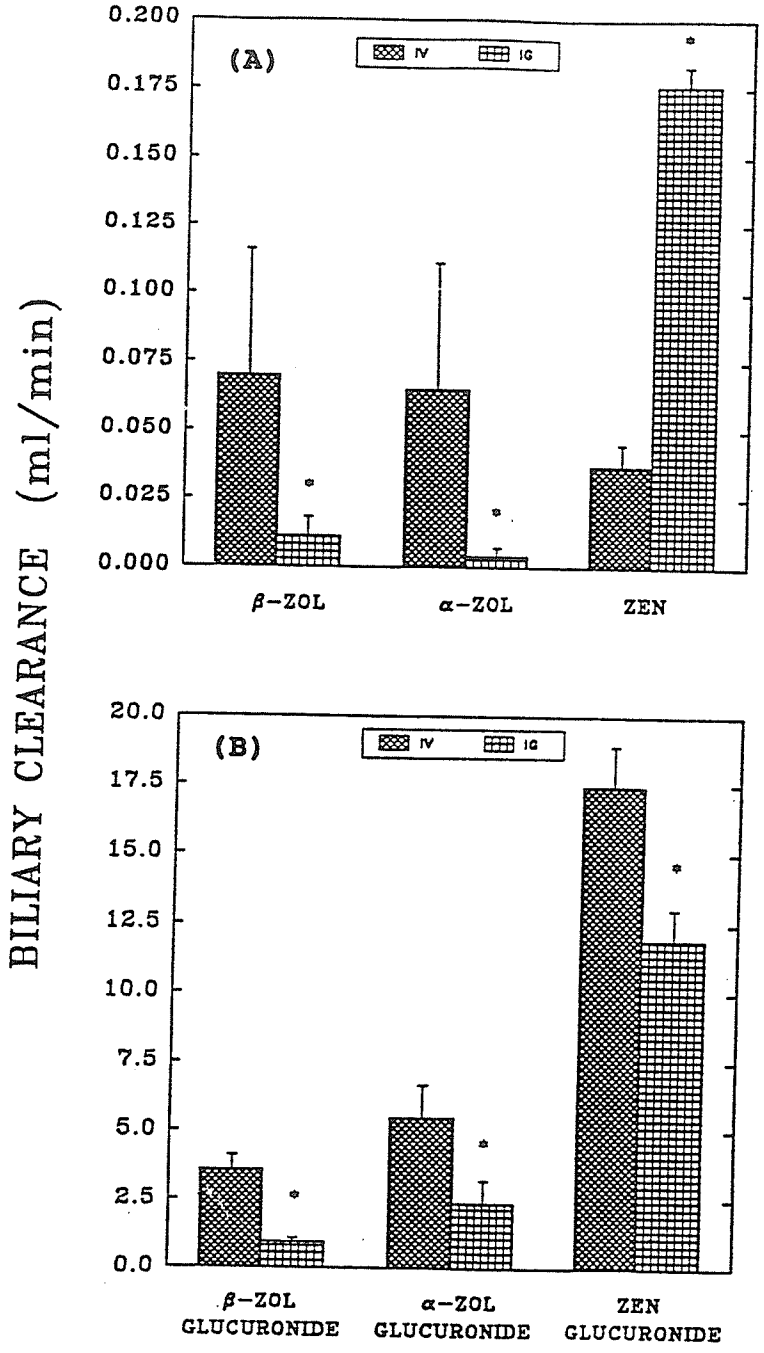



**FIGURE 23.** Biliary clearance in iv and ig dosed rats for (A) ZEN and phase I metabolites and (B) Phase II metabolites. Biliary clearance, $CL_B = X_{(0\rightarrow t)}/AUC_{(0\rightarrow t)}$, where X is the amount of the parent or metabolite excreted in bile and $AUC_{(0\rightarrow t)}$, the respective AUC in plasma for the same substrate and time. Data presented as Means ± SEM. For each compound, an '*' indicates means are significantly different, $p<0.05$.

metabolites, α- and ß-ZOL, but for ZEN, ig dosed rats had a greater $CL_{bile}$ than iv dosed rats (Fig. 23A). For phase II metabolites, $CL_{bile}$ for ß-ZOL and ZEN glucuronides was greater in iv compared to ig dosed rats while no difference was observed for α-ZOL glucuronide (Fig. 23B). For all three compounds, the phase II metabolites had a greater $CL_{bile}$ than their parent compounds with ZEN glucuronide cleared at a higher rate than either of the phase II metabolites. No differences in $CL_{M(bile)}$ were observed between ig and iv dosed rats for phase I metabolites, α- and ß-ZOL (Fig. 24A). However, for phase II metabolites, $CL_{M(bile)}$ was higher in ig compared to iv dosed rats for all three conjugates (Fig. 24B). Again, the glucuronide of the parent mycotoxin showed the greatest $CL_{M(bile)}$, and ZEN glucuronide was 5 fold higher in ig than iv dosed rats.

For phase I metabolites, $CL_{bile}$ (Fig. 23A) exceeded $CL_R$ (Fig. 25A) of ZEN in iv rats but not in ig rats. However, the route of administration had no effect on the difference between $CL_R$ and $CL_{bile}$ of phase I metabolites. For phase II metabolites, $CL_{bile}$ (Fig. 23B) exceeded $CL_R$ (Fig. 25B) for all three conjugates by both routes of administration. In iv dosed rats, $CL_{bile}$ exceeded $CL_R$ by 18, 28 and 110 times for α-ZOL, ß-ZOL and ZEN respectively. In ig dosed rats, $CL_{bile}$ exceeded $CL_R$ by 10, 48 and 50 times for α-ZOL, ß-ZOL and ZEN respectively.

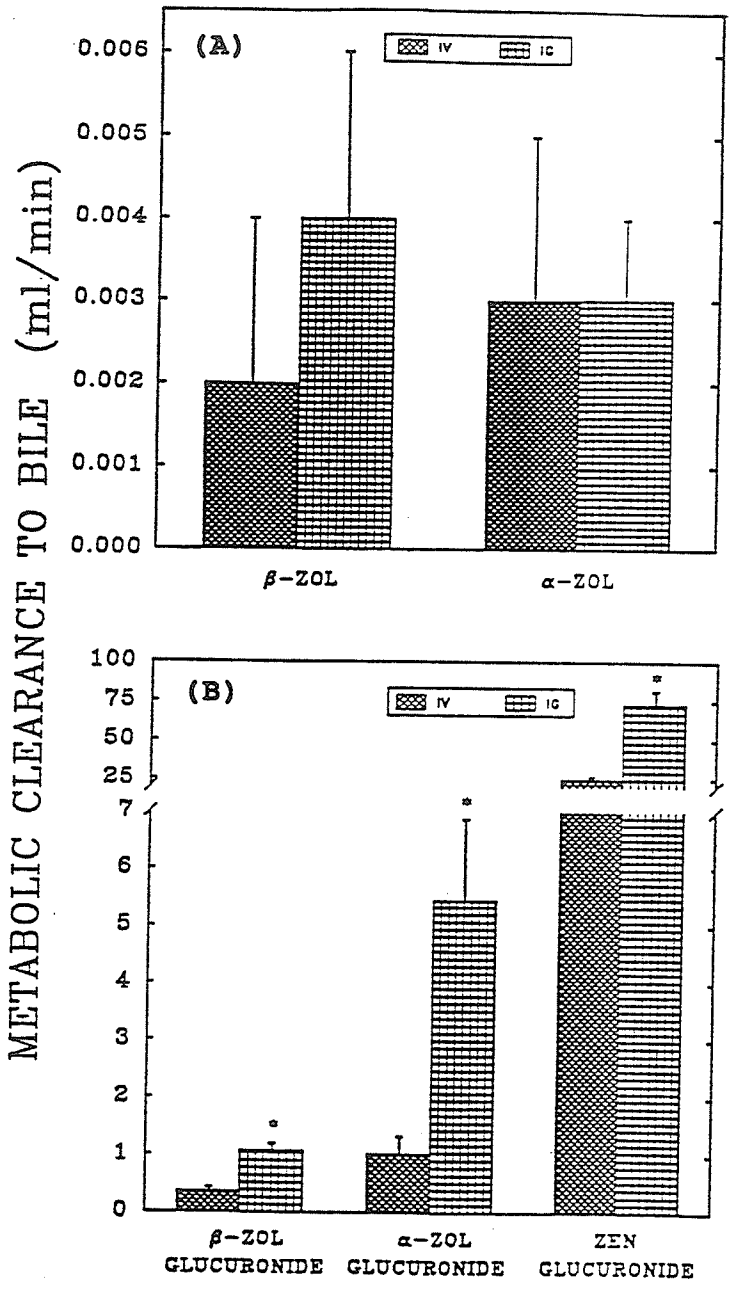



**FIGURE 24.** Metabolic clearance to bile in iv and ig dosed rats for (A) Phase I metabolites and (B) Phase II metabolites. $CL_{Meta} = X_{(0\rightarrow t)}/AUC_{ZEN(0\rightarrow t)}$, where X is the amount of the metabolite excreted in bile and $AUC_{ZEN(0\rightarrow t)}$, the respective AUC of the parent compound in plasma for the same time interval. The calculations from metabolic clearance assumes that the rate limiting step is metabolic production. Data presented as Means ± SEM. For each compound, an '*' indicates means are significantly different, $p<0.05$.

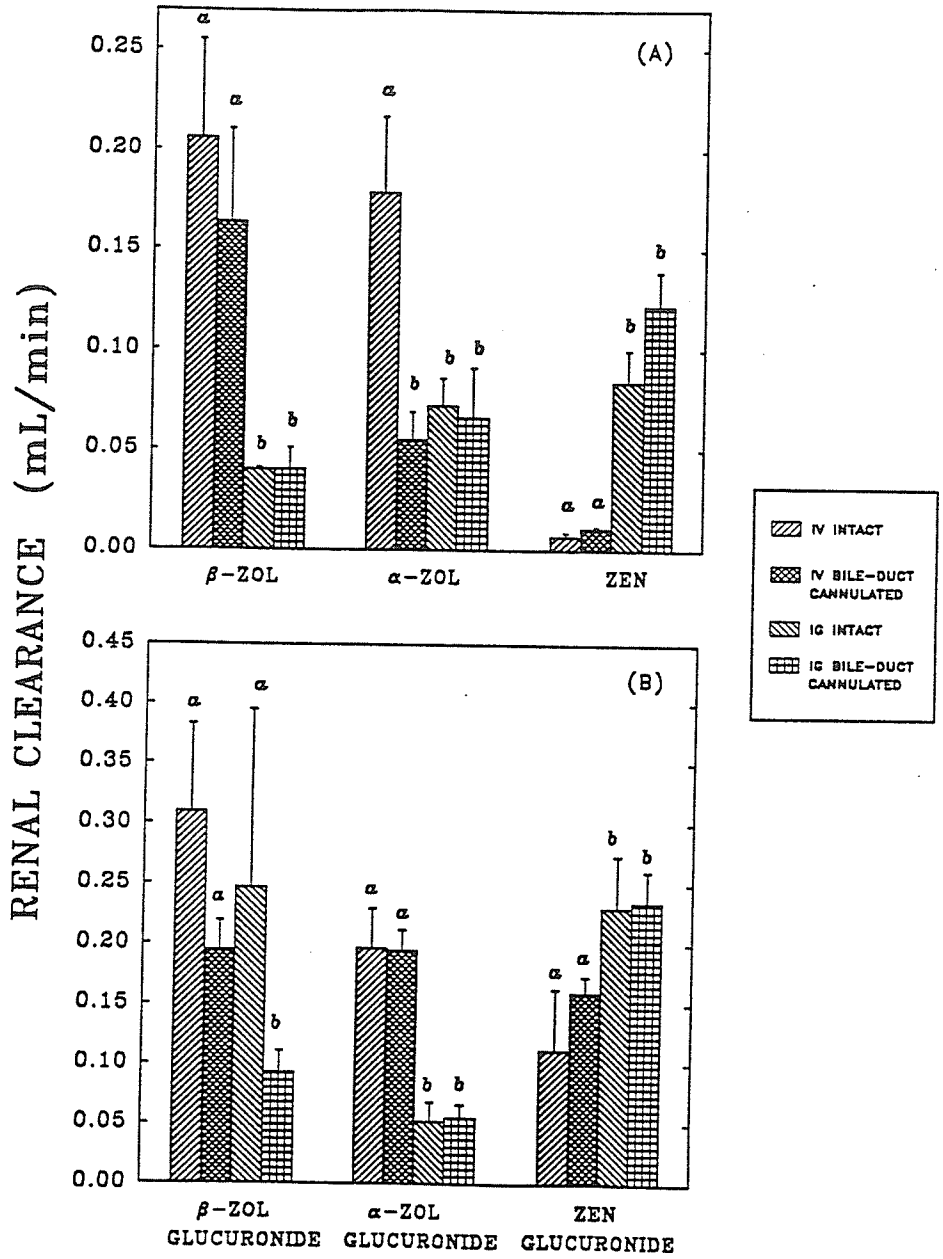



**FIGURE 25.** Renal clearance in iv and ig dosed intact and bile-duct cannulated rats for (A) ZEN and phase I metabolites and (B) Phase II metabolites. Renal clearance, $CL_R = X_{(0\rightarrow t)}/AUC_{(0\rightarrow t)}$, where X is the amount of the parent or metabolite excreted in urine and $AUC_{(0\rightarrow t)}$, the respective AUC in plasma for the same substrate and time. Data presented as Means ± SEM. For each compound, means designated with different letters are significantly different, $p<0.05$.

No differences were detected between $CL_{bile}$ (Fig. 23) and $CL_{M(bile)}$ (Fig. 24) rates for phase I metabolites for either administration route with the exception of α-ZOL in iv dosed rats. In that case the $CL_{bile}$ exceeded $CL_{M(bile)}$. For phase II metabolites in iv dosed rats, $CL_{bile}$ (Fig. 23B) exceeded $CL_{M(bile)}$ (Fig. 24B) for α- and ß-ZOL conjugates. Ig dosed rats exhibited a greater metabolic clearance to α-ZOL conjugate than $CL_{bile}$, while no difference was observed between $CL_{bile}$ and $CL_{M(bile)}$ for ß-ZOL glucuronide. For both routes of administration, $CL_{M(bile)}$ of ZEN glucuronide exceeded $CL_{bile}$. $CL_{M(bile)}$ exceeded $CL_{bile}$ in ig dosed rats by 6-fold compared to 1.5-fold in iv dosed rats.

Renal clearance data are presented in Figure 25. $CL_R$ of ZEN was greater in ig compared to iv dosed rats in both intact and bile-duct cannulated animals. $CL_R$ of phase I metabolites was greater in iv dosed rats compared to ig dosed rats with the exception of α-ZOL clearance in iv bile-duct cannulated rats which did not differ from ig dosed rats (Fig. 25A). α-ZOL had a higher $CL_R$ in iv intact rats compared to the other groups. Biliary cannulation had no effect on $CL_R$ of ZEN, ß-ZOL and phase II metabolites. In ig bile-duct cannulated rats α-ZOL $CL_R$ exceeded that for ß-ZOL. In iv dosed rats, regardless of biliary cannulation, $CL_R$ of ZEN was lower than the phase I metabolites. In ig bile-duct cannulated rats $CL_R$ of ZEN was greater than that of ß-ZOL while no other

differences were observed in ig rats.

$CL_R$ of ß-ZOL glucuronide was less in ig dosed bile-duct cannulated rats than in any other group. In iv dosed rats $CL_R$ of α-ZOL glucuronide was greater than ig dosed rats. Biliary cannulation had no effect on $CL_R$ of α-ZOL glucuronide. Ig dosed rats cleared higher amounts of ZEN glucuronide than iv dosed rats. In both ig and iv dosed intact and bile-duct cannulated rats, $CL_R$ of ZEN glucuronide was higher than that of the free parent. No differences were observed between phase I metabolites and their conjugates in intact iv dosed rats.

The metabolic clearance to urine data are presented in Figure 26. $CL_R$ (Fig. 25A) exceeded $CL_{M(urine)}$ (Fig. 26A) for ß-ZOL in both iv and ig dosed intact and bile-duct cannulated rats. The difference was greater in iv dosed rats, 70 fold in intact and 55-fold in bile-duct cannulated, and much less in ig dosed rats, 2-fold in both intact and bile-duct cannulated rats. $CL_R$ exceeded $CL_{M(urine)}$ for α-ZOL in both iv and ig dosed rats with the exception of ig bile-duct cannulated rats where no difference was observed. The difference was greater in iv dosed rats, 26 fold in intact and 18-fold in bile-duct cannulated, than in ig dosed intact rats, a 5-fold difference.

$CL_R$ (Fig. 25B) exceeded $CL_{M(urine)}$ (Fig. 26B) for both phase II metabolites in iv dosed rats whereas no difference was observed in ig dosed rats for ß-ZOL glucuronide. $CL_{M(urine)}$

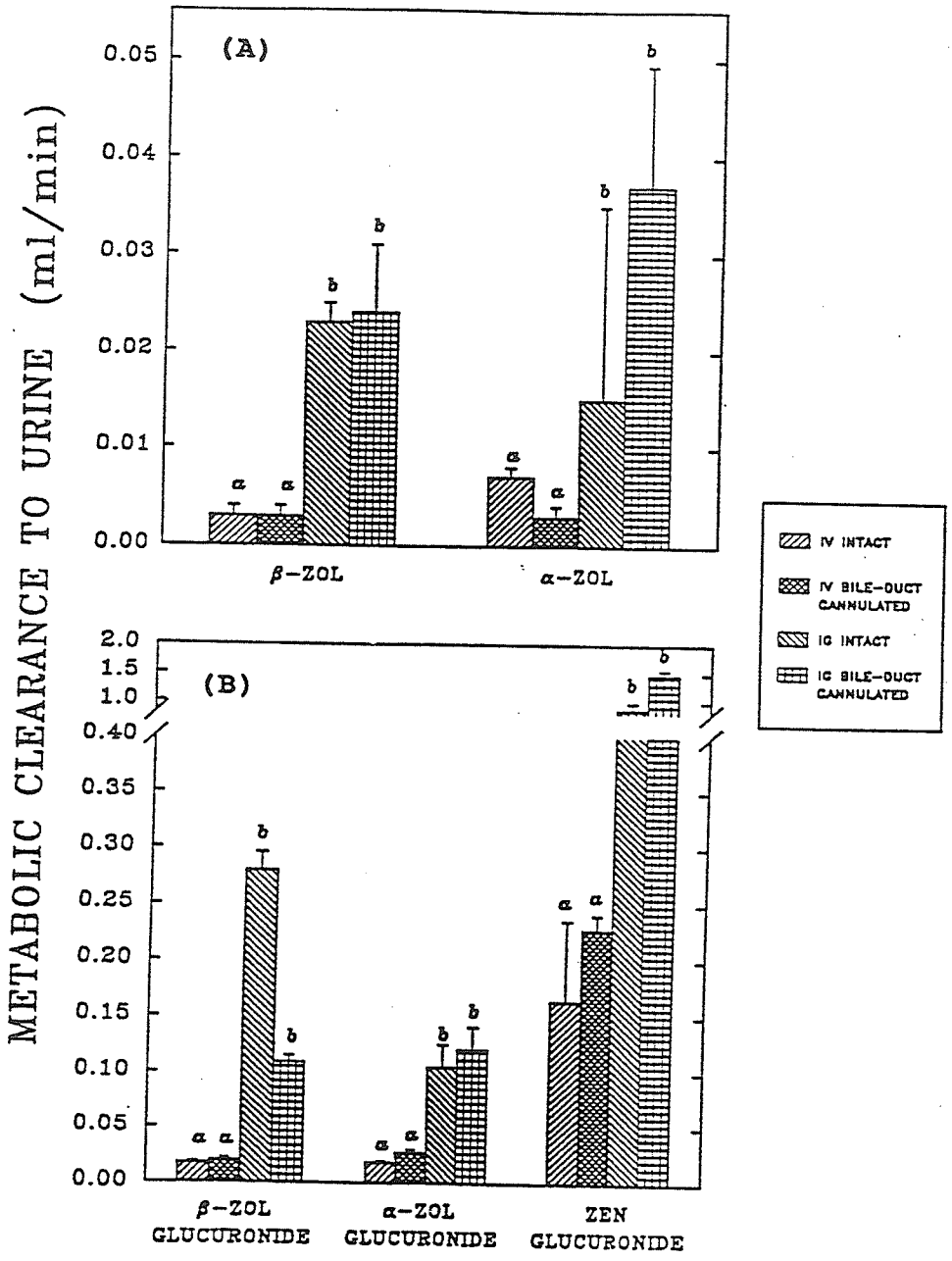



**FIGURE 26.** Metabolic clearance to urine in iv and ig dosed rats for (A) Phase I metabolites and (B) Phase II metabolites. $CL_{M_{(0\rightarrow t)}} = X_{(0\rightarrow t)}/AUC_{ZEN(0\rightarrow t)}$, where X is the amount of the metabolite excreted in urine and $AUC_{ZEN(0\rightarrow t)}$, the respective AUC of the parent compound in plasma for the same time interval. The calculations from metabolic clearance assumes that the rate limiting step is metabolic production. Data presented as Means ± SEM. For each compound, means designated with different letters are significantly different, $p<0.05$.

exceeded $CL_R$ for α-ZOL glucuronide by 2-fold in both intact and bile-duct cannulated ig dosed rats. For ZEN glucuronide, there was no difference between $CL_{M(urine)}$ and $CL_R$ in iv intact rats while in iv dosed bile-duct cannulated and ig dosed intact and bile-duct cannulated rats, $CL_{M(urine)}$ exceeded $CL_R$, by a factor of 1.5, in iv bile-duct cannulated rats and by factors of 4 and 6 in ig dosed intact and bile-duct cannulated rats, respectively.

No differences were detected between $CL_{M(urine)}$ (Fig. 26A) and $CL_{M(bile)}$ (Fig. 24A) for phase I metabolites for either route of administration. However, for phase II metabolites, $CL_{M(bile)}$ (Fig. 24B) was greater than $CL_{M(urine)}$ (Fig. 26B) for all three glucuronides in both iv and ig dosed rats. For ß-ZOL glucuronide $CL_{M(bile)}$ exceeded $CL_{M(urine)}$ by 18 and 10 fold in iv and ig dosed rats respectively. For α-ZOL $CL_{M(bile)}$ exceeded $CL_{M(urine)}$ by 38 and 45 fold in iv and ig dosed rats respectively. For ZEN glucuronide, $CL_{M(bile)}$ exceeded $CL_{M(urine)}$ by 100 fold in iv and 50-fold in ig dosed rats.

## DISCUSSION

ZEN and its metabolites α- and ß-ZOL are excreted by way of the bile as glucuronide conjugates, with only a minor contribution from the urinary route. These observation are consistent with excretion studies performed in our laboratory (Chapter 3), in which we observed that after 96 h rats excreted 2.5% of the oral dose as a glucuronide and 15% as free ZEN in urine, while the feces was the predominant route of excretion with over 52% of the dose recovered as ZEN or α-ZOL. Smith (1980b), using tritiated ZEN, reported greater urinary excretion for ZEN, with 31% and 62% of the radioactivity recovered in the urine and feces, respectively. The discrepancy between studies may be due to a dose effect. That is, Smith's ZEN dose was 100 mg ZEN $kg^{-1}$ BW, 100 times greater than that used in the present study or our previous work (Chapter 3). This higher dosage may have resulted in the saturation of biliary metabolic pathway leading to a greater urinary excretion.

It is probable that ZEN, after absorption by the gut, is conjugated by liver, eliminated via the bile into the gut, and may be subject to hydrolysis by the brush-border ß-glucuronidase enzyme or the hydrolytic gut microflora, and excreted in the feces as free ZEN (Farnworth and Trenholm,

1981). The free parent or its metabolites, however can be reabsorbed back into the circulation, transported to the liver where it can be conjugated and eliminated into bile, thus undergoing an enterohepatic circulation. It would therefore be logical to suggest because of the relatively low recovery administered ZEN in urine and the high fraction in bile, that the fate of a large proportion of biliary conjugates would be excretion via the feces after hydrolysis of glucuronides by gut microflora and or brush-border ß-glucuronidase activity. However, since we recovered only 58% of the dose in bile in ig dosed rats compared to 90% in iv dosed rats, it is possible that a fraction of the ig dose may be unabsorbed excreted unchanged in the feces. However we have previously demonstrated that the gut is capable of glucuronidating ZEN, which may after hydrolytic activity in the lower gut, be excreted as fecal ZEN without being reabsorbed, thus reducing the amount of the dose that reaches the circulation. This can be easily demonstrated by analysing the gut contents for any residual ZEN. Another explanation for the lower recovery in ig dosed rats may due to the anaesthetic which depresses GI motility, thus reducing absorption.

The AUC was substantially reduced after biliary cannulation and was less in ig compared to iv dosed rats supporting the hypothesis that biliary excretion impacts on the blood profile of ZEN as a function of route of administration. The lower AUC after an oral dose is probably

due to first-pass presystemic elimination and/or lower absorption by the gut, although absorption appears to be rapid, since peak plasma concentrations were reached by the first sampling time after the dose. The absorption however, may be incomplete, as dicussed earlier. The lower bioavailability in an oral route suggests that compared to an iv dose, the oral route is protective due to presystemic detoxification and elimination by biliary and gastrointestinal metabolic pathways. The fact that plasma AUC was greater in intact compared to bile-duct cannulated rats and that intact rats had higher peak plasma ZEN concentrations in both iv and ig routes further supports the hypothesis that the mycotoxin undergoes extensive biliary excretion and subsequent EHC. Extensive presystemic elimination by the gut and liver is supported by fact that the systemic clearance, $CL_{po}$ in ig dosed rats is greater than iv dosed rats.

We have previously shown in our laboratory, that the gut as well as the kidney is capable of glucuronidation of ZEN in *in vitro* studies with intestinal and renal microsomes (Chapter 7). This observation is supported by the $V_d$ in the current study. $V_d/f$, in ig rats in both intact and bile-duct cannulated rats, is higher than the $V_d$ observed in iv rats. This finding is suggestive of extrahepatic tissue uptake or presystemic metabolism of the mycotoxin. Further evidence to support the hypothesis that ZEN undergoes extensive presystemic elimination by the gut in the current study is

provided by metabolic clearance to bile data. In contrast to $CL_{bile}$, where iv rats cleared greater amounts of phase II metabolites in bile than ig dosed rats, the metabolic clearance to bile data was the reverse, with ig rats having greater metabolic clearance to bile rates than iv dosed rats for all three glucuronides. Therefore, our data provides further evidence to support the notion that there is extensive presystemic elimination, specifically due to the contribution of the upper GIT to overall conjugation of ZEN and its phase I metabolites. Since the mycotoxin is already being conjugated in the gut, before it reaches the liver where the majority of glucuronidation occurs, it may represent an important step in the detoxification of ZEN.

Since $CL_R$ for α- and ß-ZOL exceeds $CL_{M(urine)}$ in ig and iv dosed rats, the amount in urine reflects production regardless of biliary cannulation. However, for ß-ZOL glucuronide in ig dosed intact and bile-duct cannulated rats, $CL_R$ approximates $CL_{M(urine)}$. Therefore, ß-ZOL glucuronide in urine underestimates metabolic clearance suggesting that gastrointestinal or extrahepatic glucuronidation is occurring. $CL_{M(urine)}$ for α-ZOL glucuronide exceeds $CL_R$ in ig dosed intact and bile duct cannulated rats, again suggesting extrahepatic conjugation. $CL_{M(urine)}$ for ZEN glucuronide, in both intact and bile-duct cannulated rats, was substantially higher after ig dose, again providing evidence to support extensive presystemic elimination, or extrahepatic metabolism possibly via gut-wall

glucuronidation. ZEN glucuronide appears to be the preferred metabolite formed by the liver, in both iv and ig dosed rats, as evidenced by the $CL_{bile}$ data where ZEN glucuronide is cleared at least 3-6 times greater than the $\alpha$- and ß-ZOL conjugates. $CL_{bile}$ exceeded $CL_R$ of all phase II metabolites indicating that the renal excretion pathway is of limited quantitative significance.

Additionally, in both iv and ig dosed rats the apparent metabolic clearance of ZEN to its glucuronide exceeds the reported liver blood flow (Greenway and Stark, 1971). Therefore, the total conjugate in bile cannot derive solely from delivery of parent conjugate but reflects clearance of glucuronide formed at other sites. However, when the substrate for biliary excretion also included circulating ZEN conjugate, i.e. if we assume that biliary excretion of conjugate reflects both metabolic formation and excretion of metabolite, then $CL_{bile}$ ($CL_{parent \rightarrow conj}$) = Amount parent conjugate/$AUC_{ZEN}$ + $AUC_{conj\text{-}ZEN}$) approximates estimated blood flow, 10.16 and 10.21 ml $min^{-1}$ in iv and ig dosed rats respectively). Mean liver blood flow estimated in rats (1 ml $g^{-1}$ $min^{-1}$, Greenway and Stark, 1971, and liver/body weight ratio estimated from Sitar and Gordon, 1980) as 10.3 and 9.6 ml $min^{-1}$.

The observation that $CL_{M(bile)}$ exceeds $CL_{M(urine)}$ suggests that a large majority of ZEN metabolism occurs primarily in the liver with a contribution from the upper GIT, since venous

return from GIT would empty into the liver before the systemic circulation.

In conclusion, the predominant route for systemic elimination in the rat was biotransformation prior to excretion. These data indicate that the biliary first-pass clearance for the ig dose contributed substantially to the overall biliary clearance of ZEN. Systemic elimination of ZEN and it metabolites occurs through both biliary and urinary routes, but biliary excretion is the major and dominant route. The major excretory product of ZEN is ZEN glucuronide. ZEN undergoes substantial enterohepatic recirculation which may increase tissue estrogen receptors exposure to ZEN and contribute to ZEN toxicity (Kiritsy *et al.*, 1987). The rationale for interruption of biliary recycling by bile sequestering agents, such as ion-exchange resins, is that it ameliorates ZEN toxicity by the removal of biliary conjugates and subsequent protection from hydrolysis by gut microflora or brush-border ß-glucuronidase activity (Smith, 1982a).

# CHAPTER 9

## SUMMARY

An analytical method for determining ZEN and its metabolites in urine and feces was described. Additionally, the effect of ZEN dose level on the metabolites formed and the route of excretion was examined. ZEN dose had little effect on its metabolism, neither the route of excretion nor the metabolites formed were affected. The quantity of ZEN and metabolites excreted in feces in 96 h was about 2-4 times greater than that excreted in urine. In both the low and high dose animals, free fecal ZEN was the major metabolite. Urinary excretion was significant, accounting for 20 and 15% of the oral dose in low and high dose animals.

The production of α-ZOL is metabolically significant, in that α-ZOL is the most potent ZEN metabolite. Significant quantities of ZEN and metabolites, 15-20% of the oral dose, were excreted in the urine. In animals dosed 100 mg ZEN $kg^{-1}$ BW, 79% of the dose was recovered, 15% in the urine and 64% in the feces after 96 h. Previous reports on the routes of ZEN excretion and metabolites formed were not quantitative, therefore, this study was a significant contribution to knowledge.

The effect of dietary protein on ZEN metabolism, specifically, the effect of dietary protein on the activities of the ZEN metabolizing enzymes, ZEN metabolism and toxicity in the rat is reported. Dietary protein affected feed intake, weight gain, feed efficiency and the activity of GT. The diet containing 15% dietary protein supported the best weight gain, while no difference in weight gain between the 45 and 7.5% protein diets was observed. Feed efficiency was significantly different for all levels of dietary protein, with high protein animals having the greatest efficiency and low protein animals having the least efficiency. The activity of GT was greater in the 7.5% dietary protein group than either the 15 or 45% protein group. There was a significant effect of dietary protein on the metabolism of ZEN, with both the route of excretion and the metabolites formed being affected. In the 7.5 and 15% protein groups, free fecal ZEN was the major excretory metabolite accounting for 31 and 26.5% of the administered dose. In the 45% protein group, free urinary ZEN (41%) was the major excretory metabolite. Animals on the 7.5 and 15% protein diets excreted more total $\alpha$-ZOL than did animals on the high protein diet due to greater fecal excretion of $\alpha$-ZOL.

Total feed intake was significantly less in the 45% protein group fed 400 ppm ZEN than both the 15 to 7.5% protein

groups. However, the inclusion of ZEN in the diet did not reduce feed intake in the 45% protein group, while feed intake declined in the 15 and 7.5% protein groups. The inclusion of ZEN in the diet did not reduce feed efficiency or affect final body weight in the 45% protein group. However, in the 15 and 7.5% dietary protein groups there was a significant decrease in feed efficiency and final body weight observed as the level of dietary toxin increased. These results suggest that animals receiving ZEN in a high protein diet are less sensitive to the toxic effects of ZEN than are animals on a normal or low protein diet.

Dietary ZEN did not affect uterine weight in the 45% protein group, whereas in the 7.5 and 15% protein animals, uterine enlargement was observed. However, only the 7.5% protein animals receiving the 400 ppm ZEN diet had significantly larger uteri than other experimental animals.

The dietary regimen had a significant effect on ZEN toxicity. These data support the concept that a high protein diet ameliorates the toxic effects of dietary ZEN. There was an effect of dietary protein on the metabolism of ZEN with both the route of excretion and the metabolites formed affected. Since $\alpha$-ZOL is the most potent ZEN metabolite diminished production would reduce the toxicity of the ZEN dose. Conjugation inactivates ZEN, while the increased

urinary excretion would act to limit enterohepatic circulation with a subsequent reduction in the toxic expression of ZEN.

The effects of age and gender as well as storage conditions on GT activity towards ZEN is reported. In the initial storage study with ZEN only, microsomal GT enzyme activity towards ZEN declines significantly after 7 days storage at both temperatures with GT enzyme activity generally higher in microsomes stored at -20 than at -80°C. In a second storage study, microsomal GT enzyme activity towards ZEN, $GT_1$ and $GT_2$ substrates declined significantly with storage. GT activity towards NA and ZEN was stable for 28 days, while GT-HBP activity was stable for 14 days, after which significant declines were observed. Generally, no differences in GT activity between microsome stored at the two temperatures was observed. However, where differences were observed, enzyme activity was higher in microsomes stored at -20 compared to -80°C. After 14 days of storage, GT activity towards HBP and ZEN was significantly greater at -20 compared to -80°C. Our data indicates that GT activity is not stable as previously suggested.

The developmental pattern of GT enzyme activity demonstrate that substrate-specific sex differences are evident in the various age-groups. The developmental pattern of GT activity towards ZEN and HBP show great similarity.

Hepatic $GT_1$ enzyme activity was greater than $GT_2$ activity and ZEN in all age groups in both sexes. Immature female rats (35 day-old) had greater $GT_1$ and $GT_2$ enzyme activity than comparable males. Similarly for ZEN, young female rats had greater glucuronidation rates than male rats. GT enzyme activity diminished with age, with a 75% reduction in enzyme activity observed in 112-day old females for both $GT_1$ and $GT_2$ activity, and a 30% reduction in enzyme activity observed in 112 day-old males compared to 35 day-old rats. Old male rats had greater conjugation capacity, $GT_1$ and $GT_2$ activity, than their female counterparts. Similarly, glucuronidation rates of ZEN, was higher in old male rats compared to females. The rate of decline in GT activity with age is less in males than females, for all three substrate. The post-natal developmental pattern of GT activity is substrate, gender and age specific. Immature female rats exhibit a greater capacity for hepatic conjugation compared males. However, this trend is reversed in adult rats where males exhibit greater GT activity. The age-dependent decline is gender specific, with glucuronidation in female rats decreasing at greater rates than males, that is the decline males is more sustained and not as pronounced as in females. The development pattern is substrate dependent, that is, enzyme activity towards $GT_1$ substrate is greater than $GT_2$ substrate in all age groups.

To date, ZEN has not been classified in any of the GT isoenzyme clusters. Dietary ZEN significantly enhanced GT activity towards itself as well as $GT_1$ and $GT_2$ substrate. $GT_1$ activity, determined from NA conjugation, was significantly greater than $GT_2$ activity, determined from HBP conjugation and ZEN, in both control and treated animals. The data show that ZEN and HBP have similar glucuronidation rates and both are significantly different from NA. This finding supports the observation of the age-gender study, where we suggested that HBP and ZEN may belong to the same GT cluster and therefore are substrates for the same GT isoform.

Additional evidence to support the classification of ZEN as a $GT_2$ substrate is provided by the kinetic data, with ZEN and HBP exhibiting similar Michaelis-Menten behaviour. Kinetic data supports the concept of separate isoenzymes for $GT_1$ and $GT_2$ substrate, based on $K_m$ and $V_{MAX}$ values. The kinetic data, while not providing conclusive evidence to implicate separate isoenzymes for NA and ZEN, suggests that they are different. In addition, age-gender and induction studies suggest that ZEN behaves in a similar manner to HBP.

The data from the developmental, kinetic and induction study suggests that ZEN can be classified as a $GT_2$ substrate because of its similar behaviour to HBP, a substrate belonging to the $GT_2$ cluster.

The subcellular distribution of hepatic HSD and ZOL syntheses activities was compared. Reduction activity in the homogenate, microsomal and cytosolic preparations depends on the available substrate. The rate of ZOL production, measured directly with ZEN as substrate, was significantly less than HSD activity, using androsterone as substrate, in all liver preparations. The effect of PB pretreatment on hepatic microsomal HSD and NADPH-cytochrome P-450 reductase enzyme activities and effect of PB pretreatment on the 48-hour excretion of total urinary ZEN and ZOL was determined. In the enzyme induction study with PB, a potent inducer of xenobiotic metabolizing enzymes, hepatic microsomal cyt $P_{450}$ enzyme activity was significantly enhanced, while HSD enzyme activity was significantly reduced in PB treated rats. Excretion of total urinary ZEN and ZOL was significantly increased after PB pretreatment. These data indicate that HSD enzyme activity is not an accurate measure of ZEN reduction *in vivo* and that another enzyme or enzyme system, such as cytochrome $P_{450}$, a mixed-function oxidase, may be responsible for ZOL synthesis.

Most *in vitro* studies on ZEN focussed on hepatic metabolism, and may have contributed to the poor correlation between the *in vitro* enzyme profile and the *in vivo* metabolites formed. We suggested that this lack of correlation may be due to, in part, the fact that extrahepatic

reduction and conjugation sites for ZEN metabolism may exist and may contribute substantially to overall ZEN metabolism. The effect of dietary ZEN on hepatic and extrahepatic ZEN metabolizing activity was examined.

Dietary ZEN, significantly reduced feed efficiency compared to control animals. Total feed intake was less in the 250 ppm ZEN treated group, resulting in decreased weight gain, reduced intake and reduced feed efficiency. Microsomal GT activity was significantly greater in the 250 ppm ZEN treated rats compared to controls. Hepatic HSD activity, measured by androsterone reduction reaction, was unaffected by dietary ZEN. When hepatic ZEN reducing capacity was measured directly, using ZEN as substrate, a significant elevation of both microsomal and cytosolic ZOL synthetic activities was detected. Hepatic microsomal GT activity was significantly higher in ZEN treated rats compared to controls In rats fed 250 ppm ZEN, GT activity was significantly higher in hepatic, intestinal and renal microsomes than control rats. ZOL synthetic capacity was significantly enhanced in both intestinal microsome and cytosol preparations from rats fed 250 ppm ZEN compared to control rats. This data indicates that while the primary organ responsible for ZEN metabolism is the liver, there is a substantial contribution by extrahepatic sites to the overall metabolism of ZEN and that these sites

should be considered when evaluating the metabolism and toxicity of a xenobiotic such as ZEN.

For the first time, the pharmacokinetics of ZEN is reported. The disposition of ZEN was investigated as a function of two routes of administration, intravenous and intragastric, in bile-duct cannulated and intact rats. These disposition studies enabled us to test the working hypothesis that the mycotoxin undergoes extensive presystemic metabolism, biliary excretion and subsequent enterohepatic recirculation. The predominant route for systemic elimination in the rat was biotransformation prior to excretion. These data indicate that the biliary first-pass clearance for the ig dose contributed substantially to the overall biliary clearance of ZEN. Systemic elimination of ZEN and it metabolites occurs through both biliary and urinary routes, but biliary excretion is the major and dominant route. The major excretory product of ZEN is ZEN glucuronide. ZEN undergoes substantial enterohepatic recirculation which may increase tissue estrogen receptors exposure to ZEN and contribute to ZEN toxicity.

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